horticulturae

MDPI

Article

Enzyme-Assisted Extraction of Proteins from Cauliflower and Broccoli Waste Leaves

Tea Sedlar [1,*], Igor Pasković [2,*], Danka Dragojlović [1], Senka Popović [3], Strahinja Vidosavljević [1], Smiljana Goreta Ban [2] and Ljiljana Popović [3]

[1] Institute of Food Technology, University of Novi Sad, 21000 Novi Sad, Serbia; danka.dragojlovic@fins.uns.ac.rs (D.D.); strahinja.vidosavljevic@fins.uns.ac.rs (S.V.)
[2] Institute of Agriculture and Tourism, 52440 Poreč, Croatia; smilja@iptpo.hr
[3] Faculty of Technology Novi Sad, University of Novi Sad, 21000 Novi Sad, Serbia; madjarev@uns.ac.rs (S.P.); ljiljana04@tf.uns.ac.rs (L.P.)
* Correspondence: tea.sedlar@fins.uns.ac.rs (T.S.); paskovic@iptpo.hr (I.P.); Tel.: +381-64-90-29-232 (T.S.)

Abstract: This research presents a novel approach to protein extraction from cauliflower (CL) and broccoli (BL) waste leaves by using enzymatic pretreatment, demonstrating its effectiveness on protein yield. Enzymatic pretreatment (pH 4.5, 10 h, t = 35 °C), was performed using commercial enzyme preparations, Viscozyme® L and Vinozyme®, in three different enzyme-to-substrate ratios E/S (0.2%, 2.5%, and 4.8%) of each enzyme. Leaf proteins (LPs) were obtained with alkaline extraction at pH 10–11 and their isoelectric precipitation at pH 4, following the control sample (extraction without enzymes). Protein yield (%), which was used as a parameter to monitor enzymatic efficiency, demonstrated a direct correlation with the enzyme-to-substrate (E/S) ratio. The highest protein yields were obtained at an enzyme concentration of 4.8% for both cellulolytic and pectolytic enzyme preparations, yielding 14.90 ± 0.12% for CL and 29.88 ± 0.86% for BL. The obtained proteins were characterized by FTIR spectroscopy and SDS-PAGE electrophoresis, and these methods confirmed the enzymatic efficiency of protein isolation. Isolated LPs showed high protein content for CLP 4.8% (77.27 ± 0.14%) and BPL 4.8% (84.66 ± 0.51%), and an increase in total amino acids, while the content of essential amino acids was over 40%. Protein solubility was assessed, revealing significant improvements ($p < 0.05$) in LPs derived from CL and BL at the highest E/S ratio of 4.8%, compared to the control sample C0%. Specifically, the solubility of CLP reached 29.4 mg/mL at pH 11, while BLP achieved 36.4 mg/mL at pH 10. As a result, these leaf proteins not only meet nutritional demands but also open innovative avenues of research in food science and biotechnology.

Keywords: enzyme-assisted extraction; vegetable by-products; leaf proteins; protein characterization; amino acid composition

Academic Editors: Gianluca Caruso, Otilia Cristina Murariu, Florin Lipsa and Somesh Sharma

Received: 18 December 2024
Revised: 23 January 2025
Accepted: 28 January 2025
Published: 1 February 2025

Citation: Sedlar, T.; Pasković, I.; Dragojlović, D.; Popović, S.; Vidosavljević, S.; Goreta Ban, S.; Popović, L. Enzyme-Assisted Extraction of Proteins from Cauliflower and Broccoli Waste Leaves. *Horticulturae* **2025**, *11*, 148. https://doi.org/10.3390/horticulturae11020148

1. Introduction

With the rising demand for vegetables for human consumption over the past two decades, the rapid increase in agricultural waste and by-products has become an urgent issue [1]. The majority of these by-products generated during harvest (e.g., leaves and stems) pose a significant risk to the environment due to improper disposal. As they traditionally have been used as animal feed, recently, an additional effort has been focused on developing sustainable and renewable resource technologies to repurpose these materials.

The challenges related to the reduction of environmental pollution and the costs of storage of agroindustry waste could be solved, bearing in mind that such materials represent a

cheap source suitable for producing value-added nutritional products [2]. Furthermore, this is supported by the fact that they are considered to be a good source of bioactive components such as proteins (peptides) [3], phenolic compounds [4], glucosinolates, vitamins, minerals, and dietary fiber [5]. Over the past decade, the demand for alternative plant protein sources has risen significantly due to multiple challenges in human nutrition. Additionally, these materials are increasingly being utilized for various types of protein-based hydrolysates, which are in high demand for sustainable agriculture and for developing novel fertiliser or plant protection strategies aimed at reducing pesticide or mineral fertiliser use [6,7]. Therefore, growing attention has been directed to the recycling of protein from the by-products of the agroindustry. Broccoli and cauliflower are commonly used *Brassica* vegetables worldwide, and consumers are more focused on their edible parts (flower head). In contrast, most non-edible parts, which make up a larger quantity of plants (about 70%), such as leaves, stems, and florets, remain spread on the ground.

A previous study [8] showed that the leaf by-products of four crops (cauliflower, broccoli, cabbage, and beetroot) could be an excellent potential sources of leaf proteins. Besides their suitable functional properties, they were also digestible, and peptides released in the digestive system had antioxidant activity. Various extraction methods (solvent extraction, ultrasound-assisted extraction, acid/alkaline extraction, etc.) and isolation of proteins from leaf sources have been developed. However, due to the nature of the plant source, this protein production still presents a significant challenge. Commonly used extraction procedures for obtaining proteins from leaves have certain limitations due to low protein yield, nutritional quality, and the need for environmental sustainability [9].

Enzyme-assisted extraction emerges as a promising alternative method for obtaining high-quality protein concentrate with superior yield and nutritional and functional properties. More importantly, it is a green technology, offering mild extraction conditions and a lower environmental impact [10].

The plant cell wall is a complex structure, which makes it challenging to extract proteins [11]. To overcome this issue, different carbohydrases, proteases, and pectinases can be used to release proteins from plant cells and enhance their extraction from plant substrates [12]. Utilizing carbohydrases not only facilitates protein extraction but also boosts the protein yield. This approach, which minimizes the need for harsh chemical treatments, aligns with ecological principles, ensuring the integrity of the extracted proteins. Several studies showed the effectiveness of cellulase use [13,14]. Viscozyme® L is a commercially available enzyme complex that includes arabanase, cellulase, hemicellulase, and xylanase, and has found extensive application as a cell wall-degrading complex in various industries. A study by Zhang et al., 2016 [15] demonstrated the effectiveness of pretreatment with Viscozyme®L on protein extraction from green tea residue. Similarly, Scarabattolu et al., 2023 [16] reported its capability for enrichment of the soybean meal extraction process by more than 30%. Furthermore, enzymatic extraction using the same enzyme complex for *Moringa oleifera* defatted leaves yielded a protein concentrate that not only met the balanced amino acid composition according to FAO protein quality demands [17] but was also highly digestible, ensuring the health benefits of the protein concentrate. The improvement in protein extraction using a cellulase enzyme Celluclast 1.5 L from olive leaves, optimizing different extraction parameters, has been investigated [18]. Also, different types of pectinases are used to break down the plant cell wall to achieve better efficiency in protein extraction. The investigation of Ayça Akyüz et al. [19] reported enzyme-assisted extraction from sugar beet leaf using Pectinex Ultra SP-L (an enzyme mixture containing polygalacturonase, pectinesterase and pectin trans-eliminase, hemicellulase, and cellulose), in which protein concentrates were obtained that could have promising applications in the food industry, opening up new possibilities for protein-rich food products.

The objective of this study was to adjust the enzyme-assisted extraction method, using an enzymatic pretreatment process followed by alkaline extraction, for the effective extraction of proteins from cauliflower and broccoli waste leaves. To achieve this goal, a thorough research process was followed. Cellulolytic and pectolytic complexes (Viscozyme® L and Vinozyme® Process Novozymes) were used at three different concentrations to obtain high nutritional protein concentrates. The efficiency of the enzymatic pretreatment was monitored by measuring protein yield (%), and the total phenolic content was used to monitor protein purity. Obtained proteins were characterized by FTIR spectroscopy and SDS-PAGE electrophoresis, and protein solubility was investigated in order to determine their potential utilization in the food industry. Also, amino acid analysis was carried out to evaluate the nutritional quality of the leaf proteins obtained.

