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Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2017 14(3):485-497. **OPEN ACCESS**

Antioxidant activity of protein hydrolysates derived from blue-green algae *spirulina platensis* extracted with three different methods and treated with enzymes

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This study evaluated the hydrolysis of the biomass of *Spirulina* sp. by using three different enzymes: Pepsin, Trypsin and Papain that obtain protein hydrolysates with antioxidant application, which used as functional and nutritional food and in food supplement. Protein was extracted from *Spirulina platensis* by three different extraction methods (Sp1, Sp2 and Sp4). Protein extracts and hydrolysates were assessed by gel electrophoresis (SDS–PAGE) of samples and marker. Antioxidant activity of protein hydrolysates from *Spirulina* was investigated. Algae cells and protein extracts were rich in Arg, Lys, Asn, Ala and His. Protein hydrolyzed by trypsin ($Sp2_{Try}$) and ($Sp4_{Try}$) showed the highest antioxidant activity against 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) radical-scavenging (32.27% and 30.11%,respectively) and on 2, 2'-azinobis 3-ethyl-Benzothiazoline-6-sulphonate(ABTS) radical (56.39% and 34.26%, respectively) at sample concentration (150 µg/ml). The investigations conducted that algal biomass shows promising qualities as a novel source of protein, its quality of protein could be increased by treatments with enzymes. We think our data also demonstrate that antioxidant activities are related to amino acid sequence analysis as well as releasing of small peptides. Therefore, *Spirulina platensis* protein hydrolysates can be used as nutritional food with antioxidant properties.

Keywords: Antioxidant, *Spirulina,* DPPH, ABTS and protein hydrolysates

INTRODUCTION

Algae are the primary producers of the marine ecosystem and the food origin for other marine living creatures. Their particular metabolites such as photo-protective compounds can be used for feeding of other creatures placed in the food chain at higher levels (Mimouni et al. 2012). *Spirulina* is blue green algae or cyanobacterium filamentous, which is an unbranched and helicoidal that used as food for a long time (Ciferri, 1983). *Spirulina* is rich pigments as well as it has the capability to do photosynthesis, So that it was classified in the plant kingdom. Based on new information of its genetics, physiology, and biochemical properties it was also placed in the bacteria kingdom (Vo et al. 2013). Small peptides and free amino acids releases from proteins by enzymatic hydrolysis, this process increase the nutritional value of food proteins. In fact, proteins are first broken into these smaller molecules during metabolism before being absorbed and used for several functions in the body. For this purpose several proteases may be used, such as pancreatin (Silvestre et al, 2013). It is an enzymatic complex consisting of

enzymes secreted by the pancreas. These proteases are divided in to endo-peptidases (trypsin, chymotrypsin and elastase) and exo-peptidases (carboxy-peptidases A and B). (Morris et al, 2007, Schmidtand and Salas-Mellado 2009, Morris et al, 2011 and Cristiane et al, 2014).Park et al, (2001) reported that, lipid oxidation in food and cellular systems inhibited by using protein hydrolysates. Peptides presents in protein hydrolysate mainly have antioxidant activity. Hydrolysates generous in peptides containing hydrophobic amino acids, such as Pro, Leu, Ala, Trp and Phe, are believed to possess high antioxidant activity (Mendis et al, 2005). Therefore,the antioxidant activity of protein hydrolysates depends on amino acids and peptides composition in protein hydrolysates (Wiriyaphan et al, 2012).

Korhonen and Pihlanto, (2006) mentioned that protein hydrolysates have several pharmacological actions such as: the antioxidant ability, the inhibition of angiotensin converting enzyme, and the hypo-cholesterolemic effects. Additionally, different peptides show an immune enhancing activity and they could modulate the immune system through nutritional interventions, which is the main target of immune nutrition (Biziulevicius et al. 2006).

The expertise on microalgae protein hydrolysis has been lodge to nearly thirty years. The applied creators have been characterized by the preferential use of *Scenedesmus* and *Spirulina* proteins, as well as alkaline proteases (Morris et al, 2008).

This study aimed to cultivate *Spirulina platensis* in Zarrouk's medium, Obtain its protein extracts treated with three different enzymes (Pepsin, Papain and Trypsin) in order to analysis its protein content and amino acids profiles, Making SDS-PAGE for algae and its protein hydrolysates to recognize the molecular weights of it and estimation of antioxidant and antiviral activity for all protein hydrolysates.

MATERIALS AND METHODS

Algae Source

Spirulina platensis was obtained from the Culture in our Plant Biochemistry Department, National Research Centre, Cairo, Egypt.

