Journal of Food Biosciences and Technology,
Islamic Azad University, Science and Research Branch, Vol. 13, No. 1, 75-89, 2023
DOI:10.30495/jfbt.2022.63017.10281 https://dorl.net/dor/20.1001.1.22287086.2023.13.1.5.1

Antioxidative Activity and Functional Properties of Enzymatic Protein Hydrolysate of *Spirulina platensis*

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Received: 4 November 2021 Accepted: 13 April 2022

ABSTRACT: This study aimed to evaluate the effect of enzymatic hydrolysis of Spirulina protein on the solubility, emulsifying activity index (EAI), emulsion stability index (ESI), and DPPH and ABTS radical scavenging activity of extracted peptides. The molecular weight of spirulina protein was determined using the SDS-Page technique. The extracted protein was hydrolyzed by alcalase enzyme and the degree of hydrolysis (DH%) was determined after 2-12 h. Functional properties and antioxidant activities of extracted protein and hydrolyzed proteins were evaluated. The concentration of extracted protein was 108 mg/L and the protein content of *Spirulina* was 54% (w/w). Most of the protein bands were in the molecular weight range of 15 to 20 kDa, and the DH reached from 4.2 ± 2.1% to 14 ± 1% after 10 h of enzymatic hydrolysis. The lowest solubility was recorded for the extracted protein at pH of 4, and the highest was related to the 14% hydrolyzed protein at pH of 8. Moreover, the extracted protein had higher EAI and ESI than hydrolyzed proteins, and the effect of pH was more evident on the EAI of hydrolyzed proteins compared to ESI. The present study showed that the antioxidant activity of the protein increased with increasing degree of hydrolysis and its concentration. Moreover, the protein with a DH of 14% in all concentrations had the highest inhibition. This work presented that the current method used for extraction and enzymatic hydrolysis of *spirulina* protein led to the production of peptides that have desirable properties for use in the food industry.

Keywords: Antioxidant Activities, Protein Hydrolysate, Solubility, Spirulina.

Introduction

Nowadays, changes in people's lifestyles have increased the global demand for the use of foods that have both nutritional and medicinal value. Algae are part of a healthy diet (Tabarsa *et al*., 2012; Abreu *et al*., 2014; Fouda *et al*., 2019) that produce amazing secondary metabolites with specific biological functions. Despite the limited availability of essential amino acids, these aquatic organisms are considered a source of protein that meets all the nutritional requirements of reputable food and drug organizations

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(Mæhre *et al*., 2014; Williams & Laurens, 2010; Becker, 2007).

Successful extraction of algal proteins is greatly affected by the bioavailability of proteins, which is primarily limited by anionic polysaccharides in the cell wall. Various methods are used to improve the efficiency of algal protein extraction (Salinas-Salazar *et al*., 2019; Hayes, 2018). Conventional enzymatic and mechanical methods for protein extraction may affect the structure of algal-derived proteins due to the release of proteases from cytosolic vacuoles. Cell degradation pretreatment techniques help break down the hard cell wall of algae and increase the availability of proteins and other valuable components for protein extraction. Sonication is a new method of protein extraction (Salinas-Salazar *et al*., 2019), which has attracted the attention of researchers in maximizing protein extraction, especially from algae. This technique facilitates cell wall degradation by converting sound waves into mechanical energy and has some advantages including a relatively short-term process and no requirement to temperature and organic solvent (Cermeño *et al*., 2020).

With the advent of the bioprocessing industry and the emergence of new technologies for the use of seafood, it is possible to separate peptides through enzymatic hydrolysis. These peptides (2-20 amino acids) that have nutritional and medicinal value are usually inactive in the overall structure of proteins and must be separated from the protein structure to achieve bioactive properties (Toopcham *et* al., 2017; Fan *et al*., 2018). Marine peptides have opened a new era for the development of marine agents with many medical functions, and as a result, there is a high demand for the separation of new functional proteins or bioactive peptides from seaweed (Cian *et al*., 2012). Antimicrobial peptides isolated from marine sources can be used as ingredients in food formulations to promote consumer health and improve the shelf life of food products and as natural antibiotics in the pharmaceutical industry (Nasri *et al*., 2013; Ngo *et al*., 2012; Anjum *et al*., 2017). The use of enzymes is the most common method for hydrolysis of extracted proteins (Samarakoon & Jeon, 2012; Sedighi *et al*., 2019) and the enzyme used for this purpose must have at least one feature, namely, it can be used in the food industry (Teshnizi *et al*., 2020). The effect of the enzyme on the functional properties of the peptide is important because it has a great effect on the size of the hydrolyzed molecules and hydrophilicity (Eckert *et al*., 2019). In this case, alcalase has proved to be very efficient in the digestion of protein sources (Wang & Zhang, 2012).

