TURKISH REPUBLIC
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF FOOD ENGINEERING

IN VITRO DETERMINATION OF THE ANTIHYPERTENSIVE ACTIVITIES OF FRESH BEEF AND PASTIRMA HYDROLYSATES

Necla ÖZER

SUPERVISIOR
Assist. Prof. Dr. Abdulatef AHHMED

MSc Thesis

May, 2017
KAYSERİ

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KAYSERİ

SCIENTIFIC ETHICS SUITABILITY

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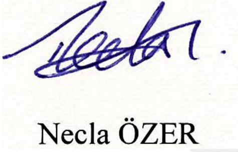

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The MSc thesis entitled **"*In vitro* Determination of The Antihypertensive Activities of Fresh Beef and Pastirma Hydrolysates**" has been prepared in accordance with Erciyes University Graduate Education and Teaching Institute Thesis Preparation and Writing Guide.

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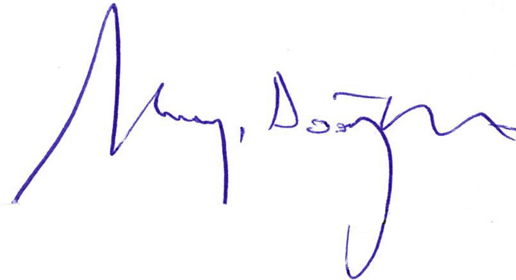

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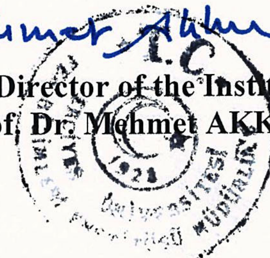

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TAZE SIĞIR ETİ VE PASTIRMA HİDROLİZATLARININ ANTİHİPERTANSİF AKTİVİTELERİNİN *İN VİTRO* ORTAMDA DEĞERLENDİRİLMESİ

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Yüksek Lisans Tezi, Mayıs 2017
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ÖZET

Son zamanlarda, gıdaların özellikle de etin biyolojik içeriği, yaşam tarzı ile ilişkili hastalıklara karşı popüler araştırma alanlarından biri olmuştur. Et ürünleri ile ilgili en çok çalışılan alanlardan biri, vücuttaki gerginlik seviyesini dengeleyen Anjiyotensin-I Dönüştürücü Enzimi (ACE) inhibe eden biyopeptitlerin hidrolizidir.

Bu çalışmada, pastırmanın geleneksel üretim sürecinde et proteinlerinin yapısında meydana gelen değişikliklerin araştırılması ve de taze ette (FM), çemenlenmemiş (PBC) ve çemenlenmiş pastırmada (PS) bulunan ACE inhibitör peptitlerin varlığının ispatlanması ve bu peptitlerin aktivitelerinin karşılaştırması amaçlanmıştır. Pastırma üretim sürecindeki farklı adımların etin protein yapısına etkisi sodyum dodesil sülfat poliakrilamit jel elektroforez (SDS-PAGE) tekniği ile gözlenmiştir. Jel görüntüleri, tüm protein fraksiyonlarının pastırma üretim süreci boyunca değiştiğini göstermektedir. Örnekler, ACE inhibe edici aktiviteyi saptamak amacıyla *in vitro* ortamda insan sindirim sistemi taklit edilerek pepsin ve tripsin enzimleri ile enzimatik sindirime maruz bırakılmıştır. Sonuçta, üç örnek de ACE'ye karşı 5 farklı konsantrasyonda inhibisyon göstermiş ve seyreltilmemiş FM, PBC ve PS örneklerinin inhibisyon oranları sırasıyla 85.55%, 62.4% ve 77.24%; IC_{50} değerleri ise sırasıyla 1.13, 4.06 ve 0.92 mg/ml olarak belirlenmiştir. Sonuçlar, örnekler arasında pastırma proteinlerinin en yüksek antihipertansif etkinliğe sahip olduğunu göstermektedir. Pastırma üretimi boyunca, protein oksidasyonunu belirten tiyol gruplarının sayısı taze ette 477 µmol/100g; çemenlenmemiş pastırma ve son ürün olan pastırmada sırasıyla 394 ve 116 µmol/100 g olarak hesaplanmıştır. Ayrıca, bromfenol mavisi ile boyama tekniği kullanılarak pastırmanın protein yüzey hidrofobikliğinin taze etinkinin 2 katı olduğu kanıtlanmıştır. Sonuç olarak, geleneksel bir et ürünü olan pastırmanın, içeriğindeki nutrasötik bileşenleri sayesinde fonksiyonel bir gıda olarak kullanılabileceği ispatlanmıştır.

Anahtar kelimeler: Pastırma; et; ACE; biyoaktif peptitler; antihipertansif efekt; hipertansiyon.

IN VITRO DETERMINATION OF THE ANTIHYPERTENSIVE ACTIVITIES OF FRESH BEEF AND PASTIRMA HYDROLYSATES

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ABSTRACT

In recent years, the effect of the biofunctional properties of foods, especially meat, on lifestyle-related diseases has been a popular area of research. Specifically, one of the most studied properties of meat products is the hydrolysis of muscle proteins into biopeptides that inhibit angiotensin-I converting enzyme (ACE), which balances tension levels in the body. The aims of this study were to investigate the nature of chemical changes in proteins during the traditional process of pastirma production, and to compare the coexistence and activity of ACE inhibitory peptides isolated from fresh meat (FM), pastirma before cemen covering (PBC), and pastirma (PS) samples. The effect of the different manufacturing phases of pastirma on the protein structure of meat was observed using Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE). Acrylamide gel images demonstrated that some protein fractions were changed during the pastirma production process. Additionally, to determine the ACE inhibitory activity of FM, PBC, and PS, samples were hydrolyzed with pepsin and trypsin to simulate the human digestive system. All 3 samples (FM, PBC and PS) showed a systematic inhibition of ACE at 5 different concentrations, while undiluted samples showed inhibition rates of 85.55%, 62.4%, and 77.24%, respectively. IC_{50} values of the samples (FM, PBC and PS) were calculated as 1.13, 4.06, and 0.92 mg/ml, respectively. Among all the samples, PS hydrolysates exhibited the highest antihypertensive activity. Regarding protein oxidation, the number of thiol groups of FM was 477 μmol/100g while non-digested PBC and PS were 394 and 116 μmol/100g. Additionally, bromophenol blue staining indicated protein hydrophobicity, showing a 2-fold increase in the pastirma processing due to denaturation of protein structure. In conclusion, it might be considered that pastirma, a traditional meat product, contains a considerable number of constituents that could be utilized as functional food and nutraceuticals. The results may signify the importance of dietary alternatives to chemicals to prevent hypertensive diseases.

