TROPICAL LIFE SCIENCES RESEARCH

Bioactive Properties of Alcalase-Derived *Spirulina platensis* Protein Hydrolysate: Amino Acid Composition, Antioxidant and Antiproliferative Activities

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Submitted: 14 March 2026; Accepted: 6 August 2026; Early view: 4 November 2026
To cite this article: Raihan Sa'id Ashim, Hartati Kartikaningsih, Kartika Dyah Palupi, Agus Budiawan Naro Putra, Zahrotul Firdaus, Janrigo Klaumegio Mere and Heder Djamaludin (in press). Bioactive properties of alcalase-derived *Spirulina platensis* protein hydrolysate: Amino acid composition, antioxidant and antiproliferative activities. *Tropical Life Sciences Research.*

Highlights

- *Spirulina platensis* protein hydrolysate achieved a high hydrolysis degree of 44.1% using alcalase.
- Hydrolysate (<20 kDa) exhibited strong antioxidant capacity in ABTS, FRAP, and DPPH assays.
- Dose-dependent cytotoxicity reduced MCF-7 breast cancer cell viability to 45.91% at 1000 ppm.

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EARLY VIEW

Bioactive Properties of Alcalase-Derived *Spirulina platensis* Protein Hydrolysate: Amino Acid Composition, Antioxidant and Antiproliferative Activities

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Abstract: *Spirulina platensis* is a microalga rich in protein and bioactive compounds, offering potential as a natural antiproliferative agent. However, in its dried biomass form, the biological activity of its native proteins remains relatively low. This study aimed to investigate the potential use of *S. platensis* as a source of bioactive peptides by analyzing the amino acid profile, antioxidant through ABTS, FRAP, DPPH assays and antiproliferative activity of *S. platensis* protein hydrolysate. Protein extraction was performed using a combination of freeze-thawing and ultrasonication methods, followed by alcalase enzymatic hydrolysis at 55 °C and pH 8 for 2 hours. The resulting hydrolysate was characterized in terms of yield, degree of hydrolysis (DH), molecular weight distribution, amino acid profile, secondary structure, and antioxidant activity (ABTS, FRAP, and DPPH assays), as well as cytotoxicity assessed by the MTT assay. The hydrolysate protein of *S. platensis* showed a DH of 44.1±0.20%, with a

dominant molecular weight fraction below 20 kDa, and antioxidant activity with IC_{50} 9.03±0.25 ppm (ABTS), 22±1.04 µg TE/mL (FRAP), and 44±2.04 ppm (DPPH). Cytotoxicity analysis revealed a dose-dependent relationship between hydrolysate concentration and cytotoxic effects on MCF-7 cells. The highest cytotoxic activity of *S. platensis* protein hydrolysate was observed at 1000 ppm, reducing cell viability to 45.91±5.62%. These findings suggest that *S. platensis* protein hydrolysate has promising potential as a natural antiproliferative candidate.

Keywords: Enzymatic proteolysis, *in vitro* Cytotoxicity, Low Molecular Peptides, Microalgal Bioactive Compounds, Radical Scavenging Activity

INTRODUCTION

Cancer is a condition in which cells in certain parts of the body undergo abnormal growth and divide continuously without control. Cancer is a non-communicable disease whose number of sufferers increases every year and is very dangerous, causing high mortality rates (Bray *et al.* 2024). Breast cancer is the most frequent cancer, and it was the second leading cause of death by cancer in women worldwide. According to Cancer Statistics 2020, breast cancer represents 30% of female cancers with 276,480 estimated new cases and more than 42,000 estimated deaths in 2020 (Burguin *et al*. 2021).

According to international consensus guidelines, current breast cancer treatment mainly includes chemotherapy, radiotherapy, targeted therapy, immunotherapy, and endocrine therapy before and after surgery. Currently, therapy targeting cancer stem cells has become a hot topic in breast cancer treatment, which is a supplement to traditional chemotherapy drugs that are unable to spread and metastasize tumors (Chen *et al*. 2012). Therefore, natural ingredients are needed as alternative candidates for cancer therapy. One of the natural ingredients that can be used is *Spirulina platensis* which is one of the natural ingredients that has antiproliferative effects and can be developed a source of bioactive peptides (Śmieszek *et al*. 2017).

Spirulina platensis is a cyanobacterium that can be consumed and is a high source of protein. However, protein in native form has low biological activity (Akbarbaglu *et al*. 2022). Therefore, enzymatic hydrolysis is needed to increase biological and nutritional activity so that it can overcome problems in its utilization. Many studies have reported on the function of bioactive peptides obtained from the enzymatic hydrolysis of various protein sources. These peptides are naturally inactive in native protein form, but after being released from the protein matrix using gastrointestinal proteases, they show nutritional and therapeutic functions including

antioxidant, antimicrobial, antiproliferative, anti-inflammatory, immunomodulatory, and hypocholesterolemic activities. Bioactive peptides from *S. platensis* protein extract obtained by freeze-thawing extraction and sonication methods then enzymatically hydrolyzed with pepsin and trypsin showed antiproliferative activity on three types of cancer cells (MCF-7, HepG-2, and SGC-7901 cell lines) (Mohammadi *et al*. 2022).

