Trends in Peptide and Protein Sciences
Volume 7 (2022): e6

DOI: http://dx.doi.org/10.22037/tpps.v7i0.38965

Original Article

Antioxidant Activity of Peptides Derived from Enzymatic Digestion of *Spirulina platensis* Protein Extract by Different Proteases

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Article history:
Received: 27 July 2022
Accepted: 21 August 2022

Keywords:
Cyanobacteria
Spirulina platensis
Protein extraction
Protease digestion
Antioxidant
DPHH

HIGHLIGHTS

- *Spirulina platensis* protein extract was digested with three types of protease.
- *Digestion of protein extract by pepsin resulted in higher antioxidant activity.*
- *Digestion by bacterial protease from Bacillus licheniformis resulted in higher yield of pep*tide formation.

ABSTRACT

One of the attractive sources of bioactive compounds is cyanobacteria and in particular, *Chlorella vulgaris* and *Spirulina platensis*. Enzymatic digestion of the *Spirulina* protein extract can result in bioactive peptides with diverse activities, including antioxidant function. This study aims to produce peptides with antioxidant properties after the enzymatic digestion of *Spirulina platensis* protein extract using three enzymes: bacterial protease, pepsin, and papain. The protein extract from *Spirulina platensis* was subjected to enzyme hydrolysis for 3 hours at 37°C (pH 7.4 for papain and bacterial protease and pH 5 for pepsin). The concentration of peptide fragments was evaluated to determine the yield of protein digestion. In order to measure the level of anti-oxidative potential of the hydrolysates, the DPPH assay was run. The results indicated that the bacterial protease led to the highest concentration of peptide fragments, while the hydrolysate obtained from pepsin digestion showed the most antioxidant activity (80%), mainly the peptides that have molecular weights less than 14 KDa. Consequently, pepsin can be a proper enzyme to produce antioxidant peptides from the protein extract of *S. platensis*. In conclusion, the results of the study confirmed that the products of enzymatic digestion by different enzymes have distinct features.

Cite this article as: Niknam, H., Fathi, F., Mahboubi, A. and M. Tabarzad, (2022). Antioxidant activity of peptides derived from enzymatic digestion of *Spirulina platensis* protein extract by different proteases. *Trends Pept. Protein Sci.*,**7**: e6.

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Introduction

Peptides extracted from natural sources have been recognized as efficient bioactive compounds for a wide

range of applications. The specific structural properties of peptides make them promising compounds in various biomedical applications. Natural peptides are usually small molecules with less than 100 amino acids, are found in animals, plants, marine organisms, fungi and bacteria, and can be extracted directly from natural sources or be derived from the proteolytic breakdown of extracted proteins (Gogineni and Hamann, 2018; Ovando et al., 2018).

One of the main sources of therapeutic peptides is microalgae and cyanobacteria. Cyanobacteria have been studied as photosynthetic prokaryotes and their therapeutic values have been reported in more than 1500 biologically active compounds. Cyanobacterial metabolites with a significant role in modulating the immune system or antimicrobial activity have also been reported, for example, bacteriocins with antimicrobial activity. Some other metabolites have shown to be a good preservative for food preparation or can be used as an antibiotic. Despite a large number of studies on cyanobacterial therapeutic potentials, a small number of their active metabolites entered the nutritional or pharmaceutical markets, however, it is growing fast (Raja et al., 2016; Saad et al., 2022).

In addition to nutritional importance, some microalgae such as *Chlorella*, *Scenedesmus*, *Arthrospira* (*Spirulina*), and *Nostoc* have shown other bioactivities, such as antioxidants, anti-inflammatory, and anti-coagulation activities (Tabarzad et al., 2020).

Spirulina is a blue-green alga that has been used as a source of proteins and other bioactive metabolites for years. It is classified as multicellular filamentous cyanobacterium that can grow in extreme conditions such as salinized or contaminated water (Soni et al., 2017; Grosshagauer et al., 2020). The hydrolysis of *Spirulina* protein content has resulted in many bioactive peptides. Several studies have shown the health benefits of *Spirulina* extracts (Montalvo, 2018; Jena and Subudhi, 2019).