2. Materials and Methods

2.1. Materials and Chemicals

Waste material (leaves) from cauliflower and broccoli were collected from three different locations—two locations originate from the western and one location from the northern part of Serbia. From each location, representative samples were taken from a large mass of waste, which were previously completely mixed to ensure homogeneity. After collection, the samples were carefully transported to the laboratory and immediately frozen at a temperature of −18 °C, where they were stored until further use, ensuring the preservation of their original quality.

Reagents and chemicals used in the experimental work were of analytical grade or better. Folin-Ciocalteau reagent, Dalton Mark VII, Coomassie Brilliant Blue G-250, and Amino Acid Standard Solution were from Sigma Chemical Company (Sigma Aldrich, St. Louis, MO, USA) and 6 M HCl from Merck (Merck, Darmstadt, Germany). Commercially available liquid enzyme preparations were used for the tests: Viscozyme® L, a multienzyme complex containing arbanase, β-glucanase, cellulase, hemicellulose, and xylanase, obtained from *Aspergillus aculeatus* (declared activity of 100 FBG/g) obtained from Sigma Chemical Company (Sigma Aldrich, St. Louis, MO, USA), and pectinase Vinozyme® Process Novozymes, Bagsværd, Denmark (declared activity 7.8 U/mL), a complex of pectinases including endo-polygalacturonase and exo-polygalacturonase.

2.2. Chemical Composition

The AOAC (1990) [20] methods were used to determine the moisture (925.10), protein (920.87) content (calculated with a nitrogen conversion factor of 6.25), fat (920.85) content, and ash (923.03) content of sample leaves and leaf proteins. Carbohydrate content was calculated by subtracting the total moisture, protein, fat, and ash content from 100% of the whole composition.

2.3. Enzyme-Assisted Extraction

The procedure to obtain leaf proteins was divided into five phases: Phase 1—application of the enzyme pretreatment of leaves, Phase 2—alkaline extraction of proteins at pH 10–11, Phase 3—precipitation of proteins at pH 4, Phase 4—decanting the supernatant and redissolving the precipitate at pH 10–11 and then re-precipitation of proteins at pH 4, Phase 5—decanting the supernatant and again dissolution of the precipitate at pH 10–11 and then re-precipitation at pH 4.

The enzyme-assisted extraction procedure was carried out with enzymatic pretreatment prior to the alkaline extraction of proteins. The enzymatic pretreatment was performed with cellulolytic and pectolytic complexes (Viscozyme® L and Vinozyme®) used at three different enzyme-to-substrate ratios (E/S) (0.2%, 2.5%, and 4.8%), selected based

on a literature report [18] to provide an optimal range for evaluation. After the enzymatic pretreatment, alkaline extraction was performed according to the previous report by Sedlar et al. [8].

Chopped fresh leaves (100 g) were squeezed in distilled water in a liquid-to-solid ratio of 7 mL/1 g. The pH of the homogenized mixture was adjusted to 4.5 with 1 M HCl at a temperature of 35 °C; mixing was achieved with a propeller mixer, thus satisfying the optimal conditions for enzymatic pretreatment. The test sample (a mixture of leaves and water with the addition of a certain amount of enzyme) and the control sample (without added enzyme) were left, under optimal conditions, to mix for 10 h. After being stirred for 10 h, alkaline extraction of soluble proteins was performed at pH 10 for broccoli leaves and pH 11 for cauliflower leaves for 30 min, based on preliminary optimization to achieve the most efficient protein extraction. The pH was adjusted using 1 M NaOH. The mixture was filtered, and the pH of the collected supernatant was adjusted to pH 4 using 1 M HCl (isoelectric precipitation). Following centrifugation (Sorvall® RC—5B Refrigerated Superspeed Centrifuge, Wilmington, Delaware, USA) at 10,000 rpm and 4 °C for 20 min, the precipitate was dried and ground.

The efficiency of the enzymatic pretreatment was monitored by measuring protein extraction yield (%), and the amount of extracted leaf protein was determined according to Bradford, M. (1976) [21]. The protein extraction yield (%) was calculated using the following, Equation (1):

$$\text{Protein extraction yield}(\%) = \frac{\text{exctracted leaf proteins } (\text{mg/g})}{\text{total protein in leaf } (\text{mg/g})} \times 100 \quad (1)$$

2.4. FTIR Spectroscopy

FTIR analysis (ATR-FTIR spectra) was performed at room temperature on a Nicolet iS10 Fourier transform infrared spectrometer. The protein powder was pressed into a 1–2 mm slice. All spectra were taken in the spectral range of 4000–500 cm^{-1} with 4.0 cm^{-1} resolution. Omnic 8.1 software (Thermo Fisher Scientific, Waltham, MA, USA) was used to operate the FTIR spectrometer and collect all the data.

2.5. SDS-PAGE Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of the anionic detergent sodium dodecyl sulphate (SDS) has proven to be a valuable method for protein separation and for the determination of their molecular masses. Protein subunits from all four protein concentrate samples were separated by the method described by Laemmli [22]. The gel system consisted of two gels: a 4% (w/v) acrylamide stacking gel (gel for samples) and a 10% (w/v) acrylamide separation gel. Samples (1 mg/mL) were prepared by dissolving in Tris/Gly buffer (pH 6.8), which contained 20 g/L SDS and 50 g/L 2-mercaptoethanol. After the preparation of gels and samples, the apparatus for electrophoresis (Multi Drive XL, Pharmacia, Uppsala, Sweden) was put into operation (20 mA for stacking gel and 40 mA for separation gel) at 25 °C until the dyed tracker reached the bottom of each gel. When electrophoresis was finished, the gels were stained using a silver staining procedure [23].

2.6. Determination of Amino Acid Composition

The amino acid composition of the samples was determined by ion-exchange chromatography. Sample preparation first involved acid hydrolysis in 6 M HCl (Merck, Darmstadt, Germany) at 110 °C for 24 h. After hydrolysis, the samples were cooled to room temperature and dissolved in 25 mL of loading buffer (pH 2.2) (Biochrom, Cambridge, UK). The samples were filtered using a 0.22 µm PTFE filter (Plano, TX, USA) and transferred to vials (Agilent Technologies, Santa Clara, CA, USA). The amino acid composition of leaf

proteins, including the control sample C0% (obtained without enzymatic pretreatment) and samples treated with 4.8% E/S enzymatic preparations, was analyzed by ion-exchange chromatography using a Biochrom 30+ Amino Analyzer (Biochrom, Cambridge, UK), following the method outlined by Spackman et al. (1958) [24]. Detection of amino acids is performed photometrically with a UV detector at 570 nm (all amino acids) and 440 nm (proline). The detection of amino acids was performed by comparing the retention times of the amino acids in standards and samples. Quantification was performed based on the area of amino acid peaks compared with the calibration curves of the standard used (Amino Acid Standard Solution, Sigma-Aldrich, St. Louis, MO, USA). All results are shown as g amino acids per 100 g leaf protein. The essential amino acid tryptophan was not determined, as it is destroyed during acid hydrolysis in 6 M HCl.

2.7. Determination of Protein Solubility

Protein solubility was determined according to the method defined by Popović et al. [24]. Solubility was observed at different pH values (pH 2–11). Suspensions of leaf protein samples obtained from enzyme-assisted extraction with a 4.8% E/S ratio, as well as control samples, were prepared in Eppendorf tubes by adding 1 mL of buffer solution to 10 mg of weighted powder. Subsequently, prepared samples were mixed in a Thermo ShakerTS-100 °C (BioSan, Riga, Latvia) for 1 h, at 900 rpm and 25 °C. The solutions were centrifuged at 14,500 rpm for 10 min (Eppendorf Mini-spin plus, Eppendorf AG., Hamburg, Germany). The supernatant was decanted, and soluble proteins from the supernatant were determined according to the Bradford method [21].

Protein solubility was determined according to the method defined by Popović et al. [25]. Solubility was observed at different pH values (pH 2–11). Suspensions of leaf protein samples obtained with enzyme-assisted extraction with 4.8% E/S ratio, as well as control samples, were prepared in Eppendorf tubes by adding 1 mL of buffer solution in 10 mg of weighted powder. The buffers used included: HCl-KCl buffer (0.1 mol/L) for pH 2, citrate phosphate buffer (0.1 mol/L) for pH 3 and 4, phosphate buffer (0.1 mol/L) for pH 5–8, and glycine buffer (0.1 mol/L) for pH 9–11. Subsequently, prepared samples were mixed in Thermo ShakerTS-100 °C (BioSan, Riga, Latvia) for 1 h, with 900 rpm, at 25 °C. The solutions were centrifuged at 14,500 rpm for 10 min (Eppendorf Mini-spin plus, Eppendorf AG., Hamburg, Germany). The supernatant was decanted, and soluble proteins from the supernatant were determined according to the Bradford method [21].