Previous studies have characterized the bioactivity of *S. platensis* protein hydrolysates produced using pepsin and pancreatin enzymes. However, research on the use of alcalase in the hydrolysis of *S. platensis* protein remains limited. In addition, only a few studies have comprehensively compared the amino acid profiles and antioxidant activities of the resulting hydrolysates using integrated FRAP, ABTS, and DPPH assays. Research on antiproliferative activity is also still scarce, particularly studies directly comparing crude *S. platensis* protein extracts with the hydrolyzed products. Therefore, this study is important to comprehensively evaluate the effect of alcalase-mediated hydrolysis on the bioactive characteristics of *S. platensis* protein. Accordingly, this study was designed to compare the amino acid profile, antioxidant activity, and antiproliferative activity of crude *S. platensis* protein extract and its alcalase-generated hydrolysate. This study aimed to investigate the potential use of *S. platensis* as a source of bioactive peptides by analyzing the amino acid profile, antioxidant through ABTS, FRAP, DPPH assay and antiproliferative activity of *S. platensis* protein hydrolysate.

MATERIAL AND METHODS

Materials used in this research included freshwater *Spirulina platensis* obtained from the Brackish-water Aquaculture Development Center (BBPBAP) Jepara, and MCF-7 breast cancer cells provided by the Genomic Laboratory of the Research Center for Medicinal Plants and Traditional Medicine, National Research and Innovation Agency (BRIN), Cibinong. Other reagents included alcalase enzyme with 50,000 U/g activity, distilled water, Biuret reagent (Merck, Germany), Bovine Serum Albumin (BSA, Merck), NaOH (Merck, Germany), HCl (Merck, Germany), ascorbic acid (Merck, Germany), ethanol (Merck, Germany), ninhydrin reagent (Smart Lab), methanol (Smart Lab A1056), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Merck, Germany), (FRAP) (Merck, Germany), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (Merck, Germany), Whatman No. 42 filter paper (Merck, Germany), GF/C paper (Merck, Germany), and MTT reagent (Sigma-Aldrich, USA).

Protein Extraction

The extraction of *S. platensis* protein was carried out using a physical method involving freeze-thawing and ultrasonication (Wang & Zhang 2016). A total of 5 g of *S. platensis* powder was dissolved in 100 mL of distilled water and homogenized using a magnetic stirrer for 30 minutes. The solution then underwent three cycles of freeze-thawing. Each cycle consisted of freezing at −20 °C for 4 hours followed by thawing at room temperature (20–25 °C) for 4 hours. Subsequently, the solution was sonicated at 40 kHz and 27 °C for 20 minutes. The protein extract was then centrifuged at 10,000 rpm and 4 °C for 30 minutes. The supernatant was filtered using Whatman GF/C filter paper with a pore size of 1.2 µm. The resulting filtrate was collected for the subsequent hydrolysis process.

Protein Hydrolysis

Protein hydrolysis of *S. platensis* was carried out through enzymatic reactions using alcalase enzyme (Wang & Zhang 2016). The *S. platensis* protein extract was mixed with alcalase enzyme (activity 50,000 U/mg) at a concentration of 1% (w/v) in a substrate volume of 100 mL. The hydrolysis process was performed at alcalase optimum operating conditions, at 55 °C and pH 8, for 2 hours in an incubator. The enzymatic reaction was terminated by heating the sample in a water bath at 90 °C for 10 minutes. The resulting hydrolysate was then centrifuged at 8000 rpm and 4 °C for 20 minutes. The obtained protein hydrolysate was subsequently freeze-dried. First, the hydrolysate was frozen using an ultra-low temperature freezer at −80 °C for 3 hours. Then, the frozen sample was placed in a freeze dryer at −55 °C and 0.04 mbar pressure for 5 days to obtain the hydrolysate in powdered form.

Yield Analysis

In this study, both the crude protein extract and the protein hydrolysate were dried into powder form using the freeze-drying method. The crude protein extract powder was weighed, and its yield was calculated using the formula: final weight divided by initial weight, then multiplied by 100%. Subsequently, to determine the yield of the *S. platensis* protein hydrolysate, the resulting hydrolysate powder was weighed and calculated using the formula: final weight divided by the initial weight of *S. platensis* material hydrolyzed using the alcalase enzyme, then multiplied by 100%. Thus, the yield values of both the crude protein extract and the protein hydrolysate from *S. platensis* were obtained (Djamaludin *et al*. 2025).

Degree of Hydrolysis Analysis

To determine the degree of hydrolysis (DH), 1 mL of ninhydrin reagent was added to the

hydrolysate protein sample, followed by heating at 80 °C for 4 min until a purple color developed. The absorbance was measured at 570 nm using a UV–Vis spectrophotometer. A blank containing distilled water and ninhydrin reagent was used for baseline correction. Glycine was used as the calibration standard to construct the standard curve. The DH value was calculated based on the amount of free amino groups released during hydrolysis. All measurements were performed in triplicate, and the results were expressed as mean ± standard deviation (SD) (Barea *et al*. 2024).

