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Research article

Radical scavenging capacity of RuBisCO bioactive peptides derived from *Dunaliella salina* and *Spirulina platensis*: An *in silico* and *in vitro* study

Seyedeh Fahimeh Razavi, Leila Zarandi Miandoab*, Elaheh Zadeh Hosseingholi, Nader Chaparzadeh

Department of Biology, Faculty of Basic Sciences, Azarbaijan Shahid Madani University, Tabriz, Iran

* **Correspondence:** Email: zarandi@azaruniv.ac.ir; Tel: 00984134327500; Fax: 00984134327500.

Abstract: Microalgae have a large high-quality protein content that can be used as human protein supplements. *Dunaliella salina* and *Spirulina platensis* have been identified as rich sources of natural bioactive compounds. We aimed to examine the antioxidant properties of bioactive peptides using the enzymatic digestion of the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme derived from *D. salina* and *S. platensis* microalgae. This was an in vitro and in silico study. Cell walls of *D. salina* and *S. platensis* were lysed, proteins were isolated, and RuBisCO fraction was concentrated. Then, the protein was enzymatically digested using pepsin, trypsin, and chymotrypsin. Finally, antioxidant activity was assessed at different stages (pre- and post-digestion). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used to assess the antioxidant potential of hydrolyzates both before and after digestion. Findings indicated that digestion over time, particularly by chymotrypsin, produced bioactive fragments with enhanced antioxidant properties. For the 0–35 protein fraction (which likely includes RuBisCO), the antioxidant potential of peptides derived from *S. platensis* was significantly greater than that from *D. salina.* We showed that chymotrypsin may be an appropriate enzyme to yield the highest peptide concentrations from the protein extracts of these microalgae with the highest antioxidant activity. Moreover, the results of digesting the RuBisCO sequence with digestive enzymes showed that antioxidant properties increased with the production of hidden bioactive peptides. This finding may lead to the application of RuBisCO protein and its derivative peptides in the food and pharmaceutical industries of *S. platensis* and *D. salina*.
Keywords: RuBisCO, microalgae; bioactive peptide; anti-DPPH; enzymatic digestion

1. Introduction

Algae, ranging from single-celled microalgae to multicellular organisms, are among the most prevalent eukaryotic organisms found in aquatic environments worldwide, including both freshwater and saltwater systems [1,2]. These versatile organisms are extensively utilized for various applications. Their pigments, proteins, lipids, and antioxidant compounds find widespread use in pharmaceutical, food, and cosmetic industries [3]. Microalgae, with their high-quality, abundant protein content and their chlorophyll, are capable of meeting human protein and metabolic requirements comprehensively [4]. Thanks to their rapid growth rates and ease of cultivation, microalgae are regarded as a sustainable strategy for future global protein production [5]. Certain microalgae species have been utilized for many years, and their nutritional and pharmacological properties have been extensively researched. Notably, *Dunaliella salina* and *Spirulina platensis* are identified as rich sources of natural bioactive compounds. The former is recognized for its high protein content, while the latter exhibits a high concentration of natural pigments per cell [6,7]. Microalgae are rich sources of natural pigments with significant biotechnological applications. Their pigment content varies based on species and cultivation conditions. For instance, chlorophylls are abundant in *Chlorella vulgaris* and *Scenedesmus dimorphus*, while β-carotene is predominantly found in *Dunaliella salina*. Astaxanthin, a potent antioxidant, is mainly derived from *Haematococcus pluvialis*, and lutein is present in *Muriellopsis* sp. and *Scenedesmus almerienses*. Fucoxanthin is extracted from *Phaeodactylum tricornutum*, and phycobiliproteins are extracted from *Spirulina platensis*. These pigments have diverse applications in food, cosmetics, and pharmaceuticals, but their concentrations are highly variable and dependent on environmental factors.

Recent research highlights that enzymatic digestion or microbial and gastrointestinal proteolysis can generate bioactive peptides from proteins. These peptides often exhibit higher antioxidant activity compared to their parent proteins [8]. Bioactive peptides, typically consisting of 2–50 amino acids, are formed during protein digestion and exhibit significant effects, including anti-inflammatory, antioxidant, antihypertensive, antidiabetic, cardioprotective, and antimicrobial activities [9]. Investigations emphasize the potential of these peptides as functional ingredients in foods, for vaccines, or as candidates for antidiabetic and anticancer drugs [10]. Consequently, identifying abundant, affordable, and accessible sources of bioactive peptides could greatly enhance human health and living standards. Additionally, since such protein-rich sources are used in animal feed for aquatic animals, livestock, and poultry, they also contribute to increased productivity in these industries [11]. RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) stands out as one of the most abundant proteins on Earth and a crucial enzyme in photosynthetic organisms. This protein consists of 16 subunits (8 large and 8 small) and includes all 20 amino acids. Its digestion through enzymatic processes can yield bioactive peptides, making it a valuable and readily available source [12]. RuBisCO exhibits several nutritional and therapeutic benefits, including improving immune function and providing anti-aging, anticancer, antihypertensive, and antidiabetic effects [13]. The large subunit, with its active site, interacts with a diverse range of substrates, prompting many in vitro and in silico studies to focus on its substrate interactions [14].