Keywords: Pastirma; meat; ACE; bioactive peptide; antihypertensive effect; hypertension.

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ABBREVIATIONS

Abbreviation	Meaning	Unit
ACE	Angiotensin Converting Enzyme	--
ATP	Adenosine Triphosphate	--
BPB	Bromophenol Blue	--
CBB	Coomassie Brilliant Blue	--
Cfu	Colony forming unit	cfu
$CuSO_4$	Copper (II) Sulfate	--
Da	Dalton	Dalton
dH_2O	Distilled Water	--
DNPH	2,4-dinitrophenylhydrazone	--
DTNB	5’-dithiabis (2-nitro-benzoicacid)	--
EDTA	Ethylenediaminetetraaceticacid	--
g	Grams	Grams
GC–MS	Gas Chromatography–Mass Spectrometry	--
GS-ATP	Guba- Straub-ATP	--
h	Hour	Hour
HCl	Hydrochloric acid	--
H_2O	Water	--
HPLC	High Pressure Liquid Chromatography	--
IC_{50}	Inhibiting Concentration $_{50}$	--
KCl	Potassium Chloride	--
kDa	Kilo Dalton	Kilo Dalton
KH_2PO_4	Monopotassium Dihydrogen Phosphate	--
K_2HPO_4	Dipotassium Hydrogen Phosphate	--
Kg	Kilo grams	Kilo grams
KNO_3	Potassium Nitrate	--
K_2SO_4	Potassium Sulfate	--
L	Liter	Liter
M	Molarity	mol/L
mA	Milliampere	Milliampere
Mb	Myoglobin	--

MHC	Myosin Heavy Chain Protein	--
mg	Milligram	Milligram
min	Minute	minute
ml	Milliliter	milliliter
mM	Millimolar	millimolar
mmol	Millimoles	Millimoles
MW	Molecular Weight	g/mol
N	Normality	eq/L
NaCl	Sodium Chloride	--
Nm	Nanometer	Nanometer
NaOH	Sodium hydroxide	--
RAS	Renin Angiotensin System	--
RP-HPLC	Reverse Phase High Pressure Liquid Chromatography	--
rpm	Revolution per minute	--
SDS-PAGE	Sodium Dodecyl Sulfate- Poly Acrylamide Gel Electrophoresis	--
sec	Second	second
TCA	Trichloroacetic Acid	--
TMAB	Total Mesophilic Aerobic Bacteria	cfu
UV-Vis	Ultra Viyole – Visible	--
v/v	volume/volume	--
WSP	Water Soluble Protein	--
w/v	weight/volume	--
µg	Microgram	Microgram
µm	Micro meter	Micro meter
µM	Micromolar	Micromolar
µmol	Micromoles	Micromoles
µl	Micro liter	Micro liter

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INTRODUCTION

Recently, scientists have become aware of the therapeutic properties of certain food ingredients to prevent diseases, since it is known that dietary proteins are a source of biologically functioning peptides. In particular, bioactive peptides that have antimicrobial, antioxidant, and antihypertensive activities are generated during the manufacture of foods. Nowadays, a large number of individuals suffer from chronic diet- and lifestyle-related diseases such as hypertension, osteoporosis, diabetes, and cancer. Many scientists believe that methods different from chemical medication should be identified and tested in the effort to reduce life-style related diseases. Therefore, potential functional and nutraceutical methods are being considered for use as natural-based treatments to minimize the suffering of individuals as the result of lifestyle-related diseases. Bioactive peptides are a novel medical alternative, and have been the focus of researchers in the last two decades.

The first study of bioactive peptides was conducted by Mellander et al, 1950 [1]. Mellander found that bioactive peptides obtained from casein contributed to bone development independently of vitamin D in rachitic babies. Over the last 20 years, interest in this topic has increased; however, most researchers focused on the production of bioactive peptides from milk proteins [2]. There are many bioactive peptides sourced from milk, soya, chicken, fish, pork, and beef proteins that have antihypertensive, antidiabetic, antimicrobial, anti-lipase, and antioxidant activity [3]. Of most interest was the discovery of antihypertensive peptides that are involved in the inhibition of angiotensin converting enzyme (ACE). It is known that ACE plays an important role in hypertension by regulating blood pressure [4].

One of the most common types of cardiovascular disease is hypertension, in which the blood pressure is persistently higher than normal values (systolic pressure 120 mmHg

and diastolic pressure 180 mmHg) [5]. In addition, hypertension is largely influenced by lifestyle-related habits, including consumption of foods containing high levels of sodium or fats, presence of high stress, and use of medications. However, consuming functional foods can be a viable option to reduce hypertension.

Therefore, ACE inhibitory peptides have become the focus of research for developing functional foods that contribute to the homeostasis of the human body. ACE is a well-characterized Zn^{4+} metallopeptidase that removes the carboxy-terminal dipeptide from the decapeptide angiotensin I to generate the potent vasoconstrictor angiotensin II. By other means but not chemical to inhibit this action, bioactive peptides are required [6]. For this reason, ACE inhibitors sourced from foods are used as therapy against hypertension. Antihypertensive drugs with ACE inhibitors are usually used for hypertension treatment. These synthetic medications have been reported to have side effects such as hypotension, angioedema, skin rashes, dizziness, tiredness, cough, and headache, as well as heart damage and stroke [7, 8].

