Advanced Research
in Microbial Metabolites & Technology
(ARMMT)
journal homepage: http://armmt.irost.ir

Antioxidant activity of alcalase hydrolysates of *Spirulina* proteins

Mina Mehdi Shishavan[1], Saeed Mirdamadi[1], Hamideh Ofoghi[1*]

[1] Department of Biotechnology, Iranian Research Organization for Science & Technology (IROST), Tehran, Iran.

Article Info

Received 11/07/2020
Received in revised form 22/12/2020
Accepted 24/12/2020

Keywords:
Spirulina,
Cell disruption,
Degree of hydrolysis,
Antioxidant activity,
Alcalase

Abstract

Spirulina microalgae are gaining increasing consideration for its pharmaceutical and nutritional (60-70% protein) virtues. Protein can be further processed to produce peptides that have functional and nutritional properties such as antihypertensive, immunomodulatory, antioxidant, hypocholesterolemic, and metal chelating activity. The purpose of this study is to investigate the relevance between antioxidant properties and the degree of hydrolysis (DH) of hydrolysates resulting from the effect of the alcalase enzyme on *Spirulina* protein at different times and the effect of time on the DH. For this purpose, *Spirulina* cells were disrupted with ultrasonication, bead milling, and water suspension methods. The resulting cell extract was hydrolyzed with serine proteases like the Alcalase enzyme. The sampling was taken every hour for up to 6 hours. DH of the samples was measured by the ortho-phthalaldehyde (OPA) method. Antioxidant activities of the hydrolysates were evaluated by assessing 2,2-diphenyl-1-picrylhydrazy (DPPH) and 2, 2′-azinobis (3-ethyl- benzothiazoline-6-sulphonate) (ABTS) radical scavenging activity. The ultrasonication method was selected for cell wall disruption. Results demonstrate that the highest increase in DH occurred in the first hour of hydrolysis. Then DH increased gradually with a low slope. The highest DH with an enzyme/substrate ratio of 1% (v/v) was 33.45% after 6 hours. Maximum DPPH radical scavenging activity occurred precisely at 120 minutes (22.71 ± 1.18 µM TE/mg protein, DH=28.29 ± 0.58). The scavenging activity of the DPPH radical increased by more than 2-fold after 2 hours of hydrolysis. The antioxidant activity of ABTS radical scavenging increased and reached 862.09 ± 22.26 µM TE/mg protein after 5 hours of hydrolysis (DH%=32.83 ± 0.87). This research indicates that along with the increase of DH, the ABTS radical scavenging activity also increases. Therefore, hydrolysis produces peptides that can scavenge DPPH and ABTS radicals at certain times. These results can be used to determine both DH and the time it occurs; therefore, the probability creation and isolation of bioactive peptides increase with high antioxidant activities. So, the hydrolysates or peptides derivative from *Spirulina* can be used as a functional food to improve and even prevent various diseases and disorders.

1. Introduction

Spirulina is a blue-green alga (family Oscillatoriaceae) (Gad et al., 2011; Heo et al., 2017), a multicellular, filamentous, and photosynthetic cyanobacterium (Vo et al., 2013)

*Corresponding author. Tel: (+9821)56276325-9
Email: ofoghi@irost.ir, hamideh2@yahoo.com
DOI: 10.22104/armmt.2020.4280.1041

that contains 50-70% proteins, essential amino acids (Al Hinai et al., 2019), vitamins, essential fatty acids, provitamin A (β-carotene), and other useful compounds (Yu et al., 2016). There is evidence that the Mayan and Aztec civilizations and communities in Central Africa have used *Spirulina* as food (Chen et al., 2016). *Spirulina* represents biologic activities and health care processes, including cholesterol reduction, immunomodulation, antioxidant and anti-cancer effects (Vo et al., 2013). *Spirulina* has gained remarkable attention for its protein content (Arora et al., 2019). This organism is categorized as a GRAS creature according to its FDA Administration Certificate (Lisboa et al., 2014). So, *Spirulina* protein is a suitable source for extracting bioactive peptides.

DH is the ratio of broken peptide bonds in a digested protein (Rutherfurd, 2010). The order of amino acids and molecular sizes of the bioactive peptides is affected by DH; therefore, biological activities of the peptides are impacted by enzymatic hydrolysis. Thus, DH is an important parameter to determine the functional attributes of bioactive peptides (Chi et al., 2015). Peptides display interesting biologic functions such as antimicrobial, antidiabetic, immunomodulation, mineral binding, and antioxidant activities (Raveschot et al., 2018).

Recently, the radical scavenging property of bioactive peptides resulting from natural sources has attracted considerable attention. For instance, bioactive peptides derived from the hydrolysis of cod, eggs, and duck meat protein possess antioxidant activity. Plant proteins are also a good source of antioxidants. Bioactive peptides isolated from pulses, walnuts, and corn gluten meal have antioxidant properties (Yu et al., 2016).

There have been only a few studies on the relationship between *Spirulina* protein and hydrolysis time and the relationship between the DH and antioxidant activities. Hence, the purpose of this study is to investigate the effect of the alcalase enzyme on the *Spirulina* protein at specified time intervals and the effect of reaction time on the proteinب s DHs. In addition, the relationship between potential antioxidant activities and the DHs was also considered. These experimental results can be applied to determine a protein's hydrolysis time when extracting biopeptides that have high antioxidant capacity.