Molecular Weight Analysis

The molecular weight of *S. platensis* protein hydrolysate was analyzed using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Mohammadi *et al*. 2022). The electrophoresis was performed using a 12.5% separating gel and a 3% stacking gel assembled between two glass plates. The hydrolysate protein was dispersed in distilled water to a final concentration of 0.2% (w/v). A 1 mL aliquot of the dispersed sample was mixed with 1 mL of bromophenol blue buffer. To activate the hydrolysate protein sample, the mixture was incubated in a water bath at 100 °C for 5 minutes. Subsequently, 20 µL of the prepared hydrolysate protein sample was loaded into the gel wells and electrophoresis was carried out until the bromophenol blue dye front reached the bottom of the gel. After electrophoresis, the gel was stained for 24 hours to visualize the protein bands.

Amino Acid Profile Analysis

Amino acid profile analysis was conducted using chromatography with a High Performance-Liquid Chromatography (HPLC) Amino Acid Analyzer (Pereira *et al*. 2019). A standard series of tryptophan was prepared in a 10 mL volumetric flask at six concentration levels (10, 20, 40, 60, 80, and 100 mg/L) to generate the calibration curve. The standard curve equation and correlation coefficient (R^2) were obtained from the linear regression analysis of peak area versus concentration. For sample preparation, 0.1 g of the hydrolysate protein sample was weighed into a 20 mL headspace vial, followed by the addition of 4.2 M NaOH solution. The mixture was hydrolyzed at 110 °C for 20 h. After hydrolysis, the sample was transferred into a 50 mL glass beaker and mixed with citrate buffer solution. The pH was adjusted to 4.25 using HCl or NaOH. The solution was quantitatively transferred into a 50 mL volumetric flask and diluted to volume with distilled water, followed by homogenization. Subsequently, the sample solution was centrifuged, and the supernatant was filtered through a 0.45 µm syringe filter into a 2 mL vial prior to HPLC analysis. Chromatographic separation was carried out using an RP-18 column (4.6 x 250 mm, 5 µm particle size). The mobile phase consisted of 0.0085 M sodium

acetate and methanol under isocratic elution conditions. The flow rate was maintained at 1.0 mL/min, with a column temperature of 25 °C. The injection volume was 20 µL. Detection was performed using a UV–Vis detector at 280 nm. The amino acid concentration in the samples was determined based on the peak area response using the calibration curve equation and expressed as mg/Kg sample. The same analytical procedure was also applied to the crude extract protein samples.

Fourier Transform Infrared Spectra Analysis

The secondary structure of *S. platensis* proteins was analyzed using Fourier Transform Infrared (FTIR) spectroscopy. Spectra were acquired with a TENSOR 27 FT-IR spectrophotometer (BRUKER, USA) over a wavenumber range of 400–4000 cm^{-1}, using 32 scans at a resolution of 4 cm^{-1}. Hydrolysate protein sample transmission spectra were obtained via potassium bromide (KBr) pellets containing 0.1% (w/w). Self-deconvolution analysis was used to identify the number and position of spectral components. Curve fitting was performed using OriginPro 8.0 software (OriginLab, USA). Secondary structure content was calculated based on the amide I region by applying non-linear Gaussian fitting to the original spectra (Benelhadj *et al*. 2023). The same analytical procedure was also applied to the crude extract protein samples.

Antioxidant Activity: DPPH Scavenging Assay

The preparation of the DPPH standard curve was carried out by diluting an ascorbic acid stock solution in methanol to obtain a series of concentrations: 0, 50, 100, 150, 200, and 250 µM/mL. For sample preparation, hydrolyzed protein and crude extract protein samples were diluted with distilled water to obtain concentrations of 5, 10, 15, 20, and 25 ppm. A volume of 0.15 mL of each standard or sample solution was transferred into a test tube, followed by the addition of 2.85 mL of DPPH solution (0.03 mg/mL). The mixture was vortexed thoroughly and incubated for 30 min at room temperature in the dark. After incubation, the absorbance was measured at 517 nm using a UV–Vis spectrophotometer. The absorbance values were plotted against ascorbic acid concentrations to construct a standard curve based on the linear regression equation $y = bx + a$, where y represents absorbance and x indicates ascorbic acid concentration. Antioxidant activity was expressed as ascorbic acid equivalent antioxidant capacity. All analyses were performed in triplicate (Djamaludin *et al.* 2025).

Antioxidant Activity: Ferric Reducing Antioxidant Power (FRAP) Assay

For sample preparation, hydrolyzed protein and crude extract protein samples were diluted with distilled water to obtain concentrations of 5, 10, 15, 20, and 25 ppm. A volume of 0.15 mL of each diluted sample was mixed with 2.85 mL of freshly prepared FRAP reagent in a test tube. The mixture was homogenized using a vortex mixer and incubated for 30 min at room temperature in the dark. After incubation, the absorbance was measured at 593 nm using a UV–Vis spectrophotometer. The antioxidant concentration in each sample was determined using a Trolox standard curve and expressed as µg TE/mL (Molina *et al*. 2020). All analyses were performed in triplicate.

Antioxidant Activity: 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonat) (ABTS) Assay

The ABTS radical solution was prepared using 7 mM ABTS and activated with potassium persulfate solution prior to analysis. For sample preparation, hydrolyzed protein and crude extract protein samples were diluted with distilled water to obtain concentrations of 5, 10, 15, 20, and 25 ppm. Ascorbic acid was used as the antioxidant standard at concentrations of 0, 50, 100, 150, 200, and 250 µM/mL to construct the calibration curve. Subsequently, 0.15 mL of standard or sample solution was mixed with 2.85 mL of ABTS reagent and incubated for 30 min at room temperature in the dark. After incubation, the absorbance was measured at 734 nm using a UV–Vis spectrophotometer. All analyses were performed in triplicate. Antioxidant activity was determined based on the ascorbic acid standard curve and expressed as ascorbic acid equivalent antioxidant capacity (Molina *et al.* 2020).

