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RESEARCH PAPER

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Unveiling the Bioactive Potential of *Colaconema formosanum*: An in Silico Exploration of Novel Peptides from Phycobiliproteins

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Abstract

Colaconema formosanum, new Rhodophyta in Taiwan. However, currently, there is little documented research on the protein bioactivity of this species. The objectives of this study were to conduct an in silico assessment of *C. formosanum* proteins as possible sources of bioactive peptides. Six proteins from *C. formosanum*, phycobiliproteins group, were selected based on LC-MS/MS analysis and these proteins were verified using the Mascot database. Subsequently, in silico analysis was started by checking the protein's physicochemical properties, followed by the bioactive peptide activities in the BIOPEP-UWM™ database. Additional parameters, including the theoretical degree of hydrolysis, value and relative frequency were also assessed. The peptides were ranked using PeptideRanker to screen for novel promising bioactive, and subsequently, an evaluation of the proteins' allergenicity and toxicity was conducted. Various bioactive activities, such as an inhibitor of ACE, DPP-IV, DPP-III, alpha-glucosidase, glutamate-carboxypeptidase, leucyltransferase, antioxidant, anti-inflammatory, and other activities were generated using in silico proteolysis of phycobiliproteins employing five proteases. In silico results indicated that phycobiliproteins from *C. formosanum* possess significant potential as a valuable reservoir of bioactive peptides. No previous reports have been made about the in silico analysis of this species. These discoveries present novel prospects for utilizing these bioactive peptides in the pharmaceutical and biotechnology industry.

Introduction

Red seaweed, classified under the phylum Rhodophyta, is a highly abundant bioactive protein reservoir that has undergone substantial research due to its potential health-promoting properties (Cotas et al., 2020). These protein sources are abundant in protein and have a superior amino acid composition similar to other familiar protein sources. Algae protein comprises bioactive constituents, including free amino acids, peptides, and phycobiliproteins (Torres & Domínguez 2019; Lafarga et al., 2020; Carpena et al., 2022; Thiviya et al., 2022). Phycobiliproteins are a group of pigmented and water-soluble proteins that are present in the majority of Rhodophyta. This protein is categorized into three primary types: allophycocyanin, phycocyanin, and phycoerythrin (Chen et al., 2022). These proteins exhibit a range of bioactivities, including antioxidant, anti-inflammatory, and immunomodulatory effects (Cian et al., 2015). Also, they have significant pharmaceutical potential and are considered a valuable component of the red seaweed's nutritional profile, these areas of research show promise for the creation of nutraceuticals and therapeutic applications (Torres & Domínguez 2019; Gamero-Vega et al., 2020).

Colaconema formosanum is a recently identified species of red algae found in Taiwan, characterized by its unique morphology and molecular features. This species, belonging to the family Colaconemataceae, is notable for its ability to infect the cortical tissues of its host, Sarcodia suae, and exhibit a range of bioactive compounds. The molecular characterization of *C. formosanum* has been extensively studied, with research focusing on its phylogenetic relationships and the potential applications of its bioactive compounds in various fields, including medicine and cosmeceuticals (Lee et al., 2021a; Lee et al., 2021b; Yeh et al., 2022; Windarto et al., 2024a; Windarto et al., 2024b)

Bioactive peptides are defined as certain protein fragments that have a beneficial impact on the functioning or circumstances of the body and may have an impact on health that goes beyond their nutritional worth (Sánchez & Vázquez 2017; Chakrabarti et al., 2018; Akbarian et al., 2022). Bioactive peptides can be obtained through various methods, including enzymatic hydrolysis (Windarto et al., 2022; Olvera-Rosales et al., 2023), microbial fermentation, chemical methods, food processing (Nong & Hsu 2022). These methods can be used individually or in combination to produce bioactive peptides with specific properties and applications.

Bioinformatics tools and databases play a crucial role in the exploration and examination of bioactive peptides obtained from food, including BIOPEP-UWM, APD3, ACEpepDB, BioPD, BioPepDB, and CAMP (Terziyski et al., 2023). These databases facilitate researchers in effectively identifying and characterizing these peptides and determining their potential bioactivities (Agyei et al., 2018; Di Leva et al., 2020; Du et al., 2023). The in silico study on bioactive peptides involves using computational methods to analyze and predict the properties and functions of peptides derived from plants, animals, and food sources. This approach is beneficial for identifying and characterizing bioactive peptides that may offer health benefits, like antioxidant, antimicrobial, or antihypertensive properties. In silico analysis of bioactive peptides typically involves several stages: protein sequence analysis, in silico proteolysis, bioinformatics tools, molecular docking, and in silico validation. The in silico study of bioactive peptides is a powerful tool for identifying and investigating peptides that may have positive effects on health, and it has significant implications for the development of pharmaceuticals, nutraceuticals, and functional foods (Langyan et al., 2021; Senadheera et al., 2022; Peredo-Lovillo et al., 2022; Tonolo et al., 2023).

Currently, there has been no research carried out on the computational assessment of bioactive peptides derived from indigenous *C. formosanum* proteins. Therefore, this study aims to assess the capability of *C. formosanum* proteins to serve as precursors for bioactive peptides through in silico approaches, including evaluating the physicochemical properties, potential precursors for bioactive peptides, toxicity, and allergenicity.

Material and Methods

C. formosanum Preparation

This study used *C. formosanum*, obtained from NTU in Taiwan. The red algae was rinsed with purified water to eliminate any particles or remains, then dried at 40°C for two hours, subsequently crushed into a fine powder, and after that stored at 4 °C.

Extraction and TCA Precipitation

For the extraction and TCA precipitation process, sodium dodecyl sulphate (SDS) solution was carefully mixed with the red algal powder at 1:4 (w/v). The cell disruption was applied using Branson Digital Sonifier® (Terra Universal Inc., LA, USA). The solution was fractionated using Hitachi centrifugation (CT15RE, Hitachi Koki Co., Ltd., Japan) and then freeze-dried. The dehydrated *C. formosanum* powder was mixed with TCA (1:3 w/v), and then dissolved at 4 °C for 12 hours. TCA was eliminated using acetone, and then the solid was freeze-dried.