Besides, studies have confirmed that *Spirulina* could significantly reduce oxidative stress. These protective and antioxidant effects are due to the presence of active metabolites, such as phycocyanins, beta-carotene, and other vitamins and minerals (Wu et al., 2016). A few clinical studies on the antioxidant activity of *Spirulina*-derived peptides and proteins exist, confirming that it can be an effective treatment for chronic obstructive pulmonary disease and skeletal muscle damage caused by oxidative stress after exercise. *Spirulina* can strongly stimulate antioxidant enzyme activity, prevent the process of lipid peroxidation, DNA damage, and can result in free radical elimination. In addition, *Spirulina* can be a protective agent against neurotoxicity, hepatotoxicity, and liver toxicity caused by oxidative stress (Ovando et al., 2018; Han et al., 2021). Different proteases and altered enzyme digestion processes may result in diverse peptide mixtures and consequently, not the same bioactivities (Nouri and Abbasi, 2018; Ovando et al., 2018; Mohammadi et al., 2022). In this study, we compared the peptide mixtures derived from enzymatic digestion by three types of proteases on *Spirulina* protein extract based on the antioxidant activity.

Materials and Methods

Cells and chemicals

Spirulina platensis powder was supplied from Algae Bioresource Development of Fars Co. (Iran). Ascorbic acid (A920902) was supplied from Merck chemical (Germany). O-Phthalaldehyde (OPA) (P1378) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (D9132) were supplied from Sigma Aldrich (USA). All other chemicals were supplied from Merck chemical (Germany). Bacterial protease from *Bacillus licheniformis* (P3910) and papain from *Carica papaya* (#76220) were supplied from Sigma-Aldrich (USA) and pepsin from porcine gastric mucus (#1.07185.0010) was from BioBasic (Canada). Peptone A (#G213) was supplied from Biobasic (Canada).

Protein extraction of S. platensis biomass

To extract *Spirulina* protein content, a similar method to Sun *et al.* was used (Sun et al., 2016). In brief, 50 g of *Spirulina* powder was dispersed in 500 mL of deionized water to make a suspension. The ultrasonic homogenizer (30 s-10 s on-off cycles, 200W, 30 min) (Topsonic, 20KHz, 400W, Farasout, Iran) and two repetitions of freeze (-70°C)-thaw (95 °C) cycles were used to enhance cell wall breakage. Then, *Spirulina* extraction mixture was centrifuged for 20 min at 8000 g and 4 °C (Sigma refrigerated ultracentrifuge, Sigma, Germany). The residual biomass was applied to the second round of extraction, similar to the previous run. The supernatants of two rounds of extraction, containing proteins, were mixed. Then, the protein content was precipitated by adding ammonium sulfate (43% w/v), keeping it at 4 °C overnight. Then, the sample was centrifuged at 15000 g at 4 °C for 20 min to form bullet-like masses. To improve the protein precipitation from extract, two sequential steps of precipitation by ammonium sulfate were also performed. The precipitate was collected and washed two times, with cold acetone (100%) to remove lipid contaminations. Finally, the precipitate was dried and dissolved in 150 mL of 50 mM phosphate buffer pH 7.4. The mixture was completely vortexed and incubated at 50 °C for 30 min to increase the protein solubility in the buffer.

Determination of total protein content by Bradford Test

To evaluate the extraction efficiency, the amount of protein in the extraction product was measured by Bradford analysis (Bradford 1976) using TaKaRa Bradford Protein Assay Kit (T9310, Japan). In brief, a serial dilution (0-1000 µg.mL^{-1}) of standard samples of bovine serum albumin (BSA) was prepared with deionized water. Then, 20 µL of standard and test samples were separately added to 1000 µL of Bradford reagent and incubated at room temperature for 5 min. Then, the absorbance of mixtures was recorded at 595 nm. As the *Spirulina* protein extract had a color that may interfere with the absorbance at 595 nm, a blank sample with the same concentration was also tested and its absorbance was extracted from the test sample absorbance. *Spirulina* protein extract was diluted 50-fold before the test started.