2.8. Determination of Total Phenolic Content

Total phenolic content (TPC) in aliquots after each phase of the experiment was determined using Folin-Coicalteu's reagent and the spectrophotometric method described by Singleton et al. (1999) [26]. The content was determined to monitor the protein purity, as phenolic compounds are known to interfere with protein extraction and analysis by forming complexes with proteins, which can affect their solubility and functionality [27]. The samples collected in each phase of the experiment (0.1 mL) were diluted with distilled water (7.9 mL), and then Folin Coicalteu's reagent (0.5 mL) and 20% sodium carbonate solution (1.5 mL) were added, while the control sample contained 8 mL of water. The samples prepared this way were left in the dark for 1 h, after which absorbance was measured at 750 nm (T80 UV–Vis Spectrophotometer; PG Instruments, Lutterworth, UK) (Singleton, 1999). The results are expressed as total phenolic content (mg/g of the sample).

2.9. Statistical Analysis

The means of replicates for results were submitted to a one-way analysis of variance (ANOVA). Significant differences between the means of the treatments were determined by Fisher's least significant differences test ($p < 0.05$) using STATISTICA 13.1 software (TIBCO

Software Inc., Hillview Avenue, Palo Alto, CA, USA). All data are the results from three repetitions, expressed as mean ± standard deviation.

3. Results and Discussion

3.1. Chemical Composition of Cauliflower and Broccoli Waste Leaves

The chemical composition of CL and BL leaves are summarized in Table 1.

Table 1. Chemical composition of cauliflower and broccoli leaves.

Sample	*Chemical Composition*	
	CL	*BL*
Moisture (%)	85.68 ± 0.30 [a]	85.43 ± 0.06 [a]
Total dry matter (%)	14.26 ± 0.34 [a]	14.54 ± 0.67 [a]
* Ash (%)	15.82 ± 0.06 [b]	16.95 ± 0.29 [a]
* Protein content (%)	31.00 ± 1.32 [a]	30.67 ± 0.57 [a]
* Lipids (%)	1.73 ± 0.07 [a]	0.63 ± 0.03 [b]
* Total sugar (%)	51.43 ± 0.68 [a]	51.75 ± 0.63 [a]

* g/100 g dry matter; CL—cauliflower leaves, BL—broccoli leaves; data are expressed as mean ± standard deviation (n = 3). Values in the rows followed by different lowercase letters are significantly different ($p < 0.05$).

Results showed that the protein content in CL and BL leaves was approximately 31%, with a moisture percentage of about 85%, consistent with literature findings [3]. This protein content is higher than those reported for Jackfruit (24.06%) [28], alfalfa (25.75%) [29], and sugar beet leaves (24.02%) [19] but comparable to the values reported for *Moringa olefira* leaves (28.7%) [30] and (31.4%) [31]. Notably, the lipid content differed significantly between the two samples, with CL exhibiting higher lipid levels (1.73 ± 0.07%) compared to BL (0.63 ± 0.03%). However, total sugar content was similar for both samples, with CL and BL containing approximately 51.43 ± 0.68% and 51.75 ± 0.63%, respectively. These differences highlight the individual compositional profiles of cauliflower and broccoli leaves. However, it is essential to note the potential variability in the chemical composition and particularly the protein content across studies, which should be considered in future investigations. This variability could be due to different geographical regions, cultivation climates, harvest time, and the difference between the cultivar species and characteristics of agricultural cultivation.

3.2. Influence of Enzyme Activity on Protein Yield and Protein Content

Previous investigation showed that protein extraction yields for broccoli and cauliflower leaves were relatively low, which makes them less suitable for the industrial-scale process [8]. Therefore, to enhance protein release from plant cells, it was necessary to promote the extraction process by performing enzymatic pretreatment prior to alkaline extraction. The cell wall is a complex structure, making it difficult to extract proteins [10]. Cellulose, hemicellulose, pectin, and glycoproteins form a structural grid around the proteins, making their release more difficult, thus giving low protein yields [19]. Therefore, three different enzyme-to-substrate ratios (E/S) (0.2%, 2.5%, and 4.8%) of each Viscozyme® L and Vinozyme® enzyme preparation were used to enhance protein extraction. Simultaneously, extraction without enzymes was carried out as a control sample C0%. According to the results from Figure 1, the efficiency of enzyme pretreatment correlates with the amount of enzyme complex applied, achieving the highest protein yield for both plant sources, with an E/S ratio of 4.8%. Initially, the protein yields were at a low level, where after the variation of enzyme concentrations, efficiency increased by 10% with enzymatic pretreatment for both sources. The improvement in protein extraction yield results from the cellulolytic and pectolytic activity of the enzymes applied [32]. The cell wall disruption

makes the intracellular materials more accessible for extraction, and the greater efficiency was primarily reflected in the BL source. Similar protein extraction yield results for CL were also observed in *Moringa oliefira* leaves (14.2%) [30] after optimizing enzyme extraction parameters. Nevertheless, these results are lower than protein yields reported for sugar beet leaves [19], where efficiency increased by 43.27% with an enzyme-assisted extraction process. The difference between extraction yields may be attributed to the different structures of sugar beet leaves, as well as differences in the enzymatic extraction conditions, including enzyme formulation and processing conditions.

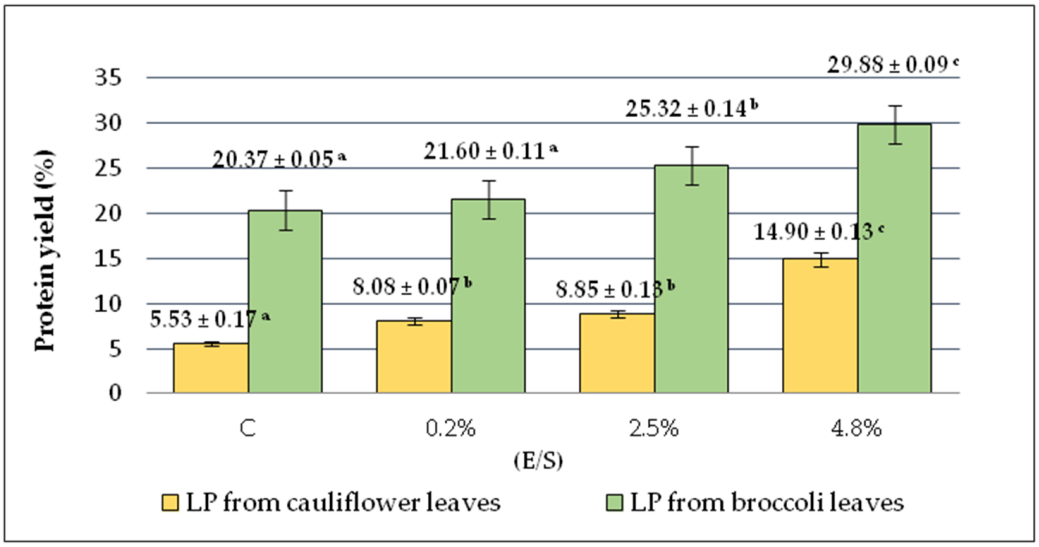


Figure 1. Protein extraction yield from CL and BL leaves for the control samples C0% and after enzyme-assisted extraction using three different E/S ratios (0.2%, 2.5%, and 4.8%). Data are expressed as mean ± standard deviation (n = 3). Values in the rows followed by different lowercase letters are significantly different ($p < 0.05$).

The protein content of leaf proteins that were isolated at different enzyme concentrations in the enzyme pretreatment is shown in Table 2. The results indicate that the higher enzyme concentrations led to a significant increase in the protein content of leaf proteins ($p < 0.05$). The decrease in protein content for CLP at an enzyme concentration of 0.2% compared to a control sample C0% may be due to insufficient enzyme concentration to break cauliflower leaves' cell wall effectively. In this case, the low concentration of the enzyme probably led to a partial reduction of protein release compared to the passive diffusion observed in the control. Thus, some proteins remain trapped or inadequately released, resulting in a lower measurable protein content. In contrast, the control sample C0% likely reflects a more stable system, where proteins are passively extracted without interference. At higher concentrations of enzymes (2.5% and 4.8%), enzymes were able to break down the matrix more efficiently, resulting in more significant protein recovery. Moreover, enzymatic pretreatment, as well as successive purification, resulted in a substantial improvement in the release of proteins from the cell matrix, marking a significant step forward in understanding enzymatic pretreatment for protein extraction.

Table 2. Protein content of isolated leaf proteins by enzyme-assisted extraction obtained with different E/S ratios.