Meat is a rich source of bioactive peptides because of its protein structure. In many studies, the protein of chicken, fish, and pork have been shown to have bioactive peptides, specifically ACE inhibitory peptides. However, there is not sufficient research regarding the purification of ACE inhibitory peptides from beef. Most research regarding bioactive peptides purified from meat have been conducted on chicken and pork.

Fresh meat and pastirma are the most commonly-consumed meat products in Turkey. Because meat proteins are broken down by proteolytic enzyme activities during the process of pastirma production, pastirma may contain novel bioactive peptides with unique physiologic effects. In particular, it is possible that bioactive peptides in pastirma could have many nutritional and therapeutic functions. However, it is not clear if their function is affected when pastirma is exposed to cooking and digestion. Little information is available regarding the nature of chemical changes in proteins during the traditional process of pastirma production. Therefore, determining the antihypertensive peptide sequences from meat products such as pastirma is a novel approach. Due to lack of information concerning the bioavailability of pastirma, this research provides valuable insight into the potential therapeutic compounds in this traditional food.

The aim of this research was to compare the existence and activity of ACE inhibitory peptides from fresh meat, dried and pressed meat, and pastirma. Detection of the ACE inhibitory peptides from pastirma proteins indicates that pastirma can be considered as a functional food. However, pastirma cannot be consumed by everyone due to its high salt content. Instead, it could be used as a model to synthesize or resource and encapsulate prodrug-type peptides to treat hypertension.

This study is unique, as it focuses on the identification of bioactive peptides that have functional and therapeutic effects on lifestyle-related diseases from traditional Turkish pastirma. However, a previous study focused on pastirma produced from different type of muscle, and their results provided valuable evidence for the health benefits of such traditional products [9]. This study investigated potential bioactive peptides that might be present in the structure of pastirma, considering that such dry cured meat products may have small peptides separated from the source protein even before they are digested by the human body.

CHAPTER 1

GENERAL INFORMATION and LITERATURE SURVEY

Recently, many new reports have been published regarding novel advances in the identification of bioactive compounds and their effects on human health. Researchers have focused on how bioactive substances can be used as functional food components. Although there is a need for additional research regarding food utility, other research has demonstrated that functional food components play a role in treating disease, including cardiovascular disease and hypertension. The disadvantage of the macronutrients present in food is that they are slowly absorbed by the digestive system. In order to increase uptake and thereby improve the advantageous effects of food components, new approaches are required to achieve maximum health benefits. Laboratory research has shown that bioactive compounds have a role in blocking and interfering with molecular level processes.

1.1. Hypertension

Cardiovascular diseases are the most common cause of death globally, killing 17.5 million people per year [10]. Hypertension, also known as high blood pressure, is one of the most common cardiovascular diseases, and is indicated by a constant rise in blood pressure under resting circumstances, which leads to damage to many tissues and organs over time.

High blood pressure is defined as increased pressure in the arteries above the values of systolic and diastolic pressure 140 mmHg and 90 mmHg, respectively. Global statistics show that high blood pressure, which has caused 9.4 million deaths and 7% of disease in 2010, is the leading risk factor for preventable death. Moreover, its global prevalence in adults aged 18 years and over was approximately 22% in 2014 [11]. Furthermore, this

prevalence is predicted to increase. In Turkey, several studies have shown that the prevalence of hypertension in the population over 40 years of age ranges between 20% and 38%, according to region [12]. Locally, this is considered to be a major public health problem, and natural means of treatment are necessary.

According to the "Causes of Death Statistics" report of the Turkish Statistical Institute, circulatory system diseases, which account for 40.3% of deaths, ranked first among causes of death in Turkey in 2015 [13]. Table 1.1 shows that 9.7% of deaths caused by diseases of the circulatory system in Turkey are because of hypertensive diseases.

Table 1.1. Distribution of deaths caused by diseases of the circulatory system by subgroups

	2015	
	Number	**(%)**
Diseases of the circulatory system	**157 965**	**100.0**
Ischaemic heart diseases	64 012	40.5
Cerebrovascular diseases	38 412	24.3
Other heart diseases	32 198	20.4
Hypertensive diseases	15 352	9.7
Other	7 991	5.1

The main cause of hypertension has not been determined, but it is known that ACE has a central role in regulating blood pressure. It is also well-known that the elasticity of the artery wall decreases and smaller blood vessels become narrower with age, which increases the heart rate [5]. Genetic factors have an important influence on hypertension. In addition, smoking, alcohol, dieting, obesity, physical inactivity, hormonal contraceptives, and stress are factors that may increase blood pressure [5, 7, 14]. Other diet-related factors associated with hypertension include unbalanced diets with high salt and saturated fat content [5]. The number, complexity, and interaction of the factors that cause hypertension have made it challenging for scientists and medical professionals to control this disease.

1.1.1. Hypertension Types

I. Primary Hypertension: present in patients those have no other illness related to hypertension, with a prevalence of 90-95%. The cause of this disease is unknown [15].

II. Secondary Hypertension: present in 5-10% of patients where hypertension arises as the result of another illness or condition, such as diabetes mellitus, obesity, high cholesterol, and dyslipidemia [15].

Antihypertensive drugs with ACE inhibitors are usually used to treat hypertension. These synthetic medications have been reported to have side effects such as hypotension, angioedema, skin rashes, dizziness, tiredness, cough, and headache, as well heart damage and stroke [7, 8]. Due to these side effects, functional foods have increasing relevance to reducing health problems. Food that contains ACE inhibitory peptides represents an alternative to chemical drugs in the treatment of hypertension.