2. Materials and methods

2.1. Materials

Materials obtained from Sigma include: ortho-phthalaldehyde (OPA), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 2′-azinobis (3-ethyl-benzothiazoline-6-sulphonate) (ABTS), 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), Alcalase® 2.4 L (declared activity of 2.4 AU/kg, density of 1.18 g/ml). *Spirulina* spp. was kindly provided by the Algae culture collection of the Food Biotechnology Research Institute, ABRII, Tabriz, Iran.

2.2. *Spirulina* Culture

Zarrouk, paoletti and Jordan media were used to culture *Spirulina*. Constituents of Zarrouk Standard culture media include (g/L): 16.8 $NaHCO_3$; 2.5 $NaNO_3$; 0.5 K_2HPO_4, 1.0 K_2SO_4; 1.0 NaCl; 0.2 $MgSO_4.7H_2O$; 0.04 $CaCl_2$; 0.01 $FeSO_4.7H_2O$; 0.08 EDTA and micronutrient solution (1mL/L). The micronutrient solution contains (g/L): H_3BO_3 2.86, $ZnSO_4.7H_2O$ 0.222, $MnCl_2.4H_2O$ 1.81, $CuSO_4.5H_2O$ 0.079, and $Na_2MoO_4.2H_2O$ 0.018 (Zarrouk, 1966) (Rosa et al., 2015). The composition of the paoletti medium is (g/L): $NaHCO_3$ 15.15, Na_2CO_3 8.89, Na_2SO_4 1.88, NaCl 0.92, K_2HPO_4 0.50, KNO_3 2.57, $MgSO_4.7H_2O$ 0.25, $CaCl_2.2H_2O$ 0.05, micronutrient solution 1.0 mL/L (2.86 H_3BO_3; 1.54 $MnSO_4.H_2O$; 0.22 $ZnSO_4.7H_2O$; 0.39 $NaMoO_4.2H_2O$; 0.079 $CuSO_4$. $5H_2O$ and 0.038 $CoCl_2.6H_2O$ and 1.0 mL/L Fe-EDTA solution (29.8 EDTA-Na_2; 24.9 $FeSO_4.7H_2O$) (Volkmann; et al., 2008). The Jourdan medium consists of (g/L) 0.05 $CO(NH_2)_2$, 0.12 $(NH_4)_2HPO_4$, 2.00 KNO_3, 0.15 $MgSO_4.7H_2O$, 0.02 $CaCl_2$, 0.02 $FeSO_4.7H_2O$, 5.00 NaCl and 8.00 $NaHCO_3$ (Ndjouondo et al., 2017) at 27 ± 2 ºC on the shaker (110 rpm, 21 days) and irradiated at 2-2.5 Klux.

2.3. Cell disruption

According to the *Spirulina* growth curve in a Zarrouk medium, biomass was collected at the logarithmic stage. Suspension of *Spirulina* powder was prepared and disrupted using three methods: water suspension, bead milling, and ultrasonication. Ultrasonication was accomplished according to the method by Carl Safi et al. (2015) with the following alteration, briefly, ultrasonication (0.1% dry *Spirulina* cell/distilled water) was done in 20 kHz for 20 minutes (50% pulse and 50% rest time) (Safi et al., 2015). Samples were placed in an ice bath during the sonication (Byreddy et al., 2015) (Ma et al., 2015). Suspension of *Spirulina* (0.067 gr biomass/100 mL distilled water) with glass beads (3 mm diameter) Vortex for 45 min in the bead milling method (Safi et al., 2015). The biomass powder was soaked in distilled water (0.1% dry *Spirulina* cell/distilled water) for 3, 24, and 72 hours at 4 ºC in the water suspension method. After disrupting the *Spirulina* cells by the above three methods, the resulting mixture was centrifuged for 15 minutes at 4 ؚC and 9116 g (Safi et al., 2015). Finally, the protein value of the supernatants was evaluated using the Lowry method (LOWRY et al., 1951). The protein content of a control group, *Spirulina* powder suspended in distilled water (0.1% dry *Spirulina* cell/distilled water), was measured immediately.

2.4. Preparation of alcalase hydrolysates

The supernatant (cell extract of *Spirulina*) was incubated at 55 ؚC, pH=8 optimum for alcalase enzyme (See, S. F., Hoo et al., 2011), agitation at 120 rpm at an enzyme/substrate ratio 1% (v/v). Sampling was carried out every hour for 6 hours. Then, the samples were boiled for15 min at 85 ؚC to stop the reaction. Samples were centrifuged, and supernatants were stored at -70 ؚC after lyophilizing (Kose & Oncel, 2015).

2.5. Degree of Hydrolysis Evaluation

The procedure defined by Morais et al. (2013) for DH measurement was used. OPA reagent was freshly prepared daily. For the preparation of the OPA reagent, 100 mM sodium tetrahydroborate 25 mL, 20% SDS 2.5 ml, a combination of OPA 40 mg and 1 mL methyl alcohol, β- mercaptoethanol 100 µL were mixed and diluted with distilled water to a volume of 50 mL. To test proteolysis with *Spirulina* protein as a Substrate, a sample (10 µL) was combined with the OPA reagent (1mL) and maintained at room temperature for 2 minutes. Then, the absorbance was read at 340 nm using a microplate reader (Biotek, USA) (Morais et al., 2013).

