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M.Sc. in Food Engineering

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GAZİANTEP UNIVERSITY

GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES

HYDROLYSIS OF BIOACTIVE PEPTIDES FROM BULGUR WASTE

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BY

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Supervisor

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by

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Hema Aso RASHID

ABSTRACT

HYDROLYSIS OF BIOACTIVE PEPTIDES FROM BULGUR WASTE

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Bulgur is one of the ready or semi-ready to eat cereals produced from wheat specifically ***Triticum durum*** variety. Residue of bulgur from manufactures are useless while, these wastes 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 extract bioactive peptides from the bulgur waste samples and indicate the activity against oxidation stress, microbial inhibition and hypertension control. The analysis made in this study were determination of protein, fat, ash and moisture content, extracting protein from the bulgur wastes, the samples hydrolyses by four diverse enzymes including pepsin, trypsin, chymotrypsin and protease in a suitable incubation conditions for entire 360 minutes. Effect of concentration, time and different types of enzyme has been investigated on each activity. The extracted protein content by alkaline processes was determined as 2.84% (w.b) by kjeldahl method. The hydrolysis rate, antioxidant activity, antimicrobial activity and antihypertensive activity show either an increases or decreases according to the incubation conditions. This study illustrate the extracting of protein content by alkaline processes and hydrolysis by four diverse enzymes and proofing the anti-oxidant, antimicrobial and antihypertensive activity of the accessible bioactive peptides in the bulgur waste samples.

Key Words: Bulgur, Bioactive Peptides, Degree of Hydrolysis, Antioxidant Activity, Antimicrobial Activity, Antihypertensive Activity

ÖZET

BULGUR ATIKLARINDAN BİYOAKTİF PEPTİTLERİN HİDROLİZİ

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Bulgur, buğdaydan özellikle ***Triticum durum*** çeşidinden elde edilen, yenmeye hazır veya yarı hazır tahıl ürünlerinden biridir. Elde edilen bulgur artıkları hayvancılık için değerlendirilirken, bu atıklar bazı gıda bileşenleri yönünden oldukça zengindir. Protein, bulgurun atıklarda kalabilen ana bileşenlerinden biridir. Bu çalışma, bulgur atığı örneklerinden protein ekstrakte etmek, hidrolize ederek biyoaktif peptitler elde etmek ve oksidasyon stresine, mikrobiyal inhibisyona ve hipertansiyon kontrolüne karşı aktivitelerini belirlemek için yapılmıştır. Bulgur atıklarından protein ekstraksiyonundan sonra numuneler, 360 dakika boyunca uygun inkübasyon koşullarında dört farklı enzimle, pepsin, tripsin, kimotripsin ve proteaz, hidrolize edildi. Bu çalışmada yapılan analizler, hidrolliz derecesi, antimikrobiyal aktvite, antihipertansif aktivite, protein, yağ, kül ve nem içeriğinin belirlenmesidir. Konsantrasyon, zaman ve çeşitli enzim türlerinin her aktivite üzerindeki etkisi araştırılmıştır. Alkali işlemlerle ekstrakte edilen protein içeriği Kjeldahl yöntemi ile % 2.84 (yaş baz) olarak belirlerdi. Hidroliz derecesi, antioksidan aktivite, antimikrobiyal aktivite ve antihipertansif aktivite inkübasyon koşullarında artma veya azalma eğilimi göstermiştir. Bu çalışma, protein içeriğinin alkali süreçlerle ekstrakte edilmesini ve dört farklı enzimle hidrolizini ve bulgur atığı örneklerindeki erişilebilir biyoaktif peptitlerin antioksidan, antimikrobiyal ve antihipertansif aktivitesini göstermiştir.

Anahtar Kelimeler: Biyoaktif Peptitler, Hidroliz Derecesi, Antioksidan Aktivite, Antimikrobiyal Aktivite, Antihipertansif Aktivite

"Dedicated to my family

My father Aso Rashid Who encouraged me to dream, to my mother Rezan Hama she is the reason behind who am I today and my little brother Aria who shines my life"

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CHAPTER 1

INTRODUCTION

1.1 Backgrounds about Bioactive Peptides in Waste of Bulgur

Nutritional and functional role of food proteins have been recognized from a long time ago. Proteins are the most plentiful biological macromolecule that available in all living cells. The building blocks of protein are amino acids with diverse number and compositions construct the simplest protein types until the most complex proteins structure and the ability to act according to the cells necessity. The amino acids have been associated by peptide or amide bond to create peptides either di-peptides, tri-peptides or polypeptides. In comparison to proteins the peptides are smaller in size and they own lower molecular weight beside the functional effects as a health preservative in the parent proteins. In the last decades, researchers interested in an inactive or encrypted substances in the peptides residue known as bioactive peptides. The biological active peptides are small fragments of protein has a positive impact on human health (Wu et al., 2008). These food derived components contains 2-20 amino acid residues in length provide physiological effects in the body besides the nutritional and pharmacological implications. The physiological positive influences of the bioactive peptides abbreviated in antioxidative, antihypertensive, antimicrobial, opioid agonistic, prebiotic, mineral binding, immunomodulatory, antithrombotic and hypo-cholesterolemic activities. Bioactive peptides released from the protein sequence to activate the profits. Therefore, the process done by use of one or more of the following methods including enzymatic hydrolysis by digestive enzymes, fermentation with proteolysis starter cultures and proteolysis by enzymes derived from microorganisms or plants (Korhonen and Pihlanto, 2006).

In the present research, bulgur waste was used as a protein source to obtain bioactive peptides concerning to availability in nature as a raw material with a high range of

consumption all over the world. Wheat is counted as one of the three major cereals that annually above 600 million tones have been harvested. Wheat kernels used to produce the bulgur by diverse techniques briefly the raw wheat kernel cooked in water then following the drying steps finally to get a desire various particle sizes the grounding methods has applied. In particular the huge amount of wheat waste remains from bulgur production of the industries as a by-product became an environmental manner because nutritionally contain important compounds such as s protein, carbohydrates and fat. While these by-products only used as a livestock for animals. This research focused on the protein as an important component especially from the waste of bulgur expected to be a crucial source of bioactive peptides.

Between the productions methods of bioactive peptides enzymatic hydrolysis by four different enzymes include pepsin, trypsin, chymotrypsin and protease was chosen as a suitable method to release the bioactive peptides from the protein residue. The hydrolysis technique accepted through the determination of degree of hydrolysis process by OPA (ortho-phthaldialdehyde) method. It is well known that the biologically active peptides are beneficial for health and many prevent cardiovascular diseases. Antihypertensive, antimicrobial and antioxidant activity of the bulgur waste demonstrated in the present research by diverse and common techniques. The inhibition quantity determined by ACE inhibitory to calculate the antihypertensive effects in the same time DPPH and ABTS methods used as an antioxidant determinates. The antimicrobial peptides identified by using each Gram negative and Gram positive bacteria to describe the inhibition activity.

1.2 The Aim of the Thesis

1. Extraction the ultimate amount of protein from the bulgur waste and specify the best method of extraction for this purpose.
2. Realizing the amount of bioactive peptides obtain through the different applying enzymes.
3. Utilizing a desirable temperature, pH, time and substrate ratio to get the encrypted peptides.
4. Quantification of the physiological activities of bioactive peptides that extracted from bulgur waste.
5. Uncover the ability to reuse the by-products remains from wheat industries.

CHAPTER 2

LITERATURE REVIEW

2.1 Protein

For hundred years ago the relationship between health and nutrition is familiar to popularity which environmentally long life term or healthy life instantaneously impacted by nutrition (Kussmann et al., 2010). In the last decades, foods that include high nutritional substances and provide a positive effect even over a long period favours by consumers despite the fundamental nutrients that provide (Hernández-Carrión et al., 2014). Proteins are the fundamental food macromolecules that accessible in all living cells have long been recognized to offer valuable nutrition to complete human diet and vital functions. The protein name means “basic material” according to Mulder and Berzelius they suggested the protein appellation in 1838 (Tracey, 1967). In the last two decades, scientific findings have been widely increased to show the nutritional importance of the protein. Protein considered as a source of essential amino acids and energy besides the functionality benefits for health. Required food proteins are from either animal or plant sources and used for economical purposes as a functional component (Periago et al., 1998). In comparison to animal proteins that have a great ability to dissolve in an aqueous medium and gelling attribute (Morr et al.,1985) plant proteins are particularly less usable by reason of the low solubility in aqueous suspension (Kong et al., 2007; Liu et al., 2011; Paraman et al., 2006; Xu et al., 2016). Nevertheless, for environmental, biological and pharmaceutical purposes it is necessary to replace animal sources with plant proteins. Contributions of plant proteins in human nutrition decrease the amount of remaining by-products from industries that only useful as livestock for animals. Other reasons favour plant sources are the limited supplements with the high cost of animal origins. Proteins collected 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). Offering a high-quality protein in the human diet becomes an interesting manner for scientists due to the functions that occur through. Proteins, lipids and carbohydrates are energy origin in the human diet while, protein provides a functional characteristic to the food and it has a primary role in human growth and maintenance. Furthermore, it constitutes permanent functions for instance caring and transferring biochemical molecules to the other side of cellular membrane beside it is the activity as enzymes (Wu et al., 2014). It regulates natural body operations by generating diverse sequences from particular amino acid interactions (Fields et al., 2009). Two amino acids joined by a covalent bond in the protein structure these bonds known as peptide bond create dipeptides while polypeptides are a mixture of peptides with diverse molecular weight to form the protein. The size of these active sequences are beginning from di-, tri- and oligo peptides until it reaches to high molecular weight polypeptides (Erdmann et al., 2008; Hern´andez-Ledesma et al., 2009). Food protein and peptides have unique biological activities and this feature offers high nutritional value to foods contain a significant amount of protein (Hartmann and Meisel, 2007; Moller et al., 2008). The reason behind the importance of peptides originate from foods is the constructions similarity compare to the internal peptides it provides the ability to interplay with the receptors in the same manner, have a major role in altering food entries, immune regulators, growth factors and antimicrobial against microorganisms (Meisel, 1998; Kamau et al., 2010). In 1950 Mellander was described food-derived peptides for the first time while working with casein phosphorylates identified that peptides improve the vitamin D-independence bone calcification in infants that infected with rachitic (Mellander, 1950). Furthermore, they own the greatest importance between the food macronutrients this contribute to the provided energy and staple materials that must be available for protein biosynthesis (Walther and Sieber, 2011; Dziuba and Dziuba, 2014).

2.2 Bioactive Peptides

The nutritional and functional advantages of proteins available in food have been discussed since the positive advantages have been acknowledged. Bioactive peptides identified as a food component that influence ultimately on human health specifically these peptides are a fragment of the protein that has a favourable effect on body

condition and function (Kitts and Weiler, 2003). 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). The biologically active peptides are inert in a latent state within the protein sequence provide numerous different health benefits. Over the past few decades, production and discovery in properties of bioactive peptides are widespread by a scientists in many articles (FitzGerald and Meisel, 2003; Korhonen and Pihlanto, 2003; Pihlanto and Korhonen, 2003; Li et al., 2004). Nowadays a database named 'Biopep' reported more than 1500 different types of bioactive peptides (Singh et al., 2014). These protein fragments have been categorized according to the origin of the initial protein and derived from different foods. The sources to obtain bioactive peptides commonly used food materials rich in protein such as plant sources such as a bean (Luna-Vital et al., 2015), rice and wheat proteins (Cavazos and Gonzalez de Mejia, 2013), wheat, broccoli (Hartmann and Meisel, 2007), soybean (Yimit et al., 2012), corn (Huang et al., 2011) beside the animal sources such as seafood (Kim and Wijesekara, 2010), fish (Yang et al., 2009; Kim and Wijesekara, 2010), cheese, beef (Jang and Lee, 2005) egg (Bhat et al., 2015), and milk (Korhonen, 2009; Nagpal et al., 2011), pork (Jang et al., 2008) in the same time microalgae (Morris et al., 2009) and fungi Brewer's yeast (Kanauchi et al., 2005), mushroom (Sun et al., 2004) described as a sources of bioactive peptides. Also, to their high nutritional value, it has been verified to supply a wide range of diverse physiological activity. These different functionalities and the beneficial health effects of these peptides include antioxidant activities, antimicrobial activities, antihypertensive actions, immune-modulatory, opiate-like, antithrombotic, mineral binding and hypo-cholesterolemic (Meisel, 1997; Hartmann and Meisel, 2007). One of the critical elements that regulate the functional characteristic of the biological activity of the peptides is the molecular weight of the bioactive peptides (Deeslie and Cheryan, 1981). The peptides chain that provides the mentioned functionalities has been described as a vital region (Meisel, 1998) In the origin of the protein sequence, these short chain of amino acids are often functionally inert, but by liberating these peptides from the native protein it obtains the active form of peptides while food consumption and processing through food hydrolysis by gastric digestive enzymes or by the action of microbial enzymes particularly during fermentation are releasing processes (Pihlanto-Leppala, 2001; Erdmann et al., 2008). The protein hydrolysis is a process

of partition the protein molecules employing enzymatic hydrolysis or chemical hydrolyzation in protein and provides smaller sequences of peptide and eventually amino acids (Neklyudov et al., 2000; Hui, 2007). The small size of the released active peptides can support the ability to transfer it to a particular position and perform their biological role. All along the food processing or digestion in the gastrointestinal the independent entities encrypted in the native protein are liberated, display it is the activity as an adjusting compound in a hormone-like performance (Korhonen and Pihlanto, 2003). Based on the peptides small size simply may cross the accessible barrier of the digestion system and get in blood circulation then transferring to the target organ. Digestion of the selected peptides to reach the particular organs through blood vessels is one of the major features to describe bioavailability thereafter, the ability of absorption and establishment of the desire properties to perform the physiological functions in the body. The amount of the bioactive peptides can cross the impediment of the intestine is insignificant that may be ignored nutritionally still, it has been shown that this small quantity may provide biologically importance in the tissue levels (Gardner, 1988, 1998) The reasons behind the low nutritional rate of the peptides are related to their chemical structure and the average number of present peptides. The main impacts on the conveying path of peptides are molecular size and hydrophobicity that identified as one of the structural attributes (Shimizu et al., 1997).

2.3 Hydrolysis of Protein: Peptide Synthesis

Physiologically active components in food, especially in the protein sequence, observed to provide high nutritional value to the hydrolysate as a consequence preparation of these biological active materials from natural sources increased either directly or by commercial methods. These small peptides are encrypted in the protein primary structure from this point of view; the nutritional role of protein has been examined as a source of bioactive peptides (Li-Chan, 2015). Protein hydrolysis decomposes protein into small fractions such as amino acids and peptides through split the peptides bonds and this breakdown done by utilizing enzymes, alkali and acids (Neklyudov et al., 2000). Decomposition mechanisms to liberate the active peptides from the parent protein achieved by three ways through the action of digestive enzymes hydrolyses by proteolytic microorganisms and by utilizing proteolytic enzymes originated from plant or microorganisms. One of the most

successful methods to derive bioactive peptides from protein origins is enzymatic hydrolysis (Thiansilakul et al., 2007; Bernardini et al., 2011). The enzymatic hydrolysis of bioactive peptides from diverse proteins to create a desired peptide chains is achieved by utilizing of the digestive enzymes and diverse proteinases including pepsin, chymotrypsin, trypsin, alcalase, thermolysin and pancreatin as well as the fungal and bacterial sources have also been used (Kilara and Panyam, 2003; Korhonen and Pihlanto, 2003). Subsequently, hydrolysed peptides exhibit numerous different activities such as developing healthier and safe substances for consumption. During hydrolysis of protein, some important structural changes appear such as enhancing the quantity of ions, decreasing the molecular weight and uncovering hydrophobic groups (Panyam and Kilara, 1996). Certain activities of the peptides are indicated by particularly location and type of amino acid leftovers from the main protein sequence (Park et al., 2001). The length of peptides typically provides properties related to the strength and ability of the bioactive peptides (Vermeirssen et al., 2004). The biological activity of the protein may increase while the peptides derived therefore, it has a positive effect on human health and may reduce the progression of chronic diseases (Udenigwe and Aluko, 2012). Attribute to numerous essential components of the peptides that hydrolyzed from food protein demonstrate crucial biological activities, for instance, antioxidant, antihypertensive, antimicrobial, immunomodulatory, lipid-lowering and anticancer activities (.Meisel 2004; Wang and Gonzalez DeMejia 2005; Korhonen and Pihlanto 2006; Pihlanto 2006; Aluko2008, 2008) These activities boosting the natural immune system and reduce the risk of chronic diseases eventually promote health. Non-communicable diseases regarded by the World Health Organization (WHO) to be a reason for mortality of 36 million people annually and it covers many diseases such as cancer, cardiovascular diseases, chronic respiratory diseases and diabetes (WHO, 2011). The factors that influence the activity of the biological peptides identified as a special feature of the used enzyme, protein origin and the process operations that may modify the natural structure of the protein (Gauthier and Pouliot, 2003). The most slight extraction method is the utilization of enzymes simultaneously the influences related to the environment are limited (Shen et al., 2008). Enzymatic hydrolyzation is preferable over the microbial fermentation as a result of short term reaction, predictability and adaptability even, it is preferred over other ways to hydrolyze protein precisely by using diverse pH. Enzymatic hydrolysis occurs under optimal condition of pH and

temperature (Udenigwe and Aluko, 2012). Despite the fact that increasing pH has a positive influence on solubility but it also possesses an adverse effect on the amino acids to reduce digestion ability and lower the proteins biological importance (Orem and Kaufman, 2011). Preparing protein hydrolysate by acidic method shows negative effects on the amino acid residues such as glutamine and asparagine hydrolyzed to their similar acids, while low pH is damage entire tryptophan and 5–10% of threonine and serine (Walker and Sweeney, 2002). On the other hand, enzymatic hydrolysis of protein can be affected by thermal treatment as a result of protein unfolding the enzyme-protein interaction may be improved (Inouye et al., 2009). Despite all the advantages that gains by the enzymatic hydrolysis non-utilization of bioactive peptides related to many reasons. Among the reasons, food manufacturing may decrease bioactivity of the peptides because during treatment changing in the molecular weight may occur or lost it is activity by contacting with other food components (Moller et al., 2008). In the same time protein hydrolyzations provide a bitter taste to the bioactive peptides this limits use of it as food additives (Kristinsson and Rasco, 2000).

