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Original Scientific Paper

Valorisation of pumpkin leaf by-product proteins through enzymatic modification in a biorefinery concept for meat emulsion application

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ABSTRACT

The valorisation of agricultural and industrial side-streams within integrated biorefinery concepts is gaining increasing importance in the context of sustainable protein production and the circular bioeconomy. In this context, waste green biomass is emerging as an alternative protein source with potential applications in food formulations. This work investigated the possibility of improving the techno-functional and antioxidant properties of protein isolated from pumpkin leaves by partial enzymatic hydrolysis.

The white protein fraction, mainly composed of RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) was isolated from green juice obtained by pressing pumpkin leaves through thermocoagulation, acid precipitation, subsequent resolubilisation and lyophilisation, and further treated with endopeptidase from *Bacillus licheniformis* in a stirred batch reactor under the reported optimal conditions (pH 8, 55 °C). Enzymatic treatment at the optimal enzyme-to-substrate ratio (*E/S* = 6.5 wt.%, 75 min, degree hydrolysis ~ 15%) markedly improved protein solubility, emulsifying capacity, interfacial functionality, and antioxidant activity, supporting the potential application of pumpkin leaf protein as natural emulsifiers and antioxidants in meat products.

1. Introduction

Nowadays, due to steadily increasing population growth and the rising demand for meat production, the search for a new generation of renewable protein sources has become highly important (*Malila et al.*, 2024). Press cakes and other residues obtained after oil extraction from stone fruit and oilseeds represent a valuable source not only for lipids for biodiesel production, but also of proteins and other high-value compounds that can be recovered and valorised. In this framework, green leaf biomass like pumpkin leaves represents an underutilised protein source with potential applications in food formulations (*Perović et al.*, 2024).

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In addition to their nutritional value, plant proteins play a key technological role in food systems (*Baig et al.*, 2025). In the meat industry, they are increasingly used as natural emulsifiers and functional ingredients, which contribute to stabilisation of emulsions, texture improvement and water and fat retention (*Bukarbayev et al.*, 2025). Their use can achieve a dual effect: reducing synthetic additives in line with clean-label trends and enriching products with proteins that enhance nutritional value. It is particularly attractive that such proteins can be obtained from waste plant biomass, promoting full utilisation of raw materials within biorefinery system and reducing waste.

Pumpkin leaves (Curcubita sp.) are one of the underutilised plant resources, although they are rich in proteins, especially chloroplast and membrane proteins that have favourable emulsifying and foaming properties. This biomass is most often discarded as agricultural waste during harvesting or processing, but within integrated biorefinery concepts, it can represent a valuable source of food protein (*Korićanac et al.*, 2025). However, some limitations such as poor solubility or insufficiently developed techno-functional properties in the native form often hinder their direct application in food systems. In this context, enzymatic modification stands out as an effective approach for improving the functional properties of native proteins including emulsifying activity, foaming ability, and water and oil binding capacity. In addition, a partial degradation of protein by proteolytic enzymes can produce oligopeptides exerting biological functions, particularly antioxidant activity. Thus the obtained hydrolysates may have significant potential as pure-declared natural antioxidants in meat emulsions, where they can partially or completely replace synthetic additives and contribute to the nutritional value of the product (*Mensah et al.*, 2024). Comparison with soy protein, which is most commonly used as a protein standard in the industry, allows for the assessment of the technological applicability of these new ingredients.

In this work, the potential of proteins isolated from pumpkin leaves to use as functional antioxidants in meat emulsion was investigated. To improve their techno-functional properties, the isolated protein fraction was subjected to partial enzymatic hydrolysis. The antioxidant and techno-functional properties of the obtained hydrolysates including solubility, emulsifying and interfacial properties were evaluated and compared with those of the unmodified pumpkin leaf protein and commercial soy protein concentrate.