Identification of *C. formosanum*

Protein Profiling

The protein composition of *C. formosanum* was analyzed by separating it using SDS-PAGE. The lyophilized *C. formosanum* was immersed in a solution containing 2% SDS and then subjected to homogenization. Electrophoresis was done twice, 30V for 30 min and 100V for 90 min. After 15 minutes in a fixing buffer, the gel was colored with Coomassie® brilliant blue R-250. Add a destaining buffer to finish staining.

In Gel Digestion of *C. formosanum*

Protein bands were analyzed using in-gel digestion to confirm the protein composition (Windarto et al., 2022). The protein bands were individually excised and dispersed in 100 µL of 50 mM DTT in 25 mM ammonium bicarbonate (ABC) at 37°C for 1 hour. The liquid above the solid residue was extracted, and the protein bands were exposed to 100 µL of 100 mM IAM in 25 mM ABC in a dark room for 30 minutes. The liquid part was extracted, and the gel was treated with 100 µL of a solution containing 50% acetonitrile (ACN) in 25 mM ABC many times to eliminate the staining (until it became colorless). The gel fragments were desiccated using 100 µL of 100% ACN for 5 minutes until the gel contracted and then subjected to centrifugation. The gel was lyophilized at 1200 rpm for 5 minutes to eliminate ACN. Subsequently, approximately 100-150 µL of a 25 mM ABC solution was introduced into the gel, and the gel was agitated with a stick. The gel was submerged in a solution with a concentration of 25 mM ABC, and

trypsin was added (1:20). The gel was then incubated at room temperature for 16 hours. The tryptic peptide was washed using 50 µL of a solution containing 50% ACN and 5% trifluoroacetic acid (TFA). The peptide was sonicated, followed by centrifugation, and stored the liquid at -20°C.

Identification using Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS)

The sample was dissolved in a solution of 5% ACN and 0.2% FA in deionized water to prepare it for further analysis using LC-MS/MS. Electrospray ionization (ESI) mode was used in mass spectrometers. The sample was injected into the LC symmetric C18 column bio basic. The Thermo-Xcalibur™ data collecting equipment was used to obtain the mass spectra data. The LC-MS/MS study used a positive ion mode ion trap analyzer. A mass spectrometry scan was conducted across a range of m/z 100 to m/z 1000 at a 200 µl/min flow rate.

Mascot Search Database

The amino acid sequence was ascertained using database-assisted identification using Mascot Distiller. The MS/MS data were processed using XCalibur software. Mascot Distiller v2.3.2.0 and Mascot search engine v2.3 (Matrix Science, UK) were used for MGF file conversion and database searching. The database parameter for protein and peptide matching involved using the *Dichotomaria marginata* database from NCBI (Windarto et al., 2022). The database was chosen because the protein of *C. formosanum* has yet to register fully, and *D. marginata* is in the same subclass (Lee & Yeh 2021). This approach is advantageous in proteomics studies. It can offer crucial information about the protein sequence of the target species, even if the protein sequence itself is not directly available (Blakeley-Ruiz & Kleiner 2022).

In Silico Analysis

Discovering the Physicochemical Characteristics of the *C. formosanum* Protein

ExPASy's ProtParam (https://web.expasy.org/protparam/) was utilized to identify the physicochemical properties of *C. formosanum* protein, such as the total amino acid (AA), the theoretical pI, formula, negatively and positively charged residues, aliphatic and instability index, and the grand average of hydropathicity (GRAVY).

Evaluation of *C. formosanum* as a Potential Precursor for Bioactive Peptides

The probability of liberating bioactive peptides for the chosen proteins was analyzed using the BIOPEP-UWM™ database (Minkiewicz et al., 2019). The segment exhibiting the most significant biological activity was selected for reporting. The database estimated the frequency of fragments with a certain activity (A) of *C. formosanum* was evaluated.

Proteolysis and Screening of Protein Sequences: in Silico

The proteins of *C. formosanum* were examined using in silico proteolysis utilizing BIOPEP's enzyme-action tool. Each protein sequence was independently subjected to hydrolysis by five distinct enzymes: chymotrypsin, trypsin, proteinase K, thermolysin, and pepsin (pH > 2). The values of frequency (A_E) and relative frequency (W) of releasing peptides by specific protease were calculated. The parameters of V and theoretical degree of hydrolysis (DHt) were also calculated. The protein sequence screening was computed using the PeptideRanker program (www.distilldeep.ucd.ie/PeptideRanker/).

Prediction Score of the Toxicity and Allergenicity

The toxicity and allergenicity of bioactive peptides were assessed using ToxinPred and AllergenFP, as described by Gupta et al., (2013) and Dimitrov et al., (2014), respectively. ToxinPred can be accessed at http://crdd.osdd.net/raghava/toxinpred/ and AllergenFP at http://ddg-pharmac.net/AllergenFP/.

Computational Screening and Characterization of Novel Peptides

The biologically active *C. formosanum* protein tripeptide fragments were carefully enumerated. BIOPEP-UWM™ displays database fragments and activity. PeptideRanker, a bioinformatics tool at http://distilldeep.ucd.ie/PeptideRanker, assessed peptide fragment bioactivity (Mooney et al., 2012). This study used a cutoff >0.5.

Results and Discussion

In silico methods can identify novel bioactive peptides; however, they have limitations. With enzyme cutting sites properly digested, in silico proteolysis was excellent. The BIOPEP database, which is updated regularly, was used for in silico proteolysis. The study results may change when additional data becomes available. Pooja et al. (2017) said that in silico proteolysis is a cost-effective method for finding bioactive peptides because it requires less enzyme, substrate, and time. This approach has excellent throughput, precision, and flexibility and can process multiple sequences quickly. Databases, bioinformatics tools, and software were used for in-silico proteolysis to release bioactive peptides from *C. formosanum* protein.

