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Exploring the bioactive potential of peptides derived from the RuBisCO protein in *Caulerpa racemosa*: an in silico approach

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Abstract

Caulerpa racemosa harbors a rich reservoir of bioactive peptides derived from RuBisCO, a photosynthetic enzyme with promising therapeutic potential. This study aimed to systematically identify and characterize bioactive peptides from *C. racemosa* RuBisCO using a multi-step in silico pipeline. Simulated proteolysis using 33 enzymes predicted peptides with 35 different biological activities using BIOPEP-UWM. In addition to traditional database screening, further computational filtering was conducted using physicochemical profiling (ExPASy ProtParam), bioactivity prediction (PeptideRanker), toxicity and allergenicity evaluation (ToxinPred, AllergenFP), and structure-based molecular docking against relevant therapeutic targets—angiotensin-I converting enzyme (ACE, PDB: 1O8A) and xanthine oxidase (XO, PDB: 3NRZ). Four peptides with high predicted bioactivity scores (>0.75) showed strong binding affinity (−169.00 to −252.29 kcal/mol) and favorable confidence scores, suggesting their possible use as dual-action therapeutic agents—with both antihypertensive and antioxidant effects. This integrative in silico approach demonstrates the therapeutic relevance of *C. racemosa* peptides and provides a framework for peptide prioritization prior to experimental validation.

Introduction

Bioactive peptides, released during proteolysis or fermentation, are protein fragments with numerous nutrients and health-promoting properties. Seaweed bioactive peptides have gained attention due to their diverse functional properties and potential health benefits. Researchers are exploring these peptide compounds, particularly from seaweed sources, to develop novel functional ingredients and therapeutic agents for dermatology, nutrition, and medicine (Bhat et al., 2015; Windarto et al., 2022; Garcia-Vaquero et al., 2022; Windarto et al., 2024a; Windarto et al., 2024b). *Caulerpa racemosa*, also known as sea grapes, is a green alga with diverse biological activities, including anti-inflammatory, antimutagenic, antinociceptive, anticancer, and cytotoxic effects. Its secondary metabolites have potential medicinal uses, but theirbiological activity is not yet fully explored, highlighting the need for further research (Ornano et al., 2014; Windarto et al., 2023; Windarto et al., 2024c).

The process of discovering bioactive peptides by traditional approaches, such as hydrolysis, purification, characterization, and activity assays, is both time-consuming and resource-intensive in terms of chemicals and labor, resulting in high costs (Cermeño et al., 2020).

In silico approaches utilize computational models and simulations to predict the efficacy and toxicity of compounds, thereby reducing the need for expensive and time-consuming animal and human trials. In silico methods can facilitate faster drug development without the need for chemical synthesis, as well as help identify compounds with potential toxicity, carcinogenicity, and mutagenic capacity, thereby ensuring that only safe andeffective compounds can advance in the development process (Zloh & Kirton, 2018; Roney & Aluwi, 2024). The BIOPEP-UWM database contains a comprehensive list of bioactive peptides and their activities. The database includes over 350 articles and thousands of bioactive peptides with dozens of distinct activities (Minkiewicz et al., 2019). The precursor protein can undergo in silico digestion by various proteolytic enzymes utilizing the ENZYME(S) ACTION tool of BIOPEP-UWM™. The resulting theoretical peptides are subsequently examined to determine if they correspond to any known bioactive peptides in the database. Furthermore, software applications such as PeptideRanker can predict the probability of a peptide being bioactive. Therefore, in silico approaches employ an empirical approach to determine the sources of precursor proteins for bioactive peptides.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is the primary enzyme responsible for the assimilation of CO_2 into the biosphere. It is found in all photoautotrophic organisms, including algae (Valegård et al., 2018). It is formed through protein synthesis, in which the genetic information encoded in DNA is translated into a series of amino acids. Amino acids are then linked together via peptide bonds to form polypeptide chains. RuBisCO is a protein that is considered complete since it contains all the necessary amino acids required for human ingestion. Its biochemical composition, organoleptic and physical characteristics make it a nutritionally beneficial food additive. In addition, RuBisCO has potential bioactivity, including antioxidant and anti-inflammatory properties, which may increase its value as a food additive (Stefano et al., 2018; Ducrocq et al., 2020; Grácio et al., 2023).

This study aimed to examine RuBisCO from *C. racemosa* to identify potential bioactive peptides. Protein sequences, biological function, and enzyme activity were tabulated using BIOPEP, PeptideRanker, and toxicity and allergenicity evaluations.

Materials and Methods

Sequences of *C. racemosa* RuBisCO

The *C. racemosa* RuBisCO large subunit sequence, consisting of 475 amino acids, was obtained from the UniProtKB database (Accession number: A0A1I9LK66) for in silico analysis. This protein is not only found in *C. racemosa*, but also in other species, such as *C. okumurae, C. cupressoides, C. serrulate*, and *C. manorensis*.

Physicochemical Properties of *C. racemosa* RuBisCO

ExPASy's ProtParam (https://web.expasy.org/protparam/) was utilized to identify the physicochemical properties of *C. racemosa* 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).

In-silico of RuBisCO bioactive peptide

The probability of liberating bioactive peptides for the chosen proteins was analyzed using the BIOPEP-UWM™ database. The segment exhibiting the most significant biological activity was chosen for reporting. The database estimated the frequency of fragments with a specific activity (A) of *C. racemosa* proteins using the provided formula:

$$A = a/N \quad (1)$$

Where "a" represents the number of fragments of a specific activity in a protein sequence, while "N" represents the total number of amino acid (AA) residues in the protein chain.

In silico proteolysis of RuBisCO

The proteins of *C. racemosa* were examined using in silico proteolysis utilizing BIOPEP's enzyme-action tool. Each protein sequence was independently subjected to hydrolysis by 33 various proteases: chymotrypsin (A), trypsin, pepsin (pH 1.3), proteinase K, pancreatic elastase, prolyl oligopeptidase, glutamyl endopeptidase (pH 4), thermolysin, chymotrypsin C, plasmin, cathepsin, clostripain, chymase, papain, ficin, leukocyte elastase, metridin, thrombin, pancreatic elastase II, stem bromelain, glutamyl endopeptidase II, oligopeptidase B, calpain 2, glycyl endopeptidase, oligopeptidase F, proteinase P1, Xaa-pro dipeptidase, pepsin (pH > 2), coccolysin, subtilisin, chymosin, ginger protease, V-8 protease (pH 7.8). The value of frequency (A_E) and relative frequency (W) of releasing peptides by specific protease were calculated using the formula:

$$A_E = d/N \quad (2)$$

$$W = AE/A \quad (3)$$

where d is the number of peptides hydrolyzing by specific proteases from the sequence, and N is the total residues of amino acid. The parameters of V and theoretical degree of hydrolysis (DHt) were also calculated using the formula:

$$DHt = (d/D) \times 100\% \quad (4)$$

"d" represents the sum of hydrolyzed peptides, while "D" represents the sum of peptide bonds in the sequence.