2.4 Cereals

Cereal grains are primary and essential in human diets around the world since along time ago. They have been used by human as a main source of energy, as well as contributed to the livestock feed. As the world's population increased the human requirements raises, therefore cereals grown in the world widely. Cereals are utilizing in many forms the most common forms of cereals are rice (***Oryza sativa***), wheat (***Triticum aestivum***), barley (***Hordeum vulgare***) and oat (***Avena sativa***), even though wheat and rice take a great part in all energies consumed by a human. Cereal production by industries prevalent universally to meets the human nutritional needs, besides economic importance. The cereal grains are a fundamental part of a balanced diet due to the vital groups of major nutrients (Truswell, 2002). Cereals can be serving in a lot of different meals depending on the cereal type or variety cultures according to the area. In the same time may serve as other products such as bread, pasta and noodles. A cereal consists of certain biological molecules that increase opportunities to gain a healthier life beside discouraging body from disease (Chaturvedi et al., 2011). In many countries recommends increasing consumption of whole cereal grains (Mckeown et al., 2013). Due to the significant importance of the

valuable food components the national nutrition guidelines place the cereals as the basis of food pyramids. Consumption of cereals affects improvements of metabolic syndrome and a group of metabolic diseases in namely obesity, hypertension, type II diabetes and dyslipidemia (Rebello et al., 2014). Together with, consuming cereals within the human diet-related to health conditions by decreasing cancer death rates this manner demonstrated in various articles specifically breast, colon and prostate cancers (Jeong et al., 2003; Liu, 2007). Cereals are a vital source of numerous special substances distinguished it for the researchers and even the consumers in the whole world. The components in cereals like proteins, carbohydrate, lipids and minerals render it to utilize as a vital product for human feeding and the byproducts be an animal feedstock. Cereal protein described as a precious source of biologically active peptides. The encrypted peptides after derivation from cereal structure have shown diverse activity such as an antihypertensive, antioxidant, antimicrobial, immunomodulatory, antithrombotic, anticancer, opiate and mineral-binding properties.

2.4.1 Wheat

One of the common types of edible cereal seeds after rice among the cereals species is wheat. Cereal has arisen in Southwest Asia especially in the river valley Tigris and Euphrates (Smith and Wayne, 1995). Cereal and wheat proteins announced as edible seeds in the grass family *Gramineae* (Bender and Bender, 1999; McKevith, 2004). Wheat (*Triticum aestivum* L) is one of the most cultivated crops that cover almost 237 million hectares yards each year and provide 420 million tons for human uses (Langer and Hill, 1991; Olabanji et al., 2004), which at the least serve one-fifth of human consumption (Ohiagu et al., 1987). A vital source of plant protein is wheat while near to 750 million tons of wheat prepared for human consumption each year and this amount is higher than other crops (U.F.A., 1990; U.F.A., 2018). Two most commercially recognized types of wheat are *Triticum turgidum durum* and *Triticum aestivum vulgare*. It has many different products related to the wheat. Longevity, pleasant flavour and gluten-forming attributes of wheat products such as pasta, noodles, bread and many other products make wheat an attractive species among the other types of cereals (Nelson, 1985). Wheat grains gathered as a source of human nutrition consisting of diverse edible and non-edible tissues including wheat germ or embryo provide energy for germination and wheat bran. Wheat germ classified

among the by-products that remain from the milling industries in contrast to wheat kernel it performing more nutritional advantageous (Haridas et al., 1980). Wheat germ contains protein, dietary fiber, minerals beside it is a precious amount of vitamin E and B group vitamins (Amado and Arrigoni, 1992). Wheat provides health benefits to the human physical state because of the component that wheat contains such as a carbohydrate, protein, lipid, minerals and vitamins. Wheat protein can be partitioned into metabolic and storage proteins (Shewry, 2003). The metabolic protein nouns as non-gluten proteins consist of globulin, albumin and amphiphilic proteins in the other hand wheat storage proteins or gluten proteins has an effect on rheological properties (Dubreil et al., 1998). Availability of proline and glutamine amino acids makes the storage proteins to be announced as prolamins. Generally, in cereal grains the protein content is between 10% to 15% range of dry grain, storage proteins show the greatest availability of the whole protein percentage (Shewry and Halford, 2002).

2.4.2 Bulgur

Produced from wheat particularly from ***Triticum durum*** variety beside the desirable taste bulgur has health benefits. Bulgur likes rice in the generation of gelatinization from the starch content, besides the rapidity in cooking it is a ready or semi-ready to eat product (Bayram, 2000). It has an extended range of ages in Middle Eastern and Turkish history with wide traditional recipes that serve main and side dish options (Quaglia, 1988; Bayram and Öner 2004). Especially in traditional Turkish diet, bulgur has significant visibility. Commercial techniques to manufacturing bulgur in Turkey are completed in two main methods which are Gaziantep or Karaman methods. The steps in the order in Gaziantep type are cleaning, cooking, drying, debranning, cracking, and milling (Babadogan, 2010). In the same time, the initial steps of cleaning and cooking are similar in both methods, major difference in this two methods of bulgur production are in the Karaman technique involve tempering of cooked and subsequently dried wheat, which is followed by de-branning, re-drying and milling steps (Turksoy et al., 2009). The populace has been interested in consuming bulgur not only for the energy or the appetite taste also availability of many benefits that affect human health. Due to the healthy nutrients bulgur is widely accepted such as a dietary fiber, minerals, B vitamins, unsaturated fatty acids and folate (Kent and Evers, 1994; Bayram, 2007).

2.5 Waste of Bulgur

The bulgur production currently in Turkey predicted to be one million tons per year in other words consumption attainment is about 15 kg per person (Anon, 2006, 2009). Bulgur production generates a quantity of waste especially in the milling processes, besides the main product. The highest levels of bulgur waste are treated like animal livestock. The amount of bulgur that consumes by human compares to the production is specified. Wheat germ is the most nutritious part in the wheat kernel and it is a foremost by-product of the industries (Haridas et al., 1980). Wheat germ is a rich provenance of protein, minerals, dietary fiber and vitamin B groups (Amado, 1992; Arrigoni, 1992). In the same time contains the low-cost functional components which stated as the phytochemicals such as flavonoids, sterols, glutathione and octacosanols (Sullivan, 1937; Pietrzak and Collins, 1996; Ai-Hooti et al., 2002). The second substantial component generates in the milling processes like a by-product from the industries is wheat bran. Wheat bran is a rich source of fiber, B vitamin groups, minerals and bioactive peptides which have a positive effect on health (Preuckler et al., 2014). Production of the main products is all industries opportunity at the same time the residues that remain after production is known as by-product sometimes are valuable especially in the food sector. From this point of view reutilization of the by-products become a manner that many producers are appeal to obtain a major step forward. Usually, by-products are transposed to animal feed that has a quite low economic value. Annually a huge amount of by-products acquired from food industries some of the derived by-products investigated to be a source of bioactive peptides (García et al., 2016). Recovery of the waste is still in a low range while it is possible to provide value to these substances. Human used cereal grains as a nutritional part of diets and a source of bioactive peptides from a long period of time which including wheat, rice, barley, corn, rye, oat, and sorghum (Malaguti et al., 2014). On the basis of raw material percentage range of the food waste from industries advertised to be 5 to 90% (Restrepo, 2006). Attempts to modifying the by-products or the remained wastes from food production industries become actual issue regenerating these residues to beneficial fresh products and operative components that economically obtain additional importance either worth for human consumption or not (Zhang et al., 2010; Mora and Toldrá, 2012). In order to reduce these residues diverse food components evaluated to provide new

opportunities for utilization. Food industries commonly utilize protein hydrolysis to create a bioactive peptide or enhancements in physiochemical property of protein in the same time develop protein nutritional quality (Chalamaiah et al., 2012). Between the whole cereal forms wheat and barley proteins are displayed to profusion diverse types of peptides that possibly have biological activities (Malaguti ***et al.***, 2014). Dietary proteins in foods have long been studied to demonstrate the bioactive peptides to display the health benefits including diabetes, inflammation, chronic diseases, obesity and prevention against cancer (Udenigwe and Aluko, 2012).

2.6 The Physiological Benefits of Bioactive Peptides

Food 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.

2.6.1 Antimicrobial Activity

The physiological functions exist by the biologically active peptides are take a wide range of activity and beneficial effects even though described to be the usual protector for the body organs. 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 Se 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 this two forms perform the mechanism of action (Hancock and Sahl, 2006). The membrane structure of Gram-negative and Gram-positive bacteria provide diverse consequences to the antimicrobial peptides include the reaction mechanism and the special characters of the bacteria (Floris et al., 2003). These peptides are apart in destroying a number of microorganisms such as bacteria and fungi. The growth of specific pathogenic microorganisms is detained by antimicrobial peptides and inhibited it is activity (Chan and Li-Chan, 2005). The antimicrobial peptides regulate the inflammation reactions despite the destroying of microorganisms therefore, the importance of these protective natural peptides increased (Devin and Hancock,

2002). Nowadays, over and above 500 diverse types of antimicrobial peptides investigated and have been separated from a great number of different organisms regards to the major role provided by innate sources (Zhang et el., 1999). Various peptides have been described to possess antimicrobial activities that own such properties like hydrophobicity and a short sequence of peptides that arranged between 10 to 50 amino acid residues (Hancock and Sahl, 2006; Agyei and Danquah, 2011; Steinstraesser et al., 2011). The main structural foundations approved are the positive charge and amphiphilic properties identifying interaction with the bacterial membrane which in their processing actions it is a general goal. The amphiphilic properties of the peptides are important because of the accessibility of tryptophan in the peptide sequence which gives the peptides to conveyance through the cytoplasmic membrane (Chan et al., 2006). The specific peptide that exist the antimicrobial activity is hydrophobic α-helical peptides in the same time predominantly peptides with amphipathic and cationic properties possess the effect (Epand and Vogel, 1999). The different properties of the peptides support it is an activity as antimicrobial peptides from this point of view, the positive charge of the peptides recognized to react with the negative charge on the outer shell of bacteria and the hydrophobic properties facilitate the peptides to pass through the membrane (Wieprecht et al., 1997). As well these charges construct hydrogen bond with ionic elements of the cell membrane whereas the molecules net supplier is the availability of arginine in the peptide sequence ultimately influences the peptide - cytoplasmic membrane (Powers and Hancock, 2003).

2.6.2 Antioxidant Activity

The ordinary protection system in the body wipe out the free radicals but indefinite conditions malfunctions against reactive radicals take a place to confront and overcome the defence mechanism (Kaneto et al., 1999). This abbreviated word ROS incorporates numerous molecules consisting of oxygen and extremely active including singlet oxygen, free radicals superoxide ($O2^{-\cdot}$), nonradical form like hydrogen peroxide ($H2O2$), hydroxyl radicals ($HO^{\cdot}$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^{-}$) (L¨u et al., 2010). In normal cases body exhibit the ROS in low concentration to kill microorganisms and other beneficial functions. The ROS created during oxygen metabolism in aerobic creatures as a normal by-product and take a significant part in the physiological operations which provoke the signal

conversions and maintenance of metabolic equilibrium (Rahman et al., 2018; Zheng, et al., 2018). The amount of ROS is limited in the body while it may increase and cause attacks against biomolecules such as proteins, enzymes, lipids, DNA and RNA resulting damages in the cells in the same time provoke chronic diseases (Amarowicz et al., 2004). The excess amount is harmful provoke the oxidative stress in the living cells. Varied diseases diagnosed by means of the noxious free radicals reactions which infect the body cells and tissues (Leanderson et al., 1997). In spite of cancer, rheumatoid arthritis and senescence infections arise by the excess and out of control amounts of derived oxygen from free radicals deteriorate natural reactions in the body thus provoke many other diseases such as Alzheimer's and Parkinson's (Ali et al., 2008; DiMatteo and Esposito, 2003).As well as the lack of antioxidant balances encourages oxidative stresses and generates ROS aggregate as a consequence the cell detriment occurs and develop certain health disturbance including diabetes mellitus, coronary heart diseases, inflammatory diseases and cancer hepatic diseases (Carocho et al., 2018; Gogineni and Hamann, 2018). From this point of view, the beneficial health effects of antioxidants performed as a body protector against the ROS molecules. Demonstrated by many studies that the antioxidant peptides usually consists of 5-16 amino acid fractions that their composition, hydrophobicity and structure associate with the diverse levels of functionality (Saadi et al., 2015). Probably the availability of these peptides in natural foods has been discussed by many studies. Consuming natural antioxidant peptides directly from food intakes regarded to preserve body cells against impairments and offer limitation to the oxidation reaction (Vioque et al., 2006). Antioxidant activity is a property discovered in peptides or protein hydrolysates from wheat protein (Kumagai, 2010), corn protein (Kong and Xiong, 2006; Li et al., 2008), soy protein (Singh et al., 2014; Wang and De Mejia, 2005), potato protein (Wang and Xiong, 2005; Pihlanto et al., 2007), casein (Phelan et al., 2009), whey protein(Pihlanto-Leppälä, 2000; Saito, 2008), egg protein (Liu et al., 2010; Bhat et al., 2015), muscle protein (Ryan et al., 2011; Lafarga and Hayes,2014) and gelatin (Mendis et al., 2005) in plant or animal sources (Liu et al., 2016). The principle of the antioxidants actions is prevention oxidative reactions by scavenging free radicals (You et al., 2009). Accessibility of these free radicals to the medium induces many unfavourable alterations. Within these changes exhibited because of the free radicals are related to the food quality immediately has a negative impact including altering in texture and flavour (Liceaga-Gesualdo and

Li-Chan, 1999). Therefore, antioxidants are vital biological molecules to block oxidations by free radicals and protect health system. Antioxidant activity resolutely related to the amino acid composition and size or molecular weight which usually preferred low amount (Chen et al., 2010). The molecular size of the antioxidant peptides has a main impact on the bioactivities because decreasing molecular size promotes interaction with the free radicals to avoid lipid peroxidation (Wang et al., 2012; Tao et al., 2018). Regarding to the amino acid compositions presence at the suitable positions adjusts the antioxidant activity. The specific activity of the antioxidant peptides awarded by some particular amino acid sequences such as histidine and the aromatic amino acids in spite of the fact that peptides working mechanisms as antioxidant definitely unidentified (Suetsuna et al., 2000). Also, availability of amino acids like tryptophan, phenylalanine, isoleucine, methionine, cysteine, tyrosine, histidine, leucine, lysine and valine in the peptide chains reinforce the antioxidant activity (Saadi et al., 2015). Presence of the amino acids such as valine and leucine at the N-terminus of the peptide sequence also displays antioxidant activities (Medina-Godoy et al., 1981). As mentioned before, besides the amino acid composition and structure there is another factor to associate the diverse function of the peptides. The factor has a potent influence on the activity of the antioxidant peptides is hydrophobicity as a result of reachability to hydrophobic targets (Chen et al., 1998). The peptides capability to manage the oxidation processes inside the human body has been discussed based on the potential importance existed (Samaranayaka and Li-Chan, 2011). In food industries synthetic antioxidants are used to decrease the oxidative stresses and increase the nutritional value of the food. To preserve the food quality numerous artificial antioxidants applied as an illustration butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG) has been used (Verhagen et al., 1990; Kim and Wijesekara, 2010). Artificial antioxidants economical and profitable in the same time effective but exhibit some harmful and poisonous influences especially in human nutrition (Fukushima and Tsuda, 1985). As long as, these antioxidants induce undesirable impacts such as carcinogenesis, destroy liver cells and obviously supply consumers anxious (Chi et al., 2015; Sila and Bougatef, 2016). Nevertheless, adding a significant quantity of these artificial antioxidants induce difficulties in the stability of the food (Kahl and Kappus, 1993; McCarthy et al., 2001; Shahidi et al., 1992). Nowadays, the main interests of the scientists are developing natural antioxidants to

reduce the effects of artificial types. Consuming derived peptides originated from natural sources may defer the inception of the diseases by decreasing oxidative damages occur and responding to inflammatory reactions (Halliwell, 2012).