A notable property of bioactive peptides is their ability to neutralize free radicals, which are highly reactive due to their extra electron. Free radicals can interact with biological molecules like proteins, fatty acids, and nucleic acids, potentially causing genetic mutations or cell death. Thus, natural or synthetic antioxidants, as well as enzymatic systems that degrade free radicals, are crucial for cellular

protection [15]. Studies suggest peptides containing aromatic and hydrophobic amino acids, such as proline and isoleucine, are particularly effective at scavenging free radicals. Unlike synthetic antioxidants, natural bioactive peptides are cost-effective, safe, and well-suited for use in the food and pharmaceutical industries [8]. In recent years, bioinformatics tools and databases have emerged as efficient, cost-effective methods for identifying proteolytic systems capable of releasing bioactive peptides from various protein precursors [16]. This study aimed to evaluate the antioxidant properties of bioactive peptides using the enzymatic digestion of the RuBisCO enzyme derived from *D. salina* and *S. platensis* microalgae.

2. Materials and methods

This was an in silico and in vitro study. The process involved lysing the cell walls of *D. salina* and *S. platensis*, isolating and concentrating the RuBisCO fraction, and enzymatically digesting the protein using pepsin, trypsin, and chymotrypsin. Antioxidant activity was assessed at different stages (pre- and post-digestion), and the results were compared and analyzed. To effectively measure the antioxidant activity of RuBisCO and its peptides following enzymatic digestion, understanding the interaction between RuBisCO and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (a well-known oxidizing agent) is essential. This interaction provides insights into the protein's antioxidant capabilities. Additionally, determining the three-dimensional structure of RuBisCO prior to hydrolysis is critical for analyzing how its structural features influence its functionality. For laboratory testing, obtaining preliminary data is equally important. This includes information on the amino acid composition, molecular weight, and the quantity of potentially bioactive peptides both before and after enzymatic digestion. Such data aids in assessing the protein's characteristics and the efficacy of peptide generation, providing a comprehensive understanding of the bioactive potential and antioxidant properties of RuBisCO and its derived peptides

2.1. In silico phase

2.1.1. Bioinformatics analysis

UniProt databases (www.uniprot.org), ExPASy portal (https://web.expasy.org/protparam/), BIOPEP (https://biochemia.uwm.edu.pl/biopep-uwm/), SWISS-MODEL (https://swissmodel.expasy.org/), PROCHECK web server (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/), Ramachandran plot server (https://saves.mbi.ucla.edu/) PubChem database (https://pubchem.ncbi.nlm.nih.gov/), CASTP web server (http://cast.engr.uic.edu), Molegro Virtual Docker software, PYMOL software, CHIMERA 1.15 software, and AutoDock 4.2 docking software were used in this study.

2.1.2. Sequence information and amino acid composition

The primary sequence of the large subunit of RuBisCO protein in *D. salina* (D0FXZ7) and *S. platensis* (D4ZVW7) was acquired from the UniProtKB database and used for further analysis. Sequence alignments were performed with ClustalW2 (http://www.clustal.org/clustal2/) [17]. The homology of sequence alignments was attained from UniProtKB/Swiss-Prot. The phylogenetic tree

was constructed using ClustalW2 [18]. The amino acid sequence compositions of the large subunit of RuBisCO in *D. salina* and *S. platensis* were analyzed using the ProtParam tool. ProtParam is an in silico analysis program designed to compute the physical and chemical properties of a protein or peptide based on its amino acid sequence [19]. The analysis included determining the total amino acid composition, molecular weight, and isoelectric point (PI) of the large subunit. The findings from this investigation were documented and reported.

2.1.3. Frequency analysis of bioactive peptides

The frequency of bioactive peptide fragments within the primary sequence of the RuBisCO large subunit and the potential biological activity of the protein were assessed using the BIOPEP database [20]. These calculations were based on two equations, respectively:

$$\text{Equation 1: } A = \frac{a}{N},$$

$$\text{Equation 2: } B = \frac{\sum_{i=1}^{K} \frac{a_i}{EC_{50i}}}{N},$$

where: N is the total number of amino acids in the protein, a is the number of bioactive peptide fragments hidden in the protein sequence, a_i is the number of repetitions of the i-th bioactive fragment in the protein sequence, EC_{50i} is the concentration of the i-th bioactive peptide required for half-maximum inhibitory activity, and κ is the number of distinct bioactive fragments with specific activity.

2.1.4. Proteolysis and protein modeling

RuBisCO proteolysis was carried out with the use of the BIOPEP web server and its enzyme action tool. Digestive proteases such as chymotrypsin, trypsin, and pepsin were used to hydrolyze the large subunit of RuBisCO from *D. salina* and *S. platensis*. The frequency of occurrence of fragments with certain activity by enzymes (A_E) and the relative frequency of occurrence of fragments with given activity by enzymes (W) were calculated as:

$$\text{Equation 3: } A_E = \frac{d}{N},$$

$$\text{Equation 4: } \mathrm{W} = \frac{\mathrm{A_E}}{\mathrm{A}},$$

where d is the number of peptides with activity considered released by the enzyme, N is the number of amino acids in the protein, AE is the frequency of release of fragments with given activity by the selected enzymes, and A is the frequency of occurrence of bioactive peptide fragments in the primary sequence [21].

The SWISS-MODEL software was employed to predict the three-dimensional structure of the large subunit of RuBisCO from *D. salina* and *S. platensis*. The SWISS-MODEL is a comparative modeling tool that generates structural predictions by comparing the target protein sequence with known protein structures. When the sequence similarity between the target and template proteins exceeds 50%, the predicted structure is generally of high quality. In this research, after downing the RuBisCO protein sequence of microalgae from the UNIPROT web server, through the SWISS-MODEL software, the crystallographic model of RuBisCO large subunit from *Chlamydomonas*

reinhardtii, with more than 90% similarity, was proposed and the results were reported. The sequence was then verified and certified by the PROCHECK web service using the number of permitted angles in the Ramachandran plot and the torsion angles of the protein bonds [22].