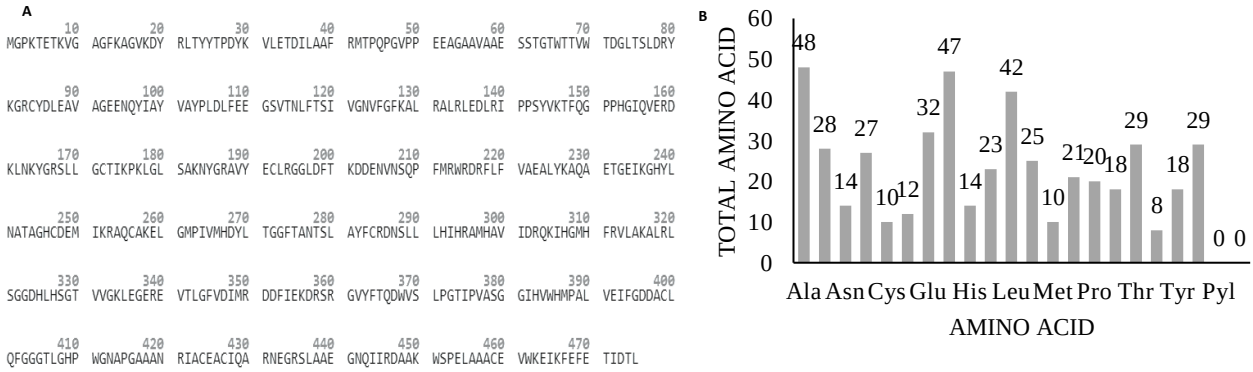


Figure 1. (A) RuBisCO protein sequence of *C. racemose* and (B) distributions of amino acids.

The bioactivity score of tripeptides and tetrapeptides

The peptide fragments from *C. racemosa* proteins, which possess established biological activity, were manually enumerated. BIOPEP-UWM™ displays the fragments, including the activities available in the database. In this study, the sequence of tripeptide and tetrapeptides residues was screened and considered for potential biological activity. The bioactivity score of the peptide fragments was measured using PeptideRanker, a bioinformatics tool available at http://distilldeep.ucd.ie/PeptideRanker. The cutoff inthis study was >0.75 to find the tripeptide and tetrapeptide fragments that may have a high probability of having bioactivities.

Probability score prediction of RuBisCO *C. racemosa*

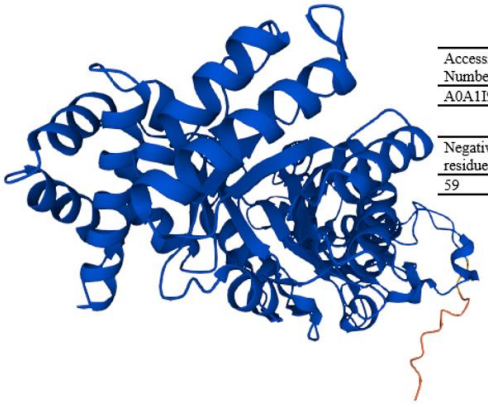
The toxicity and allergenicity of bioactive peptides were assessed using ToxinPred and AllergenFP, as described by Gupta et al. (2013) and Dimitrov et al. (2014), respectively. ToxinPred is available at http://crdd.osdd.net/raghava/toxinpred/ and AllergenFP at http://ddg-pharmac.net/AllergenFP/.

Molecular docking simulation

Peptides derived from *C. racemosa* RuBisCo were screened based on predicted bioactivity scores, and the top four peptides with scores >0.75 were selected for molecular docking. Their 3D structures were constructed and energy-minimized using Avogadro software with the MMFF94 force field (Hanwell et al., 2012). Docking was performed using the HDOCK server (Yan et al., 2020) against two target proteins: angiotensin-converting enzyme (ACE, PDB ID: 1O8A) for antihypertensive activity (Natesh et al., 2003), and xanthine oxidase (XO, PDB ID: 3NRZ) for antioxidant activity (Okamoto et al., 2004). Protein structures were preprocessed by removing water molecules and heteroatoms. Docking results were ranked based on binding energy (kcal/mol), confidence score, and ligand RMSD, and the best-scoring poses were analyzed to evaluate interaction potential.

Results and Discussion

Various bioinformatics software, tools, and databases were used to perform in silico proteolysis and release different bioactive peptides from the RuBisCO sequences of *C. racemosa* proteins. The UniProtKB database was used to query the sequences of RuBisCO of *C. racemosa*. The sequence obtained has similarities (100%) to the RuBisCO sequence in other species, *C. okumurae*, *C. cupressoides*, *C. serrulata*, and *C. manorensis*. RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) is considered the most abundant protein on Earth, found in all green leaves, and responsible for photosynthesis. The composition of this substance is widely regarded as very suitable for human consumption because of its exceptional nutritional content and ability to be used in a wide range of food applications (Grácio et al., 2023; Nawaz et al.,



Accession Number	Number of AA	Molecular Weight	Theoretical pI	Formula
A0A1I9LK66	475	52515.82	6.19	$C_{2348}H_{3636}N_{646}O_{685}S_{20}$

Negatively charged residues (Asp + Glu)	Positively charged residues (Arg + Lys)	Instability index	Aliphatic index	GRAVY
59	53	35.18	81.18	-0.227

Figure 2. RuBisCO protein structure and its physicochemical properties.

2024). The sequence of the RuBisCO large subunit from *C. racemosa* was obtained from UniProtKB, and the distribution of its amino acids is shown in Figure 1.

Figure 2 shows the physicochemical properties of RuBisCO from *C. racemosa*. The total number of peptides is 475, with a molecular weight of 52515.82 Da, and the theoretical isoelectric point (pI) is 6.19. The number of negatively charged residues (Asp + Glu) are 59, and the positively charged residues (Arg + Lys) are 53. Furthermore, the instability index of 35.18 indicates that the protein sequences exhibit stability. These sequences will undergo further evaluation in wet-lab studies to assess their stability. Because RuBisCO sequences have a high aliphatic index (81.18), they are classified as thermostable proteins, meaning all sequences resist degradation at high temperatures. The GRAVY rating (-0.227) indicates that the proteins have a hydrophobic nature and are positively graded. The importance of these parameters lies in their ability to provide a comprehensive understanding of a peptide's physical and chemical properties. This information is essential for designing and optimizing peptides for specific applications, such as medicine, nutrition, and biotechnology (Kaur et al., 2020; Roshanak et al., 2023).

The sequences were analyzed using the BIOPEP database to assess the potential of RuBisCO protein sequences from *C. racemosa* as precursors for bioactive peptides. The results of the in-silico approach of the bioactive peptides from RuBisCO and its activities can be seen in Figure 3. Most of the bioactive peptides in RuBisCO from *C. racemosa* play a role as DPP-IV inhibitor, ACE inhibitor, antioxidant, and DPP-III inhibitor. This result aligns with the hydrophobic nature of the peptide RuBisCO in *C. racemosa*. RuBisCO is a valuable source of bioactive peptides that possess qualities similar to opioids, enhance memory, fight cancer, reduce inflammation, stimulate appetite, provide antioxidant effects, and lower blood pressure (Stefano et al., 2018; Kose, 2021).