	Enzyme Activity			
Protein Content (%)	**C0%**	**E/S 0.2%**	**E/S 2.5%**	**E/S 4.8%**
CLP (%)	53.81 ± 0.29 ^{c}	48.23 ± 0.55 ^{d}	66.36 ± 0.12 ^{b}	77.27 ± 0.14 ^{a}
BLP (%)	52.63 ± 0.41 ^{c}	52.03 ± 0.43 ^{c}	63.46 ± 1.07 ^{b}	84.66 ± 0.51 ^{a}

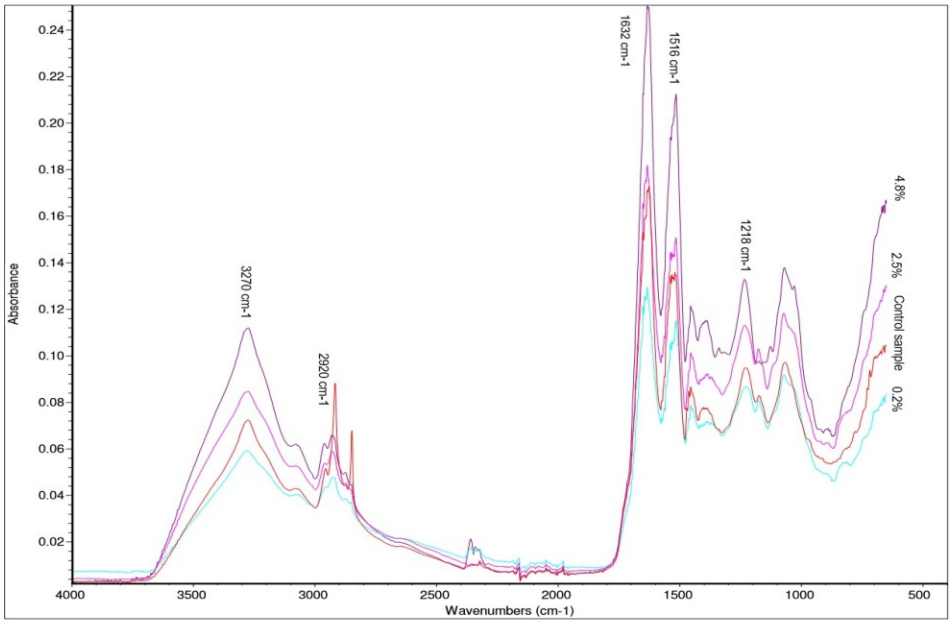
CLP—cauliflower leaf protein, BLP—broccoli leaf protein; C0%—extraction without enzymes, E/S 0.2%—enzyme-assisted extraction with 0.2% enzyme preparations, E/S 2.5%—enzyme-assisted extraction with 2.5% enzyme preparations, E/S 4.8%—enzyme-assisted extraction with 4.8% enzyme preparations; data are expressed as mean ± standard deviation (n = 3). Values in the rows followed by different lowercase letters are significantly different ($p < 0.05$).

3.3. FTIR

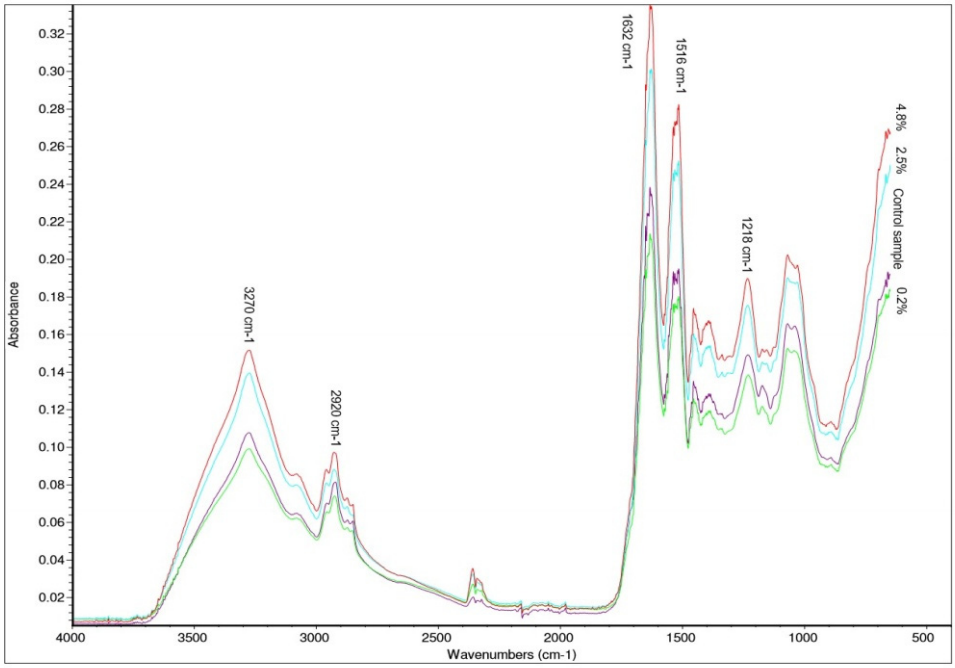
FTIR is an efficient technique used to assess the secondary structure of a protein, and spectra within the range 400–4000 cm^{-1} were used to identify the functional groups of BLP and CLP and monitor the influence of the concentration of the applied enzymatic complex. The FTIR spectra of all leaf proteins extracted using different enzyme concentrations and control samples are shown in Figure 2.

The characteristic infrared absorption band of these proteins (BLP—A and CLP—B) mainly includes amide A band (3270 cm^{-1}), amide B band (2920 cm^{-1}), amide I band (1632 cm^{-1}), amide II band (1516 cm^{-1}), and amide III band (1218 cm^{-1}). The amide A band is often related to the stretching vibrations of the amino groups and the hydrogen bond on the polypeptide, usually at a wavelength of 3000–3700 cm^{-1}, as well as peaks in the 2800–3000 cm^{-1} region resulting from adsorption of C–H bond stretching [33]. Amide bands I, II, and III influence the understanding of protein secondary structure. The amide I band results from the stretching vibration of the carbonyl group, whereas the amide II band results from the stretching vibration of the amino group [34]. As the concentration of enzymes increased, the characteristic peaks of the amide A, amide B, amide I, II, and III bands showed higher absorption intensity. The same bonds have more vigorous adsorption intensity at higher E/S ratios due to a more significant number of the same bonds in leaf proteins. BLP and CLP spectra display an exception with an E/S ratio of 0.2%, which has a lower peak absorption value than the control. However, spectra of both leaf proteins obtained with an E/S ratio of 4.8% gave the highest peak absorption value, consistent with their protein content.

Although BLP was higher in protein content compared to CLP, lower absorbance in FTIR spectra may be influenced by several factors, such as the presence of different components like fiber, polyphenols, and others. Also, protein interactions with polyphenols or carbohydrates could differ between broccoli and cauliflower, potentially affecting the FTIR peak intensity associated with amide bonds (typically observed around 1.650 cm^{-1} for amide I and 1.550 cm^{-1} for amide II regions), which can interfere with protein signal strength. Therefore, this technique confirms the presence of characteristic protein peaks, but variations in absorbance intensity between samples with similar protein contents are possible and may reflect differences in sample composition, protein–matrix interactions, or sample preparation techniques.



(**a**)



(**b**)

Figure 2. Fourier transform infrared spectra (FTIR) of (**a**) broccoli leaf protein (BLP), red spectrum—control sample, light blue spectrum—BLP spectrum obtained with 0.2% E/S ratio enzymatic pretreatment, pink spectrum—BLP spectrum obtained with 2.5% E/S ratio enzymatic pretreatment, purple spectrum—BLP spectrum obtained with 4.8% E/S ratio enzymatic pretreatment; and (**b**) cauliflower leaf protein (CLP), purple spectrum—control sample, green spectrum—CLP spectrum obtained with 0.2% E/S ratio enzymatic pretreatment, light blue spectrum—CLP spectrum obtained with 2.5% E/S ratio enzymatic pretreatment, red spectrum—CLP spectrum obtained with 4.8% E/S ratio enzymatic pretreatment.

3.4. SDS-Electrophoresis

The electrophoretic profile of leaf proteins obtained with enzyme-assisted extraction, along with control samples, are presented in Figure 3.