2.1.5. Docking analysis

The DPPH molecule was obtained from the PubChem database and verified by Molegro Virtual Docker software. Then, by minimizing energy in Chem3D software, all bonds and angles were examined to prepare for docking. Furthermore, the sequence and structure of RuBisCO protein modeled by SWISS-MODEL software were analyzed in Molegro. This software removes excessive ligands, cofactors, and water molecules from the crystal structure [23]. The final sequence was structurally edited before docking in CHIMERA 1.15 software. After that, using the *Minimize Structure* tool, the best and most dependable spatial structure of the protein was selected, and the docking process was performed using AutoDock 4.2. This software is among the most trustworthy software for drug discovery, molecular docking, and computational experiments. One of the features of this software is its high accuracy and high speed compared to other molecular docking software [24].

2.2. In vitro phase

2.2.1. Sample preparation

S. platensis was purchased from the Amol University Technology Centre in Iran, and *D. salina* was generously provided to our research team by the Biotechnology Research Centre of Northwest Iran. All salts were purchased from Merck (Germany), and all enzymes were purchased from Sigma-Aldrich (USA). *D. salina* was cultivated in modified Johnson's medium (ASW) [25], while *S. platensis* was cultivated in Zarrouk's medium [26]. The levels of acidity, light, humidity, and temperature of each strain were adjusted based on optimal values [27]. In order to draw the growth curve and investigate the growth rate of *S. platensis* and *D. salina,* respectively, under optimal growth, sampling was done periodically from the culture containers, and the growth curve was drawn based on the most pigment absorption. We measured the growth of *S. platensis* at 560 nm [28] and *D. salina* at 660 nm [29] using the UNICO Vis 2100 spectrophotometer. All salts and solutions, including PBS buffer (NaCl, Na_2HPO_4, KCl, KH_2PO_4), ammonium sulfate, acetone solution, acetic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ninhydrin, and all required for protein gel electrophoresis [sodium dodecyl sulfate, acrylamide, bis-acrylamide, tetramethyl ethylenediamine (TEMED), tris base, ammonium persulfate (APS), bromophenol blue, and glycerol] were purchased from Merck (Germany).

2.2.2. Protein extraction and analysis

In brief, to extract protein content, 5 g of sample cell pellet was dissolved in 50 mL of PBS buffer (0.01 M, pH 7.4) to make a suspension. To enhance cell wall breakage, an ultrasonic homogenizer (30 s/10 s on/off cycles, 200 W, 30 min) in an ice bath (Bandelin HD2070 Ultrasonic Homogenizer-Sonicator, Germany) and four repetitions of freeze/thaw cycles (−20 °C, 4 h/40°C) were used. Then, the extract was centrifuged at 13,000× g for 20 min at 4°C (PIP Universal centrifuge PIT320 series high-end). This step was repeated twice. Then, the protein content was precipitated by

adding ammonium sulfate (40% w/v), keeping it at 4℃ overnight. Then, the sample was centrifuged at 2,500 × g for 20 min at 4℃ to form bullet-like masses. To improve protein precipitation, precipitation by ammonium sulfate was performed in two consecutive steps. The precipitate was collected and washed two times with cold acetone (100%) to remove lipid contamination. Finally, the precipitate was dried and dissolved in 150 mL of 50 mM phosphate buffer pH 7.5 and stored in a freezer at −20℃ [30,31]. To measure protein concentration in the protein solutions, 100 μL of the protein solution was mixed with 2 μL of distilled water. Then, 900 μL of Bradford reagent was added, and after 5 min at room temperature, the absorbance was measured at 595 nm. The standard curve for total protein was prepared using bovine serum albumin (BSA) at concentrations of 200, 300, 400, 500, and 600 μg/mL [32].

To analyze the protein profile of samples and qualitatively compare the results with quantitative data from spectrophotometry, a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) test with polyacrylamide gel (stacking gel 5% and running gel 12%) was used. Samples were mixed with sample buffer in a ratio of 1:4 and placed in a Bain-Marie for 5 min at 94℃ [33]. Then, samples were loaded in the acrylamide gel next to the ladder protein (pre-stained protein ladder Cinnagen, 180–11 kDa). Akhtariran-PST2000 electrophoresis power supply was used for this purpose. After loading each sample in the wells, the electrophoresis voltage was maintained at 80 V, and the current intensity was set to 2 A. Then, the gel was placed in the coloring solution for one day and night on the Heidolph Rotmax 120 model shaker. The gel containing protein bands was decolorized in the decolorizing solution for 5 h. Photographing of the gels was done with HP Scanjet G3010 scanner. In order to purify and find the specific protein fraction and band of RuBisCO protein present in the protein solution, the sedimentation method with concentrations of ammonium sulfate was used. To this aim, a pre-weighed amount of ammonium sulfate was slowly added while gently stirring for 5–10 min until the desired saturation. Stirring continued for an additional 10–30 min, and then the solution was cooled for 15–30 min. Centrifugation was performed at 20,000 × g for 15 min at 4℃. The remaining pellet was re-dissolved in buffer, and more pre-weighed ammonium sulfate was added to reach the desired secondary saturation, allowing proteins to precipitate between the initial and secondary fractions. Undissolved material was removed by centrifugation, and ammonium sulfate was eliminated from the protein solution using a dialysis bag. The final contents were stored for subsequent assays [34].