Previous studies showed that the enzymatic hydrolysis process under controlled conditions can improve the functional properties of a protein and affect its size, solubility, and emulsification, and antioxidant properties (Chang *et al*., 2017; Gomes & Kurozawa, 2020). Afify *et al*. (2018) extracted protein from the green microalgae, Scenedesmus obliquus, by three methods (solvent), hydrolyzed it using three enzymes (pepsin, trypsin, papain), and showed that the resulting peptides had variable antioxidant and antiviral effects (Afify *et al*., 2018). Another previous study showed that the amount of protein extracted from Chlorella sorokiniana and Phaeodactylum tricornutum was 39.2% and 35.2% (wt%), respectively, and had different emulsification properties at different pHs (Ebert *et al*., 2019). Recently, Zhu et al. (2021) evaluated the effect of different proteases on amino acids, structure, and antioxidant properties of protein from

Haematococcus pluvialis. The results of their study showed that alcalase had the highest degree of hydrolysis and peptide efficiency (Zhu *et al*., 2021).

Spirulina is filamentous microalgae containing more than 50% protein by dry weight (Mostolizadeh *et al*., 2020) and has extensive nutritional and therapeutic properties (Martínez Andrade *et al*., 2018; Sharma *et al*., 2015). Given the abundance of this microalga and the potential for the production of valuable products from these materials, this study aimed to evaluate the effect of enzymatic hydrolysis of spirulina protein on the solubility, emulsification, and antioxidant capacity of the resulting peptides for use in the food industry.

Materials and Methods

- *Extraction of protein*

Pure Spirulina Powder (Spirulina platensis) was purchased from Noordaro Company (Gonbad Kavous, Golestan, Iran), and stored in a sealed plastic package at room temperature. First, 1 g of Spirulina algae powder was added to 100 mL of distilled water (Parimi *et al*., 2015; Alzahrani *et al*., 2018) and was shaken at 400 rpm for 30 min. Then, this mixture was sonicated (20 kHz, 25% power level, and 750 W) in an ice bath for 5 min (Sony Vibra-Cell, John Morris Auckland, New Zealand). The sonicated mixture was then centrifuged (8000 rpm for 30 min) and the supernatant was collected. The supernatant containing protein was desalted using ammonium sulfate salts and then dialyzed in ammonium bicarbonate buffer (100 mM) at pH: 8 for 8 h (Alzahrani *et al*., 2018; Wang & Zhang, 2012). The second step of dialysis was carried out in distilled water for 2 h and then centrifuged at 5000 rpm for 15 min.

- *Measurement of soluble protein*

The Lowry method was used to measure the amount of protein in the supernatant with some modifications (Lowry *et al*., 1951). Briefly, 2.5 ml of a solution containing 1% copper sulfate, 2% sodium potassium tartrate, 0.1 N sodium, and 2% sodium carbonate was mixed with the obtained supernatant (500 µL), vortexed for 10 min, and kept in the dark at room temperature. Then, diluted Folin solution (250 µL) was added to the prepared samples and vortexed. Finally, the absorbance value was determined at 750 nm and the concentration was measured by drawing the standard curve of bovine serum albumin (BSA) protein.

- *Enzymatic hydrolysis*

Alcalase enzyme was used for enzymatic hydrolysis in this study. The extracted protein was resuspended in ammonium bicarbonate buffer with pH of 8 to obtain a solution with a protein concentration of 2 mg/L. The alcalase enzyme (2.4 Anson units per gram) were purchased from Sigma–Aldrich, Inc. (St. Louis, MO, USA))was then added to reach the concentration of 2% and this enzymatic reaction was performed at 45°C and pH of 8 (pH was kept constant using 0.1 N NaOH). In order to evaluate the effect of reaction time, the degree of enzymatic hydrolysis was determined after 2, 4, 6, 8, 10, and 12 h of reaction time (Wang & Zhang, 2012). To stop the enzymatic activity, the solution was heated at 90°C for 10 min. After centrifuging at 4000 g for 20 min at 4°C, the supernatant was collected and stored at -80°C until the next test.

- *Degree of hydrolysis*

The degree of enzymatic hydrolysis of protein extracted from Spirulina was determined according to the method described by Church et al. (1983) with some modifications (Church *et al*., 1983).

The sample or standard (10 µL) was mixed with 200 µL of freshly prepared O-phthaldehyde solution in a 96-well plate, incubated in the dark for 2 min, and read using spectrophotometry (Enspire Multimode Plate Reader, Perkin Elmer, MA, USA). Amino acid concentration resulting from enzymatic hydrolysis was determined by drawing the L-leucine standard curve (Alzahrani *et al*., 2018). The DH was then calculated using following equation;

$$DH\ \% = h/\ h_{tot} \times 100$$

where h_{tot} is the total number of peptide bonds per protein equivalent, and h is the number of hydrolysed bonds. h_{tot} is dependent on the amino acid composition of the raw material.

- ***SDS-Page***

The molecular weight of spirulina protein was determined using the SDS-Page technique by the Lameli method (Laemmli, 1970). Briefly, the extracted protein (25 µL) was transferred to electrophoresis wells containing 75.3% acrylamide-bisacrylamide and 12% acrylamide-bisacrylamide, and the electrophoresis was carried out at the current intensity of 1 mA for 3 h (PROTEIN Π xi SLABGEL, BIO-RAD, USA). The decolorization operation was performed using a solution containing 0.0625 g of Brilliant Blue Comasi Paint, 7% pure acetic acid, and 20% methanol for 12 h and then with a solution containing 7% pure acetic acid and 5% methanol for 12 h. The BIO-RAD protein marker was used to determine the molecular weight.

