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ORIGINAL PAPER

# Modeling and optimization of pea protein hydrolysis: linking process parameters to technological functionality

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**Abstract**
The limited technological functionality of plant proteins often hinders their application in food systems. Although enzymatic hydrolysis is widely promoted as a "green" strategy for improving these properties, a systematic understanding of how process parameters translate into specific functional outcomes remains limited. This study investigated the link between hydrolysis process parameters (enzyme content (Alcalase 2.4 L), hydrolysis time, and substrate concentration), technological properties (solubility, water- and oil-holding capacities, emulsifying and foaming properties) and structural features (molecular weight distribution, free-SH groups, surface hydrophobicity) of a pea protein concentrate (PPC) using a Box–Behnken design. Enzyme concentration emerged as the predominant factor significantly influencing all functional responses. Experimental data revealed three distinct hydrolysis ranges (limited, intermediate, and extended), each associated with specific and predictable technological performances. These relationships were integrated into a functionality map that enables the selection of hydrolysis conditions according to the desired functional profile and the identification of trade-offs. Limited hydrolysis (DH < 10%) improved the oil-holding and foaming properties, whereas extended hydrolysis (DH > 20%) enhanced solubility. Beyond these empirical findings, the study provides a transferable process design approach applicable to other protein matrices.

**Keywords** Pea proteins · Hydrolysis degree · Alcalase 2.4L · Technological properties · Experimental design · Response surface methodology

## Introduction

Feeding the ever-expanding global population while facing increasing ecosystem degradation and climate change represents a world priority [38]. In this context, rebalancing between animal and alternative proteins in human diets is indicated as a potential solution to mitigate the effects of cattle production, encouraging consumers to adopt healthier diets. Among alternative proteins, those derived from pulses have been increasingly investigated as nutritious and sustainable sources of protein. Notably, pea proteins from *Pisum sativum* L. have received significant attention due to their high yield, low production costs, reduced allergenic responses, and high digestibility [6, 43]. In addition, pea plants do not necessitate genetic modification for growth, unlike in soybean production, which enhances the appeal of pea plants as a clean option [29]. Nevertheless, the use of pea proteins in food formulation remains limited due to their inadequate technological properties (i.e., solubility, water and oil-holding capacity, emulsifying, and foaming abilities) [46]. This is particularly evident when pea proteins are used as substitutes for animal proteins due to their different chemical structures (a lower number of -SH groups) and compositions (a higher content of globular proteins) [10, 20]. Moreover, the extraction process often induces structural modifications in pea protein chains, which further reduces their technological properties [34]. To address these issues, over the last few years, several physical, biological, and chemical strategies have been proposed to modify protein structure and/or conformation, thereby modulating

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technological properties [16]. Among biological strategies, enzymatic hydrolysis represents a green, safe, and efficient approach that could enhance the technological properties of proteins by cleaving peptide bonds while maintaining their nutritional value [19, 48, 53]. The result is the generation of a series of fragments with different molecular weights, ionizable groups, and technological functionalities [48]. By precisely setting the hydrolysis parameters, it may be possible to tailor the degree of hydrolysis (DH) of plant proteins, thereby modulating their functionalities. Limited hydrolysis is expected to enhance the technological properties of proteins, whereas a further extension of hydrolysis is reported to have the opposite effect due to an excessive reduction in peptide size [1, 51]. In this regard, the literature reports contrasting results on pea proteins. For instance, [18] and [40] observed an increase in the solubility of pea protein hydrolysates (PPHs) with DH > 2% obtained using different enzymes (i.e., Flavorzyme, Neutrase, Alcalase, papain, Esperase, and trypsin). In contrast, [6] and [24] reported that, for the same matrix hydrolyzed by trypsin, Protamex, chymosin or Alcalase, the properties decreased compared with those of unhydrolyzed samples, independent of the DH. However, [40] revealed a marked improvement in both the emulsion activity index (EAI) and the emulsion stability index (ESI) of PPHs with 6% DH, while [18] observed a substantial reduction in the emulsifying properties of PPHs when the DH was between 3 and 10%. This discrepancy can be attributed to the factors affecting the hydrolysis process. The latter is strictly dependent on substrate properties and concentration, enzyme type and content, hydrolysis pH, temperature, and time. For instance, [14] simultaneously considered temperature, substrate concentration, and the enzyme-to-substrate ratio to optimize the hydrolysis process to obtain antioxidant hydrolysates [14].

Despite the growing use of enzymatic hydrolysis as a sustainable process to enhance protein technological properties, there is a lack of clear and systematic guidance on how enzymatic hydrolysis parameters translate into specific and predictable functionalities. This knowledge could allow the rational design of protein ingredients with tailored properties.

This study aimed to explore the relationship between different hydrolysis parameters (i.e., enzyme content ([E]), hydrolysis time ($t_i$), and substrate concentration ([S]) on the technological properties solubility, water- and oil-holding capacities, emulsifying and foaming properties) and structural features (hydrolysis degree, molecular weight distribution, free-SH groups, surface hydrophobicity) of a pea protein concentrate (PPC) via the Box-Behnken design approach. The hydrolysis process was conducted by Alcalase 2.4 L, a serine endopeptidase, being one of the most widely used proteolytic enzymes for plant protein hydrolysis, including pea proteins, due to its broad substrate specificity, remarkable stability, full activity over a wide pH range, and suitability for industrial applications [11, 15, 17, 45, 52]. A heatmap, intended as an operative tool to drive the selection of proper hydrolysis conditions according to the desired functionalities of the pea hydrolysates, was ultimately developed.

**Table 1** Numerical summary of the considered levels for each tested factor

| Level | Factor | | |
|---|---|---|---|
| | [S] (%w/w) | [E] (%w/w) | $t_i$ (min) |
| −1 | 3.0 | 1 | 10 |
| 0 | 6.5 | 5 | 125 |
| +1 | 10.0 | 10 | 240 |

## Materials and methods

### Materials

Pea protein concentrate (82% w/w protein content) was obtained from Raab Vitalfood GmbH (Rohrbach, Germany). Alcalase® 2.4 L (Protease from *B. licheniformis*), 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, (Ethylenedinitrilo) tetraacetic 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). Ethanol absolute and N, N-dimethylformamide were purchased from VWR International (Milan, Italy). Ultrapure water was obtained by the Milli-Q® Advantage A10 system (Millipore S.A.S., Molsheim, France). All other chemicals used were of analytical reagent grade.