1. Materials and methods

2.1. Preparation of leaf protein isolate

The extraction of protein from fresh pumpkin leaves (*Cucurbita pepo* var. *oleifera*; September 2023 harvest, JS&O d.o.o., Novo Miloševo, Serbia) involved mechanical screw-pressing, thermal coagulation and isoelectric precipitation, as described with slight modification (*Perović et al*., 2024). Fresh leaves (150 g) were pressed using a twin-screw press (Angel Juicer 8500) to obtain green juice and a fibrous press cake. The juice was incubated at 55 °C for 30 min, cooled, and centrifuged (7800 rpm, 10 min, 4 °C) to remove pigments and membrane proteins. The supernatant ("brown juice") was adjusted to pH 4.5 with 2 M HCl and stored at 2-8 °C for 12-16 h without stirring. The precipitate, white protein fraction, was collected by centrifugation, neutralised to pH 7, and freeze-dried to obtain the protein powder. The total nitrogen content was determined by the Kjeldahl method (AOAC 2001.11).

2.2. Enzymatic hydrolysis

The white protein fraction was hydrolysed with Alcalase® 2.4 L (EC 3.4.21.62, endoprotease from *Bacillus licheniformis*, 2.4 Anson Units (AU)/g; Sigma Aldrich, St. Louis, USA) in a stirred batch reactor at pH 8 and 55 °C. The protease was added to 200 mL of the reaction mixture at different enzyme-to-substrate ratios (E/S = 0.5-10 wt%). The degree of hydrolysis (*DH*), defined as the percentage of peptide bonds cleaved, was calculated based on base consumption using the pH-stat method (*Knežević-Jugović et al*., 2022).

2.3. Evaluation of leaf protein interfacial properties

Emulsifying properties were analysed turbidimetrically (*Moure et al.*, 2002). Oil-in-water emulsions (1 wt.% protein) were formulated with sunflower oil in a 3:1 volume ratio and homogenised at 9500 rpm for 1 min. The absorbance of the 100-fold diluted emulsions in 0.05 M phosphate buffer (pH 7, 0.1% SDS) was measured at 500 nm. The emulsion activity index (*EAI*) was calculated as follows:

$$EAI\ \left(\frac{m^2}{g}\right) = \frac{2 \cdot T \cdot A_0 \cdot d_f}{\theta \cdot c \cdot 10{,}000} \qquad (1)$$

where T is turbidity (2.303 × 1 cm^2); A, absorbance; θ, oil phase volume fraction; c, protein mass

per unit volume of aqueous phase (g); and d_f = 100, the dilution factor.

For determining emulsion stability index (*ESI*), the prepared emulsions were kept for 10 min at 20 °C and analysed as previously described. *ESI* was calculated using the formula:

$$ESI\ (h) = \frac{A_0}{(A_0 - A_t) \cdot t} \qquad (2)$$

where t represents the time interval observed for emulsion stability.

Surface and interfacial tension were measured using a Krüss K100 tensiometer (Wilhelmy plate method) to assess protein adsorption and protein-lipid interactions at the interface. Interfacial tension was determined between the aqueous protein solution and sunflower oil, while surface tension was monitored at the air-water interface. A platinum Wilhelmy plate was immersed 3 mm into 10 g of the aqueous phase, followed by gentle addition of 25 g of the oil phase. Measurements were conducted after 10 min of stabilisation.

2.4. Determination of protein solubility at various pH

Protein samples were dispersed in water and stirred for 30 min. The pH was adjusted to 2, 4, 6, 8, and 10 using 0.2 M NaOH or HCl, then diluted to a protein concentration of 0.5 mg/cm^3, and stirred (250 rpm, 30 min). After centrifugation (12,000 rpm, 10 min), supernatants were collected. Solubility was expressed as the percentage of soluble protein relative to the initial concentration. Protein content was determined by the modified Lowry method (*Hartee*, 1972).

2.5. Determination of antioxidant activity of leaf protein and hydrolysates

The antioxidant capacity was evaluated through ABTS radical scavenging and ferrous ion chelating assays, as described (*Knežević-Jugović et al*., 2022). Lyophilised powders were dissolved in deionized water to a concentration of 2 mg/cm^3. For the ABTS assay, 0.01 cm^3 of the protein solution was mixed with 1 cm^3 of freshly prepared ABTS$^+$ radical solution, incubated for 5 min in darkness, and absorbance measured at 734 nm.

For the metal-chelating assay, 0.2 cm^3 of the protein solution was mixed with 0.8 cm^3 of water and 0.10 cm^3 of 2 mM iron (II) chloride, incubated for 3 min, followed by addition of 0.20 cm^3 of 5 mM ferrozine and a further 10-min incubation. Absorbance was measured at 562 nm.