Cultivation conditions

The culture of *Spirulina platensis* was inoculated initially in prepared Zarrouk's medium, by adding the microalgae into each culture flask from the stock (Optical density at 640 nm = 0.50) to get 10% suspension of *Spirulina platensis* (Zarrouk, 1966). All the flasks were kept under fluorescent lights (light: dark = 24 h: 0 h) in the Culture Laboratory. The culture flasks were continuously aerated using electric aerator. Sampling was carried out at every 48h from each flask to observe cell density, chlorophyll a, and optical density of culture media. The amount of inseminated algae in samples was 5 mg dried substance per 2 liter of the Zarrouk's medium was used as control medium. The algae were cultured at optimal conditions using mono white color (3500 ± 350 Lux) with a photoperiod of 24h, at 30 ± 2ºC and pH=9-11. Algae were collected from the medium after 14 days when they were at the end of their logarithmic growth phase and when they were at their maximum nutritional value and density(Reichert et al. 2006).

Growth Measurement

Biomass concentration (g/L) was calculated by measuring optical density at 640 nm to produce a standard curve relating dry weight of *Spirulina platensis* biomass (g/L) respectively, to optical density. To monitor biomass changes in cultures, samples were taken every 48 h using aseptic technique (Payer, 1971).

Harvesting

At the end of each batch run, replicate cultures were pooled, filtered, washed with distilled water to remove soluble salts, centrifuged at 6,000 x g for 15 min at 4˚C and frozen at -20˚C.

Determination of dry weight

A known weight of the cells (0.5 g) was dried in oven at 100°C for 16 h, after filtration and washing, the filter paper was placed in a desiccator, cooled to room temperature. The mass was recorded by using analytical balance until constant weight (AOAC, 1990).

Specific growth rate (SGR, µg/ day) of microalgae

The specific growth rate (SGR µg/ day) of cultured microalgae was calculated by the following equation (Boyle, 1977):

$SGR\ (\mu g/\ day) = \ln (X_1 - X_2)/ t_2 - t_1$: (1)

Where,

X_1 = Biomass concentration at the end of selected time interval.

X_2 = Biomass concentration at the beginning of selected time interval, and

$t_2 - t_1$ = Elapsed time between selected time in the day.

Chlorophyll a

Optical densities of the prepared samples were determined at 664, 647 and 630 nm wave length by using UV spectrophotometer. A blank with 100% acetone was run simultaneously. Chlorophyll a content was calculated by the following formula (Vernon, 1960):

Chlorophyll a (mg*/*l) = 11.85 (OD 664)-1.54 (OD 647)-0.08 (OD 630). (2)

Extraction of protein

Protein was extracted from *Spirulina platensis* by using three different extraction methods:

Extraction with NaOH (2N), pH 12:

Freeze-dried biomass (0.5 g) was added to 25 mL of NaOH (2N); pH 12, then stirred for 2 h at 40 °C. The supernatant was separated from the pellet by centrifugation at 10,000 g for 10 min at 20 °C and adjusted to pH 3 with HCl (1N) in order to precipitate the proteins. The protein isolate (Sp1) was collected after centrifugation at 10,000 x g for 10 min at 20 °C and the pellets were neutralized with NaOH (0.1N) (Safi et al. 2014). Samples were taken for protein analysis. Solubilized protein was dialyzed against water for 24h.

Extraction with NaOH (2N), pH 11 and pH adjusted to 5.5:

Proteins were extracted using the alkali solubilization method described by Hultin and Kelleher, (2000).The dried algae was mixed and homogenized with nine parts cold (6 ºC) deionized water for each part of algae using a Bio-Homogenizer at a high speed for 1 min. In the alkaline solubilization step, the pH of the homogenized sample was adjusted to 11.0 using NaOH (2N) and incubated at 4 ºC for 30 min. After centrifuging the mixture at10,000 g for 20 min at 4 ºC, the supernatant containing the soluble proteins was separated by centrifugation at 10,000 x g for 10 min at 20 °C and adjusted to pH 5.5 using HCl (1N) to precipitate the proteins. The mixture was then centrifuged at 10,000×g for 20 min at 4 ºC to recover the proteins in the pelleted material (Sp2).

Extraction with ethanol:

Freeze dried algae was homogenized for 3 min in ethanol solution. Then mashed sample was extracted by ethanol with the same extraction condition of Wang et al. (2012) (concentration of ethanol 90%, liquid–material ratio 20:1,extraction temperature 40 ºC, and extraction time 80 min). After extraction, the pH of the homogenate was adjusted to9.0 by 0.1 M NaOH, and the ethanol concentration was diluted to 60% with distilled water. The diluent homogenate was stirred and pre-incubated at 25ºC for 30 min, and centrifuged at 12, 000 g for 15 min. After removing ethanol from the resulting supernatant under vacuum, ethanol-soluble proteins (Sp4) were salt out by saturated NaCl at 25 ºC for 12 h and collected by centrifugation at 12,000 xg for 15 min. The lipid in the precipitate was removed by diethyl ether for 24 h, and solvent was removed by centrifugation at 15,000 xg for 15 min. (Wang et al. 2014).

