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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Characteristics and Biological Activities of Bioactive Peptides Derived from Bulgur Waste*

Bulgur Atıklarından Elde Edilen Biyoaktif Peptitlerin Özellikleri ve Biyolojik Aktiviteleri

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Abstract

Bulgur is one of the ready or semi-ready to eat cereals produced from wheat specifically Triticum durum variety. Residues of bulgur processing are known as bulgur waste that rich in some food components. Protein is one of the main components of bulgur that may remain in the wastes. This study was carried out to obtain and investigate the properties of bioactive peptides of bulgur waste proteins. Protein isolated from bulgur waste was hydrolyzed enzymatically to bioactive peptides and their potential activity against oxidation stress, microbial inhibition and hypertension control was determined. The bulgur waste proteins extracted from samples were hydrolyzed at different time intervals using pepsin, trypsin, chymotrypsin and protease under the optimum conditions of enzymes and o-phthalaldehyde (OPA) method was used to determine the degree of hydrolyses. The highest rate of hydrolysis efficiency was observed by protease as 10.08% at 240 min treatment while, the highest antioxidant capacity was measured with chymotrypsin (526.35% at 240 min) by 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and with trypsin (151.93 % at 240 min) by 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. Trypsin hydrolysates showed the highest antibacterial activity against *Escherichia coli* whereas pepsin hydrolysates exhibited the highest activity against *Staphylococcus aureus*. It has been observed that trypsin, chymotrypsin and protease hydrolysates have higher antihypertensive effects than protease hydrolyzates. The highest antihypertensive effect was obtained with protein hydrolyzate obtained by hydrolysis with chymotrypsin for 180 minutes. As a result, the novel peptides indicated to offer the selected biological effects, suitable to use as a food additive for different purposes in industrial applications.

Keywords: Bulgur waste, Bioactive peptides, Antioxidant activity, Antimicrobial activity, Antihypertensive activity

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*This study was summarized from the Hema Aso RASHID's MSc thesis.

Öz

Bulgur, buğdayın özellikle *Triticum durum* çeşidinden üretilen, yemeye hazır veya yemeye yarı hazır tahıllardan biridir. Bulgur işleme sırasında oluşan kalıntılar, bazı gıda bileşenleri açısından zengin bir içeriği sahip olup bulgur atığı olarak bilinmektedir. Protein, bulgur işlemi sonrası atıklarda yer alan ana bileşenlerden biridir. Bu çalışma bulgur üretimi sonrası oluşan bulgur atığı proteinlerinden biyoaktif peptitlerin elde edilmesi ve özelliklerinin araştırılması amacıyla yapılmıştır. Bulgur atıklarından izole edilen protein, enzimatik olarak biyoaktif peptitlere hidroliz edildi. Aynı zamanda bulgur atıklarından izole edilmiş ve hidroliz edilen proteinin oksidasyon stresine, mikrobiyal inhibisyona ve hipertansiyon kontrolüne karşı potansiyel aktiviteleri belirlendi. İlk olarak bulgur atığı proteinleri ekstrakte edilmiş ve ardından enzimlerin kendilerine özgü optimum koşulları altında pepsin, trypsin, kimotripsin ve proteaz kullanılarak farklı zaman aralıklarında hidrolize edilmiştir. Bulgur atığı proteinlerinin enzimatik hidroliz derecesinin belirlenmesinde o-ftalaldehit (OPA) yöntemi kullanılmıştır. En yüksek hidroliz etkinliği 240 dk muamelesinde %10,08 ile proteaz ile gözlenirken, en yüksek antioksidan kapasite 2,2'-azino-bis(3-etilbenzotiazolin-6-sülfonik asit) (ABTS) yöntemi ile kimotripsin (240 dk'da %526,35) ve 2,2-difenil-1-pikrilhidrazil (DPPH) yöntemi ile de trypsin (240 dakikada %151.93) olarak ölçülmüştür. Enzimatik hidrolizle elde edilen protein hidrolizatlarının antimikrobiyal etkisine bakıldığında, Tripsin hidrolizatları Escherichia coli'ye karşı en yüksek antibakteriyel aktiviteyi gösterirken, pepsin hidrolizatları Staphylococcus aureus'a karşı en yüksek aktiviteyi gösterdi. Tripsin, kimotripsin ve proteaz hidrolizatlarının antihipertansif etkilerinin proteaz hidrolizatlarına göre daha yüksek olduğu görülmüştür. En yüksek antihipertansif etki, kimotripsin ile 180 dakika boyunca hidroliz yoluyla elde edilen protein hidrolizatı ile elde edildi. Sonuç olarak, yeni peptitlerin, endüstriyel uygulamalarda farklı amaçlarla gıda katkı maddesi olarak kullanılmaya uygun, farklı biyoaktif özelliklere sahip, amaca yönelik seçilmiş biyolojik etkiler sunduğu belirlendi.

