Chemical Profile and Antioxidant Activity of Various Cysteine-Proteases' Impact on *Spirulina* Protein Hydrolysate

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Abstract: Spirulina *is a type of microalgae that contains many useful compounds having antioxidant properties. It has low biological activity and limited protein content when used in dry form. Proteins can be broken down through a hydrolysis reaction to increase their bioactivity, producing smaller peptides and free amino acids. This study aims to evaluate the effects of two cysteine-protease enzymes, bromelain and papain, on the hydrolysis of* Spirulina *protein. The research examined how these enzymes affect the degree of hydrolysis, protein content, molecular weight, and antioxidant activity of the resulting protein hydrolysate. A non-factorial, completely randomized design was used with three replicates per treatment. The results showed that the type of enzyme used significantly influenced all measured parameters. Bromelain was found to be more effective than papain.* Spirulina *protein hydrolyzed with bromelain had 32.15 ± 0.74% protein, 48.51 ± 0.94% hydrolysis, a density of 0.786 mg/mL, and 29.64 ± 0.82 ppm antioxidant activity. It also contained 18 types of amino acids, totaling 14.41 g/kg. The most efficient of physical extraction methods—particularly the combination of freeze-thaw and ultrasonication—for obtaining high-yield, high-quality protein from* Spirulina. *Further purification is needed to obtain the smallest peptide.*

Keywords: *amino acids; bromelain; enzyme; microalgae; papain*

■ INTRODUCTION

Spirulina, a blue-green algal species that belongs to the Cyanobacteria group, is generally recognized as safe (GRAS) and can be used as a source for nutraceutical goods and functional foods [1]. *Spirulina* has a variety of primary and secondary metabolite compounds. It can

contain up to 75.7% protein by weight (dry biomass) [2]. Vitamins, β-carotene, α-tocopherol, essential fatty acids (omega-3: EPA and DHA), essential amino acids, and several minerals are also present in *Spirulina* [3-4]. *Spirulina*'s biological properties include lowering blood pressure, cholesterol profiles, and blood sugar levels; and preventing fatty liver disease, anemia, and viral replication. The amount of protein in *Spirulina* directly affects its biological activity. As a result, it can be used as a raw material in both the food and non-food industries.

To improve the bioavailability of *Spirulina* protein, an extraction procedure might be used to acquire the protein. However, the biological value of *Spirulina* protein is lower than that of chicken egg protein, which is 68.00 and 94.70%, respectively. *Spirulina* cannot be digested by humans due to its 10% cellulose-containing cell shell [5]. According to Carrizzo et al. [6], the procedure of extracting the protein from *Spirulina* can improve the stability of bioactive peptides that are created during the hydrolysis process in the digestive system, resulting in more optimal usage. A protein hydrolysis procedure can be used to create protein that the body can digest more readily. Despite being high in nutrients, the digestion of *Spirulina* can vary. According to certain research, protein hydrolysis may increase digestibility by increasing the protein's absorption capacity [7].

Both chemical and enzymatic methods can be used to hydrolyze proteins. Compared to chemical hydrolysis, enzymatic protein hydrolysis is thought to be more beneficial. Certain amino acids may sustain structural damage as a result of the chemical hydrolysis process. Compared to chemical hydrolysis, enzymatic hydrolysis is preferable due to its better-regulated procedure and end products. Furthermore, because enzymes break down particular bonds in molecules more selectively than chemical catalysts, fewer undesirable by-products are produced [8]. Protease enzymes, either one type (single hydrolysis) or a combination of multiple types (double hydrolysis), are used to carry out enzymatic protein breakdown. Numerous factors, including pH, temperature, and enzyme concentration, affect the high activity of enzymes. Protease enzymes are chosen for the protein hydrolysis process based on their specifications, ideal pH, heat stability, the impact of activators and inhibitors, cost, and availability [9].

