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ANGIOTENSIN-I-CONVERTING ENZYME INHIBITORY AND ANTIOXIDANT ACTIVITY OF TRYPTIC *SPIRULINA PLATENSIS* PROTEIN HYDROLYSATES: EFFECT OF HYDROLYSIS AND *IN VITRO* GASTROINTESTINAL DIGESTION

ABSTRACT

In this study, protein hydrolysates derived from *Spirulina platensis* protein (SPPHs) using trypsin were investigated in terms of angiotensin I-converting enzyme (ACE) inhibitory activity, antioxidant activity and total phenolic content (TPC) and subjected to an *in vitro* digestion model using human gastric and duodenal fluids. Moreover, the effects of hydrolysis time and enzyme/substrate (E/S) ratio on the degree of hydrolysis (DH) of the hydrolysates were determined. The maximum DH (%) was found as 25.03±0.89% with the combination of E/S ratio of 3:100, hydrolysis time of 8 hours ($p<0.05$). The highest ACE inhibitory activity value was observed as 21.79±1.52% for initial SPPHs, prepared within the hydrolysis conditions of E/S ratio of 3:100 and hydrolysis time of 8 h. In general, the increase in E/S ratio and hydrolysis time resulted in an increase in the DH and in an improved ACE inhibitory activity of both initial and the GI digested samples ($p<0.05$). After digestion by pepsin, TPC of the digests was in the range of 28.87±0.32 and 40.28±1.05mg caffeic acid equivalent/g dry weight. However, further digestion by pancreatin led TPC of the final GI digest between 19.85±1.24 and 29.00±1.00mg caffeic acid equivalent/g dry weight. Moreover, the antioxidant activity of further digested SPPHs by gastric and intestinal proteases remained generally stable after *in vitro* treatment.

Keywords: Angiotensin I-converting Enzyme, Antioxidant Activity, Bioaccessibility, Enzymatic Hydrolysis, *Spirulina platensis*

1. INTRODUCTION

Spirulina platensis, a blue-green microalgae belonging family *Oscillatoriaceae*, is a rich source of nutraceuticals with high levels of amino acids, polyunsaturated fatty acids, polysaccharides, phytochemicals and vitamin contents [1 and 3]. Spirulina has been declared as "the best for tomorrow, by the United Nations World Health Organisation in 1996 and it is the only blue green algae that is commercially cultivated for food use today [4]. Among other microalgae species, *Spirulina platensis* is gaining increasing attention mainly due to its remarkable protein concentration of 60-70% protein (dry weight), being higher than any other natural food as well as providing 18 amino acids including all essential ones [5 and 7]. Protein

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hydrolysates are important sources of multifunctional bioactive peptides that are released *in vitro* or *in vivo* from animal or plant proteins and could be utilized in food formulations for health promoting purposes and/or to enhance the shelf life of foods [8 and 9]. Beyond nutrition, these hydrolysates have additional physiological functions in humans such as antioxidative, antihypertansive, antimicrobial, cholesterol-lowering, immunomodulatory and opioid properties [10 and 13]. Enzymatic hydrolysis enables the release of these peptides which are inactive within the sequence of parent protein [14 and 15]. It has been suggested that non-protein compounds such as phenolics and pigments need to be taken into account when evaluating the antioxidant activities of some protein hydrolysates like rapeseed [16]. *S. platensis* contains phycobiliproteins as light-harvesting protein–pigment complexes [17]. Although the phenolic content and antioxidant capacity of *S. platensis* has been well described in previous studies, there is no study present regarding with the phenolic content of protein hydrolysates. Several studies have been reported a number of peptides having antioxidative [18], antibacterial [19] antihypertansive [20 and 22], antitumor [23], antiproliferative [24] and iron chelating [25] activities derived from *S. platensis* using proteases such as alcalase, pepsin, papain, trypsin, protamex, protease K etc. Since proteases exhibit varying specifities for the polypeptide chains, different enzymes hydrolyse proteins into different degrees and thereby yield diverse functionalities [26]. The hydrolysis of protein is calculated in terms of degree of hydrolysis (DH) [27]. Degree of hydrolysis effects on the size and the amino acid composition of the peptide chains. Moreover, the biological activity of protein hydrolysates is dependent on DH [11].

2. RESEARCH SIGNIFICANCE

Antioxidative and antihypertensive activities have previously been found in *S. platensis* protein hydrolysates that were produced by commercial enzyme preparations such as Alcalase, Papain, Flavourzyme, Pepsin, Protamex, and Protease K [18, 20 and 21]. Trypsin is a serine protease that hydrolyzes peptide chains at the carboxyl side of arginine and lysine residues [28]. In the literature, antioxidative and angiotensin I-converting enzyme (ACE) inhibitory peptides have been identified in tryptic hydrolysates from Nile tilapia skin [29], sea cucumber [30] and whey [31]. Peptides having antiproliferative [24], antitumor [23], anti-atherosclerotic [32] and antibacterial [19] activities have been identified from *Spirulina* species subjected to trypsin hydrolysis. In the present work, the effects of hydrolysis time (4, 6 and 8 hours) and enzyme to substrate ratio (1:100, 2:100 and 3:100) on the degree of hydrolysis and total phenolic content, antioxidative and ACE-inhibitory activities were determined in tryptic *S. platensis* protein hydrolysates (SPPHs). Moreover, an in vitro digestion model was used to simulate the human gastrointestinal (GI) digestion system in order to investigate the degradation and the performance of protein hydrolysates in the digestive tract. The digests were also assessed for their phenolic contents, antioxidant and ACE inhibitory activities.