2.2.3. Enzymatic digestion

In order to simulate the events that happen in the digestive tract, the RuBisCO protein solution was digested by three enzymes: trypsin, pepsin, and chymotrypsin. Specifically, protein solutions were first hydrolyzed by pepsin [ratio of enzyme to substrate (E/S) 6% w/w, pH 2, 37℃, and reaction time 2 h]; then, the reaction was stopped at 85℃ for 15 min. After inactivation, the solutions were hydrolyzed by trypsin (E/S 3% w/w, 37 °C, pH 8, and reaction time 3 h). After inactivation at 85℃ for 15 min, solutions were hydrolyzed by chymotrypsin (E/S 5% w/w, 37 °C, pH 8, and reaction time 3 h). The final reaction was stopped by heating the mixture in a boiling water bath for 10 min [35]. The cooled hydrolysates were then subjected to centrifugation at 10,000 rpm for 20 min at 4℃. In each step, sampling was done to determine the anti-DPPH power of the produced fragment and to measure the progress of the reaction and the degree of hydrolysis (DH) by ninhydrin test at 570 nm with a glycine standard diagram.

2.2.4. Antioxidant assay

To determine the anti-DPPH activity of the protein solution before and after enzymatic digestion, 1 mL of each solution was added to 1 mL of a 0.002% DPPH solution in ethanol, and the resulting solution was vortexed for 10 s for 30 min in the dark. Finally, the absorbance of the solution was measured at 517 nm [36]. Ascorbic acid was used as a positive control. DPPH-free radical inhibition activity (I) was calculated by Eq 5:

$$\text{Equation 5: } I(\%) = \frac{A\ blank - A\ sample}{A\ blank} \times 100,$$

where *I* is the DPPH-free radical inhibition activity, A blank is the absorbance of blank, and A sample is the absorbance of the sample.

2.3. Statistical analysis

All the steps were repeated three times; SPSS software, version 17, was used for the statistical analysis of the results obtained from the laboratory section. Results were reported as mean ± standard deviation. Duncan's test was chosen to compare the results between different samples and a significance level of 0.05 was considered.

3. Results

3.1. In silico

3.1.1. Bioinformatics

The alignment results between *D. salina* and *S. platensis* RuBisCO sequences with *C. reinhardtii* showed that there is more than 80% similarity between the RuBisCO sequences of these microalgae. The structure of the RuBisCO large subunit of *D. salina* and *S. platensis* was modeled with acceptable accuracy, and the results showed that the sequence of *D. salina* is evolutionarily closer to *C. reinhardtii* (Figure 1A). Other in silico investigations showed that glycine is the most abundant amino acid in the RuBisCO protein of these microalgae and that this protein is also rich in alanine, leucine, valine, threonine, glutamic acid, arginine, and aspartic acid. Additionally, the PI of this protein was approximately 6.33 and 6.04 for *D. salina* and *S. platensis*, respectively (Table 1). The occurrence rate of hidden bioactive peptides (A) and protein potential biological activity (B) calculated for the RuBisCO protein showed that these values are higher in *S. platensis* than *D. salina*, which can be due to the difference in the amino acid composition or in the coding sequence of the antioxidant amino acids in the large chain (Table 1S). In general, proteins with hydrophobic and aromatic amino acids (especially proline amino acids) in their structure have better antioxidant activity, so the type of amino acids has a major effect on their activity.

Table 1. Physicochemical properties of RuBisCO large subunit.

Amino acid composition	*D. salina*	*S. platensis*
Ala (A)	43	39
Arg (R)	29	29
Asn (N)	15	16
Asp (D)	26	27
Cys (C)	11	11
Gln (Q)	12	13
Glu (E)	31	34
Gly (G)	50	45
His (H)	15	15
Ile (I)	19	23
Leu (L)	40	38
Lys (K)	23	24
Met (M)	11	15
Phe (F)	20	25
Pro (P)	21	23
Ser (S)	17	17
Thr (T)	31	32
Trp (W)	8	9
Tyr (Y)	18	14
Val (V)	3	27
Total number of aa	443	476
Molecular weight (kDa)	52.45	53.27
Theoretical PI	6.33	6.04

3.1.2. Sequence information and amino acid composition

In this study, by comparing the sequence of the large chain of RuBisCO protein, Pro142 and Met116 residues in *S. platensis* were replaced by serine and leucine amino acids in *D. salina*. These results, along with other factors, may indicate differences in the reported antioxidant properties (Table 2S).

3.1.3. Frequency analysis of bioactive peptides

An in silico proteolytic digestion of the large subunit of RuBisCO with BIOPEP and Peptide-cutter tools with the digestive enzymes pepsin, trypsin, and chymotrypsin showed that, potentially, 243 regions in *D. salina* and 260 regions in *S. platensis* are cut. In addition, the results of the investigation of these web servers showed that chymotrypsin enzyme shows a greater ability to break the sequence and produce peptide fragments in both microalgae, compared to pepsin and trypsin enzymes (Table 2). Examining the amount of W (the relative frequency of occurrence of fragments with the given activity by the enzyme) and A_E (the frequency of the occurrence of fragments with a given activity by the enzyme) showed that potential peptides with antioxidant properties are created more by enzymatic digestion in *S. platensis* (Table 3). This may be due to the presence of more

antioxidant amino acids and more active hidden antioxidant peptides in *S. platensis* (Table 2S)**.**

Table 2. Possible cleavage sites of enzymes in the large subunit sequence of RuBisCO protein.