1.2. Bioactive Peptides

Because it has been shown that dietary proteins are a source of biologically functioning peptides, functional foods are believed to have therapeutic properties. Bioactive peptides sourced from animal or vegetable origins may have a role in regulation of human metabolism beyond their effect when consumed. In other words, bioactive peptides, which result from enzymatic hydrolysis of food proteins during digestion, have additional functional properties. Exhibiting biological effects at measurable physiological levels and having a beneficial effect to health are the most desirable properties of bioactive peptides [8].

Bioactive peptides are inactive while they are in the structure of the source protein. They rarely appear as peptide sequences, as they are normally digested by enzymes such as pepsin, trypsin, and chymotrypsin [16]. Bioactive peptides usually range in length between 2-30 amino acids [17]. Bioactive peptides that are sourced from food show physiological effects such as antihypertensive, anti-oxidative, antimicrobial, prebiotic, mineral binding, antithrombotic, hypocholesterolemic, and immunomodulatory effects [18].

A review of the literature indicated that few studies have been performed on the bioactive peptides of beef proteins, particularly in the dried cured meat product, pastirma, with the exception of the studies conducted by Ahhmed et al. [9] and Toldra [19]. Isolation and identification of antihypertensive bioactive peptides from dried cured beef product is an important endeavor, particularly for Turkish society. The study conducted by Ahhmed et al. [9] on the muscle protein of pastirma showed it may contain novel peptide sequences that are created due to exposure to the production processes [20]. It is predicted that pastirma has potential bioactive peptides, particularly antihypertensive peptides, since a great deal of protein decomposition takes place during processing. Therefore, this study, which examined the ACE inhibitory peptides that may exist in pastirma and beef meat, is of significant relevance.

1.2.1. Production of Bioactive Peptides

While there are many methods for releasing bioactive peptides from foods, isolation by digestive enzymes remains the most preferred method to simulate the human stomach. Many bioactive peptides sourced from meat proteins have been produced by enzymatic hydrolysis. Proteases used to produce peptides are sourced from bacteria, animal, and plant tissues. However, pepsin and trypsin are the two most common enzymes that are used for meats, notably pork meat [21, 22]. There are many other ways to generate peptides, including microwave, high pressure, and ultrasonic treatment; however, none of these adequately simulate the human digestive system.

1.2.2. Identification of Bioactive Peptides

As discussed above, because bioactive peptides are inactive while they are in the polymer structure of crude protein, proteins must be hydrolyzed for their release. After the hydrolysis of food protein, the bioactivity of hydrolysates is determined. The hydrolysates are fractioned according to peptide size using ultrafiltration for purification and identification, and the various bioactivities of hydrolysates are regulated. Reverse phase high performance liquid chromatography (RP-HPLC) or gel permeation chromatography are commonly used to purify and separate peptides based on molecular weight [23, 24]. Activity is checked on the sample collected at the peak of the spectrum. An aliquot of this sample is injected into HPLC to purify the sample. Mass spectrometry

and protein sequencing are combined to identify peptide fractions. Then, the amino acid sequence obtained is used to produce a synthetic version of the peptide. Finally, the analyses are repeated to determine bioactivity [25].

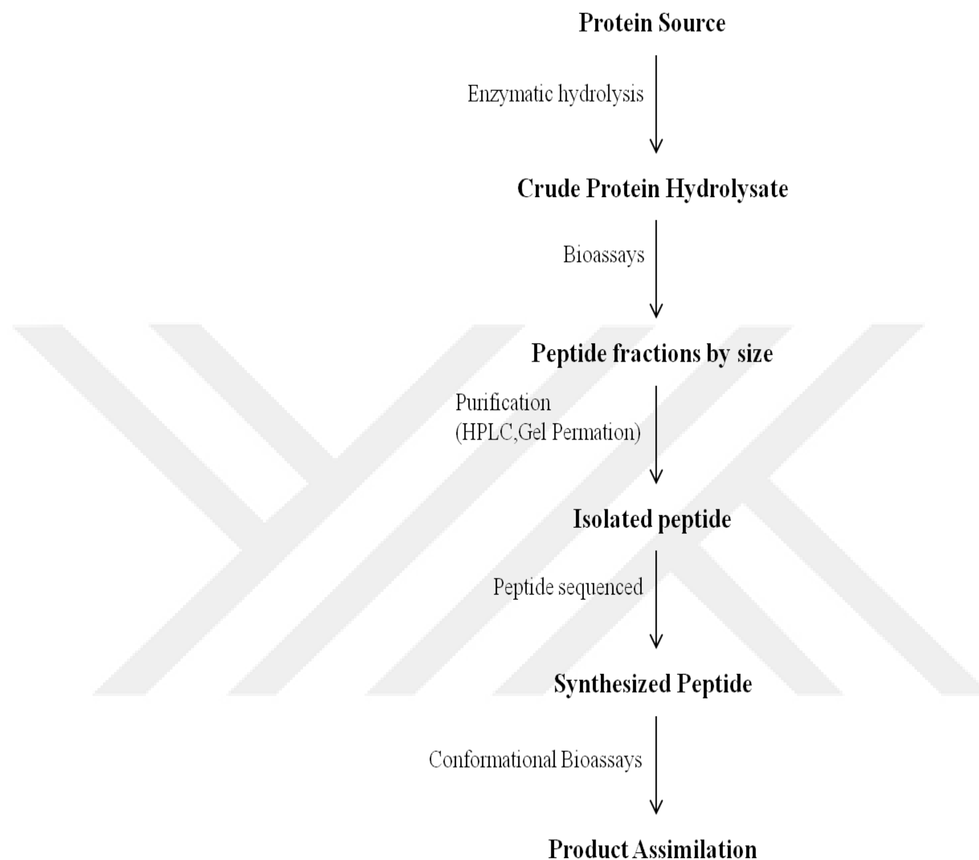

Figure 1.1. General scheme shows how to isolate and identify procedure of bioactive peptides from food proteins, adapted from Arihara & Ohata [22].