Enzymatic digestion of protein extract

According to the results of Bradford, there is 40 mg of protein in each sample used for enzyme digestion. The ratio of 2% (w/w) of protease/protein was applied. In brief, 80 µL of each enzyme stock (pepsin, papain, and bacterial protease at 10 mg.mL^{-1}) was added to each sample. The enzyme digestion was performed in 50mM phosphate buffer pH 7.4 for papain and bacterial protease and 50 mM phosphate buffer pH 5 for pepsin. The samples were incubated for 3 h at 37 °C in Shaker Water Bath (WNB 7-45, Memmert, Germany), and then, placed at 95 °C for 30 min to stop the digestion process. Finally, the samples were evaluated by OPA test to determine the yield of enzymatic digestion. The obtained peptide mixture was then dialyzed at 4°C for 72 h in 50 mL BPS pH 7 using a dialysis membrane, cutoff 14 KDa (#D0405, sigma, USA). Both compartments were then, freeze-dried and dissolve again in BPS pH 7.

Determination of peptide content by OPA test

To prepare the OPA reagent, 99 mL of boric acid 3% pH 10.5, 1 mL of 50 mg/mL^{-1} of O-phthaldehyde (OPA) in ethanol and 50 µL of 2-mercaptoethanol were mixed (Ru¨tti et al., 2009). The peptone serial dilutions were used as standards. The first standard was prepared at a concentration of 1 mg.mL^{-1}. Then, it was serially diluted to the final concentration of 0.0156 mg.mL^{-1}. In a 96 wells microplate, 20 µL of standard peptone or test sample and 200 µL of OPA reagent were mixed. After one-hour of incubation at room temperature in dark, fluorescence (excitation at 340 nm and emission at 455 nm) was recorded by fluorimeter (Cytation3, Agilent, USA). A calibration curve for standards was drawn and the amount of peptide in the test samples was calculated using the calibration curve.

Evaluation of antioxidant activity using DPPH assay

First, all samples were diluted to the protein concentration of 0.01 mg.mL^{-1}. Then, three dilutions of 1/10, 1/20 and 1/40 were prepared from the enzymatic digestion samples. A solution of ascorbic acid at 1 mg.mL^{-1} was used as the positive control. Fifty µL of each sample were mixed with 50 µL of DPPH methanolic solution (0.1 mM). For negative control, 50 µL of absolute ethanol and 50 µL DPPH reagent were mixed. As the samples have color, one blank sample was prepared for each test sample, containing 50 µL phosphate buffers and 50 µL samples. Finally, the absorbance of the samples at 520 nm was recorded by ELISA reader (Garni medical engineering Co., Iran). Then, the antioxidant activity was calculated using the Eq. 1 (de Torre et al., 2019):
(Eq. 1)

$$\text{Antioxidant activity} = [1 - \frac{(A_s - A_b)}{(A_c)}] \times 100$$

where, A_s is the absorbance of sample, A_b is the absorbance of blank, and A_c is the absorbance of the control (DPPH reagent without sample).

Statistical analysis

All experiments were performed triplicated and mean ± SD was calculated using Excel 2016. Differences between means of antioxidant activities of different samples were analyzed by one-way ANOVA test followed by Tukey post hoc test using IBM SPSS ™ 21. A p-value less than 0.05 was considered significant.

Results and Discussion

Yield of protein extraction from Spirulina platensis biomass

A part of protein extract was not easily dissolved in phosphate buffer. In conclusion, there are several large insoluble proteins in the extract. Therefore, final centrifugation was performed to remove the insoluble parts before Bradford test. The total protein in soluble content was measured by Bradford test. The results showed that the yield of soluble protein purification was around 10% (w/w) of dry biomass. However, more than twofold protein content was detected in the insoluble part, which was about 30% (w/w) of dry biomass (according to the dried weight of precipitate).