### Preparation of pea protein hydrolysates

Hydrolysis was conducted in accordance with [22] with some modifications. PPC was suspended in ultrapure water and stirred overnight at 4 °C to make proteins fully hydrated. Afterwards, the hydrolysis process was developed by using microbial protease Alcalase 2.4 L. Samples were prepared considering different substrate and enzyme concentrations, as well as the time of reaction, according to a Box-Behnken design, as reported in Table 1. For each sample, a batch of 200 mL was prepared. Each batch underwent hydrolysis for a predetermined duration (10, 125, and 240 min), under stirring in a water bath at 50 °C and pH 8, optimum conditions for the enzyme. The enzymatic reaction was stopped

by heating the sample at 90 °C for 15 min. After cooling at room temperature, the pH was adjusted to 7.0 with 1 M NaOH. Samples were then frozen at −40 °C and freeze-dried (Epsilon 2-4LSCplus, Christ, Osterode am Harz, Germany), leading to the pea protein hydrolysate samples (PPHs). PPHs were vacuum sealed in high-barrier plastic pouches and stored at room temperature until analysis.

## Analytical determinations

### Degree of hydrolysis

The degree of hydrolysis (DH) was measured with O-phthaldialdehyde (OPA) by the modified method of [7]. PPHs, prepared at 1% (w/v) in ultrapure water, were stirred overnight to guarantee complete hydration and subsequently centrifuged at 20,000×*g* for 20 min (D3024, DLAB Scientific, Beijing, China). Then, 150 µL of the sample was mixed with 3 mL OPA reagent (0.2 M sodium tetraborate, 5 mL of 20% (w/w) sodium dodecyl sulfate (SDS), 5.7 mM dithiothreitol, 80 mg of OPA dissolved in 2 mL absolute ethanol, and a final volume of 100 mL ultrapure water). The reaction mix was incubated in the dark for 2 min, and the absorbance was measured at 340 nm with a spectrophotometer (UV-1800, Shimadzu Kyoto, Japan). The total content of free amino groups in the sample was determined by acid hydrolysis of PPC using 2.5 mL of 6 M HCl at 110 °C for 24 h followed by the addition of 7.5 mL 2 M NaOH. A calibration curve was obtained from standard L-leucine solutions. 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 PPHs and $NH_{2total}$ is the total content of free amino groups in the unhydrolyzed sample obtained upon total acid hydrolysis.

### Molecular weight distribution

The molecular weight (MW) distribution was determined by size-exclusion chromatography as described by [22]. The equipment used was an LC-4000 HPLC (Jasco Europe, Cremella, Italy), and the samples were separated on a TSKgel 2000 SWXL column (Tosoh Bioscience, Griesheim, Germany), thermostated at 30 °C. The isocratic elution medium consisted of water/acetonitrile/TFA (55/45/0.1, v/v/v) at a flow rate of 0.5 mL/min. 10 µL of each PPHs, solubilized in ultrapure water at 1 mg/mL, were centrifuged (20,000×*g* for 20 min) and then injected into the HPLC system. Detection was carried out at 220 nm. BSA (66,463 Da), β-Lg (18,400 Da), aprotinin (6,511 Da), C-peptide (3,183 Da), L-glutathione oxidized (612 Da), and L-Ser (106 Da) were used as calibration standards. Chromatograms were divided into four regions based on previous studies [13] and visual inspection of the elution profiles. Among the standards, β-lactoglobulin, C-peptide, and oxidised L-glutathione were considered the most representative for the MW intervals relevant to this study, yielding four ranges that reflect the main shifts in peptide size during hydrolysis. The relative content of each MW range is expressed as the percentage of the chromatographic peak area [44]. Data analysis was performed using ChromNav software (Jasco Europe, Cremella, Italy).

### Surface hydrophobicity

Surface hydrophobicity was measured as described by [43] using the fluorescent probe 8-anilino-1-nephthalenesulfonic acid (ANS). Briefly, PPC and PPHs were prepared at a protein concentration of 0.025% (w/v) with potassium phosphate buffer (50 mM, pH 6.8). The solutions were then diluted to four concentrations ranging from 0.005% to 0.020% (w/v), and an aliquot (0.6 mL) of ANS solution (8 mM ANS in 50 mM potassium phosphate buffer, pH 6.8) was added to 10 mL of the sample, vortexed, and kept for 15 min in the dark. The diluted protein solutions were excited at 390 nm, and the signal intensity was measured at 480 nm using a Cary Eclipse spectrofluorometer (Agilent Technologies, Santa Clara, CA, USA).

The relative fluorescence intensity (RFI) was computed by the following equation:

$$RFI = \frac{(F - F_0)}{F_0} \quad (2)$$

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 the RFI versus protein concentration, calculated by linear regression.

### Free sulfhydryl groups (free-SH)

The content of free sulfhydryl groups in the samples was determined using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) reagent, according to the slightly modified method of [35]. The SDS-TGE buffer was freshly prepared before each experiment by mixing 5 mL of 25% (w/v) SDS solution and 45 mL of TGE (containing 86 mM Tris base, 92 mM glycine, and 4 mM EDTA, pH 8), degassing for 30 min,

and gently flushing with nitrogen for 15 min. The freeze-dried samples (5 mg) were dissolved in 1.5 mL SDS-TGE buffer and incubated for 40 min. Finally, 30 µL of 10 mM DTNB was added to the sample solutions, and the absorbance was determined at 421 nm after 30 min of incubation in the dark. The free sulfhydryl content was calculated as follows:

$$\text{SH groups } \left(\mu\text{mol/g}_{\text{protein}}\right) = (73.53 \times \text{A}_{412}/\text{C}) \quad (3)$$

where $A_{412}$ is the absorbance at 412 nm, C is the concentration of the sample in mg/mL, and 73.53 derives from $10^6/13{,}600\ M^{-1}\ cm^{-1}$.