- ***Protein solubility***

The solubility of hydrolyzed and extracted proteins was determined according to the method described by Jamdar et al. (2010). After preparing the protein solution (10 mg/mL) and adjusting the pH in the range of 2 to 11, the solution was centrifuged at 10,000 g for 10 min. Then, the nitrogen content of the supernatant (diluted) was measured by the Kjeldahl method. Briefly, the sample was digested by concentrated sulfuric acid containing copper catalysts at 420°C for 2 h and then cooled. After adding water and calculating the percentage of dissolved nitrogen, the percentage of soluble protein was obtained by multiplying this percentage by a global factor (6.25) (Jamdar *et al*., 2010).

- ***Emulsifying properties***

The emulsifying properties of proteins were determined according to Pierce & Kinsella (1978) method (Pearce & Kinsella, 1978). Vegetable oil (10 ml) and 30 ml of protein solution (1%) were mixed and the pH was adjusted in the range of 2 to 11. The mixture was homogenized using a homogenizer (Polytron, Lucerne, Switzerland) at 20,000 rpm for 1 min. A sample (50 µL) of the emulsion was taken from the bottom of the vessel at the beginning of homogenization (t_0) and after 10 min of homogenization (t_{10}). These samples were mixed with 5 mL of sodium dodecyl sulfate (0.1%) solution and the adsorption rate (A_0 and A_{10}) of the diluted solution was measured at the wavelength of 500 nm using a spectrophotometric device (UV-1601 Shimdazo, Kyoto, Japan). The emulsifying activity index (EAI) and emulsion stability index (ESI) were calculated according to the following equation; (Klompong *et al*., 2007).

$$EAI\ (m2\ /\ g) = \frac{2\times 2.303\times A500}{0.25\times \text{protein weight}\ (g)}$$

ESI (min) = ESI (min) =A0 ×Δt/ΔA

In which, ΔA is A_{10}- A_0 and Δt is 10 min.

- ***DPPH radical scavenging activity***

The percentage of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging by protein solution was measured according to Jamdar et al. (2010). The different concentrations (0.4 to 2 mg/mL) of sample solution (0.5 ml) were mixed with 2.5 ml of DPPH dissolved in ethanol (0.02 M). The reaction mixture was incubated in the dark at room temperature for 30 min. The adsorption of the resulting solution was measured at 517 nm (methanol was used as a control in this experiment). The radical inhibition capacity of the tested samples (with three replications) was calculated using the following equation; (Jamdar *et al*., 2010).

$$\text{DPPH radical scavenging \%} = [A_{control}/[A_{sample} - A_{control}] \times 100$$

- ***ABTS radical scavenging activity***

The percentage of 2,2-azino-bis-ethyl benzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity was measured using the method described by (Wang & Zhang, 2012). The ABTS radical cation was formed by mixing the ABTS stock solution (7 mM) with potassium sulfate (2.4 mM) and the resulting mixture was kept in the dark at room temperature for 16 h. ABTS radical solution was diluted in 5 mM PBS buffer at pH: 7.4 to reach absorption of 0.7± 0.02 at 734 nm. Diluted ABTS radical solution (1mL) was mixed with 1 mL of different concentrations of samples. After 10 min, the absorbance was read at 734 nm and ABTS inhibition activity of the samples was calculated using the following equation; (Wang & Zhang, 2012).

$$\text{ABTS inhibition activity} = \left[\frac{\text{A control} - \text{A sample}}{\text{A control}}\right] \times 100\%$$

- ***Statistical analysis***

One-way analysis of variance (ANOVA) was performed using SPSS (ver.20) software. Analytical data were obtained from analyses of three samples for each individual treatment in chemical assays. Differences among mean values were examined by Duncan's test ($p \leq 0.05$) significance level.

Results and Discussion

- ***Extracted protein concentration***

The concentration of extracted protein from Spirulina was determined by preparing different concentrations of bovine serum albumin (BSA) as the standard, measuring its absorption at 750 nm, and drawing the standard curve. According to the formula obtained, the concentration of extracted protein was 108 mg/L and the protein content of *Spirulina* was 54% (w/w). The present study tried to extract and purify Spirulina protein using sonication and dialysis methods.. Compared to similar previous studies trying to extract protein from different species of microalgae, the efficiency obtained in the present study for the extraction of protein was acceptable. Parimi *et al*. (2015) optimized the efficiency of protein extracted from Spirulina using a high-pressure and alkaline-acid method, which resulted in the extraction of protein with the efficiency of 60% (w/w) (Parimi *et al*., 2015). The lower extraction rate in the present study was due to having different aims (they aimed to generate biofuel feedstock with reduced nitrogen content) and consequently the use of different methods for protein extraction. On the other hand, Bashir *et al*. (2016) used the alkaline-acid solvent method and the freeze-drying method to extract protein from spirulina algae, which resulted in the extraction of protein with an efficiency of 52% (w/w) (Bashir *et al*., 2016). Moreover, Moraes *et al*. (2011) used 6 methods for the extraction, among of

which the ultrasound method resulted in the highest efficiency of extraction of phycocyanin from Spirulina (56% higher than other methods) (Moraes *et al*., 2011) which confirmed to use this method in the current study.