Cytotoxicity Analysis on MCF-7 Cancer Cells

MCF-7 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin, and maintained at 37 °C in a humidified incubator containing 5% CO_2. Cells were harvested and seeded into 96-well plates at a density of $1x10^4$ cells/well in 100 µL medium, followed by incubation for 24 h to allow cell attachment. The hydrolyzed protein and crude extract protein samples were prepared in culture medium and tested at concentrations of 62.5, 125, 250, 500, and 1000 ppm. The maximum concentration was limited to 1000 ppm to avoid excessive sample turbidity and interference with absorbance measurements during the MTT assay. Untreated cells served as the negative control, while medium without cells was used as the blank control. After treatment for 24 h, the culture medium was removed, and the wells were rinsed with PBS. Subsequently, 100 µL of MTT reagent solution [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] at a working concentration of 0.5 mg/mL was added to each well, including media controls. The plate was incubated for 3–4 h at 37 °C in a 5% CO_2 atmosphere until purple-blue formazan crystals

formed. Cell morphology was observed under an inverted microscope prior to the addition of 100 µL of 10% Sodium Dodecyl Sulfate (SDS) in 0.1 N HCl as a solubilizing solution. The plate was wrapped in aluminum foil and incubated overnight at room temperature in the dark. Absorbance was measured at 595 nm using an ELISA reader, and IC_{50} values were calculated based on cell viability percentages. An identical analytical procedure was performed for both the hydrolyzed protein and crude extract protein samples. All analyses were conducted in triplicate (Putra *et al*. 2025).

Statistical Analysis

Residual data from the experimental results were analyzed to evaluate the assumptions of normality and homogeneity. When the statistical test yielded a $p > 0.05$, the residuals were considered normally distributed and homogeneous. Conversely, a $p < 0.05$ indicated that the data were not normally distributed and not homogeneous. If the assumptions of normality and homogeneity were met, a parametric statistical analysis was performed using one-way Analysis of Variance (ANOVA), followed by Tukey's post hoc test to determine significant differences between treatment groups. All analyses were conducted using SPSS software version 26 (IBM Corp., Armonk, NY, USA).

RESULTS

Yield

Based on the experimental results, it was observed that the extraction of crude protein from *S. platensis* using a combined freeze–thawing and ultrasonication method yielded a total of 0.8±0.02 g of crude protein from an initial 5 grams of dry biomass. This amount corresponds to an extraction efficiency of 16%, indicating that the applied physical disruption techniques were able to release a measurable portion of intracellular protein. Furthermore, during the subsequent enzymatic hydrolysis stage, the use of the alcalase enzyme to the extracted protein of *S. platensis* resulted in the formation of a protein hydrolysate with a final mass of 0.61±0.02 g. This outcome reflects a hydrolysis yield of 76.25%, demonstrating that alcalase was highly effective in converting the extracted protein into soluble hydrolysate fractions.

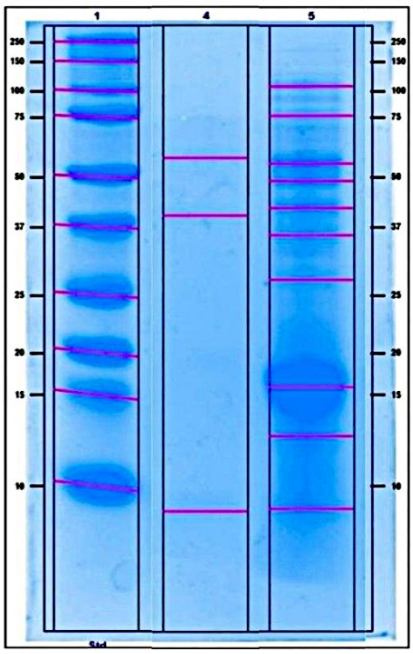


Figure 1. Profile of molecular weight (1. marker stain; 4. protein hydrolysate; 5. crude protein extract).

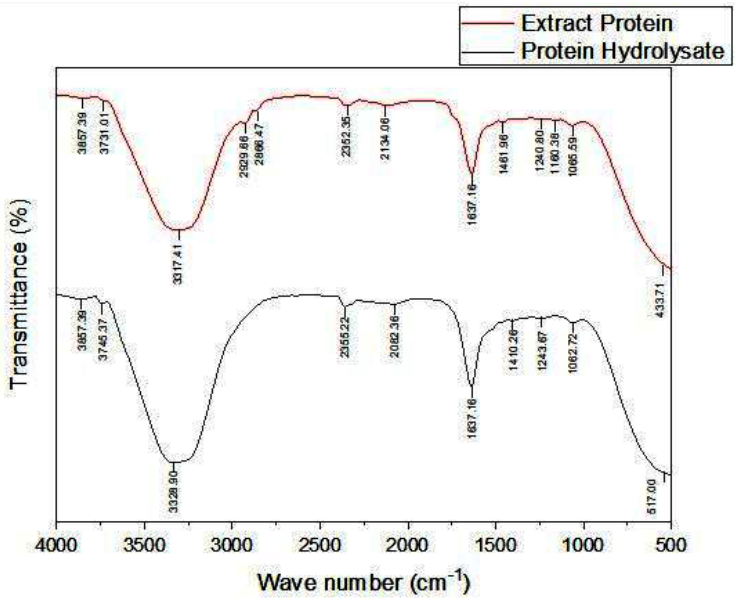


Figure 2. FTIR spectra of crude extract and protein hydrolysate of *S. platensis*.