2.6.3 Antihypertensive Activity

Throughout the ages, the relationship between nutrition and health has a reputation which regards serious ecological protection to human health (Kussmann et al., 2010). Reducing chronic diseases effects and promoting health become an interesting manner to achieve healthier live for human being. Alteration in a normal grade of blood pressure synthesis a common chronic health disorder described as hypertension (Chobanian, 2003); this type of disease diagnosed in more than 1 billion people worldwide (WHO, 2011). Angiotensin-converting enzyme (ACE) promotes several reactions in the body meanwhile, two of the significant actions is related to blood pressure first transforming inactive decapeptide angiotensin I into a potent vasoconstriction agent and salt-conserving octapeptide that known as angiotensin II further disabled the bradykinin nonapeptides which means vasodilation the factor that encourages blood pressure decreasing (Gvras and Gavras, 1999). The ACE has a crucial role in promoting health and regulating blood pressure. One of the certain regulators that manage the volume of blood vascular is angiotensin II by fluid retention and sodium retention widen the blood vessels (Padfield et al., 1977; Brown et al., 1998). The blood vessels restrict relaxation and get a tough shrinkage because of the angiotensin II all these abnormalities lead to high blood pressure (Mora et al., 2014). Food proteins have a particular nutritional role in human diet furthermore, the primary structure contains smaller residues known as peptides that encrypted the biologically active peptides in their sequence may show higher activity even than the parent protein. 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 Mohan, 2014). Away from foods the initial ACE inhibitory detected by scientists were from the snake venom (Ferreira et al., 1970) since then developed and several numbers of ACE inhibitors have been defined and generated the most well-known nowadays is captopril. ACE inhibitory peptides released from many food types for

instance wheat germ (Matsui et al.,1999) , c-zein from corn (Miyoshi et al., 1995; Yano et al., 1996) , sunflower (Megias et al., 2004), soybean (Shin et al., 1995; Shin et al., 2001), pea (Vermeirssen et al., 2003), chickpea (Yust et al., 2003), horde in from barley (Gobbetti et al., 1997), garlic (Suetsuna, 1998), dried bonito (Fujita and Yoshikawa, 1999), oilseeds such as rapeseed (Pedroche et al., 2004; Yoshie-Stark et al., 2006), gelatin (Oshima et al., 1979) and ovalbumin (Yoshikawa et al., 2000). Inhibitory property of the peptides is controlling by the main structure characteristics that related to the C-terminal tripeptides (Maruyama et al., 1987), in the same time it is defined in studies that ACE connecting site revealed it is the preference towards the hydrophobic amino acids at each of it is positions (Meisel, 1993a). The characteristics of different amino acids offer the inhibition effect. Existence of some amino acids such as valine, tyrosine, tryptophan, proline, lysine, phenylalanine, arginine, leucine and isoleucine has a major impact on the connection between ACE and peptides to supply the inhibition properties (Lo´pez-Fandin˜o et al., 2006; Murray and FitzGerald, 2007). The peptides that inhibit ACE instantly offers a hypertensive effect in the same time it is effect experienced in the other body systems such as immune and nervous system activity (Meisel, 1993b). Artificial inhibitors developed as a medication to handle hypertension or heart failure and inhibit ACE and renin such as captopril, alcacepril, enalapril, lisinopril and aliskiren (renin) (Ondetti et al., 1977; Gradman et al., 2008). The artificial ACE inhibitors instantly obstruct the activity of ACE while the natural peptides derived from food interact with it to display it is a function from this point of view angiotensin I is unreachable to produce angiotensin II (Ahhmed and Muguruma, 2010). The side effects of the majority of synthetic drugs that are utilized for hypertension minimized in headache, nausea, taste disturbance, allergic reactions and inflammatory responses (Vigersky et al ., 2006; Wijesekara and Kim, 2010; Singh et al., 2017). By the side of bioactive peptides evolution food proteins recognized as a most familiar peptide type in the same time, a vital and the virtually effective origin of ACE inhibitory peptides (FitzGerald et al., 2004).

2.7 Bioavailability and Production of Bioactive Peptides

Proteins extracted from plants are a distinctive essential food component that developed to enhance current foods in promoting health, technological treatment and nutrition (Duranti, 2006). The need to replace animal proteins with plant proteins to

obtain bioactive peptides increased basis on the environmental and medical advantages. To provide physiological impact to the body the bioactive peptides may inter blood circulation safely after absorption in the intestine or may offer some positive effects in the digestion pathways (Erdmann et al., 2008). The amount of nutrients available for absorption and utilization respect to the whole amount is described as the bioavailability (Fuller and Tomé, 2005). The physiological factors are gastrointestinal (GI) motility, transit time, intestinal permeability, pH, transporters and enzymes (Balimane et al., 2000; Pauletti et al., 1997). On the contrary, the physiochemical and sensory properties connected to the functional characteristics of protein in the foods (Friedman, 1996; Vercruysse et al., 2005). The physiochemical properties of the nutrients regulate the bioavailability such as size, lipophilicity, solubility, molecular weight and charge. Most of the known bioactive peptides may own multifunctional properties (Meisel, 2004). While the meaning of functional is the description of those food forms which has a positive impact on body conditions and maintenance of general health wellness although provide prevention treatment towards chronic diseases (Bigliardi and Galati, 2013). World Health Organization demonstrated that chronic disease becomes a vital illness nowadays and may increase the death rate in the future or following decades (WHO, 2017). Several studies showed that protein has many health benefits and has a vital role in diet towards obesity problems. Diet rich in whole grains, unsaturated fats and proteins provide better weight handling and lower the chronic disease's effect (Vincent-Baudry et al., 2005; Dixit et al., 2011).

The cardiovascular system of the human body needs these biological active peptides to bear the alterations that occur for instance the organism impediment that each peptide must pass through in the gastrointestinal system and remain active while digestion and absorption (Baro et al., 2001). While during the metabolism period the peptide bonds are cleaved and synthesis a small sequence to be ready for absorption and offer the diverse functions in the body (Hinsberger and Sandhu, 2004; Gilbert et al., 2008). The peptides emulsified in the digestion system which provide their absorption in a high portion take a place inside the intestine (Davis and Reeves, 2002). The small size of the peptides provides the ability to handle digestion system and pass through the epithelial barriers into capillary blood vessels in order to reach to the certain position that may perform their biological ability (Vermeirssen et al.,

2002). The porosity of the compounds via capillary of the circulatory body system and it is the ability to solubilizing in lipids identify it is proficiency to penetrate the intestinal lymphatic system (Deak and Csaky, 1984).

The amino acids have been applied to manufacture edible and non-edible products which including animal feed, flavorant or possible to use it as a constituent element in the chemical industry (Scott et al., 2007). The standard properties of dietary proteins are controlled by the amino acid composition, it is digestion and absorption in the body nevertheless, and released peptides are a factor to define protein quality (Awati et al., 2009).

The amounts of available amino acid substances specify the nutritional characteristics of proteins simultaneously the physiological properties that allow the consumption of amino acids during digestion and absorption (Friedman, 1996; Korhonen and Pihlanto, 2006). The ability to release the peptides is one of the characters that define the quality of protein in conjunction with the composition, digestion and absorption of amino acids (Awati et al., 2009). Some of the proteins and peptides may pass the digestion processes directly absorb by the intestine and penetrate the intestinal lymphatic system. A number of amino acids believed to be valuable physiologically including histidine, lysine, tyrosine, arginine, tryptophan and glutamine (Marshall, 1994). Cereal proteins that own high quality hide an incorporated series of amino acids in the parent protein chain show biological activity and positive role once they liberated (de Mejia et al., 2012; Zambrowicz et al., 2013).

The regular techniques that commonly used to generate bioactive peptides described as enzymatic hydrolysis especially by utilizing digestion enzymes, food protein fermentation with proteolytic starter cultures and extracted plant or microorganisms proteolysis enzymes (Korhonen and Pihlanto, 2006). Nowadays, chemical synthesis, DNA technology or enzymatic synthesis used as a major method to prepare bioactive peptides in substantial quantities (Gill, 1996). Even with the most common methods to generate peptides with biological activities is the enzymatic process. The bioactive peptides establishment and degradation is influenced by the digestion enzymes and this explained by a number of studies in vitro (Svedberg et al., 1985). The encrypted sequence of peptides within the parent protein manages most of the physiological

actions that carry out by protein once they liberated (Rizzello et al., 2016). To develop enzymatic hydrolysates one or more of the microbial protease or digestive enzymes must use which include pepsin, alcalase, chymotrypsin, trypsin, thermolysin, and pancreatin (Korhonen and Pihlanto, 2006). Bioactive peptides are commonly liberated from food proteins through the hydrolysis by using commercial or digestive enzymes (Lassoued et al., 2015). The ordinary manufacturing of bioactive peptides by food industries is enzymatic hydrolysis which familiar to use extensively (Lafarga and Hayes, 2014; López-Barrios et al., 2014). Insufficient hydrolysis conditions the protein hydrolysate decomposes the bond between peptides to generate smaller peptides or free amino acids (Vioque et al., 2001). The intestinal brush borders offer enzymes to extracellularly hydrolyze the protein that passes through in the most cases the peptides that contain three or more amino acids (Euston et al., 2001). The amount of enzymes is one of the vital manners that must be stated to provide an attractive function, highest characteristics biologically and economically reduce the costs and inhibit waste of the enzyme (Shahidi et al., 1995). The bioactive peptides decomposed by enzymatic hydrolysis provide numerous different physiological characteristics (Saadi et al., 2015). Impacts of protein hydrolysate discussed by many researchers some studies perceive the influence of single peptides while the rest of them investigate combined and different peptides (Fitzgerald et al., 2005; Izumi et al., 2009). The protein hydrolysate is acting as a simple medicine to treat diseases related to metabolism system and it may be an origin to nutritional/medicinal preparations (Schmidl et al., 1994). On the contrary, the absorption and hydrolysis of short peptides less than three amino acids occur later. The proficiency of enzymes to hydrolysis peptides depends upon the composition of amino acids (Robert and Zaloga, 1994).The factors influence recovers of protein and hydrolysis are selecting the suitable type of enzyme and substrate, the ratio of solvent and stir speed must be appropriate to the reaction and the hydrolysis requirement also must be controlled such as a pH, time, temperature and enzyme/substrate ratio (Paul el et al., 2004; Shahidi et al., 1995). By the side of the factors that influence the enzyme activity, there are some other factors which must not be ignored including ions effectiveness on the enzyme activity of the enzyme while providing less activity and prevent to reach a product (Gonzàlez-Tello et al., 1994). The ratio of whole number of peptides cleaved during the hydrolysis is determined as a degree of hydrolysis. It is one of the other characteristics that have a

major role on the bioactivity and practical activity of the hydrolysates is the degree of hydrolysate (Nielsen et al., 2001). Protein hydrolysates concept achieve advancement in food industries to enhance protein nutritional property, adjust protein physicochemical properties and create bioactive peptides (Chalamaiah et al., 2012). The particular degree of hydrolysis that obtained from the hydrolysis of protein develops a source of precious peptides distinguished by the nutritional value and great bioactivity (Clemente, 2000; Hartman and Miesel, 2007). To decrease the establishment of an inappropriate product the enzymatic hydrolysis must achieve by controlling the pH and temperature conditions (Vioque et al., 2001). In food and pharmaceutical industries favours enzymatic hydrolysis technique because of the safety gained from this method otherwise the final output possible to contain toxic chemicals or may leave some organic solvents as leftovers (Sila and Bougatef, 2016). The high cost of enzymes minimized the usage field by the food industries. By-products and microorganisms in the past few years become a worth source to extract the proteinases to reduce the high cost of the enzymatic reaction (Agyei and Danquah, 2011). The proteases to hydrolyze food proteins classified to guide the utilization of enzymes. There are two types of proteases endopeptidases which include chymotrypsin, trypsin and elastase while, the second type is exo-peptidases that include A and B carboxy peptidases (Andriamihaja et al., 2013). These classifications handled according to the cleaved peptide position or action site. Exopeptidases breakdown the peptide bonds from the ends of poly peptide chains while, the endo peptidases liberate the peptide bonds from the internal part of the chain. To extract the bioactive peptides by enzymatic hydrolysis of the great options is utilizing pepsin as a digestive enzyme because in the beginning of digestion pepsin start to breakdown protein in stomach. To manage the optimal situation for pepsin must complete the hydrolysis under acidic pH to stimulate the stomach environments subsequently alkaline pH may adjust the hydrolysis to encourage the intestinal surrounding. Decomposition of protein sequence by enzymes such as a Pepsin starts from carboxylic part of tryptophan, leucine, phenylalanine, tyrosine and glutamic acid fractions (Lin et al., 2012). Trypsin and α-chymotrypsin are digestive enzyme graciously breakdown protein and peptides amide bonds. Beside the long chain and comparable structure each of the two enzymes, trypsin and chymotrypsin are serine proteases distinguished in substrates specific nature (Gutfreund et al., 1956; Ma Wet et al., 2005). The protease enzyme particularly chymotrypsin begins from the C-

terminal of the tryptophan, tyrosine and phenylalanine amino acids on the peptide to breakdown the chains (Fink et al., 1976). Hydrolysis of the polypeptides by these enzymes occur in the small intestine surrounding by an alkaline environment to create free amino acids or smaller peptide sequences. One of the most competent enzymes that described to use in the preparation of biologically active peptides and protein hydrolysates is alcalase (Saadi et al., 2015). The wide range of pH and temperature provide the alcalase particular specificity than other proteases.

CHAPTER 3

MATERIAL AND METHODS

3.1 Material

The waste of bulgur was supplied from an industry from Turkey. Enzymes used in this study were trypsin from porcine pancreases (1 BAEE Unit of Trypsin activity will produce 0.001absorbance increase per minute at 253nm) while, 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^{o}C) while, 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), Cupper sulfate($CuSO_4$), 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), Ethyl acetate, sodium chloride (NaCl) and borate buffer (H_3BO_3).

3.2 Experimental Setup

The bulgur waste samples used for determination of protein, fat, moisture and ash content. After characterization of samples, they were hydrolyzed by four different enzymes as illustrated in Table 3.1. Each of the hydrolyzed samples examined antioxidant activity, antimicrobial activity and antihypertensive activity.

3.3 Methods

3.2.1 Determination of Moisture Content in the Waste of Bulgur

The waste of bulgur was grinded to obtain softer form. Moisture content was determined by applying temperature to approximately 3 g of the bulgur waste samples in the oven (JSOF-100, Korea); 105^{o}C was used until constant weight reached (AOAC, 1990). Lost weight explained as the amount of moisture that the sample maintained.

3.2.2 Determination of Ash Content

The total ash contents of the bulgur waste sample were determined by a method stated by AOAC (1990). Shortly, 3 grams of the sample was burned in the crucibles for 5 hours at 550^{o}C to obtain a grey color of ash and constant weight. After cooling in room temperature the ash content was calculated according to the given equation:

$$\text{Ash } \% = \frac{weight\ of\ ash}{weight\ of\ the\ sample} \times 100 \qquad (3.1)$$

3.2.3 Protein Content Determination

By use of Kjeldahl method (AOAC, 1990) the protein content of the milled samples was founded. The first step of Kjeldahl method was digestion to break down all nitrogen bonds and convert them to ammonium ions (NH_4^+) while, 1 g of the sample was added in the Kjeldahl tube in the same time 12 mL of sulfuric acid 95 %, 7 grams of potassium sulfate were added to increase the boiling point of sulfuric acid and small quantity of copper sulfate in order to increase the speed and efficiency of the process the black foam produced. Digestion was done in 400°C for 40 min the foam decomposes and a clear liquid appear as the final reaction of digestion allowed to cool down to room temperature. The second step of the Kjeldahl processes was distillation done by Kjeldahl protein device (FOSS, 2200 KT A DIST UNIT W BOR. PUMP, Hoganas, Sweden) some alkali added to convert ammonium into ammonia and finally the procedure completed by titration the sample with an acid solution such as hydrochloric acid HCl (0.1N). The specific conversion factor for the bulgur wastes was 5.7.

Table 3.1 Hydrolysis conditions of bulgur waste protein

Enzymes	Time	Temperature	Unit	Buffer
Pepsin	6 hours	37 ^{o}C	3 units 9 units	Sodium Citrate buffer at pH 2
Trypsin	6 hours	37 ^{o}C	3 units 9units	Tris buffer at pH 8
Chymotrypsin	6 hours	37 ^{o}C	3 units 9 units	Tris buffer at pH 8
Protease	6 hours	50 ^{o}C	3 units 9 units	Tris buffer at pH 8

3.2.4 Fat Content Determination

Dried and weighted extraction flasks were used for determination of the fat quantity in the bulgur waste samples according to AOAC (1990) procedures. About 5 grams of the sample was put in the dry thimbles and stabilized in the dried extraction flasks that was filled with 150 mL of hexane in the same time it contains 2 or 3 glass beads to decrease the eruptions. The soxhlet instrument (Gerhardt, SE-416, Germany) was used after 2 hours and 45 minutes of extraction the samples cooled down to room temperature and weighted.

3.2.5 Extraction of Protein and Enzymatic Digestions

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 later on centrifuged at 10000 rpm for 20 minutes at the room temperature. The extracted protein was concentrated to obtain 2.86% protein by freeze dryer (CHRIST ALPHA 1-4 LD Plus, Germany) within 3 hours the sample condensed and kept in -18°C until next use.

The concentrated protein was subjected to enzymatic hydrolysis by use of 1 mL of protein sample was diluted in 1 mL deionized water with desirable amount of enzymes the suspension pH adjusted by buffers. The different incubation conditions were applied for each enzyme has been obtained in shaking incubator (INNOVA 40, New Brunswick, New Jersey, USA) as illustrated in Table 3.1 Inactivation of enzyme activity was achieved after hydrolyses by incubating at 95^{o}C for 20 minutes

in boiling water bath. Cooling in room temperature was the next step of hydrolyses and finally the supernant was ready after separating by centrifuged for 15 min at 6000 rpm in a centrifuge (Hettich, werk Nr., Germany).