Enzymatic hydrolysis

Protein extracts from previous methods (Sp1, Sp2 and Sp4) were mixed with 0.05 M phosphate buffer solution (PBS, pH 7.4) at liquid–material ratio 500:1. The mixtures were digested with pepsin, trypsin and papain 1.2% at 37 and 50 ºC, pH 2.0, 7.0, 8.0 respectively, for 2 h (Adler-Nissen, 1986). After hydrolysis, the samples were heated in a boiling water bath for 20 min to inactivate the enzyme. The hydrolysates were centrifuged at 12,000 x g and 4°C for 15 min and the supernatants were lyophilized and stored at -20 ºC.

Total Nitrogen

The total nitrogen was evaluated using semi-micro kjeldahl method, specification 950. Method 20.87-37.1.22 (AOAC, 1995).Approximately 2g of sample were weighed into a digestion flask together with a combined catalyst of 5g K_2SO_4 and $CuSO_4$and 15ml of concentrated H_2SO_4. The percentage nitrogen was evaluated and converted into protein percentage using the conversion factors obtained for each microalga in another study.

Determination of protein by Bradford method

Protein content of all extracts was estimated according to (Bradford, 1976) at 595 nm, using coomassie blue G-250 as a protein binding dye. Bovine serum albumin (BSA) was used as a protein standard. Protein concentrations in the samples were calculated from the calibration curve, in µg protein in µl extract.

Amino acid analysis

The procedure for amino acid analysis was essentially the same reported by (Gerloff et al. 1965) Small samples (30 – 50 mg) were weighed

into glass tubes and 2 ml of 6 N HCl were added for each mg of crude protein (nitrogen × 5.89) in the samples. Tubes were opened and the hydrolysates were dried at 40 ºC in a rotary evaporator. The residue was dissolved in a known amount of citrate buffer and an aliquot was analyzed for its amino acid content with a Beckman Spinco amino acid analyzer.

Electrophoresis

The protein extracts were separated by 12 % SDS-PAGE with a Mini Protean II Dual Slab Cell (Bio-Rad) according to Laemmli (1970).Samples with protein quantity equivalent was loaded for all variants. Gels were stained with Coomassie brilliant blue R-250.

Antioxidant activity of protein extracts and its hydrolysates

Determination of antioxidant activity of protein extracts and its hydrolysates by DPPH· free radical-scavenging activity:

The DPPH· (2, 2- diphenyl -1- picrylhydrazyl) free radical-scavenging activities of protein extracts were determined according to the method described by Molyneux (2004).Briefly, a 0.1 mM of ethanolic DPPH· solution was prepared. The initial absorbance of DPPH· in ethanol absolute was measured at 517 nm and did not change throughout the period of assay. An aliquot (0.1 ml) of each sample (with appropriate dilution if necessary) was added to 3.0 ml of ethanolic DPPH solution. Discolorations were measured at 517 nm after incubation for 30 min in the dark. Measurements were performed at least in triplicate. The percentage of DPPH which was scavenged was calculated using the following formula:

$$\text{Scavenging\%} = [1 - (A_{\text{sample}} - A_{\text{blank}}/A_{\text{control}})] \times 100\% \quad (3)$$

Here, ethanol (3.0 ml) plus sample solution (0.1 ml) were used as a blank and 3 ml of DPPH–ethanol solution plus ethanol absolute (0.1 ml) were also used as a negative control.

Determination of antioxidant activity of protein extracts and its hydrolysates by $ABTS^{\cdot+}$ free radical-scavenging activity:

The antioxidant activity of compounds was determined by the $ABTS^{\cdot+}$ (2, 2`- azinobis (3-ethyl- benzothiazoline -6- sulfonic acid) radical cation decolorization assay (Re et al. 1999),involving preformed $ABTS^{\cdot+}$ radical cation. A solution of (7 mM) ABTS was obtained and next $ABTS^{\cdot+}$ radical cation was obtained by reacting ABTS with potassium persulfate; this mixture was stored in the dark at room temperature for 16 h before use. Before the assay, the mixture was diluted with ethanol absolute at a ratio of 1:100 to give an absorbance at λ=734 nm of 0.70 ± 0.02. All compounds were dissolved in ethanol absolute at a concentration of 50, 100 and 150 µg/ml were added to ethanolic $ABTS^{\cdot+}$ to measure absorbance after 6 min. Antioxidant activity measurements were carried out in triplicate and expressed as percentage of the absorbance of the uninhibited radical solution according to the following equation:

$$\%\ \text{inhibition}\ (\lambda=734\ \text{nm}) = (1 - Abs_c/Abs_0) \times 100 \quad (4)$$

Abs_c: Absorbance of ethanolic $ABTS^{\cdot+}$.

Abs_0: Absorbance of samples.