3. EXPERIMENTAL METHOD

S. platensis powder (SPP) was obtained from a local manufacturer in Turkey (Akuatik Fisheries and Cosmetics Products Ltd., Adana, Turkey). All chemicals were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

S. platensis protein concentrate (SPPC) was extracted by modifications of Benelhadj and coworkers's method [6] while tryptic *S. platensis* protein hydrolysates (SPPH) was prepared using the hydrolysis method described by Ahn et al. [12] *In vitro* gastrointestinal digestion was simulated according to a previously published method by Swieca et al. (2013) [65]. The degree of hydrolysis (DH) of SPPH was determined based on the method described by Jadhav et al. (2013). The DH was calculated using the following equation:

$$\text{DH\%} = \left(\frac{\text{10\% TCA soluble nitrogen in the sample}}{\text{total nitrogen in the sample}}\right) \times 100 \quad (1)$$

Total phenolic content (TPC) of the protein concentrates was determined by the Folin-Ciocalteu method according to Ling (2014) and results were expressed as milligram caffeic acid equivalent per g dry weight [33]. Total antioxidant activity was determined by Cupric ion reducing antioxidant capacity (CUPRAC) [42] and Ferric ion reducing antioxidant power (FRAP) (Benzie and Strain, 1996) assays. Results were expressed as milligram Trolox equivalent per g dry weight. ACE inhibitory activity was measured spectrophotometrically according to the method of Ahn et al. (2012) using equation (2) [12]. The assay mixture without protein hydrolysate was used as control. A 100% inhibitory activity would indicate complete inhibition of the enzyme and no increase in the absorbance. Statistical analysis was performed to data using a statistical package (Minitab, Version 17, Minitab Inc., State College, PA). The differences between mean values were compared using Tukey test.

$$\text{ACE inhibition (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (2)$$

4. FINDINGS AND DISCUSSIONS

4.1. Changes in the DH

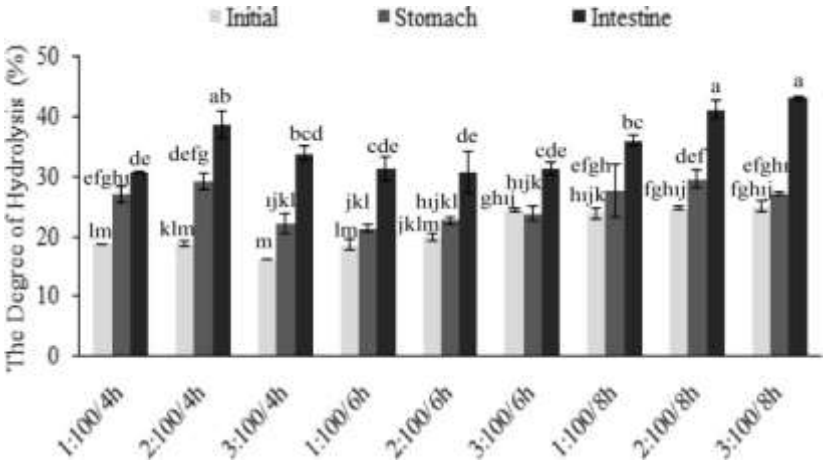
The effects of hydrolysis time and E/S ratio on the DH% of the hydrolysates were shown Table 1. Moreover, we evaluated the progression of DH after each simulated digestion step for the tryptic hydrolysates. The initial DH of SPPHs varied between 18.57% and 25.03%. The maximum DH was found as 25.03±0.89% with the combination of E/S ratio of 3:100, hydrolysis time of 8h, while the minimum DH (%) was obtained 12.78±0.09% for the hydrolysate with E/S ratio of 3:100, hydrolysis time of 4h (Table 1). Obvious changes in initial DH% were observed depending on the hydrolysis time and E/S ratio ($p<0.05$). Trypsin is an endopeptidase that cleaves peptide bonds at the C-terminal side of lysine and arginine [34]. In accordance with our study, the DH% increased with increasing hydrolysis time in the study of Ovissipour et al. (2009). According to Margot et al. (1997), the proportional relation between DH and E/S ratio was reported in tryptic whey protein hydrolysates [35]. Additionally, in the hydrolysis of Pisane and Propulse isolates by trypsin, higher DH values were found at ascending E/S ratios [36].