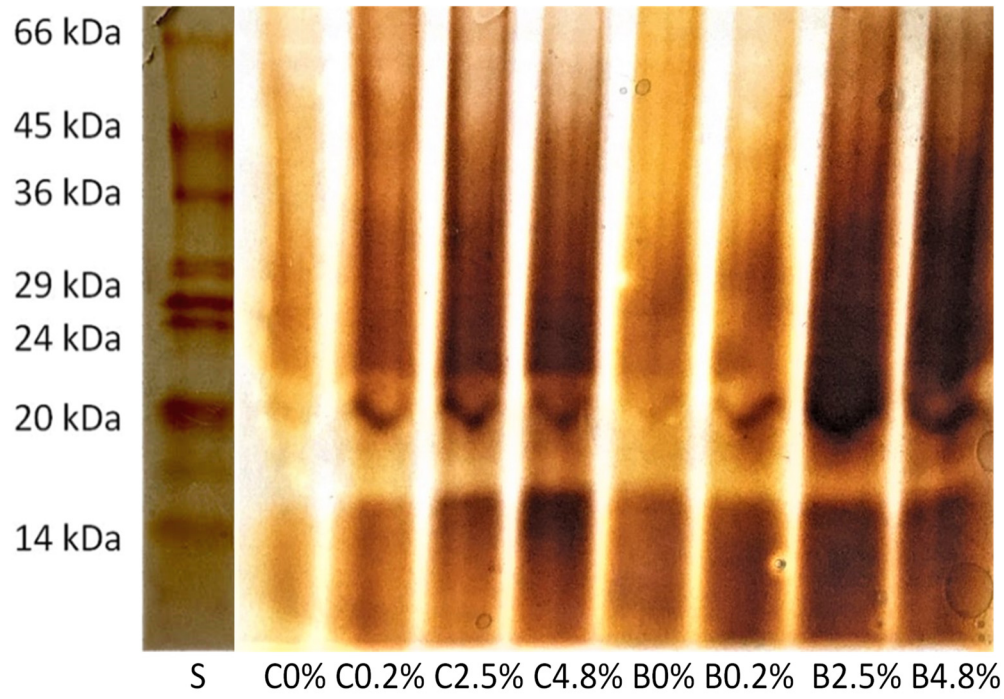


Figure 3. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for cauliflower and broccoli leaf protein (C), (B), obtained using three different E/S ratios (0.2%, 2.5%, and 4.8%), along with standard protein markers (lane S) and control samples (C0%, B0%).

SDS-PAGE was used to show the distribution of protein fractions and the influence of different enzyme concentrations on protein release. Notably, the 4.8% E/S ratio was found to be particularly effective, leading to a significant increase in protein yield. This result suggests that the 4.8% E/S ratio can lead to better cell wall destruction and, thus, help release a larger amount of protein. According to Figure 3, this enzymatic influence is most reflected in BLP, where the highest band intensity was recorded.

All samples are characterized by the presence of bands of both smaller and larger molecular masses, indicating a comprehensive protein analysis. The presence of a protein band at 20 kDa is common to all tested samples, most likely belonging to the globulin fraction of proteins, and 14 kDa, which corresponds to the albumin fraction of proteins. These results are consistent with the previous report by Sedlar et al. [8]. Other protein bands were also detected, corresponding to molecular weights of about 25 kDa, 30 kDa, 32 kDa, and 45 kDa.

3.5. Amino Acid Composition

The amino acid composition of LPs was use for the evaluation of protein quality. Table 3 provides detailed information of the amino acid composition of CLP and BLP, both obtained with an E/S ratio of 4.8%, along with the control samples.

The total amino acid content showed a significant increase ($p < 0.05$) in both LP samples in comparison to the control sample. Among all samples, the most abundant amino acid was aspartic acid, followed by leucine, one of the branched-chain essential amino acids necessary for hemoglobin formation [35]. However, the amount of essential amino acids as a percentage of total amino acid content was noted to be above 40% within all samples (especially CLP 4.8%—42.60% and BLP 4.8–43.62%). Also, the ratio of essential amino acids to non-essential amino acids exceeds 0.6 (0.65–0.78). Both observed parameters were above the standard values recommended by the FAO/WHO [36], which are typically within the range of 0.6–0.7 depending on dietary requirements for specific age groups and physiological states. A similar report was found for soluble leaf protein from cauliflower

by-products [3]. Although the isolated proteins are rich in essential amino acids such as threonine and leucine, the absence of phenylalanine has been identified as a limiting factor in nutritional terms. Thus, despite the differences in the amino acid content, it is worth mentioning that all types of studied leaf proteins could be regarded as high-quality protein sources, and despite some limitations in specific amino acid profiles, they significantly contribute to balanced food nutrition when complemented with other protein sources.

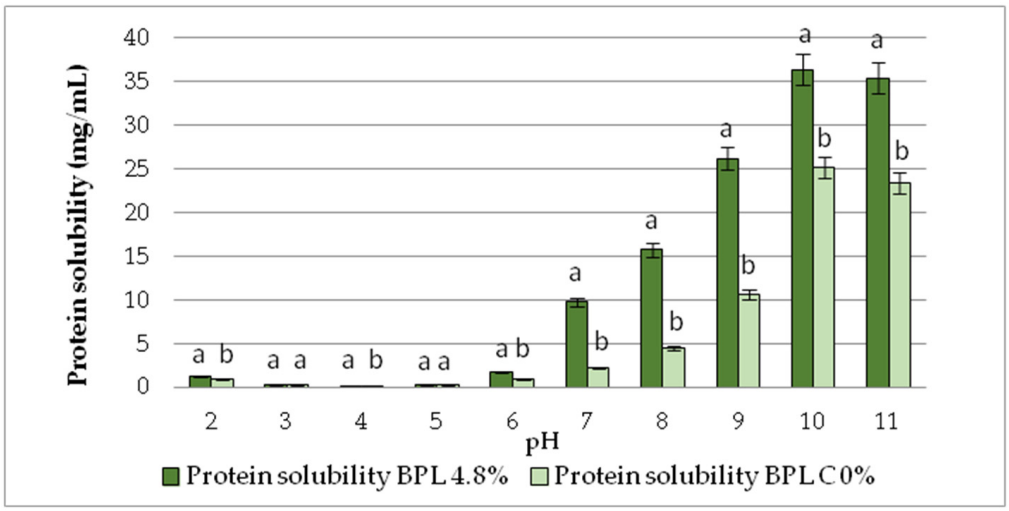
Table 3. Amino acid composition of leaf proteins of cauliflower and broccoli obtained with enzymatic pretreatment along with control samples (g/100 g LP).

Amino Acid Content (g/100 g LP)	C0% CLP	Samples CLP E/S 4.8%	C0% BLP	BLP E/S 4.8%
Threonine *	2.25 ± 0.05 ^{a}	3.60 ± 0.04 ^{b}	2.69 ± 0.04 ^{a}	3.55 ± 0.04 ^{b}
Valine *	3.02 ± 0.04 ^{a}	3.87 ± 0.02 ^{b}	3.57 ± 0.03 ^{a}	3.60 ± 0.03 ^{a}
Methionine *	1.13 ± 0.03 ^{a}	1.16 ± 0.03 ^{a}	1.05 ± 0.05 ^{a}	0.85 ± 0.05 ^{b}
Isoleucine *	2.60 ± 0.05 ^{a}	3.40 ± 0.05 ^{b}	3.14 ± 0.04 ^{a}	3.41 ± 0.04 ^{b}
Leucine *	3.66 ± 0.04 ^{a}	4.15 ± 0.02 ^{b}	4.12 ± 0.02 ^{a}	3.82 ± 0.02 ^{b}
Phenylalanine *	nd	nd	nd	nd
Histidine *	1.26 ± 0.02 ^{a}	1.18 ± 0.05 ^{a}	1.30 ± 0.03 ^{a}	1.26 ± 0.03 ^{a}
Lysine *	2.92 ± 0.03 ^{a}	3.11 ± 0.03 ^{b}	0.02 ± 0.05 ^{a}	2.94 ± 0.05 ^{b}
Aspartic acid	4.73 ± 0.05 ^{a}	6.83 ± 0.04 ^{b}	5.43 ± 0.04 ^{a}	6.19 ± 0.03 ^{b}
Serine	1.92 ± 0.05 ^{a}	3.12 ± 0.02 ^{b}	2.16 ± 0.03 ^{a}	2.88 ± 0.04 ^{b}
Glutamic acid	3.24 ± 0.03 ^{a}	3.52 ± 0.05 ^{a}	3.78 ± 0.05 ^{a}	3.56 ± 0.05 ^{a}
Proline	0.32 ± 0.04 ^{a}	0.07 ± 0.03 ^{b}	0.13 ± 0.02 ^{a}	0.02 ± 0.02 ^{b}
Glycine	2.77 ± 0.02 ^{a}	3.46 ± 0.04 ^{b}	3.07 ± 0.03 ^{a}	3.19 ± 0.04 ^{a}
Alanine	2.70 ± 0.05 ^{a}	3.06 ± 0.02 ^{b}	2.71 ± 0.05 ^{a}	2.76 ± 0.05 ^{a}
Cystine	0.11 ± 0.03 ^{a}	0.13 ± 0.05 ^{a}	0.12 ± 0.04 ^{a}	0.26 ± 0.03 ^{b}
Tyrosine	2.43 ± 0.04 ^{a}	4.26 ± 0.03 ^{b}	3.28 ± 0.02 ^{a}	3.29 ± 0.04 ^{a}
Arginine	3.13 ± 0.03 ^{a}	3.13 ± 0.04 ^{a}	3.68 ± 0.03 ^{a}	2.96 ± 0.03 ^{b}
TAA	38.19 ± 0.60 ^{a}	48.05 ± 0.56 ^{b}	40.25 ± 0.57 ^{a}	44.54 ± 0.59 ^{b}
ΣEAA *	16.84 ± 0.26 ^{a}	20.47 ± 0.24 ^{b}	15.88 ± 0.26 ^{a}	19.43 ± 0.26 ^{b}
ΣNEAA	21.35 ± 0.34 ^{a}	27.58 ± 0.32 ^{b}	24.37 ± 0.31 ^{a}	25.11 ± 0.33 ^{b}
ΣEAA/ΣNEAA	0.78	0.74	0.65	0.77