Table 1 illustrates the biological activities of the active fragments. Based on Table 1, the RuBisCO sequence has the most biological activity as an ACE inhibitor, DPP-IV inhibitor, antioxidant, and DPP-III inhibitor. Several studies have shown that RuBisCO has activity as an ACE inhibitor, antioxidant, and dipeptidyl peptidase inhibitor IV and III (Udenigwe et al., 2017; Stefano et al., 2018; Ito et al., 2021).

In silico proteolysis was employed to analyze the proteins of RuBisCO from *C. racemosa* using BIOPEP's enzyme-action instrument. The 33 distinct enzymes independently subjected the RuBisCO sequence to hydrolysis. Proteases can break down proteins into

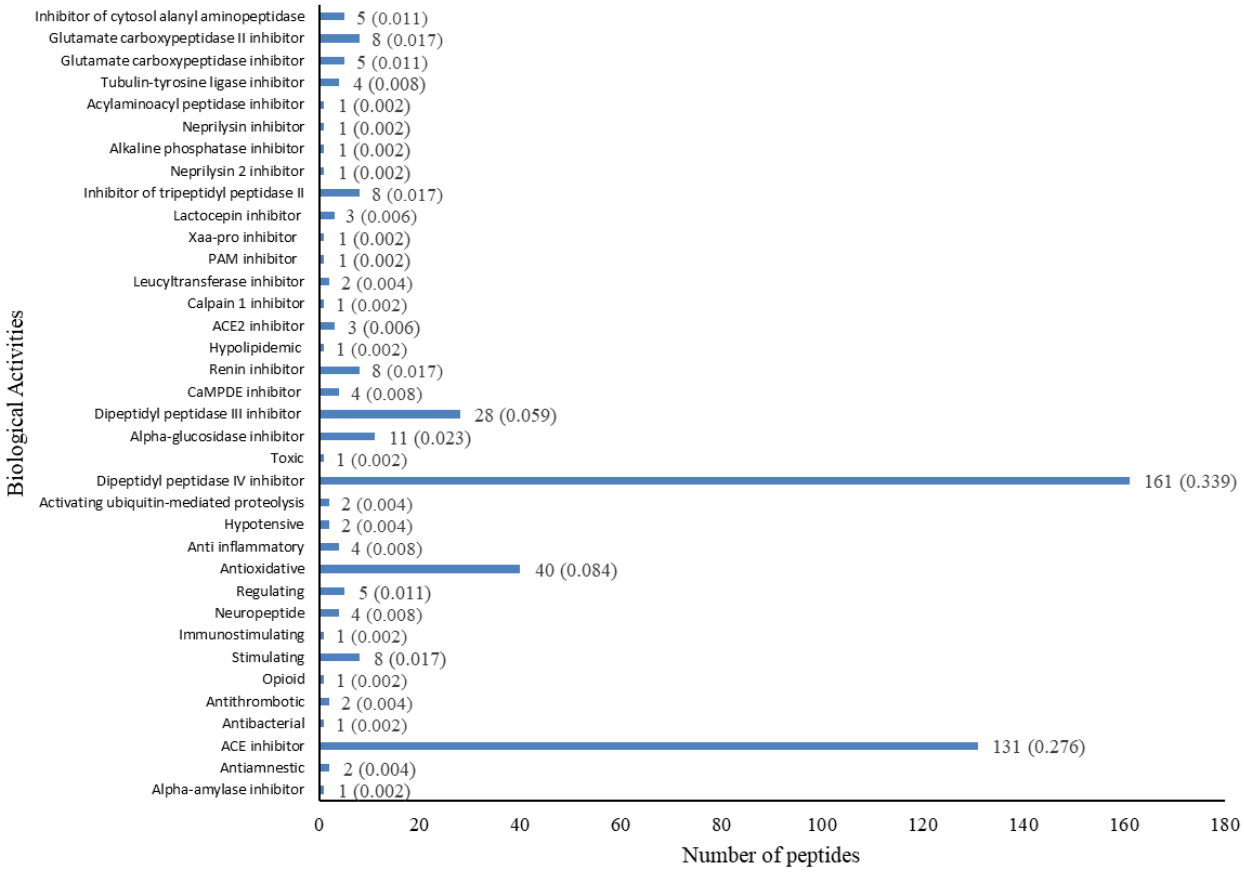


Figure 3. Total number of RuBisCO from *C. racemosa* as bioactive peptides. Numbers in brackets indicate the frequency of bioactive peptide activity. RuBisCO: Ribulose bisphosphate Carboxylase-Oxygenase; ACE: Angiotensin Converting Enzyme; PAM: Protein associated with Myc; CaMPDE: Calmodulin-dependent phosphodiesterase.