Degree of hydrolysis

Based on these values, the calculated degree of hydrolysis (DH) for the *S. platensis* hydrolysate was 44.1±0.20%. This relatively high DH indicates that a substantial proportion of peptide bonds in the protein substrate were cleaved during the hydrolysis process, resulting

in the formation of smaller peptides and free amino acids. A higher DH is generally associated with improved solubility, digestibility, and potential bioactivity of the hydrolysate, which may enhance its functional and nutritional properties in food or nutraceutical applications. Furthermore, the extent of hydrolysis suggests that the enzymatic treatment was effective in breaking down the protein matrix of *S. platensis*, facilitating the release of bioactive peptide fractions.

Molecular weight

The molecular weight analysis results of crude protein extract and protein hydrolysate of *Spirulina platensis* using SDS-PAGE are presented in Fig. 1. In lane 1, the marker stain displayed 10 bands, with the tenth band showing the lowest molecular weight of 10.0 kDa (Rf = 0.761), and the first band showing the highest molecular weight of 250.0 kDa (Rf = 0.027). The molecular weight analysis of the *Spirulina platensis* protein hydrolysate, produced by enzymatic hydrolysis with alcalase, is shown in lane 4, which shows the formation of 3 bands. The first band had the highest molecular weight at 70.9 kDa with an Rf value of 0.219, while the third band had the lowest molecular weight at 6.7 kDa with an Rf value of 0.803. Meanwhile, the molecular weight analysis of the crude protein extract from *S. platensis*, shown in lane 5, revealed 10 bands. The first band had the highest molecular weight of 114.3 kDa (Rf = 0.101), while the tenth band had the lowest molecular weight of 6.8 kDa (Rf = 0.799). The SDS-PAGE molecular weight analysis yielded a lane length of 7.54 mm and a linear regression equation of $y = –1.75x + 2.23$ with an R^2 value of 0.952598.

Amino acid profile

The results of this study indicated changes in amino acid concentrations after hydrolysis. Several amino acids showed increased levels after enzymatic hydrolysis using alcalase, including L-alanine (600.51 to 640.44 mg/kg), L-arginine (490.77 to 573.82 mg/kg), glycine (457.08 to 538.52 mg/kg), L-valine (398.95 to 486.5 mg/kg), L-serine (398.95 to 589.48 mg/kg), and L-threonine (357 to 432.59 mg/kg) (Table 1). This increase indicates that hydrolysis successfully broke peptide bonds within the protein structure, releasing more free amino acids. Conversely, decreases were observed in L-aspartic acid (682.76 to 656.67 mg/kg), L-glutamic acid (1322 to 1240.94 mg/kg), and L-proline (233.88 to 268.84 mg/kg). Among these, L-glutamic acid was the most abundant amino acid in both crude extract and hydrolysate, while L-serine showed a notable increase, indicating that hydrolysis using alcalase significantly facilitates the release of L-serine from the polypeptide chain.

Table 1. Amino acid profile of crude extract and protein hydrolysate of *S. platensis*.

Amino acid type	Amino acid	Amino acid content	
		Crude extract protein (mg/Kg)	Hydrolysate Protein (mg/Kg)
Hydrophobic (non-polar)	L-Proline	233.88	268.84
	L-Isoleucine	314.20	346.85
	L-Valine	398.95	486.5
	L-Leucine	428.42	490.14
	Glycine	457.08	538.52
	L-Alanine	600.51	640.44
Hydrophilic (polar/charged)	L-Threonine	357	432.59
	L-Lysine	377.75	336.07
	L-Serine	398.95	589.48
	L-Arginine	490.77	573.83
	L-Aspartic Acid	682.72	656.67
	L-Glutamic Acid	1322	1240.94
Aromatic	L-Phenylalanine	476.07	476.07
	L-Tyrosine	608.01	608.01

Fourier transform infrared spectra

The Fourier Transform Infrared (FTIR) spectra demonstrated clear differences between the crude protein extract and the alcalase-hydrolyzed protein derived from *S. platensis*, as illustrated in Fig. 2. The FTIR spectrum of the crude protein extract exhibited a broad absorption band around 3275 cm^{-1}, which is attributed to O–H and N–H stretching vibrations, along with well-defined amide I and amide II bands located at approximately 1638 cm^{-1} and within the range of 1510–1530 cm^{-1}, respectively. These characteristic peaks indicate the presence of intact protein structures in the untreated extract. Conversely, the hydrolyzed protein sample showed noticeable shifts in peak positions and variations in the intensity of the amide bands, reflecting the cleavage of peptide bonds and modifications in the secondary structure of the protein as a result of enzymatic hydrolysis. Moreover, an increase in absorption intensity near 3270 cm^{-1} was observed in the protein hydrolysate, which can be associated with the formation of free amino and amine groups originating from smaller peptide fragments. These spectral alterations confirm the effective action of alcalase in degrading complex protein molecules into low-molecular-weight peptides, which may contribute to the enhancement of functional properties, including antioxidant and antiproliferative activities.