The standard curve of L-Leucine (0-4 mg/mL) was used to determine the amount of α-amino acids (Choe et al., 2020). The total number of amine groups in *Spirulina* protein hydrolysates were determined by acid digestion (6 N hydrochloric acid at 120 ^{0}C, 24 h) (Mustățea et al., 2019). The DH percent of hydrolysates was computed with the following formula:

$$(\text{DH})\ \% = \frac{L1-L0}{Lmax-L0}$$

Where L1 is the number of free amine groups liberated after hydrolysis, L_0 is the number of free amine groups in the original *Spirulina* hydrolysate, and Lmax is the whole amount of free amine groups in the original *Spirulina* hydrolysate generated after 24 h hydrolysis with HCl 6 N at 120 $^{\circ}$C (Sangsawad et al., 2017).

2.6. Measurement of antioxidant activity

2.6.1. DPPH radical scavenging activity assay

The DPPH radical scavenging activity of the hydrolysate was determined according to the method described by Xueran Geng et al. (2016). 0.004% DPPH was dissolved in methanol giving a primary absorbance of 0.70 ± 0.02 at 517 nm. Twenty microliter of the hydrolysate (sample) with 180 microliter of DPPH solution in methanol (0.004%) was mixed in a 96-well microplate. The mixture was incubated in a dark place at room temperature for thirty minutes, and then its absorbance was read with a microplate reader device (Biotek, USA) at 517 nm. According to the following formula the DPPH radical cleansing activity of the sample was computed:

$$\text{Radical scavenging activity (\%)} = \frac{Abs\ blank - Abs\ sample}{Abs\ blank} \times 100$$

Where Abs blank is the absorbance without the sample and Abs sample is the absorbance with the sample (Geng et al., 2016) (Q. Zhang et al., 2018).

Trolox, at a concentration of 0-300 µM, was used to draw the standard curve (Gad et al., 2011).

2.6.2. ABTS radical scavenging activity assay

Scavenging of the ABTS radical was measured by an absorbance decrease at 734 nm using a spectrometer. The Trolox standard curve with 0ت 600 µM of Trolox was drawn and used to express the trolox equivalent antioxidant capacity (TEAC).

The antioxidant activity of the hydrolysates was determined by the ABTS radical cation decolorization method. A solution of (7 mM) ABTS was prepared and the next ABTS radical cation was obtained by reacting ABTS with potassium persulfate (2.45 mM); this combination (ABTS + $K_2S_2O_8$) was maintained for 12-16 h in darkness. Before the assay, the absorbance of the solution was adjusted to 0.7 ± 0.02 at 734 nanometer with 5 mM phosphate buffer pH=7.4 (Xu et al., 2018). 25 µl sample (distilled water for blank) was blended with one ml (ABTS + $K_2S_2O_8$). The absorbency was measured using a spectrometer (T80+ UV/VIS Spectrometer, PG Instruments Ltd, United Kingdom). The percentage of antioxidant activity was calculated with the following equation:

$$\text{Antioxidant activity (\%)} = \left(\frac{A\ blank - A\ sample}{A\ blank}\right) \times 100$$

Where A blank is the absorption without the sample and A sample is the absorption in the attendance of the sample (Afify et al., 2017) (Ko et al., 2012). The antioxidant activity assay was accomplished in triplicate and represented as µM TE/mg protein.

2.7. Statistical analysis

For statistical analysis ANOVA in Minitab were used to determine significant differences between groups and the p value of less than 0.05 was considered as a statistical significance. Test results were presented as means of experiments done in triplicate ± standard deviation.

3. Results and Discussion

3.1. *Spirulina* culture

Spirulina growth curves in different media are shown in Fig. 3.1. By using this diagram, the logarithmic growth stage was determined and biomass was harvested at this stage. The dry weight biomass of *Spirulina* is shown in Fig. 3.2. The biomass dry weight obtained was 1.94 ± 0.11 g/L in Paoletti, 2.07 ± 0.10 g/L in Jordan, and 2.22 ± 0.19 g/L in Zarrouk media.

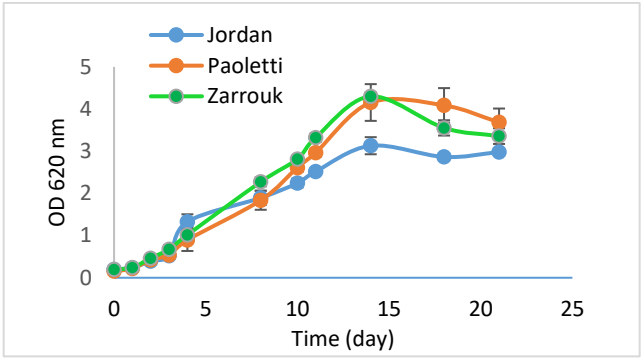

Fig. 3.1. *Spirulin*a was grown in three standard culture media (Pauleti, Jordan, and Zarrouk). The growth curve was drawn based on the absorbance at 620 nm. The results are mean ± SD from experiments performed in triplicate.