### Solubility

Solubility was determined by the method described by [37]. The samples were suspended in ultrapure water (3% w/w), stirred at room temperature for 12 h, and centrifuged at 20,000×*g* for 20 min. The supernatant was discarded, and the pellet was dried overnight in a vacuum oven (Vuotomatic 50, Bicasa, Milan, Italy). The solubility was calculated by exactly weighing the remaining insoluble precipitate by using the following formula:

$$\text{Protein solubility } (\%) = (\text{S}-\text{DIF})/\text{S} \times 100 \quad (4)$$

where S is the weight of the initial sample and DIF is the weight of the dried insoluble fraction.

### Water and oil holding capacities (WHC and OHC)

The water-holding capacity (WHC) and oil-holding capacity (OHC) of PPC and PPHs were measured according to [39], with slight modifications. An aliquot of 0.1 g of freeze-dried sample was suspended in 1 mL of ultrapure water or sunflower oil in a 2 mL Eppendorf tube, vortexed, and centrifuged at 20,000×*g* at 20 °C for 30 min. The supernatant was drained, and the pellet obtained was weighed. WHC and OHC were calculated using the following equation:

$$WHC/OHC\ (g/g_{protein}) = \frac{P-S}{S} \times 100 \quad (5)$$

where P is the precipitate weight (g) and S is the weight (g on a dry basis) of the freeze-dried samples.

### Foaming properties

Sample dispersions (10 mL, $V_0$) at a 1% (w/v) protein concentration were hydrated at 4 °C for 12 h. The foam was generated by mixing the sample with an Ultra-Turrax (Ika-Werke, DI 25 Basic, Staufen, Germany) at 8,000 rpm for 3 min in a 50 mL graduated cylinder, and the mixture was measured and visually monitored immediately after the foaming process ($V_1$) and after 1 h ($V_{60}$). The foaming activity (FAI) and foam stability (FSI) indices were calculated as follows:

$$FAI\ (\%) = (V_1/V_0) \times 100 \quad (6)$$

$$FSI\ (\%) = (V_{60}/V_0) \times 100 \quad (7)$$

### Emulsifying properties

The method of [2] was used. The samples were dispersed in ultrapure water (0.01 g/mL) and stirred overnight at 4 °C. Emulsions were prepared by adding sunflower oil (9:1 w/v sample: oil ratio). The mixture was then homogenized with an Ultra-Turrax (IKA-Werke, DI 25 Basic, Staufen, Germany) at 10,000 rpm for 3 min. Aliquots of 250 µL of the emulsions were taken from the bottom of the emulsion container, diluted (1:100, v/v) in 0.1% SDS solution and used to measure the absorbance at 500 nm. The emulsifying activity (EAI) and stability (ESI) indices [36] were calculated as follows:

$$EAI\left(\frac{\text{m}^2}{\text{g}}\right) = \frac{2 \times 2.303 \times A_0 \times DF}{C \times (1-\theta) \times \Phi \times 10000} \quad (8)$$

$$ESI\,(\text{min}) = \frac{A_0}{A_0 - A_{120}} \times 120 \quad (9)$$

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), $A_0$, and $A_{120}$ are the absorbances of the diluted emulsion at time 0 and after 120 min, respectively.

## Experimental design and statistical analysis

The experimental design was selected to capture multifactor interactions and enable visualization of technological outcomes in a form directly usable for process optimization. The effect of three independent variables, namely substrate concentration [S], enzyme [E], and hydrolysis time ($t_i$),

on various responses of interest was studied by the Box-Behnken design (BBD). The experimental design was built and analyzed with R (The R Foundation for The Statistical Computing, Vienna, Austria, version 4.3.2) within the RStudio environment (version 2023.09.1 – Build 494) using the *tidyverse* package (version 2.0.0) for data handling [47], and the *rsm* package (version 2.10.4) which covers the most standard first- and second-order designs and methods for one response variable [28]. In the present study, based on the literature, three levels were set for each factor, encoded as −1, 0, and + 1 to cover the span of interest (Table 1). The design was carried out once, and the central point was repeated 5 times to estimate the experimental error. Experimental run execution was randomized. To model the surface response from experimental observations, a generic quadratic polynomial regression equation was used:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_i x_i^2 + \sum_{j=i+1}^{k} \sum_{i=1}^{k-1} \beta_{ij} x_i x_j + \varepsilon_{ij} \quad (10)$$

where $Y$ is the predicted response, $\beta_0$ is the intercept of the surface response model, $\beta_i$ are the linear coefficients, $\beta_{ii}$ are the quadratic coefficients, $\beta_{ij}$ represents the interaction coefficients, $x_i$ and $x_j$ are the input variables, and $\varepsilon$ is the error term. The $\beta_{ij}$ term is extremely important because it defines how two variables concur to significantly influence the predicted response (i.e., $Y$).

The model was validated by performing the hydrolysis process twice under the following conditions: [S]: 6.5%; [E]: 10%; and $t_i$: 240 min. The assays were performed in triplicate. To measure the error between the experimental observations and the predicted values, the relative percent difference ($\bar{E}\,\%$) was calculated through the following equation:

$$\bar{E}\% = \frac{|X_i - X_p|}{X_i} \quad (11)$$

where $X_i$ is the experimental value and $X_p$ is the predicted value.

Response surface plots and a qualitative heatmap of the functional properties were generated by using the package plotly (version 4.10.4, [41] and Origin Pro 9 software (OriginLab, Northampton, MA), respectively.

## Results and discussion

### Degree of hydrolysis and structural characterization of pea protein hydrolysates

The response surface methodology (RSM) technique, utilising a Box-Behnken design, was used to conduct a 3-factor, 3-level experiment to investigate how enzymatic process parameters influence the characteristics of pea protein hydrolysates. The selected factors were substrate concentration [S], enzyme concentration [E], and hydrolysis time (ti). Based on the literature, the minimum and maximum levels (Table 1) were chosen according to commonly used conditions for pea protein hydrolysis, defining the range of interest.

A comprehensive set of 17 experimental runs is detailed in Table S1. The estimated regression coefficients obtained from fitting the degree of hydrolysis (DH), molecular weight distribution (MW), free sulfhydryl groups (-SH) and surface hydrophobicity ($H_0$) data of the pea protein hydrolysates (PPHs) are reported in Table 2.