Protease enzymes can be obtained from plants, animals, and microorganisms. Protease enzymes derived from plants can be obtained from papaya (papain) and pineapple (bromelain)–including the cysteine-protease enzyme group. The bromelain enzyme used in the protein hydrolysis process has a fairly broad cutting specificity for the amino acid residues that make up the substrate, namely arginine, lysine, tyrosine, and phenylalanine, so that it can produce a high degree of hydrolysis [10]. The papain enzyme used in the protein hydrolysis process has the advantages of being more stable to temperature and pH, easily available, available in large quantities, and affordable [9].

Nevertheless, little is known about the effects of using cysteine-protease enzymes, particularly papain and bromelain, on the chemical profile and antioxidant activity of the hydrolyzed *Spirulina* protein. The objective of this study is to analyze the chemical composition and antioxidant capacity of the hydrolyzed *Spirulina* protein using a variety of cysteine-protease enzymes, specifically papain and bromelain.

■ EXPERIMENTAL SECTION

Materials

The materials used in this study included dry *Spirulina* biomass obtained from a cultivation center in Jepara, bromelain and papain enzymes (100,000 U/g, Pangbo brand), Bovine Serum Albumin, NaOH, HCl, ascorbic acid, and ethanol (Merck, Germany), ninhydrin reagent, methanol, DPPH (Smart Lab); Whatman No. 42 filter paper; and aluminum foil.

Instrumentation

The instruments used in this study included a UV-vis double-beam spectrophotometer (UV-1280, Shimadzu), a probe sonicator (UCD-950, 1000 W/220 V/ 50 Hz), a freezer (Polytron), a centrifuge (SL40R, Thermo Scientific), and an HPLC amino acid analyzer with an RP-18 column, utilizing an isocratic pump system.

Procedure

Extraction of Spirulina *protein*

The process of extracting *Spirulina* protein was carried out using two methods, namely extraction with physical and chemical methods. The physical extraction method was carried out by a combination of freeze-thawing and ultrasonication methods [11], while extraction with chemical methods was carried out by utilizing the solubility properties of proteins in alkali and the isoelectric point of proteins [12]. *Spirulina* powder, as much as 5 g was dissolved in 100 mL of distilled water and stirred for 30 min. Then, the solution was subjected to a freeze-thawing process for 3 cycles. The freeze-thawing process consists of freezing at a temperature of –20 °C for 4 h and thawing at room temperature for 4 h. Furthermore, the solution was ultrasonicated with a power of 570 W for 20 min. The protein extract solution was then centrifuged at 10,000 rpm at 4 °C for 30 min. The sample was then filtered, and the resulting filtrate was stored for hydrolysis and protein concentration analysis.

Spirulina powder, as much as 2 g was dissolved in 100 mL of 1 M NaOH pH 11. The solution was then stirred for 30 min at 40 °C. Furthermore, centrifugation was carried out at 10,000 rpm at 20 °C for 10 min and filtered to separate the filtrate and pellets. The filtrate obtained was then precipitated using 1% HCl until it reached pH 4.0. The sample was then centrifuged again at 10,000 rpm at 20 °C for 10 min. The pellets obtained were then dissolved in 100 mL of distilled water for hydrolysis and protein concentration analysis.

Hydrolysis of Spirulina *protein*

Spirulina protein hydrolysis was carried out through enzymatic reactions using different protease enzymes, referring to the research method conducted by Sun et al. [13]. *Spirulina* protein extract samples were added with protease enzymes, namely papain (EC 3.4.22.2) and bromelain (EC.3.4.22.4) at a concentration of 4% (w/v) with a substrate volume of 100 mL. The temperature and pH of the sample were adjusted to the optimum working conditions of each enzyme. The optimum temperature and pH of the papain were 60 °C and pH 6, while for the bromelain, they were 50 °C and pH 7.0. The hydrolysis process was carried out for 4 h in an incubator. The enzymatic reaction was stopped by heating the sample in a water bath at 80 °C for 20 min. The hydrolysate obtained was then centrifuged at a speed of 8000 rpm at 4 °C for 20 min. The resulting protein hydrolysate was then stored in frozen storage and tested.