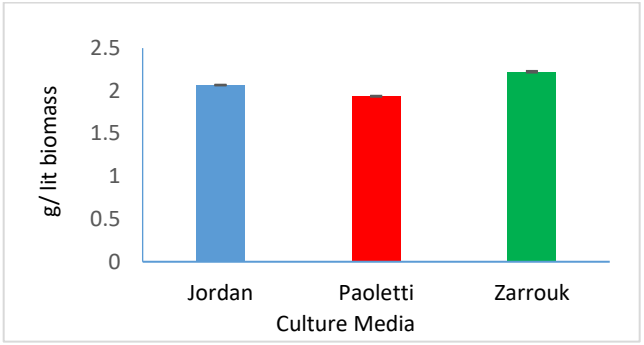

Fig. 3.2. Comparison of the dry weight biomass of *Spirulina* (g/L) obtained from *Spirulina* growth in three standard culture media (Jordan, Paoletti, and Zarrouk). The results are mean ± SD from experiments performed in triplicate.

The amount of biomass obtained from the three types of culture media was not significantly different. The amount of biomass in the Zarrouk medium was more than other culture media; hence, this culture medium was selected to continue the experiments. Our results are consistent with the results of Nyabuto et al. (Nyabuto et al., 2015).

3.2. Protein content of different cell disruption methods

The protein value of the three different cell disruption methods is indicated in Fig. 3.3.

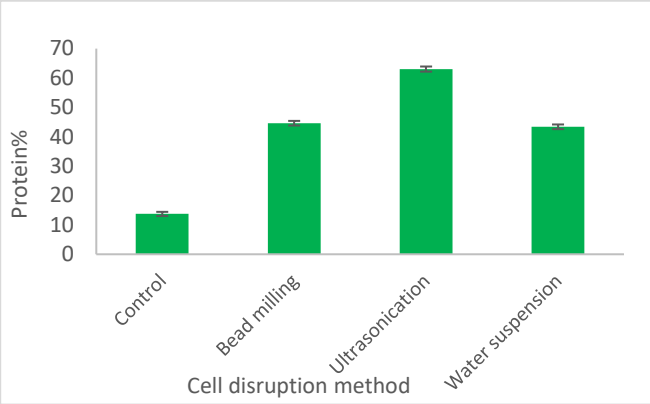

Fig. 3.3. Different cell disruption methods (bead milling, ultrasonication, and water suspension) and the percentage of protein obtained from each method. Control is without any treatment. The results are mean ± SD from experiments performed in triplicate.

Table 3. 1. Spirulina protein content after cell disrupting (the protein value of Spirulina cell extract). The results are mean ± SD from experiments performed in triplicate.

Cell disruption method	mg/ml protein
Bead milling	0.297 ± 0.005
Ultrasonication	0.630 ± 0.009
Water suspension	0.433 ± 0.008
Control	0.137 ± 0.007

The protein value of the control group is 0.137 ± 0.007 mg/mL. The amount of protein released during water suspension of 3, 24, and 72 hours was 0.413 ± 0.013, 0.416 ± 0.012, and 0.433 ± 0.008 mg/mL, respectively. Comparison of the values shows no significant difference between 3, 24, and 72 hours soaking in distilled water ($p \geq 0.05$). The amount of protein released using the bead milling method was 0.297 ± 0.005 mg/mL. A comparison of water suspension and bead milling methods indicate that these differences are not significant ($p \geq 0.05$).

The amount of protein released using the ultrasonication method (0.630 ± 0.008 mg/mL) was higher than the other methods. The ultrasonication method results were remarkably different from the other methods ($p \leq 0.05$). In these experiments, the amount of protein released is important to aid in choosing the method of cell disruption. Among these methods, ultrasonication was the most effective method to disrupt the cell wall and release protein; so, it was chosen to continue the experiments. Our results correspond with the results in Safi et al. (2014), which showed that ultrasonication was effective for the disruption of *Spirulina platensis* cell wall (Safi et al., 2014).

3.3. Degree of hydrolysis

The DH is shown in Fig. 3.4. Measurement of DH using OPA reagent demonstrates that the highest increase in DH (25.08 ± 0.75) occurred in the first hour of hydrolysis. Then the DH increases gradually with low slope changes to (33.45 ± 1.22).

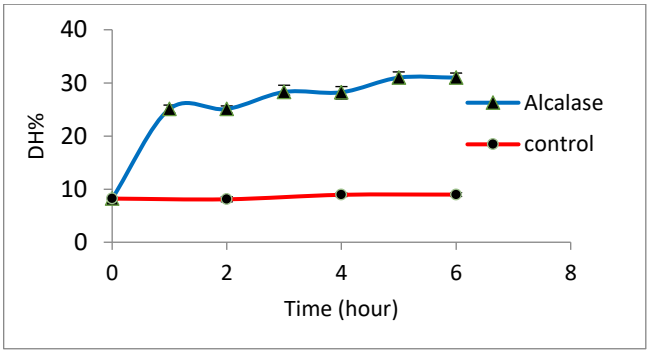

Fig. 3.4. DH of *Spirulina* proteins with alcalase enzyme at different times. The control is untreated with enzyme. The experimental data were represented as the Average ± SD carried out in three replicates.

Chang-Feng Chi et al. (2015) also observed the highest DH in skipjack tuna dark muscle after 1 hour with alcalase enzyme. Changes in the DH were minor after 1 hour (Chi et al., 2015). There were considerable statistical differences of DH between the 0 hour and the first hour of *Spirulina* protein treated with alcalase ($P \leq 0.05$). Our results are consistent with Bochao Zhang and Xuewu Zhang experiments (DH= 31.2% after 10 h of hydrolysis) (B. Zhang & Zhang, 2013). Also, these results are similar to Guerra A. et al., (DH= 40.81% after 60 min hydrolysis of mung bean protein) (Carlos M. Guerra A., 2017).