Independence between [E] and [S] was detected, suggesting that the interaction between these factors may be observed out of the selected span of interest. However, it should be noted that such a lack of interaction does not affect the aptness or reliability of the model. The adopted experimental design was intended to assess the optimal processing conditions for guiding hydrolysate production from pea proteins with tailored technological properties, rather than investigating how [E] and [S] influence the rate of enzymatic product formation. A significant interaction between [E] and $t_i$ was detected (Table 2). Additionally, the overall model's fit for each data set can be considered quite satisfactory, as demonstrated by the adjusted $R^2$ and the lack of fit.

Fig. 1a shows the three-dimensional response surface plotting the DH on the Z-axis against $t_i$ and [E] while maintaining constant [S] at the central value (5% w/w).

As expected, the DH of PPHs progressively increased with increasing [E] and $t_i$, reaching a maximum DH of approximately 30%. The literature reports different results regarding the maximum DH of PPHs obtained by Alcalase hydrolysis, ranging from 15 to 50% [3, 40]. This discrepancy between the results could be attributed to differences in other parameters used for hydrolysis (i.e., time, protein: enzyme ratio, PPC purity and composition).

As expected, hydrolysis also caused a progressive reduction in peptide dimensions by increasing the processing factors. Native proteins and polypeptides with MW > 18 kDa were present only in the non-hydrolyzed PPC, accounting for 72.4% of the total protein and in the hydrolysate with the lowest DH among all samples. In this hydrolysate (DH < 7%), intact proteins represented 37% of the total protein, reflecting

**Table 2** Estimated regression coefficients of the second-order model for degree of hydrolysis (DH), molecular weight distribution (MW), free sulfhydryl groups (-SH) and surface hydrophobicity ($H_0$) of pea protein hydrolysates (PPHs)

| Variable | DH (%) | MW 18−6 kDa | 6−3 kDa | 3-0.6.6 kDa | <0.6 kDa | free -SH (µmol/g) | $H_0$ (-) |
|---|---|---|---|---|---|---|---|
| Intercept | 2.006e-10*** | 0.009** | 0.002** | 1.018e-09*** | 4.833e-09*** | 1.745e-08*** | 0.006** |
| [S] | 0.458 | 0.355 | 0.817 | 0.542 | 0.156 | 0.302 | 0.315 |
| [E] | 2.994e-08*** | 4.087e-06*** | 0.013* | 2.309e-06*** | 1.269e-06*** | 0.031* | 0.001*** |
| $t_i$ | 1.302e-06*** | 7.169e-05*** | 0.070 | 0.000*** | 1.861e-05*** | 0.448 | 0.031* |
| [S] × [E] | 0.251 | 0.291 | 0.922 | 0.775 | 0.570 | 0.753 | 0.531 |
| [S] × $t_i$ | 0.007** | 0.203 | 0.535 | 0.050* | 0.630 | 0.018* | 0.764 |
| [E] × $t_i$ | 3.963e-05*** | 0.001*** | 0.039* | 0.003** | 0.035* | 0.023* | 0.978 |
| $[S]^2$ | 0.021* | 0.259 | 0.401 | 0.574 | 0.656 | 0.131 | 0.388 |
| $[E]^2$ | 2.452e-05*** | 0.818 | 0.559 | 9.082e-05*** | 0.001*** | 0.206 | 0.086 |
| $t_i^2$ | 0.007** | 0.191 | 0.361 | 0.004** | 0.001** | 0.005** | 0.012* |
| $R^2$ adj | 0.986 | 0.943 | 0.500 | 0.956 | 0.962 | 0.721 | 0.761 |
| Lack of fit | 0.209 | 0.021 | 0.001*** | 0.011* | 0.188 | 0.056 | 0.054 |

* $p<0.05$

** $p<0.01$

*** $p<0.001$

limited proteolysis and partial preservation of native protein structure. These findings indicated that the main pea proteins, convicilin (77 kDa), vicilin subunits (50 kDa, 39 kDa, and 28 kDa), vicilin minor subunits (32 kDa), and legumin (25 and 22 kDa) were hydrolyzed entirely at the beginning of the enzymatic hydrolysis in almost all PPH samples [27, 30]. Concomitantly, peptides with lower dimensions were formed (Fig. 1 and Fig. S1). The effects of [E] and $t_i$, which are the significant factors (Table 2), on MW ranges of 18−6 kDa and <0.6 kDa are shown in Fig. 1b and c, respectively. The amount of peptides with 18−6 kDa (Fig. 1b) gradually decreased with increasing [E] and $t_i$, while concomitantly, peptides with lower MWs were formed. When the highest DH was detected (around 30%), peptides with a MW of 18−6 kDa were absent (Fig. 1b), while those with a MW of 6−3 kDa were less than 10% (Fig. S1a). Under these conditions, the predominance of peptides presented MWs<3 kDa. In fact, peptides with MW 3-0.6.6 kDa (Fig. S1b) and MW<0.6 kDa (Fig. 1c) accounted for approximately 55% and 40%, respectively.

To further investigate the structural modifications occurring on PPHs upon hydrolysis, free -SH groups were measured. The effect of the interaction between [E] and $t_i$ is shown in Fig. 2a.

The amount of free -SH groups of PPHs ranged from approximately 3 µmol/g to 6 µmol/g, indicating an increased exposure of -SH groups upon hydrolysis and with respect to PPC (1.6±0.2 µmol/g) (Table S1). This increasing trend was maintained until reaching approximately 6% of [E] and 150 min of ti, while further increases in these parameters did not modify the -SH content. These results can be attributed to the unfolding of proteins and the consequent disruption of their tertiary structure, which exposes -SH groups [25].

Regarding surface hydrophobicity, PPC exhibited a higher surface hydrophobicity index ($H_0$) (3348.4±12.3; Table S1) as compared to PPHs. The response surface plot (Fig. 2b) indicated that $H_0$ plummeted with increasing [E] rather than $t_i$. This finding can be correlated with the breakdown of hydrophobic areas, followed by an increase in the exposure of ionizable groups when small proteins and oligopeptides are formed [8, 49]. Moreover, $H_0$ could be negatively affected by the formation of hydrophobic links between small polypeptides, leading to protein refolding and reburying of hydrophobic sites within a new structure [4]. These hypotheses were also confirmed by other authors who observed the same trend when hydrolysis was conducted on pea and lentil proteins [4, 49].