Antioxidant activity: DPPH scavenging

Based on the results of one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test, the samples were classified into three statistically significant groups, as indicated by different superscript letters in Table 2 ($p < 0.05$). All measurements were performed in triplicate (n = 3), and the data are presented as mean ± standard deviation. The IC_{50} values were calculated from the linear regression equation obtained by plotting sample concentration against percentage of DPPH radical inhibition, with correlation coefficients (R^2) exceeding 0.99 for all calibration curves. Among the tested samples, the crude protein extract of *S. platensis* exhibited the highest IC_{50} value (185±7.25 ppm), indicating relatively weak antioxidant activity due to the higher concentration required to inhibit 50% of DPPH free radicals. In contrast, the *S. platensis* protein hydrolysate showed a significantly lower IC_{50} value (44±2.04 ppm), demonstrating a marked improvement in antioxidant activity after enzymatic hydrolysis. The enhanced antioxidant capacity of the hydrolysate may be attributed to the release of bioactive peptides and amino acids with higher radical-scavenging potential during the hydrolysis process. Ascorbic acid, used as the positive control, exhibited an IC_{50} value of 39±1.18 ppm, confirming the validity of the assay system.

Table 2. IC_{50} of crude extract, protein hydrolysate of *S. platensis* and ascorbic acid (positive control).

Sample	IC_{50} (ppm)	Antioxidant Activity
Crude Protein	185±7.25[a]	Weak
Protein Hydrolysate	44±2.04[b]	Very Strong
Ascorbic acid	39±1.18[b]	Very Strong

Note: The result in the same column followed by the different letter which significantly differ by Tukey's test ($p < 0.05$).

Antioxidant activity: Ferric reducing antioxidant power (FRAP)

Based on the results of one-way analysis of variance (ANOVA) followed by Tukey's post-hoc multiple comparison test, the samples were classified into three statistically different groups based on their antioxidant activity, as presented in Table 3. Different superscript letters in the table indicate significant differences among samples at $p < 0.05$. All measurements were performed in triplicate (n = 3), and the data are expressed as mean ± standard deviation. The *S. platensis* protein hydrolysate exhibited a significantly lower IC_{50} value (22±1.04 µg TE/mL) compared with the crude protein extract (39±1.18 µg TE/mL), indicating that enzymatic hydrolysis improved the antioxidant capacity of the protein. The enhanced antioxidant activity

of the hydrolysate may be associated with the release of low-molecular-weight peptides and amino acid residues with higher reducing capacity during hydrolysis. Based on the IC_{50} classification criteria, both the crude extract and hydrolysate were categorized as having very strong antioxidant activity.

Table 3. IC_{50} of crude extract, protein hydrolysate of *Spirulina platensis* and Trolox (positive control).

Sample	IC_{50} (µg TE/mL)	Antioxidant Activity
Crude Protein	39±1.18[a]	Very Strong
Protein Hydrolysate	22±1.04[b]	Very Strong
Trolox	12±0.24[c]	Very Strong

Note: The result in the same column followed by the different letter which significantly differ by Tukey's test ($p < 0.05$).

Antioxidant activity: 2,2'-azino-bis(3-etilbenzotiazolin-6-sulfonat) (ABTS)

Based on the results of one-way ANOVA followed by Tukey's post hoc test (n = 3), as presented in Table 4, significant differences in antioxidant activity were observed among the tested samples. Different superscript letters in the table indicate statistically significant differences at $p < 0.05$. The IC_{50} values were calculated from the linear regression equation obtained by plotting sample concentration against ABTS radical scavenging activity (% inhibition), with correlation coefficient (R^2) values exceeding 0.99 for all standard and sample calibration curves. Prior to analysis, each sample was prepared at several concentrations and reacted with ABTS solution under controlled conditions as described in the method section. Among the tested samples, the crude protein extract of *S. platensis* exhibited the highest IC_{50} value (76.88±1.17 ppm), indicating lower antioxidant activity compared to the hydrolyzed protein sample. In contrast, the *S. platensis* protein hydrolysate showed a significantly lower IC_{50} value (9.03±0.25 ppm), suggesting that enzymatic hydrolysis enhanced the antioxidant activity of the protein. The improved antioxidant activity of the hydrolysate may be associated with the release of low-molecular-weight peptides and amino acids during hydrolysis, which are known to contribute to higher radical scavenging activity. Ascorbic acid, used as the positive control, showed the lowest IC_{50} value (1.79±0.05 ppm), confirming the validity of the assay system.