- ***Properties of hydrolyzed protein***
- ***Degree of protein hydrolysis***

The results of enzymatic hydrolysis of Spirulina protein are shown in Figure 1. After 2 h of exposure to alcalase, the average percentage of enzymatic hydrolysis was 4.2 ± 2.1%. As the duration of enzymatic hydrolysis increased, this percentage reached to 14 ± 1% (after 10 h). The results showed a significant difference between 2 and 4 h ($P < 0.05$) but this difference was not significant between 4, 6, and 8 h. In the present study, alcalase was used for enzymatic hydrolysis of Spirulina protein. Hydrolyzed products resulting from the enzymatic breakdown of proteins have more effective medical applications than extracted proteins or free amino acids, and today much attention has been paid to the production of food protein hydrolyzates using proteolytic enzymes (Wang & Zhang, 2012). According to the results, the maximum DH was 14% after 10 h, which is an acceptable efficiency compared to the results of similar studies on Chlorella microalgae (Wang & Zhang, 2012) with the maximum DH of 18% under optimal conditions.

- ***Molecular weight***

Electrophoresis using the SDS-Page technique showed that most of the protein bands were in the weight range of 15 to 20 kDa. Also, another band of this protein had a molecular weight in the range of 50 to 75 kDa. However, other bands of this protein can also be seen in the range of 37 to 50 kDa (Figure 2). The SDS-Page technique showed approximately three main bands, the most obvious of which had the molecular weight of 15 to 20 kDa.

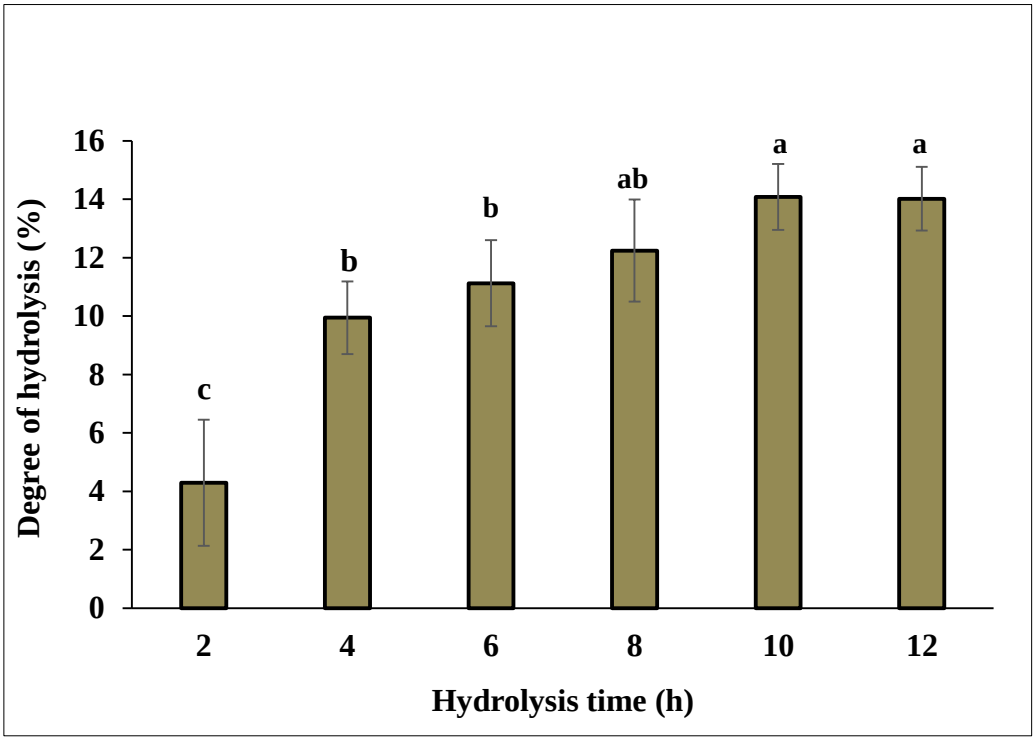

Fig. 1. Degree of hydrolysis (DH) of Spirulina protein by alcalase enzyme for 2 to 12 h. The same letters on each column indicate that there is no significant difference between treatments.

Some previous studies also reported this band and related it to the alpha chain of phycocyanin C with many therapeutic effects (Liu & Kokare, 2017). In addition, a band of 37 to 50 kDa observed in the current study seems to be related to chlorophyll protein (Rajakumar & Muthukumar, 2018). Benelhadj et al. (2016) extracted protein from spirulina that had a molecular weight of 10 to 50 kDa. The two main bands in their study were related to Biliproteins namely phycocyanin-C (19.5 kD alpha subunit and 21.5 kDa beta subunit) and allophycocyanin (19.6 kDa and 17.7 kDa for alpha and beta subunits) (Benelhadj *et al*., 2016).