Anahtar Kelimeler: Bulgur atığı, Biyoaktif peptitler, Antioksidan aktivite, Antimikrobiyal aktivite, Antihipertansif aktivite

1. Introduction

For hundred years ago the relationship between health and nutrition are familiar to popularity which environmentally long-life term or healthy life instantaneously impacted by nutrition (Kussmann et al., 2010). Proteins are fundamental food macromolecules that accessible in all living cells which offer valuable nutrition as a source of essential amino acids and energy either from animal or plant sources. Collected proteins from the plant sources are preferable for human consumption and promoting health because contains absolute bioactive and medicinal compounds when compared to animal proteins (Sarmadi and Ismail, 2010). Food protein and peptides have unique biological activities and this feature offers high nutritional value to foods containing significant amount of protein (Hartmann and Meisel, 2007; Moller et al., 2008). In the last decades, researchers interested in an inactive or encrypted substance in the peptide's residue known as bioactive peptides. These peptides identified as a food component that influence ultimately on human health specifically that are a fragment of the protein that has a favorable effect on body condition and function (Kitts and Weiler, 2003). The biologically active peptides are inert in a latent state within the protein sequence provide numerous different health benefits. The structure and composition of these short chains of peptides residue regulate the activity of the bioactive peptides that generally consists of 2-20 amino acids (Erdmann et al., 2008). Within the positive physiological influences of the bioactive peptides abbreviated in antioxidative, antihypertensive, antimicrobial, opioid agonistic, prebiotic, mineral binding, immunomodulatory, antithrombotic and hypo-cholesterolemic activities. The peptides chain that provides the mentioned functionalities released from the protein sequence to obtain the active form of it.

Bioactive peptides are a certain protein fraction that except it is ordinary appropriate nutrition benefits demonstrate pharmacological features in the human body (Hartmann and Meisel, 2007). These beneficial health effects show a wide range of physiological effects including antioxidant, antihypertensive and antimicrobial activities. The valuable health effects of antioxidants performed as a body protector against the reactive oxygen species (ROS) molecules. Many studies demonstrated that the antioxidant peptides usually have 5-16 amino acid fractions. And their composition, hydrophobicity and structure associate with the diverse levels of functionality (Saadi et al., 2015). The principle of the antioxidants actions is prevention of oxidative reactions by scavenging free radicals (You et al., 2009; Demirci et al., 2021; Tahmaz et al., 2022). Therefore, antioxidants are vital biological molecules to block oxidations by free radicals and protect health system. Biologically active peptides in protein sequence may show higher activity even than the parent protein. The angiotensin-converting enzyme (ACE) inhibitors have a crucial role in promoting health and regulating blood pressure. Diets that are rich in bioactive peptides pretended to cure hypertension in the first stages in other word heal pre-hypertensive (Pins and Keenan, 2006; Fluegel et al., 2010). Peptides performed high capability in food to inhibit ACE activity from this point of view the peptides known as antihypertensive peptides utilized to prevent or handling hypertension (Udenigwe and Aluko, 2012). These peptides are apart in destroying a number of microorganisms such as bacteria and fungi, too. One of the most natural and crucial portions of the immunity is the availability of antimicrobial peptides on the surface of internal organs especially small intestine and lungs incessantly exhibited a number of possible pathogens (Douglas et al., 2001). Prevention and manage to spread of diseases in the body by bioactive peptides that behave like an antimicrobial agent and react with the hosts or by promoting definite response of the immune system in these two forms perform the mechanism of action (Hancock and Sahl, 2006).