3.2.6 Degree of Hydrolyses

The hydrolysis efficiency was detected by using o-phthalaldehyde (OPA) assay. For this reason, 3 mL deionized water was mixed up with 75 µl OPA reagent and 10 µl of hydrolyzed waste of bulgur sample shake about 5 seconds then after two minutes the absorbance was read at 340 nm and the percentage degree of hydrolysis was calculated by percentage of hydrolyses as given in Equation 3.2:

$$\text{DH } (\%) = \frac{(ABS\ x\ 1{,}934\ x\ d)}{c} \qquad (3.2)$$

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

3.2.7 The Physiological Properties of the Hydrolyzed Peptides

3.2.7.1 Antimicrobial Activity

The antimicrobial peptides indicated by gram negative and gram positive bacteria species include *Escherichia coli* (25322) and *Staphylococcus aureus* (25923). These microorganisms were retained on nutrient agar plated and recovered by sub-culturing in nutrient broth for 24 hours. About 0.1 mL of each microorganism stocks was inoculated on plate count agar (LAB 149) for 15 minutes. The papers with 1cm diameter were prepared and drown in the hydrolyzed bulgur waste samples for around one minute then semi-dried in room temperature. Semi-dry papers were stabilized on the plate count agar that contain microorganisms and incubate for 48 hours at 37^{o}C to verify inhibition zone.

3.2.7.2 DPPH Radical Scavenging Activity

The antioxidant activity of the hydrolyzed bulgur waste was determined by using 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 milliliters of each samples and was kept at room temperature for 30 minutes in the dark place. Meanwhile, the UV visible

spectrophotometer (OPTIMA SP-3000nano, Tokyo, Japan) was used at 517 nm to read the absorbance of the samples. DPPH radical scavenging activity calculated by equation (3.3),

$$\text{DPPH radical scavenging activity } \% = [1- \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}] \times 100 \qquad (3.3)$$

While, absorbance of control was obtain through use of ethanol and DPPH without the sample.

3.2.7.3 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 10mM phosphate buffered saline pH 7.4 to adjust the absorbance at 0.8±0.1 at 734 nm. After these modifications 1 mL of the hydrolyzed sample was mixed with 1 mL of diluted ABTS stock solution. Only 10 min needed for the sample to be rested at room temperature and the absorbance was measured by UV visible spectrophotometer (SP-3000nano, OPTIMA, Tokyo, Japan) at 734 nm then calculated by:

$$\text{ABTS scavenging activity } (\%) = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100 \qquad (3.4)$$

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

3.2.7.4 Antihypertensive Activity

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) (Sigma-Aldrich). The reaction was started by the addition of 20 µL of ACE solution (0.1 U/mL) incubated at 37^{o}C for 45 minutes. The reaction was stopped by adding 200 µL of 1M HCl.

Then, to separate the released hippuric acid 1.5 mL of ethyl acetate was added and centrifuged at 2500 rpm for 10 minutes. 1 mL of the supernatant was transferred into a test tube and removing the ethyl acetate was achieved by vacuum evaporate in 60^{o}C for 60 minutes. The evaporated sample was dissolved in 2 mL deionized water and the absorbance was determined at 228 nm using spectrophotometry (SP-3000nano, OPTIMA, Tokyo, Japan). The inhibition activity indicated by the equation of ACE activity %:

$$\text{ACE inhibitory activity } \% = [\frac{(Aa-A)}{(Aa-Ab)}] * 100 \quad (3.5)$$

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.

3.2.8 Statistical Analysis

One, two and three ways analysis of variances (ANOVA) were applied by SPSS (19) to indicate the significance difference between time of hydrolysis, type of enzyme and concentration 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, type of enzyme, and concentrations of the enzymes has been used as an independent parameter. They compared with three ways of ANOVA using degree of hydrolysis, DPPH, ABTS, ACE and inhibition of *Staphylococcus aureus* and *Escherichia coli.*

By two ways of ANOVA hydrolysis time, enzyme type and concentration of the enzymes were chosen as independent parameters. Compare with two ways of ANOVA by utilizing degree of hydrolysis, DPPH, ABTS, ACE and inhibition of *Staphylococcus aureus* and *Escherichia coli.*

Time of hydrolysis, type of enzyme, and concentrations of the enzymes 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.*

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Raw Material Characterization

The bulgur waste contains both bran and flour which remains from the wheat. These residues include different amount of moisture content may change according to manufacturing processes. The existing amount of moisture content in the bulgur waste samples was found to be 12.15% while, the whole bulgur samples had 11.2 % of moisture according to Zeynep et al. (2011) and these values were average of six replicates.

The amount of ash that available in the waste of bulgur was 3.55 % (d.b). This compares to the whole bulgur results were only 0.92 % (d.b) (Zeynep et al., 2011) show a huge difference in the waste components.

The quantity of the available fat in the bulgur waste was found to be 4.58 %. The difference between fat components in the accurate waste contrast to the whole bulgur is very large where only 1.2 % fat is obtained from the whole bulgur samples.

The main component that contains the bioactive peptides is the protein. The extracted amount of protein from the bulgur waste by o.1N NaOH solution was determined by Kjeldahl method showed 2.84 % (w.b). Whereas, the amount of protein that available in the waste sample were 16.34 % according to the dry base matter. At the same time, the protein content in the whole bulgur samples reported by Njoku et al., (2007) was from10.6% to 10.84% (d.b). Production of bulgur from the wheat sources contains major components include carbohydrate and protein that used for human consumption from this point of view, remains constituents in the waste samples were diverse in distribution. Due to the remaining components

within the waste show higher availability that increase in protein, fat and ash content amounts in waste samples compare to the whole bulgur samples.

4.2 Degree of Hydrolysis

Degree of hydrolysis defines the cleaved peptide bonds that fractionated from the protein during hydrolysis. Identifying the degree of hydrolysis is one of the main popular guides to define the functional characteristics of the hydrolyzed proteins (Kristinsson and Rasco, 2000). Finding the appropriate enzymes to convert the size of peptides versus to time was the purpose behind usage of degree of hydrolysis. Indications of the degree of hydrolysis with ortho-phthalaldehyde methods illustrate the enzymes ability to generate smaller peptides and display the activity of the biological peptides. The percentage of degree of hydrolysis results are given in Figure 4.1 that illustrate the effect of the concentration at 3 units of each of the utilized pepsin, trypsin, chymotrypsin and protease enzymes at the entire 360 minutes of hydrolysis. In contrast, Figure 4.2 shows the usage of 9 units of the enzymes activities at the same hydrolysis treatment conditions for concentrated protein from bulgur waste that displayed at Table 3.1.

Generally, the combined results explained by three way ANOVA at the whole 360 minutes and the four utilized enzymes in both concentrations showed a significant ($P<0.05$) increasing effect on degree of hydrolysis. It is expected that increasing concentration of enzymes and time of hydrolysis straightly shows an effect on enzymes activation to breakdown peptide bonds and raise significantly ($P<0.05$) the amount of peptide production. The ANOVA results shown in the Table 4.3.The highest hydrolysis percentage of pepsin, trypsin, chymotrypsin and protease enzymes were 2.1%, 8.2%, 4.5% and 10.6%, respectively, by using 3 units of enzymes per gram of waste sample. Meanwhile, 9 units of the enzyme had 8.1%, -0.4%, 11.3% and 2.9% activity per gram of waste samples according to the enzyme type in varying time at the same incubation condition.

The degree of hydrolysis explained by two way ANOVA for the entire enzymes pepsin, trypsin, chymotrypsin and protease in both 3 units and 9 units concentrations significantly increased ($P<0.05$). In the same time, with in the 15, 30, 60, 120, 180, 240, and 360 minutes of incubation period hydrolyses rate

developed significantly (P<0.05) for the hydrolyzed bulgur samples. The improved consequences were due to the specificity of the enzymes at the same time the availability of suitable enzyme quantity to degrade the accessible protein in the bulgur samples. Because numerous factors describe the peptides and bioactive peptides generate by enzymatic hydrolysis such as processing requirement, enzyme specification, hydrolysis rate and the specificity of the resultant peptides from hydrolysis which include amino acid composition, hydrophobicity and molecular size (Udenigwe and Aluko, 2012). Nevertheless, the hydrolysis efficiency was decreased in 300 minutes and non-significantly (P>0.05) affect the incubation because of the insufficient enzymes concentrations especially Trypsin that explained in Table 4.2.

Changing the enzymes have a significant (P<0.05) impact on hydrolysis rate pepsin, chymotrypsin and protease influenced instantly on the hydrolysis rate since displayed a development rate significantly. Nevertheless, trypsin showed non-significant (P>0.05) effect on the rate of hydrolysis. The concentration effect with a consistent of enzyme type and time of incubation was found to increase significantly (P<0.05).

The results displayed typical changes by one way ANOVA that show increment or reduction in hydrolysis percentage within the 360 minutes demand a specific declaration. The entire various enzymes at both concentrations together performed to rise the hydrolysis activity significantly (P<0.05). Nevertheless, non-significant results observed in some specific points. To begin with, the enzymes specificity show 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). The effect of both concentrations 3 units and 9 units by pepsin raised significantly (P<0.05). Also, time showed significant changes that increased during the whole period of incubation by utilizing 3 units of pepsin enzyme even though, in 120 minutes the degree of hydrolysis was the highest rate. While, usage of 9 units of pepsin enzyme provide a diverse sort of reaction which by enhancing time of incubation the rate of hydrolysis increased in the entire processes Table 4.1 illustrate the process. The hydrolysis of wheat by pepsin enzymes was reported by Nagy et al (2009) that enhancing time increases the degree of hydrolysis within entire 120 minutes.

Trypsin's impact on the hydrolysis was various in the results may be due to the ability of peptides to degrade in the specific sides. Trypsin usually cleaves primary amino acids of the lysine and arginine (Salami et al., 2008). The enzyme quantity in both various concentrations were affected significantly ($P<0.05$) and increased the degree of hydrolysis. While, degree of hydrolysis was not affected by time factor during 360 minutes of hydrolysis which the reactions decrease provides a non-significant means ($P>0.05$) to the hydrolysis particularly by utilizing 3 units of trypsin enzyme. Thereby, regarding to lowering in the hydrolysis Guerard et al. (2002) considered that the decreasing in degree of hydrolysis may be due to diminishing the concentration of existing hydrolysis peptides, deactivation of the enzymes and inhibition of the enzymes. Deficiency of the specific peptide bonds for hydrolysis during a particular point of time may also lower degree of hydrolysis ratio. Even though, the usage of 9 units of trypsin enzyme give a significant ($P<0.05$) result were provided to the hydrolysis incubation period at the whole 360 minutes as shown in Table 4.1.

Mainly refractions 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).The various enzymes attack the available amino acids in the structure of bulgur waste samples in particular positions from these considerations diverse results arises between enzyme hydrolysis. Time influenced the hydrolysis rate of bulgur waste samples with chymotrypsin increased significantly ($P<0.05$) with time during the incubation. Both concentrations of chymotrypsin incubation at 15, 30, 60, 300 and 360 minutes raised the degree of hydrolysis significantly ($P<0.05$). Whereas, the hydrolysis was not significant and decreased at 120, 180 and 240 minutes specifically in 180 minutes the degree of hydrolysis was at the lowest rate of reaction. As described at Table 4.1. 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; Silvestreet al., 2012).

The ultimate enzyme used in hydrolysis of bulgur waste was protease. The utilized enzyme displayed a significant ($P<0.05$) result in both 3 and 9 units of enzyme concentrations at the whole 360 minutes of incubation. The degree of hydrolysis

increased gradually at a significant ($P<0.05$) rate with increasing time and amount of protease in both concentrations described in Table 4.1. 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 the each proteases (Baldyga an Bourne, 1999).

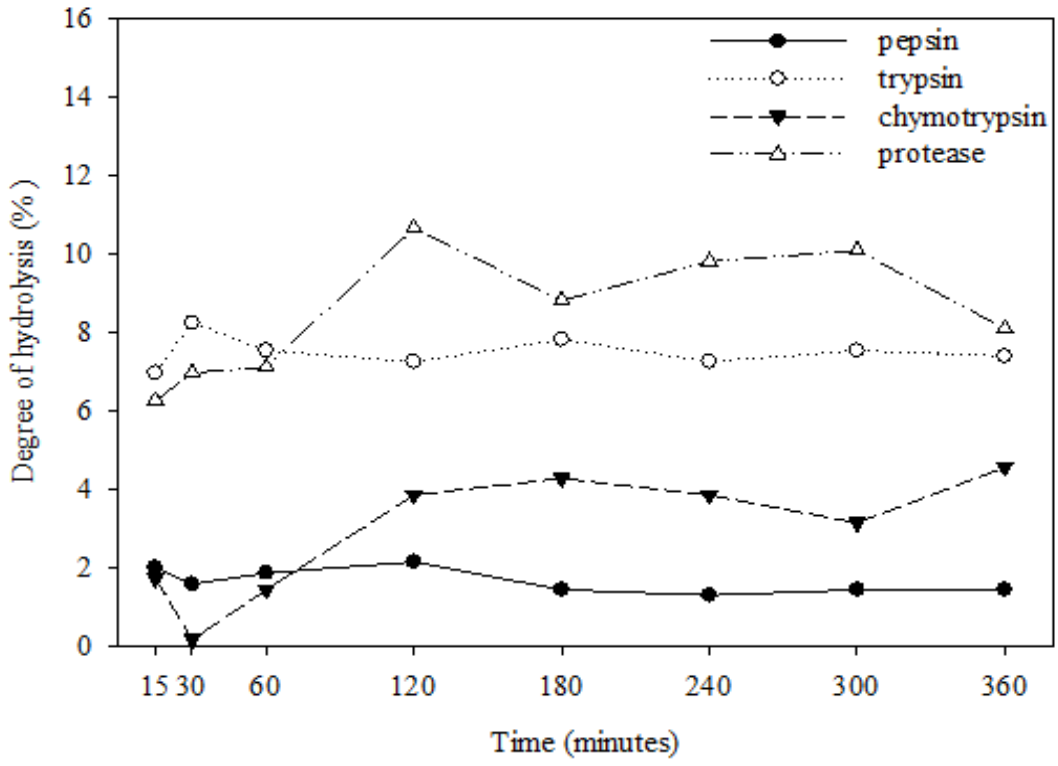

Figure 4.1 Degree of hydrolysis determination by applying 3 units of utilized enzymes

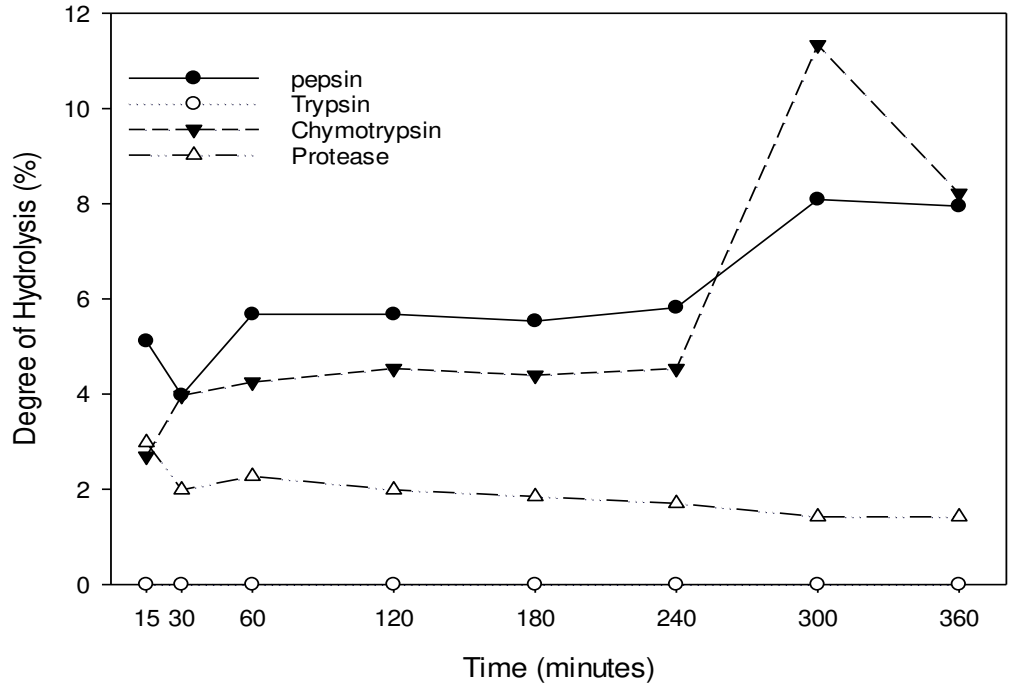

Figure 4.2 Degree of Hydrolysis determination by applying 9 units of utilized enzymes

Table 4.1 Determination of degree of hydrolysis by one way ANOVA

Time (minutes)	Pepsin		Trypsin		Chymotrypsin		Protease	
	3	9	3	9	3	9	3	9
15	1.99 ± 0.10^{aAX}	5.11 ± 0.26^{aAY}	6.96 ± 0.35^{aB}	-0.85 ± -0.04^{aBY}	1.70 ± 0.09^{CX}	2.69 ± 0.13^{CY}	6.25 ± 0.31^{aDX}	2.98 ± 0.15^{aDY}
30	1.56 ± 0.08^{bAX}	3.97 ± 0.20^{bAY}	8.23 ± 0.41^{bB}	-0.70 ± -0.03^{bBY}	0.14 ± 0.01^{bCX}	3.97 ± 0.20^{bCY}	6.96 ± 0.35^{bDX}	1.99 ± 0.10^{bDY}
60	1.85 ± 0.09^{cAX}	5.68 ± 0.28^{cAY}	7.52 ± 0.38^{cB}	-0.42 ± -0.02^{cBY}	1.42 ± 0.07^{cCX}	4.25 ± 0.21^{cCY}	7.10 ± 0.35^{cDX}	2.27 ± 0.11^{cDY}
120	2.13 ± 0.11^{dAX}	5.68 ± 0.28^{dAY}	7.24 ± 0.36^{dB}	-1.27 ± -0.06^{dBY}	3.83 ± 0.19^{CX}	4.53 ± 0.23^{CY}	10.65 ± 0.53^{dDX}	$1.99 \pm 0.\ 10^{dDY}$
180	1.42 ± 0.07^{eAX}	5.54 ± 0.28^{eAY}	7.81 ± 0.39^{eB}	-0.85 ± -0.04^{eBY}	4.26 ± 0.21^{CX}	4.39 ± 0.22^{CY}	8.80 ± 0.44^{eDX}	1.85 ± 0.09^{eDY}
240	1.28 ± 0.06^{fAX}	5.82 ± 0.29^{fAY}	7.24 ± 0.36^{fB}	-0.85 ± -0.04^{fBY}	3.83 ± 0.19^{CX}	4.53 ± 0.23^{CY}	9.79 ± 0.49^{fDX}	1.70 ± 0.09^{fDY}
300	1.42 ± 0.07^{gAX}	8.09 ± 0.40^{gAY}	7.52 ± 0.38^{gB}	-0.85 ± -0.04^{gBY}	3.12 ± 0.16^{gCX}	11.34 ± 0.57^{gCY}	10.08 ± 0.50^{gDX}	1.42 ± 0.07^{gDY}
360	1.42 ± 0.07^{hAX}	7.95 ± 0.40^{hAY}	7.38 ± 0.37^{hB}	-0.56 ± -0.02^{hBY}	4.54 ± 0.23^{hCX}	8.22 ± 0.41^{hCY}	8.09 ± 0.40^{hDX}	1.42 ± 0.07^{hDY}

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 of pepsin, trypsin, chymotrypsin and protease enzymes on degree of hydrolysis by one way ANOVA at $\alpha = 0.05$ level.