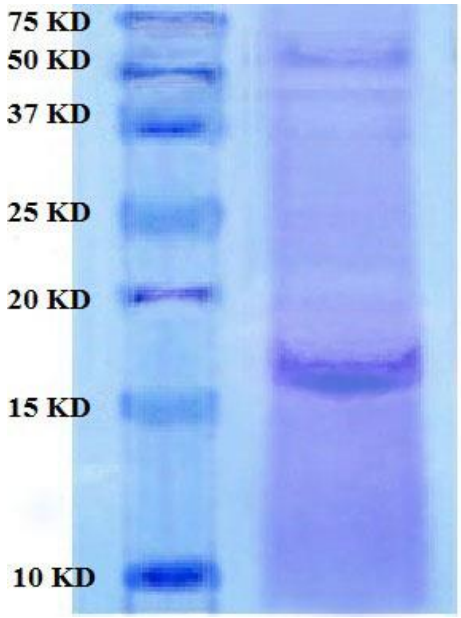

Fig. 2. SDS-PAGE protein pattern of Spirulina.

- ***Protein solubility***

The solubility of protein extracted from Spirulina and hydrolyzed with DH of 4% and 14% at different pHs is shown in Figure 3 . The solubility of this protein at acidic pHs is less than its solubility at neutral and alkaline pHs. However, the lowest solubility of the protein was recorded at pH of 4. On the other hand, the solubility of the extracted protein, hydrolyzates with DH of 4% and 14% at this pH shows a significant difference ($P < 0.05$) there fore the highest solubility was recorded for hydrolysate with DH of 14%. Moreover, no significant difference was observed in the protein solubility in the pHs of 8 to 11 ($P<0.05$). In the present study, alkaline pH increased the solubility of Spirulina protein. Measurement of this property is of great importance because protein should be soluble in water to be usable in food systems (Agarwal *et al*., 2015). The interaction of the protein with water is the main factor that determines its dissolution behavior. The process of protein extraction can lead to changes in protein structure and function. Sometimes, irreversible changes can lead to loss of protein quality, bioactivity, and solubility. Protein solubility depends on its physicochemical properties and affects functional properties such as emulsification, foam formation, and jelling properties (Bashir *et al*., 2016).

In the present study, the solubility of extracted and hydrolyzed protein of Spirulina was in the range of 5.7% to 77%. The lowest solubility was recorded for the extracted protein at pH of 4, and the highest was related to the 14% hydrolyzed protein at pH of 8. These results show that both pH and protein properties (degree of hydrolysis) also affect the solubility of the protein. The less solubility was recorded at pH of 4 because this point is probably the isoelectric point of this protein (Chen *et al*., 2019). The effect of pH on protein solubility is due to the change in electrostatic repulsion force between protein molecules and hydration of charged groups. A similar result was reported in a previous study, in which the lowest solubility of protein extracted from the microalga Nannochloropsis oculata was observed at pH of 4 and the highest at pH 7 of 10 (Cavonius *et al*., 2015).

However, some studies did not report an isoelectric point for the solubility of the extracted protein, such as the solubility of the protein extracted from the microalgae Tetraselimis Sp. This may be due to the different methods used to extract the protein (Schwenzfeier *et al*., 2011). The protein extraction method may also lead to changes in protein solubility as a result of using acidic or alkaline pH and sonication (Pereira *et al*., 2018). The low solubility of the protein extracted from Spirulina may also be due to its greater agglomeration during the drying and heating process. In general, the low solubility of Spirulina protein, especially at the isoelectric point, can be a sign of no cell wall breakage during the sonication process (Bashir *et al*., 2016). Another factor that affects the solubility of the protein is the percentage of lipid in extracted protein, which decreases the solubility of the protein at neutral pH (Sanmartín *et al*., 2013). Compared to other proteins widely used in the food industry, such as soy and cheese proteins that have a solubility of about 73% and 50% at neutral pH, respectively (Pereira *et al*., 2018), Spirulina-derived protein in the present study has a good solubility to be used in the food industry.

- ***Emulsifying properties***

The results of the emulsifying activity index (EAI) of extracted and hydrolyzed proteins at different pHs are shown in Table 1. No significant difference was observed between the extracted proteins and hydrolyzed proteins (4% and 14%) at alkaline pHs ($P > 0.05$) but this case was significant at acidic pHs ($P < 0.05$) in terms of EAI. There was a significant difference between the extracted protein, 4% hydrolyzed protein, and 14% hydrolyzed protein at pH of 7 ($P < 0.05$). However, this effect was not observed at pHs >8.