Cereal grains are essential in human diet. They are used for different purposes such as energy supply, as well as contributing to the nutrition of animals. Cereal protein described as a precious source of biologically active peptides. Particularly, bulgur is produced from Triticum durum wheat variety beside the desirable taste it has many health benefits. Bulgur production especially in the milling processes generates a quantity of waste like wheat germ and wheat bran, besides the main product. Wheat germ contains protein, dietary fiber, minerals and precious amount of vitamin E and B group vitamins (Amado and Arrigoni, 1992). Also, wheat bran is a rich source of fiber, B vitamin groups and minerals (Preuckler et al., 2014).

This article explains the current knowledge about bioactive peptides. It was carried out (1) to obtain bioactive peptides from bulgur waste products by hydrolysis with different enzymes, (2) to determine the biological activities of obtained bioactive peptides and (3) to reveal the reuse capability of by-products from wheat industries.

2. Materials and Methods

2.1. Materials

The waste of bulgur was supplied from a factory in Gaziantep, Türkiye. Enzymes used in this study were trypsin from porcine pancreases (1 BAEE Unit of Trypsin activity will produce 0.001 absorbance increase per minute at 253 nm, BAEE is Benzyl L-arginine ethyl ester), pepsin from porcine gastric mucosa (1 Unit will produce 0.001 absorbance increase per minute at 280 nm), α-chymotrypsin from bovine pancreas (1 Unit will hydrolyze 1µmole BTEE per minute at pH 7.8 and 25°C, BTEE is N-Benzoyl-L-tyrosine ethyl ester) and protease from Bacillus specious (1 Unit of protease is the amount of enzyme needed to produce 1 mg of tyrosine per minute at 660 nm). Sodium hydroxide (NaOH), Potassium sulfate (K_2SO_4), Cuppersulfate ($CuSO_4$), sulfuric acid (H_2SO_4), hydrochloric acid (HCl), Tris(hydroxymethyl)aminomethane (Tris), tri-sodium citrate dehydrate, o-phthalaldehyde (OPA), 2,2′-azino-bis(3- ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), ethanol, Angiotensin Converting Enzyme from rabbit lung (ACE), N-Hippuryl-His-Leu hydrate – powder were purchased from Sigma-Aldrich Company (USA).

2.2. Determination of Bulgur Waste Characteristics

Moisture, ash, fat and protein contents of wastes of bulgur were determined according to the AOAC (1990) with at least duplicate runs. Moisture content was determined by heating at 105°C in oven until constant weight was obtained. Protein content was determined by Kjeldahl method with digestion (400°C for 40 min), distillation (FOSS, 2200 Hoganas, Sweden) and titration steps (nitrogen factor 5.7). Ash content was determined by burning the sample at 550°C up to constant weight. Fat content was determined by Soxhelet (Gerhardt, SE-416, Germany) extraction method.

2.3. Extraction of Bulgur Waste Protein

The accessible quantity of protein from the wastes of bulgur was extracted by alkali solution treated by 0.1N NaOH and the sample to solvent ratio was (1:10 w/v). The sample was stirred within the buffer for two hours and then centrifuged at 10000 rpm (Eppendorf centrifuge 5810R) for 20 minutes at the room temperature. The extracted protein was concentrated by freeze dryer (CHRIST ALPHA 1-4 LD Plus, Germany) and kept in -18°C until next use.