## Technological properties

The technological properties of the PPHs were further analyzed, and the regression coefficients are reported in Table 3. These trends are interpreted in light of the structural modifications accompanying hydrolysis (peptide size reduction, exposure of -SH groups, and changes in $H_0$), which provide mechanistic support to the structure–functionality relationships discussed below.

[E] was the most significant factor for all the considered properties, [S] was relevant for solubility and the emulsion stability index (ESI), while $t_i$ affected solubility, the emulsion ability index (EAI) and the foaming stability index (FSI). Overall, the fitting procedure resulted in an acceptable fit based on the adjusted $R^2$ and the lack of fit (Table 3).

As expected, hydrolysis promoted an increase in solubility of at least 20% with respect to that of PPC, which was 21.9±0.2% (Table S1). These results are in agreement with

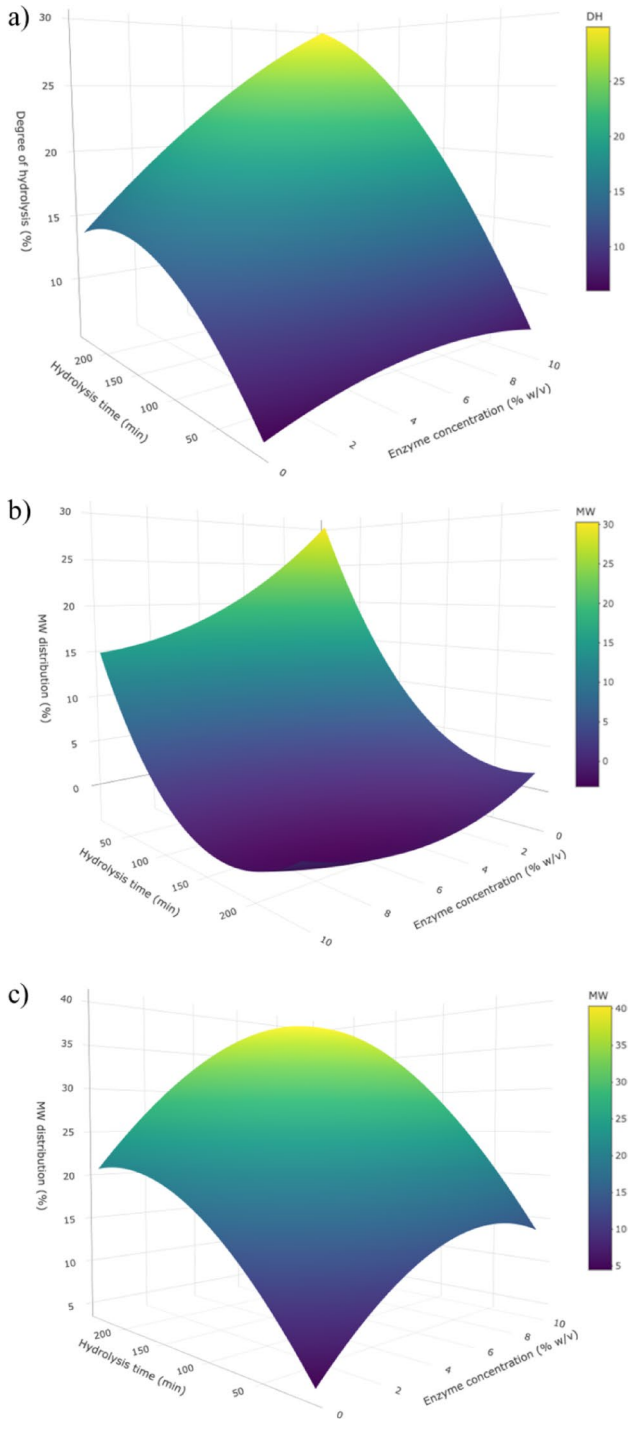


**Fig. 1** 3D response surface analysis plot showing the effect of enzyme concentration and hydrolysis time on the degree of hydrolysis (DH) (**a**) and the molecular weight distribution (MW) in the range 18–6 kDa (**b**) and < 0.6 kDa (**c**) of pea protein hydrolysates (PPHs). A constant value (central point) was imposed on the third independent variable of the BBD

those reported by other authors who observed a solubility increase when hydrolysis was conducted on pea proteins [17, 42]. Different factors may affect solubility. On the one hand, the breakdown of large proteins into low MW peptides (Fig. 1b and c, Fig. S1a and b) probably exposed new ionisable amino and carboxyl groups with consequent increase in hydrophilicity (Fig. 2b). These modifications promoted interactions with water through hydrogen bonding [5, 48]. On the other hand, protein solubility has been reported to correlate with enzyme type. Alcalase, the enzyme used in this study, targets explicitly peptide bonds containing tyrosine, glutamic acid, or leucine at the carboxyl side, thereby increasing solubility [45].

The WHC of the PPHs (Fig. 3a) resulted in lower values compared to PPC (3.5 ± 0.2 $g_{water}/g_{sample}$), especially when [E] increased. This behavior was probably due to the decrease in MW (Fig. 1b and c, Fig. S1a and S1b), which impairs the ability of the peptides to physically entrap water due to their small size [21, 48]. Moreover, the steep increase in solubility (Table S1) together with the reduction of the insoluble fraction that is physically able to retain water likely contributed to this behaviour, as water holding is predominantly associated with the presence of insoluble material [31, 33].

Contrary to WHC, the OHC of the PPHs (Fig. 3b) was greater than that of the PPC (0.9 ± 0.0 gwater/gsample), ranging between 1.1 and 2.4 g/g. This can be attributed to the excellent fat-binding ability of non-polar amino acid side chains, as well as to the greater flexibility of peptides and polypeptides resulting from size reduction (Fig. 1b and c) after hydrolysis [9].