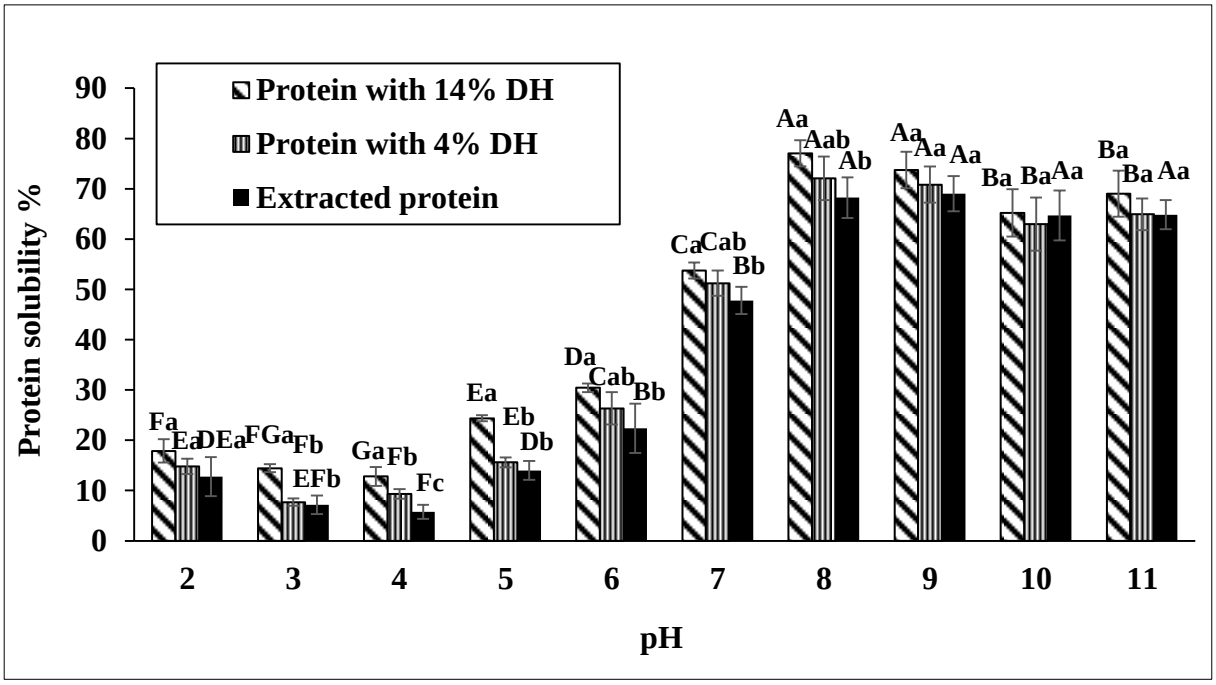

Fig. 3. The solubility of protein extracted and hydrolyzed (4% and 14%) at different pHs. The same small letters on each column indicate no significant difference between the treatments, and the same capital letters indicate no significant difference between the treatments at different pHs (effect of pH on protein solubility).

Table 1. Emulsification activity index of extracted proteins and hydrolyzed proteins (DH of 4% and 14%) at different pHs

pH	EAI (m^2/g)		
	Protein hydrolyzed (14%)	**Protein hydrolyzed (4%)**	**Extracted protein**
2	15.6 ± 3.12^{Bb}	17.6 ± 1.98^{Cb}	45.9 ± 2.56^{ABa}
3	16.2 ± 1.8^{Bb}	16.9 ± 2.256^{Cb}	43.6 ± 2.25^{ABCa}
4	13.8 ± 1.5^{Bb}	13.8 ± 1.59^{Cb}	40.2 ± 1.75^{Ca}
5	14.1 ± 1.15^{bC}	15.23 ± 1.05^{Cb}	44.93 ± 1.68^{BCa}
6	17.5 ± 1.46^{Bb}	49 ± 2.93^{Aa}	45.23 ± 2.05^{ABa}
7	18.5 ± 1.2^{Bc}	35.23 ± 2.15^{Bb}	46.96 ± 1.51^{ABa}
8	16.9 ± 2.2^{Bb}	17.36 ± 1.85^{Cb}	47.26 ± 2.3^{Aa}
9	46.9 ± 1.95^{Aa}	45.73 ± 2.47^{Aa}	47.2 ± 2.35^{Aa}
10	48.3 ± 2.37^{Aa}	47.3 ± 0.7^{Aa}	47.3 ± 2.7^{Aa}
11	47.9 ± 1.4^{Aa}	46.3 ± 2.46^{Aa}	46.3 ± 1.4^{Aa}

The same small letters indicate no significant difference between the three groups at the same pH, and the same capital letters indicate no significant difference between each group at different pHs (effect of pH on EAI).

According to Table 2, there is no significant difference between the three groups at each pH based on the emulsion stability index (ESI). However, the ESI of the extracted protein shows a significant increase at pHs 2 and 3 compared to the ESI of hydrolysates. On the other hand, the ESIs at pH 2 and 3 had significant differences with other ESIs at other pHs ($P < 0.05$).