2.4. Enzymatic Hydrolysis of Bulgur Waste Protein

The extracted and concentrated protein was subjected to enzymatic hydrolysis by use of 1 mL of protein mixture diluted in 1 mL deionized water with proteolytic enzymes. Protein hydrolysis was performed about 4 hours in shaking incubator (INNOVA 40, New Brunswick, New Jersey, USA) using 3 units of trypsin (by use of sodium citrate buffer at pH 2 and at 37°C), chymotrypsin (using Tris buffer at pH 8 and at 37°C), pepsin (using Tris buffer at pH 8 and at 37°C) or protease (using Tris buffer at pH 8 and at 50°C) enzymes. These mentioned hydrolysis conditions were regulated according to the optimum conditions. Enzymatic hydrolysis reaction was terminated by heating at 95°C for 20 min, followed by cooling to room temperature in an ice bath. Then the hydrolysates were centrifuged at 6000 rpm for 15 min and the collected supernatant was stored at -18°C until use.

2.5. Degree of Hydrolyses

The degree of hydrolysis was determined by using OPA method (Spellman et al., 2003). About 3 mL deionized water was mixed with 75 µl OPA reagent and 10 µl of sample and shaked about 5 seconds then after two minutes the absorbance was read at 340 nm. The degree of hydrolysis was calculated by Equation 1:

$$DH\ (\%) = ((ABS\ x\ 1.934\ x\ d))/c \quad \text{(Eq. 1)}$$

where ABS is the absorbance of samples, d is the dilution factor, and c the protein content of the sample (g/L).

2.6. Antimicrobial Activity of Hydrolyzed Peptides

Escherichia coli (25322 ATCC) and *Staphylococcus aureus* (25923 ATCC) were used to determine the antimicrobial properties of peptides against Gram-negative and Gram-positive bacterial species. These microorganisms were retained on nutrient agar plate and recovered by sub-culturing in nutrient broth for 24 hours. About 0.1 mL stock solution of each microorganism was inoculated on plate count agar. The sterile paper discs, 1cm diameter, were prepared and dipped into the hydrolyzed samples for around one minute then semi-dried in room

temperature. Semi-dry paper discs were stabilized on the plate count agar that contain microorganisms and incubated for 48 hours at 37°C to verify inhibition zone diameter (mm).

2.7. Antioxidant Activity of Hydrolyzed Peptides

It is necessary to combine more than one method to evaluate the antioxidant activity of foodstuffs (Nuutila et al., 2003; Georgetti et al., 2006). Antioxidant activities of the hydrolyzed peptides were determined by the methods of DPPH and ABTS radical scavenging activity.

2.7.1. DPPH Radical Scavenging Activity

The antioxidant activity of the hydrolyzed peptides was determined by use of DPPH free radical scavenging activity illustrated by Yang et al. (2008). DPPH radical stock solution, 0.1 mM, was prepared in 95% ethanol. Two milliliters of stock solution was mixed well with two milliliter of each sample and kept at room temperature for 30 minutes in the dark place. Absorbance values of the samples were read at 517 nm by UV visible spectrophotometer (OPTIMA SP-3000nano, Tokyo, Japan). Absorbance value of control was obtained through use of ethanol and DPPH without the sample. DPPH radical scavenging activity was calculated by Equation 2.

$$DPPH\ radical\ scavenging\ activity\ \% = [1 - (Absorbance\ of\ sample)/(Absorbance\ of\ control)] \times 100 \quad \text{(Eq. 2)}$$

2.7.2. ABTS Radical Scavenging Activity

ABTS radical scavenging activity was measured according to the method described by Re et al. (1999) with some modifications. ABTS stock solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate at a ratio of 1:1 and it was left in dark place at room temperature for 12-16 hours. Before analysis, the mixture was diluted with 10 mM phosphate buffered saline solution at pH 7.4 to adjust the absorbance at 0.8±0.1 at 734nm. After these modifications 1 mL of the hydrolyzed sample was mixed with 1 mL of diluted ABTS stock solution. Mixture was rested for 10 min at room temperature and the absorbance was measured by UV visible spectrophotometer (OPTIMA, SP-3000nano, Tokyo, Japan) at 734 nm. ABTS scavenging activity was then calculated by the Equation 3:

$$ABTS\ scavenging\ activity\ (\%) = (Absorbance\ of\ control - Absorbance\ of\ sample)/(Absorbance\ of\ control) \times 100 \quad \text{(Eq. 3)}$$

where, absorbance of the control determined through replacing the sample by 1 mL deionized water.