Interfacial properties were then evaluated. EAI significantly decreased with [E] and $t_i$, while ESI was positively dependent on [S] and [E] (Fig. S2 and Table 3). EAI remained comparable to that of PPC (2.3 ± 0.2 g/m2) only when DH < 10%, while further increases in DH caused a negative deflection of the property. This behaviour reflects the changes in peptide MW distribution occurring during hydrolysis (Fig. 1b and c). It can be assumed that samples still containing a substantial portion of high-molecular-weight proteins and peptides (MW > 18 kDa) were more effective in promoting emulsion formation, as previously observed [12]. On the other hand, small peptides, intended as those with low MW or those with less than 20 residues, led to a marked decrease in emulsifying properties. These small peptides are generally ineffective in lowering the interfacial tension between oil and water because of their limited ability to unfold and their reduced flexibility [26], In addition, the marked reduction in surface hydrophobicity observed as the hydrolysis progressed (Fig. 2) further weakens small peptides affinity for the oil–water interface, contributing to the loss of emulsifying capacity [32]. Taken

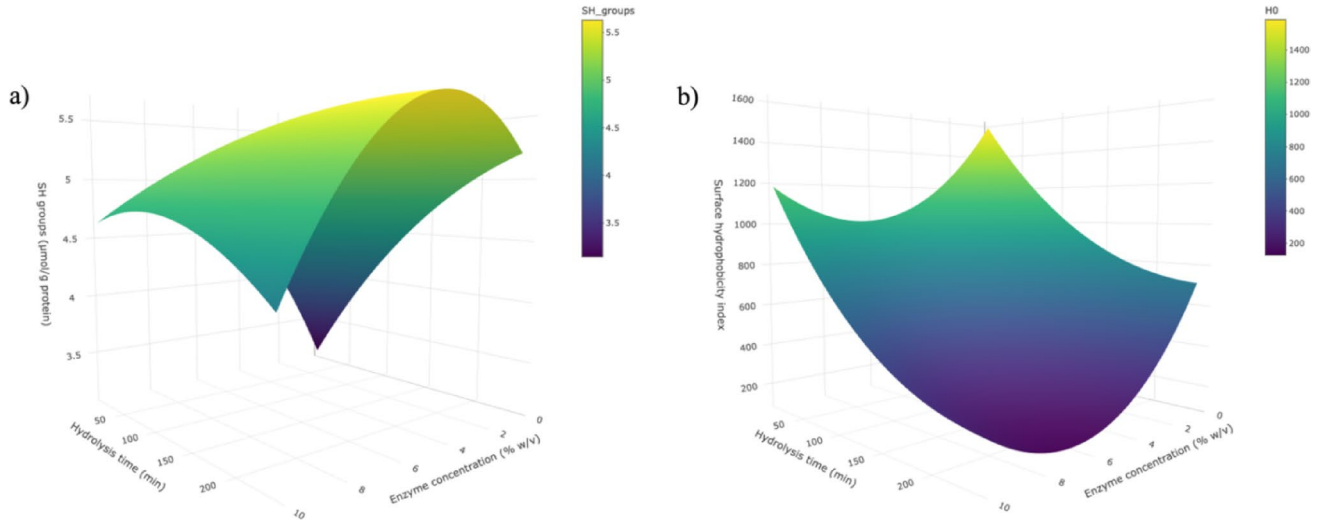


**Fig. 2** 3D response surface analysis plot showing the effect of enzyme concentration and hydrolysis time on free -SH groups (**a**) and surface hydrophobicity index (**b**) of pea protein hydrolysates (PPHs). A constant value (central point) was imposed on the third independent variable of the BBD

**Table 3** Estimated regression coefficients of the second-order model for solubility, water holding capacity (WHC), oil holding capacity (OHC), emulsifying activity and stability indices (EAI and ESI) and foaming activity and stability indices (FAI and FSI) of pea protein hydrolysates (PPHs)

| Variable | Solubility (%) | WHC (g/g) | OHC (g/g) | EAI ($g/m^2$) | ESI (min) | FAI (%) | FSI (%) |
|---|---|---|---|---|---|---|---|
| Intercept | 9.61e-09*** | 4.68e-05*** | 5.40e-07*** | 7.12e-08*** | 5.04e-09*** | 2.24e-06*** | 1.51e-05*** |
| [S] | 0.016* | 0.194 | 0.794 | 0.914 | 0.022* | 0.064 | 0.110 |
| [E] | 0.005** | 0.002** | 0.001*** | 4.36e-05*** | 0.002** | 0.002** | 0.001*** |
| $t_i$ | 0.032* | 0.514 | 0.058 | 0.002** | 0.153 | 0.064 | 0.002** |
| [S] × [E] | 0.061 | 0.869 | 0.579 | 0.896 | 0.375 | 0.229 | 0.458 |
| [S] × $t_i$ | 0.864 | 0.544 | 0.450 | 0.444 | 0.033* | 0.176 | 0.114 |
| [E] × $t_i$ | 0.750 | 0.589 | 0.360 | 0.157 | 0.139 | 0.400 | 0.759 |
| $[S]^2$ | 0.198 | 0.092 | 0.024* | 0.580 | 0.510 | 0.032* | 0.142 |
| $[E]^2$ | 0.289 | 0.112 | 0.376 | 0.002** | 0.000*** | 0.002** | 0.008** |
| $t_i^2$ | 0.135 | 0.447 | 0.472 | 0.239 | 0.012* | 0.647 | 0.118 |
| $R^2$ adj | 0.692 | 0.915 | 0.700 | 0.884 | 0.857 | 0.780 | 0.829 |
| Lack of fit | 0.012* | 0.060 | 0.334 | 0.679 | 0.064 | 0.320 | 0.392 |

*$p<0.05$

**$p<0.01$

***$p<0.001$

together, these results indicate that emulsifying properties are governed by the presence of high MW proteins and/or peptides, which contribute to the formation of interfacial films, and the increase in small peptides with lower surface hydrophobicity that reduce the interfacial behaviours, rather than by the degree of hydrolysis alone.

The foaming ability index (FAI) (Fig. 3c) was greater than that of PPC (77.1 ± 8.8%; Table S1) across all hydrolysates, indicating that proteolysis generally favoured foam formation. Foaming stability index (FSI) (Fig. 3d and Table S1) also improved when the DH was lower than 20%, whereas extended hydrolysis depleted this property. Such behavior underlines the pivotal role of DH in affecting interfacial properties [26].