Table 1. The effect of hydrolysis time and E/S ratio on the degree of hydrolysis (%) of SPPHs during enzymatic hydrolysis with trypsin

E/S Ratio	Hydrolysis Time, Hours		
	4	6	8
1/100	18.69±0.11 bA	18.57±0.89 bB	23.91±0.89 aA
2/100	18.80±0.44 bA	19.91±0.67 bB	24.69±0.33 aA
3/100	16.24±0.11 aB	24.47±0.44 aA	25.03±0.89 aA

Means with the different letter within a column (a, b, c,..) and a row (A, B,..) are significantly different (ANOVA, Tukey test, p<0.05)

After pepsin digestion in the gastric phase, the DH of the digests ranged from 21.39±0.62 to 29.22±1.48 (p<0.05). On the other hand, the DH of pancreatic digests became 30.73±3.41-43.12±0.18 after intestinal digestion (p<0.05) (Graph 1). Pancreatin enzyme contains enzymatic components including trypsin, amylase, lipase, ribonuclease, and protease. Pancreatin has been reported to be responsible for further degradation of the peptides to low-molecular weight peptides, oligopeptides and possible amino acids [37]. In our study, pepsin enzyme caused the relatively low degree of hydrolysis in comparison to trypsin and pancreatin enzymes. Bamdad et al. (2011) observed that the efficient hydrolysis flavourzyme and alcalase demonstrates their higher proteolytic activity than pepsin towards hordein. The relatively low degree of hydrolysis of pepsin hydrolysates has been attributed to pepsin's inability to hydrolyze the proline peptide bond efficiently [38 and 40].



Graph 1. Changes in the degree of hydrolysis (%) of the SPPH before and after *in vitro* digestion

Abbreviations
1:100/4h: Enzyme/substrate ratio of 1:100, hydrolysis time of 4 h
2:100/4h: Enzyme/substrate ratio of 2:100, hydrolysis time of 4 h
3:100/4h: Enzyme/substrate ratio of 3:100, hydrolysis time of 4 h
1:100/6h: Enzyme/substrate ratio of 1:100, hydrolysis time of 6 h
2:100/6h: Enzyme/substrate ratio of 2:100, hydrolysis time of 6 h
3:100/6h: Enzyme/substrate ratio of 3:100, hydrolysis time of 6 h
1:100/8h: Enzyme/substrate ratio of 1:100, hydrolysis time of 8 h
2:100/8h: Enzyme/substrate ratio of 2:100, hydrolysis time of 8 h
3:100/8h: Enzyme/substrate ratio of 3:100, hydrolysis time of 8 h
Means with the different letter within a column (a, b, c,..) are significantly different (ANOVA, Tukey test, p<0.05)

4.2. Changes in the Antioxidant Activity

Phenolic compounds have been related directly and indirectly with antiradical activity [41]. Castel et al. (2014) reported higher antioxidant activity together with higher phenolic content for amaranth protein concentrates. A significant TPC ranged from

23.97±2.56 to 41.11±2.71 mg caffeic acid equivalent (CAE)/g dry weight (dw) was observed for the tryptic protein hydrolysates, as shown in Table 2. The effects of hydrolysis time and enzyme to substrate ratio were found significant on TPC ($p<0.05$). The increasing effect of time on TPC can be explained by revelation of phenolic compounds during enzymatic hydrolysis.

Table 2. Total phenolic content (mg CAE/g dw) and antioxidant activity (mg TE/g dw) before and after GI digestion for SPPH

Sample	Initial	Stomach	Intestine
Total phenolic content (mg CAE/g dw)			
1:100, 4 h	34.52±2.35 aABCD	27.34±0.03 abC	21.54±0.46 bBC
2:100, 4 h	35.57±3.02 aABC	31.09±0.39 abBC	23.75±0.97 bABC
3:100, 4 h	31.18±0.94 bBCD	36.28±0.34 aAB	26.20±0.82 cAB
1:100, 6 h	30.18±0.48 bCD	40.28±1.05 aA	23.20±2.03 bABC
2:100, 6 h	23.97±2.56 bD	36.69±0.58 aAB	26.15±0.68 bAB
3:100, 6 h	29.14±1.63 aCD	28.87±0.32 aC	24.44±1.15 aABC
1:100, 8 h	37.16±0.25 aABC	32.73±3.59 aBC	29.00±1.00 aBC
2:100, 8 h	41.11±2.71 aAB	34.62±1.03 aABC	19.85±1.24 bC
3:100, 8 h	43.71±0.22 aA	36.27±0.64 bAB	26.04±0.91 cAB
Total antioxidant activity (mg TE/g dw)			
CUPRAC			
1:100, 4 h	6.33±1.54 aA	8.94±2.39 aA	10.53±0.60 aA
2:100, 4 h	13.48±0.23 aA	8.64±0.25 aA	8.11±1.72 aA
3:100, 4 h	12.94±0.46 aA	10.01±0.20 bA	7.61±0.01 cA
1:100, 6 h	9.25±0.31 bA	10.53±0.89 abA	17.02±2.04 aA
2:100, 6 h	7.10±1.38 bA	17.13±0.65 aA	11.18±0.41 bA
3:100, 6 h	8.48±0.78 cA	16.23±1.95 bA	20.24±0.60 aA
1:100, 8 h	13.71±0.15 aA	11.71±2.35 aA	14.12±2.81 aA
2:100, 8 h	7.94±1.07 aA	11.41±1.02 aA	15.85±1.95 aA
3:100, 8 h	8.16±1.09 cA	12.59±1.16 bA	17.71±1.68 aA
FRAP			
1:100, 4 h	2.93±0.26 bA	5.20±1.93 aA	1.94±0.15 bA
2:100, 4 h	4.91±1.65 aA	4.68±0.86 aA	2.16±0.14 aA
3:100, 4 h	2.58±0.11 aA	2.67±0.04 aA	2.70±0.04 aA
1:100, 6 h	2.90±0.34 aA	1.67±0.01 aA	4.51±1.23 aA
2:100, 6 h	3.80±0.44 aA	4.36±0.08 aA	2.03±0.25 bA
3:100, 6 h	3.14±0.15 bA	3.94±0.05 aA	2.26±0.05 cA
1:100, 8 h	2.75±0.80 aA	3.77±0.19 aA	2.35±0.04 aA
2:100, 8 h	3.01±0.11 abA	1.55±0.33 bA	4.98±0.53 aA
3:100, 8 h	3.57±0.20 bA	3.36±0.15 cA	3.71±0.68 aA