Capital letters (X and Y) show significant difference of 3 units and 9 units of each enzyme on degree of hydrolysis by one way ANOVA at $\alpha = 0.05$ level.

Table 4.2 Determination of degree of hydrolysis by two ways ANOVA

Time	Enzyme type and Concentration	Enzyme type	Time and Concentration	Concentration	Time and Enzyme type
15	Pepsin ^{CX} Trypsin ^{BX} Chymotrypsin ^{AX} Protease ^{DX}	Pepsin	15^{bX} 30^{aX} 60^{bX} 120^{bX} 180^{bX} 240^{bX} 300^{cX} 360^{cX}	3 units and 9 units of Pepsin	3^{aA} 9^{abD} 3^{aA} 9^{aD} 3^{aA} 9^{cD} 3^{aC} 9^{dcD} 3^{aBC} 9^{bcD} 3^{aBC} 9^{bcD} 3^{aBC} 9^{bcD} 3^{aB} 9^{eD}
30	Pepsin ^{BX} Trypsin ^{CX} Chymotrypsin ^{AX} Protease ^{DX}				
60	Pepsin ^{BX} Trypsin ^{BX} Chymotrypsin ^{AX} Protease ^{CX}	Trypsin	15^{aX} 30^{bX} 60^{abX} 120^{aX} 180^{abX} 240^{abX} 300^{abX} 360^{abX}	3 units and 9 units of Trypsin	3^{aC} 9^{abA} 3^{aC} 9^{aA} 3^{aC} 9^{cA} 3^{cC} 9^{bcA} 3^{bcC} 9^{bcA} 3^{bcC} 9^{bcA} 3^{bcC} 9^{eA} 3^{bC} 9^{dA}
120	Pepsin ^{BX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{CX}				
180	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{CX}	Chymotrypsin	15^{aX} 30^{aX} 60^{bX} 120^{cX} 180^{cX} 240^{cX} 300^{dX} 360^{eX}	3 units and 9 units of Chymotrypsin	3^{aA} 9^{abC} 3^{aA} 9^{aC} 3^{aA} 9^{cC} 3^{aA} 9^{bcC} 3^{aA} 9^{bcC} 3^{aA} 9^{bcC} 3^{aA} 9^{eC} 3^{aA} 9^{dC}
240	Pepsin ^{ABX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{CX}				
300	Pepsin ^{BX} Trypsin ^{AX} Chymotrypsin ^{DX} Protease ^{CX}	Protease	15^{abX} 30^{aX} 60^{abX} 120^{dX} 180^{bcX} 240^{cdX} $300^{c\ dX}$ 360^{abX}	3 units and 9 units of Protease	3^{aA} 9^{abD} 3^{aA} 9^{aD} 3^{aA} 9^{cD} 3^{aA} 9^{bcD} 3^{aA} 9^{bcD} 3^{aA} 9^{bcD} 3^{aA} 9^{eC} 3^{aA} 9^{dC}
360	Pepsin ^{BX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{CX}				

Small letters show significant difference of time on degree of hydrolysis by two way ANOVA at α = 0.05 level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on degree of hydrolysis by two way ANOVA at α = 0.05 level.

Capital letters (X) show significant difference of 3 units and 9 units amount of each enzyme on degree of hydrolysis by two way ANOVA at α = 0.05 level.

Table 4.3 Determination of degree of hydrolysis by three ways ANOVA

Time	Type of Enzyme	concentration
15^{a}	Pepsin ^{AB}	3 units ^{X}
30^{a}		
60^{ab}	Trypsin ^{A}	
120^{bc}		
180^{bc}	Chymotrypsin ^{B}	9 units ^{X}
240^{bc}		
300^{d}	Protease ^{C}	
360^{cd}		

Small letters show significant difference of time on degree of hydrolysis by three way ANOVA at $\alpha = 0.05$ level.

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

Capital letters (X) show significant difference of 3 units and 9 units amount of each enzyme on degree of hydrolysis by three way ANOVA at $\alpha = 0.05$ level.

4.3 Antimicrobial Activity

The antimicrobial activities have been demonstrated to exhibit the protein fractions ability to inhibit growth of microorganisms. The four diverse enzymes start to break down the protein fractions to obtain smaller peptides. The entire hydrolyzed samples examined against Gram positive bacteria (*Staphylococcus aureus)* and Gram negative bacteria (*Escherichia coli).* Different diameters of growth inhibition zone (mm) of each portion evaluated. Tables 4.4- 4.5 illustrate the results that describe effect of 3 units and 9 units respectively of utilized enzymes for determining antimicrobial activity.

All the examined factors including time, concentration and different types of enzymes on the activity of the bioactive peptides from the bulgur waste samples by three way ANOVA against the microbial activity of Gram positive bacteria *Staphylococcus aureus* had positively significant ($P<0.05$) as illustrated in Table 4.8. In divers to Gram negative *Escherichia coli* that provide non-significant ($P>0.05$) effect as shown in Table 4.11 due to changes in the enzyme concentrations. While, time and the different types of the utilized enzymes provide a significant ($P<0.05$) increasing during the digestion period.

Effects of each three analyzed factors indicated by stabilizing one of the described factors to explain remain components by applying two ways of ANOVA. *Staphylococcus aureus* shows significantly ($P<0.05$) positive inhibition towards the microbial growth by usage of three different enzymes and in both concentrations. Also, during the 300 minutes of digestion the created peptides inhibit microbial growth significantly ($P<0.05$) but in the last digestion point the ability to inhibit microbial growth has been disappeared non-significantly ($P>0.05$) as described in Table 4.7. Although, peptides produced by *Escherichia coli* provided a positive ability to hinder growth of microorganisms significantly ($P<0.05$) shown in Table 4.10 and reinforce immune system in the body.

In practical term, the growth inhibition by *Staphylococcus aureus* against both concentrations 3 and 9 units of the enzymes increased significantly ($P<0.05$) as declared in Table 4.6. Also, *Escherichia coli* showed significant ($P<0.05$) inhibition capability by utilizing trypsin and chymotrypsin while non-significant ($P>0.05$) inhibition observed by pepsin and protease declared in Table 4.9. The inhibitions

towards gram positive bacteria were greater significantly (P<0.05) than gram negative and interestingly by utilizing pepsin and protease for enzymatic hydrolysis. The resistivity of gram negatives versus antimicrobial contents regards to the cell wall structure (Lambert et al., 2001; Ördög et al., 2004).

Table 4.4 Effect of 3 units of enzyme hydrolysis on Gram positive and Gram negative bacteria

TIME	PEPSIN		TRYPSIN		CHMOTRYPSIN		PROTEASE	
3 U	*E. coli*	*S. aureas*	*E. coli*	*S. aureas*	*E. coli*	*S. aureas*	*E. coli*	*S. aureas*
15	+	++	++	+++	+++	++	++	+++++
30	+	+++	++	+++	+	++	++	++
60	+	++++	+	++	+	+	++	+++
120	+	++++	++	+++	+	+++	+	++
180	+	++++	++	+++	+	+++	++	++
240	++	++	+++	++	+	++	+	++
300	+	+++	+	+++	+	+	++	+
360	+	+	+	+	+	+	++	+

Table 4.5 Effect of 9 units of enzyme hydrolysis on Gram positive and Gram negative bacteria

TIME	PEPSIN		TRYPSIN		CHMOTRYPSIN		PROTEASE	
9U	*E. coli*	*S. aureas*	*E. coli*	*S. aureas*	*E. coli*	*S. aureas*	*E. coli*	*S. aureas*
15	+	-	+	-	++	++	+++	-
30	+	-	+	+	++	++	++	-
60	+	+	+	+	++	+	++	+
120	+	+	+	++	++	+	++	+
180	+	+	+	++	+	+	++	+
240	+	+	+	++	+++	+	++	+
300	+	+	+	++	+	+	++	+
360	-	+	+	++	+	+	+	+

Table 4.6 Determination of microbial inhibition diameter (mm) by Gram positive *Staphylococcus aureus* from one way ANOVA

Time (minutes)	Pepsin		Trypsin		Chymotrypsin		Protease	
	3	9	3	9	3	9	3	9
15	1.00 ± 0.05^{aAX}	0.10 ± 0.01^{aAY}	2.00 ± 0.10^{aBX}	0.50 ± 0.03^{aBY}	1.00 ± 0.05^{aCX}	1.20 ± 0.06^{aCY}	4.00 ± 0.20^{aDX}	0.20 ± 0.01^{aDY}
30	1.50 ± 0.08^{bAX}	0.10 ± 0.01^{bAY}	2.00 ± 0.10^{bBX}	0.50 ± 0.03^{bBY}	1.00 ± 0.05^{bCX}	1.00 ± 0.05^{bCY}	0.50 ± 0.03^{bDX}	0.10 ± 0.01^{bDY}
60	3.00 ± 0.15^{cAX}	0.10 ± 0.01^{cAY}	1.20 ± 0.06^{cBX}	0.50 ± 0.03^{cBY}	0.50 ± 0.03^{cCX}	0.50 ± 0.03^{cCY}	1.50 ± 0.08^{cDX}	0.10 ± 0.01^{cDY}
120	2.00 ± 0.10^{dAX}	0.10 ± 0.01^{dAY}	2.00 ± 0.10^{dBX}	1.00 ± 0.05^{dBY}	2.00 ± 0.10^{dCX}	0.50 ± 0.03^{dCY}	1.00 ± 0.05^{dDX}	0.20 ± 0.01^{dDY}
180	3.00 ± 0.15^{eAX}	0.10 ± 0.01^{eAY}	2.00 ± 0.10^{eBX}	1.00 ± 0.05^{eBY}	1.50 ± 0.08^{eCX}	0.50 ± 0.03^{eCY}	1.20 ± 0.06^{eDX}	0.10 ± 0.01^{eDY}
240	0.10 ± 0.01^{fAX}	0.30 ± 0.02^{fAY}	1.20 ± 0.06^{fBX}	1.00 ± 0.05^{fBY}	1.00 ± 0.05^{fCX}	0.20 ± 0.01^{fCY}	0.50 ± 0.03^{fDX}	$0.\ 50 \pm 0.03^{fDY}$
300	2.00 ± 0.10^{gAX}	0.20 ± 0.01^{gAY}	$2.00 \pm 0.10g^{BX}$	0.10 ± 0.01^{gBY}	0.50 ± 0.03^{gCX}	0.20 ± 0.01^{gCY}	0.50 ± 0.03^{gDX}	0.30 ± 0.02^{gDY}
360	0.30 ± 0.02^{hAX}	0.30 ± 0.02^{hAY}	0.50 ± 0.03^{hBX}	1.00 ± 0.05^{hBY}	0.50 ± 0.03^{hCX}	0.30 ± 0.02^{hCY}	0.50 ± 0.03^{hDX}	0.30 ± 0.02^{hDY}

Small letters show significant difference between time on antimicrobial activity by one way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference between pepsin, trypsin, chymotrypsin and protease enzymes on antimicrobial activity for one way ANOVA at $\alpha = 0.05$ level.

Capital letters (X and Y) show significant difference between 3 units and 9 units amount of each enzyme on antimicrobial activity for one way ANOVA at $\alpha = 0.05$ level.

Table 4.7 Determination of microbial inhibition diameter (mm) by Gram positive *Staphylococcus aureus* from two ways of ANOVA

Time	Enzyme type and Concentration	Enzyme type	Time and Concentration	Concentration	Time and Enzyme type
15	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{BX} Protease ^{CX}	Pepsin	15^{bX} 30^{cX} 60^{eX} 120^{dX}	3 units and 9 units of Pepsin	3^{fD} 9^{cA} 3^{cD} 9^{dA} 3^{dD} 9^{bA} 3^{eD} 9^{deA}
30	Pepsin ^{BX} Trypsin ^{DX} Chymotrypsin ^{CX} Protease ^{AX}		180^{eX} 240^{bX} 300^{dX} 360^{aX}		3^{fD} 9^{dA} 3^{bD} 9^{fA} 3^{cD} 9^{aA} 3^{aD} 9^{efA}
60	Pepsin ^{DX} Trypsin ^{BX} Chymotrypsin ^{CX} Protease ^{BX}	Trypsin	15^{bX} 30^{cX} 60^{aX} 120^{dX}	3 units and 9 units of Trypsin	3^{fC} 9^{cD} 3^{cC} 9^{dD} 3^{dC} 9^{bD} 3^{eC} 9^{deD}
120	Pepsin ^{BX} Trypsin ^{DX} Chymotrypsin ^{CX} Protease ^{AX}		180^{dX} 240^{bX} 300^{bX} 360^{aX}		3^{fC} 9^{dD} 3^{bC} 9^{fD} 3^{cC} 9^{aD} 3^{aC} 9^{efD}
180	Pepsin ^{CX} Trypsin ^{CX} Chymotrypsin ^{BX} Protease ^{AX}	Chymotrypsin	15^{dX} 30^{dX} 60^{bX} 120^{eX}	3 units and 9 units of Chymotrypsin	3^{fA} 9^{cC} 3^{cA} 9^{dC} 3^{dA} 9^{bC} 3^{eA} 9^{deC}
240	Pepsin ^{BX} Trypsin ^{CX} Chymotrypsin ^{BX} Protease ^{AX}		180^{dX} 240^{cX} 300^{aX} 360^{aX}		3^{fA} 9^{dC} 3^{bA} 9^{fC} 3^{cA} 9^{aC} 3^{aA} 9^{efC}
300	Pepsin ^{BX} Trypsin ^{BX} Chymotrypsin ^{AX} Protease ^{AX}	Protease	15^{fX} 30^{aX} 60^{eX} 120^{cdX}	3 units and 9 units of Protease	3^{fB} 9^{cB} 3^{cB} 9^{dB} 3^{dB} 9^{bB} 3^{eB} 9^{deB}
360	Pepsin ^{AX} Trypsin ^{CX} Chymotrypsin ^{BX} Protease ^{BX}		$180d^{X}$ 240^{bcX} 300^{abX} 360^{abX}		3^{fB} 9^{dB} 3^{bB} 9^{fB} 3^{cB} 9^{aB} 3^{aB} 9^{efB}

Small letters show significant difference of time on antimicrobial activity by two way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antimicrobial activity by two way ANOVA at $\alpha = 0.05$ level.

Capital letters (X and Y) show significant difference of 3 units and 9 units of each enzyme on antimicrobial activity by two way ANOVA at $\alpha = 0.05$ level.

Table 4.8 Determination of microbial inhibition diameter (mm) by Gram positive *Staphylococcus aureus* from three ways of ANOVA

Time	Type of Enzyme	concentration
15^{c}	Pepsin ^{AB}	3 units ^{X}
30^{bc}		
60^{bc}	Trypsin ^{B}	
120^{c}		
180^{c}	Chymotrypsin ^{A}	9 units ^{X}
240^{ab}		
300^{ab}	Protease ^{A}	
360^{a}		

Small letters show significant difference of time on antimicrobial activity by three way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antimicrobial activity by three way ANOVA at $\alpha = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antimicrobial activity by three way ANOVA at $\alpha = 0.05$ level.