1.3. Mechanism of ACE and inhibitory peptides

ACE is a circulating transmembrane dipeptidyl peptidase that is capable of cleaving any peptide [5]. It plays an important role in the renin-angiotensin system (RAS) and consequently on regulation of blood pressure. In addition, it catalyzes the transformation of the inactive form of angiotensin I to active angiotensin II (Figure 1.2), and angiotensin II to angiotensin III, which can lead to death. Angiotensin II directly causes contraction of vascular smooth muscle cells. Thus, if the RAS is overactive, it causes an increase in blood pressure. Furthermore, ACE deactivates the vasodilator peptide, bradykinin, which is responsible for enlarging blood vessels; hence, it contributes to a decrease in blood pressure [3, 5, 8, 27].

Chemical or food active compounds are used to inactivate ACE because of its adverse effects on blood pressure. ACE inhibitory medications effectively inactivate ACE, but they have many side effects such as hypotension, angioedema, skin rashes, dizziness, tiredness, cough, headache, and heart damage [7, 8]. Instead of these medications, peptides that have similar inhibitory effects may be considered, as they have fewer side effects. Bioactive peptides with the ability to inhibit ACE activity act as a competitor to the RAS, since ACE prefers the ACE inhibitory peptide instead of Angiotensin I. There are two ways in which an antihypertensive peptide can inactivate ACE, either by binding to the ACE active site or to the inhibitor site. In either way, it prevents Angiotensin I from binding to the enzyme (Figure 1.3).

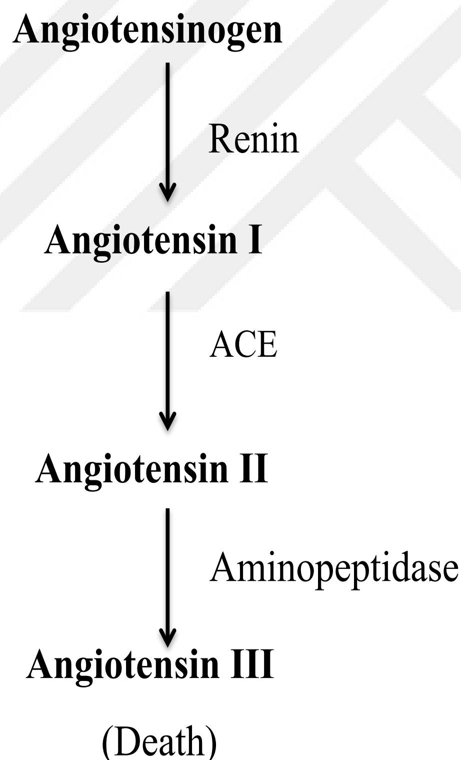

Figure 1.2. The Renin-Angiotensin System [4]

Normally, different gastrointestinal enzymes of suitable pH levels digest proteins, and subsequently the intestinal villi absorb ACE inhibitory peptides, where they interact with ACE and block it in the bloodstream. The bioactive peptides are classified into 3 types: the “true inhibitor-type”, the “substrate-type”, which has a weak inhibitory activity, and the “prodrug-type”, which is converted to the “true inhibitor-type”. In general, ACE inhibitory peptides derived from meat are the “true inhibitor-type” [3, 28].

These peptides show more activity after incubation with ACE than they do in *in vitro* conditions, indicating that these peptides are converted to the true inhibitor-type, which exhibit an increased activity after being hydrolyzed by ACE. *In vivo* studies of antihypertensive peptides showed that only true inhibitor-type peptides and prodrug-type peptides reduced systolic blood pressure in spontaneously hypertensive rats (SHR) [26]. The strength of an ACE inhibitor is usually measured by the concentration that leads to 50 percent inhibition of ACE activity, and it expressed analytically as the IC_{50} value [27]. The lower the IC_{50} value indicates the stronger the inhibition of ACE activity of the peptide.