3.4 Antioxidant activity

The antioxidant activities of the enzymatic hydrolysates are presented in Figs. 3.5. and 3.6. The DPPH radical neutralization activity of *Spirulina* protein hydrolysates was between 8.96 ± 1.30 and 22.71 ± 1.18 µM TE/mg protein. It almost doubled (22.71 ± 1.18 µM TE/mg protein after 2 h, DH=28.29 ± 0.58) compared to the 0 hour (8.96 ± 1.30 µM TE/mg protein, DH=8.22 ± 0.21) and control.

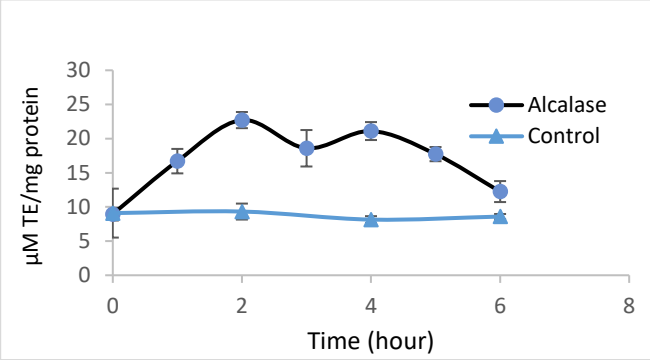

Fig. 3.5. DPPH radical neutralizing activity of hydrolysates at different times (1 h to 6 h). The experimental data were expressed as the mean ± SD carried out in three replicates.

The results illustrate that the antioxidant activity was gradually enhanced with increasing DH (up to 4-hour hydrolysis, DH% increased from 25% to about 30%), but when the DH increased further (more than 30%), the DPPH radical scavenging activity decreased. Yu et al. (2009) also reported this outcome (You et al., 2009). The DPPH, a free radical, in ethanol indicated maximum absorbance at 517 nanometers. When the DPPH radical encounters the hydrolysates, the absorbance reduces. As a result, the hydrolysates could stop the radical chain reaction by donating an electron to the free radicals (You et al., 2009).

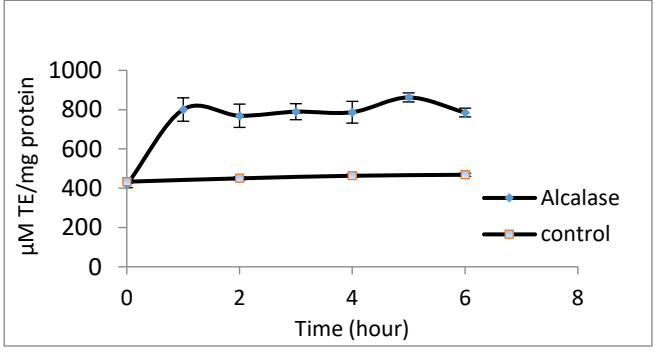

Fig. 3.6. ABTS radical scavenging activity of crude hydrolysates. The experimental data were represented as the mean ± SD carried out in three replicates.

The ABTS radical scavenging activity of *Spirulina* protein hydrolysates were between 417.08 ± 14.36 (0 h) and 862.09 ± 22.26 µM TE/mg protein (5 h). Antioxidant activity increased with increasing DH (from 1 h to 5 h). Y Zhang et al. (2011) reported the antioxidant activities of peanut peptides increased with raising DH (Y. Zhang et al., 2011).

The ABTS-antiradical activity of hydrolysates increased significantly due to enzymatic hydrolysis. The maximum activity was 862.09 ± 22.26 µM TE/mg protein after 5 h hydrolysis with DH=32.83 ± 0.87. According to the Tukey method, the antioxidant activity at 0 hours is significantly different from the antioxidant activity at other times (1 to 6 h) ($P \leq 0.05$). Compared to the 0 hour, the antioxidant activity increased at these times. The hydrolysate at 5 h is significantly different from hydrolysates at 2, 4, and 6 h. Therefore, protein hydrolysis with alcalase enzyme might produce biopeptides with property donor electron or hydrogen to free radicals and convert them to permanent products (Agustini et al., 2015) (Merculieff et al., 2014). Their results confirmed the obtained data in more recent research.

DPPH and ABTS radicals were neutralized by protein hydrolysates. The neutralization might be controlled by a difference in hydrophobicity, the chain length of biopeptides, the amino acid composition of peptides, and the amino acid side chain (Ulagesan et al., 2018). An important factor for antiradical activities is the amino acid composition in the protein hydrolysate. The protein hydrolysate of Skipjack tuna has high radical scavenging activity because of the attendance of aromatic and hydrophobic amino acids (Ulagesan et al., 2018). Hydrolysates containing Isoleucine, Proline, and Leucine hydrophobic amino acids have high antioxidant activity (Memarpoor-Yazdia et al., 2013). Also, the existence of hydroxyl and sulfhydryl groups in Ser and Cys amino acid causes an antioxidant effect. Electron-deficient radicals can get protons from aromatic amino acids (Kim et al., 2019).