Analysis of protein content

Protein content analysis was carried out on dry biomass samples of *Spirulina* before extraction and on *Spirulina* protein extract. Protein content analysis used the Biuret method, referring to Yathisha et al. [14]. A 2 mL protein sample was placed in a test tube and 6 mL of biuret reagent was added, then vortexed for 1 min. The solution was then incubated for 30 min and its absorbance was measured using a spectrophotometer at a wavelength of 540 nm. Protein concentration was calculated based on a standard curve made using a series of bovine serum albumin concentrations. The protein concentration obtained was then converted into a protein yield value.

Analysis of hydrolysis degree

Spirulina protein hydrolysate was then subjected to spectrophotometric hydrolysis degree analysis referring to research conducted by Mohammadi et al. [15]. Each sample of *Spirulina* protein hydrolysate from the treatment was put into a test tube as much as 5 mL, and 1 mL of ninhydrin reagent was added. All sample solutions were then measured for absorbance at 570 nm.

Analysis of antioxidant activity

Antioxidant activity analysis was carried out on dry biomass samples, protein extracts and *Spirulina* protein hydrolysates. Free radical inhibition testing using DPPH refers to the previous research [16]. Each type of *Spirulina* sample with concentrations of 25, 50, 75, 100, 125, and 150 ppm was put into a test tube, and then 0.1 mM DPPH reagent was added with a ratio of 1:1 (v/v). The solution was incubated in the dark for 30 min. Absorbance measurements were carried out at a wavelength of 517 nm.

Analysis of molecular weight of Spirulina *protein and hydrolysates*

SDS-PAGE was used to characterize the components of *Spirulina* protein and its hydrolysates after enzymatic hydrolysis by bromelain and papain

[17]. A sample was prepared to perform SDS-PAGE analysis by adding a sample buffer into the *Spirulina* protein and hydrolysate the *Spirulina* ratio to 1:1 (v/v). The sample was heated to 100 °C for 5 min. A separating SDS-polyacrylamide gel (12.5%) was prepared between two glass plates, and the gel was solidified for 30 min. A stacking gel (3%) was injected into the space between the glass plates. An aliquot of 20 µL of the *Spirulina* protein and the hydrolysates protein *Spirulina* was poured into the wells of the stacking gel. Then, the gel was positioned in the electrophoresis set-up and set at 120 V for 30 min. The gel was removed from the plates and stored overnight in a staining solution. The protein bands were then visualized on the gels.

Analysis of amino acids

Amino acids were analyzed using HPLC amino acid analyzer [18]. Tryptophan standards were prepared in a 4.2 M NaOH solution with a minimum concentration of six points. A sample of 0.1 g was weighed and dissolved in 20 mL of 4.2 M NaOH. Furthermore, the solution was heated to 110 °C for 20 h for hydrolysis. The sample was adjusted to pH 4.25 using HCl or NaOH solution, then diluted to a volume of 50 mL using distilled water. This homogeneous solution was transferred as much as 2 mL into a centrifuge vial, filtered using a 0.45 µm syringe filter, and injected into the HPLC system. The analysis used an RP-18 column with a mobile phase of 0.0085 M sodium acetate and methanol on an isocratic pump system. The determination of amino acid concentration was performed using a standard calibration curve based on the equation y = bx + ay = bx + ay = bx + a, shown in Eq. (1):

$$\text{L}-\text{Tryptophan concentration} = \frac{A_{sp} - a}{b} \times FP \times \frac{V_s}{V_{sp}} \quad (1)$$

where A_{sp} is the sample area, a is the intercept, b is the slope of the calibration curve, FP is the dilution factor, V_s is the final volume of the sample solution, and V_{sp} is the injection volume of the sample.

Statistical analysis

The research data were analyzed using a t-test and analysis of variance (ANOVA) with α 0.05 and continued with the Tukey post hoc test if significantly different. Analysis was conducted using the Minitab 19 Program.

■ RESULTS AND DISCUSSION

Protein Content of *Spirulina* Crude Extract

Physical protein extraction incorporates freeze-thaw and ultrasonication methods, whereas chemical protein extraction uses the solubility of proteins in alkali and their isoelectric points. The protein content of the *Spirulina* crude protein extract was ascertained using the biuret technique. The results of the levels of crude protein extract from *Spirulina* are shown in Table 1.