The sample underwent SDS-PAGE-based protein profiling to determine the protein it originated from and evaluate its complexity, quantity, and distribution in

C. formosanum. The SDS-PAGE gel demonstrated the existence of two distinct protein bands (Figure 1a). The proteins were described through in-gel trypsin digestion, LC-MS/MS analysis (Figure 1b), and mascot search. The study indicated that the protein phycoerythrin subunits- a and b- were the predominant proteins detected in the most prominent band, with a molecular weight of approximately 17.68 kDa.

The investigation conducted by Zhao et al., (2013) revealed that the bands within the molecular weight range of 16-20 kDa were recognized as phycobiliproteins (α and β subunits), more especially phycoerythrins. The SDS-PAGE profiles of phycoerythrin (B, upper) in the study of Aslam et al., (2019) showed a range of molecular weights between 6.5 and 116 kDa; phycobiliproteins from Porphyra ranged from 10-100 kDa (Huang et al., 2021); B-phycoerythrin from *Rhodosorus marinus* show an MW of approximately 7 kDa for the α subunit and 18 kDa for the β subunit (Básaca-Loya et al., 2009). This indicates that the phycoerythrin protein comprises multiple subunits with varying molecular weights (Aslam et al., 2019). SDS-PAGE results reveal phycoerythrin and red algal phycobiliprotein content and structure. By studying their molecular weight distribution, they help identify, characterize, and understand these proteins' subunits and functions.

LC-MS/MS and Mascot search are powerful tools for identifying peptides and proteins in biological samples. The results of the Mascot search are ranked based on the confidence of the match, and the accession number can be used to retrieve additional information about the identified protein. Table 1 shows the result of the Mascot search and the predicted protein from *C. formosanum*.

According to the results, most of the proteins in *C. formosanum* belong to phycobiliproteins with molecular masses ranging from 17441.7 to 18415.98 g/mol, including phycoerythrin subunit a, phycocyanin beta subunit, phycoerythrin subunit b, phycocyanin alpha subunit, allophycocyanin alpha subunit, and allophycocyanin beta subunit. These results aligned with SDS-PAGE, which showed that the band of most proteins was 16-20 kDa. Physicochemical properties of *C. formosanum* were identified using ExPASy's ProtParam. The result of the physicochemical parameters of predicted *C. formosanum* peptide sequences are shown in Table 2. Based on the result, the number of amino acids from six predicted proteins ranged from 38 – 114 residues. All predicted proteins' isoelectric point (pI) values were above 4 and less than 11. The instability index of all predicted protein sequences from *C. formosanum* was less than 40, and the aliphatic index was high (above 50). The protein sequences exhibit hydrophobic characteristics and are positively charged due to the GRAVY values falling from -2 to +2. These properties are crucial in understanding the behavior and function of a protein, as they influence its interactions with other molecules and its overall biological activity.

Information regarding the bioactive peptide compounds of *C. formosanum* and their activities has yet to be completely known. The BIOPEP database is an effective peptide bioactivity tool. It can model protein breakdown and predict peptide biological activity by studying their amino acid sequence (Minkiewicz et al., 2019; Pearman et al., 2020). Table 3 shows the total number of potential bioactive peptides from *C. formosanum* proteins.

Phycobiliproteins, such as phycoerythrin, phycocyanin, and allophycocyanin, are the most common proteins in red seaweed. The "A" value(s) in the BIOPEP-UWM^{TM} database showed how often a specific protein contains encrypted bioactive peptides (Minkiewicz et al., 2019). This is important because it shows how usually each fragment has bioactive properties. The data showed that the DPP-IV inhibitor was most likely to be released from the *C. formosanum* proteins (165), followed by the ACE inhibitor (137). Phycoerythrin subunit a showed the highest number of activities (92). Based on the result, the predicted peptide sequence from *C. formosanum* had 450 bioactive activities in total. The bioactive peptide activities of *C. formosanum* can be seen in Figure 2.

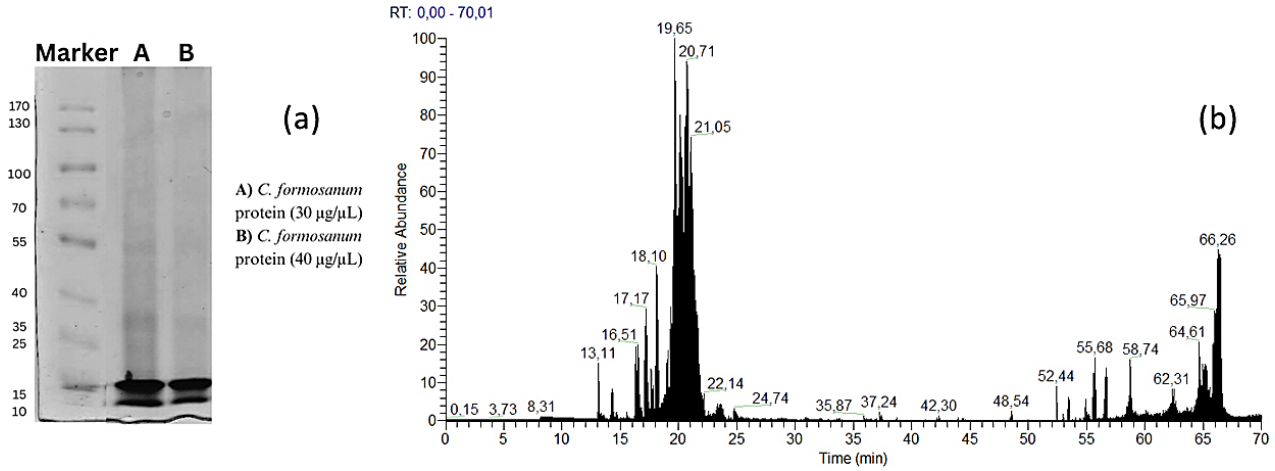

Figure 1. (a) SDS-PAGE and (b) LC-MS/MS Chromatogram of *C. formosanum* protein.