2.8. Antihypertensive Activity of Hydrolyzed Peptides

The antihypertensive activity was measured by a modified method as described by Cushman and Cheung (1971). About 40 µL of sample was added to a mixture of buffered substrate solution that containing 100 µL of borate buffer pH 8.3 contains 300 mM NaCl and 6 mM hippurylhistidyl-leucine (HHL). The reaction was started by the addition of 20 µL of ACE solution (0.1 U/mL) incubated at 37°C for 45 minutes. The reaction was stopped by adding 200 µL of 1M HCl. Then, 1.5 mL of ethyl acetate was added to separate the released hippuric acid and centrifuged at 2500 rpm for 10 minutes. About 1 mL of the supernatant was transferred into a test tube and removing of the ethyl acetate was achieved by vacuum evaporate at 60°C for 60 minutes. The remaining sample was dissolved in 2 mL deionized water and the absorbance was determined at 228 nm using spectrophotometer. The inhibition activity was calculated by Equation 4:

$$ACE\ inhibitory\ activity\ \% = [(Aa - A)/(Aa - Ab)] * 100 \quad \text{(Eq. 4)}$$

where, Aa is the absorbance of the replaced sample by borate buffer, Ab is the absorbance of the replaced sample and ACE by borate buffer, and A is the absorbance of sample, ACE and HHL.

2.9. Statistical Analysis

Oneway of analysis of variances (ANOVA) was applied by SPSS (19) to indicate the significance difference between time of hydrolysis and type of enzyme at $\alpha = 0.05$ level. Duncan's multiple range tests was also carried out to determine difference between studied groups. Time of hydrolysis and type of enzyme has been used as an independent parameter. They compared with one way ANOVA using degree of hydrolysis, DPPH, ABTS, ACE and inhibition of *Staphylococcus aureus* and *Escherichia coli*.

3. Results and Discussion

The bulgur waste contains both bran and flour which remains from the wheat. These residues can contain varying amounts of moisture, which may vary according to the manufacturing processes. The moisture content of the bulgur waste samples was found to be 12.15%, while the whole bulgur samples had 11.21 % of moisture similar to that reported in Tacer Caba et al. (2011).

The amount of ash in the waste of bulgur was 3.55 % (d.b) whereas the whole bulgur contains only 0.92 % (d.b). Large variations in the ash content of the main product and waste components have been reported previously (Tacer Caba et al, 2011).

The quantity of fat of the bulgur waste was found to be 4.58 % while the whole bulgur samples had 1.20%. The high fat content of bulgur waste could be due to the presence of the main oil-containing part, germ, in the waste.

The main component turned to the bioactive peptides is the protein. The protein content of bulgur waste was determined by Kjeldahl method as 2.92 % (d.b). Whereas, the amount of protein in the whole bulgur sample was 16.34 % according to the dry base matter. At the same time, the protein content in the whole bulgur reported by Özboy and Köksel (1998) was varied from 11.5% to 16.6% (d.b). The bulgur waste components were similar to whole bulgur from wheat sources that contain fat, carbohydrate, minerals and protein. Changes in the moisture, ash, oil and protein contents of the wastes and whole bulgur may have resulted from the distribution of the components.

3.1. Degree of Hydrolysis

Identifying the degree of hydrolysis is one of the main popular guides to define the functional characteristics of the cleaved peptide bonds from the hydrolyzed proteins (Kristinsson and Rasco, 2000). The enzymes specificity shows effectiveness on the hydrolysis processes such as pepsin cleaves peptide bonds of protein from carboxylic sides of glutamic acid, tryptophan, phenylalanine, leucine and tyrosine (Lin et al., 2012).