Table 1. Biological activities of the active fragments

Enzyme	ACE-i	AP-i	αg-i	Anti-a	Anti-c	Anti-o	Anti-t	CaMPDE-i	DP3-i	DP4-i	GC2-i	Hypolipid	TP2-i	Lact-i	Npr-i	PAM-i	Reg	Ren-i	Stim	TubTL-i	XP-i
Chymotrypsin (A)	RL, VF, VW, VAY, AY, PL, RW, GF, GM, GL, GH, KY, IAY, DY, DF, EF, DL	GF	VW			AY, PW, RW, TY, VW, IAY		EF	RW, GF, IH	SL, GL, PL, AY, GF, GH, IH, KY, PW, QF, QY, RL, RM, RW, TY, VF, VN, VW	DF	EF	VF, GF	PL			DY, SL	EF, QF		AY	PL
Trypsin	GR, QK, YK, DR				YK				YK	WR, DR, PK, YK											
Pepsin (pH 1.3)	RL, GF, GL, DF, EF, DL	GF						EF	GF	GL, GF, QF, RL	DF	EF	GF					EF, QF			
Proteinase K	RL, AY, GP, RW, GF, GM, GL, GV, SY, KF, KL, KP, EI, DY, TP, DF, QGP, QP, EF, DL	GF		GP		AY, EL, RW, KP	GP	KF, EF	RW, GF, RV, HF	GP, TP, SP, KP, QP, AL, SL, GL, AY, EI, GF, GV, HF, HI, HV, KF, KV, QF, QV, RI, RL, RM, RW, SY, TL, TY	DF	EF	GF				DY, GP, SL	KF, EF, QF		AY	
Pancreatic Elastase	RL, HY, PL, RA, KG, FG, MG, HG, EG, EA, PG, DG, KL, KA, EI, RG, DL		EA	PG		PEL	PG		KA	KA, RA, PL, WT, EG, EI, ES, ET, HI, HS, HV, HY, KG, KT, KV, MG, NA, NL, NT, NV, PG, PV, QA, QV, RG, RI, RL				PL	FG	PG	PG	FT			PL
Prolyl Oligopeptidase	MGP, QP									QP											
Glutamyl Endopeptidase (pH 4)	GE								GE	GE	FE, GE										
Thermolysin	YP, IP, FR, VG, AG, FG, LG, YK, VE, LQ, LN, LR		YP, VE, LR		YK	LH			LR, YK, YR, IH, FR	APG, IP, YP, AE, AG, AT, FR, IH, IQ, LH, LN, LT, VD, VE, VG, VS, VT, YD, YK, YR	AE, FE				FG			FT, LR			
Chymotrypsin C	RL, VW, VAY, AY, GP, RW, AP, GM, GL, GE, SY, KY, KL, VE, KE, IAY, DY, TP, DL		VW, VE	GP		AY, RW, TY, VW, IAY	GP		RW, GE	GP, AP, TP, SP, AL, SL, GL, AE, AY, GE, KE, KY, RL, RW, SY, TY, VE, VN, VW	AE, GE, FE						DY, GP, SL			AY	
Plasmin	GR, DR, QK, YK				YK				YK	WR, DR, PK, YK											
Cathepsin	RL, VAY, AY, PL, GF, GM, GL, GH, IAY, DY, DF, EF, DL	GF				AY, TY, IAY		EF	GF, IH	GL, PL, AY, GF, GH, IH, QF, RL, RM, TY	DF	EF	GF	PL			DY	EF, QF		AY	PL
Clostripain	DR									WR, DR											
Chymase	RL, VAY, AY, PL, GF, GL, MRW, IAY, DF, EF, DL	GF				AY, TY, IAY		EF	GF	GL, PL, AY, GF, QF, RL, TY	DF	EF	GF	PL				EF, QF		AY	PL
Papain	AF, AG, MG, QG, SG, EG, PG, DG, KL, YK, AR, AV, DF, EF, ER, DR, DL			PG	YK	YYT, PEL	PG		MR, YK	APG, AL, SL, WR, WT, AE, AG, AT, AV, DR, EG, ET, MG, MR, NL, PG, QF, QG, VL, YK	AE, DD, DF	EF	AF			PG	PG, SL	EF, QF	VL		
Ficin	VY, VAY, AY, PL, VK, VG, AG, MG, QG, TG, EG, PG, QK, NY, NK, TF, IAY, DY, DF, EF, ER, DR, DL		WS	PG		AY, EL, PWG, PEL, TY, VY,		EF	MR, TF, IH, VY	AL, PL, WR, WS, AG, AY, DR, EG, IH, MG, MH, MR, NY, PG, PK, QF, QG, TF, TG, TK, TL, TS, TY, VG, VK, VL, VY	DF	EF	VY	PL		PG	DY, PG	EF, QF, TF	VL	AY	PL

					IAY, PG															
Leukocyte Elastase	RL, RA, GA, GL, GV, GT, EA, KA, EI, DGL, YV, DL		YPL, EA		YYT, PEL			KA	KA, GA, RA, GL, WT, EI, ES, ET, GV, HI, HS, HV, KT, KV, NA, NL, NT, PV, QA, QV, RI, RL, YV							FT			
Metridin	RL, VAY, AY, PL, GF, GL, MRW, IAY, DF, EF, DL	GF			AY, TY, IAY		EF	GF	GL, PL, AY, GF, QF, RL, TY	DF	EF	GF	PL			EF, QF		AY	PL
Thrombin																				
Pancreatic Elastase II	RL, GF, GM, GL, DF, EF, DL	GF					EF	GF	HF, GL, GF, HF, QF, RL, RM	DF	EF	GF				EF, QF			
Stem Bromelain	IA, DA, MG, QG, EG, EA, PG, DG, KL, KA, EV, DF, YV, EF, ER, DR, DL		YPL, EA	PG	YYT, PEL, YF	PG	EF	MR, YF, DA, KA	KA, IA, WR, WT, DR, EG, ES, ET, EV, HS, KT, KV, MG, MR, NA, NL, NR, NT, NV, PG, QA, QF, QG, YF, YV	DA, DF	EF			PG	PG	EF, NR, QF	IV		
Glutamyl Endopeptidase II																				
Oligopeptidase B	GR, QK, YK, DR			YK				YK, FL, YI	WR, DR, PK, YK											
Calpain 2	IPP, YG, VK, VG, AG, FG, MG, SG, EG, PG, DG, YK, NK, AR, PQ, AV, FQ, ST, ER, DR, DL		IPP	PG	YK		PG	YK, FL, YI	APG, FL, AL, SL, WR, WT, AE, AG, AT, AV, DN, DR, EG, ET, FQ, IQ, MG, NL, NQ, PG, PK, PQ, VG, VK, VL, YG, YI, YK	AE, DD			FG	PG	PG, SL	FT	VL	YG	
Glycyl Endopeptidase	AG, MG								AG, MG											
Oligopeptidase F	RL, GF, GL, DF, EF, DL	GF					EF	GF	GL, GF, QF, RL	DF	EF	GF				EF, QF			
Proteinase P1	AY, RA, GM, GH, GV, MG, GK				AY				RA, AY, GH, GV, MG, RI, SV, TN							FT	IV	AY	
Xaa-Pro Dipeptidase	MG								MG											
Pepsin (pH > 2)	RL, RY, VF, RF, VY, HY, PL, VK, IA, IP, RA, IF, VG, HL, SG, PG, IE, VE, PQ, IL, RG, VM		VE	PG	HL, VY, RY	PG		RF, HL, HF, IH, PF, VY	VA, PA, HA, IP, IA, RA, HL, SL, PL, WT, HD, HF, HY, IH, IL, IM, IQ, PF, PG, PK, PQ, RG, RL, RM, RN, VD, VE, VF, VG, VK, VL, VM, VN, VT, VY	IE		VA, VF, VY	PL	VF	PG	PG, SL		VL		PL
Coccolysin	YP, AG, FG, LG, YK, LVE, LQ, LN, AV, YV, LR		YP, LR		YK	LH		LR, YK, YR	APG, YP, AE, AG, AT, AV, IQ, LH, LN, LT, YD, YK, YR, YV	AE, FE				FG		FT, LR			
Subtilisin	RL, VF, VW, VY, VAY, AY, PL, GF, GL, GT, EA, MRW, IAY, DF, RG, EF, DL		VW, EA			AY, PEL, TY, VY, VW, IAY	EF	GF, VY	GL, PL, AY, GF, HS, QF, RG, RL, TS, TT, TY, VF, VL, VS, VW, VY	DF	EF	VF, VY, GF	PL			EF, QF	VL	AY	PL
Chymosin																				
Ginger Protease																				
V-8 Protease (pH 7.8)	GE				KD			GE	GE, TD, TL	FE, GE										