Table 1. The result of the Mascot search and the predicted protein from *C. formosanum*

Observed	Mr (expt)	Mr (calc)	Peptide Sequence	Score	Protein
475.2137	948.4129	948.4123	R.DIDHYMR.I	1194	Phycoerythrin subunit a
965.4200	964.4127	964.4073	R.DIDHYMR.I		
968.4124	967.4051	967.4069	K.EAGDACFAK.Y		
1037.5731	1036.5658	1036.5665	K.LAGNHEAVVK.E		
566.2500	1130.4855	1130.4840	K.NPGEAGDSQEK.V		
1131.4932	1130.4859	1130.4840	K.NPGEAGDSQEK.V		
726.3612	1450.7078	1450.7086	K.SVMTTTISAADAAGR.F		
1451.7153	1450.7080	1450.7086	K.SVMTTTISAADAAGR.F		
734.3593	1466.7041	1466.7035	K.SVMTTTISAADAAGR.F		
1467.7118	1466.7045	1466.7035	K.SVMTTTISAADAAGR.F		
736.8545	1471.6945	1471.6903	K.NPGEAGDSQEKVNK.C		
889.4376	1776.8607	1776.8642	R.FPSSSDLESIQGNIQR.A		
893.4185	1784.8225	1784.8217	K.YSYLKNPGEAGDSQEK.V		
595.9483	1784.8232	1784.8217	K.YSYLKNPGEAGDSQEK.V		
829.4518	828.4445	828.4454	K.VVAQADAR.G	250	Phycocyanin beta subunit
875.4652	874.4579	874.4582	R.DMEIVLR.Y		
709.3725	1416.7305	1416.7321	K.INSNASAIVTNSAR.A		
967.5142	1933.0139	1933.0157	R.ETYQALGTPGTSVAVAIQK.M		
709.6790	2126.0152	2126.0201	R.GEFLSNTQLDALATMVSEGK.K		
1064.0149	2126.0152	2126.0201	R.GEFLSNTQLDALATMVSEGK.K		
408.2343	814.4540	814.4548	R.DGEIILR.Y	203	Phycoerythrin subunit b
815.4613	814.4540	814.4548	R.DGEIILR.Y		
831.4565	830.4493	830.4498	R.VVVNSDAK.A		
416.2319	830.4493	830.4498	R.VVVNSDAK.A		
839.4086	838.4013	838.4007	MLDAFSR.V		
855.4011	854.3938	854.395	MLDAFSR.V		
893.4465	892.4393	892.4403	K.FIADGNTR.L		
646.8458	1291.6770	1291.6772	K.AAYVGGSDLQALKK.F		
1292.6845	1291.6772	1291.6772	K.AAYVGGSDLQALKK.F		
710.8938	1419.7731	1419.7722	K.AAYVGGSDLQALKK.F		
803.9241	1605.8336	1605.8362	K.ETYIALGVPTNSSVR.A		
831.4567	830.4494	830.4497	R.AAASLEAAK.S	93	Phycocyanin alpha subunit
438.7296	875.4446	875.4461	K.SLTNSAQR.L		
758.8839	1515.7532	1515.7529	K.TPITEAIASADSQGR.F		
893.4845	892.4772	892.4767	K.SFVLSGQR.R	91	Allophycocyanin alpha subunit
957.4665	956.4593	956.4603	R.DLDYYLR.L		
1045.5265	1044.5192	1044.5200	K.SIVNADAEAR.Y		
523.2669	1044.5193	1044.5200	K.SIVNADAEAR.Y		
525.2666	1048.5186	1048.5189	R.YLSPGELDR.I		
529.3027	1056.5909	1056.5927	R.IAQILTENR.E		
1057.5996	1056.5923	1056.5927	R.IAQILTENR.E		
957.4665	956.4593	956.4603	R.DLDYYLR.Y	70	Allophycocyanin beta subunit
676.3456	1350.6766	1350.6779	K.YLDDNSVEKLR.G		
880.9447	1759.8747	1759.8774	MQDAITSVINAADVQGK.Y		

Table 2. Physicochemical properties of predicted protein sequences from *C. formosanum*

Predicted Protein	Number of AA	Theoretical pI	Formula	Negatively charged residue (Asp + Glu)	Positively charged residues (Arg + Lys)	Instability index	Aliphatic index	GRAVY
Phycoerythrin subunit a	114	6.93	$C_{529}H_{845}N_{157}O_{179S4}$	18	18	38.88	54.04	-0.939
Phycocyanin beta subunit	78	9.87	$C_{358}H_{603}N_{109}O_{115S3}$	7	11	2.60	86.41	-0.227
Phycoerythrin subunit b	70	9.89	$C_{342}H_{560}N_{98}O_{100S1}$	7	12	16.23	97.57	-0.083
Phycocyanin alpha subunit	38	10.27	$C_{167}H_{285}N_{53}O_{57}$	3	6	16.52	75.00	-0.413
Allophycocyanin alpha subunit	53	9.69	$C_{278}H_{454}N_{86}O_{83}$	8	11	39.26	99.43	-0.732
Allophycocyanin beta subunit	40	4.92	$C_{207}H_{324}N_{56}O_{67}S_1$	7	6	15.02	87.75	-0.730

Bioactive peptides derived from macroalgae have been discovered to possess an inhibitory effect against dipeptidyl peptidase IV (DPP-IV), such as *Laminaria digitata* (Purcell et al., 2023) *Ulva* spp. (Cain et al., 2022); ACE inhibitory peptides from *Palmaria palmata* (Furuta et al., 2016), *Acrochaetium* sp. (Windarto et al., 2022); antioxidant peptides from *Palmaria palmata* (Beaulieu et al., 2016), *Colaconema formosanum* (Windarto et al., 2024); Alpha-glucosidase inhibitor peptides from *Cystoseira wrightiana*, *Ecklonia cava*, and *Ishige okamurae* (Ryu et al., 2023); neuropeptide from *Ulva lactuca* (Amin et al., 2022); anticancer peptide from *Enteromorpha prolifera* (Lin et al., 2022); anti-inflammatory peptide from *Amansia multifida* (Mesquita et al., 2021); antiviral peptide from *Grateloupia chiangii* (Hwang et al., 2020); and antimicrobial peptide from *Gracilaria fisheri* (Boonsri et al., 2017).