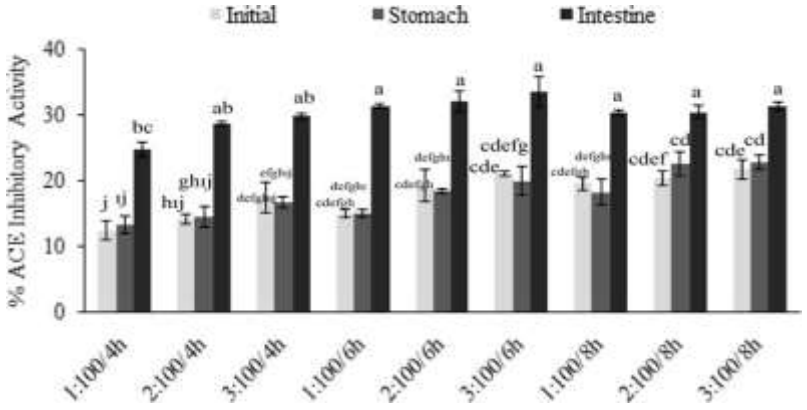
The data presented consist of average values ± standard deviation of three independent batches. Total phenolic content is expressed as mg of CAE per g of dw of sample, respectively. Different capital letters in the columns or small letters in the rows represent statistically significant differences (ANOVA, Tukey test, $p<0.05$).

CUPRAC assay give satisfactory results in determining the antioxidant status of food and biological fluids as being superior in regard to its realistic pH close to the physiological pH and applicability to lipophilic antioxidants as well as hydrophilic ones [42]. Protease treatments, especially trypsin have been reported to enhance the copper reducing capacity of whey [43]. On the other hand, FRAP assay has been reported to correlated well with ABTS radical scavenging assay results for protein hydrolysates derived from threadfin bream surimi wastes [44]. The cupric reducing antioxidant capacity of the tryptic SPPHs were ranged between 6.33±1.54 and 13.71±0.15mg TE/g dw (Table 2). Among SPPHs, the antioxidant activity did not indicated a regular profile in both FRAP and CUPRAC assays, where the values showed alternating increases and decreases. During the enzymatic hydrolysis, peptides having antioxidant activities could be perpetually formed and degraded, based on their molecular structure which is primarily influenced by hydrolysis conditions [37]. This data agreed with the findings that the antioxidant activities of egg white protein hydrolysates 6 h of hydrolysis [45]. The DH is usually used as a parameter as an indicator of the proteolysis process of protein hydrolysates [46]. The DH% is associated with antioxidant activity for most protein hydrolysates [47]. In other words, higher DH% values may yield smaller or shorter peptide chains producing greater antioxidant activity. In the literature, a number of studies exist in which the antioxidant properties have been both positively or negatively correlated with DH of the hydrolysates. Increase of the relative antioxidant activities of SPPHs was independent from the degree of hydrolysis. Hwang and others (2010) also reported that increase of the relative antioxidant activities of peanut protein hydrolysates was not time dependent and the activity did not increase along with the degree of hydrolysis.

After the simulated GI digestion, the antioxidant activity of SPPHs did not differ significantly from that of initial for some samples ($p>0.05$), whereas changes in antioxidant activity was significant for others ($p<0.05$), as can be seen in Table 2. The antioxidant activity of further digested SPPHs by gastric and intestinal proteases remained generally stable after in vitro treatment as shown Table 2, suggesting that peptides present in hydrolysates may be partially degraded into smaller peptides being effective hydrogen or electron donors. Small-sized peptides have also been reported to have a better chance of escaping digestion in GI tract and can be easily absorbed to attain the target organs [48]. Previous articles have also reported some hydrolysates that still presented their antioxidant activity after GI digestion derived from loach [49], flaxseed [50], casein [51], and hake by-products [52]. The antioxidant activity is defined as a synergistic effect that arisen from an amino acid with antioxidant potential and their position in the peptide sequence [53]. It is appropriate to note that pepsin and pancreatin digestion favor the release of peptides having bioactivities and phenolics linked to proteins as well [54]. After digestion by pepsin, TPC of the digests was in the range of 28.87±0.32mg CAE/g dw and 40.28±1.05mg CAE/g dw. However, further digestion by pancreatin led TPC of the final GI digest between 19.85±1.24mg CAE/g dw and 29.00±1.00mg CAE/g dw. Total phenolic content (mg CAE/g dw) before and after pepsin and pancreatin digestion of SPPHs are shown in Table 2. The decrease in TPC after GI may result from the interactions between peptides and phenolics since hydrolysis process has an effect on protein-polyphenol interactions [55].