Spirulina that was extracted by combining freeze-thaw and ultrasonication produced more protein than those that were extracted with alkali. A combination of freeze-thaw and ultrasonication techniques produced 75.43% protein. A yield of 23.68% was obtained from protein extracted using alkali. Previous work conducted a physical protein extraction of *Spirulina* using a combination of freeze-thaw and ultrasonication techniques, yielding an 84.8% protein extract [11]. In the study by Parimi et al. [17], the protein yield of the *Spirulina* protein extract made with alkali was 60.7%, whereas this number is higher.

In protein extraction, a combination of freeze-thaw and ultrasonication techniques yields a greater yield because of more efficient cell wall disintegration. *Spirulina* protein extracted using a combination of freeze-thaw and ultrasonication techniques yielded an 84.8% protein yield, according to Wang and Zhang [11]. The freeze-thaw method of protein extraction involves the formation of ice crystals during freezing and stretching during thawing. Repetitive stretching and ice crystal production in cells can disrupt the cell wall and eventually break it [18]. High-frequency sound waves used

Table 1. %Yield of crude protein extract of *Spirulina*

Sample	Extraction method	Concentration (mg/mL)	Yield (%)
Protein extract	Physical	3.77 ± 0.45^{a}	75.43 ± 8.95^{a}
	Chemical	1.18 ± 0.33^{b}	23.68 ± 6.63^{b}

in the ultrasonication process break down cell walls [19]. According to Zhu et al. [20], the probe sonicator's mechanical energy creates cavitation bubbles, eventually bursting and sending shock waves radiating outward from the sample. Cell wall damage results from shock waves altering the permeability of cell walls.

The protein yield of chemically extracted *Spirulina* protein extract is lower than that of physically extracted protein. According to research by Safi et al. [12], a protein yield of 15.8% was obtained from the chemically isolated protein of *Haematococcus pluvialis* microalgae. Protein extraction with high pH might alter the functional characteristics of the protein and could result in unfavorable changes in protein structure or even protein denaturation, even though alkaline treatment can normally increase protein solubility [18]. Because alkaline extraction can result in protein denaturation, lower protein purity, color darkening, and the creation of undesirable chemicals, Das et al. [21] support the idea that high pH can degrade protein quality.

Degree of Hydrolysis

The percentage of free amino groups produced during the hydrolysis process relative to the total amount of nitrogen in the substrate is known as the degree of hydrolysis (DH) [22]. Variations in enzyme types, enzyme and substrate ratios, pH, duration, and hydrolysis temperature are some of the variables that influence DH [23]. Different protease enzymes, specifically papain and bromelain enzymes, were used in this study's hydrolysis process on *Spirulina* protein extracts obtained through physical extraction. Table 2 displays the DH of the protein extracts from *Spirulina* that were hydrolyzed using various protease enzymes.

Because it generates a larger DH value, the bromelain enzyme is more effective for use in the protein hydrolysis process, according to the analysis of the *Spirulina* protein hydrolysate. For the amino acid residues that comprise the substrate, arginine, phenylalanine, lysine, and tyrosine, the bromelain enzyme has a broad cutting specificity and can break down proteins 1000 times their weight [10]. This is in line with studies by El-Moataaz et al. [24], which found that arginine, phenylalanine, tyrosine, and lysine are all reasonably abundant in *Spirulina*, with successive values of 44.91, 23.78, 19.74, and 19.10 mg/100 g.

The reason for the low degree of hydrolysis of the papain-hydrolyzed *Spirulina* protein may be that the enzyme has already reached its maximum phase of activity earlier in the hydrolysis process, meaning that DH does not increase until the process is finished. Within 10 min, hydrolysis usually accelerates dramatically; after 15 to 20 min, it slows down and stabilizes. The rate of hydrolysis gradually drops as the DH stabilizes [25]. According to Limsukon et al. [26], although they can speed up the hydrolysis process, catalysts don't always have the same effect. The catalyst may become less effective as the reaction goes on because of things like catalyst deactivation or the presence of byproducts that impede the catalyst. Because so many products are formed, the enzyme's ability to hydrolyze the substrate is inhibited, resulting in the stationary phase. The active side of the substrate protein will be closed by the amino acids produced during the hydrolysis process, preventing the enzyme from carrying on with the hydrolysis.