Table 3. Total number of *C. formosanum* proteins as bioactive peptides

Activities	Number of proteins as bioactive peptides						Total
	Phycoerythrin subunit a	Phycocyanin beta subunit	Phycoerythrin subunit b	Phycocyanin alpha subunit	Allophycocyanin alpha subunit	Allophycocyanin beta subunit	
ACE inhibitor	28 (0.245)	24 (0.307)	28 (0.400)	12 (0.315)	26 (0.490)	19 (0.475)	**137**
DPP-IV inhibitor	38 (0.333)	38 (0.487)	33 (0.471)	15 (0.394)	23 (0.434)	18 (0.450)	**165**
DPP-III inhibitor	7 (0.061)	5 (0.064)	5 (0.071)	1 (0.026)	6 (0.113)	4 (0.100)	**28**
Antioxidative	2 (0.017)	2 (0.025)	4 (0.057)	-	6 (0.113)	5 (0.125)	**19**
α-glucosidase inhibitor	2 (0.017)	2 (0.025)	2 (0.028)	2 (0.052)	3 (0.056)	3 (0.075)	**14**
Neuropeptide	1 (0.008)	-	1 (0.014)	-	4 (0.075)	1 (0.025)	**7**
Glutamate carboxypeptidase inhibitor	3 (0.026)	2 (0.025)	2 (0.028)	-	3 (0.056)	2 (0.050)	**12**
Inhibitor of cytosol alanyl aminopeptidase	1 (0.008)	-	1 (0.014)	2 (0.052)	-	1 (0.025)	**5**
Leucyltransferase inhibitor	1 (0.008)	1 (0.013)	-	1 (0.026)	1 (0.018)	1 (0.025)	**5**
activating ubiquitin-mediated proteolysis	2 (0.017)	2 (0.025)	1 (0.014)	1 (0.026)	-	-	**6**
Other activities	7 (0.061)	13 (0.166)	11 (0.157)	2 (0.052)	15 (0.283)	4 (0.100)	**52**
Total	**92**	**89**	**88**	**36**	**87**	**58**	**450**

Numbers in brackets indicate the frequency of bioactive peptide activity

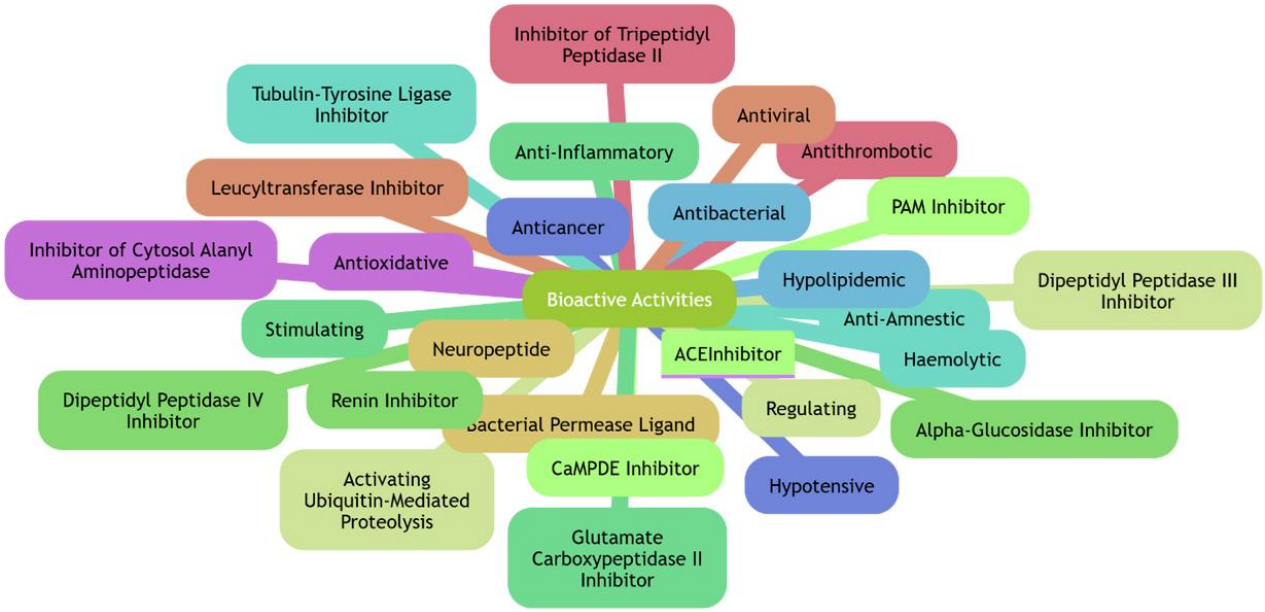

Figure 2. Various biological activities from *C. formosanum*-derived peptides.

Multiple proteases were utilized in this study. The exact cleavage sites and subsequent peptides are determined by the type of enzyme that influences the cleavage of the peptide sequence, making it a key factor. For instance, trypsin, which is a serine protease, cleaves peptides after arginine and lysine residues, whereas chymotrypsin, another serine protease, cleaves peptides after tyrosine, phenylalanine, and tryptophan residues. Thermolysin, a metalloprotease, cleaves peptides after glutamic acid and aspartic acid residues, whereas pepsin, a gastric protease, cleaves peptides after aspartic acid and glutamic acid residues. Proteinase K, a serine protease, cleaves peptides after various amino acids, including arginine, lysine, and glutamic acid. These enzymes have distinct specificities and can be used to generate specific peptides with desired properties, making them valuable tools in peptide synthesis and analysis (Butré et al., 2015; Baharin et al., 2022; Ceuleers et al., 2021; Zhang et al., 2023). The list of bioactive peptides with their biological activities derived from in-silico hydrolysis and its degree (DHt) can be seen in Table 4.