Statistical analysis

Results are expressed as mean ± standard deviation (SD). Comparison between the mean values of different parameters in the different concentrations was done using one way analysis of variance (ANOVA). Excel 2010 was used for data analysis. P value equal to or less than 0.05 was considered significant.

RESULTSAND DISSCUSSION

Spirulina platensis must absorb nitrate from the media, because it does not contain a heterocyst necessary for nitrogen fixation and cultivation requires substantial inputs of soluble nitrogen. Sodium nitrate is essential for biomass production. Zarrouk medium has high soluble nitrate used to grown *Spirulina platensis*. As shown in Figure 1, pH value changed from initial 9 to 10.5 on 14th day of experimentation. Richmond et al. (1980) noticed that the definitive pH values achieved in the workout was 11.2 for jar cultures. *Spirulina* is living in alkaline media; values above 10.3 were display to be harmful. *Spirulina* species normally cultivated in alkaline medium with pH 8.5 to 11 and this prevents the growth of bacteria, algae, yeasts and fungi (Parada et al. 1998).

Zarrouk medium observed an average output of biomass, from second day growth increased on, receiving a final cell concentration of 5.86 $g.L^{-1}$ on 14thday according to following equation:

Biomass = (O.D. *1.0955) + 0.506

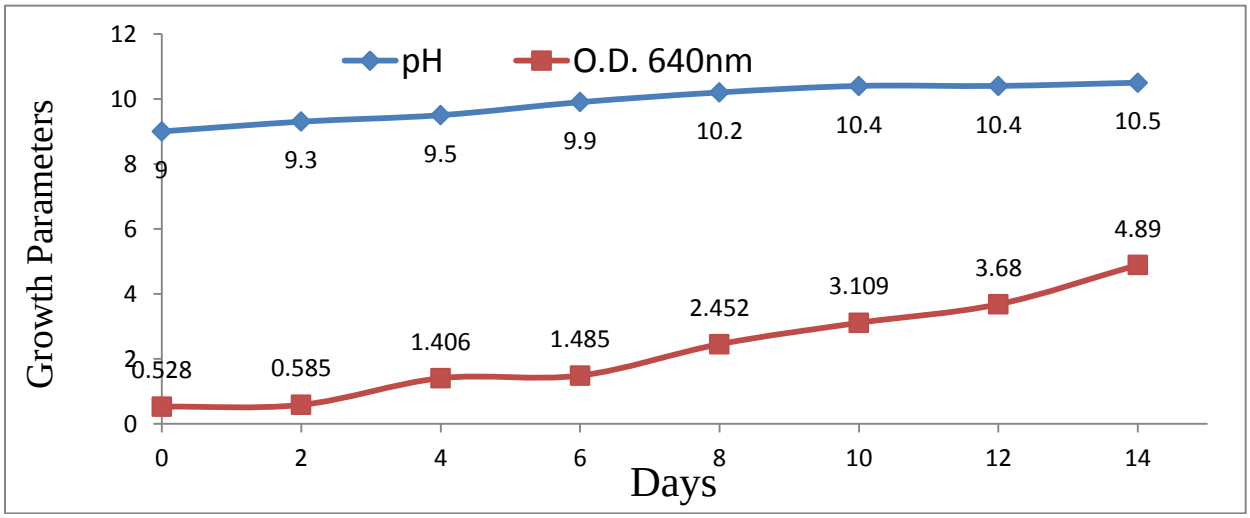

Fig. 1 Growth parameters of Zarrouk media containing *Spirulina platensis*

Growth rate, chlorophyll-a, total biomass, %N and protein content:

As shown in Table 1, specific growth rate (SGR,) of cell and chlorophyll a were recorded 0.075 µg.day^{-1}and 6.5 µg.ml^{-1}, respectively. Appearance of culture also shifted from light green to dark green in proportion to the increasing cell mass. Total biomass of *Spirulina sp.* was recorded 576.32 mg/l. The results presented here are in agreement with the studies by Reichert et al. (2005) on semi-continuous cultivation of the microalga *Spirulina sp.* These results are in agreement with data presented by Van Liere et al. (1975) and Van Liere, (1979) who reported that lower SGR observed in batch cultures than continuous cultures. Nutrient replenishment in continuous culture, circulation of algae in a permanent light environment is conditions lacking in batch culture where obtainable light and nutrients decrease with time as thickness of the culture increases.