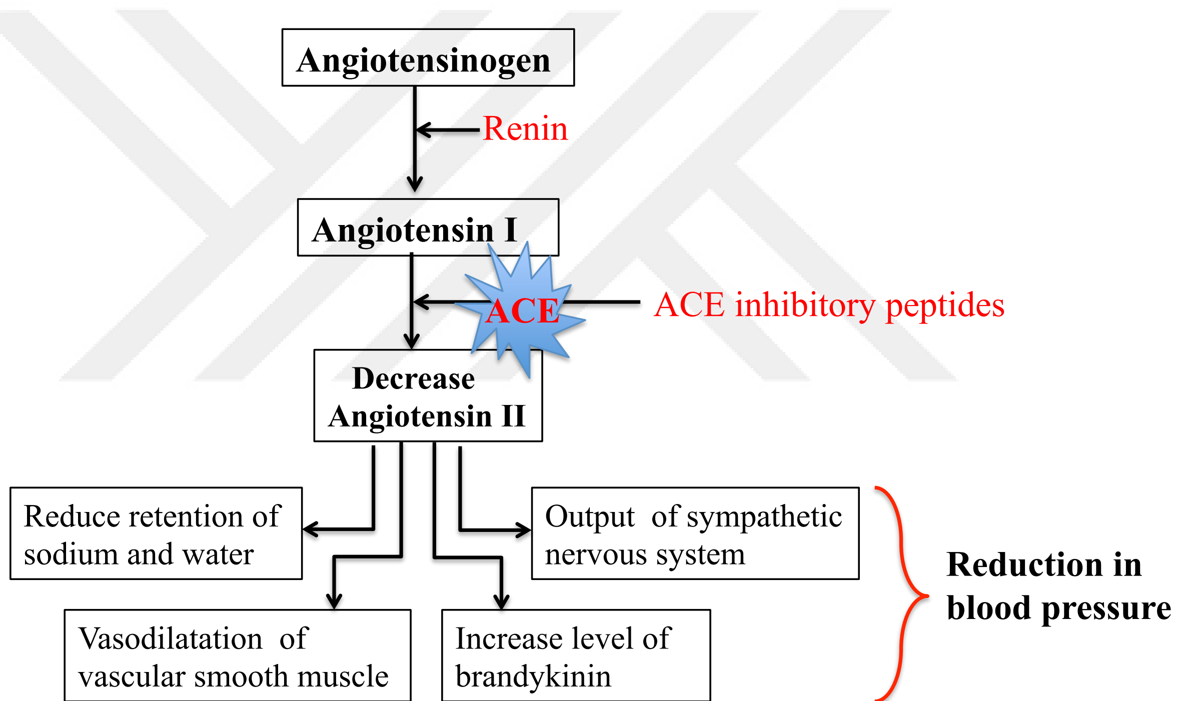

Figure 1.3. Action mechanism of ACE inhibitory peptides [4]

1.4. Meat

Meat is obtained from different muscles of animals that have various protein structures and amino acid sequences due to genetic variation. In addition, meat contains different fractional proteins, such as myofibrillar (myosin, actin, and troponin), sarcoplasmic, and stroma proteins. For this reason, more research is necessary to differentiate muscle types and muscle proteins. Additional studies using diversified methods of hydrolyzing different muscle types may be required to maximize the strength of the bioactivity theory.

For example, Arihara et al. (2010) found two active peptides from pork (*Biceps femoris*) as a result of hydrolysis of myosin protein by thermolysin. One was myopentapeptide A, which has an MNPPK (Met-Asn-Pro-Pro-Lys) amino acid sequence, while the other was myopentapeptide B, which has an ITTNP (Ile-Tht-Asn-Pro) amino acid sequence. Antihypertensive effects of peptides were tested on rats *in vivo* as follows: each peptide was dosed at 1 mg/kg body weight and after 6 h, blood pressure decreased to 23.4 ± 3.0 mmHg and 21.0 ± 3.1 mmHg for myopentapeptide A and myopentapeptide B, respectively. After 24 h, blood pressure of the experimental group was lower than that of the control group. This study showed that myopentapeptide A and myopentapeptide B, both isolated from pork muscle, are potential *in vivo* antihypertensive peptides [29].

Muguruma et al. (2009) isolated the KRVITY (Lys-Arg-Val-Ile-Thr-Tyr) (M6) peptide by hydrolysis of myosin B with pepsin in pork (*longissimus dorsi*). *In vivo* studies of rats demonstrated that after oral administration of M6, the systolic blood pressure of rats decreased by 12 mmHg in 3 h and 23 mmHg in 6 h, indicating that M6 significantly reduces blood pressure in mammals. The maximum reduction in blood pressure was achieved between 3 and 6 h after oral administration, indicating that M6 is an important peptide in this respect, and is a potential prodrug [28].

In addition, Ahhmed et al. [9] determined the presence of antihypertensive biopeptides on pastirma produced from M. *Longissimus dorsi* in 2015. The muscle used for pastirma production in their study was different from the pastirma muscle used in the current study. *In vitro* studies showed that fresh meat had a higher ACE inhibitory activity, with an IC_{50} value of 0.68 mg/ml, while pastirma had an IC_{50} value of 0.78 mg/ml [9].

Thereafter, it was shown that enzymatic hydrolysates of food proteins contain different ACE inhibitory peptides, including from casein [30], whey protein [31], fish and porcine protein [32], chicken muscle and egg protein [32], hemoglobin [33], blood plasma proteins [34], gelatin [35], buckwheat protein [36], wheat germ [37], corn gluten [38], soybean protein [39], garlic [40], and algae [41]. Several studies of meat muscle proteins from pork, chicken, and fish have been published. However, the first identified ACE inhibitory peptide from beef hydrolysate was reported to be a hexapeptide with the amino acid sequence VLAQYK (Val-Leu-Ala-Gln-Tyr-Lys); this peptide had an IC_{50} value of 32.06 μM, as reported by Jang et al. [42]. Later, the same group found different

bioactive peptides in bovine muscle hydrolysates [43]. However, there is still a lack of information on the different types of muscles and protein fractions of beef. Furthermore, as can be seen from the literature, there is a lack of effort to find antihypertensive peptides in processed meat products, such as dried-cured meat products. In particular, there are very few studies of traditional Turkish beef products.

1.5. Pastirma

The Turkish region of Anatolia has a long history of wealthy food culture, with a diet high in meat. Some traditional meat products such as pastirma are consumed in excess. Pastirma is a dry cured meat product that is commonly produced in Kayseri, Turkey. It is a popular product produced from whole beef muscle [44]. Pastirma used to be produced and consumed exclusively in the Kayseri region of Turkey, but now it is available in retail markets throughout Turkey [45].

1.5.1. Production of Pastirma

For pastırma production, muscles obtained from beef carcasses are cut into acceptable amounts, then fat layers and connective tissiue are removed. For traditional production, the first step of the process is the dry curing in which meat is treated with a curing mixture ($NaCl + KNO_3$) [45]. Each side of muscles are salted for 1 day. After dry curing, the muscles are washed with water in order to remove excess salt from the surface. Then the muscless are dried in the open air and pressed with the help of squeezing equipment, which presses 25 kg weight per kg of meat [44]. Furthermore, the muscles are hanged for second drying process in the shade for 3 days at 15–20 ◦C. After the product is pressed again, the surface of the muscle is covered with a paste (cemening). This paste is called as ‘cemen’ and contains 12%, 20%, 13% and 55% of milled fenugreek seeds, crushed garlic, red pepper and water, respectively [20]. Cemen covered muscles are cured again for 1 day in hot weather, and 1-2 days in cooler weather. After that, the cemen is left a thin layer and the product is dried again for 2 days. Finally, the pastirma is ready to sent to the market.