No. of cleavages	Microalgae/Name of enzyme
D. salina/pepsin	87
D. salina/trypsin	51
D. salina/chymotrypsin	105
S. platensis/pepsin	100
S. platensis/trypsin	51
S. platensis/chymotrypsin	109

Table 3. Frequency of occurrence of digested fragments with certain activities and relative frequency of occurrence of fragments

Microalgae/large subunit	Activity	W	A_E
D. salina	Antioxidative	0.1165	0.0105
S. platensis	Antioxidative	0.1459	0.0111

3.1.5. Proteolysis and protein modeling

After checking the RCSB database, it was found that the 3D structure of the RuBisCO protein in *S. platensis* and *D. salina* has not been crystallographed; thus, the 3D structure of the two proteins was designed using modeling tools. Additional investigations showed that the most similar protein with the available crystallographic structure is the *C. reinhardtii* RuBisCO protein, which has 84% similarity with the *S. platensis* and 95% similarity with *D. salina.* Thus, this structure is the best model for this process. On the other hand, after performing modeling with SWISS-MODEL, the GMQE numerical index was calculated for the built models, with values ranging between 0 and 1; the closer this number is to 1, the higher the quality of the structure (Table 3S). This value was higher than 0.9 for both microalgae. To check the correctness of the structure of the simulated proteins, the correct spatial position of the backbone atoms of the amino acid chain and their side chain in the Ramachandran diagram was evaluated through PROCHECK software (Figure 1B), and more than 96% of the amino acids were located in the right position.

3.1.6. Docking

After determining protein structure, it was necessary to prepare the structure of DPPH as a free radical agent interacting with reducing agents. The 3D structure of this chemical compound was extracted from PubChem database and, after editing and optimizing, the structure in the docking process molecular was used (Figure 1C). After preparing the RuBisCO protein and DPPH ligand and determining the active site of the protein by the CASTP server, the most optimal mode of connection between the two was performed at the predicted location through the AutoDock 4.2 software during the docking process (Figure 1D–E). The result of this connection and the amino acids involved in this interaction are shown in Table 4S. The interaction between the amino acids involved in the connection and DPPH atoms is shown in 2D in Discovery Studio Visualization software (Figure 1F).

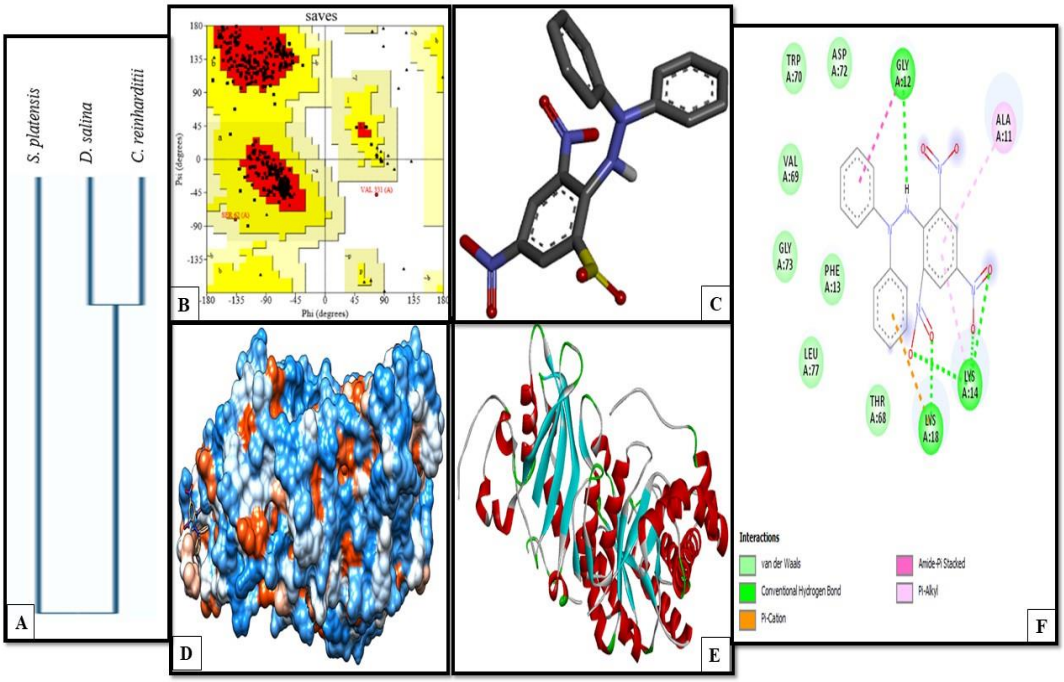

Figure 1. A. Alignment of the large subunit of RuBisCO protein in microalgae. **B.** Ramachandran diagram of the modeled RuBisCO protein. **C.** DPPH ligand. **D.** RuBisCO protein large subunit modeled after structural editing. **E.** 3D docking of the RuBisCO large subunit modeled with the DPPH ligand. **F.** Specific interactions of modeled RuBisCO large subunit and DPPH ligand.

3.2. In vitro

3.2.1. Protein analysis

After drawing the growth curve of *S. platensis* and *D. salina*, sampling was done at the stage of maximum protein production and before entering the death stage. The protein content extracted in phosphate buffer from both microalgae (Figure 1S) showed that the average total protein in *S. platensis* was higher than in *D. salina* microalgae. This difference is quite plausible due to differences in microalgal species. For enzymatic digestion studies on the large subunit of RuBisCO, it is necessary to separate this protein from the protein content obtained from cell lysate, which was used by the precipitation technique with ammonium sulfate in the fractions 0-20 (F0-F20) and F20-F40 of both microalgae. The corresponding band identified was selected for the complete extraction of RuBisCO from the protein content of F0-F35, a large part of which belongs to the 52–53 kDa band of the large subunit of RuBisCO. Since the major component of this fraction is the large subunit of RuBisCO, this value was first measured, followed by the volume and concentration (Figure 2).