Table 4.9 Determination of microbial inhibition diameter (mm) by Gram negative *Escherichia coli* from one way ANOVA

Time (minutes)	Pepsin		Trypsin		Chymotrypsin		Protease	
	3	9	3	9	3	9	3	9
15	0.2 ± 0.01^{aX}	0.5 ± 0.03^{aY}	1.5 ± 0.08^{aBX}	0.5 ± 0.03^{aBY}	2 ± 0.10^{aCX}	1.5 ± 0.08^{aCY}	1 ± 0.05^{aX}	1.5 ± 0.03^{aX}
30	0.1 ± 0.01^{bX}	0.5 ± 0.03^{bY}	1 ± 0.05^{bBX}	0.2 ± 0.01^{bBY}	0.3 ± 0.02^{bCX}	1 ± 0.05^{bCY}	1 ± 0.05^{bX}	1 ± 0.05^{bX}
60	0.1 ± 0.01^{cX}	0.5 ± 0.03^{cY}	0.5 ± 0.03^{cBX}	0.1 ± 0.01^{cBY}	0.2 ± 0.01^{cCX}	1 ± 0.05^{cCY}	1 ± 0.05^{cX}	1 ± 0.05^{cX}
120	0.1 ± 0.01^{dX}	0.5 ± 0.03^{dY}	1.5 ± 0.08^{dBX}	0.5 ± 0.03^{dBY}	0.1 ± 0.01^{dCX}	1.2 ± 0.06^{dCY}	0.5 ± 0.03^{dX}	1 ± 0.05^{dX}
180	0.5 ± 0.03^{eX}	0.5 ± 0.03^{eY}	1 ± 0.05^{eBX}	0.1 ± 0.01^{eBY}	0.1 ± 0.01^{eCX}	0.5 ± 0.03^{eCY}	1 ± 0.05^{eX}	0.5 ± 0.03^{eX}
240	0.1 ± 0.01^{fX}	0.1 ± 0.01^{fY}	2 ± 0.10^{fBX}	0.5 ± 0.03^{fBY}	0.1 ± 0.01^{fCX}	2 ± 0.10^{fCY}	0.5 ± 0.03^{fX}	1.5 ± 0.08^{fX}
300	0.1 ± 0.01^{gX}	0.2 ± 0.01^{gY}	0.2 ± 0.01^{gBX}	0.5 ± 0.03^{gBY}	0.3 ± 0.02^{gCX}	0.5 ± 0.03^{gCY}	0.5 ± 0.03^{gX}	1 ± 0.05^{gX}
360	0.2 ± 0.01^{hX}	0.1 ± 0.01^{hY}	0.2 ± 0.01^{hBX}	0.2 ± 0.01^{hBY}	0.1 ± 0.01^{hCX}	0.5 ± 0.03^{hCY}	1 ± 0.05^{hX}	0.5 ± 0.03^{hX}

Small letters show significant difference of time on antimicrobial activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antimicrobial activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antimicrobial activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Table 4.10 Determination of microbial inhibition diameter (mm) by Gram negative *Escherichia coli* from two ways of ANOVA

Time	Enzyme type and Concentration	Enzyme type	Time and Concentration	Concentration	Time and Enzyme type
15	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{DX} Protease ^{CX}	Pepsin	15^{cX} 30^{bX} 60^{bX} 120^{bX} 180^{eX} 240^{dX} 300^{aX} 360^{aX}	3 units and 9 units of Pepsin	3^{fA} 9^{cA} 3^{dA} 9^{dA} 3^{cA} 9^{bA} 3^{dA} 9^{deA} 3^{dA} 9^{dA} 3^{eA} 9^{fA} 3^{aA} 9^{aA} 3^{bA} 9^{efA}
30	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{BX} Protease ^{CX}				
60	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{CX}	Trypsin	15^{dX} 30^{cX} 60^{bX} 120^{dX} 180^{cX} 240^{eX} 300^{aX} 360^{aX}	3 units and 9 units of Trypsin	3^{fD} 9^{cD} 3^{dD} 9^{dD} 3^{cD} 9^{bD} 3^{dD} 9^{deD} 3^{dD} 9^{dD} 3^{eD} 9^{fD} 3^{aD} 9^{aD} 3^{bD} 9^{efD}
120	Pepsin ^{AX} Trypsin ^{DX} Chymotrypsin ^{BX} Protease ^{CX}				
180	Pepsin ^{BX} Trypsin ^{BX} Chymotrypsin ^{AX} Protease ^{DX}	Chymotrypsin	15^{dX} 30^{bX} 60^{bX} 120^{bX} 180^{aX} 240^{cX} 300^{aX} 360^{aX}	3 units and 9 units of Chymotrypsin	3^{fB} 9^{cC} 3^{dB} 9^{dC} 3^{cB} 9^{bC} 3^{dB} 9^{deC} 3^{dB} 9^{dC} 3^{eB} 9^{fC} 3^{aB} 9^{aC} 3^{bB} 9^{efC}
240	Pepsin ^{AX} Trypsin ^{DX} Chymotrypsin ^{BX} Protease ^{BX}				
300	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{CX}	Protease	15^{cX} 30^{bX} 60^{bX} 120^{aX} 180^{aX} 240^{bX} 300^{aX} 360^{aX}	3 units and 9 units of Protease	3^{fC} 9^{cB} 3^{dC} 9^{dB} 3^{cC} 9^{bB} 3^{dC} 9^{deB} 3^{dC} 9^{dB} 3^{eC} 9^{fB} 3^{aC} 9^{aB} 3^{bC} 9^{efB}
360	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{CX}				

Small letters show significant difference of time on antimicrobial activity by two way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antimicrobial activity by way ANOVA at $\alpha = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antimicrobial activity by two way ANOVA at $\alpha = 0.05$ level.

Table 4.11 Determination of microbial inhibition diameter (mm) by Gram negative *Escherichia coli* from three ways of ANOVA

Time	Type of Enzyme	concentration
15[a]	Pepsin [A]	3 units [Y]
30[b]		
60[b]	Trypsin [B]	
120[b]		
180[c]	Chymotrypsin [B]	9 units [Y]
240[c]		
300[c]	Protease [C]	
360[c]		

Small letters show significant difference of time on antimicrobial activity by three way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antimicrobial activity by three way ANOVA at $\alpha = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antimicrobial activity by three way ANOVA at $\alpha = 0.05$ level.

4.4 Antioxidant Activity

The oxidative stresses detected by the position, evaluated target and type of generated ROS from this point of view, To evaluate the antioxidant activity determinations of the food stuffs combining of more than one method is necessitating (Georgetti et al., 2006; Nuutila et al., 2003). Consequently, two different spectrophotometeric systems selected including DPPH and ABTS methods to verify the antioxidant activity of the hydrloysed bulgur waste samples. Both methods consist of estimations of the discoloration with the free radicals and convert the samples to colorless product. Numerically the difference between DPPH and ABTS are considerable which possibly regards to some reasons including the wavelength measured at diverse absorbance, DPPH and free radical scavenging's mechanism of action and finally the antioxidant reading decrease by reacting DPPH with certain phenols.

4.4.1 DPPH

Several positive effects may appear during enzymatic hydrolyses such as improvement of radical scavenging activity which directly related to antioxidant activity of hydrolyses (Aleman et al., 2012). The antioxidants are available in many foods and whole grain is one of the rich sources in particular germ and bran fragments (Martínez-Tome et al., 2004; Pérez-Jiménez and Saura-Calixto 2005). The primary considerations that related directly to the rate of antioxidant activity are the type of enzymes utilized in hydrolysis. The outcomes obtained from the hydrolyses bulgur waste samples described in Figures 4.3- 4.4 illustrate the effect of time and concentration on the free radicals and DPPH property on the bulgur hydrolyses by 3 units and 9 units of each enzyme respectively. The enzyme concentrations that used in enzymatic hydrolysis were 3 units and 9 units of each enzyme particularly. By combining effects of whole factors on bulgur waste hydrolyses samples as a result of three way ANOVA in Table 4.14 significant ($P<0.05$) rate was observed. Except time that create a non-significant ($P>0.05$) impact on the antioxidant activity.

Additionally, significant ($P<0.05$) impacts observed while the time changed with constant state of the enzyme type and each enzymes concentration when two way ANOVA applied declared in Table 4.13. The most afflicted enzyme was trypsin which non-significantly ($P>0.05$) influenced at constant time and concentration. The

three units of the entire four utilized enzymes also provide a non-significant (P>0.05) effect which antioxidant activity decreased by DPPH at the existed concentration.

In particular, by applying one way ANOVA the DPPH activity to describe the antioxidant ability of the bulgur waste hydrolyses by trypsin, chymotrypsin and protease were not significant (P>0.05) by using three units of the enzyme. Despite pepsin that showed a positive ability creates an anti-oxidative peptide significantly (P<0.05) with small quantity of the enzymes. On the other hand, hydrolysis the sample by 9 units of the accessible enzymes were entirely significant (P<0.05) during the whole hydrolysis time as discussed in Table 4.12. The high quantity of the enzyme offered a limited antioxidant activity in contrast to 3 units enzymes that provides a magnificence activity against the oxidation components.

The non-significant (P>0.05) effect of the 3 units of enzymes emerged after 240 minutes of incubation especially in 360 minutes were the lowest antioxidant ability of the hydrolyzed samples. A particular reason behind the reduction in DPPH rate may be due to the high level of enzymatic hydrolysis in other word inaccessibility to hydrolyze peptides beside availability of high amount of hydrophilic amino acids (You et al., 2009). Besides, 3 units of the enzyme and 9 units significantly (P<0.05) affected the diverse enzymes including pepsin, trypsin, chymotrypsin and protease ability against the oxidation throughout the incubation term. During the same period enhancing antioxidant activity within some particular times perhaps it regards to the side chains of the peptide residue may contain amino acids that encourage immediately reaction between the hydrolyzed components and DPPH free radicals such as hydrophobic amino acids (Je et al., 2005).

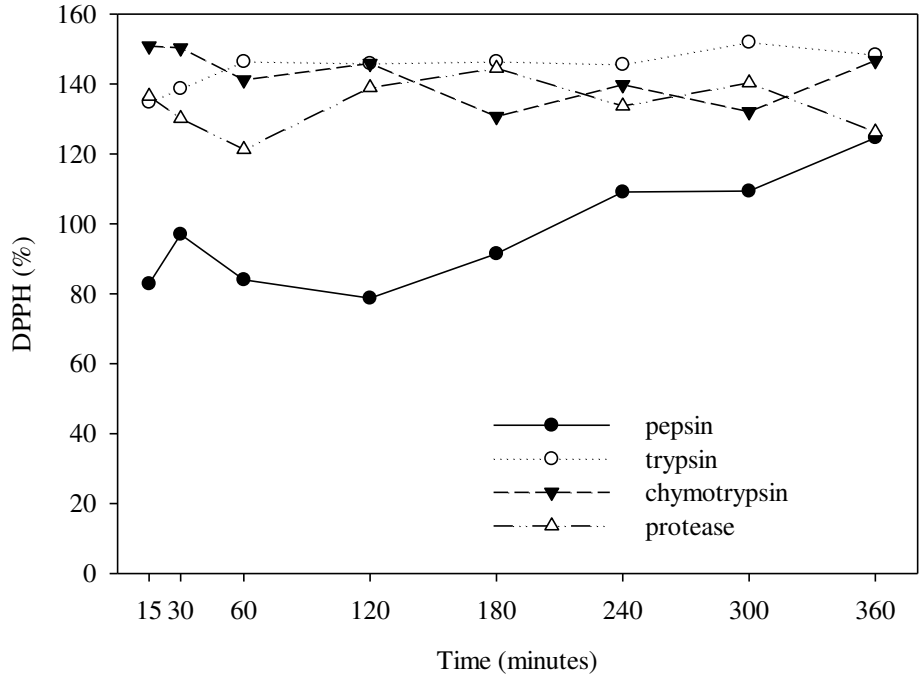

Figure 4.3 Determination the percentages of antioxidant activity by DPPH with 3 units concentration of enzymes

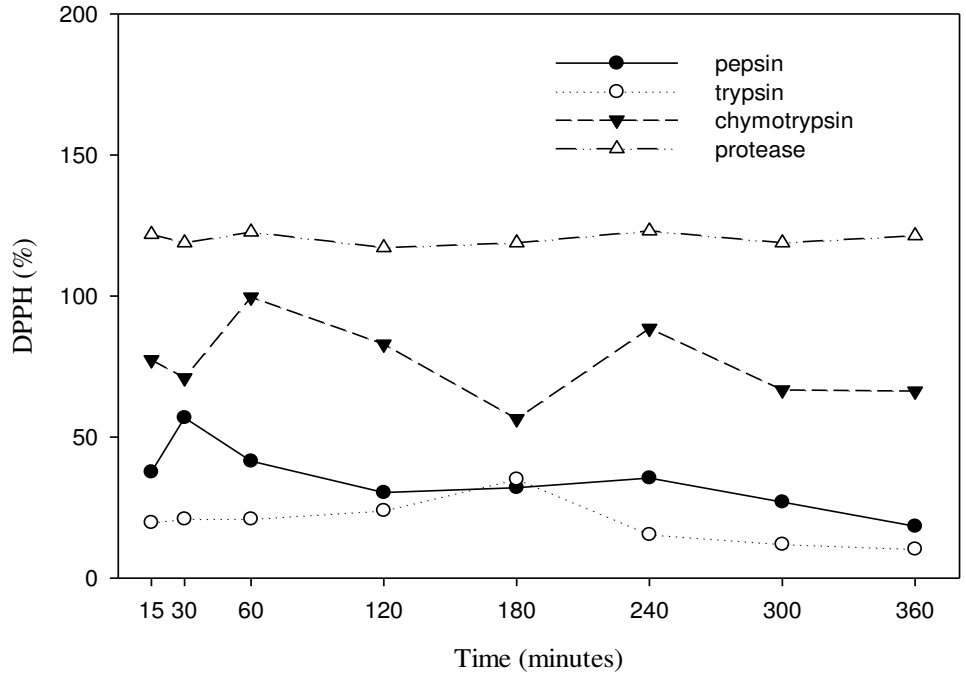

Figure 4.4 Determination the percentages of antioxidant activity by DPPH with 9 units concentrations of enzymes.

Table 4.12 Determination of antioxidant activity by DPPH method from one way ANOVA

Time (minutes)	Pepsin		Trypsin		Chymotrypsin		Protease	
	3	9	3	9	3	9	3	9
15	82.87 ± 4.14^{aAX}	37.61 ± 1.88^{aAY}	134.81 ± 6.74^{aX}	19.66 ± 0.98^{aBY}	136.46 ± 6.82^{aX}	77.35 ± 3.87^{aCY}	136.46 ± 6.82^{aX}	121.79 ± 6.09^{aDY}
30	96.96 ± 4.85^{bAX}	56.84 ± 2.84^{bAY}	138.67 ± 6.93^{bX}	20.94 ± 1.05^{bBY}	150.28 ± 7.51^{bX}	70.94 ± 3.55^{bCY}	130.11 ± 6.51^{bX}	118.80 ± 5.94^{bDY}
60	83.98 ± 4.20^{cAX}	41.45 ± 2.07^{cAY}	146.41 ± 7.32^{cX}	20.94 ± 1.05^{cBY}	141.16 ± 7.06^{cX}	99.57 ± 4.98^{cCY}	121.27 ± 6.06^{cX}	122.65 ± 6.13^{cDY}
120	78.73 ± 3.94^{dAX}	30.34 ± 1.52^{dAY}	145.86 ± 7.29^{dX}	23.93 ± 1.20^{dBY}	145.86 ± 7.29^{dX}	82.91 ± 4.15^{dCY}	138.95 ± 6.95^{dX}	117.09 ± 5.85^{dDY}
180	91.44 ± 4.57^{eAX}	32.05 ± 1.60^{eAY}	146.41 ± 7.32^{eX}	35.04 ± 1.75^{eBY}	130.66 ± 6.53^{eX}	56.41 ± 2.82^{eCY}	144.48 ± 7.22^{eX}	118.80 ± 5.94^{eDY}
240	109.12 ± 5.46^{AX}	35.47 ± 1.77^{fAY}	145.58 ± 7.28^{X}	15.38 ± 0.77^{fBY}	139.78 ± 6.99^{X}	88.46 ± 4.42^{fCY}	133.70 ± 6.69^{X}	123.08 ± 6.15^{fDY}
300	109.39 ± 5.47^{fAX}	26.92 ± 1.35^{gAY}	151.93 ± 7.60^{gX}	11.97 ± 0.60^{gBY}	132.04 ± 6.60^{gX}	66.67 ± 3.33^{gCY}	140.33 ± 7.02^{gX}	118.80 ± 5.94^{gDY}
360	124.59 ± 6.23^{AX}	18.38 ± 0.92^{hAY}	148.34 ± 7.42^{X}	10.26 ± 0.51^{hBY}	146.69 ± 7.33^{X}	66.24 ± 3.31^{hCY}	126.24 ± 6.31^{X}	121.37 ± 6.07^{hDY}

Small letters show significant difference of time on antioxidant activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antioxidant activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antioxidant activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Table 4.13 Determination of antioxidant activity by DPPH method from two ways ANOVA

Time	Enzyme type and Concentration	Enzyme type	Time and Concentration	Concentration	Time and Enzyme type
15	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{CX} Protease ^{BX}	Pepsin	15^{aX} 30^{dX} 60^{abX} 120^{aX} 180^{abX} 240^{cdX} 300^{bcX} 360^{cdX}	3 units and 9 units of Pepsin	3^{abA} 9^{dB} 3^{abA} 9^{eB} 3^{aA} 9^{eB} 3^{abA} 9^{cdB} 3^{abA} 9^{cB} 3^{abA} 9^{dB} 3^{abA} 9^{bB} 3^{bA} 9^{aB}
30	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{DX} Protease ^{CX}				
60	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{CX} Protease ^{BX}	Trypsin	15^{a} 30^{ab} 60^{ab} 120^{ab} 180^{ab} 240^{ab} 300^{b} 360^{ab}	3 units and 9 units of Trypsin	3^{abC} 9^{cA} 3^{abC} 9^{eA} 3^{cC} 9^{eA} 3^{abC} 9^{cdA} 3^{abC} 9^{cA} 3^{abC} 9^{dA} 3^{abC} 9^{bA} 3^{bC} 9^{aA}
120	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{CX} Protease ^{BX}				
180	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{BX} Protease ^{BX}	Chymotrypsin	15^{cdX} 30^{bcdX} 60^{dX} 120^{cdX} 180^{aX} 240^{cdX} 300^{abX} 360^{bcX}	3 units and 9 units of Chymotrypsin	3^{abC} 9^{dC} 3^{abC} 9^{eC} 3^{cC} 9^{eC} 3^{abC} 9^{cdC} 3^{abC} 9^{cC} 3^{abC} 9^{dC} 3^{abC} 9^{bC} 3^{bC} 9^{aC}
240	Pepsin ^{AX} Trypsin ^{ABX} Chymotrypsin ^{CX} Protease ^{BX}				
300	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{CX} Protease ^{BX}	Protease	15^{bcX} 30^{cX} 60^{abX} 120^{bcX} 180^{bcX} 240^{bcX} 300^{bcX} 360^{aX}	3 units and 9 units of Protease	3^{abB} 9^{dB} 3^{abB} 9^{eB} 3^{aB} 9^{eB} 3^{abB} 9^{cdB} 3^{abB} 9^{cB} 3^{abB} 9^{dB} 3^{abB} 9^{bB} 3^{bB} 9^{aB}
360	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{AX}				

Small letters show significant difference of time on antioxidant activity by two way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of Pepsin, Trypsin, Chymotrypsin and protease enzymes on antioxidant activity by two way ANOVA at $\alpha = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antioxidant activity by two way ANOVA at $\alpha = 0.05$ level.