Bulgur waste protein was hydrolyzed for 4 h under the optimum conditions of enzymes including pepsin, trypsin, chymotrypsin and protease. The degree of hydrolysis was determined by the OPA method (*Table 1*). OPA method illustrates the enzymes ability to generate smaller peptides and display the activity of the biological peptides. When pepsin was used, the degree of hydrolysis decreased after 120 min with time, the hydrolysis was the highest at 120 min. The hydrolysis of wheat by pepsin enzymes was reported by Nagy et al. (2009) that increasing time increased the degree of hydrolysis within entire 120 minutes as parallel to our results. Trypsin usually cleaves primary amino acids of the lysine and arginine (Salami et al., 2008). Degree of hydrolysis was not affected ($P>0.05$) by hydrolysis time when trypsin was used.

Mainly breakage of the long polypeptides to smaller peptides by chymotrypsin start by the availability of lateral chains as phenylalanine, leucine, tryptophan and tyrosine at C-terminal of hydrophobic or aromatic amino acids (Salami et al., 2008). Various enzymes attack to different the amino acids sequence of protein or peptides in the structure of bulgur waste that resulted in different degree of hydrolysis and products. The degree of hydrolysis of bulgur waste proteins with chymotrypsin increased significantly ($P<0.05$) with time during 4 h incubation. The extraction of protein from the main source, the sort of utilized enzymes for hydrolysis, the available amount of peptides in desirable concentration and the degree of hydrolysis may affect the activity of every single peptide (Wang et al., 2010).

The protease enzyme was also used in the hydrolysis of bulgur waste proteins. Protease had significant effect ($P<0.05$) on the rate of proteolysis. The degree of hydrolysis increased gradually ($P<0.05$) with time (*Table 1*). Protease cleaves peptide bonds on aromatic amino acids of leucine, phenylalanine, tyrosine and tryptophan (Rao et al., 1998). The notable proficiencies of protease examined by previous studies to provide high proportions of hydrolysis rate in protein especially for legume proteins (Yust et al., 2003; Li et al., 2005). The great activity may regard to the ability to hydrolyze the denatured proteins by the side of original protein, the suitable range of pH and temperature, the specificity of the substrate and stability versus auto-proteolysis alongside with the other general difficulties for of each proteases (Baldyga and Bourne, 1999).

When the effects of four enzymes were compared with respect to degree of hydrolysis, it can be said that degree of hydrolysis increased with time when pepsin, trypsin, chymotrypsin and protease were used.

Table 1. Degree of hydrolysis, DPPH, ABTS and ACE activity of bulgur waste hydrolysate obtained by treatment of different proteolytic enzymes

	Time (min)	Pepsin	Trypsin	Chymotrypsin	Protease
Degree of Hydrolysis (%)	60	1.85 ± 0.09^{bA}	7.52 ± 0.38^{aB}	1.42 ± 0.07^{aA}	7.10 ± 0.35^{aB}
	120	2.13 ± 0.11^{cA}	7.24 ± 0.36^{aC}	3.83 ± 0.19^{bB}	8.80 ± 0.44^{bC}
	180	1.42 ± 0.07^{aA}	7.81 ± 0.39^{aC}	4.26 ± 0.21^{cB}	9.79 ± 0.49^{cD}
	240	1.28 ± 0.06^{aA}	7.24 ± 0.36^{aC}	3.99 ± 0.19^{bB}	10.08 ± 0.40^{cD}
DPPH radical scavenging activity (%)	60	83.98 ± 4.20^{abA}	146.41 ± 7.32^{aB}	141.16 ± 7.06^{aB}	121.27 ± 6.06^{aC}
	120	78.73 ± 3.94^{aA}	145.86 ± 7.29^{aB}	145.86 ± 7.29^{aB}	138.95 ± 6.95^{bB}
	180	91.44 ± 4.57^{abA}	146.41 ± 7.32^{aB}	130.66 ± 6.53^{aC}	144.48 ± 7.22^{cB}
	240	109.12 ± 5.46^{cA}	151.93 ± 7.60^{aB}	139.78 ± 6.99^{aC}	133.70 ± 6.69^{bC}
ABTS scavenging activity (%)	60	258.79 ± 12.94^{aA}	405.73 ± 20.29^{aB}	443.01 ± 22.15^{aB}	379.01 ± 18.95^{aC}
	120	346.51 ± 17.33^{bA}	403.53 ± 20.18^{aAB}	477.00 ± 23.85^{abB}	449.35 ± 22.47^{bB}
	180	354.19 ± 17.71^{bA}	409.02 ± 20.45^{aB}	487.97 ± 24.40^{bC}	412.61 ± 20.63^{abB}
	240	387.08 ± 19.35^{bA}	398.28 ± 19.46^{aA}	526.35 ± 26.32^{cB}	486.10 ± 24.31^{cB}
ACE inhibitory activity (%)	60	8.71 ± 0.44^{abA}	20.43 ± 1.02^{bB}	21.14 ± 1.06^{aB}	21.00 ± 1.05^{aB}
	120	8.57 ± 0.43^{abA}	17.57 ± 0.88^{aB}	17.57 ± 0.37^{bB}	23.43 ± 0.17^{abC}
	180	9.29 ± 0.46^{bcA}	27.86 ± 0.39^{cB}	28.29 ± 1.41^{cB}	22.29 ± 0.11^{aC}
	240	10.86 ± 0.54^{cA}	24.14 ± 1.21^{cB}	16.14 ± 0.80^{bC}	24.00 ± 0.35^{bB}