In contrast to Wang and Zhang's study [11], which hydrolyzed *Spirulina platensis* using papain enzyme at an enzyme ratio of 4% to the substrate for 8 h and produced a DH of 11.12%, the DH generated in our study was greater. The work of various proteolytic enzymes throughout the hydrolysis process may alter the protein's structure and composition, resulting in variations in the DH generated. Furthermore, the number of peptide bonds that the enzyme can access on the substrate varies depending on the type of enzyme [27].

Table 2. The degree of hydrolysis of *Spirulina* hydrolysate protein

Sample	Cysteine-Protease	Degree of hydrolysis (%)
Protein hydrolysate	Bromelain	32.15 ± 0.74^{a}
	Papain	22.55 ± 1.97^{b}

Different levels of peptide bond breakdown are caused by variations in the kind and amount of protein in the *Spirulina* substrate, the activity of each protease utilized throughout the hydrolysis process, and the presence of inhibitors that stop the enzyme from contacting the substrate [15].

Protein Content

The amount of biological activity in *Spirulina* can be determined by looking at its protein content. The biuret method was used to determine the amount of protein present in *Spirulina*'s dry biomass, protein extract, and protein hydrolysate. Table 3 displays the findings from the primary study's protein content. According to the test results, the dry biomass of *Spirulina* contains 3.51 mg/mL of protein, whereas the dry biomass of *Spirulina* that undergoes a physical protein extraction process has a higher protein concentration of 4.05 mg/mL. The protein concentration is reduced to 2.43 and 2.31 mg/mL by hydrolyzing of the *Spirulina* protein with bromelain and papain enzymes. A separation with non-protein components causes the protein extract to have a higher protein concentration than the dry biomass. According to Ketemepi et al. [28], the protein extraction process is done to obtain products with high protein content because they have lost non-protein components like fat, carbohydrates, minerals, and water. As a result, the product's protein content is higher than that of the original raw materials. Proteins that undergo hydrolysis have a lower protein content than before hydrolysis because cysteine-protease hydrolysis breaks down peptide bonds in proteins into peptides and free amino acids [29].

Compared to protein extract hydrolyzed with papain enzyme, *Spirulina* protein extract hydrolyzed with bromelain enzyme has a higher protein concentration. This outcome is not directly correlated with the degree of hydrolysis that occurs; the breakdown of protein into amino acids should result in a lower concentration of protein, the more hydrolyzed the protein is. But this also happened in a study by Tejano et al. [30], where they used pepsin, bromelain, and thermolysin enzymes to hydrolyze the protein of *Chlorella sorokiniana*. The hydrolysis degree was 8.16, 15.93, and 18.08%, respectively, and the protein content was 54.73, 63.87, and 84.64%. As a result of hydrolyzing insoluble proteins into soluble nitrogen compounds, which subsequently break down into simpler molecules like peptides and amino acids, protein hydrolysate's protein content rises [31].

Antioxidant Activity

Hydrolysis of the protein source *Spirulina* can increase its possible antioxidant action. The IC_{50} value, or the concentration of the substance required to capture 50% of DPPH free radicals, is used to quantify antioxidant activity. A compound's antioxidant activity increases with decreasing IC_{50} value [32]. Table 4 displays

Table 3. Protein content of dry biomass, crude extract, protein hydrolysate (bromelain), and protein hydrolysate (papain)

Samples	Concentration (mg/mL)	Yield (%)
Dry biomass	3.51 ± 0.12^{b}	70.11 ± 2.48^{b}
Protein extract	4.05 ± 0.07^{a}	81.06 ± 1.43^{a}
Protein hydrolysate (bromelain)	2.43 ± 0.05^{c}	48.51 ± 0.94^{c}
Protein hydrolysate (papain)	2.31 ± 0.03^{c}	46.11 ± 0.65^{c}

Note: The results in the same column followed by the same letter do not significantly differ by Tukey's test ($P < 0.05$)

Table 4. The IC_{50} of dry biomass, protein extract, and protein hydrolysate of *Spirulina*

Samples	IC_{50} (ppm)	Antioxidant category
Dry biomass	178.35 ± 5.91^{a}	Weak
Protein extract	112.50 ± 3.62^{b}	Moderate
Protein hydrolysate (bromelain)	29.64 ± 0.82^{d}	Very strong
Protein hydrolysate (papain)	69.34 ± 1.67^{c}	Strong

Note: The results in the same column followed by the same letter do not significantly differ by Tukey's test ($p < 0.05$)

the IC_{50} values for *Spirulina*'s dry biomass, protein extract, and protein hydrolysate.