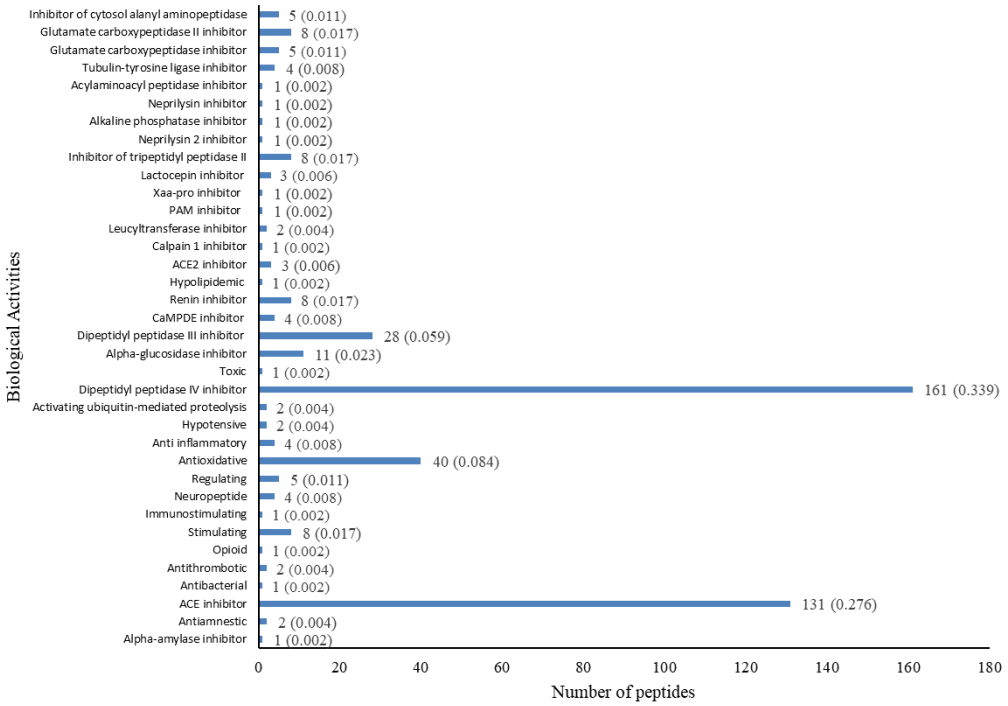


Figure 4. Number of active fragments for each protease.

peptides and work specifically by recognizing and cleaving specific peptide bonds. This specificity is due to the unique structure and function of the protease enzyme. Various proteases are known to exhibit specificity in their substrate recognition and cleavage. Based on the results, pepsin (pH >2) could release the most active fragments, followed by calpain 2, ficin, pancreatic elastase, stem bromelain, and proteinase K, respectively. Meanwhile, thrombin, glutamyl endopeptidase II, chymosin, and ginger protease could not release active fragments from the RuBisCO protein (Figure 4). Pepsin is a protease that is active at a pH greater than 2. Pepsin's optimal pH range is between 1.5 and 2.5, allowing it to efficiently cleave peptide bonds in proteins. At this pH, the enzyme's active site is optimized for substrate binding and catalysis, releasing large amounts of peptide fragments (Mostashari et al., 2022). RuBisCO is primarily broken down by proteases such as calpain 2 and ficin, which are active at different pH ranges and are involved in various cellular processes. Thrombin, chymosin, and ginger protease cannot break down the RuBisCO protein because they are not explicitly designed to target RuBisCO.

The degree of hydrolysis (DHt) is a critical factor contributing to the peptides' composition and functional properties. A high DHt value indicates that a significant percentage of peptide bonds in the protein have been cleaved during hydrolysis. This indicates that the protein has undergone hydrolysis, forming smaller peptides and individual amino acids. Consequently, there is an increased abundance of free amino acids and a decrease in the protein's molecular weight, which improves their solubility and bioavailability, and increases their bioactivity. The DHt of each hydrolysis using various enzymes can be seen in Table 2.

Different enzymes have different specificities and efficiencies, which can affect the extent of peptide bond cleavage and the resulting degree of hydrolysis (Baharuddin et al., 2016; Sbroggio et al., 2016; Langyan et al., 2021). Table 2 showed that pepsin (pH >2) had the highest DHt (71.73%); meanwhile, thrombin, chymosin, ginger protease, and glutamyl endopeptidase II could not hydrolyse the RuBisCO protein. Pepsin is most active

Table 2. The degree of hydrolysis (DHt) of each enzyme on RuBisCO protein

Enzymes	DHt (%)	Enzymes	DHt (%)	Enzymes	DHt (%)
Chymotrypsin (A)	26.5823	Clostripain	5.9072	Calpain 2	46.2025
Trypsin	11.1814	Chymase	18.5654	Glycyl endopeptidase	9.9156
Pepsin (pH 1.3)	13.0802	Papain	43.8819	Oligopeptidase F	13.0802
Proteinase K	35.865	Ficin	44.7257	Proteinase P1	36.9198
Pancreatic Elastase	53.3755	Leukocyte Elastase	39.6624	Xaa-Pro Dipeptidase	2.7426
Prolyl Oligopeptidase	4.2194	Metridin	18.5654	Pepsin (pH > 2)	71.7300
Glutamyl Endopeptidase (pH 4)	6.7511	Thrombin	0	Coccolysin	32.0675
Thermolysin	38.1857	Pancreatic Elastase II	15.1899	Subtilisin	27.0042
Chymotrypsin (C)	32.7004	Stem Bromelain	55.0633	Chymosin	0
Plasmin	11.1814	Glutamyl Endopeptidase II	0	Ginger Protease	0
Cathepsin	21.9409	OligopeptidaseB	11.1814	V-8 Protease (pH 7.8)	12.4473

at pH 1.5 to 2.5, allowing it to efficiently cleave peptide bonds in proteins. At this specific pH level, the enzyme's active site is tailored to bind with the substrate and facilitate catalytic reactions efficiently, resulting in a significant amount of hydrolysis. The catalytic mechanism, stability, and specificity also play a role in pepsin's ability to produce high DHt and release active peptide fragments. Pancreatic elastase also has a high DHt (53.37%) due to its specific properties and mechanisms of action; it is stable, it has elastin, which increases the DHt, has a pH optimum between 7.5-8.5 which is close to the gastrointestinal tract pH (Graszkiewicz et al., 2010; Capurso et al., 2019). Thrombin and glutamyl endopeptidase II cannot hydrolyze proteins because they are precise for their respective substrates. Thrombin can only hydrolyze fibrinogen, and glutamyl endopeptidase II can only hydrolyze glutamyl bonds. They cannot recognize and bind to other proteins, preventing them from hydrolysing them (Andreatta et al., 1971; Stennicke & Breddam, 2012).