Table 4. IC_{50} of crude extract, protein hydrolysate of *S. platensis* and ascorbic acid (positive

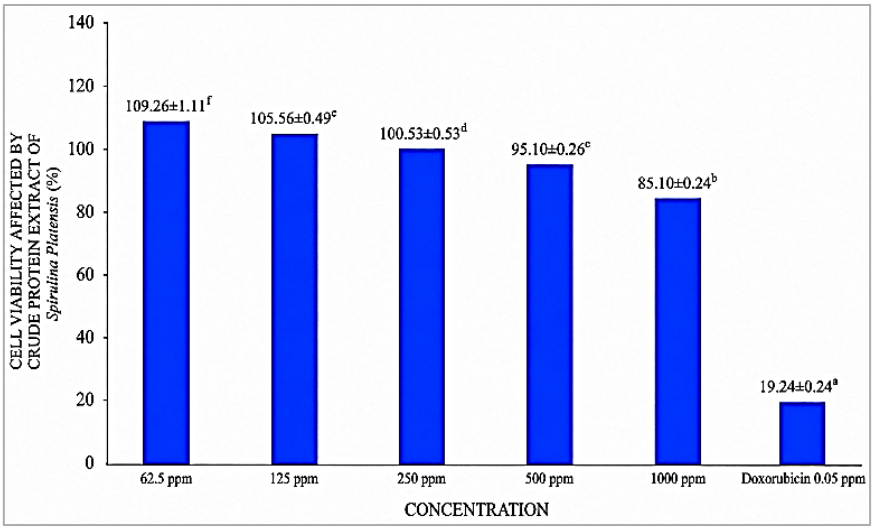
control).

Sample	IC_{50} (ppm)	Antioxidant Activity
Crude Protein	76.88±1.17[a]	Strong
Protein Hydrolysate	9.03±0.25[b]	Very Strong
Ascorbic acid	1.79±0.05[c]	Very Strong

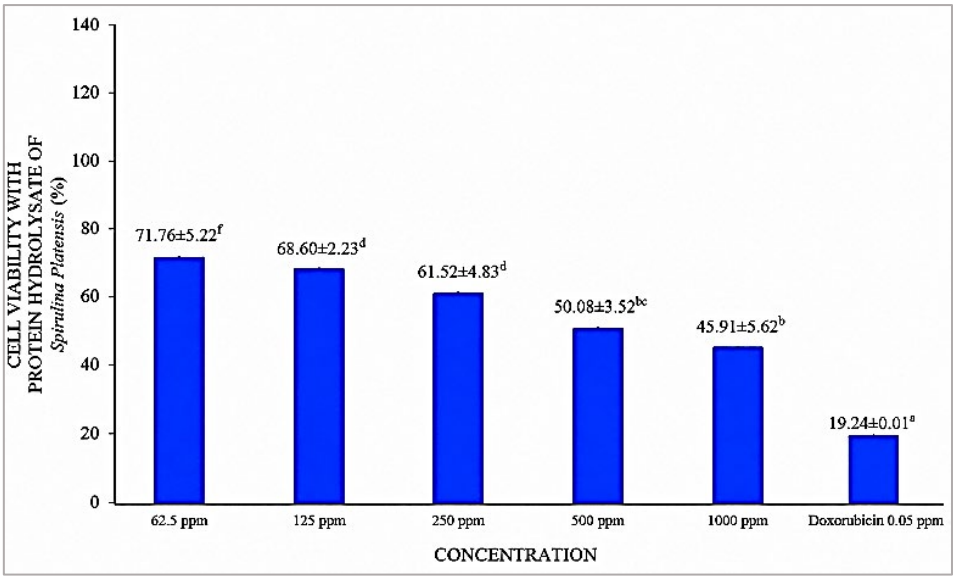
Note: The results in the same column followed by the same letter do not significantly differ by Tukey's test ($p < 0.05$).

Cytotoxicity

The antiproliferative activity of *S. platensis* crude protein extract and protein hydrolysate against MCF-7 breast cancer cells was evaluated using ANOVA, which showed that increasing concentrations significantly affected cell viability ($p < 0.05$) (Fig. 3). For the crude protein extract, Tukey's post-hoc test revealed significant differences among concentration groups. The highest cell viability was observed at 62.5 ppm (109.27%), followed by 125 ppm (105.57%) and 250 ppm (100.53%). Cell viability decreased with increasing concentration, reaching 95.10% at 500 ppm and 85.08% at 1000 ppm. The positive control, doxorubicin, showed markedly lower viability, indicating strong cytotoxic activity.



(a)



(b)

Figure 3. Cell viability (%) of MCF-7 breast cancer cells treated by crude protein extract (a) and protein hydrolysate (b) of *S. platensis*. Note: The results in the same bar graph followed by the same letter do not significantly differ by Tukey's test ($p < 0.05$).

Similarly, the protein hydrolysate exhibited a significant concentration-dependent reduction in MCF-7 cell viability ($p < 0.05$). The highest viability was recorded at 62.5 ppm (71.76±5.22%), followed by 125 ppm (68.60±2.23%) and 250 ppm (61.52±4.83%). Further increases in concentration resulted in lower viability values of 50.08±3.52% at 500 ppm and 45.91±5.62% at 1000 ppm. Doxorubicin again produced the lowest cell viability (19.24±0.01%), confirming its strong cytotoxic effect. The protein hydrolysate was more effective than the crude protein extract in reducing MCF-7 cell viability across all tested concentrations. At 1000 ppm, the hydrolysate reduced cell viability to 45.91±5.62%, compared to 85.10±0.24% for the crude protein extract. This enhanced antiproliferative activity is likely associated with protein hydrolysis, which generates smaller bioactive peptides that may interact more effectively with cellular targets, resulting in stronger cytotoxic and antiproliferative effects against MCF-7 breast cancer cells.