Released peptides from the screening process are dipeptides. The types of biological activity of peptides found are various, including ACE inhibitors, DPP-IV inhibitors, antioxidants, and other activities. In addition, the degree of hydrolysis was also carried out. This metric quantifies the extent to which the protein has undergone hydrolysis, forming smaller peptides and free amino acids (AAs) (Rutherfurd, 2010). The DH is an essential parameter in protein hydrolysis as it affects the composition and properties of the resulting peptides. A higher DH can result in more free AAs and smaller peptides, which can benefit specific applications such as

Table 4. Bioactive peptide with its biological activities and its degree (DHt)

Protein	Enzyme	Released Peptide	Activities	DHt (%)
Phycoerythrin subunit a	Chymotrypsin	SY	ACE-i, DPP-IVi	17.6991
		KL	ACE-i	
	Trypsin	YK	Ac, ACE-i, DPP-IIIi	15.9292
		VK, EK	ACE-i, DPP-IVi	
	Proteinase K	SY	ACE-i, DPP-IVi	23.0088
		KL	ACE-i	
		RI	DPP-IVi	
	Thermolysin	YK	Ac, ACE-i, DPP-IVi, DPP-IIIi	30.0885
		AD	DPP-IVi, αG-i	
		YS	DPP-IIIi	
	Pepsin (pH >2)	PG	Reg, At, Aa, ACE-i, DPP-IVi, PAM-i	74.3363
		RF	ACE-i, DPP-IIIi, Leu-i	
		HY	ACE-i, DPP-IVi, Anti-inf	
		CF	ACE-i	
		VK, SY, VM	ACE-i, DPP-IVi	
		RA	ACE-i, Ubi, DPP-IVi	
		HE	DPP-IVi, GC-i	
		IQ, VN	DPP-IVi	
Phycoerythrin subunit b	Chymotrypsin	RY	ACE-i, Ao	23.1884
	Trypsin	VK	ACE-i, DPP-IVi	17.3913
		LK	Ao	
		YR	DPP-IVi, DPP-IIIi, Neuro	
	Proteinase K	RY	ACE-i, Ao	36.2319
		RA	ACE-i, Ubi, DPP-IVi	
		GV	ACE-i, DPP-IVi	
		KF	ACE-i, DPP-IVi, Ren-i, CaMP-i	
		AL	DPP-IVi	
		RV	DPP-IIIi	
	Thermolysin	LR	Ren-i, ACE-i, DPP-IIIi, αG-i	46.3768
		LKK	Ac	
		VK, VR	ACE-i, DPP-IVi	
		LG, LQ	ACE-i	
		LK	Ao	
		YR	DPP-IVi, DPP-IIIi, Neuro	
	Pepsin (pH > 2)	RL	ACE-i, DPP-IVi	75.3623
		RY	ACE-i, Ao	
		VK, IA, VG	ACE-i, DPP-IVi	
		IL	ACE-i, Stim, Neuro	
		OL, VN	DPP-IVi	
Allophycocyanin beta subunit	Chymotrypsin	DY	Reg, ACE-i	28.2051
		RY	ACE-i, Ao	
		KY	ACE-i, DPP-IVi	
	Trypsin	LR	Ren-i, ACE-i, αG-i	15.3846
		YK	Ac, ACE-i, DPP-IVi, DPP-IIIi	
	Proteinase K	DY	Reg, ACE-i	35.8974
		RY	ACE-i, Ao	
		KY	ACE-i, DPP-IVi	
	Thermolysin	LR	Ren-i, ACE-i, DPP-IIIi, αG-i	43.5897
		YK	Ac, ACE-i, DPP-IVi, DPP-IIIi	
		AD	DPP-IVi, αG-i	
		IN	DPP-IVi	
	Pepsin (pH > 2)	RY	ACE-i, Ao	82.0513
		VE	ACE-i, DPP-IVi, αG-i	
		RG	ACE-i, DPP-IVi, Leu-i	
		IN	DPP-IVi	
		VQ	DPP-IVi	
Phycocyanin beta subunit	Chymotrypsin	RY	ACE-i, Ao	19.4805
	Trypsin	YK	Ac, ACE-i, DPP-IVi, DPP-IIIi	12.9870
		GR, AR	ACE-i	
		MR	DPP-IVi, DPP-IIIi	
	Proteinase K	RY	ACE-i, Ao	29.8701
		EI, AV	ACE-i, DPP-IVi	
		AI	ACE-i	
		KI, KV	DPP-IVi	
	Thermolysin	LR	Ren-i, ACE-i, DPP-IIIi, αG-i	40.2597
		YK	Ac,ACE-i, DPP-IVi, DPP-IIIi	
		AR	ACE-i	
		AD	DPP-IVi, αG-i	
		AS, YQ	DPP-IVi	
	Pepsin (pH > 2)	PG	Reg, DPP-IVi, ACE-i, At, Aa, PAM-i	74.0260
		VA	DPP-IVi, TP2-i	
		RA	DPP-IVi, ACE-i, Ubi	
		IN, IQ	DPP-IVi	
		RG	DPP-IVi, ACE-i, Leu-i	
		VL	DPP-IVi, Stim	
		VT	DPP-IVi	
		RY	ACE-i, Ao	