4.3. Changes in the ACE Inhibitory Activity

Different authors have obtained ACE-inhibiting peptides by hydrolysis of *S. platensis* proteins in vivo [56] and also *in vitro* [21, 57 and 58]. Pepsin has been shown to generate ACE inhibitory peptides from algae protein [49, 57 and 59]. Ko et al. (2012) observed that alcalase-proteolytic hydrolysate exhibited the highest ACE inhibitory activity among them. The SPPHs showed ACE-inhibiting ability in the range from 12.50±1.40% to 21.79±1.52%, as shown in Graphic 2. The comparison of our data with those obtained by other researchers is a complex issue, because the observed differences in the ACE-inhibitory activity may be arisen from a variety of reasons, such as the protein extraction procedure, as well as the differences in the peptide mixture composition, presence of impurities, related to changes in the parameters of the digestion process (enzyme, pH, time, temperature, substrate/enzyme ratio, etc.), or the different analytical method used for the determination of the ACE-inhibitory activity [60].



Graphic 2. Changes in ACE% inhibitory activity before and after GI digestion for each SPPHs prepared in different E/S ratio and hydrolysis time. Means with the different letter within a column (a, b, c,..) are significantly different (ANOVA, Tukey test, $p<0.05$)

The increase of E/S ratio and hydrolysis time resulted in an increase in the DH and in an improved ACE inhibitory activity of both initial and the GI digested samples ($p<0.05$). The highest %ACE inhibitory activity value was 21.79±1.52% in initial SPPHs, prepared in hydrolysis conditions with E/S ratio of 3:100 and hydrolysis time of 8 h. An increase in enzyme concentration and/or hydrolysis time was shown to result in improved ACE inhibitory activity for fish protein hydrolysates [61]. In order to display an antihypertensive effect in vivo, the ACE inhibitory peptides are required to be resistant to gastrointestinal degradation and also to be absorbed into the bloodstream in their intact form [62 and 63]. Peptides can be degraded during digestion and their biological activity can be activated or inactivated [64]. According to the results, the effect of stomach digestion on the ACE inhibitory activity of SPPHs was insignificant as a general trend ($p>0.05$), as shown in Graphic 2. However, further digestion in the intestine brought the ACE inhibitory activity of the final GI digests to 24.85±1.01-33.60±2.40 ($p<0.05$), indicating that some peptides with stronger ACE inhibitory activities were produced during the simulated GI digestion. Previous studies have also reported that peptides derived from rapeseed [16], egg white lysozyme [62], and ham [64] still exhibited ACE inhibitory activity after GI digestion.

5. CONCLUSION

The degree of bioactivity generated in the SPPHs varied and was dependent on the hydrolysis time, enzyme to substrate ratio and type of protease. According to the results, the antioxidant and ACE inhibitory potency of SPPHs still showed relevant bioactivities after in vitro digestion by gastrointestinal proteases. It can be noted that the antioxidant and ACE inhibitory activity observed in SPPHs could be due to the sum of the activities of various peptides and phenolics present in *Spirulina*. Isolation of purified peptides and determination of amino acid sequence of peptides present in the hydrolysates having ACE inhibitory activity and antioxidant activity should be justified for future studies in order to elucidate possible structure-activity correlations at amino acid level on potency of bioactivities.

REFERENCES

1. Wang, L., Pan, B., Sheng, J., Xu, J., and Hu, Q., (2007). Antioxidant Activity of Spirulina Platensis Extracts By Supercritical Carbon Dioxide Extraction. Food Chemistry. 105(1): p. 36-41.
2. Lupatini, A.L., Colla, L.M., Canan, C., and Colla, E., (2016). Potential Application of Microalgae Spirulina Platensis as A Protein Source. Journal of the Science of Food and Agriculture. 97(3):724-732
3. Zhang, L., Chen, L., Wang, J., Chen, Y., Gao, X., Zhang, Z., and Liu, T., (2015). Attached Cultivation for Improving The Biomass Productivity of Spirulina Platensis. Bioresource technology. 181:136-142.
4. Gad, A.S., Khadrawy, Y.A., El-Nekeety, A.A., Mohamed, S.R., Hassan, N.S., and Abdel-Wahhab, M.A., (2011). Antioxidant Activity and Hepatoprotective Effects of Whey Protein and Spirulina in Rats. Nutrition. 27(5):582-589.
5. Kim, S.K. and Kang, K.H., (2011). Medicinal Effects of Peptides from Marine Microalgae. Advances in food and nutrition research. 64:313-323.
6. Benelhadj, S., Gharsallaoui, A., Degraeve, P., Attia H. and Ghorbel, D. (2016). Effect of Ph on The Functional Properties of Arthrospira (Spirulina) Platensis Protein Isolate. Food Chemistry. 194:1056- 1063.
7. Fradique, M., Batista, A.P., Nunes, M.C., Gouveia, L., Bandarra N.M., and Raymundo, A., (2010). Incorporation of Chlorella Vulgaris and Spirulina Maxima Biomass in Pasta Products. Part 1: Preparation and evaluation. Journal of the Science of Food and Agriculture. 90(10):1656-1664.
8. Di Bernardini, R., Harnedy, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A.M., and Hayes, M., (2011). Antioxidant and Antimicrobial Peptidic Hydrolysates from Muscle Protein Sources and By-Products. Food Chemistry. 124(4):1296-1307.
9. Carrasco-Castilla, J., Hernández-Álvarez, A.J., Jiménez-Martínez, C., Jacinto-Hernández, C., Alaiz, M., Girón-Calle, J., Vioque, J., and Dávila-Ortiz, G., (2012). Antioxidant and Metal Chelating Activities of Phaseolus Vulgaris L. Var. Jamapa Protein Isolates, Phaseolin and Lectin Hydrolysates. Food Chemistry. 131(4):1157-1164.
10. Hartmann, R. and Meisel, H., (2007). Food-Derived Peptides with Biological Activity: From Research to Food Applications. Current opinion in biotechnology. 18(2):163-169.
11. Jamdar, S., Rajalakshmi, V., Pednekar, M., Juan, F., Yardi, V., and Sharma, A., (2010). Influence of Degree of Hydrolysis on