* Small letters show significant difference of time on degree of hydrolysis by one way ANOVA at $\alpha = 0.05$ level. Capital letters (A, B, C and D) show significant difference among pepsin, trypsin, chymotrypsin and protease enzymes at each time on degree of hydrolysis at $\alpha = 0.05$ level.

3.2. Antioxidant Activity of Hydrolyzed Peptides

3.2.1. DPPH Radical Scavenging Activity

Several positive effects can occur during or after enzymatic hydrolysis such as improvement of radical scavenging activity which directly related to antioxidant activity of hydrolyses (Aleman et al., 2011). The primary consideration that related directly to the rate of antioxidant activity is the type of enzymes utilized in hydrolysis. The DPPH activity of the bulgur waste hydrolysate obtained with different hydrolysis times, by trypsin and chymotrypsin were non-significant ($P>0.05$). However, pepsin and protease showed a significantly positive ability to produce an anti-oxidative peptide during the hydrolysis period ($P<0.05$) (*Table 1*). Winata and Lorenz (1996) found that the addition of protein isolates to milk was enhanced the antioxidant activity of milk.

3.2.2. ABTS Radical Scavenging Activity

The use of enzymes for hydrolysis resulted in the formation of different peptides having various antioxidant activities, as given in *Table 1*. Such as, chymotrypsin exhibited the most significant ($P<0.05$) increase on the formation of bioactive peptides leading to have high ABTS activity. These outcomes may be due to the capability of chymotrypsin to cleave peptides only at the C-terminal of leucine, tryptophan, phenylalanine, and tyrosine of hydrophobic or aromatic amino acids. The type and amount of free amino acids, the types of enzymes, and the solubility of the hydrolysate affect the formation of the bioactive peptide that scavenge ABTS radicals (Phanturat et al., 2010).

3.3. Antihypertensive Activity

Angiotensin-I converting enzyme (ACE) take a part in adjustment of human blood pressure (Raghavan and Kristinsson, 2009). The presence of hydrophobic amino acids in the C-terminal side particularly proline, phenylalanine, tryptophan and tyrosine have a great impact on the ACE inhibitory activity (Haque and Chand, 2008). Besides, the availability of isoleucine and valine at the N-terminal provide a positive influence on the ACE inhibitory activity. *Table 1* summarizes the changes in ACE inhibitory activity of the hydrolysates. Bioactive peptides produced from pepsin, protease, trypsin and chymotrypsin hydrolysis had significant ($P<0.05$) effect on the formation of antihypertensive active peptides.