Our findings align with the literature, which reports that structural modifications induced by enzymatic hydrolysis improve foaming properties. The observed increase in solubility (Table S1) significantly affected these results, as more proteins migrated to the interface, favouring foaming formation [43]. Moreover, the reduction in protein MW previously discussed (Fig. 1b and c) enabled the easier orientation of the molecules at the air-water interface [6, 23]. Finally, the increase in exposed -SH groups observed upon hydrolysis (Fig. 2a) may also have contributed to the improved foaming properties, as reported by [50]. In contrast, extended hydrolysis produces very small peptides (MW < 1 kDa) that, despite being highly soluble, lack the molecular size, flexibility, and interfacial activity required to stabilise foam lamellae [21]. Taken together, these observations indicate

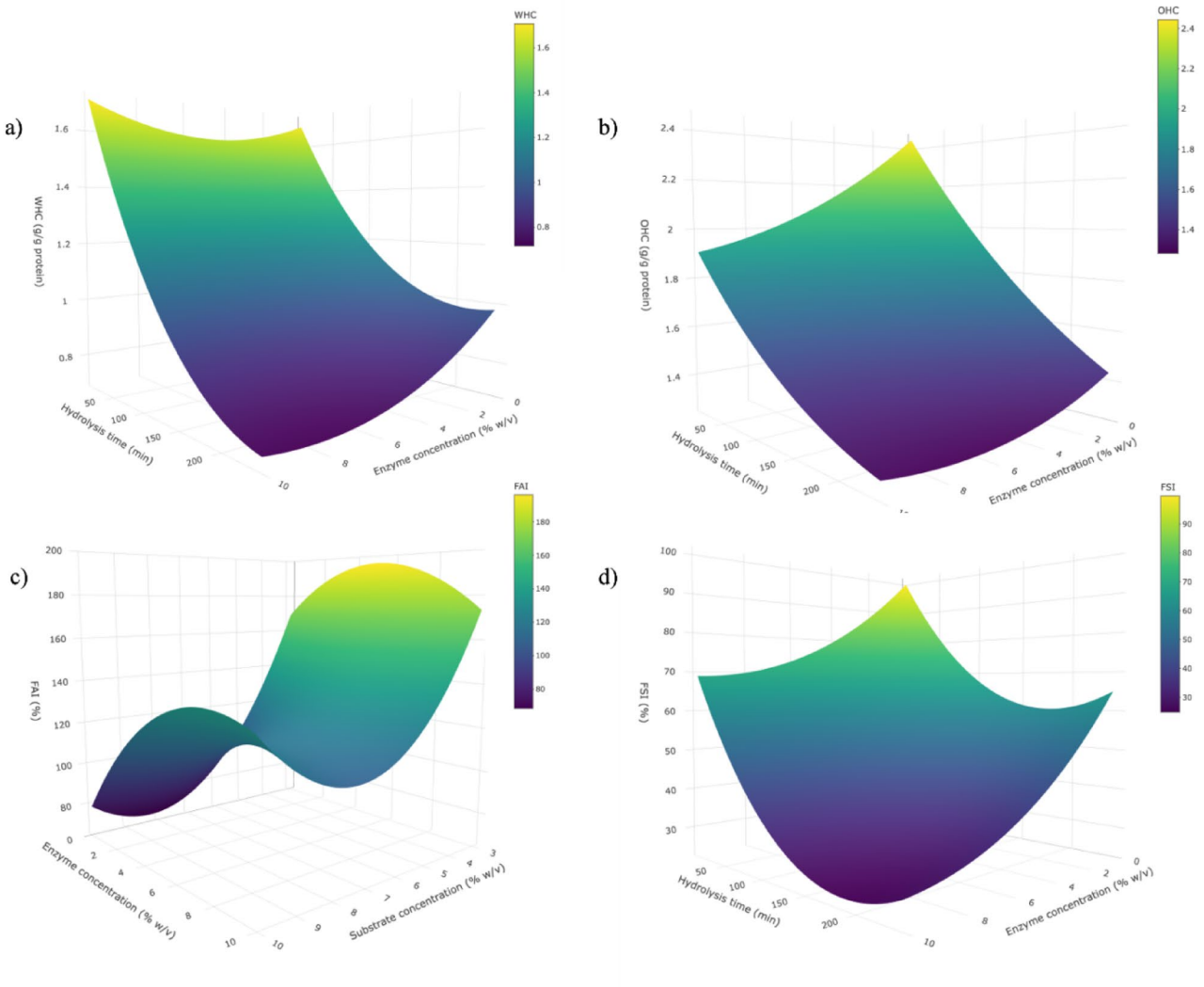


**Fig. 3** 3D response surface analysis plot showing the effect of [E] and $t_i$ on water holding capacity (WHC) (**a**), oil holding capacity (OHC) (**b**), and foaming stability index (FSI) (**d**) and the effect of [S] and [E] on foaming ability index (FAI) (**c**). A constant value (central point) was imposed on the third independent variable of the BBD

**Table 4** Experimental and predicted values of the responses

| Response | Experimental value | Predicted value | $\bar{E}\%$ |
|---|---|---|---|
| DH (%) | 28.32±0.34 | 29.94 | 5.72 |
| SH (µmol/g) | 3.96±0.24 | 4.21 | 6.31 |
| $H_0$ (-) | 331.5±0.9 | 295.2 | 10.95 |
| Solubility (%) | 68.2±3.7 | 65.4 | 4.11 |
| WHC (g/g) | 0.62±0.20 | 0.73 | 16.11 |
| OHC (g/g) | 1.66±0.02 | 1.31 | 21.08 |
| EAI ($m^2$/g) | 1.17±0.03 | 1.15 | 1.71 |
| ESI (min) | 503.7±0.4 | 480 | 4.71 |
| FAI (%) | 114.6±2.9 | 113.2 | 1.22 |
| FSI (%) | 28.85±2.24 | 28.51 | 1.17 |

that foaming behavior depends on the balance between enhanced solubility, favoring interfacial adsorption, and the maintenance of sufficiently large peptide species to form and stabilise interfacial films.