Table 4.14 Determination of antioxidant activity by DPPH method from three ways ANOVA

Time	Type of Enzyme	concentration
15^{ab}	Pepsin ^{A}	3 units ^{X}
30^{b}		
60^{ab}	Trypsin ^{B}	
120^{ab}		
180^{ab}	Chymotrypsin ^{C}	9 units ^{X}
240^{ab}		
300^{ab}	Protease ^{B}	
360^{a}		

Small letters show significant difference of time on antioxidant activity by three way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of Pepsin, Trypsin, Chymotrypsin and protease enzymes on antioxidant activity by three way ANOVA at $\alpha = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antioxidant activity by three way ANOVA at $\alpha = 0.05$ level.

4.4.2 ABTS

One of the most common systems to indicate the antioxidant activity of the bioactive peptides is the usage of blue ABTS. During the reaction became neutral and colorless as a response toward the antioxidant peptides or the amount of scavenged ABTS. The hydrolysis samples scavenge ABTS radicals according to types of peptides, free amino acid content, enzyme content, solubility of hydrolyses and on the contrary to DPPH degree of hydrolysis also may influence the reaction (Phanturat et al., 2010). The results that gained from the hydrolysis of bulgur wastes are illustrated in Figures 4.5- 4.6 for each 3 units and 9 units of hydrolyzed samples respectively. The antioxidant ability of the samples influenced by various factors includes time, concentration and four different types of the enzymes. According to the entire factors by three way ANOVA that shown in Table 4.17 enzyme types provide a significant ($P<0.05$) ability to the reaction. While, time and association of concentration with the time were non-significantly ($P>0.05$) influenced the hydrolysis of bulgur waste ordinarily.

Consequence of enzymes and concentrations together on the ABTS activity significantly ($P<0.05$) increased corresponding to time at 15, 30 and 60 minutes hydrolysis as described in Table 4.16 by applying to two way ANOVA. Whereas, after 120 minutes of digestion by the diverse enzymes until the end of the applied time non-significant ($P>0.05$) impacts and decreasing in antioxidant activity ability appeared. On the other hand, trypsin reacted non-significantly ($P>0.05$) toward changes in concentration and time together. In the same time, protease concentration do not provide an ability to create antioxidant peptides and non-significantly ($P>0.05$) affected. Though, each of pepsin, chymotrypsin enzymes displayed an increase significantly ($P<0.05$) of antioxidant activity by ABTS method. The alteration in concentration show a positive influence on the antioxidant activity by ABTS significantly ($P<0.05$).

More characteristically, digestion by 3 units of the utilized enzymes shows a varied antioxidant activity in the Table 4.15. For instance, pepsin and chymotrypsin exhibited a significant ($P<0.05$) increase on the reaction while trypsin and protease show non-significant ($P>0.05$) alteration. At the same time, usage of 9 units also omits the sufficient activity of pepsin, trypsin and protease and non-significantly

(P>0.05) affected the reaction. Simply chymotrypsin was able to provide anti-oxidation activity significantly (P<0.05). These alterations may regard to the decreasing number of available hydrophilic antioxidants that respond ABTS along with the improvement of liberate hydrophobic amino acids (You et al., 2009).

The utilized pepsin, trypsin, chymotrypsin and protease enzymes to digest the bulgur waste samples with only 3 units obtained a significant (P<0.05) ability at 15, 30, 60 and 240 minutes of hydrolysis time. While, non-significant (P>0.05) antioxidant activity observed at 120, 180, 300 and 360 minutes of hydrolysis. The usage of 9 units of available enzymes provides a different antioxidant activity where, at 15, 30, 60, 120, 240 and 300 minutes significantly (P<0.05) the reactions completed. Compare to, the 180 and 360 minutes that non-significantly (P>0.05) altered.

Each concentration of 3 and 9 units of the enzymes pepsin, trypsin and protease affected significantly (P<0.05) on antioxidant ability by ABTS. Only non-significantly (P>0.05) lowering in digestions ability observed in chymotrypsin. These consequences may regard 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.

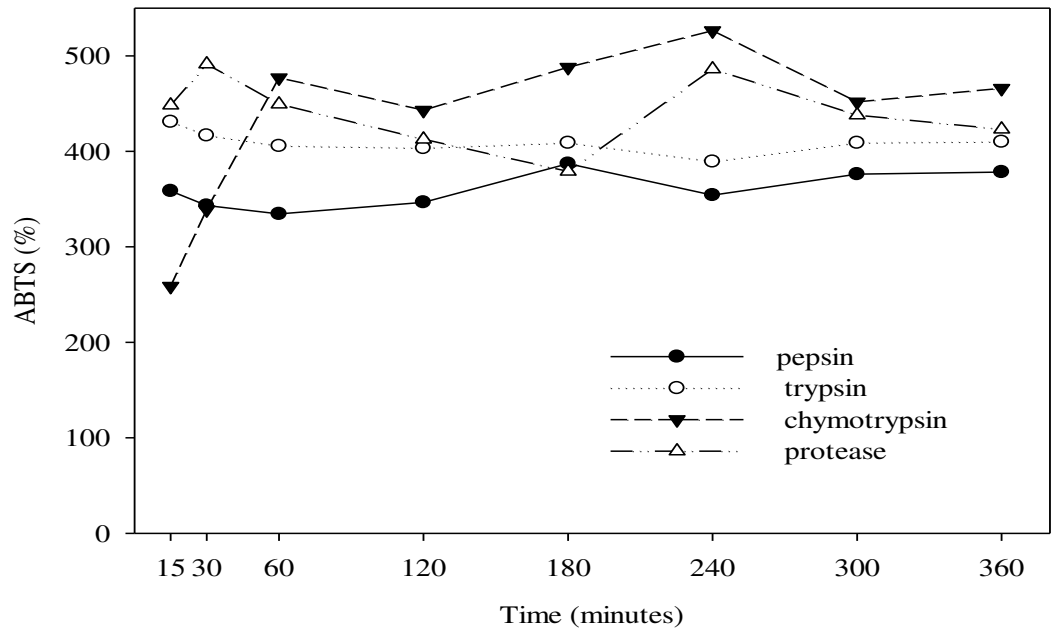

Figure 4.5 Antioxidant determinations by ABTS with 3 units enzyme concentration

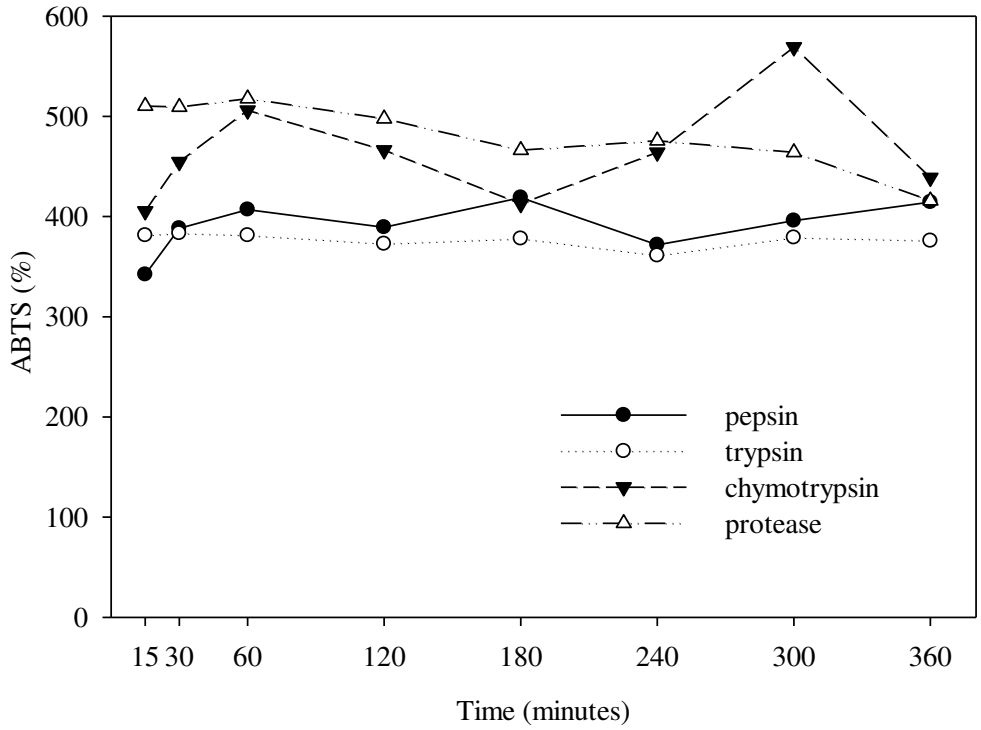

Figure 4.6 Antioxidant determinations by ABTS with 9 units enzyme concentration

Table 4.15 Determination of antioxidant activity by ABTS method from one way ANOVA

Time (minutes)	Pepsin		Trypsin		Chymotrypsin		Protease	
	3	9	3	9	3	9	3	9
15	358.57 ± 17.93^{aAX}	342.13 ± 17.11^{aA}	430.95 ± 21.55^{aB}	381.11 ± 19.06^{aB}	258.79 ± 12.94^{aX}	405.26 ± 20.26^{aY}	448.30 ± 22.42^{aD}	510.25 ± 25.51^{aD}
30	343.22 ± 17.16^{bAX}	388.18 ± 19.41^{bA}	416.69 ± 20.83^{bB}	383.21 ± 19.16^{bB}	338.84 ± 16.94^{bX}	454.60 ± 22.73^{bY}	491.35 ± 24.57^{bD}	509.20 ± 25.46^{bD}
60	258.79 ± 12.94^{cAX}	406.82 ± 20.34^{cA}	405.73 ± 20.29^{cB}	381.11 ± 19.06^{cB}	477.00 ± 23.85^{cX}	506.05 ± 25.30^{cY}	449.35 ± 22.47^{cD}	517.60 ± 25.88^{cD}
120	346.51 ± 17.33^{AX}	389.28 ± 9.46^{dA}	403.53 ± 20.18^{B}	372.71 ± 18.64^{dB}	443.01 ± 22.15^{X}	466.15 ± 23.31^{dY}	412.61 ± 20.63^{D}	497.65 ± 24.88^{dD}
180	387.08 ± 19.35^{AX}	418.88 ± 20.94^{A}	409.02 ± 20.45^{B}	377.96 ± 18.90^{B}	487.97 ± 24.40^{X}	412.61 ± 20.63^{Y}	379.01 ± 18.95^{D}	466.15 ± 23.31^{D}
240	354.19 ± 17.71^{fAX}	371.73 ± 18.59^{fA}	389.28 ± 19.46^{fB}	361.16 ± 18.06^{fB}	526.35 ± 26.32^{fX}	464.05 ± 23.20^{fY}	486.10 ± 24.31^{fD}	475.60 ± 23.78^{fD}
300	376.12 ± 18.81^{AX}	395.86 ± 19.79^{gA}	409.02 ± 20.45^{B}	379.01 ± 18.95^{gB}	451.78 ± 22.59^{X}	569.04 ± 28.45^{gY}	437.81 ± 21.89^{D}	464.05 ± 23.20^{gD}
360	378.31 ± 18.92^{X}	414.50 ± 20.72^{A}	410.11 ± 20.51^{B}	375.86 ± 18.79^{B}	466.04 ± 23.30^{X}	438.86 ± 21.94^{Y}	423.11 ± 21.16^{D}	415.76 ± 20.79^{D}

Small letters show significant difference of time on antioxidant activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antioxidant activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antioxidant activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Table 4.16 Determination of antioxidant activity by ABTS method from two ways ANOVA

Time	Enzyme type and Concentration	Enzyme type	Time and Concentration	Concentration	Time and Enzyme type
15	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{AX} Protease ^{CX}	Pepsin	15^{abX} 30^{abcX} 60^{aX} 120^{abcX} 180^{cX} 240^{abcX} 300^{bcX} 360^{cX}	3 units and 9 units of Pepsin	3^{aA} 9^{aA} 3^{abA} 9^{abA} 3^{abA} 9^{bA} 3^{bcA} 9^{abA} 3^{cA} 9^{abA} 3^{bcA} 9^{abA} 3^{bcA} 9^{bA} 3^{bcA} 9^{aA}
30	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{AX} Protease ^{BX}				
60	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{CX} Protease ^{CX}	Trypsin	15^{a} 30^{a} 60^{a} 120^{a} 180^{a} 240^{a} 300^{a} 360^{a}	3 units and 9 units of Trypsin	3^{aB} 9^{aA} 3^{abB} 9^{abA} 3^{abB} 9^{bA} 3^{bcB} 9^{abA} 3^{cB} 9^{abA} 3^{bcB} 9^{abA} 3^{bcB} 9^{bA} 3^{bcB} 9^{aA}
120	Pepsin ^{A} Trypsin ^{A} Chymotrypsin ^{B} Protease ^{B}				
180	Pepsin ^{AB} Trypsin ^{A} Chymotrypsin ^{B} Protease ^{AB}	Chymotrypsin	15^{aX} 30^{bX} 60^{cdX} 120^{cX} 180^{cX} 240^{cdX} 300^{dX} 360^{cX}	3 units and 9 units of Chymotrypsin	3^{aC} 9^{aB} 3^{abC} 9^{abB} 3^{abC} 9^{bB} 3^{bcC} 9^{abB} 3^{cC} 9^{abB} 3^{bcC} 9^{abB} 3^{bcC} 9^{bB} 3^{bcC} 9^{aB}
240	Pepsin ^{A} Trypsin ^{A} Chymotrypsin ^{B} Protease ^{B}				
300	Pepsin ^{AX} Trypsin ^{AX} Chymotrypsin ^{CX} Protease ^{BX}	Protease	15^{bX} 30^{bX} 60^{bX} 120^{abX} 180^{aX} 240^{bX} 300^{abX} 360^{aX}	3 units and 9 units of Protease	3^{aC} 9^{aB} 3^{abC} 9^{abB} 3^{abC} 9^{bB} 3^{bcC} 9^{abB} 3^{cC} 9^{abB} 3^{bcC} 9^{abB} 3^{bcC} 9^{bB} 3^{bcC} 9^{aB}
360	Pepsin ^{A} Trypsin ^{A} Chymotrypsin ^{B} Protease ^{AB}				

Small letters show significant difference of time on antioxidant activity by two way ANOVA at α = 0.05 level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antioxidant activity by two way ANOVA at α = 0.05 level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antioxidant activity by two way ANOVA at α = 0.05 level.

Table 4.17 Determination of antioxidant activity by ABTS method from three ways ANOVA

Time	Type of Enzyme	concentration
15[a]	Pepsin [A]	3 units [X]
30[ab]		
60 [b]	Trypsin [B]	
120 [ab]		
180 [ab]	Chymotrypsin [C]	9 units [X]
240 [b]		
300 [ab]	Protease [C]	
360[ab]		

Small letters show significant difference of time on antioxidant activity by three way ANOVA at α = 0.05 level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antioxidant activity by three way ANOVA at α = 0.05 level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antioxidant activity by three way ANOVA at α = 0.05 level

4.5 Antihypertensive Activity

Angiotensin-I converting enzyme (ACE) take a part in adjustment of human blood pressure (Raghavan and Kristinsson, 2009; Pfeffer, 1993). The presence of hydrophobic amino acids in the C-terminal side of the amino acids particularly proline, phenylalanine, tryptophan and tyrosine has a great impact on the ACE inhibitory activity (Haque and Chand, 2008). Furthermore, the availability of isoleucine and valine at the N-terminal positions provide a positive influence. Figures 4.7 – 4.8 summarizes the changes in ACE inhibitor activity of the bulgur waste samples. Generally, by applying three way ANOVA the bear of each factor such as a time and concentration displayed a significant ($P<0.05$) impact on the hydrolysis as shown in Table 4.20. Meanwhile, type of the enzymes influenced non-significantly ($P>0.05$) on the reaction.