Comparison of the yield of enzymatic digestion of protein extract

The type of enzymes used in protein hydrolysis can significantly affect the yield of peptide production and the biological activities of resulted peptide mixture, due to

the size, quantity, and composition of amino acids in peptides after digestion. This diversity is the result of the selective activity of enzymes on particular amino acids and peptide bound. Three enzymes—papain, pepsin, and bacterial protease from *Bacillus licheniformis*—were used in this study to hydrolyze protein samples.

The aspartic acid protease, pepsin, is responsible for breaking the connection between aromatic and hydrophobic amino acids. Peptide bonds including phenylalanine, tryptophan, and tyrosine are efficiently broken down by pepsin (Fruton, 2002). *Bacillus licheniformis*'s bacterial protease (P3910), according to the manufacturer's description, exhibits broad specificity and a predilection for a large uncharged residue in the P1 position. It is active in an alkaline environment. Compared to other proteases, papain might more thoroughly digest the majority of protein substrates. Papain has a wide range of activity and can break peptide bonds between basic amino acids, leucine, or glycine. Additionally, it catalyzes the hydrolysis of amides and esters. While valine is not accepted at the P1 position, papain favors the amino acid with a long hydrophobic side chain at the P2 position (Verma, et al. 2016).

It was revealed that the insoluble sample contained twice as much protein as the soluble part did. Therefore, the insoluble sample was suspended in PBS and diluted to make the protein concentration close to the concentration of the soluble part. Then, enzymatic digestions of both samples were carried out. The yield of hydrolysis was indicated by the increased fold in free amine groups as an index for peptides formation, using OPA test. The OPA method is one of the methods used to measure hydrolysis yield (Rutherfurd 2010). The reaction between the free amine group in amino acids and OPA is the basis of fluorescence detection (Mohajeri, et al. 2019). The peptide concentration of soluble and non-soluble parts of protein extract had calculated as 886.3068 $\mu g.mL^{-1}$ and 1120.051 $\mu g.mL^{-1}$, respectively, as indicated in Table 1. After digestion by pepsin, bacterial protease, and papain, the free amine groups raised up. After enzymatic digestion, the peptide concentration in the soluble fraction raised up to 1094.731 $\mu g.mL^{-1}$ in the presence of bacterial protease, which was about 23.5 % increase in peptides content, while they were 917.1326, and 945.5477 $\mu g.mL^{-1}$ in the presence of papain and pepsin, respectively, which showed the lesser amount of hydrolysis than bacterial protease. After being enzymatically digested by bacterial protease, the peptide concentration in the insoluble fraction raised up to 1276.528 $\mu g.mL^{-1}$ which showed about 12 % increase. Two other enzymes did not significantly affect the proteins in the insoluble fraction, and there were non-significant changes in the concentrations of the peptides before and after papain and pepsin's enzymatic digestion of the insoluble part of protein extract.

Table 1. Concentration of peptides of the microalgae Spirulina platensis

Sample	Concentration ($\mu g.mL^{-1}$)
Soluble fraction+ protease	1094.731 (± 109.2)
Soluble fraction + papain	917.1326 (± 89.2)
Soluble fraction + pepsin	945.5477 (± 65.5)
Soluble fraction	886.3068 (± 24.1)
Insoluble fraction+ protease	1276.528 (± 96.3)
Insoluble fraction	1120.051 (± 120.1)

Comparison of the antioxidant activity of different enzymes

In the presence of a hydrogen donor or an antioxidant agent, the DPPH radical is transformed to DPPH-H, a non-radical component, lowering the color intensity of the solution. Because this mechanism conforms to the Beer-Lambert equation, there is a direct correlation between the antioxidant concentration and the decrease of DPPH radicals (Rizzello, et al. 2017). The antioxidant activity of the different samples from various enzymatic digestions was reported in Table 2 and Fig. 1.