The A_E, W, V, and B_E parameters are crucial in understanding the efficiency of bioactive peptides. These parameters are derived from the peptide sequence and provide valuable insights into the structural and functional properties of the peptide. In this study, we selected the six most potential biological activities based on the results obtained: ACE inhibitor, alpha-glucosidase inhibitor, antioxidant, renin inhibitor, dipeptidyl peptidase III and IV inhibitor. The sum of A_E,

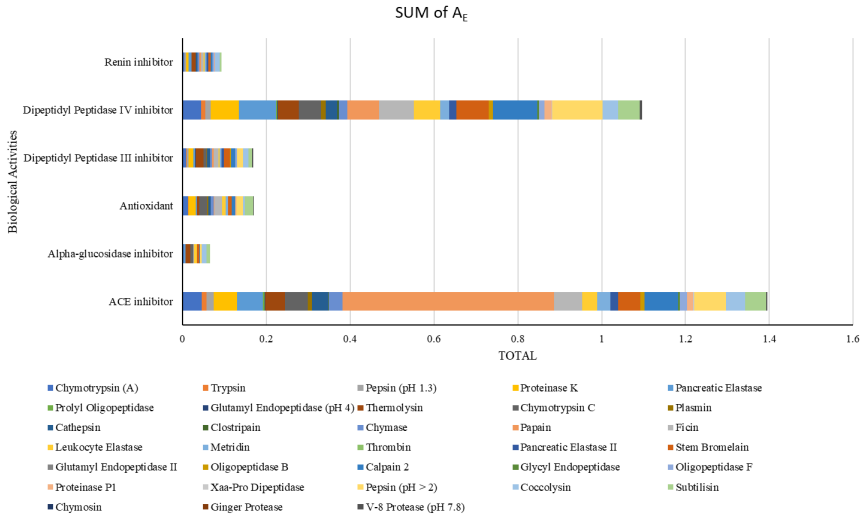


Figure 5. The sum of A_E of each protease for selected biological activities. A_E: Frequency of release fragments.

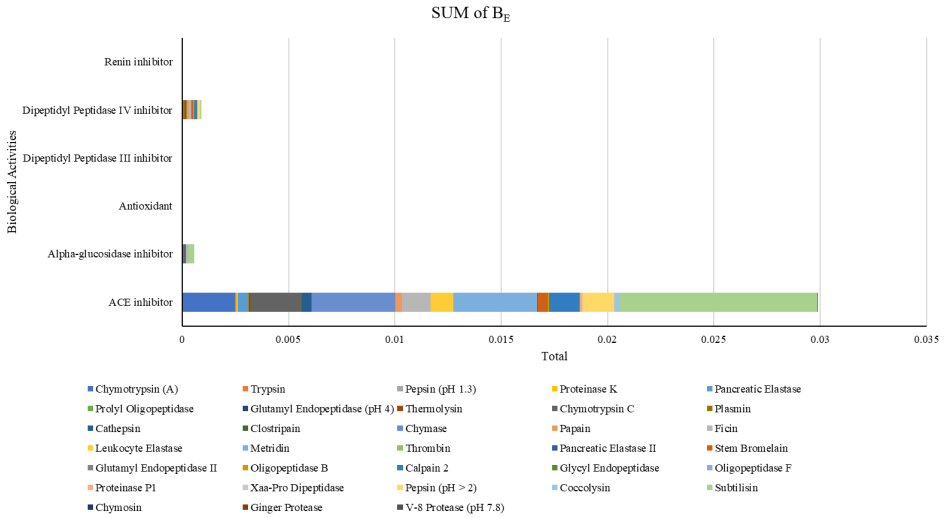


Figure 6. The sum of B_E of each protease for selected biological activities. B_E: Activity of fragments released by proteolytic enzymes.

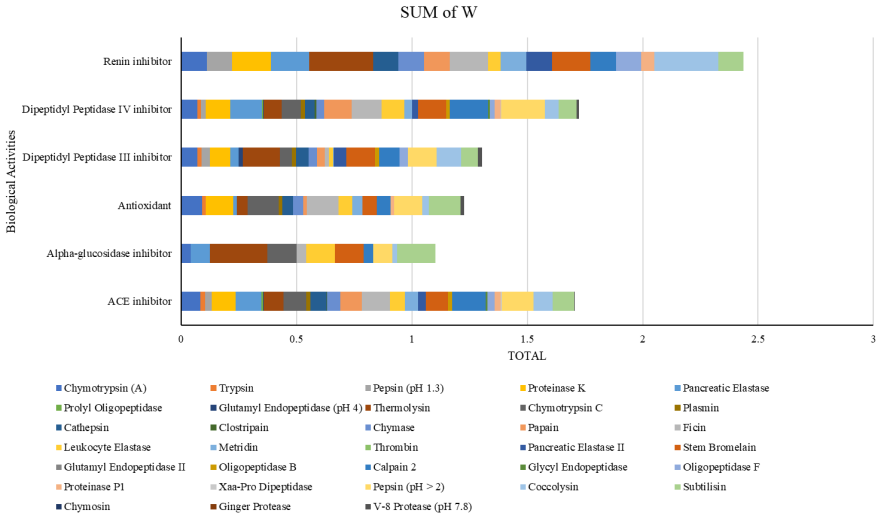


Figure 7. The sum of W of each protease for selected biological activities. W: Relative frequency of release fragments.

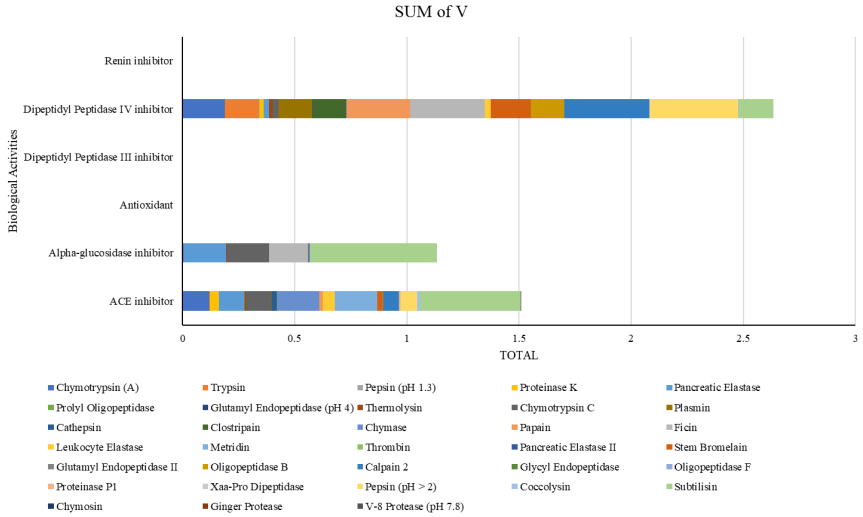


Figure 8. The sum of V of each protease for selected biological activities. V: Relative activity of fragments released by proteolytic enzymes.

B_E, W, and V values for each protease in each biological activity are presented in Figure 5-6-7-8.