Functional Properties, Antioxidant Activity and ACE Inhibitory Activity of Peanut Protein Hydrolysate. Food Chemistry. 121(1):178-184.
12. Ahn, C.B., Jeon, Y.J. Kim, Y.T., and Je, J.Y., (2012). Angiotensin I Converting Enzyme (ACE) Inhibitory Peptides from Salmon Byproduct Protein Hydrolysate by Alcalase Hydrolysis. Process Biochemistry. 47(12):2240-2245.
13. Udenigwe, C.C., (2014). Bioinformatics Approaches, Prospects and Challenges of Food Bioactive Peptide Research. Trends in Food Science & Technology. 36(2):137-143.
14. Korhonen, H., (2009). Milk-Derived Bioactive Peptides: From Science to Applications. Journal of Functional Foods. 1(2):177-187.
15. Power, O., Jakeman, P., and FitzGerald, R., (2013). Antioxidative Peptides: Enzymatic Production, In Vitro and In Vivo Antioxidant Activity and Potential Applications of Milk-Derived Antioxidative Peptides. Amino Acids. 44(3):797-820.
16. Yu, J., Hu, Y., Xue, M., Dun, Y., Li, S., Peng, N., Liang, Y., and Zhao, S., (2016). Purification and Identification of Antioxidant Peptides from Enzymatic Hydrolysate of Spirulina Platensis. Journal of Microbiolgy and Biotechnolgy. 26(7):1216-1223.
17. Sun, Y., Chang, R., Li, Q., and Li, B., (2016). Isolation and Characterization of an Antibacterial Peptide from Protein Hydrolysates of Spirulina Platensis. European Food Research and Technology. 242(5):685-692.
18. Pan, H., She, X., Wu, H., Ma, J., Ren, D., and Lu, J., (2015). Long-Term Regulation of The Local Renin–Angiotensin System in The Myocardium of Spontaneously Hypertensive Rats By Feeding Bioactive Peptides Derived From Spirulina Platensis. Journal of agricultural and food chemistry. 63(35): p. 7765-7774.
19. Lu, J., Ren, D.F., Xue, Y.L., Sawano, Y., Miyakawa, T., and Tanokura, M., (2010). Isolation of an Antihypertensive Peptide From Alcalase Digest of Spirulina Platensis. Journal of agricultural and food chemistry. 58(12):7166-7171.
20. He, H.L., Chen, X.L. Wu, H., Sun, C.Y. Zhang, Y.Z., and Zhou, B.C., (2007). High Throughput and Rapid Screening of Marine Protein Hydrolysates Enriched in Peptides with Angiotensin-I-Converting Enzyme Inhibitory Activity by Capillary Electrophoresis. Bioresource Technology. 98(18):3499-3505.
21. Wang, Z. and Zhang, X., (2016). Characterization and Antitumor Activity of Protein Hydrolysates from Arthrospira Platensis (Spirulina Platensis) Using Two-Step Hydrolysis. Journal of Applied Phycology. 28(6):3379-3385.
22. Wang, Z. and Zhang, X., (2017). Isolation and Identification of Anti-Proliferative Peptides from Spirulina Platensis Using Three-Step Hydrolysis. Journal of the Science of Food and Agriculture. 97(3):918-922.
23. Kim, N.H., Jung, S.H., Kim, J., Kim, S.H., Ahn, H.J., and Song, K.B., (2014). Purification of an Iron-Chelating Peptide from Spirulina Protein Hydrolysates. Journal of the Korean Society for Applied Biological Chemistry. 57(1):91-95.
24. Adjonu, R., Doran, G., Torley, P., and Agboola, S., (2013). Screening of Whey Protein Isolate Hydrolysates for Their Dual Functionality: Influence of Heat Pre-Treatment and Enzyme Specificity. Food chemistry. 136(3):1435-1443.