Table 2. Antioxidant activities of peptide mixtures derived from different enzymes digestion compared to ascorbic acid as the standard

Peptide concentration ($\mu g.mL^{-1}$)		Antioxidant activity (%)			
		protease digest	papain digest	pepsin digest	Ascorbic acid
10	Mean	35.0000	50.1667	80.1333	57.4333
	n	3	3	3	3
	SD	0.80000	0.76376	1.00664	0.60277
1	Mean	27.4000	42.3667	55.1667	35.1000
	n	3	3	3	3
	SD	0.85440	0.70946	0.96090	0.95394
0.5	Mean	40.2333	30.2000	37.5333	32.7333
	n	3	3	3	3
	SD	0.68069	0.81854	0.55076	0.25166
0.25	Mean	45.0667	17.6333	25.2000	27.2667
	n	3	3	3	3
	SD	0.20817	0.41633	0.81854	0.25166

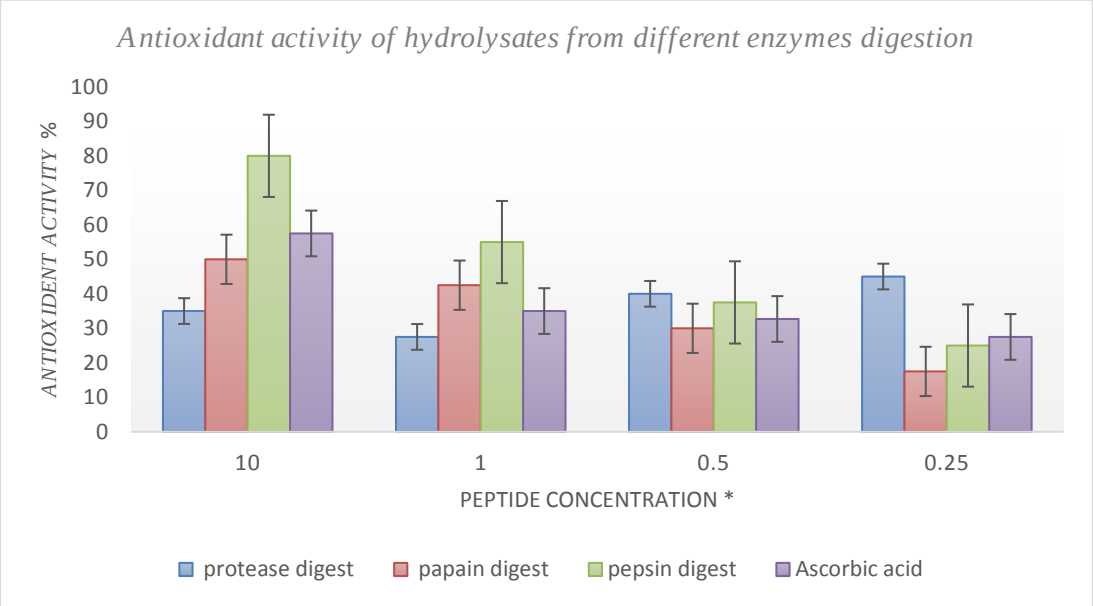

Figure 1. The bar chart shows the percentage of antioxidant properties of peptides produced by enzymatic digestion in the presence of pepsin, papain, and bacterial protease enzymes. The percentage of antioxidant activity of the insoluble fraction after enzymatic digestion is presented and the percentage of antioxidant activity of ascorbic acid was considered as a positive control sample.* Note that the concentration is in $\mu g.mL^{-1}$. There are significant differences between the antioxidant activity of samples and between the samples and ascorbic acid (p-value < 0.0001).

Comparing the mean antioxidant activities of the samples confirmed that they are significantly different (p-value < 0.0001). Consequent to the results, more than enzyme type, the concentration of peptide mixture also affects the antioxidant activity. By reducing the peptide concentration in the sample, the antioxidant properties of the sample decreased but not in a linear manner. This trend can be seen in almost all samples. Results revealed that the pepsin enzyme's digestion at the highest concentration, 10 $\mu g.mL^{-1}$, resulted in the highest antioxidant activity. It reduced free radicals by 80%. The positive control sample, ascorbic acid, showed 57.5% antioxidant activity at the same concentration of 10 $\mu g.mL^{-1}$. The papain product and the protease product in the highest concentration showed 50%, and 35% antioxidant activity, respectively. In the protein concentration of 10 and 1 $\mu g.mL^{-1}$, pepsin digestion resulted in higher antioxidant activity, but in the concentration of 0.5 and 0.25 $\mu g.mL^{-1}$, bacterial protease digestion resulted in more antioxidant activity. In addition, the peptide fraction with a molecular weight of less than 14KDa showed higher antioxidant activity (Table 3) which means the more potent antioxidant peptides may have smaller sequences.