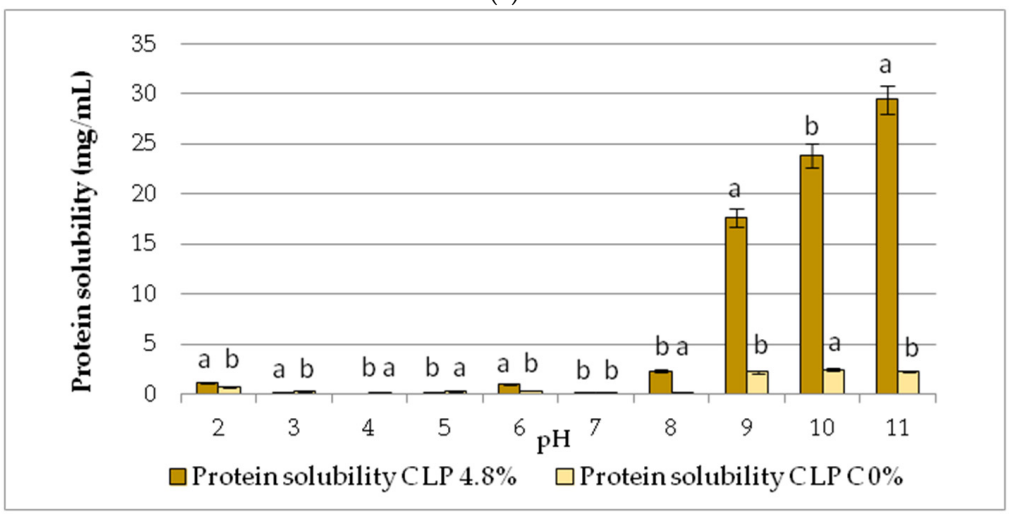
nd: not detected, * essential amino acids, ΣEAA *: total essential amino acids, ΣNEAA: total non-essential amino acids, ΣEAA/ΣNEAA: essential to non-essential amino acid ratios. C0% CLP—control sample: cauliflower leaf protein obtained without enzymatic pretreatment, CLP E/S 4.8%—cauliflower leaf protein obtained with enzymatic pretreatment with 4.8% E/S enzymatic preparations, C0% BLP—control sample: broccoli leaf protein obtained without enzymatic pretreatment, BLP E/S 4.8%—broccoli leaf protein obtained with enzymatic pretreatment with 4.8% E/S enzyme preparations; data are expressed as mean ± standard deviation (n = 3). Values in the rows followed by different lowercase letters are significantly different ($p < 0.05$).

3.6. Protein Solubility

Protein solubility was determined for leaf protein samples obtained with the 4.8% E/S ratio along with control samples. The leaf proteins obtained with an enzyme concentration of 4.8% exhibited the highest solubility. Their pH-dependent solubility profile (pH 2–pH 11) is shown in Figure 4.



(**a**)



(**b**)

Figure 4. Solubility profile of leaf proteins obtained by enzyme-assisted extraction: (**a**) BLP with E/S 4.8% and control sample BLP C0%—extraction without enzymes and (**b**) CLP with E/S 4.8% and control sample CLP C0%—extraction without enzymes. Data are expressed as mean ± standard deviation (n = 3). Values in the rows followed by different lowercase letters are significantly different ($p < 0.05$).

Generally, solubility is better in the alkaline than in the acidic region where the minimum solubility occurs between pH 2 and 6 (isoelectric point—pI). Following pI, the solubility of leaf proteins showed a linear increase in an alkaline environment (pH 8–pH 11), which is similar to the literature report for protein solubility of alfalfa leaf proteins [37]. Moreover, there was a significant improvement ($p < 0.05$) compared to the control sample (the solubility of CLP reached 29.4 mg/mL at pH 11, while BLP achieved 36.4 mg/mL at pH 10). This difference in solubility values may be attributed to the distinct protein compositions of CLP and BLP, as well as variations in their structural properties, cell wall structure, and amino acid composition, which influence their behavior in alkaline conditions. Higher concentrations of enzymes gave better results for leaf protein solubility, leading to improved functional properties in terms of emulsification, foam, or gelling [38]. Compared to the previous report [8], the solubility of the CLP and BLP was significantly higher in the alkaline medium, while there was no significant difference in the acidic medium. One of the reasons for better solubility is that enzymatic pretreatment does not disrupt the initial structure of the protein due to the mild conditions in which it acts [39].

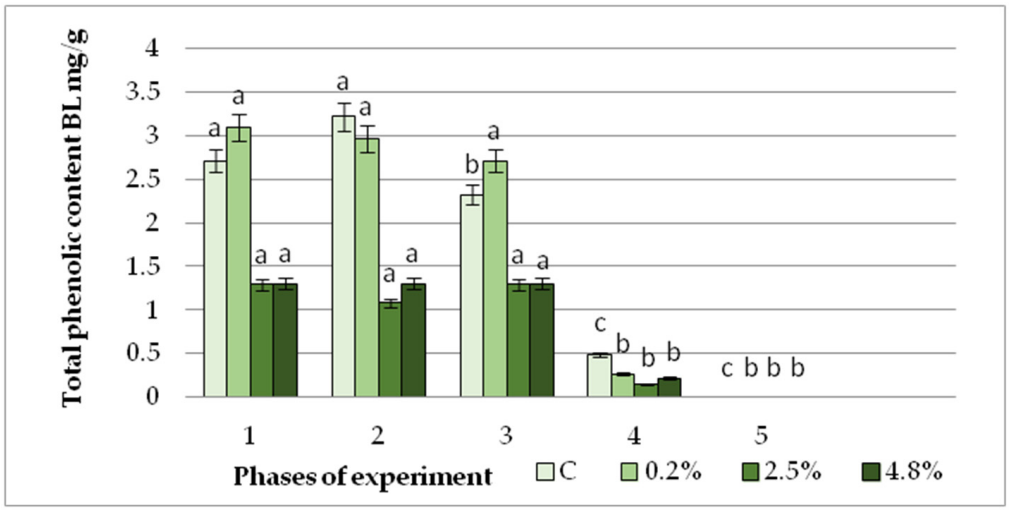
Moreover, the solubility is higher because of the higher protein purity. These findings also underscore the positive correlation between protein content, yield and solubility, providing a potent basis for further research.