Tabe 4. *Continued*

Protein	Enzyme	Released Peptide	Activities	DHt (%)
Phycocyanin alpha subunit	Chymotrypsin	TN	DPP-IVi	10.8108
	Trypsin	LK	Ao	16.2162
		SK	DPP-IVi	
	Proteinase K	-	-	-
	Thermolysin	AS	DPP-IVi	40.5405
	Pepsin	RL, IA	ACE-i, DPP-IVi	70.2703
		RF	ACE-i, DPP-IIIi, Leu-i	
		RA	ACE-i, Ubi, DPP-IVi	
		SL	DPP-IVi, Reg	
		SK	DPP-IVi	
Allophycocyanin alpha subunit	Chymotrypsin	DY	Reg, ACE-i	26.9231
		RL	ACE-i, DPP-IVi, Ao	
		RY	ACE-i	
		VL	Stim, DPP-IVi	
	Trypsin	IR	ACE-i, Ao, Ren-i, CaMP-i, DPP-IVi	21.1538
		LK	Ao	
		YR	DPP-IVi, DPP-IIIi, Neuro	
	Proteinase K	DY	Reg, ACE-i	36.5385
		RL	ACE-i, DPP-IVi	
		RY	ACE-i, Ao	
		SP, RI	DPP-IVi	
	Thermolysin	LR	Ren-i, ACE-i, DPP-IIIi, αG-i	42.3077
		IR	Ren-i, ACE-i, Ao, DPP-IVi	
		AR	ACE-i	
		AD	DPP-IVi, αG-i	
		AE	DPP-IVi, GC-i	
		KS, VN	DPP-IVi	
		YR	DPP-IVi, DPP-IIIi, Neuro	
	Pepsin (pH > 2)	RL	ACE-i, DPP-IVi	69.2308
		IR	ACE-i, Ao, CaMP-i, DPP-IVi	
		RY	ACE-i, Ao	
		IA	ACE-i, DPP-IVi	
		SG	ACE-i	
		SF	ACE-i, Ren-i, DPP-IVi	
		IL	ACE-i, DPP-IVi, Neuro, Stim	
		VL	DPP-IVi, Stim	
		VN	DPP-IVi	

Note: ACE-i: ACE inhibitor, DPP-IVi: Dipeptidyl peptidase IV inhibitor, DPP-IIIi: Dipeptidyl peptidase III inhibitor, Reg: Regulating, Stim: Stimulating, CaMP-i: CaMPDE inhibitor, Ren-i: Renin inhibitor, αG-i: Alpha-glucosidase inhibitor, Neuro: Neuropeptide, Aa: Antiamnestic, At: Antithrombotic, Ubi: Activating ubiquitin-mediated proteolysis, Ac: Anticancer, Ao: Antioxidant, Anti-inf: Anti-inflammatory, Leu-i: Leucyltransferase inhibitor, GC-i: Glutamate carboxypeptidase inhibitor, TP2-i: Tripeptidyl peptidase II inhibitor, PAM-i: PAM inhibitor

nutritional supplements or pharmaceuticals. On the other hand, a lower DH can lead to a higher proportion of larger peptides and intact proteins, which may be more suitable for different applications (Hou et al., 2017).

According to this study's findings, pepsin is the most potent enzyme that cleavages the sequence and has many biological activities, followed by thermolysin. López-Ferrer et al., (2011) stated that pepsin selectively cleaves peptide bonds after hydrophobic amino acids such as leucine and phenylalanine. However, it can also cleave at other positions with less specificity. Pepsin has been used to generate peptides with various biological activities (Castañeda-Valbuena et al., 2022), ACE inhibitor from *Chlorella vulgaris* and *Spirulina platensis* (Suetsuna & Chen 2001), antioxidant from algae protein waste (Sheih et al., 2009), antithrombotic from *Porphyra yezoensis* (Indumathi & Mehta 2016). Thermolysin selectively identifies and hydrolyzes peptide bonds next to hydrophobic amino acids, particularly isoleucine, leucine, valine, and phenylalanine. This selectivity facilitates the effective breakdown of proteins into smaller peptides that may demonstrate diverse biological functions (Nourmohammadi & Mahoonak, 2018). Thermolysin has been utilized to produce peptides exhibiting diverse biological functions, such as ACE inhibitory, antioxidant, and DPP-IV inhibitory peptides from *G. chorda* (Mune et al., 2023), and ACE inhibitory pentapeptides thermolysin digestion of porcine myosin (Arihara et al., 2001). Moreover, the combination of pepsin-thermolysin was shown to produce a competitive ACE inhibitory peptide from *C. formosanum* (Windarto et al., 2024b).

The value frequency (A_E), relative frequency (W), V, and B_E parameters are used in protein identification to determine the probability of a protein sequence being a specific protein. These parameters are calculated based on the frequency of amino acids in the protein sequence and are used to compare the sequence to known protein sequences in a database. The total A_E, W, B_E, and V for each protein and its bioactivities can be seen in Figure 3.

The PeptideRanker is a server that uses predictive algorithms to estimate the probability of discovering new bioactive peptides by assessing the likelihood of a given peptide sequence being bioactive. In this study, the cutoff was set to > 0.5. According to Coscueta et al., (2022), a peptide that scores over the PeptideRanker threshold (0.5) is identified as bioactive. Thus, novel peptides with a bioactivity score >0.5 were considered for analysis as they could be potentially bioactive. The list of predicted bioactive tripeptides and their allergenicity can be seen in Table 5.