The peptides were further evaluated and ranked based on their bioactivity ratings to identify novel bioactive peptides with specific effects. The PeptideRanker is a server that employs predictive algorithms to determine the likelihood of a particular peptide sequence being bioactive, thereby estimating the likelihood of discovering new bioactive peptides. The cutoff value for this investigation was established at greater than 0.75. A peptide that exceeds the PeptideRanker threshold (0.5) is classified as bioactive (Coscueta et al., 2022). Consequently, due to their prospective bioactivity, novel tripeptides and tetrapeptides with a bioactivity score greater than 0.75 were selected for analysis. Table 3 displays the predicted bioactive peptide and the allergenicity.

Tripeptide and tetrapeptide are more stable than longer peptides, which can be broken down by digestive enzymes, making them more likely to survive gastrointestinal digestion and reach the bloodstream; they can be designed to target specific biological pathways or receptors, allowing for more precise and effective bioactivity; more cost-effective option for bioactive peptide development and ease to synthesis (Daliri et al., 2017; Du & Li, 2022). Based on the results, some peptides are probable allergens with Tanimoto scores between 0.5-0.8. The information on the allergenicity of a peptide is crucial for ensuring the safety and efficacy of peptide-based treatments. The Tanimoto score is an essential tool in identifying the allergenicity of a peptide by predicting the potential for cross-reactivity between different allergens. The Tanimoto score ranges from 0 to 1, where 1 indicates identical sequences, and 0 indicates no similarity. The Tanimoto score can help identify these similarities and predict the potential for cross-reactivity (Karlsson et al., 2016; Shao et al., 2021). In this study, we identified a bioactive peptide derived from RuBisCo *C. racemosa*, which exhibited a high bioactivity prediction score (>0.75), indicating strong potential for biological activity. Interestingly, this same peptide sequence was also detected in other species, suggesting its conserved nature and potential functional importance across different taxa that summarized in Table 4. Short-chain bioactive peptides such as RDRF, AYF, RCY, IPP, PFMR,

Table 3. The PeptideRanker of the bioactive peptide by various proteases (> 0.75) and its allergenicity

Enzyme	Peptide	Score	Allerg	TS
Chymotrypsin (A)	AAF	0.833	NON	0.83
	RDRF	0.826	NON	0.83
	SQPF	0.879	YES	0.83
	TGGF	0.844	NON	0.84
Trypsin				
Pepsin (pH 1.3)	AAF	0.833	NON	0.83
	AYF	0.9153	NON	0.84
	TGGF	0.8441	NON	0.84
Proteinase K	AAF	0.833	NON	0.83
	GAGF	0.934	NON	0.82
	HGM	0.78	NON	0.84
	RDRF	0.826	NON	0.83
	TGGF	0.844	NON	0.84
Pancreatic Elastase	FQG	0.88	NON	0.84
	FRMT	0.84	NON	0.83
	HPWG	0.951	YES	0.83
	MPI	0.799	NON	0.83
	PPHG	0.796	YES	0.83
	QFG	0.919	NON	0.85
	RCY	0.752	NON	0.83
Glutamyl Endopeptidase (pH 4)				
Thermolysin	LGMP	0.879	NON	0.81
Chymotrypsin C				
Plasmin				
Cathepsin	AAF	0.833	NON	0.83
	TGGF	0.844	NON	0.84
Clostripain				
Chymase	AAF	0.833	NON	0.83
	GHPW	0.954	YES	0.8
	MRW	0.978	NON	0.83
	RDRF	0.826	NON	0.83
	TGGF	0.844	NON	0.84
Papain	AYF	0.915	NON	0.84
	HPWG	0.951	YES	0.83
	MHF	0.963	NON	0.84
	PPHG	0.796	YES	0.83
	QPF	0.954	NON	0.85
Ficin	AAF	0.833	NON	0.83
	MPAL	0.801	NON	0.81
	PPH	0.765	YES	0.83
	PWG	0.985	NON	0.83
	QPF	0.954	NON	0.85
Leukocyte Elastase	FRMT	0.84	NON	0.83
	GFV	0.825	NON	0.82
	GGFT	0.853	YES	0.84
	GMPI	0.845	NON	0.82
	YPL	0.792	YES	0.82
Metridin	AAF	0.8333	NON	0.83
	GHPW	0.954	YES	0.8
	MRW	0.978	NON	0.83
	RDRF	0.826	NON	0.83
	TGGF	0.844	NON	0.84
Thrombin				
Pancreatic Elastase II	AAF	0.8333	NON	0.83
	AYF	0.915	NON	0.84
	TGGF	0.844	NON	0.84
Stem Bromelain	HPWG	0.951	YES	0.83
	MHF	0.963	NON	0.84
	PPHG	0.796	YES	0.83
	QPF	0.954	NON	0.85
	YPL	0.792	YES	0.82
Glutamyl Endopeptidase II				
Oligopeptidase B				
Calpain 2	AFR	0.91	NON	0.83
	HPWG	0.951	YES	0.83
	IPP	0.766	YES	0.82
	MHFR	0.902	YES	0.82
	PFMR	0.974	YES	0.81
	PPHG	0.796	YES	0.83
Glycyl Endopeptidase	HPWG	0.951	YES	0.83
	PPHG	0.796	YES	0.83
Oligopeptidase F	AAF	0.8333	NON	0.83
	AYF	0.915	NON	0.84
	TGGF	0.844	NON	0.84
Proteinase P1	GFV	0.825	NON	0.82
	WGNA	0.771	YES	0.82
	WHM	0.966	NON	0.82
Xaa-Pro Dipeptidase				
Pepsin (pH > 2)	HPWG	0.951	YES	0.83
	PPHG	0.796	YES	0.83
	RCY	0.752	NON	0.83
	VWHM	0.81	NON	0.83
Coccolysin	LGMP	0.879	NON	0.81
Subtilisin	AAF	0.833	NON	0.83
	GHPW	0.954	YES	0.8
	GMPI	0.845	NON	0.82
	MRW	0.978	NON	0.83
	QPF	0.954	NON	0.83
	RDRF	0.826	NON	0.85
	TGGF	0.844	NON	0.83
Chymosin				
Ginger Protease				
V-8 Protease (pH 7.8)				

*Allerg: Allergenicity

*TS: Tanimoto Score

Table 4. Bioactive peptides from RuBisCO and its activities

Peptide	Sequence	Source	Reported Bioactivity	Reference
RDRF	RDRF	*Ulva australis*	Collagenase inhibitor, antibacterial	Kang et al (2023)
AYF	AYFPEL	Milk proteins	Antihypertensive	Contreras et al. (2010)
RCY	RCY	Natural compound B16	Tyrosinase inhibitors	Hsiao et al. (2014)
IPP	IPP	Milk casein	ACE inhibitor	Hirota et al. (2007); Adams et al. (2020)
PFMR	PFMR	*Ulva lactuca*	ACE inhibitor	Amin et al (2021)
YPL	YPLDLF	Spinach RuBisCo	Opioid activity	Hirata et al. (2007)

and YPL exhibit a range of biological activities due to their specific amino acid composition, sequence, and structural characteristics. These peptides often contain hydrophobic (e.g., Phe, Ile, Leu, Tyr) and positively charged residues (e.g., Arg, Lys), which enhance their interaction with target enzymes or reactive species. For example, IPP (Ile-Pro-Pro) is a well-characterized tripeptide with proven ACE-inhibitory and antihypertensive effects, attributed to its proline-rich sequence and ability to bind the ACE active site (Nakamura et al., 1995; Seppo et al., 2003). Similarly, RCY and AYF contain aromatic and sulfur-containing residues, which are known to contribute to antioxidant activity through free radical scavenging (Erdmann et al., 2008). Multifunctionality is common among short peptides (<5 residues) because they can adopt diverse conformations and interact with various biological targets, leading to overlapping antioxidant, antihypertensive, anti-inflammatory, or anticancer effects (Udenigwe & Aluko, 2012). This versatility makes such peptides promising candidates for nutraceutical or pharmaceutical applications.