Muscle and protein structures undergo many physicochemical changes during the pastirma production process [20]. During this period, free amino acid and free fatty acids are obtained from the hydrolyzation of muscle proteins and lipids by the

endogenous enzymes in muscle [46], and also by the curing process. However, in the case of dry cured beef products like pastirma, the key factor that activates endogenous enzymes, which act on muscle proteins, has not been determined to date. The proteolytic activity during meat processing generates a large amount of peptides and free amino acids through proteolysis mechanisms by calpains, cathepsins, and peptidases [47]. The most interesting peptides are the bioactive peptides that potentially provide health benefits. In addition, the most attractive peptides possess unique physiologic activities such as antihypertensive activity. Many studies have been conducted on the ACE inhibitory properties of peptides derived from food. One of the most relevant activities reported in meat is the inhibition of ACE. This enzyme participates in the RAS by being converted into angiotensin II, which constricts arteries and, as a consequence, increases blood pressure. Thus, inhibiting ACE is a potential way to reduce blood pressure [5]. ACE inhibitory peptides are obtained by hydrolysis of muscle proteins using proteases such as pepsin, trypsin, and α-chymotrypsin. The peptides obtained are purified by using chromatographic techniques or ultrafiltration. Several peptides from the hydrolysates of chicken collagen, chickpea, fish, and dairy products with *in vitro* ACE inhibitory activity have been reported. There are few reports regarding antihypertensive peptides derived from cured meat products. The study by Escudero et al. was conducted on "Spanish Ham", a dried cured meat product produced from pork [48]. Their results revealed an antihypertensive peptide with an AAATP (Ala-Ala-Ala-Thr-Pro) sequence and an IC_{50} value of 100 μM. Because of these promising results, this study focused on obtaining antihypertensive peptides from pastirma made from beef muscle.

During pastirma production, because endogenous proteolytic enzymes denature meat proteins, it is thought that new bioactive peptides that have therapeutic effects may be generated. Therefore, this thesis focused on the determination of bioactive peptides that exist in the traditional Turkish meat product pastirma.

This research was carried out with the aim of the following concepts;

- This thesis was aimed to examine the changes in the protein structure of meat during pastirma production.

- The results of this study were expected to show that meat itself becomes a biologically active food through gastric and intestinal digestion.
- This research was conducted to provide evidence that pastirma may contain a significant number of functional food nutraceuticals that can be used as clinical therapeutics. Traditional pharmaceuticals contain chemical ingredients that can cause several side effects, while functional foods have minimal side effects. Because, the existence of bioactive peptides that have ACE inhibitory activity in pastirma have not been studied to date, with exception of the studies of Ahhmed et al. [9] and Toldra [19], this study was performed to validate the value of functional food as an alternative to chemicals in the treatment of hypertensive diseases.
- The results of this research were expected to highlight the importance of pastırma in respect to bioactive components.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIAL

Research analyses were carried out on samples sourced from 30 months old male cow (Species: Montofon). Two groups of muscles were prepared for each experiment: one group was analyzed as fresh meat; the other group was processed into pastirma. For some experiments, pastirma meat was taken from production line just before covering cemen that is a mix of spices (12, 20, 13 and 55% of fenugreek, garlic, red pepper and water, respectively). The meat was salted and pressed. Meanwhile, in order to observe the effect of cemen; some experiments are conducted with three different samples; fresh meat (FM), pastirma before cemen process (PBC) and pastirma (PS). Fresh and processed meat samples were sourced from the same animals and muscle type is selected as *Biceps femoris*. Muscles were used 48-hour post-mortem and the pH values of muscles before processing was 5.6. The pastirma was manufactured with using traditional methods in a local producer ‘Şahin-Melek Et ve Et Ürünleri’ in Kayseri province. Because pastirma processing took one month, meanwhile all fresh samples were kept at -80°C until the experiments

2.2. METHODS

2.2.1. Physicochemical Analysis

2.2.1.1. pH Determination

For pH determination, 5 grams of fresh meat, pastirma before cemen covering and pastirma samples were chopped and homogenized at 20 ml distilled water with using Silent crusher M (Heidolph, Germany) for 3 minutes with time interval (15 sec) [49]. After homogenization, pH is measured by pH meter (Mettler Toledo, Switzerland),

which is calibrated at two points (pH=4.0 and pH=7.0) (Mettler Toledo pH meter) [50].

2.2.2. Protein Analysis

2.2.2.1. Protein extraction

Proteins were extracted from all samples (FM, PBC and PS) by using two different solutions. The first solution was a low-ionic-strength solution (50 mmol/L imidazole–HCl, pH 6.0, 2 mmol/L Ethylenediaminetetraaceticacid (EDTA)) that extracts the proteins defined as water-soluble proteins (WSP) which includes enzymes [20]. The second solution was Guba-Straub-adenosine triphosphate solution (GS-ATP), which is a high-ionic-strength solution, (0.09 mol/L KH_2PO_4, 0.06 mol/L K_2HPO_4, 0.3 mol/L KCl, 1 mmol/L ATP, pH 6.5). The latter is prepared to extract all muscle proteins including heavy protein such as actomyosin complex. Twenty-eight grams of the solutions and 2 g of all three samples were homogenized separately three times for 30 seconds by using Silent crusher M (Heidolph, Germany). After homogenization, the mixtures were filtered by filter paper (Advantech, Japan) [51].

2.2.2.1.1. Protein Concentration Analysis

Protein concentration (mg/ml) of samples of which proteins were extracted was done by Biuret method [52]. Absorbance of samples was measured by Agilent type (Cary 60 UV-Vis, USA) spectrometer at an absorbance 540nm with each sample evaluated in triplicate. Protein concentration of FM, PBC and PS samples were evaluated as both digested and non-digested.