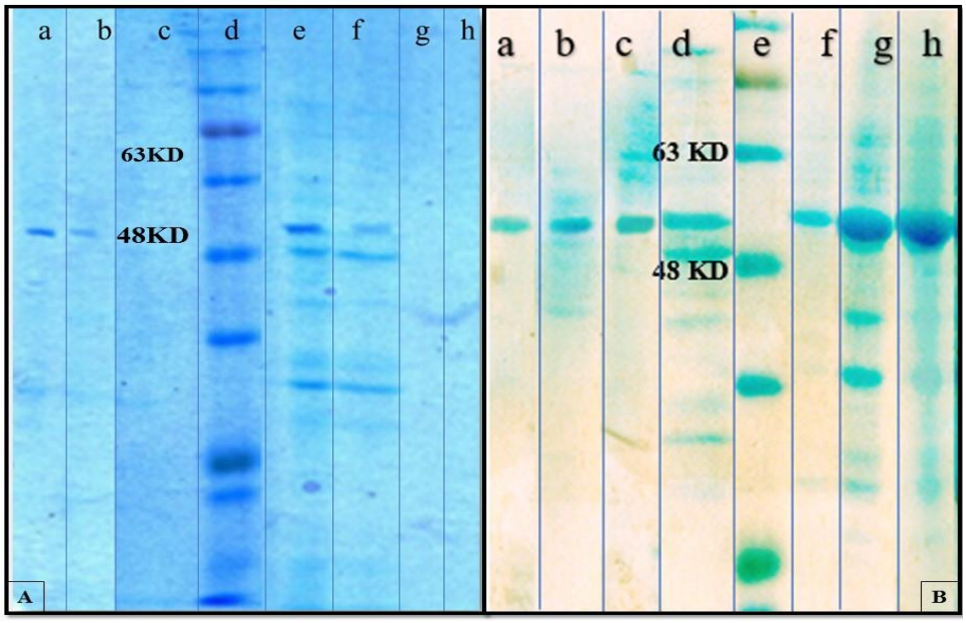

Figure 2. A. Acrylamide gel of ammonium sulfate precipitation fractions. a: *D. salina* F0-F20; b: *D. salina* F20-F40; c: *D. salina* F60-F40; d: Protein ladder (pre-stained protein ladder cinnagen, 11–180 kDa); f: *S. platensis* F0-F20; g: *S. platensis* F20-F40; h: *S. platensis* F40-F60. **B.** a: *S. platensis* F0-F20; b: *S. platensis* F20-F40; c: *S. platensis* F0-F35; d: *S. platensis* control sample; e: protein ladder (pre-stained protein ladder cinnagen, 11–180 kDa); f: *D. salina* F0-F20 fraction; g: *D. salina* F20-F40 fraction; h: *D. salina* F0-F35 fraction; j: *D. salina* control sample.

3.2.2. Enzymatic digestion

Samples were digested by pepsin, trypsin, and chymotrypsin enzymes. After each stage, the amount of protein in the fraction 0–35 decreased. So, the evidence shows that the protein digestion of samples is performed at a high speed. In addition, on average, chymotrypsin had a higher digestive power than the others in the production of peptide fragments (Figure 3).

Measuring the amount of hydrolysis and free amino acids produced by digestion with enzymes in F0–F35 showed that, like the digestion results, the production of free amino acids with chymotrypsin is higher than that with pepsin and trypsin. Data show the greater ability of chymotrypsin to produce bioactive peptide fragments. In addition, the comparison of the digestion results and the amount of free amino acids produced between the two microalgae showed that the production of peptide fragments is higher in *D. salina* than *S. platensis*, which is probably due to the presence of sequences with more suitable sites for enzymatic cleavage (Figure 4). Also, the effect of enzyme type on the production of free amino acids was significant at a p-value of 0.001.

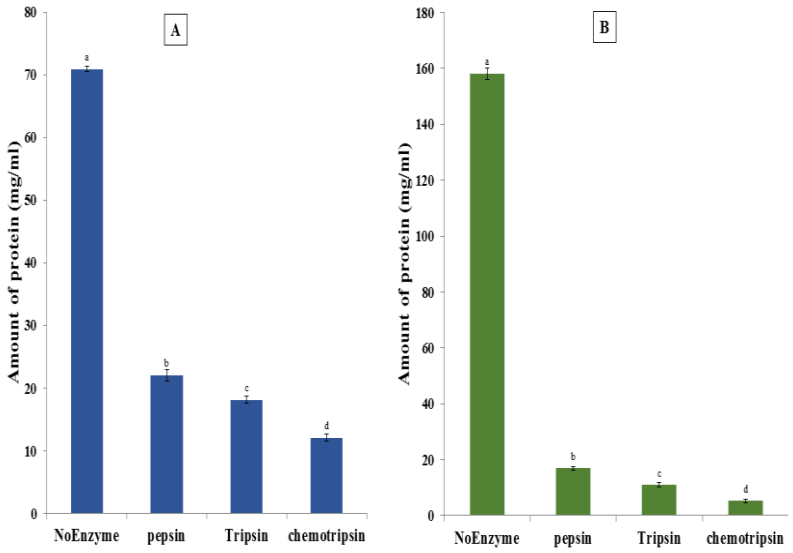

Figure 3. Comparison of protein content after digestion of F0–F35 in (**A**) *S. platensis* and (**B**) *D. salina.*