The results of the present study showed that the extracted protein had higher emulsifying activity and emulsion stability than hydrolyzed proteins. In these two cases, the effect of pH was more evident on the EAI of hydrolyzed proteins, and only pH: 2 and 3 showed a significant effect on the ESI. The EAI is defined as the area of the water/oil contact point that stabilizes per unit weight of protein, and the ESI is defined as the time required to reach turbidity that is half the initial turbidity of the emulsion (Chatterjee *et al*., 2015). In general, more amounts of higher molecular weight peptides or hydrophobic peptides play a role in emulsion stability. On the other hand, hydrolysis further reduces the emulsifying properties. The mechanism of formation of an emulsion system is the adsorption of peptides on the surface of newly formed oil droplets during homogenization and the formation of a protective membrane that prevents fat droplets from coagulating. Proteins with a higher DH have lower ESI and EAI due to their small peptide size. Small peptides migrate rapidly and are adsorbed on the surface but have less ability to reduce the surface tension because they cannotopen and twist at the surface to lead to emulsion stability, like large peptides (Schröder *et al*., 2017). In addition to the size of the peptide, both the hydrophobic and hydrophilic properties of peptides are also important for emulsification. In addition, the flexibility of the protein or peptide structure may be a critical factor in controlling emulsifying properties (Klompong *et al*., 2007). In the present study, the lowest emulsifying activity of the hydrolyzed protein was recorded at pH: 4, the point at which solubility is minimized and was considered as the isoelectric point. EAI and ESI usually increase as the pH moves away from the isoelectric point (Klompong *et al*., 2007), which was observed in the present study.

Table 2. Emulsion stability index for extracted protein and hydrolyzed proteins

pH	ESI (min)		
	Protein hydrolyzed (14%)	Protein hydrolyzed (4%)	Extracted protein
2	11.26 ± 1.2 ^{Bb}	11.8 ± 1.98 ^{Bb}	15.43 ±0.85 ^{Ba}
3	12.13 ± 0.95 ^{Bb}	12.7 ± 1.06 ^{Bb}	17.36 ±1.05 ^{Ba}
4	21.66 ± 1.5 ^{Ab}	21.8 ±1.84 ^{Ab}	23.8 ±2.07 ^{Aa}
5	21.6 ± 1.05 ^{Ab}	22.53 ± 0.98 ^{Aa}	21.6 ±1.7 ^{Aa}
6	23 ±1.33 ^{Aa}	23 ±1.85 ^{Aa}	22.23 ± 1.7 ^{Aa}
7	22.4 ± 1.63 ^{Aa}	23.13 ±1.34 ^{Aa}	22.5 ±0.95 ^{Aa}
8	20.2 ±1.6 ^{Aa}	21.16 ±1.62 ^{Aa}	22.6 ±0.95 ^{Aa}
9	21.7 ±2 ^{Aa}	21.86 ±.77 ^{Aa}	22 ±1.9 ^{Aa}
10	22.46±1.26 ^{Aa}	23.16 ±1.53 ^{Aa}	23.16 ±1.5 ^{Aa}
11	22.7 ±1.58 ^{Aa}	22.73 ±1.48 ^{Aa}	22.9 ±1.4 ^{Aa}

The same small letters on each column indicate no significant difference between the three groups at the same pH, and the same capital letters indicate no significant difference between each group at different pHs (effect of pH on ESI).

- ***DPPH and ABTS radical scavenging activity***

As shown in Figure 4 a, increasing the concentration of hydrolyzed and extracted proteins leads to the increase of the DPPH radical inhibition. Moreover, Spirulina protein with a DH of 14% shows the highest DPPH radical inhibition, and extracted protein shows the lowest inhibition. However, there was no significant difference between the DPPH radical inhibition of the extracted protein and the 4% hydrolyzed protein at all concentrations ($P>0.05$).

Due to the results of Figure 4 a and b, it is clear that the ABTS and DPPH radical inhibition of extracted and hydrolyzed proteins are similar. Thus, the protein with a DH of 14% in all concentrations had the highest inhibition, and extracted protein had the lowest inhibition. There is no significant effect in the inhibition of ABTS by the extracted protein ($P>0.05$) by increasing the protein concentration from 1.2 mg/mL to 1.6 mg/mL.

In general, the antioxidant properties of Spirulina protein increased with increasing DH of the protein and its concentration in the current study. Studies on the activity and structure of antioxidant peptides show that the size of peptides and the type of amino acids in the peptide sequence are the most important factors involved in peptide activity (Nwachukwu & Aluko, 2019). Smaller peptides are more likely to react more effectively with free radicals. A previous study showed that small peptides with 2-10 amino acids have greater antioxidant potential than the protein from which they are derived or large polypeptides. In other words, enzymatic hydrolysis leads to the release of active peptides with the ability to trap free radicals by breaking the peptide bond (Wang & Zhang, 2012). Previous studies have shown that the degree of hydrolysis is an important factor in the preparation of bioactive peptides. Pereira *et al*. (2019) reported that proteins that were enzymatically hydrolyzed for a longer time have the highest ability to scavenge DPPH. However, their results also showed that an excessive increase in enzymatic hydrolysis time of the protein reduced the activity of radical DPPH scavenging. Excessive hydrolysis destroys the bioactivity of peptides by altering the specific structures of amino acid chains, ultimately reducing their activity in free radical scavenging (Pereira *et al*., 2019). Moreover, Alzahrani *et al*. (2018) investigated the biological activities of three microalgae proteins,

including Spirulina, and evaluated the effect of hydrolysis of alcalase, flavourzyme, and trypsin enzymes on functional properties. The results of their study showed that the resulting peptides from enzymatic hydrolysis by alcalase had the highest antioxidant activity due to their phenolic content (Alzahrani *et al*., 2018).