Alcalaseضض 2.4 L (a protease enzyme) has an extensive substrate specificity and hydrolyzes both native and denatured proteins, is active in a wide range of pH and temperature, and is resistant to auto proteolysis (Awuor1 et al., 2017). Electron donor peptides are probably hydrolyzed due to an increased DH caused by increasing enzyme activity time. A hydrophobicity test indicated an increasing hydrophobicity trend up to 4 h of hydrolysis, which decreased for 6-h hydrolysis (Corrذa et al., 2014).

Antioxidant activity of the hydrolysates was shown to increase with increasing hydrolysis times. However, after a few hours of hydrolysis, the differences were not significantly different (Corrذa et al., 2014). This might illustrate a lesser availability of breakable peptide bonds or diminution of the peptides liberated from proteins because of substrate competition (Corrذa et al., 2014).

4. Conclusions

The experiments indicate that the antioxidant activity of Spirulina protein hydrolysates increases with digestion by alcalase enzyme. Hydrolysates with a DH above 25% to 32% indicate high antioxidant activities. A suitable DH for ABTS and DPPH radicals scavenging activity is 32% and 28%, respectively. However, a high DH does not always lead to more radical scavenging activity. The high antiradical activity of Spirulina protein hydrolysates is due to the attendance of peptides that are created by hydrolysis with alcalase enzyme. Recognition of the appropriate hydrolysis time and DH increases the possibility of creating and separating peptides with high antioxidant activity from Spirulina protein. All these results can be used to continue work and extract biopeptides with high radical scavenging activity. In the future, extraction and identification of special bioactive peptides will be necessary. Spirulina bioactive peptides can be utilized in diet supplements, infant foods, functional foods, and to prevent oxidation and microbial degradation in foods.

Conflict of Interest

All authors declare that they have no competing interests.

Acknowledgements

The authors are thankful to the Iranian Research Organization for Science and Technology (IROST) for laboratory facilities.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Open access

References

[1] Afify, A. E. M. M. R., Abd El Baky, H. H., El Baroty, G. S., El Baz, F. K., & Murad, S. A. (2017). Antioxidant activity of protein hydrolysates derived from blue-green algae spirulina platensis extracted with three different methods and treated with enzymes. *Bioscience Research*, *14*(3), 485-497.

[2] Agustini, T. W., Suzery, M., Sutrisnanto, D., Maنf, W. F., & Hadiyanto. (2015). Comparative Study of Bioactive Substances Extracted from Fresh and Dried Spirulina sp. *Procedia Environmental Sciences*, *23*(December), 282-289. https://doi.org/10.1016/j.proenv.2015.01.042

[3] Al Hinai, M., Al Kalbani, A., Al Rubkhi, B., Al Kalbani, U., & Walke, S. (2019). Protein extraction from spirulina platensis. *International Journal of Innovative Technology and Exploring Engineering*, *8*(12), 1524-1530. https://doi.org/10.35940/ijitee.L3110.1081219

[4] Arora, R., Sudhakar, K., & Rana, R. S. (2019). Comparative study on the growth performance of Spirulina platensis on modifying culture media. *Energy Reports*, *5*, 327-336. https://doi.org/10.1016/j.egyr.2019.02.009

[5] Awuor1, O. L., Kirwa, M. E., Betty, M., & Jackim, M. F. (2017). Optimization of Alcalase hydrolysis conditions for production of Dagaa (Rastrineobola argentea) Protein hydrolysate with antioxidative properties. *Industrial Chemistry*, *3*(1) 1000122Ind). https://doi.org/10.4172/2469-9764.1000122

[6] Byreddy, A. R., Gupta, A., Barrow, C. J., & Puri, M. (2015). Comparison of Cell Disruption Methods for Improving Lipid Extraction from Thraustochytrid Strains. *Marine Drugs*, *13*, 5111-5127. https://doi.org/10.3390/md13085111

[7] Carlos M. Guerra A., W. M. and J. J. M. A. (2017). Antioxidant potential use of bioactive peptides derived from mung bean hydrolysates (Vigna Radiata). *African Journal of Food Science*, *11(3)*, 67-73. https://doi.org/10.5897/AJFS2016.1511

[8] Chen, Y.-H., Chang, G.-K., Kuo, S.-M., Huang, S.-Y., Hu, I.-C., Lo, Y.-L., & Shih, S.-R. (2016). Well-tolerated Spirulina extract inhibits influenza virus replication and reduces virus-induced mortality. *Scientific Reports*. https://doi.org/10.1038/srep24253

[9] Chi, C. F., Hu, F. Y., Wang, B., Li, Z. R., & Luo, H. Y. (2015). Influence of amino acid compositions and peptide profiles on antioxidant capacities of two protein hydrolysates from skipjack tuna (Katsuwonus pelamis) dark muscle. *Marine Drugs*, *13*(5), 2580-2601. https://doi.org/10.3390/md13052580

[10] Choe, J., Park, B., Lee, H. J., & Jo, C. (2020). Potential Antioxidant and Angiotensin I-converting Enzyme Inhibitory Activity in Crust of Dry-aged Beef. *Scientific Reports*, *10*(1), 1-8. https://doi.org/10.1038/s41598-020-64861-0

[11] Corrêa, A. P. F., Daroitb, D. J., Fontoura, R., Meira, S. M. M., Segalin, J., & Brandelli, A. (2014). Hydrolysates of sheep cheese whey as a source of bioactive peptides with antioxidant and angiotensin-converting enzyme inhibitory activities. *Peptides*, *61*, 48-55. https://doi.org/10.1016/j.peptides.2014.09.001