## Model validation and results rationalisation

To confirm the validity of the model, a separate hydrolysis experiment was conducted under the following conditions: [S]: 6.5%; [E]: 10%; and $t_i$: 240 min. Table 4 shows the analytical results of the characterization of this sample in comparison to those predicted by the model.

The relative percent difference between the experimental and predicted values confirmed the suitability and validity of the developed model, being the $\bar{E}\%$ highly acceptable (i.e., lower than 7%) in most cases. The only exception was represented by OHC, which $\bar{E}\%$ was 21%. However, considering the variability associated with enzymatic hydrolysis, the results obtained are satisfactory.

The results were ultimately rationalized in a road map of functionalities to qualitatively depict the trend of the

**Table 5** Processing conditions in terms of enzyme concentration ([E]) and hydrolysis time ($t_i$) to obtain different hydrolysis extents

| Hydrolysis extent | DH | Processing conditions |
|---|---|---|
| Limited | < 10% | [E] < 0.5%<br>$t_i$ up to 240 min |
| Intermediate | 10% < DH < 20% | 0.5< [E] < 5.5%<br>$t_i$ <137 min |
| Extended | > 20% | [E] > 5.5%<br>$t_i$ >137 min |

considered properties of PPC and PPHs at different extents of hydrolysis.

To this aim, the extent of the hydrolysis was classified as limited (DH < 10%), intermediate (10% < DH < 20%) or extended (DH > 20%).

The BBD equation was used to pinpoint the conditions of [E] and $t_i$, required to provide these hydrolysis extensions (Table 5), while [S] was excluded as not significant (Table 3). When limited hydrolysis was conducted, the only processing parameter to consider was [E]. In the other cases, $t_i$ should also be set, being the limit between intermediate and extended hydrolysis approximately 140 min.

The extent of hydrolysis was thus related to the technological functionalities of PPC and PPHs, as shown in the qualitative heatmap in Fig. 4. Beyond serving as a visual tool, this heatmap also functions as a process design resource, translating hydrolysis conditions into specific functional outcomes. By combining various technological responses into one framework, this approach helps identify trade-offs (for instance, maximizing solubility while reducing foam destabilization) and offers a digital approach to optimize processes.

Figure 4 clearly highlights the positive or negative impact of hydrolysis in comparison with the reference PPC. The WHC was worse in PPHs than in PPC. EAI remained unchanged during limited and intermediate hydrolysis, whereas the ESI was significantly enhanced under intermediate and extensive conditions. The OHC and foaming properties (FAI and FSI) of PPHs were greater than those of PPC. In particular, these properties were favored when limited hydrolysis was applied, while further increases in hydrolysis extent progressively reduced them. Finally, a noticeable increase in solubility occurred when intermediate and even more extended hydrolysis was performed. This map could help in the design of the hydrolysis process needed to obtain a novel PPH ingredient with target functionalities.

## Conclusions

The results of this study highlighted that enzymatic hydrolysis can be tailored to obtain pea protein-based ingredients with target technological functionalities. By modelling the selected process parameters as a function of hydrolysate characteristics, the enzyme concentration emerged as the most significant parameter impacting protein functionalities. The experimental dataset was then translated into a functionality map that connects hydrolysis extent (limited, intermediate, and extended) with specific performances in solubility, interfacial properties, and water/oil interaction capacities. This appears to be a useful tool that enables selection of hydrolysis conditions based on the desired functional profile.

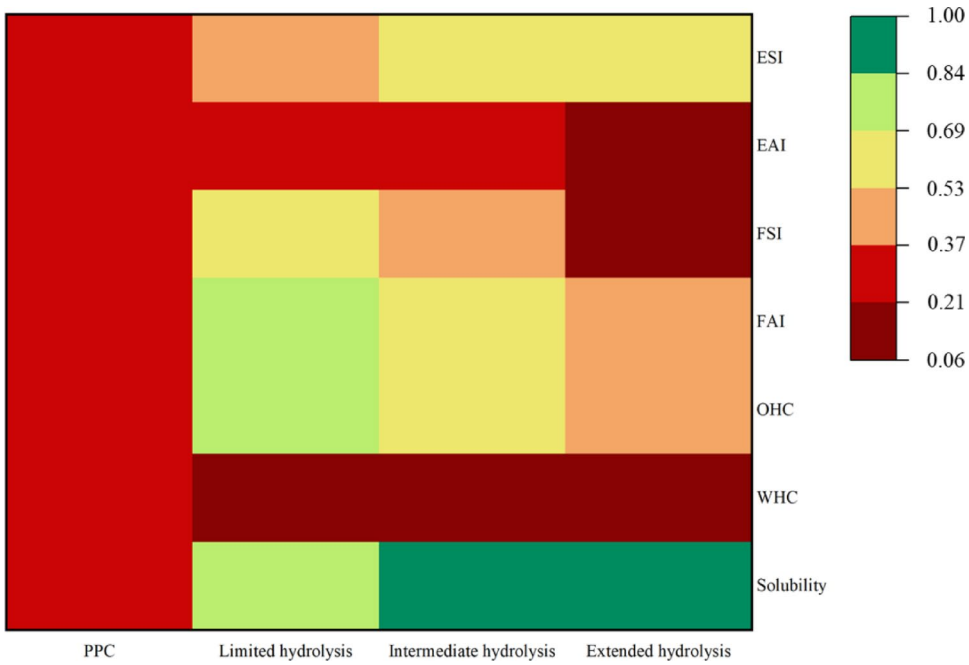


**Fig. 4** Qualitative heatmap representation of the functional properties of PPC and PPHs as a function of different hydrolysis extents using a colorimetric scale. Dark red and dark green represent the lowest and highest magnitudes of the functional property, respectively

Limited hydrolysis (DH < 10%) promoted OHC and foaming properties. In contrast, extended hydrolysis (DH > 20%) enhanced solubility. Although developed for pea protein, the proposed strategy and the resulting design tool might be fully transferable to other protein sources, supporting the engineering of novel protein ingredients with application-specific functionalities.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00217-026-05037-4.

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**Data availability** No datasets were generated or analysed during the current study.

## Declarations

**Conflict of interest** The authors declare no competing interests.



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