Consistent with the results of other studies, this study indicated that, depending on the type of enzyme used for protein hydrolysis, bioactive peptides with different degrees of antioxidant activity could be obtained. For example, Hamishehkar and coworkers extracted bioactive peptides from *Spirulina platensis* protein extract using pancreatin and pepsin. They discovered pepsin hydrolysates had higher hydrolysis yield and antioxidant activity than pancreatin hydrolysates. Compared to the native protein, pepsin and pancreatin hydrolyzed samples greatly reduced lipid oxidation in emulsions (Mohammadi, et al. 2022). In another study, the digestibility of proteins extracted from *Schizochytrium limacinum* was investigated in simulated stomach phase and simulated small intestine phase conditions, using pepsin and the combination of α-chymotrypsin and trypsin, respectively. They showed that the non-hydrolyzed protein had a 43.25% DPPH radical scavenging activity. The DPPH free radical scavenging activity of protein hydrolysates was boosted by pepsin to 61.5%, and 73.8% after further trypsin and chymotrypsin hydrolysis (Moaveni, et al. 2022).

Table 3. The percentage of antioxidant activity of samples with different molecular weights

Samples	Antioxidant activity (Mean ± SD) (%)
Pepsin digestion >14 KDa	66.05 (± 1.02)
Papain digestion >14 KDa	69.13 (± 0.89)
Protease (bacterial) digestion >14 KDa	34.57 (± 0.99)
Pepsin digestion <14 KDa	82.71 (± 1.3)
Papain digestion <14 KDa	33.95 (± 1.1)
Protease (bacterial) digestion <14 KDa	19.13 (± 0.68)
Pepsin digestion Total	72.84 (± 0.98)
Papain digestion Total	44.44 (± 1.03)
Protease (bacterial) digestion Total	53.1(± 0.87)
Crude protein extract	62.96 (± 1.2)
Ascorbic acid	79.63 (± 0.62)

Conclusion

Cyanobacteria and marine microalgae have been rich in bioactive compounds and have been considered for a wide range of biomedical applications, nutritional supplements, and the discovery of therapeutic agents. *Spirulina* is one of the cyanobacteria that is an outstanding source of bioactive compounds, particularly bioactive peptides. Therefore, finding the new bioactive peptides, especially anti-oxidative ones, extracted from the *Spirulina* protein content is an intriguing topic that is interesting for many scientists. In this study, we evaluate if using different enzymes will produce diverse peptides with various potentials. In determining the optimum enzyme for enzymatic digestion, three enzymes were assessed to produce a mixture of peptides with varying levels of antioxidant activity. The findings suggested that pepsin could be the best option to break down the *Spirulina* protein to produce anti-oxidative peptides with the highest level of free radicals scavenging. The antioxidant effects of the peptides produced by the enzymatic digestion of these enzymes should be examined in more timescales in future research. Moreover, the effect of additional factors like temperature on the efficiency of enzymatic digestion and peptide mixture can be studied to obtain more precise information about the most potent bioactive peptide.

Ethical Statement

This work did not contain any animal or human studies performed by the authors.

Authors' Contribution

M. Tabarzad designed and supervised the project. H. Niknam and F. Fathi performed the experiments and analyzed the results. A. Mahboubi advised the methods and data analysis. F. Fathi drafted the manuscript. The final manuscript was reviewed and confirmed by M. Tabarzad.

Funding

This work was supported by Protein Technology Research Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran (Grant No. 27392, accepted by Ethical committee of Shahid Beheshti University of Medical Sciences, Tehran, Iran: IR.SBMU.PHARMACY.REC.1399.377)

Competing Interests

The authors declare that they have no conflicts of interest.

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