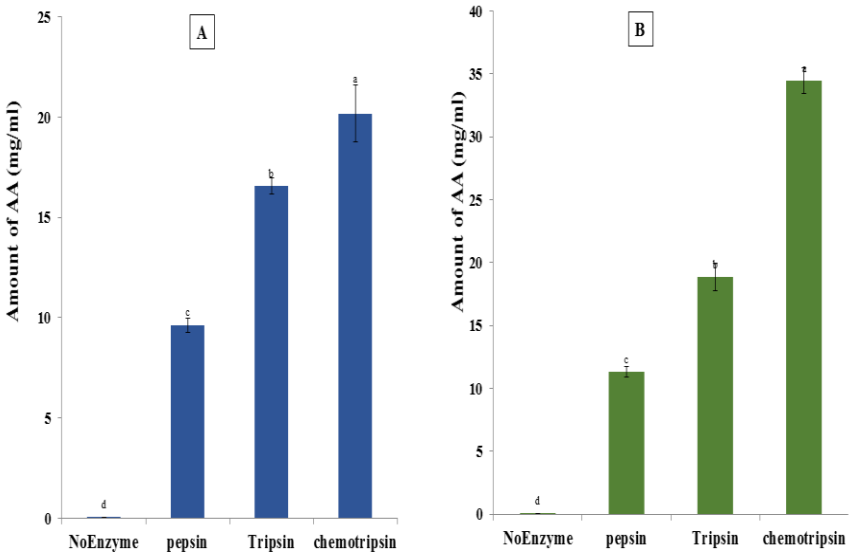

Figure 4. Free amino acid content obtained from the digestion of fraction 0–35 in (**A**) *S. platensis* and (**B**) *D. salina.*

3.2.3. Antioxidant assay

The antioxidant activity of protein and peptide sequences was measured by DPPH radical scavenging activity. On average, the total protein extracted from *S. platensis* showed 43% inhibitory activity. This was approximately 12% in *D. salina* (Figure 2S). During the reaction and while increasing the effect of digestive enzymes on fraction 0–35, bioactive fragments with more antioxidant properties were produced because chymotrypsin has more parts with higher antioxidant properties (Figure 3S). Also, statistical data showed that the effect of the type of microalgae on DPPH free radical

inhibitory percentage activity and the effect of digestive enzymes on DPPH free radical inhibitory activity percentage in *S. platensis* and *D. salina* was significant at a p-value of 0.001. Furthermore, antioxidant properties of bioactive peptides derived from the digestion of fraction 0–35 in *S. platensis* were reported to be far higher than the peptides derived from the digestion of the same fraction in *D. salina*. In the control sample without digesting the selected fraction, the antioxidant power of *D. salina* was higher than that of *S. platensis* (Figure 5).

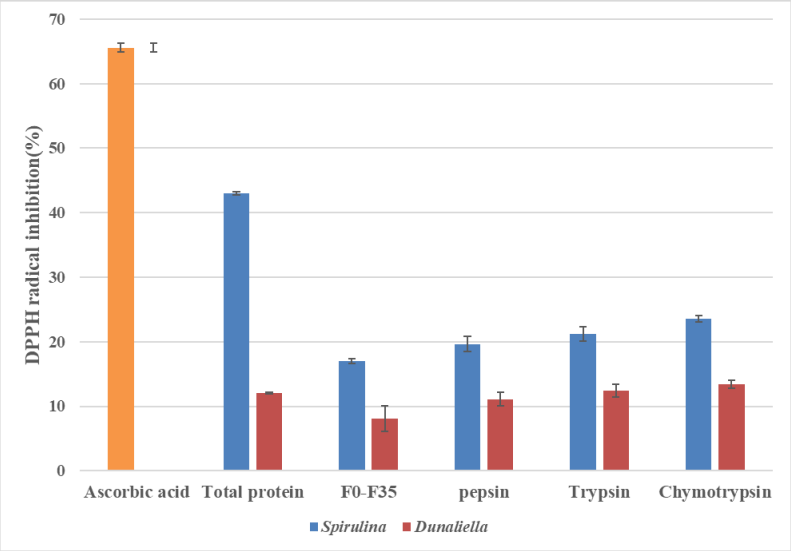

Figure 5. DPPH free radical inhibition percentage of total protein *S. platensis* and *D. salina*. DPPH free radical inhibition percentage of RuBisCO from *S. platensis* and *D. salina* and peptides derived from F0–F35 in *S. platensis* and *D. salina*.

4. Discussion

In this study, the antioxidant activity of total protein extracts and RuBisCO protein from *D. salina* and *S. platensis* was measured before and after enzymatic digestion with pepsin, trypsin, and chymotrypsin. Results indicated that digestion over time, particularly by chymotrypsin, produced bioactive fragments with enhanced antioxidant properties. For the 0–35 protein fraction (which likely includes RuBisCO), the antioxidant potential of peptides derived from *S. platensis* was significantly greater than that of peptides from *D. salina*. These findings highlight the potential of RuBisCO-derived peptides as antioxidant agents, especially due to the ease of microalgae cultivation compared to other protein sources. However, while this study demonstrates in vitro antioxidant activity, further research is needed to validate these findings in in vivo models. Future studies should investigate the bioavailability, metabolic stability, and pharmacokinetics of these peptides to determine their efficacy in biological systems.