The statistical analysis revealed that, according to ANOVA, there were significant differences ($p < 0.05$) between the samples. The samples were divided into three significant categories using Tukey's additional test: (1) Protein hydrolysate containing papain (69.34 ± 1.67 ppm) and protein extract (112.50 ± 3.62 ppm) had strong antioxidant activity, (2) protein hydrolysate containing bromelain enzyme had the lowest IC_{50} value (29.64 ± 0.82 ppm), and (3) dry biomass had the highest IC_{50} value (178.35 ± 5.91 ppm), which is categorized as moderate antioxidant activity. When compared to papain hydrolysate and other samples, bromelain hydrolysate exhibited notable variations, suggesting that bromelain is more successful at boosting antioxidant activity. Bromelain has biological action as an enzyme, particularly as a treatment for inflammatory conditions [33]. It has been demonstrated that the hydrolysis procedure boosts antioxidant activity in comparison to protein extracts and dry biomass. This is because hydrolysis produces bioactive peptides, which are better able to donate protons to free radicals. Additionally, the hydrolysis process breaks down proteins' tertiary structure, increasing the accessibility of amino acid residues [34]. According to Lisboa et al. [35], protein hydrolysis can raise the percentage of inhibition by up to 8.5 times compared to intact protein.

Using ANOVA, the impact of protease on antioxidant activity was also examined. When hydrolyzed using the enzyme bromelain, the IC_{50} values were lower than those of papain ($p < 0.05$). Variations in the resultant peptides' molecular weight and amino acid sequence can account for this discrepancy. Because of their high hydrophobic amino acid concentration, peptides with low molecular weight typically exhibit greater antioxidant action [36]. It is thought that several amino acids, including tyrosine, tryptophan, methionine, lysine, and cysteine, which function as electron donors, metal chelators, or hydroperoxide reducers, contribute to the mechanism of antioxidant activity of bioactive peptides [37]. Furthermore, through the use of phenol, indole, or imidazole groups, aromatic amino acids like phenylalanine, tyrosine, tryptophan, and histidine efficiently absorb free radicals [38]. At the same time, negatively charged amino acids like glutamic acid and aspartic acid also contribute to antioxidant activity by donating excess electrons [39].

Molecular Weight of *Spirulina* Crude Protein and Hydrolysate

For the protein hydrolysate samples of *Spirulina* protein extract, hydrolysate protein *Spirulina* using bromelain hydrolysis, and hydrolysate protein *Spirulina* using papain hydrolysis, the results of the SDS-PAGE analysis were as follows: concentrations of 0.547, 0.786, and 0.756 mg/mL, respectively; the dominant protein bands on the gel displayed protein fragments with low molecular weights, indicating a successful hydrolysis process using the bromelain enzyme. This profile supports the potential of bioactive peptides as immunostimulant candidates because low-weight peptide molecules are more readily absorbed by cells and are more effective in modulating immune responses. The molecular weight profile of the protein hydrolysate is shown in Fig. 1.