2.6. Statistical analysis

All measurements were performed in triplicate, and results are expressed as mean ± SD. Statistical analysis was performed using OriginPro 9.0, and differences between means were evaluated by one-way ANOVA followed by Tukey's test at the 5% significance level.

2. Results and discussion

3.1. Enzymatic hydrolysis of the white protein fraction

The green juice was separated into two protein fractions after thermocoagulation and centrifugation: a water-soluble (white protein), and a water-insoluble (green protein) fraction. The Sankey chart illustrating the protein flow and fraction distribution during the protein isolation is shown in Fig. 1.

The lyophilised pumpkin leaves contained 28.63±1.54% protein (dry weight basis), which is comparable or slightly higher than that of other leaf sources like spinach (28%) or sugar beet leaves (19.4% to 24.02±0.18%) (*Akyüz et al.*, 2021). The protein recovery yield was 10.20% (total yield of 2.15%), similar to other plant sources (*Liese et al*., 2023), but considerably lower than that reported for duckweed (*Nieuwland et al.*, 2021). The obtained white protein fraction contained 94.43% dry matter, mainly consisting of soluble RuBisCO protein with 84.45% protein on a dry mass basis. This high protein contents confirms the efficiency of the isolation method. Ribulose-1,5-bisphosphate carboxylase/oxygenase (EC.4.1.1.29; RuBisCO) is an enzyme that plays a vital role in photosynthesis, representing the major soluble protein fraction in leaves, accounting for 80–90% of total protein. In higher plants, it predominantly occurs as form I, composed of eight large (L, 52 kDa) and eight small (S, 14-15 kDa) subunits, designated as the L8S8 structure. Although RuBisCO has been reported to possess good gelation and emulsifying properties (*Martin et al.*, 2019; *Gracio et al*. 2023), our preliminary results indicated that its solubility and emulsifying capacity were insufficient for direct implementation in food products. Therefore, the protein was subjected to enzymatic hydrolysis.

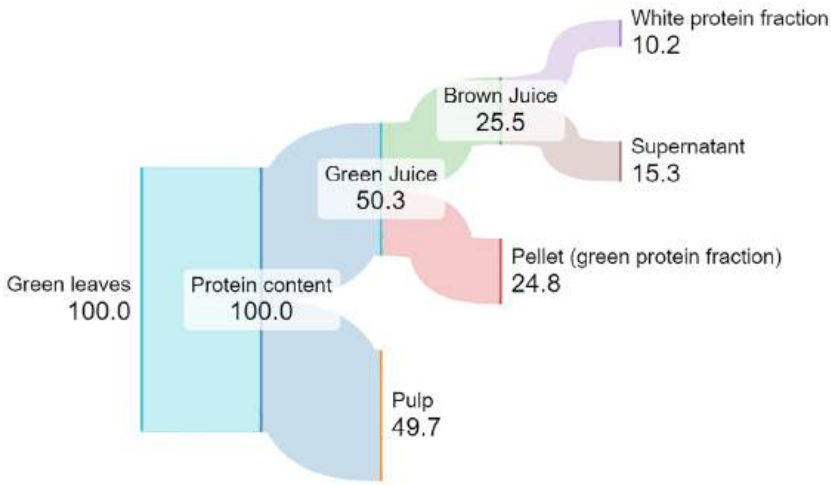

Figure 1. The Sankey chart illustrating the protein flow and fraction distribution during the protein isolation

Enzymatic hydrolysis is a key method for producing protein hydrolysates and peptides, offering precise process control, high specificity, and solvent-free, non-toxic products. Besides improved functional properties, the obtained hydrolysates may also exhibit antioxidant activity due to the release of cryptic bioactive peptides from the parent protein chain. The kinetics of enzymatic hydrolysis of the white protein fraction at *E*/*S* ratio ranging from 0.5 to 10 wt% is presented in Fig. 2. The antioxidant activity of the obtained hydrolysates was also examined to select the optimal degree of hydrolysis. This property is particularly relevant for meat products, as oxidative changes that occur during storage or as a result of microbial contamination reduce the quality, safety and shelf life of products. In this context, the use of antioxidants as food additives is a common approach in the meat industry. However, consumers' increasing concern over synthetic additives like butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), due to their potential health risks, has driven the search for natural alternatives.