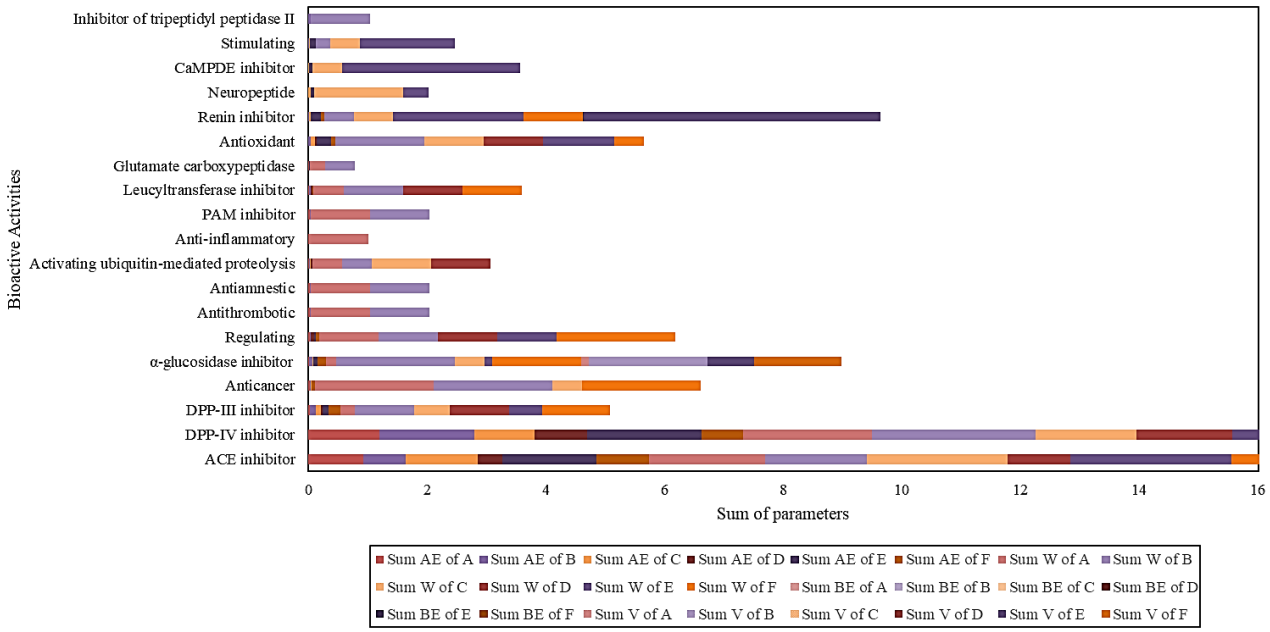

Figure 3. The Sum of A_E, W, B_E and V for all proteins and their activities (A: phycoerythrin subunit a, B: phycocyanin beta subunit, C: phycoerythrin subunit b, D: phycocyanin alpha subunit, E: allophycocyanin alpha subunit, F: allophycocyanin beta subunit).

Table 5. Bioactive peptides score by PeptideRanker (Cutoff> 0.5) and its allergenicity

Protein	Protease	Peptide Sequence	Score	Allergenicity
Phycoerythrin subunit a	Thermolysin	AGR	0.548	Non-allergen
	Pepsin (pH > 2)	CRF	0.984	Allergen
Phycoerythrin subunit b	Thermolysin	FSR	0.831	Non-allergen
	Chymotrypsin	KKF	0.532	Non-allergen
Allophycocyanin beta subunit	Chymotrypsin	RGM	0.848	Allergen
	Proteinase K	TSV	0.780	Non-allergen

According to the results, phycoerythrin subunits (a and b) and allophycocyanin beta subunit had potential bioactive peptides. PeptideRanker was used to assess the activity of peptide fragments generated through in-silico hydrolysis of phycobiliproteins, which have yet to be previously reported. PeptideRanker probability scores of peptide fragments obtained from the phycobiliproteins are shown in Table 5. The cutoff score (>0.5) was used to choose peptides with a high probability of bioactivity specifically. The tripeptide CRF achieved the highest score of 0.984. The following peptides had high scores: RGM (0.848), FSR (0.831), TSV (0.780), AGR (0.548), and KKF (0.532). Further research is required to verify the bioactivities of specific peptides that have not been previously reported but have obtained high scores due to in-silico hydrolysis. Furthermore, the probable toxicity profile analysis has verified the safety of utilizing these bioactive peptides, with only a few exceptions. Coscueta et al., (2022) stated that as the score increases, the likelihood of bioactivity also increases. Generally, a score above 0.5 indicates bioactivity, with scores above 0.8 being robust indicators.

The toxicity and allergenicity profile of proteins and peptides involves evaluating their potential adverse effects on human health. This includes assessing their ability to cause allergic reactions, trigger immune responses, and induce toxicity. The probability of toxicity and allergenicity profile of the protein can be seen in Table 6.

Based on Table 6, all the proteins were non-toxins, except the sequence of NKCRFPSSD that cleavage from phycoerythrin subunit a. The protein was also non-allergen, except for the phycocyanin beta subunit, which was probably an allergen. Understanding the toxicity and allergenicity of proteins and peptides is crucial for ensuring the safety of food and feed products, pharmaceuticals, and biotechnology applications (Perçin & Karakaya, 2020). This research relies on in-silico analysis carried out through a bioinformatics approach. In addition, the in silico bioactive peptide theory must be confirmed through in vitro and in vivo tests in the laboratory.

Conclusion

Bioinformatics techniques have been extensively employed to thoroughly and cost-effectively investigate and uncover bioactive peptides generated from plant proteins. The in silico method was used to expedite

Table 6. Probability of toxicity and allergenicity profile

Protein	Peptide sequence	SVM Score	Prediction	Allergenicity
Phycoerythrin subunit a	All	-Ve	Non-Toxin	Non-allergen
	NKCRFPSSD	0.05	Toxin	
Phycocyanin beta subunit	All	-Ve	Non-Toxin	Allergen
Phycoerythrin subunit b	All	-Ve	Non-Toxin	Non-allergen
Phycocyanin alpha subunit	All	-Ve	Non-Toxin	Non-allergen
Allophycocyanin alpha subunit	All	-Ve	Non-Toxin	Non-allergen
Allophycocyanin beta subunit	All	-Ve	Non-Toxin	Non-allergen

screening novel bioactive peptides derived from algae, thereby diminishing the time needed for this procedure. The majority of proteins found in *C. formosanum* are classified as phycobiliproteins, specifically including phycoerythrin subunit a, phycocyanin beta subunit, phycoerythrin subunit b, phycocyanin alpha subunit, allophycocyanin alpha subunit, and allophycocyanin beta subunit. The results of computational proteolysis demonstrated that phycobiliproteins exhibit 27 bioactive activities, with the majority of the bioactive peptides generated exerting inhibitory effects on ACE and DPP-IV. Most peptides derived from *C. formosanum* exhibited neither toxic nor allergenic properties. Computational studies are necessary for making predictions, followed by experimental confirmation in a laboratory setting to enhance the understanding of the role of bioactive peptides derived from macroalgae. To maximize the advantages for humankind, it is crucial to employ computational and experimental research techniques to reveal the untapped potential of marine resources.

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Author Contribution

First Author: Investigation, Methodology, Formal analysis, Data curation, Validation, Visualization, Writing – original draft, Writing – review & editing. Second Author: Resources. Third Author: Project administration, Funding acquisition.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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