25. Kristinsson, H.G. and Rasco, B.A., (2000). Fish Protein Hydrolysates: Production, Biochemical, and Functional Properties. Critical Reviews in Food Science and Nutrition. 40(1):43-81.
26. Kishimura, H., Tokuda, Y., Yabe, M., Klomklao, S., Benjakul, S., and Ando, S., (2007). Trypsins from the Pyloric Ceca of Jacopever (Sebastes Schlegelii) and Elkhorn Sculpin (Alcichthys Alcicornis): Isolation and Characterization. Food Chemistry. 100(4):1490-1495.
27. Choonpicharn, S., Jaturasitha, S., Rakariyatham, N., Suree, N., and Niamsup, H., (2015). Antioxidant and Antihypertensive Activity of Gelatin Hydrolysate from Nile Tilapia Skin. Journal of food science and technology. 52(5):3134-3139.
28. Abedin, M.Z., Karim, A.A., Latiff, A.A., Gan, C.Y., Ghazali, F.C., Barzideh, Z., Ferdosh, S., Akanda, M.J.H., Zzaman, W., and Karim, M.R., (2014). Biochemical and Radical-Scavenging Properties of Sea Cucumber (Stichopus Vastus) Collagen Hydrolysates. Natural product research. 28(16):1302-1305.
29. Chatterjee, A., Kanawjia, S., Khetra, Y., and Saini, P., (2015). Discordance Between in Silico and in Vitro Analyses of ACE Inhibitory and Antioxidative Peptides from Mixed Milk Tryptic Whey Protein Hydrolysate. Journal of Food Science and Technology. 52(9):5621- 5630.
30. Vo, T.S. and Kim, S.K., (2013). Down-Regulation of Histamine-Induced Endothelial Cell Activation as Potential Anti-Atherosclerotic Activity of Peptides from Spirulina Maxima. European Journal of Pharmaceutical Sciences. 50(2):198-207.
31. Mäkinen, S., Johannson, T., Gerd, E.V., Pihlava, J.M., and Pihlanto, A., (2012). Angiotensin I-Converting Enzyme Inhibitory and Antioxidant Properties of Rapeseed Hydrolysates. Journal of Functional Foods. 4(3):575-583.
32. Estrada, J.P., Bescos, P.B., and Del Fresno, A.V., (2001). Antioxidant Activity of Different Fractions of Spirulina Platensis Protean Extract. Il farmaco. 56(5):497-500.
33. Ling, A.L.M., (2014). Antioxidant Activity, Total Phenolic and Flavonoid Contents of Selected Commercial Seaweeds of Sabah, Malaysia. International Journal of Pharmaceutical and Phytopharmacological Research. 3(3).
34. Chanput, W., Theerakulkait, C., and Nakai, S., (2009). Antioxidative Properties of Partially Purified Barley Hordein, Rice Bran Protein Fractions and Their Hydrolysates. Journal of Cereal Science. 49(3):422-428.
35. Margot, A., Flaschel, E., and Renken, A., (1997). Empirical Kinetic Models for Tryptic Whey-Protein Hydrolysis. Process Biochemistry. 32(3):217-223.
36. Karamac, M., Amarowicz, R., and Kostyra, H., (2002). Effect of Temperature and Enzyme/Substrate Ratio on the Hydrolysis of Pea Protein Isolates by Trypsin. Czech Journal of Food Sciences. 20(1):1-6.
37. Vaštag, Ž., Popović, L., Popović, S., Peričin-Starčević, I., and Krimer-Malešević, V., (2013). In Vitro Study on Digestion of Pumpkin Oil Cake Protein Hydrolysate: Evaluation of Impact on Bioactive Properties. International journal of Food Sciences and Nutrition. 64(4):452-460.
38. Jadhav, S., Lutz, S., Ghorpade, V., and Salunkhe, D., (1998). Barley: Chemistry and Value-Added Processing. Critical Reviews in Food Science. 38(2):123-171.
39. Hausch, F., Shan, L., Santiago, N.A., Gray, G.M., and Khosla, C., (2002). Intestinal Digestive Resistance of Immunodominant

Gliadin Peptides. American Journal of Physiology-Gastrointestinal and Liver Physiology. 283(4):G996-G1003.
40. Simpson, D.J., (2001). Proteolytic Degradation of Cereal Prolamins—the Problem With Proline. Plant Science. 161(5):825-838.
41. Balogh, E., Hegedűs, A., and Stefanovits-Bányai, É., (2010). Application of and Correlation among Antioxidant and Antiradical Assays for Characterizing Antioxidant Capacity of Berries. Scientia horticulturae. 125(3):332-336.
42. Apak, R., Güçlü, K., Özyürek, M., and Karademir, S.E., (2004). Novel Total Antioxidant Capacity Index for Dietary Polyphenols And Vitamins C and E, Using Their Cupric Ion Reducing Capability In The Presence of Neocuproine: CUPRAC method. Journal of Agricultural and Food Chemistry. 52(26):7970-7981.
43. Önay-Uçar, E., Arda, N., Pekmez, M., Yılmaz, A.M., Böke-Sarıkahya, N., Kırmızıgül, S., and Yalçın, A.S., (2014). Comparison of Antioxidant Capacity, Protein Profile and Carbohydrate Content of Whey Protein Fractions. Food Chemistry. 150:34-40.
44. Wiriyaphan, C., Chitsomboon, B. and Yongsawadigul, J. (2012). Antioxidant Activity Of Protein Hydrolysates Derived from Threadfin Bream Surimi Byproducts. Food Chemistry. 132(1): p. 104-111.
45. De Castro, R.J.S. and Sato, H.H., (2015). A Response Surface Approach on Optimization of Hydrolysis Parameters for the Production of Egg White Protein Hydrolysates with Antioxidant Activities. Biocatalysis and Agricultural Biotechnology. 4(1):55-62.
46. Hsu, K.C., (2010). Purification of Antioxidative Peptides Prepared From Enzymatic Hydrolysates of Tuna Dark Muscle By-Product. Food Chemistry. 122(1):42-48.
47. Liu, Q., Kong, B., Xiong, Y.L., and Xia, X., (2010). Antioxidant Activity and Functional Properties of Porcine Plasma Protein Hydrolysate as Influenced by the Degree of Hydrolysis. Food Chemistry. 118(2):403-410.
48. Foltz, M., Van Buren, L., Klaffke, W., and Duchateau, G.S., (2009). Modeling of the Relationship between Dipeptide Structure and Dipeptide Stability, Permeability, and ACE Inhibitory Activity. Journal of food science. 74(7):H243-H251.
49. You, L., Zhao, M., Regenstein, J.M., and Ren, J., (2010). Changes in the Antioxidant Activity of Loach (Misgurnus Anguillicaudatus) Protein Hydrolysates during a Simulated Gastrointestinal Digestion. Food Chemistry. 120(3):810-816.
50. Silva, F., O'Callagahan, Y., O'Brien, N., and Netto, F., (2013). Antioxidant Capacity of Flaxseed Products: The Effect of in Vitro Digestion. Plant Foods for Human Nutrition. 68(1):24-30.
51. Ao, J. and Li, B., (2013). Stability and Antioxidative Activities of Casein Peptide Fractions During Simulated Gastrointestinal Digestion in Vitro: Charge Properties of Peptides Affect Digestive Stability. Food Research International. 52(1):334-341.
52. Teixeira, B., Pires, C., Nunes, M.L., and Batista, I., (2016). Effect of In Vitro Gastrointestinal Digestion on The Antioxidant Activity of Protein Hydrolysates Prepared from Cape Hake By-Products. International Journal of Food Science and Technology. 51(12):2528-2536.
53. Samaranayaka, A.G. and Li-Chan, E.C., (2011). Food-Derived Peptidic Antioxidants: A Review of Their Production, Assessment,