Table 1: Specific growth rate (µg.day^{-1}) of cell, chlorophyll a (chll-a) and total biomass (mg.l^{-1}.day^{-1}) of *Spirulina platensis* grown in Zarrouk´s media

Parameters	*Spirulina platensis*
SGR of cell	0.075± 0.01
SGR of Chll-a	6.5± 0.01
%N	7.34± 0.012
%Crude protein	46.02± 0.011
N-T-P factor*	6.27
Total biomass	576.32

*N-T-P: nitrogen to protein factor

Spirulina has high capacity for nitrate utilization; therefor it includes at least 10-fold protein higher than rice (Kronzucker et al. 2000; Ali et al. 2007). Total protein content was determined from the elemental analysis which observed the value of total nitrogen and the conversion factor found for each crude microalga. Cultures can be affected in different ways due to nutrient deficiency, especially nitrogen (N). In N-sufficient growth mediums, carbohydrate synthesis is limited, while protein production is supported. In contrast, in N-deficient mediums, protein production drops and carbohydrate synthesis increases (Fernandez-Reiriz et al. 1989). Protein content was high and harmonious with literature values, ranging from 45 to 58% dry weight in *Spirulina sp.* which is 46.02 %. Comparable values for total protein, ranging from 46% to 50% in dry weight, were reported by Wong and Chan, (1980) it was 45.6 %. As shown in Table 2, % protein with Bradford was 44.45% in Sp1 extract, 44.55% in Sp2 extract and 45.46% in Sp4 extract.

Table 2: Soluble protein content of *Spirulina platensis* extracted by different methods

Protein extracts	% protein
Sp1	44.45 ± 0.01
Sp2	44.55 ± 0.02
Sp4	45.46 ± 0.01

Sp1: Proteins were extracted using NaOH pH 12.
Sp2: Proteins were extracted using NaOH pH 11and pH adjusted to 5.5
Sp4: Proteins were extracted using ethanol.

Amino acids content:

S. platensis contains broad spectrum of amino acids. The most common amino acid of the dry matter was Glutamic acid followed by Aspartic acid. The most abundant essential amino acid was Isoleucine. Phenyl alanine is also present in relatively high doses; therefore, people with phenyl ketonuria should obviate *Spirulina*. For those prefer a whole food supplement to artificial nutrient sources, the concentrated nutritional profile of *Spirulina* occurs naturally is ideal (Pinero Estrada et al. 2002; Chamorro et al. 2002; Islk et al. 2006 and Al-Attar, 2010).Amino acids profile from crude microalgae offered high content of non-essential amino acids, such as arginine, alanine and asparagine. Moreover, it is contain essential amino acids such as lysine, histidine and therionine. Danesi et al. (2002) and Campanella et al. (1999) were found similar values. However, Arginine being the highest in *Spirulina* extract Sp1 (8.153 µmol / 100 mg d.w). Nevertheless, Table 3 showed that the amount of all amino acids in all protein extracts was reduced according to the extraction method. It should however be noted that the total nitrogen estimation includes other nitrogenous compounds, such as intracellular inorganic materials. Pigments, nucleic acids, glucosamine and amines that could account for about 10% of the overall nitrogen content in microalgae

Table 3. Amino acid content (µmol / 100 mg dw) of *S. platensis*

Amino acid	***Spirulina platensis***			
	Algae	Sp1	Sp2	Sp4
Histidine	2.92	2.63	2.346	2.07
Arginine	7.89	8.153	6.317	5.13
Therionine	2.06	1.901	1.915	1.89
Alanine	2.68	2.406	2.448	2.16
Proline	0.49	0.421	0.17	0.15
Tyrosine	1.41	1.179	1.224	1.08
Valine	1.63	1.463	1.371	1.21
Methionine	1.47	1.45	1.19	1.05
Isoleucine	1.74	1.496	1.451	1.28
Leucine	0.84	0.754	0.487	0.43
Phenylalanine	1.34	1.152	1.077	0.95
lysine	2.13	1.918	2.131	1.88
Aspargine	2.38	2.152	2.391	2.11
Aspartic acid	1.68	1.448	1.609	1.42
Glutamic acid	1.48	1.46	1.379	1.25

Sp1: Proteins were extracted using NaOH pH 12.
Sp2: Proteins were extracted using NaOH pH 11 and pH adjusted to 5.5.
Sp4: Proteins were extracted using ethanol

Electrophoresis protein profiles:

Asillustrated in Fig. 2, qualitative comparisons of protein extracts patterns obtained from *Spirulina platensis*, first extract (Sp1) patterns showed nine major bands per lane within a range molecular weight varying between 219kDa and 59kDa .While the other methods of extraction obtained four major bands per lane in the second method (Sp2), molecular weight range from 225kDa and 52kDa and the third method (Sp4) gave also ten bands per lane molecular weight range from 237kDa and 59kDa.

On the other hand, Table 4 observed that, protein extracts treated with enzymes obtained from Sp1 by using papain showed thirteen major bands per lane, molecular weight range from 250kDa to 59KDa.While, hydrolysis with trypsin obtained twelve bands per lane, from 253KDa to 58KDa. However, hydrolysis with pepsin obtained twelve bands per lane, but molecular weight range from 250KDa to 39KDa. Based on the SDS-PAGE data in Fig. 2 and Table 4 protein extracts treated with enzymes obtained from Sp2 after hydrolysis with papain twelve bands were obtained, from 253KDa to 32KDa .Also, hydrolysis with trypsin obtained twelve bands per lane, from 253KDa to 32KDa. However, hydrolysis with pepsin obtained twelve bands per lane also, but molecular weight range from 253KDa to 48KDa.