Applying two way ANOVAdescribed in Table 4.19 provide the effect of time and various enzymes indicate a significant ($P<0.05$) effect on the activity of antihypertensive on bio-peptides. The enzyme type of pepsin, trypsin, chymotrypsin and protease had also significant ($P<0.05$) effect on the digestion. The alterations occurred in the both terminals of the amino acids during hydrolysis time and even the available number of amino acids provides a various anti-hypertension activity. In the same situation, a significant ($P<0.05$) enlargement recorded during the entire 360 minutes of hydrolysis except the 120 minute that proved to be non-significant ($P>0.05$). The degradation of ACE inhibitory proficiency may be recognized by the protein amount and the rate of hydrolysis which inaccessible quantity of peptides decline the reaction of ACE inhibitors.

The impact one way ANOVA of the applied factors such as a concentration provide that the usage of 3 units and 9 units of each enzyme indicated to be significant ($P<0.05$). The antihypertensive peptides at 15, 30, 60, 120, 180, 240, 300 and 360 minutes were also own the significant ($P<0.05$) ability to produce antihypertensive peptides from bulgur waste hydrolysis as discussed in Table 4.18.

The effect of pepsin, trypsin and chymotrypsin at 3 units and 9 units of the enzyme observed a significant ($P<0.05$) ability on the antihypertensive activity. Excludes proteases that proved to be non-significant ($P>0.05$) towards hypertension and inability to create peptides. The inhibition activities of the antihypertensive peptides

mainly improved by accessibilities of each Arginine and Lysine amino acids on the C-terminal side (Cheung et al., 1980; Ariyoshi, 1993). As mentioned, trypsin cleaves the peptides at the C-terminal side of the lysine and arginine. From this point of view, the deficiency in both stated amino acids at C-terminal of the bulgur waste hydrolysis may lead the declined results of trypsin and reduction in antihypertensive ability.

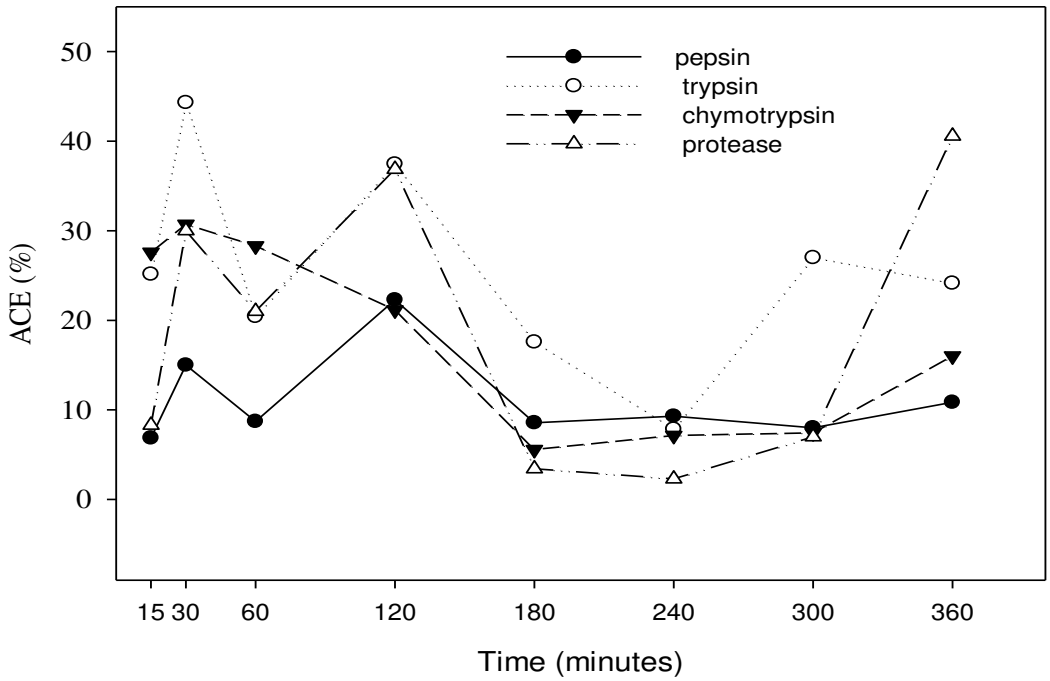

Figure 4.7 Indication of ACE activity by utilizing 3 units enzyme

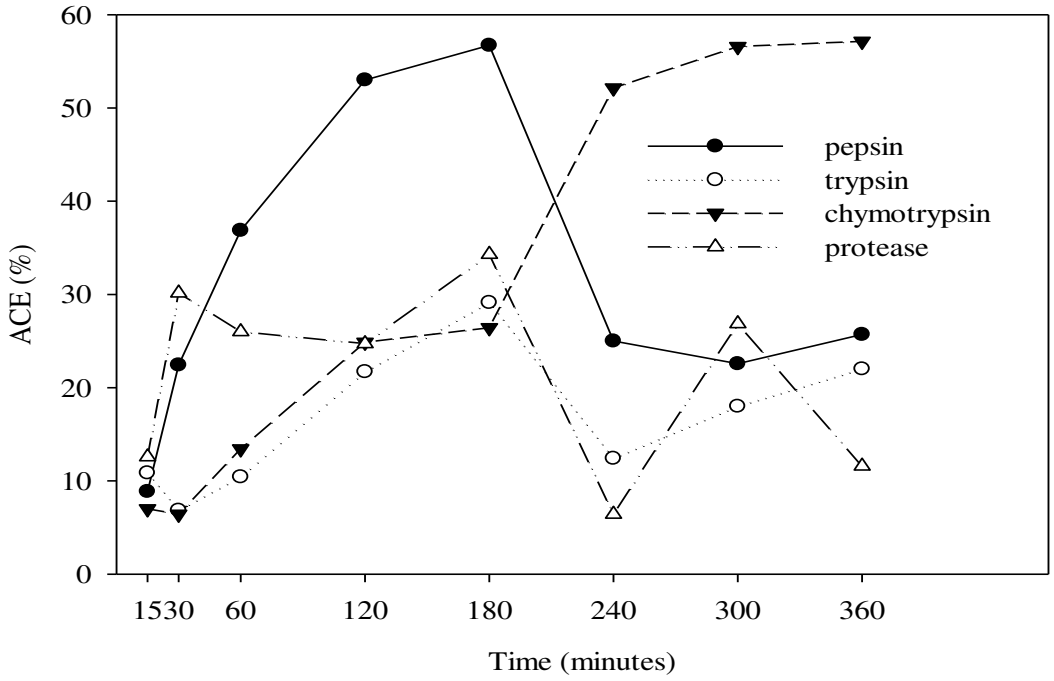

Figure 4.8 Indication of ACE activity by utilizing 9 units enzyme

Table 4.18 Determination of antihypertensive activity by ACE method from one way ANOVA

Time (minutes)	Pepsin		Trypsin		Chymotrypsin		Protease	
	3	9	3	9	3	9	3	9
15	6.86 ± 0.34^{aAX}	8.86 ± 0.44^{aAY}	25.14 ± 1.26^{aBX}	10.86 ± 0.54^{aBY}	27.57 ± 1.38^{aCX}	7.00 ± 0.35^{aCY}	8.29 ± 0.41^{DX}	12.57 ± 0.63^{DY}
30	15.00 ± 0.75^{bAX}	22.43 ± 1.12^{bAY}	44.29 ± 2.21^{bBX}	6.86 ± 0.34^{bBY}	30.71 ± 1.54^{bCX}	6.43 ± 0.32^{bCY}	30.00 ± 1.50^{DX}	30.14 ± 1.51^{DY}
60	8.71 ± 0.44^{cAX}	36.86 ± 1.84^{cAY}	20.43 ± 1.02^{cBX}	10.43 ± 0.52^{cBY}	28.29 ± 1.41^{cCX}	13.43 ± 0.67^{cCY}	21.00 ± 1.05^{DX}	26.00 ± 1.30^{DY}
120	22.29 ± 1.11^{dAX}	53.00 ± 2.65^{dAY}	37.43 ± 1.87^{dBX}	21.71 ± 1.09^{dBY}	21.14 ± 1.06^{dCX}	24.86 ± 1.24^{dCY}	36.86 ± 1.84^{DX}	24.71 ± 1.24^{DY}
180	8.57 ± 0.43^{eAX}	56.71 ± 2.84^{eAY}	17.57 ± 0.88^{eBX}	29.14 ± 1.46^{eBY}	5.57 ± 0.28^{eCX}	26.43 ± 1.32^{eCY}	3.43 ± 0.17^{DX}	34.29 ± 1.71^{DY}
240	9.29 ± 0.46^{fAX}	25.00 ± 1.25^{fAY}	7.86 ± 0.39^{fBX}	12.43 ± 0.62^{fBY}	7.14 ± 0.36^{fCX}	52.14 ± 2.61^{fCY}	2.29 ± 0.11^{DX}	6.43 ± 0.32^{DY}
300	8.00 ± 0.40^{gAX}	22.57 ± 1.13^{gAY}	27.00 ± 1.35^{gBX}	18.00 ± 0.90^{gBY}	7.43 ± 0.37^{gCX}	56.57 ± 2.83^{gCY}	7.00 ± 0.35^{DX}	26.86 ± 1.34^{DY}
360	10.86 ± 0.54^{hAX}	25.71 ± 1.29^{hAY}	24.14 ± 1.21^{hBX}	22.00 ± 1.10^{hBY}	16.00 ± 0.80^{hCX}	57.14 ± 2.86^{hCY}	40.57 ± 2.03^{DX}	11.57 ± 0.58^{DY}

Small letters show significant difference of time on antihypertensive activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antihypertensive activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antihypertensive activity by one way ANOVA at $\boldsymbol{\alpha} = 0.05$ level.

Table 4.19 Determination of antihypertensive activity by ACE method from two ways ANOVA

Time	Enzyme type and Concentration	Enzyme type	Time and Concentration	Concentration	Time and Enzyme type
15	Pepsin ^{AX} Trypsin ^{CX} Chymotrypsin ^{CX} Protease ^{BX}	Pepsin	15^{aX} 30^{cX} 60^{dX} 120^{fX} 180^{eX} 240^{bcX} 300^{bX} 360^{cX}	3 units and 9 units of Pepsin	3^{dA} 9^{aC} 3^{gA} 9^{bC} 3^{eA} 9^{eC} 3^{gA} 9^{abA} 3^{bA} 9^{fC} 3^{aA} 9^{dC} 3^{cA} 9^{eC} 3^{eA} 9^{eC}
30	Pepsin ^{AX} Trypsin ^{BX} Chymotrypsin ^{AX} Protease ^{CX}				
60	Pepsin ^{BX} Trypsin ^{AX} Chymotrypsin ^{BX} Protease ^{BX}	Trypsin	15^{cX} 30^{eX} 60^{bX} 120^{fX} 180^{deX} 240^{dX} 300^{deX} 360^{eX}	3 units and 9 units of Trypsin	3^{dC} 9^{aA} 3^{gC} 9^{bA} 3^{eC} 9^{cA} 3^{gC} 9^{eA} 3^{bC} 9^{fA} 3^{aC} 9^{dA} 3^{cC} 9^{eA} 3^{eC} 9^{eA}
120	Pepsin ^{C} Trypsin ^{B} Chymotrypsin ^{A} Protease ^{B}				
180	Pepsin ^{C} Trypsin ^{B} Chymotrypsin ^{A} Protease ^{A}	Chymotrypsin	15^{aX} 30^{abX} 60^{bcX} 120^{cX} 180^{aX} 240^{dX} 300^{dX} 360^{eX}	3 units and 9 units of Chymotrypsin	3^{dB} 9^{aC} 3^{gB} 9^{bC} 3^{eB} 9^{cC} 3^{gB} 9^{eC} 3^{bB} 9^{fC} 3^{aB} 9^{dC} 3^{cB} 9^{eC} 3^{eB} 9^{eC}
240	Pepsin ^{C} Trypsin ^{B} Chymotrypsin ^{D} Protease ^{A}				
300	Pepsin ^{A} Trypsin ^{B} Chymotrypsin ^{C} Protease ^{A}	Protease	15^{bX} 30^{fX} 60^{dX} 120^{fX} 180^{cX} 240^{aX} 300^{cX} 360^{eX}	3 units and 9 units of Protease	3^{dB} 9^{aB} 3^{gB} 9^{bB} 3^{eB} 9^{cB} 3^{gB} 9^{eB} 3^{bB} 9^{fB} 3^{aB} 9^{dB} 3^{cB} 9^{eB} 3^{eB} 9^{eB}
360	Pepsin ^{A} Trypsin ^{B} Chymotrypsin ^{C} Protease ^{A}				

Small letters show significant difference of time on antihypertensive activity by two way ANOVA at $\alpha = 0.05$ level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antihypertensive activity by two way ANOVA at $\alpha = 0.05$ level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antihypertensive activity by two way ANOVA at $\alpha = 0.05$ level.

Table 4.20 Determination of antihypertensive activity by ACE method from three ways ANOVA

Time	Type of Enzyme	concentration
15[a]	Pepsin [A]	3 units [X]
30[c]		
60 [bc]	Trypsin [A]	
120 [d]		
180 [c]	Chymotrypsin [A]	9 units [X]
240 [ab]		
300 [ab]	Protease [A]	
360[cd]		

Small letters show significant difference of time on antihypertensive activity by three way ANOVA at α = 0.05 level.

Capital letters (A, B, C and D) show significant difference of pepsin, trypsin, chymotrypsin and protease enzymes on antihypertensive activity by three way ANOVA at α = 0.05 level.

Capital letters (X) show significant difference of 3 units and 9 units of each enzyme on antihypertensive activity by three way ANOVA at α = 0.05 level.

CHAPTER 5

CONCLUSION

In this study, the waste of bulgur analyzed to discover available amounts of protein especially bioactive peptides. In particular the extracted protein from bulgur waste samples was performing to determine bioactive peptides such as; the inhibition of oxidation, microbial growth and hypertension.

The following results were investigated from bulgur waste samples.
Raw material determination:

- The available amount of moisture content in the bulgur waste was found to be 12.15%.
- The ash content determined to be 3.55 % on dry base matter.
- The fat content in the bulgur waste was indicated to be 4.58 %.
- The amount of protein showed 2.84 % in the bulgur waste samples.

Degree of hydrolysis:

- Two different concentrations of each enzyme indicated at 360 minutes of hydrolysis utilizing 37 C^o for each pepsin, trypsin, chymotrypsin enzymes and 50 C^o for protease respectively as hydrolysis temperature.
- By applying three different ways of ANOVA exclusively the results shows diverse hydrolysis ability.
- The effect of time and enzyme concentration was positive on the hydrolysis rate by Pepsin and Protease enzymes in one way ANOVA in diverse to Trypsin that 3 units concentration was not sufficient for hydrolysis and time provide inadequate ability to hydrolyze the bulgur waste by Chymotrypsin.
- By applying the results in two ways ANOVA all the enzymes proved to be enough to hydrolyze the bulgur waste samples except Trypsin that show

inactivity according to time changing also, except 300 minutes of hydrolysis the time was whole enough for hydrolysis.

- While, the best results has been seen by applying three ways of ANOVA that the results show positive ability towards hydrolyses in both concentrations, diverse enzymes and through the whole period of hydrolyses.

Antimicrobial activity:

- Bioactive peptides were to determine inhibition zone of microbial growth on the Gram positive *Staphylococcus aureus* and Gram negative bacteria *Escherichia coli*.
- The indications gathered by Gram positive bacteria Staphylococcus *aureus* offered an increase in inhibition zone through the 360 minutes of incubation. In the same time both concentrations had a positive effect on antimicrobial activity of the bulgur waste samples by applying one, two and three ways of ANOVA.
- On the other hand, Gram negative bacteria *Escherichia coli* provide diverse abilities which at pair of enzyme concentrations trypsin and chymotrypsin's inhibition ability increased by applying one way ANOVA while, hydrolysis by pepsin and protease to produce bioactive peptides with antimicrobial capability has been failed because of the insufficient enzyme concentrations.
- Two ways of ANOVA shows a positive effect through all circumstances.
- By providing three ways ANOVA both concentrations show non-sufficient ability of Gram-negative bacteria.

Antioxidant activity:

- Two different methods has been used to indicate antioxidant activity were ABTS and DPPH.
- DPPH provide a fit results towards the three factors by utilizing 9 units of each enzyme meanwhile, only pepsin was show an activity by using 3 units which after 180 minutes of incubation the antioxidant disappeared.
- Two ways of ANOVA provide a positive ability of 9 units of the enzymes except trypsin were non-sufficient to create antioxidant peptides and decreasing ability were because of the low amount of concentration or enzyme type.
- Time affected three ways of ANOVA negatively towards whole effects.

- ABTS ability to create antioxidant peptides was positive by one way ANOVA only by pepsin in the first three period of time because of the not sufficient concentration. In the same time, Chymotrypsin enzyme effect negatively on the antioxidant activity.
- By two ways ANOVA till one hour incubation the antioxidant activity of Pepsin and Chymotrypsin were precise by both concentration. The enzyme type affect was not suitable to make bioactive peptides with antioxidant activity.
- Three ways of ANOVA was non-acceptable because of time through the whole processes.

Antihypertensive activity:

- The pair concentrations of each pepsin, trypsin and chymotrypsin increased versus to time by both concentrations through applying one way of ANOVA. But, time affected protease negatively anti-hypertension ability was not shown.
- The anti-hypertension activity of the produced peptides from bulgur waste samples hydrolysis showed a positive effect by two ways ANOVA in both concentrations except the 120 minutes of incubation show decreasing in activity.
- By applying three ways of ANOVA the enzyme time affecte the reaction negatively and does not show any effect towards the hypertension while, time and concentrations effect were sufficient.

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