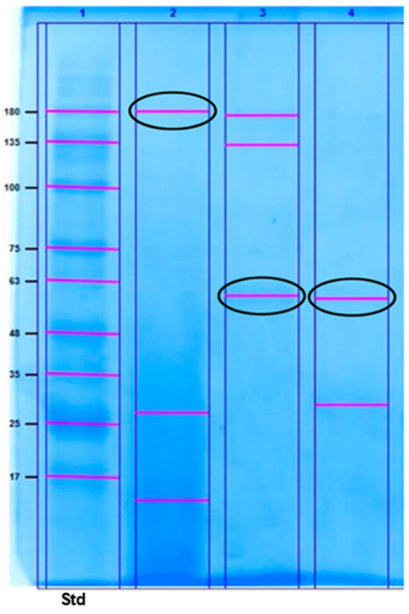

Fig 1. SDS-Polyacrylamide gel electrophoretogram of the *Spirulina* protein before and after hydrolysis by bromelain and papain. (1) standard marker stain, (2) *Spirulina* protein extract, (3) hydrolysate protein *Spirulina* using bromelain hydrolysis, (4) hydrolysate protein *Spirulina* using papain hydrolysis

Previous research by Rajakumar and Muthukumar [40] validates similar findings. In their study, SDS-PAGE showed the presence of low molecular weight protein bands, dominated by peptide fragments, as a result of protein hydrolysis of Spirulina protein bands in the 19-22 kDa range, consistent with the α/β bili-protein subunit of *Spirulina platensis*, were identified after sonication treatment and processing using a biphasic system. Furthermore, the structure and biological activity of proteins isolated under neutral circumstances were preserved. These results support the idea that bioactive peptides can be preserved during hydrolysis or extraction. The presence of this low-molecular-weight protein band supports the previously extensively researched potential bioactivity of hydrolyzed peptides, including immunostimulatory activity.

Amino Acids Profile

Table 5 shows the results of the amino acid profile analysis of *Spirulina* protein hydrolysate that was hydrolyzed using the bromelain enzyme. The amino acid profile of food protein is a significant indicator of the nutritional value of the food [15]. *Spirulina* protein hydrolysate contains a variety of amino acid types because of the breakdown of proteins during the hydrolysis process.

Eight types of necessary amino acids and 10 types of non-essential amino acids make up the 18 types of amino acids found in the hydrolyzed *Spirulina* protein hydrolysate, according to the amino acid analysis results. A variety of 18 to 20 different types of essential and non-essential amino acids will be present in the hydrolysate products of a flawless hydrolysis process [41]. Isoleucine, leucine, lysine, methionine, tryptophan, valine, phenylalanine, and threonine are among the essential amino acids found in *Spirulina* protein hydrolysate. Alanine, arginine, aspartic acid, glycine, glutamic acid, cysteine, proline, serine, tyrosine, and histidine are among the non-essential amino acids that are present in *Spirulina* protein hydrolysates. Aspartic acid, glutamic acid, histidine, serine, arginine, glycine, threonine, alanine, methionine, cysteine, valine, phenylalanine, tyrosine, leucine, isoleucine, lysine, and tryptophan are among the 17-type amino acids found in the hydrolyzed *Spirulina* protein hydrolysate by the pepsin enzyme, according to research by Mohammadi et al. [15].

Table 5. Amino acid profile of *Spirulina* protein hydrolysate using bromelain enzyme

Amino acid	Concentration (mg/kg)
Alanine	1212.52
Arginine	626.49
Aspartic acid	1340.89
Glycine	805.08
Glutamic acid	2034.84
Isoleucine	861.33
Leucine	1240.82
Lysine	386.33
Methionine	11.15
Tryptophan	789.61
Valine	918.41
Phenylalanine	672.19
Proline	402.34
Serine	500.38
Threonine	908.13
Histidine	295.11

Spirulina protein hydrolysate hydrolyzed by the bromelain enzyme contains 14409.31 mg/kg of total amino acids. Among the amino acid types found in *Spirulina* protein hydrolysate, glutamic acid has the highest concentration (2034.84 mg/kg), while methionine has the lowest concentration (11.15 mg/kg). Protein, a complex molecule rich in important amino acids including arginine (5.83 ± 0.470 g/100 g protein), aspartic acid (7.35 ± 0.450 g/100 g protein), cysteine (0.13 ± 0.001 g/100 g protein), glutamic acid (11.05 ± 0.850 g/100 g protein), glycine (11.75 ± 0.84 g/100 g protein), and methionine (1.56 ± 0.065 g/100 g protein) [42]. Up to 47% of the protein weight in *Spirulina* is made up of several important amino acids. Leucine, valine, and isoleucine have the greatest essential amino acid levels, but sulfur amino acids like cysteine and methionine have the lowest [43].