Moreover, protein extracts treated with enzymes obtained from Sp4 after hydrolysis with papain eleven bands were obtained, from 253KDa to 54KDa .While; hydrolysis with trypsin obtained ten bands per lane, from 259KDa to 59KDa. However, hydrolysis with pepsin obtained eleven bands per lane also, but molecular weight range from 259-KDa to 51-KDa. Protein profiles of investigated green freshwater and blue-green algae are mainly formed by polypeptides associated with photosynthesis. Protein bands from interval 4-36 kDa are predominantly formed by polypeptides originating from photosystems I and II. Polypeptides from thylakoid membranes, light-harvesting complex proteins (LHC IIc, LHCPa, LHCPb) and phytochelatins, and polypeptides of P700-chlorophyll a-protein 1 from photosystem I are displayed as protein bands 8, 10, 15 and 18 kDa; molecular weight 9 kDa could belong to protein CPIII - Chlorophyll a protein; and the area around 22 kDa may indicate protein CP22 (Brandt et al. 1982; Funk et al. 1994; Hoj and Moller, 1986; Ikeuchi and Inoue, 1988; Morishige and Thornber, 1994; Osman et al. 2004).

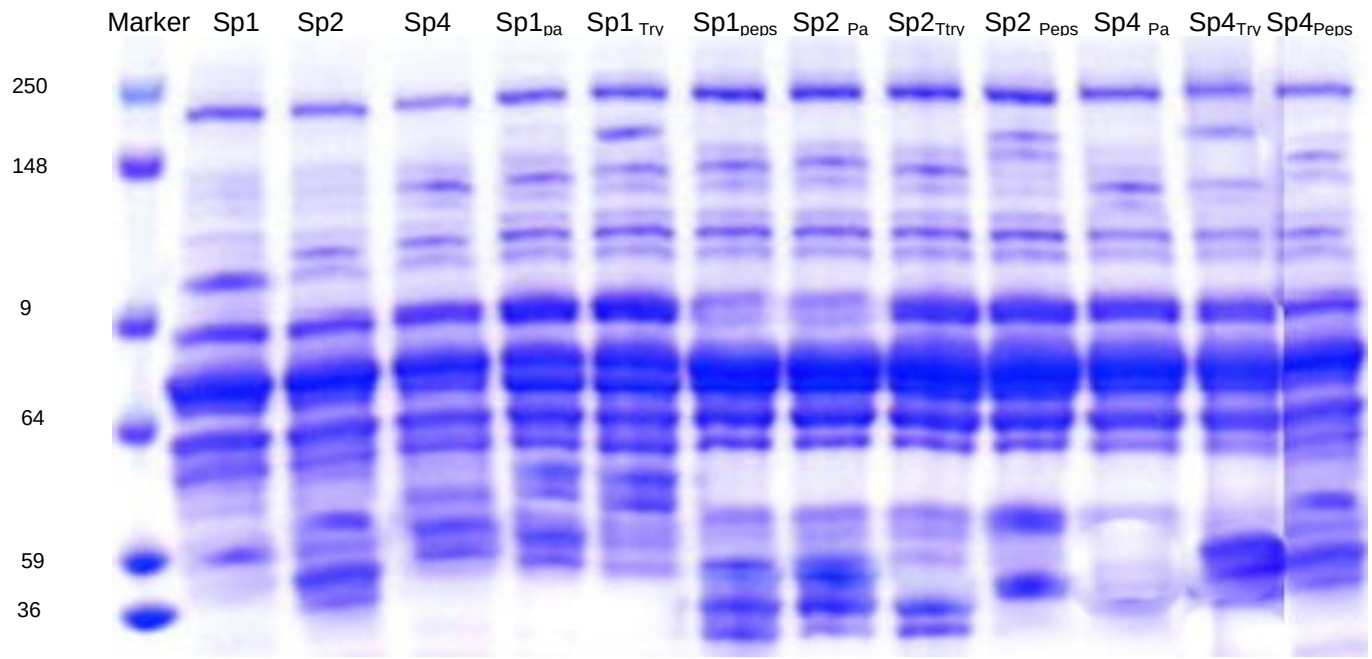

Figure. 2: SDS-PAGE profile of *Spirulina platensis*, where :(Sp1) Algae extracted with first method, (Sp2)Algae extracted with second method,(Sp4)Algae extracted with third method, ($Sp1_{pa}$), ($Sp2_{pa}$) and ($Sp4_{pa}$) extracts hydrolyzed with papain, ($Sp1_{try}$), ($Sp2_{try}$) and ($Sp4_{try}$) extracts hydrolyzed with trypsin, ($Sp1_{pep}$), ($Sp2_{pep}$) and ($Sp4_{pep}$) extracts hydrolyzed with pepsin