The environmental sustainability of microalgae, coupled with their rapid growth and high protein yield, makes them an attractive alternative to traditional protein sources, which require more resources and time to cultivate. Utilizing digestive enzymes for hydrolysis could yield novel antioxidant peptides for pharmaceutical and food industries. Hydrolysis with endopeptidases such as pepsin, trypsin, and chymotrypsin is believed to improve the bioavailability of active peptides and protect them from

further digestion in the gastrointestinal tract. Additionally, evidence suggests that enzymatic digestion may produce peptides with superior biological activity. Further optimization of enzymatic conditions, such as temperature, pH, and enzyme concentration, could further enhance peptide yield and activity. To bridge the gap between laboratory findings and practical applications, future research should also focus on optimizing enzymatic hydrolysis conditions, such as temperature, pH, and enzyme concentration, to maximize the yield of bioactive peptides while maintaining their structural integrity.

Bioactive peptides, typically composed of 5–20 amino acid residues, are initially inactive within their parent protein structures. However, upon enzymatic digestion in the stomach and intestine, they are released and exhibit significant biological effects [37]. Due to their numerous health benefits, these peptides are increasingly included in the pharmaceutical and dietary sectors [10]. Global researchers are actively exploring rich protein sources to extract these bioactive peptides efficiently for industrial applications. This demand has driven efforts to identify sustainable protein sources and develop cost-effective, high-efficiency extraction techniques using diverse organisms, including bacteria, fungi, plants, and animals [38,39].

Among these sources, microalgae have emerged as a prominent subject of study due to their affordability and sustainability. They are recognized for producing bioactive peptides with a variety of beneficial properties, including antioxidant, antidiabetic, antihypertensive, anticancer, antimicrobial, and anti-inflammatory effects [40,41]. Particularly, *S. platensis* and *D. salina* are noted for their high protein content and robust antioxidant properties. Recent advancements in microalgae cultivation, such as optimizing growth conditions and the use of bioreactors, have further improved the yield of bioactive peptides. Despite these advantages, challenges remain in scaling up production. Industrial application of these peptides requires overcoming hurdles related to production costs, large-scale extraction techniques, and ensuring consistent bioactivity in final formulations. Implementing advanced bioreactor technologies, optimizing biomass harvesting, and improving protein purification strategies could enhance efficiency and lower costs [42].

Chronic diseases linked to oxidative stress caused by excessive free radicals pose significant threats to human health, damaging essential biomolecules like DNA, proteins, and lipids. A critical strategy to mitigate these effects is the use of enzymatic or molecular antioxidant compounds to neutralize free radicals [43,44]. Oxidative stress is increasingly linked to the development of neurodegenerative diseases, cardiovascular conditions, and certain types of cancer, which further highlights the importance of bioactive peptides in combating these conditions. Advances in bioinformatics have further accelerated this research. Tools and databases in proteomics have streamlined the discovery of optimal proteolytic systems and the identification of peptide sources, complementing experimental studies [45,46]. Bioinformatics tools like the BIOPEP database have also enabled the prediction of peptide bioactivity, allowing researchers to efficiently pinpoint peptides with potential therapeutic effects from large protein sequences. Although this study establishes a foundation for RuBisCO-derived antioxidant peptides, future research should explore their practical applications in functional food development, pharmaceutical formulations, and natural food preservatives. The potential of these peptides as additives in nutraceuticals, anti-aging products, or even active components in wound healing therapies should also be investigated. Additionally, regulatory and safety assessments will be necessary before commercialization. Addressing these aspects could facilitate the transition from experimental studies to real-world applications, unlocking the full potential of RuBisCO-derived peptides in health and industry.

The study's limitations include reliance on computational models for RuBisCO's 3D structure,

which may not fully reflect the actual protein structure due to the lack of crystallized data for *D. salina* and *S. platensis*. The observed antioxidant activity in vitro may not directly translate to in vivo effects, and variability in protein extraction and digestion methods could affect the results. Additionally, the use of pepsin, trypsin, and chymotrypsin may not fully replicate human digestive processes, potentially influencing the accuracy of the proteolysis and peptide production profiles. Another limitation of this study is the exclusive assessment of anti-DPPH activity as the sole measure of antioxidant capacity. To address these limitations, future studies should incorporate in vivo validation, explore alternative enzymatic hydrolysis methods that mimic human digestion more closely, incorporate additional assays, such as 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) or ferric reducing antioxidant power (FRAP), and investigate the long-term stability and activity of these peptides under real physiological conditions.

5. Conclusions

This research reveals the prominent radical scavenging capacity of proteins and peptides from protein fractions 0–35 derived from *S. platensis* and *D. salina*. Simulated digestive conditions and bioinformatics analysis demonstrated that chymotrypsin was the most effective enzyme for digesting these proteins and producing bioactive peptides with anti-DPPH activity properties, outperforming trypsin and pepsin. Notably, *S. platensis* showed stronger antioxidant effects than *D. salina*. Given their high protein content, ease of cultivation, and ability to produce bioactive peptides, these microalgae present a sustainable and valuable resource for the development of pharmaceutical and food-based antioxidants. Enzymatic hydrolysis, particularly with chymotrypsin, proves to be a promising method for extracting bioactive peptides, suggesting a natural and efficient approach to improving human health and reducing disease risk through functional foods and nutraceuticals.

Author contributions

SFR conceived and conducted the study, gathered the data and drafted the manuscript; SFR and LZM conducted statistical analysis and interpretation of data, depicted the graphs, and supervised the study. NCh supervised the study. EZH was the consultant. SFR and LZM revised the final manuscript for important intellectual content.

All authors read and approved the final manuscript.

Use of Generative-AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

Conflict of interest

All authors declare no conflicts of interest in this paper.

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