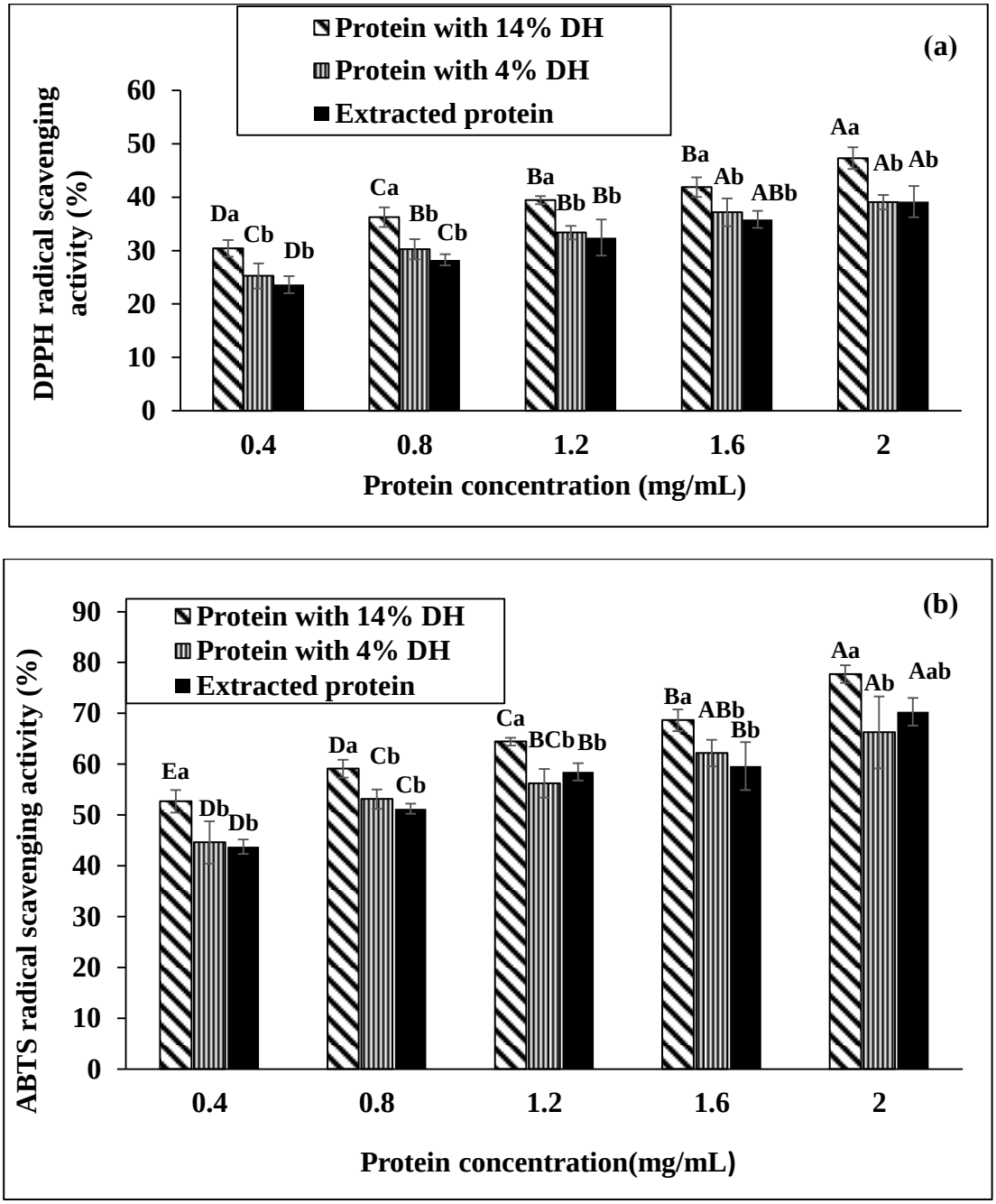

Fig. 4. DPPH (a) and ABTS (b) radical scavenging activity by different concentrations of extracted and hydrolyzed proteins. The same small letters on the columns indicate no significant difference between the three groups in each concentration, and the same capital letters indicate no significant difference in each group at different concentrations (comparison based on protein concentration).

Conclusion

Protein (15 to 20 kDa) was extracted from Spirulina using sonication and dialysis methods which had a concentration of 108 mg/mL.This work reported that the use of the alcalase enzyme resulted in a maximum hydrolysis rate of 14% after 10 hours. Due to the importance of protein solubility in the food industry, we investigated the effect of alkaline and acidic pH on solubility, The results demonstrated an improvement in the solubility of the extracted and hydrolyzed protein of spirulina under alkaline conditions and these conditions led to an increase in the emulsifying activity of hydrolyzed proteins. Our results presented that the extracted protein had higher EAI and ESI than hydrolyzed proteins, however, increasing DH and the concentration of Spirulina protein increased its antioxidant capacity. In general, the present study showed that the hydrolyzed protein obtained from Spirulina has desirable properties for use in various areas of the food industry. However, more research and studies are needed to further enhance these features.

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