[12] Gad, A. S., KHadrawy, Y. A., El-nekeety, A. A., Mohamed, S. R., Hassan, N. S., & Abdel-wahhab, M. A. (2011). Antioxidant activity and hepatoprotective effects of whey protein and Spirulina in rats. *Nutrition*, *27*(5), 582-589. https://doi.org/10.1016/j.nut.2010.04.002

[13] Geng, X., Tian, G., Zhang, W., Zhao, Y., Zhao, L., & Wang, H. (2016). Peptide with Angiotensin Converting Enzyme Inhibitory and Antioxidative Activities and Antihypertensive Effects in Spontaneously Hypertensive Rats. *Nature Publishing Group*, *January*, 1-9. https:// doi.org/10.1038/srep24130

[14] Heo, S., Ko, S., Kim, C. S. U., Oh, G., Ryu, B., Qian, Z. J. I., Kim, G., Park, W. O. N. S. U. N., Choi, I., Tuong, T. H. I., Phan, V. Y., Heo, S., Kang, D., Yi, M., & Jung, W. (2017). A heptameric peptide purified from Spirulina sp . gastrointestinal hydrolysate inhibits angiotensin I-converting enzyme- and angiotensin II-induced vascular dysfunction in human endothelial cells. *International Journal of Molecular Medicine*, *39*, 1072-1082. https://doi.org/10.3892/ijmm.2017.2941

[15] Kim, J. M., Liceaga, A. M., & Yoon, K. Y. (2019). Purification and identification of an antioxidant peptide from perilla seed (Perilla frutescens) meal protein hydrolysate. *Food Science & Nutrition*, 1645-1655. https://doi.org/10.1002/fsn3.998

[16] Ko, S. C., Kim, D., & Jeon, Y. J. (2012). Protective effect of a novel antioxidative peptide purified from a marine Chlorella ellipsoidea protein against free radical-induced oxidative stress. *Food and Chemical Toxicology*, *50*(7), 2294-2302. https://doi.org/10.1016/j.fct.2012.04.022

[17] Kose, A., & Oncel, S. S. (2015). Properties of microalgal enzymatic protein hydrolysates: Biochemical composition, protein distribution and FTIR characteristics. *Biotechnology Reports*, *6*, 137-143.

https://doi.org/10.1016/j.btre.2015.02.005

[18] Lisboa, C. R., Pereira, A. M., Ferreira, S. P., & Costa, J. A. V. (2014). Utilisation Of Spirulinasp . And Chlorellapyrenoidosa Biomass For The Productionof Enzymatic Protein Hydrolysates. *International Journal of Engineering Research and Applications*, *4*(5), 29-38.

[19] Lowry, O. H., ROSEBROUGH, N. J., FARR, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, *193*(1), 265-275. https://doi.org/10.1016/0922-338X(96)89160-4

[20] Ma, N. L., Teh, K. Y., Lam, S. S., Kaben, A. M., & Cha, T. S. (2015). Optimization of cell disruption methods for efficient recovery of bioactive metabolites via NMR of three freshwater microalgae (chlorophyta). *Bioresource Technology*. https://doi.org/org/10.1016/j.biortech.2015.03.036

[21] Memarpoor-Yazdia, M., Mahakia, H., & Zare-Zardinib, H. (2013). Antioxidant activity of protein hydrolysates and purified peptides from Zizyphus jujuba fruits. *JOURNAL OF FUNCTIONAL FOODS*, *5*, 62-70.

[22] Merculieff, Z., Ramnath, S., Sankoli, S. M., Venkataramegowda, S., Murthy, G. S., & Ceballos, R. M. (2014). Phytochemical, antioxidant and antibacterial potential of Elaeagnus kologa (Schlecht.) leaf. *Asian Pacific Journal of Tropical Medicine*, *7*(S1), S599-S602. https://doi.org/10.1016/S1995-7645(14)60295-9

[23] Morais, H. A., Silvestre, M. P. C., Silva, V. D. M., Silva, M. R., Simões e Silva, A. C., & Silveira, J. N. (2013). Correlation between the degree of hydrolysis and the peptide profile of whey protein concentrate hydrolysates: Effect of the enzyme type and reaction time. In *American Journal of Food Technology* (Vol. 8, Issue 1, pp. 1-16). https://doi.org/10.3923/ajft.2013.1.16

[24] Mustățea, G., Ungureanu, E. L., & Iorga, E. (2019). Protein acidic hydrolysis for amino acids analysis in food - progress over time: A short review. *Journal of Hygienic Engineering and Design*, *26*(April), 81–87.