Table 4: Molecular weight (KD) of protein bands in *Spirulina platensis* protein extracts and its hydrolysates

Lanes	Marker	Sp1	Sp2	Sp4	Sp1 pa	Sp1 Try	Sp1 peps	Sp2 Pa	Sp2 Try	Sp2 Peps	Sp4 Pa	Sp4 Try	Sp4 Peps
Bands	K.D.												
1	250	219	225	237	250	253	250	253	253	246	253	259	259
2	148	143	146	141	144	191	151	155	147	186	141	193	161
3	98	123	119	122	125	148	125	125	125	163	124	141	125
4	64	110	98	101	118	126	119	104	119	130	119	125	119
5	59	95	79	83	101	103	105	81	101	124	103	119	103
6	36	76	64	68	86	85	81	77	80	118	82	103	86
7		63	63	63	77	77	77	68	67	101	77	82	70
8		62	60	61	68	68	68	63	63	80	67	68	63
9		59	59	60	63	63	63	60	60	67	63	63	61
10			52	59	62	62	60	52	59	63	60	59	60
11					61	61	58	39	39	60	54		59
12					60	58	39	32	32	48			51
13					59								

Protein bands around 40 and 70 kDa may belong to the enzyme magnesium chelatase (termed I and D); moreover, bands 75 kDa are adherent to protein Toc75 (Gamini Kannangara and Wettstein, 2010; Inoue and Potter, 2004). Polypeptides from photosystem I complex (PSI-200) are displayed as protein bands 58 and 62 kDa (Malkin, 1986). The presence of bands around 100 and 110 kDa may be induced by chlorophyll a protein CPIV and P700-chlorophyll a protein 1 from photosystem I (Brandt et al. 1982; Hoj and Moller, 1986).

Antioxidant activity:

According to Yu et al. (2002) two or more radical suits are demanded to assess the radical-scavenging activity because the radical system used for antioxidant estimation may give different results. In this study, DPPH and ABTS assays were used. The radical scavenging activity of the extracts was higher in the ABTS assay than the DPPH assay at 150 µg/ml.

Antioxidant activity using DPPH:

Evaluation of Protein hydrolysates capacity to scavenge DPPH and ABTS radicals antioxidant was determined. *Spirulina* is well known by its high content in proteins. Among them, phycobiliproteins are known by its pharmaceutical and antioxidant properties. These proteins were characterized by Simó et al, (2005). All extracts treated with enzymes achieved were qualified for scavenging DPPH radicals presented in Table 5. DPPH radical-scavenging capacities ranged from 26.74% to 32.27% at sample concentration 150µg/ml. The highest antioxidant capacity was 32.27% obtained with samples extracted with the second method and hydrolyzed with Trypsin ($Sp2_{Try}$) and samples extracted with the third method and hydrolyzed with Trypsin ($Sp4_{Try}$) it was 30.11%, while the lowest capacity was obtained for ($Sp1_{Try}$) first protein extract hydrolyzed with Trypsin 26.74%. *Spirulina* contains an enormous range of other nutrients such as γ-linolenic acid, b-carotene and proteins. This will be an additional health benefit as antioxidant agent.

Antioxidant activity using ABTS:

All protein extracts and hydrolysates exhibited good antioxidant capacity with the ABTS radical scavenging assay. Actually, antioxidant capacities ranging from 30.58% to 68.41% at sample concentration 150 µg/ml showed by Table 6. The highest antioxidant capacity appeared when we used Trypsin in the first extraction method 68.41%

Table 5: Antioxidant activity (%) of *Spirulina* protein extracts and hydrolysates by DPPH (Data are means of triplicate ± SD.)

Extracts	Sample Concentrations (µg/ml)		
&Hydrolysates	50	100	150
Sp1	25.61 ± 0.52	27.63 ± 0.59	28.42 ± 0.57
Sp1 Pa	26.38 ± 0.30	28.11 ± 0.19	29.84 ± 0.27
Sp1 Pep	25.26 ± 0.58	27.73 ± 0.30	29.71 ± 0.16
Sp1 Try	20.53 ± 0.16	23.43 ± 0.38	26.74 ± 0.20
Sp2	25.47 ± 0.32	26.61 ± 0.45	27.97 ± 0.41
Sp2 Pa	25.18 ± 0.18	27.07 ± 0.12	28.52 ± 0.47
Sp2 Pep	25.62 ± 0.79	27.49 ± 0.43	29.80 ± 0.19
Sp2 Try	28.61 ± 0.43	30.35 ± 0.23	32.27 ± 0.15
Sp4	27.30 ± 0.37	28.07 ± 0.13	28.88 ± 0.24
Sp4 Pa	28.34 ± 0.06	28.84 ± 0.23	28.30 ± 0.25
Sp4 Pep	28.30 ± 0.25	28.94 ± 0.10	29.38 ± 0.13
Sp4 Try	28.01 ± 0.50	29.40 ± 0.13	30.11 ± 0.29
BHA (butylatedhydroxyanisol)	88.54 ±0.020	95.22 ±0.02	99.41 ±0.025
BHT (butylatedhydroxytoluene)	85.57 ±0.025	94.76 ±0.031	98.15 ±0.046

Data are means of triplicate ± SD.