and Potential Applications. Journal of Functional Foods. 3(4):229-254.
54. Cian, R.E., Caballero, M.S., Sabbag, N., González, R.J., and Drago, S.R., (2014). Bio-Accessibility of Bioactive Compounds (ACE Inhibitors and Antioxidants) from Extruded Maize Products Added with A Red Seaweed Porphyra Columbina. LWT-Food Science and Technology. 55(1):51-58.
55. Cian, R.E., Martínez-Augustin, O., and Drago, S.R., (2012). Bioactive Properties of Peptides Obtained By Enzymatic Hydrolysis from Protein Byproducts of Porphyra Columbina. Food Research International. 49(1):364-372.
56. Lu, J., Sawano, Y., Miyakawa, T., Xue, Y.L., Cai, M.Y., Egashira, Y., Ren, D.F., and Tanokura, M., (2010). One-Week Antihypertensive Effect of Ile-Gln-Pro in Spontaneously Hypertensive Rats. Journal of Agricultural and Food Chemistry. 59(2):559-563.
57. Sheih, I.C., Fang, T.J., and Wu, T.K., (2009). Isolation and Characterisation of A Novel Angiotensin I-Converting Enzyme (ACE) Inhibitory Peptide From the Algae Protein Waste. Food Chemistry. 115(1):279-284.
58. Suetsuna, K. and Chen, J.R., (2001). Identification of Antihypertensive Peptides from Peptic Digest of Two Microalgae, Chlorella Vulgaris and Spirulina Platensis. Marine Biotechnology. 3(4):305-309.
59. Suetsuna, K. and Nakano, T. (2000). Identification of an Antihypertensive Peptide from Peptic Digest of Wakame (Undaria Pinnatifida). The Journal of Nutritional Biochemistry. 11(9):450-454.
60. Boschin, G., Scigliuolo, G.M., Resta, D., and Arnoldi, A., (2014). ACE- Inhibitory Activity of Enzymatic Protein Hydrolysates from Lupin and Other Legumes. Food Chemistry. 145:34-40.
61. Matsui, T., Matsufuji, H., Seki, E., Osajima, K., Nakashima, M., and Osajima, Y., (1993). Inhibition of Angiotensin I-Converting Enzyme By Bacillus Licheniformis Alkaline Protease Hydrolyzates Derived From Sardine Muscle. Bioscience, Biotechnology, and Biochemistry. 57(6):922-925.
62. Rao, S., Sun, J., Liu, Y., Zeng, H., Su, Y., and Yang, Y., (2012). ACE Inhibitory Peptides and Antioxidant Peptides Derived from in Vitro Digestion Hydrolysate of Hen Egg White Lysozyme. Food Chemistry. 135(3):1245-1252.
63. Vermeirssen, V., van der Bent, A., Van Camp, J., van Amerongen, A., and Verstraete, W., (2004). A Quantitative In Silico Analysis Calculates the Angiotensin I Converting Enzyme (ACE) Inhibitory Activity in Pea and Whey Protein Digests. Biochimie. 86(3):231-239.
64. Escudero, E., Mora, L., and Toldrá, F., (2014). Stability of ACE Inhibitory Ham Peptides against Heat Treatment and in Vitro Digestion. Food chemistry. 161:305-311.
65. Świeca, M., Gawlik-Dziki, U., Dziki, D., Baraniak, B., and Czyż, J., (2013). The Influence of Protein–Flavonoid Interactions on Protein Digestibility in Vitro and the Antioxidant Quality of Breads Enriched with Onion Skin. Food Chemistry, 141(1):451-458.