The hydrolysis curves indicate a high enzyme specificity toward the leaf protein, as a relatively high degree of hydrolysis (6.3-16.7%) was achieved, depending on the *E*/*S* ratio. The used enzyme, Alcalase, is known for its endopeptidase activity, meaning that it attacks peptide bonds within the interior of the molecule, randomly producing polypeptides of lower molecular mass. It preferentially cleaves peptide bonds between hydrophobic and aromatic amino acid residues (*Jovanović et al*., 2018). The results revealed that with an increasing amount of enzyme (*E*/*S* ratio), the initial reaction rate also increased proportionally up to 0.28 mAU, and continued to rise further to 0.33 mAU, but not in a linear manner. The release of soluble proteins increased with reaction time and correlated with the degree of hydrolysis, reaching a plateau after around 120 min. The curve of initial reaction rate obtained from the tangents to the hydrolysis curves, showed a maximum, indicating enzyme saturation, thus *E*/*S* ratios of 6.5% and 8% were optimal, with 6.5% selected as the most efficient and economical.

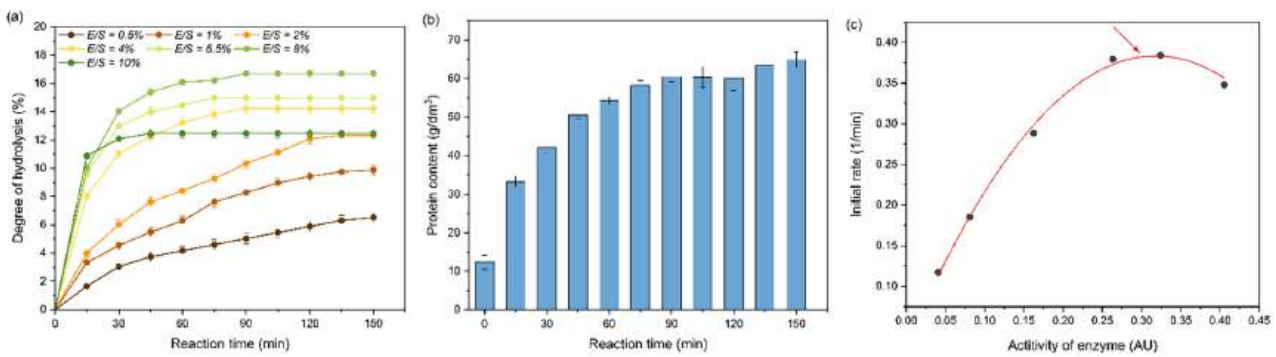

Figure 1. Time course of enzymatic hydrolysis at different *E/S* ratios: (a) degree of hydrolysis and (b) protein content as a function of reaction time, and (c) initial reaction rate (*V*o) as a function of enzyme activity (AU).

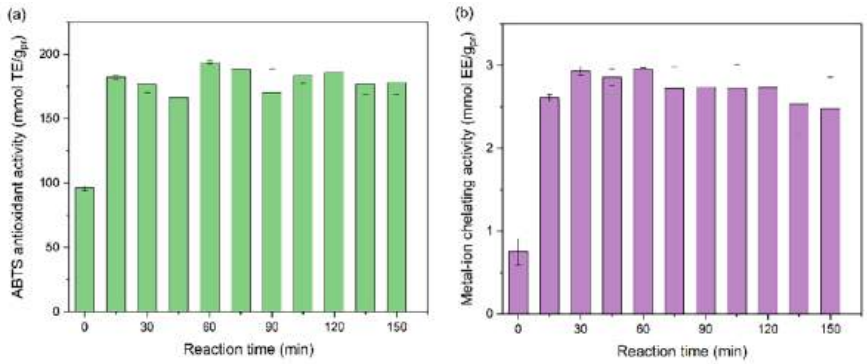

Figure 3. Antioxidant activity of leaf protein hydrolysates determined by (a) ABTS method, and (b) Methal-chelating ion method

These findings agree well with the antioxidant activity results obtained by both methods (Fig. 3). All hydrolysates obtained at different degrees of hydrolysis showed higher ABTS and metal ion chelating activity compared to the initial leaf protein. The antioxidant activity was closely related to the degree and extent of hydrolysis, as prolonged hydrolysis generated shorter peptide fragments capable of electron transfer (*Cotabarren* et al., 2019). The ABTS activity increased significantly after only 15 min of hydrolysis and remained stable up to 150 min. The hydrolysates obtained after 75 min showed the highest chelating activity compared to the native protein. However, no significant differences among the hydrolysates were observed in their metal ion chelating values during the first 120 min of hydrolysis, whereas the values decreased significantly thereafter. Based on these results, the hydrolysate obtained after 75 min and *E*/*S* = 6.5% was selected for further analysis of functional properties.