Table 6: Antioxidant activity (%) of *Spirulina* protein extracts and hydrolysates by ABTS (Data are means of triplicate ± SD)

Extracts	Sample Concentrations (µg/ml)		
& Hydrolysates	50	100	150
Sp1	30.64 ± 1.31	47.17 ± 2.50	67.49 ± 3.18
Sp1 Pa	46.88 ± 0.72	61.87 ± 1.26	63.67 ± 2.68
Sp1 Pep	27.82 ± 0.39	47.20 ± 1.28	66.85 ± 1.19
Sp1 Try	28.38 ± 0.75	65.21 ± 0.64	68.41 ± 0.55
Sp2	23.68 ± 0.66	40.13 ± 1.30	53.65 ± 1.57
Sp2 Pa	25.18 ± 0.18	27.07 ± 0.12	28.52 ± 0.47
Sp2 Pep	28.61 ± 0.43	30.35 ± 0.23	32.27 ± 0.15
Sp2 Try	24.75 ± 0.99	44.72 ± 1.19	56.39 ± 1.43
Sp4	17.37 ± 1.33	25.96 ± 0.23	27.12 ± 0.18
Sp4 Pa	26.44 ± 0.94	28.96 ± 0.30	30.85 ± 0.43
Sp4 Pep	19.49 ± 0.55	24.27 ± 0.48	32.91 ± 0.70
Sp4 Try	19.05 ± 0.40	20.95 ± 0.60	34.26 ± 0.55
Trolox	92.35 ±0.020	95.66 ±0.020	98.23 ±0.038

Data are means of triplicate ± SD.

In contrast, Wang et al. (1998) reported that some antioxidant compounds as found in phenolic compounds from sage which presenting ABTS scavenging activity may not have activity with DPPH. The difference between the two tests could be by virtue of the different technique in scavenging DPPH and ABTS radicals. In the DPPH test, the antioxidant effect was probable to be by virtue of the hydrogen donating ability of the extract (Conforti et al. 2005). The ABTS test is a measure of the activity of the antioxidant in scavenging proton radicals through donation of electrons (Mathew and Abraham, 2004). Furthermore, factors such as the solubility of the extract in different systems and stereo-selectivity of the radicals may also affect its capacity to quench different radicals (Benedetti et al. 2004).Chang et al. (2007) confirmed that low molecular weight hydrolysates have more power to be in possession ROS scavenging activities than high molecular weight hydrolysates. In addition, Sheih, et al. (2009) showed that pepsin hydrolysate from *Chlorella vulgaris* protein waste, generated during production of recap has efficacious antioxidative activity. Previous studies have shown that low molecular weight hydrolysates generally possess higher radical scavenging capacity than high molecular weight hydrolysates (Gallegos-Tintoré et al. 2015), since small peptides can more easily access the oxidative mechanism to donate protons to free radicals, and stabilize oxygen reactive species through a direct electron transfer, thereby exhibiting better antioxidant capacity (Sheih et al. 2009; Cian et al. 2013; Fan et al. 2014).

CONCLUSION

In order to distinguish *Spirulina* protein extracts treated with enzymes gained from this study, Protein hydrolysates afford high antioxidant activity. These results showed up that *Spirulina* protein hydrolysates perhaps contain substances that were proton donors and could react with free radicals to shift them to stable diamagnetic molecules.

A mixture of free amino acids, di, tri and oligo-peptides observed from the enzymatic hydrolysis of proteins. This modifies the functional characteristics of proteins and increases the number of polar groups and the solubility of the hydrolysate, which usually improves the workable quality (Schmidt and Salas-Mellado, 2009). Protein products contain enzymatic hydrolysis of proteins which used in dietary extensions, increasing the feasible and nutritional features of foods, particularly due to composing of bioactive peptides during the hydrolysis procedure. These protein extracts treated with enzymes with efficacious activity, such as those gained in this study, are more easily digested and absorbed by the human body compared to intact proteins.

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CONFLICT OF INTEREST

The authors have not declared any conflict of interests

ACKNOWLEGEMENT

We thank National Research Center, Project No.10090001for financially supporting this research.

AUTHOR CONTRIBUTIONS

AMRA designed the electrophoresis and reviewed the manuscript. HHA and FKE designed the algae cultivation and enzymatic assays. GSE and designed the antioxidant and analysis and reviewed the manuscript. SAM performed the experimental part and pre-wrote the manuscript. All authors read and approved the final version.

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