DISCUSSION

The antiproliferative efficacy of *S. platensis* observed in this study is intrinsically linked to its biochemical profile, as characterized by FTIR, SDS-PAGE, and amino acid analysis. FTIR spectra confirmed the presence of functional groups, specifically Amide I and II, which are characteristic markers of bioactive peptides. These structural findings are supported by SDS-

PAGE results, which revealed a dominance of low-molecular-weight peptides (< 3 kDa). The prevalence of these small fractions is crucial, as smaller peptides possess enhanced cellular permeability and structural stability, allowing them to bypass cellular barriers and effectively reach intracellular targets (Wang & Zhang 2016a). The amino acid profile further elucidates the mechanism of action. The presence of aromatic residues (tyrosine, phenylalanine) and hydrophilic residues (serine, threonine) facilitates π-π interactions and hydrogen bonding with cancer cell membranes, leading to destabilization and mitochondrial dysfunction (Jiménez & López 2023; Otero & Verdasco 2023). While crude proteins also contain these amino acids, the enzymatic hydrolysis process employed in this study is the key differentiator; hydrolysis liberates these residues from the complex protein matrix, significantly increasing their bioavailability and active concentration compared to the unhydrolyzed form. This explains why the protein hydrolysates in this study exhibited more pronounced cytotoxic effects than the crude extracts reported in previous literature.

The antioxidant capacity of *S. platensis* peptides was evaluated through three complementary assays, each revealing different facets of their protective mechanism. The DPPH and ABTS assays primarily measure the ability of peptides to scavenge free radicals through Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET), respectively. The variance in these results arises from the chemical environment of the assays; ABTS is applicable to both hydrophilic and lipophilic systems, whereas DPPH is more sensitive to hydrophobic compounds. Conversely, the FRAP assay measures reducing power (Fe^{3+} to Fe^{2+}), indicating the peptides' ability to act as electron donors to terminate oxidative chain reactions.

Compared to known commercial antioxidants, these *Spirulina*-derived peptides demonstrate competitive radical scavenging activity (Zeng *et al*. 2020). From a therapeutic perspective, excessive oxidative stress contributes to tumor initiation and progression by inducing DNA damage and genetic instability (Wang & Zhang 2017). Therefore, the ability of these peptides to lower intracellular ROS levels and neutralize free radicals not only protects normal cells but also facilitates apoptosis in tumor cells by stabilizing the cellular redox environment (Di Carlo & Sorrentino 2024; Ma *et al*. 2021; Tajvidi *et al*. 2021).

In the MTT assay, *S. platensis* extracts exerted concentration-dependent cytotoxic effects on MCF-7 breast cancer cells, resulting in a progressive reduction in cell viability. At the tested doses, the samples significantly inhibited cancer cell proliferation while exhibiting minimal toxicity toward normal human embryonic kidney (HEK293) cells (Bougatef *et al*. 2025). This selectivity is driven by the amino acid composition; positively charged residues (lysine and arginine) enhance electrostatic interactions with the negatively charged phosphatidylserine

exposed on cancer cell membranes, increasing permeability and facilitating intracellular uptake (Moustafa *et al*. 2021). Furthermore, histidine residues promote membrane destabilization in acidic tumor microenvironments, while aspartic and glutamic acids contribute to cell cycle arrest (Meloni *et al*. 2020).

Mechanistically, these peptides suppress proliferation by inhibiting the BCR-ABL signaling cascade and downregulating the PI3K/Akt pathway, both essential for tumor survival (Prabakaran *et al*. 2020). They further disrupt mitochondrial membrane potential, triggering cytochrome c release and activation of caspase-dependent intrinsic apoptosis pathways (Śmieszek *et al*. 2017b). Regarding the assay conditions, while some studies Gonzales *et al*. (2010) show that IC_{50} values decrease with prolonged incubation, it is important to clarify that incubation time was kept constant in the present study. This was intentional to focus specifically on the dose-response relationship and the efficacy of specific peptide fractions.
The high concentration of hydrophobic residues, such as leucine, valine, and isoleucine, enhances peptide affinity for lipid-rich cancer membranes, leading to direct membrane lysis (Insuasty-Cepeda *et al*. 2023). Ultimately, the balanced amino acid profile and the formation of small, bioactive peptides through gastrointestinal or alcalase enzymes are the key determinants of the potent antiproliferative potential of S. platensis protein hydrolysates (Aini & Darmanto 2025; Wang & Zhang 2016b).

CONCLUSION

This study confirms that *Spirulina platensis* protein hydrolysates produced by alcalase exhibit significant, concentration-dependent cytotoxic activity against MCF-7 breast cancer cells, supported by their physicochemical characteristics, strong antioxidant activity, and structural modifications resulting from successful protein hydrolysis, which together suggest that antioxidant mechanisms may contribute to the observed cytotoxic effects. Further studies are required to identify active peptides, clarify mechanisms of action, and validate these effects through *in vivo* experiments before practical application can be considered.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was funded by Universitas Brawijaya, Indonesia, through the Universitas Brawijaya Student Academic Research Scheme (UB STARS) Program 2024, Grant No. 02886/UN10.A0101/B/RT.01.00/2024. The authors would like to thank the Faculty of Fisheries and Marine Science, Universitas Brawijaya, for providing laboratory facilities and technical support. The authors also acknowledge the Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research and Innovation Agency (BRIN), Cibinong, for facilitating this study.

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