[25] Ndjouondo, G. P., Dibong, S. D., Wamba, F. O., & Taffouo, V. D. (2017). Growth, Productivity and Some Physico-chemical Factors of Spirulina platensis Cultivation as Influenced by Nutrients Change. *International Journal of Botany*, *13 (2)*, 67-74. https://doi.org/10.3923/ijb.2017.67.74

[26] Nyabuto, D. K., Cao, K., Mariga, A. M., Kibue, G. W., He, M., & Wang, C. (2015). *Growth performance and biochemical analysis of the genus Spirulina under different physical and chemical environmental factors*. *10*(36), 3614 -3624. https://doi.org/10.5897/AJAR2015.10210

[27] Raveschot, C., Cudennec, B., Coutte, F., Flahaut, C., Fremont, M., Drider, D., & Dhulster, P. (2018). Production of bioactive peptides by lactobacillus species: From gene to application. *Frontiers in Microbiology*, *9*(OCT), 1-14. https:// doi.org/10.3389/fmicb.2018.02354

[28] Rosa, C. Da, Priscila, A., Santos, T. D., Radmann, M., Santana, B., & Vieira Costa, J. A. (2015). Production of Spirulina in semicontinuous cultivation using medium recycle. *Journal of Engineering Research and Applications Www.Ijera.Com*, *5*(4), 36-42. www.ijera.com

[29] Rutherfurd, S. M. (2010). Methodology for determining degree of hydrolysis of proteins in hydrolysates: A Review. *Journal of AOAC International*, *93*(5), 1515-1522.

[30] Safi, C., Frances, C., Ursu, A. V., Laroche, C., Pouzet, C., Vaca-Garcia, C., & Pontalier, P. Y. (2015). Understanding the effect of cell disruption methods on the diffusion of chlorella vulgaris proteins and pigments in the aqueous phase. *Algal Research*, *8*, 61-68. https://doi.org/10.1016/j.algal.2015.01.002

[31] Safi, C., Ursu, A. V., Laroche, C., Zebib, B., Merah, O., Pontalier, P. Y., & Vaca-Garcia, C. (2014). Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods. *Algal Research*, *3*(1), 61-65. https:// doi.org/10.1016/j.algal.2013.12.004

[32] Sangsawad, P., Roytrakul, S., & Yongsawatdigul, J. (2017). Angiotensin

converting enzyme (ACE) inhibitory peptides derived from the simulated in vitro gastrointestinal digestion of cooked chicken breast. *Journal of Functional Foods*, *29*, 77-83. https://doi.org/10.1016/j.jff.2016.12.005

[33] See, S. F., Hoo, L. L., And, & Babji, A. S. (2011). Optimization of enzymatic hydrolysis of Salmon (Salmo salar) skin by Alcalase. *International Food Research Journal*, *18(4)*, 1359-1365.

[34] Ulagesan, S., Kuppusamy, A., & Kim, H. J. (2018). *Antimicrobial and antioxidant activities of protein hydrolysate from terrestrial snail Cryptozona bistrialis*. *8*(12), 12-19. https://doi.org/10.7324/JAPS.2018.81202

[35] Vo, T.-S., Ryu, B., & Kim, S.-K. (2013). Purification of novel anti-inflammatory peptides from enzymatic hydrolysate of the edible microalgal *Spirulina maxima*. *Journal of functional food*. *5*, 1 3 3 6 -1 3 4 6. https://doi.org/ org/10.1016/j.jff.2013.05.001

[36]Volkmann;, H., Imianovsky;, U., Oliveira;, J. L. B., & SantبAnna, E. S. (2008). Cultivation of arthrospira (spirulina) platensis in desalinator wastewater and salinated synthetic medium: Protein content and amino-acid profile. *Brazilian Journal of Microbiology*, 98-101.

[37] Xu, X., Guo, S., Hao, X., Ma, H., Bai, Y., & Huang, Y. (2018). Improving antioxidant and antiproliferative activities of colla corii asini hydrolysates using ginkgo biloba extracts. *Food Science and Nutrition*, *6*(4), 765-772. https://doi.org/10.1002/fsn3.587

[38] You, L., Zhao, M., Cui, C., Zhao, H., & Yang, B. (2009). Effect of degree of hydrolysis on the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates. *Innovative Food Science and Emerging Technologies*, *10*, 235-240.
https://doi.org/10.1016/j.ifset.2008.08.007

[39] Yu, J., Hu, Y., Xue, M., Dun, Y., Li, S., Peng, N., Liang, Y., & Zhao, S. (2016). Purification and identification of antioxidant peptides from enzymatic hydrolysate of Spirulina platensis. *Journal of Microbiology and Biotechnology*, *26*(7), 1216-1223.
https://doi.org/10.4014/jmb.1601.01033

[40] Zarrouk, C. (1966). *Contribution à l'étude d'une cyanophycée : influence de divers facteurs physiques et chimiques sur la croissance et la photosynthèse de Spirulina maxima (Setch et Gardner) Geitler*. University of Paris.

[41] Zhang, B., & Zhang, X. (2013). Separation and nanoencapsulation of antitumor polypeptide from Spirulina platensis. *Biotechnology Progress*, *29*(5), 1230-1238.
https://doi.org/10.1002/btpr.1769

[42] Zhang, Q., Tong, X., Sui, X., Wang, Z., & Qi, B. (2018). Antioxidant activity and protective effects of Alcalase-hydrolyzed soybean hydrolysate in human intestinal epithelial Caco-2 cells Food Research International Antioxidant activity and protective e ff ects of Alcalase-hydrolyzed soybean hydrolysate in huma. *Food Research International*, *111*(May), 256-264. https://doi.org/10.1016/j.foodres.2018.05.046

[43] Zhang, Y., Zhang, H., Wang, L., Guo, X., Qi, X., & Qian, H. (2011). Influence of the degree of hydrolysis (DH) on antioxidant properties and radical-scavenging activities of peanut peptides prepared from fermented peanut meal. *Eur Food Res Technol*, *232*, 941-950.
https://doi.org/10.1007/s00217-011-1466-0