3.7. Total Phenolic Content

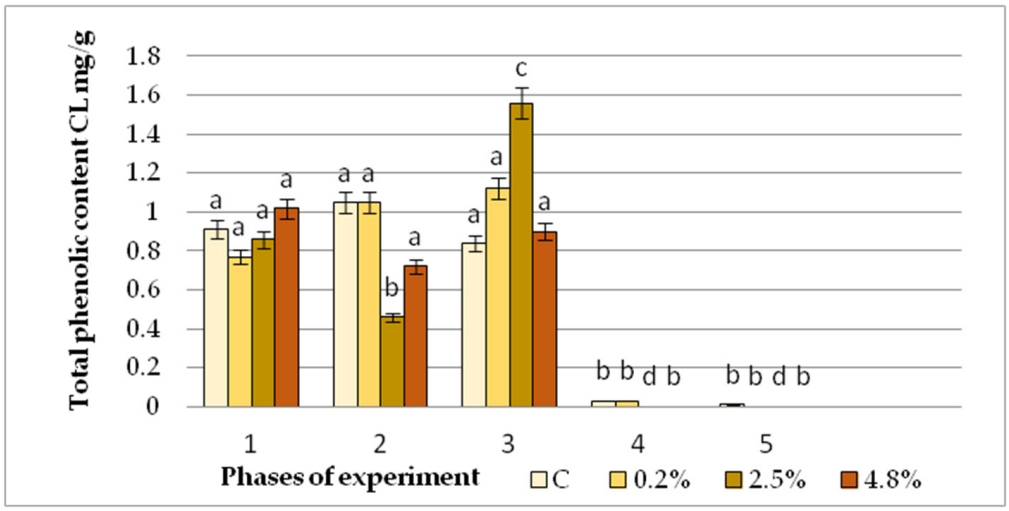
Enzyme-assisted extraction is a method for isolating phenolic compounds and proteins from plant-based materials. Enzymes effectively facilitate the release of these bioactive compounds by targeting structural components of the cell wall, such as pectin, cellulose, and hemicellulose. Operating under mild conditions and preserving proteins and phenolic compounds' structural and functional properties make this approach more valuable; the process also facilitates extraction and provides a sustainable method for the use of plant-based sources. Enzymatic treatments have demonstrated their effectiveness, making them an integral part of strategies to optimize the recovery of functional compounds from vegetal raw materials.

Protein functionality is mainly determined by the solubility and purity of the sample, as well as bound compounds such as polyphenolics. These are usually the cause of problems concerning dark color, unpleasant taste, and poor digestibility precisely because of the formation of polyphenol–protein complexes. The total phenolic content measured during each phase of the experiment is presented in Figure 5.

Determination of the total phenolic content was used to investigate the degree of protein purity. In the initial phase of the experiment (enzymatic pretreatment), a significant release ($p < 0.05$) of phenolic compounds was observed in cauliflower leaves at an enzyme concentration of 2.5% (1.56 ± 0.11), while for the broccoli leaves, a notable release was detected already with an enzyme concentration of 0.2% (3.09 ± 0.12 mg/g). This suggests that the specific enzyme concentration effectively breaks down phenolic structures in cauliflower and broccoli leaves during enzymatic pretreatment, liberating a substantial portion of these compounds. The higher release of phenolic compounds in broccoli leaves without enzyme and at 0.2% enzyme concentration could be attributed to their specific matrix composition, which likely contains more readily extractable phenolic compounds weakly bound within the cell matrix or associated with soluble fractions. In contrast, at higher enzyme concentrations, phenolic compounds may interact with proteins or polysaccharides, forming complexes that reduce their free phenolic content in the extract. Lower phenolic release within cauliflower leaf samples may indicate a stronger binding of phenolic to cellular structures or a different composition that resists enzymatic breakdown. In subsequent phases, particularly during alkaline extraction, a notable release of free phenolic compound was recorded. Alkaline conditions facilitate the breakdown of protein–phenolic and cell wall bonds, enabling the efficient extraction of bound phenolic compounds across both cauliflower and broccoli leaf samples. This approach, combining enzyme treatment and alkaline extraction, demonstrates the efficiency of using staged processes to optimize phenolic release, potentially enhancing both the purity and yield of proteins, which can be nutritive ingredients in food and biochemical applications. However, results indicate that in Phases 4 and 5 (purification phases), the total phenolic content decreases drastically, suggesting that the protein is, to a large extent, free of phenolic components.



(**a**)



(**b**)

Figure 5. Total phenolic content for samples of (**a**) broccoli leaves (BL) and (**b**) cauliflower leaves (CL) during each phase of the experiment obtained during enzyme-assisted extraction, with three different E/S ratios (0.2%, 2.5%, and 4.8%), along with control sample C, E/S ratio 0%. Phase 1—application of enzyme pretreatment to the leaves, Phase 2—alkaline extraction of proteins at pH 10–11, Phase 3—precipitation of proteins at pH 4, Phase 4—decanting the supernatant and redissolving the precipitate at pH 10–11 and then re-precipitation of proteins at pH 4, Phase 5—decanting the supernatant and again dissolution of the precipitate at pH 10-11 and then re-precipitation at pH 4. Data are expressed as mean ± standard deviation (n = 3). Values in the rows followed by different lowercase letters are significantly different ($p < 0.05$).

4. Conclusions

This study shows that enzymatic pretreatment using pectolytic and cellulolytic enzymes significantly increases protein extraction efficiency from cauliflower and broccoli waste leaves, transforming agricultural by-products into high-value nutritional resources. The maximum protein yield was achieved at the highest ratio of enzyme to substrate (E/S 4.8%), where the isolated proteins showed a high content of total and essential amino acids and improved solubility. Characterization of the obtained proteins confirms their functionality and nutritional value, indicating their potential for application in food industries. This research contributes to the sustainable use of plant by-products and opens up opportunities for the development of innovative products that meet modern nutritional and sustainability requirements. Applying such methods can contribute to reducing waste and enrich the

market with functional proteins, making them a key element in the transition to a circular economy in the food industry.

Author Contributions: Conceptualization, T.S. and L.P.; methodology and software, T.S., L.P., D.D. and S.V.; validation, L.P. and I.P.; formal analysis, T.S. and L.P.; investigation, T.S. and L.P.; resources, T.S.; data curation, T.S., D.D. and S.V.; writing—original draft preparation, T.S. and L.P.; writing—review and editing, T.S., L.P., I.P., D.D., S.P., S.V. and S.G.B.; visualization, L.P., I.P., S.G.B., S.V., D.D. and S.P.; supervision, L.P. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by the Ministry of Science, Technological Development, and Innovations; the Republic of Serbia (contracts nos. 451-03-66/2024-03/200222 and 451-03-66/2024-03/200134) and was supported in part by the Croatian Science Foundation (HRZZ) and Slovenian Research Agency (ARIS) under the Weave initiative project nos. HRZZ IP-2022-10-8305 and ARIS N4-0346 (PROGRESS).

Data Availability Statement: All data are contained within the article.

Acknowledgments: We especially thank Marija Polić Pasković and Ivan Dlačić, authors of the graphical abstract.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Madurwar, M.V.; Ralegaonkar, R.V.; Mandavgane, S.A. Application of agro-waste for sustainable construction materials: A review. *Constr. Build. Mater.* **2013**, *38*, 872–878. [CrossRef]
2. Caliceti, C.; Capriotti, A.L.; Calabria, D.; Bonvicini, F.; Zenezini Chiozzi, R.; Montone, C.M.; Piovesana, S.; Zangheri, M.; Mirasoli, M.; Simoni, P.; et al. Peptides from cauliflower by-products, obtained by an efficient, ecosustainable, and semi-industrial method, exert protective effects on endothelial function. *Oxid. Med. Cell. Longev.* **2019**, *2019*, 1046504. [CrossRef] [PubMed]
3. Xu, Y.; Li, Y.; Bao, T.; Zheng, X.; Chen, W.; Wang, J. A recyclable protein resource derived from cauliflower by-products: Potential biological activities of protein hydrolysates. *Food Chem.* **2017**, *221*, 114–122. [CrossRef]
4. Huynh, N.T.; Smagghe, G.; Gonzales, G.B.; Van Camp, J.; Raes, K. Enzyme-assisted extraction enhancing the phenolic release from cauliflower (*Brassica oleracea* L. var. *botrytis*) outer leaves. *J. Agric. Food Chem.* **2014**, *62*, 7468–7476. [CrossRef]
5. Shilpi, A.; Shilpi, A.; Shivhare, U.S.; Basu, S. Supercritical CO_2 Extraction of Compounds with Antioxidant Activity from Fruits and Vegetables Waste—A Review. *Fmfi* **2013**, *2*, 43–62.
6. Pasković, I.; Popović, L.; Pongrac, P.; Polić Pasković, M.; Kos, T.; Jovanov, P.; Franić, M. Protein Hydrolysates—Production, Effects on Plant Metabolism, and Use in Agriculture. *Horticulturae* **2024**, *10*, 1041. [CrossRef]
7. Mark, J.; Fantke, P.; Soheilifard, F.; Alcon, F.; Contreras, J.; Abrantes, N.; Campos, I.; Baldi, I.; Bureau, M.; Alaoui, A.; et al. Selected farm-level crop protection practices in Europe and Argentina: Opportunities for moving toward sustainable use of pesticides. *J. Clean. Prod.* **2024**, 143577. [CrossRef]
8. Sedlar, T.; Čakarević, J.; Tomić, J.; Popović, L.j. Vegetable By-Products as New Sources of Functional Proteins. *Plant Food Hum. Nutr.* **2020**, *76*, 31–36. [CrossRef]
9. Zhang, C.; Sanders, J.P.M.; Bruins, M.E. Critical Parameters in Cost-Effective Alkaline Extraction for High Protein Yield from Leaves. *Biomass Bioenergy* **2014**, *67*, 466–472. [CrossRef]
10. Nadar, S.S.; Rao, P.; Rathod, V.K. Enzyme-Assisted Extraction of Biomolecules as an Approach to Novel Extraction Technology: A Review. *Food Res. Int.* **2018**, *108*, 309–330. [CrossRef]
11. Alvarez, S.; Naldrett, M.J. Plant Structure and Specificity—Challenges and Sample Preparation Considerations for Proteomics. In *Modern Proteomics—Sample Preparation, Analysis and Practical Applications*; Springer Series; Springer: Cham, Switzerland, 2016. [CrossRef]
12. Li, S.; Wei, X.; He, Y.; Zhang, Z. Use of carbohydrase to enhance protein extraction efficiency and antioxidative properties of oat bran protein hydrolysates. *Food Res. Int.* **2012**, *46*, 69–75. [CrossRef]
13. Ansharullah; Hourigan, J.A.; Chesterman, C.F. Application of Carbohydrases in Extracting Protein from Rice Bran. *J. Sci. Food Agric.* **1997**, *74*, 141–146. [CrossRef]
14. Rosset, M.; Acquaro Junior, V.R.; Beléia, A.D.P. Protein Extraction from Defatted Soybean Flour with *Viscozyme* L. Pretreatment. *J. Food Process. Preserv.* **2014**, *38*, 784–790. [CrossRef]
15. Zhang, C.; van Krimpen, M.M.; Sanders, J.P.M.; Bruins, M.E. Improving Yield and Composition of Protein Concentrates from Green Tea Residue in an Agri-Food Supply Chain: Effect of Pre-Treatment. *Food Bioprod. Process.* **2016**, *100*, 92–101. [CrossRef]