The toxicity of the RuBisCO protein from *C. racemosa* can be seen in Table 5. Most of the peptides found in RuBisCO protein from *C. racemosa* are not toxic; only seven fragments are predicted as toxins. The released active peptides from various enzymes with a PeptideRanker score > 0.75 are not toxins. The information on the toxicity of a peptide is crucial for ensuring the safety, efficacy, cost-effectiveness, regulatory compliance, and improved understanding of peptide-based therapeutics. Accurate toxicity prediction is essential for successfully developing and applying peptide-based treatments (Wei et al., 2022; Zhao et al., 2022). While many previous studies on RuBisCO-derived peptides focus on sequence-based prediction alone (e.g., using BIOPEP), this study advances the field by integrating structural-based molecular docking, enabling an assessment of binding affinity and interaction strength with real protein targets. For instance, the tripeptide IPP, previously shown to lower blood pressure in clinical trials (Nakamura et al., 1995), displayed a comparable docking score in our model, validating the predictive approach. This reinforces the value of combining peptide screening with structure-based computational validation for therapeutic peptide discovery.

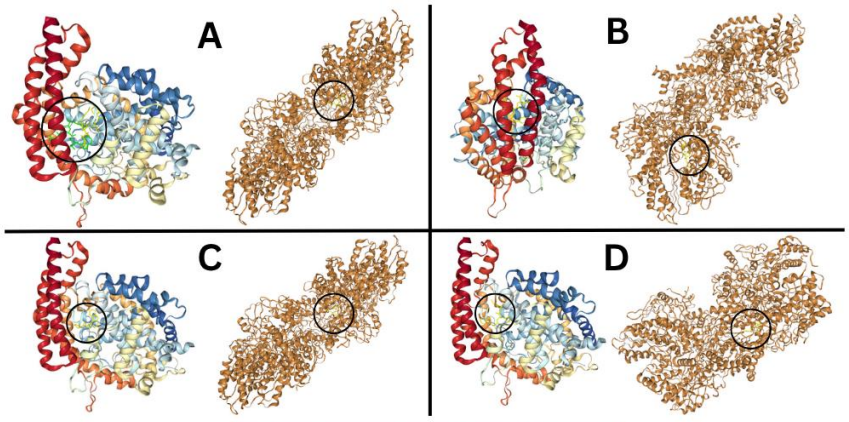
This study used the top four peptides with the highest score (> 0.75) as the receptor for molecular docking. The molecular docking results revealed that the selected peptides exhibited strong binding affinities toward both ACE and xanthine oxidase (Figure 9) with docking scores ranging from −169.00 to −252.29 kcal/mol and −164.59 to −221.89 kcal/mol, respectively. These values suggest favorable interactions, especially since more negative docking scores correlate with stronger binding (Meng et al., 2011). The confidence scores for both targets (up to 0.8855 for ACE and 0.8081 for XO) further support the reliability of the predicted binding poses. While the ligand RMSD values were relatively high, particularly for ACE (up to 82.13 Å), such variability is expected in flexible peptide docking and does not necessarily indicate poor binding (Yan et al., 2017). These findings imply that the peptides possess potential dual bioactivity, acting as both ACE inhibitors and antioxidants, aligning with previous studies highlighting the multifunctionality of marine-derived peptides (Ngo et al., 2011).

Table 5. The prediction of toxicity from RuBisCO protein of *C. racemosa*

Fragments	SVM Score	Predicted Toxicity
AGHCDEMIKR	0.04	Toxin
DRYKGRCYDL	0.15	Toxin
GDDACLQFGG	0.03	Toxin
GHCDEMIKRA	0.09	Toxin
HCDEMIKRAQ	0.19	Toxin
NATAGHCDEM	0.05	Toxin
RYKGRCYDLE	0.20	Toxin
All the released of bioactive peptides (> 0.75)	-Ve	Non-Toxic

Conclusions

This study provides a comprehensive in silico analysis of the bioactive potential of peptides derived from the RuBisCO protein in *C. racemosa*. By integrating multiple bioinformatic tools, including BIOPEP-UWM, PeptideRanker, ToxinPred, AllergenFP, ProtParam, and molecular docking simulation using HDOCK we identified a diverse array of peptides with predicted



PEPTIDE	ACTIVITIES	DOCKING SCORE	CONFIDENCE SCORE	LIGAND RMSD (Å)
MRW	ACE inhibitor	-252.29	0.8855	74.62
	Antioxidant	-221.89	0.8081	54.57
PFMR	ACE inhibitor	-237.69	0.8524	77.17
	Antioxidant	-217.78	0.7950	42.35
PWG	ACE inhibitor	-169.00	0.5939	82.13
	Antioxidant	-164.59	0.5724	60.53
WHM	ACE inhibitor	-221.65	0.8074	79.84
	Antioxidant	-197.46	0.7210	55.10

Figure 9. Molecular docking and score of peptides as ACE inhibitor (left) and antioxidants (right). A) MPW; B) PFMR; C) PWG; and D) WHM. The circle shows the binding of the peptides to the receptor.

biological activities, notably ACE and DPP-IV inhibition. Several peptides demonstrated high activity scores and were predicted to be non-toxic and non-allergenic, indicating their promise as nutraceutical or therapeutic candidates. This study also provides novel insight by demonstrating that peptides from C. *racemosa* RuBisCO are capable of dual inhibitory effects on ACE and XO, as confirmed through a combination of in silico enzymatic digestion, bioactivity scoring, and molecular docking. Importantly, we emphasize that all results presented here are based solely on in silico predictions. While these computational approaches offer valuable preliminary insights and significantly streamline the discovery process, experimental validation is essential to confirm the actual bioactivity, safety, and efficacy of the identified peptides. Future work should involve in vitro enzymatic hydrolysis, peptide isolation, and functional assays to validate the predicted activities and assess their real-world potential. Such validation would not only confirm the therapeutic relevance of these peptides but also strengthen their applicability in functional food or pharmaceutical development.

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Author Contributions

First Author: Conceptualization, Data Curation, Formal Analysis, Methodology, Writing-original draft, writing-review and editing; Second Author: Project Administration, Funding Acquisition; Third Author: Resources, Software; and Fourth Author: Investigation and Formal Analysis.

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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