Methionine is a sulfurous amino acid that is necessary for metabolism and the detoxification of toxic substances in the human body; free amino acids and peptides found in hydrolysate have been identified as potential antioxidant compounds; glutamic acid, a non-

essential amino acid, is important for brain function and memory enhancement and may also help build muscle. Free radicals can be neutralized by hydrophobic amino acids like valine, alanine, proline, leucine, and methionine, as well as aromatic amino acids like tyrosine, histidine, and phenylalanine [44]. Protease activity or the purposeful addition of particular enzymes can yield amino acids and bioactive peptides; each of these enzymes generates distinct peptide fragments with particular amino acid sequences and bioactivity [35].

Glutamic acid is the most prevalent of the 18 different types of amino acids found in *Spirulina* protein hydrolysate. Known for its antioxidant properties, glutamic acid is oxidized when hydrogen peroxide, a crucial component in the production of free radicals, is reduced. The protein hydrolysate of *Spirulina* also contains several hydrophobic amino acids, including leucine, alanine, valine, isoleucine, phenylalanine, proline, tryptophan, and methionine. After oral medication, proline-containing peptides are often more easily absorbed, more resistant to breakdown by digestive enzymes, and more likely to raise plasma levels [45]. The antioxidant content of these hydrophobic amino acids is substantial. Peptide sequences that contain hydrophobic amino acids are more bioavailable because they can interact with hydrophobic targets, like cell membranes [46]. According to this data, hydrolyzed *Spirulina* protein may be a viable natural antioxidant source.

■ CONCLUSION

The most efficient of physical extraction methods—particularly the combination of freeze-thaw and ultrasonication—for obtaining high-yield, high-quality protein from *Spirulina*. The degree of hydrolysis, protein content, molecular weight, and antioxidant activity of protein hydrolysate are all impacted by the different kinds of cysteine-protease enzymes. The resulting protein extracts demonstrated enhanced bioactivity, as evidenced by increased antioxidant potential and low-molecular-weight bioactive peptides. The enzyme bromelain may be more effective at hydrolysing the protein found in *Spirulina*. Using bromelain, the *Spirulina* protein hydrolysate's hydrolysis degree, protein content, molecular weight, and antioxidant activity (IC_{50}) were 32.15 ± 0.74%, 48.51 ± 0.94%, 0.786 mg/mL, and 29.64 ± 0.82 ppm, respectively. Enzymatic hydrolysis using bromelain significantly improved the antioxidant activity of the protein hydrolysates. Using papain, the results for the *Spirulina* protein hydrolysate were 22.55 ± 1.97%, 46.11 ± 0.65%, 0.756 mg/mL, and 69.34 ± 1.67 ppm. The hydrolyzed *Spirulina* protein, which was hydrolyzed using bromelain, comprised 14409.31 mg/kg of 18 different types of essential and non-essential amino acids. Glutamic acid was the amino acid type with the highest concentration in the *Spirulina* protein hydrolysate, at around 2034.84 mg/kg. Future research should optimize extraction and hydrolysis parameters, such as freeze-thaw cycles, sonication time, enzyme concentration, and hydrolysis duration, to further increase yield and bioactivity. Additionally, *in vivo* studies are necessary to validate these protein hydrolysates' immunomodulatory and antioxidant effects in biological systems.

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■ CONFLICT OF INTEREST

The authors declare that they hold no competing interests.

■ AUTHOR CONTRIBUTIONS

Heder Djamaludin: Conceptualization, Investigation, Methodology, Writing – original draft, Resources, Writing – review and editing. Inayatussakinah Inayatussakinah, Andhika Alfanda Kusdiyarlistio, and Zidan Armanda: Investigation. Dinia Rizqi Dwijayanti and Inggit Kresna Maharsih: Investigation, Writing – original draft, and Visualization. Kartika Dyah Palupi, Pamungkas Rizki Ferdian, Rizki Rabeca Elfirta, and Hartoyo Notonegoro: Data Analysis and Visualization, and Writing – original

draft. All authors have read and approved the final manuscript.

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