16. Scarabattoli, L.; Sangiorgio, S.; Romagnuolo, F.; Gelati, L.; Cavuoto, D.; Rabuffetti, M.; Morelli, C.F.; Lupinelli, S.; Speranza, G. Use of Carbohydrases to Promote Protein Extraction from Rice Bran and Soybean Meal: A Comparative Study. *LWT—Food Sci. Technol.* **2023**, 115060. [CrossRef]
17. Benhammouche, T.; Melo, A.; Martins, Z.; Faria, M.A.; Pinho, S.C.M.; Ferreira, I.M.L.P.V.O.; Zaidi, F. Nutritional Quality of Protein Concentrates from *Moringa Oleifera* Leaves and In Vitro Digestibility. *Food Chem.* **2021**, *348*, 128858. [CrossRef]
18. Vergara-Barberán, M.; Lerma-García, M.J.; Herrero-Martínez, J.M.; Simó-Alfonso, E.F. Use of an Enzyme-Assisted Method to Improve Protein Extraction from Olive Leaves. *Food Chem.* **2015**, *169*, 28–33. [CrossRef]
19. Akyüz, A.; Ersus, S. Optimization of Enzyme-Assisted Extraction of Protein from the Sugar Beet (*Beta vulgaris* L.) Leaves for Alternative Plant Protein Concentrate Production. *Food Chem.* **2021**, *335*, 127673. [CrossRef]
20. AOAC International. *Official Methods of Analysis of AOAC International*, 15th ed.; Association of Official Analytical Chemists: Arlington, VA, USA, 1990.
21. Bradford, M. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248–254. [CrossRef]
22. Laemmli, U.K. Cleavage of Structural Proteins during the Assembly of Head of Bacteriophage T4. *Nature* **1970**, *227*, 680–685. [CrossRef]
23. Čakarević, J.; Vidović, S.; Vladić, J.; Gavarić, A.; Jokić, S.; Pavlović, N.; Blažić, M.; Popović, L. Production of bio-functional protein through revalorization of apricot kernel cake. *Foods* **2019**, *8*, 318. [CrossRef] [PubMed]
24. Spackman, D.H.; Stein, W.H.; Moore, S. Automatic Recording Apparatus for Use in the Chromatography of Amino Acids. *Anal. Chem.* **1958**, *30*, 1190–1206. [CrossRef]
25. Popović, L.; Peričin, D.; Vaštag, Ž.; Popović, S.; Krimer, V.; Torbica, A.M. Antioxidative and Functional Properties of Pumpkin Oil Cake Globulin Hydrolysates. *J. Am. Oil Chem. Soc.* **2013**, *90*, 1157–1165. [CrossRef]
26. Singleton, V.L.; Orthofer, R.; Lamuela-Raventós, R.M. Analysis of Total Phenols and Other Oxidation Substrates and Antioxidants by Means of Folin-Ciocalteu Reagent. *Methods Enzymol.* **1999**, *299*, 152–178.
27. Jaroslaw Czubinski, Krzysztof Dwiecki, A review of methods used for investigation of protein–phenolic compound interactions. *Int. J. Food Sci. Technol.* **2017**, *52*, 573–585. [CrossRef]
28. Calderon-Chiu, C.; Calderón-Santoyo, M.; Herman-Lara, E.; Ragazzo-Sanchez, J.A. Jackfruit (*Artocarpus heterophyllus* Lam) Leaf as a New Source to Obtain Protein Hydrolysates: Physicochemical Characterization, Techno-Functional Properties and Antioxidant Capacity. *Food Hydrocoll.* **2021**, *112*, 106319. [CrossRef]
29. Xie, Z.; Huang, J.; Xu, X.; Jin, Z. Antioxidant Activity of Peptides Isolated from Alfalfa Leaf Protein Hydrolysate. *Food Chem.* **2008**, *111*, 370–376. [CrossRef]
30. Teixeira, E.M.B.; Carvalho, M.R.B.; Neves, V.A.; Silva, M.A.; Arantes-Pereira, L. Chemical Characteristics and Fractionation of Proteins from Moringa oleifera Lam. *Leaves. Food Chem.* **2014**, *147*, 51–54. [CrossRef]
31. Nouman, W.; Anwar, F.; Gull, T.; Newton, A.; Rosa, E.; Domínguez-Perles, R. Profiling of Polyphenolics, Nutrients and Antioxidant Potential of Germplasm's Leaves from Seven Cultivars of Moringa oleifera Lam. *Ind. Crops Prod.* **2016**, *83*, 166–176. [CrossRef]
32. Momen, S.; Alavi, F.; Aider, M. Alkali-Mediated Treatments for Extraction and Functional Modification of Proteins: Critical and Application Review. *Trends Food Sci. Technol.* **2021**, *110*, 778–797. [CrossRef]
33. Secundo, F.; Guerrieri, N. ATR-FT/IR Study on the Interactions between Gliadins and Dextrin and Their Effects on Protein Secondary Structure. *J. Agric. Food Chem.* **2005**, *53*, 1757–1764. [CrossRef] [PubMed]
34. Cardamone, J.M. Investigating the Microstructure of Keratin Extracted from Wool: Peptide Sequence (MALDI-TOF/TOF) and Protein Conformation (FTIR). *Mol. Struct.* **2010**, *972*, 87–93. [CrossRef]
35. PubChem. *Asparagine*. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/asparagine (accessed on 17 December 2024).
36. World Health Organization. *Protein and Amino Acid Requirements in Human Nutrition 2007 Report of a Joint WHO/FAO/UNU Expert Consultation*; WHO Technical Report Series; WHO: Geneva, Switzerland, 2007.
37. Hadidi, M.; Baradaran Khaksar, F.; Pagan, J.; Ibarz, A. Application of Ultrasound-Ultrafiltration-Assisted Alkaline Isoelectric Precipitation (UUAAIP) Technique for Producing Alfalfa Protein Isolate for Human Consumption: Optimization, Comparison, Physicochemical, and Functional Properties. *Food Res. Int.* **2020**, *130*, 108907. [CrossRef] [PubMed]

38. Kumar, M.; Tomar, M.; Potkule, J.; Verma, R.; Punia, S.; Mahapatra, A.; Belwal, T.; Dahuja, A.; Joshi, S.; Berwal, M.K.; et al. Advances in the Plant Protein Extraction: Mechanism and Recommendations. *Food Hydrocoll.* **2021**, *115*, 106595. [CrossRef]
39. Nikbakht Nasrabadi, M.; Sedaghat Doost, A.; Mezzenga, R. Modification Approaches of Plant-Based Proteins to Improve Their Techno-Functionality and Use in Food Products. *Food Hydrocoll.* **2021**, *118*, 106789. [CrossRef]