3.2. Functional properties of leaf protein hydrolysates

Compounds with strong antioxidant activity can extend the shelf life of meat products, enhance food safety and enrich them with bioactive ingredients. To be applied in meat formulations, hydrolysates must exhibit favourable solubility, emulsifying and related functional properties. Solubility is the key parameter influencing all other functionalities, including emulsification. The solubility results of the white protein fraction before and after enzymatic hydrolysis are presented in Fig. 4, while emulsifying and interfacial properties are summarised in Table 1.

It appeared that solubility increased significantly at pH 4-12 compared to the native protein. This enhancement may result from the reduction in molecular weight and the increase in polar groups caused by enzymatic hydrolysis, leading to the formation of smaller, more hydrophilic and soluble oligopeptide units. Since the isoelectric point of pumpkin leaf protein is around 4.6, the observed improvement in solubility at acidic pH values is particularly important, as many meat products have pH levels in this range. Furthermore, the obtained hydrolysate exhibited improved emulsifying properties, in terms of both *EAI* and *ESI*, compared to the intact leaf protein. Although these values are still lower than those achieved with soy protein concentrate (*Jovanovic et al*., 2018), the *EAI* values cannot be directly compared, as different protein concentrations were used in the experiments. The improved emulsifying properties of the hydrolysates correlated well with increased surface activity and reduced interfacial tension, indicating that smaller peptides were able to diffuse rapidly toward the interface. Overall, the enzymatic hydrolysis enhanced both solubility and interfacial activity, confirming its potential for application in meat emulsion system.

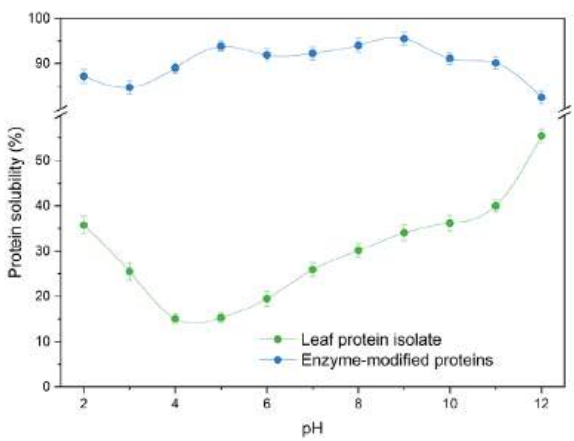

Figure 4. Protein solubility as a function of pH for leaf protein isolate and its enzymatic hydrolysate

Table 1. Emulsifying capacity and interfacial properties of leaf protein isolate and its enzymatic hydrolysate at two protein concentrations

Samples	*c*, mg/ml	*EAI*, m^2/g	*ESI*, min	Surface tension, mN/m	Interfacial tension, mN/m
Leaf protein-white fraction	1	12.95 ± 0.44	24.90 ± 3.45	23.7 ± 0.11	11.6 ± 0.02
	5	15.22 ± 0.52	26.40 ± 4.25	1.6 ± 0.33	5.8 ± 0.07
Enzymatically modified leaf protein	1	60.98 ± 0.69	28.44 ± 2.52	29.0 ± 0.09	10.7 ± 0.04
	5	64.85 ± 1.87	36.30 ± 3.04	29.0 ± 0.08	10.3 ± 0.13

3. Conclusion

Green biomass, such as waste pumpkin leaves generated after oilseed processing, has enormous potential as an alternative protein source. Enzymatic hydrolysis of the isolated white protein improved its solubility, interfacial functionality and antioxidant activity. The hydrolysate produced under optimal conditions (*E*/*S* ratio = 6.5%; DH ~ 15%; time 75 min) demonstrated suitability as a natural emulsifier and antioxidant, supporting its use as a value-added ingredient in functional meat products within a sustainable biorefinery concept.

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