3.4. Antimicrobial Activity of Hydrolyzed Peptides

The antimicrobial activities have been demonstrated to exhibit the protein fractions ability to inhibit growth of microorganisms. The four diverse enzymes break down the protein fractions into smaller peptides. The entire hydrolyzed samples examined against Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli*) by evaluating the growth inhibition zone (mm) of each portion.

The protein hydrolysates showed antimicrobial activity on both *Escherichia coli,* and *Staphylococcus aureus*. *Table 2* shows the effects of each protein hydrolysates on the *Escherichia coli* and *Staphylococcus aureus* by the extent of inhibition zone with increasing hydrolysis time. The least antimicrobial effect against *Escherichia coli* was observed in peptides obtained by hydrolysis of pepsin and chymotrypsin while the highest effect was observed in peptides obtained as a result of hydrolysis with trypsin for 240 minutes. Bulgur waste protein hydrolysates were found to be more effective against *Staphylococcus aureus* than that of *Escherichia coli* (*Table 2*). All of the hydrolysates obtained from proteolysis of bulgur waste protein by trypsin, chymotrypsin, pepsin and protease showed antimicrobial effect against *Staphylococcus aureus*. The most effective one was the hydrolysate obtained as a result of hydrolysis with pepsin for 60-180 minutes. Bueno-Gavila et al (2019) reported that the antimicrobial hydrolysate obtained from bovine casein had the highest antimicrobial activity against *Enterococcus faecalis*. The enrichment of milk with antimicrobial peptides was significantly reduced the bacterial load level to an acceptable level (Sivakumar and Dhanalakshmi, 2016).

Table 2. Effect of bioactive peptides obtained from bulgur waste by treatment of different proteolytic enzymes on Staphylococcus aureus and Escherichia coli with increasing hydrolysis time*

Time (min)	Pepsin		Trypsin		Chymotrypsin		Protease	
	E. coli	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
60	+	++++	+	++	+	+	++	+++
120	+	++++	++	+++	+	+++	+	++
180	+	++++	++	+++	+	+++	++	++
240	++	++	+++	++	+	++	+	++

*+ less effective, ++ moderate effective, +++ effective, ++++ high effective

4. Conclusions

In this study, the waste of bulgur analyzed to discover available amounts of protein especially bioactive peptides. The moisture, ash, fat and protein contents of the bulgur waste were found as 12.15%, 3.55 %, 4.58 % and 2.92 %, respectively. Bioactive peptides were obtained by hydrolyzing of bulgur waste with proteolytic enzymes pepsin, trypsin, chymotrypsin and protease. The highest rate of hydrolysis was found by use of trypsin and protease. Increasing hydrolysis time increased degree of hydrolysis when protease was used. Bioactive peptides provided by trypsin hydrolysis can easily inhibit Gram negative bacteria, *Escherichia coli*. All of the bulgur waste protein hydrolysates obtained by trypsin, chymotrypsin, pepsin and protease hydrolysis showed antimicrobial activity against *Staphylococcus aureus*. It was found that all bioactive peptides have ABTS scavenging activity and the highest activity belongs to the peptides obtained by chymotrypsin hydrolysis. The higher DPPH activity of the bulgur waste hydrolysate obtained by trypsin and chymotrypsin than that of other proteolytic enzymes ($P<0.05$). Antihypertensive activity of the pepsin, trypsin and chymotrypsin increased versus with time. As a result, the bioactive peptides can be produced to offer the selected biological effects, suitable to use as a food additives for industrial applications.

Ethical Statement

There is no need to obtain permission from the ethics committee for this study.

Conflicts of Interest

We declare that there is no conflict of interest between us as the article authors.

Authorship Contribution Statement

Concept: Bozkurt, H., Aykaç, Ç.; Design: Bozkurt, H., Aykaç, Ç.; Data Collection or Processing: Rashid, H.A.; Statistical Analyses: Rashid, H.A., Bozkurt, H.; Literature Search: Rashid, H.A., Aykaç, Ç.; Writing, Review and Editing: Rashid, H.A., Bozkurt, H., Aykaç, Ç.

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