2.2.2.1.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) Analysis

Molecular weights of native proteins and also digested proteins sourced from the three samples were determined by SDS–PAGE using a gradient slab gel (7.5–20% acryl). Protein fractions were dyed with β-mercaptoethanol-bromophenol blue and electrophoretic seperation was obtained by an amplyfier set at 36mA/Gel constant current. Consequently, gels were destained by a buffer contains: H_2O, methanol and acetic acid for about 2-3 h to clear the gel and become ready for imaging After separation for 90 min, gels were dyed with %0.25 (w/v) Coomassie Brilliant Blue R-

250 (CBB). Excess CBB %50 (v/v) and methanol %10 (v/v) were removed by acetic acid [28].

2.2.3. Protein Oxidation

2.2.3.1. Determination of total sulfhydryl groups

A half ml of extracted protein samples of FM, PBC and PS were mixed with tris-HCL buffer A (mixture of trizmahydrochloride, sodium dodecyl sulphate (SDS), urea, Ethylenediaminetetraaceticacid (EDTA) and 250 ml of distilled water, pH 6.0) and Ellman's reagent (4 mg of 5, 5'-dithiobis (2-nitro-benzoicacid), tris-HCl). As a consequence, the mixtures were kept for an hour in a dark place at the room temperature; they were centrifuged for 7 min. In order to determine the absorbance of the supernatant, an aqueous of 3 ml of each sample was checked by a spectrophotometer (UV-1800, UV spectrophotometer, SHIMADZU) at an absorbance of 412 nm [53, 54].

Following equation was used to calculate sulfhydryl (SH) groups:

Total µmol SH/g = 73.53 x A / P
73.53: a constant factor.
A: Absorbance of sample
P: Protein concentration of sample (mg/ml)

2.2.3.2. Determination of free sulfide molecules

An aqueous (0.2 ml) of extracted protein samples was mixed with buffer A, and β-mercaptoethanol and DTNB (5,5'-dithiobis-(2-nitrobenzoic acid)) buffer (DTNB and methanol). The mixture was left at room tempareture in dark place for an hour. After a cold TCA (50%) buffer (Trichloraaceticaacid (TCA) and distilled water) was added to the mixture in order to reach a final concentration 10%; they are centrifuged for 5 min. Then, buffer C (SDS, EDTA, urea, trizma-hyrochlorade and distilled water) was added. Absorbance of samples was determined by a spectrophotometer (UV-1800, UV spectrophotometer, SHIMADZU) at 412 nm [53, 54].

Again the following equation was used to calculate sulfhydryl (SH) groups:

Total µmol SH/g = 73.53 x A / P
73.53: a constant factor.
A: Absorbance of sample
P: Protein concentration of sample (mg/ml)
Finally:

Free of sulfide molecules µmol S/g = µmol total SH/g - µmol free SH/g

2.2.3.3. Surface Hydrophobicity

An aqueous (400 µl) of extracted protein samples was mixed with 80 µl buffer BPB. After vortex, the mixtures are left dark place in room temperature for 10 min. After 4 min centrifuge at 6200 rpm, the supernatant of the mixtures were separated. Then 2700 µl of H_2O was added to the supernatant. After vortex, absorbance of samples was determined by a spectrophotometer (UV-1800, UV spectrophotometer, SHIMADZU) at 495nm [55].

Again the following equation was used to calculate surface hydrophobicity:
BPB bond (µg/ml) = 200 µg x (Abs. s. – Abs. c.) / Abs. c.
Abs. s.: Absorbance of sample
Abs. c.: Absorbance of control

2.2.4. Bioactive Peptit Analysis

2.2.4.1. Hydrolysate Preparation

Fifty grams of pastirma were chopped into small pieces and added to 130 ml of distilled water in which later followed by processing for 5 minutes in food blender (IKA Waring Commercial Blender, Germany) in order to imitate to mechanical digestion. Then, the mixture was homogenized for 10 min by using the homogenizer Silent crusher M (Heidolph, Almanya) surrounded with ice [32]. The process was similarly subjected on the FM and PBC samples.

2.2.4.2. Digestion

Homogenate from the samples were subjected to incubation at 70°C for 30 min in a water bath (Memmert, Germany) on account of cooking simulation. After incubation, the pH was adjusted with 1 M HCl, pepsin (Sigma–Aldrich, Inc. St. Louis, MO, USA)

from porcine gastric mucosa was added in order to simulate the digestion process in human body. The mixture was then incubated for digestion in a shaking incubator at 37°C. In order to inactivate the enzyme, samples were boiled to 10 min at 100°C. At the end of the process, the pH of the mixture was again adjusted to alkaline with 1 M NaOH. After pH adjustment, trypsin was added and the samples were incubated once again at 37°C for 2 h. Then the samples were heated at 100 ^{o}C for 10 min to terminate enzyme activity. The reaction mixture was centrifuged for 10 min at 3500 rpm and the supernatant was then collected by passing through a cheesecloth to remove fats. Obtained filtrate was passed through a cellulose membrane filter (0.45µm) and kept at -80°C until ACE inhibitory experiment [32].

2.2.4.3. SDS PAGE Analysis of Hydrolysates

SDS-PAGE views of hydrolysated samples by enzymes were obtained by the method as described at 2.2.1.2.

2.2.4.4. ACE Inhibitory Activity Assay for Hydrolysate

Hydrolysates, which were kept at –80 ^{0}C, were used in ACE inhibitory activity analysis. The ACE inhibitory activity was determined by using the method of Cushman and Cheung (1971) [56]. This method was partially modified by Katayama et al. (2004) [57]. Principally, this assay is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (Hip–His–Leu) catalyzed by ACE. A sample solution of peptide was mixed with Hip–His–Leu (Nacalai Tesque Inc., Kyoto, Japan) as substrate containing sodium borate buffer and NaCl and then pre-incubated at 37°C for 5 min. The reaction was initiated by the addition of rabbit lung ACE (Sigma–Aldrich, Co., MO. USA) in a buffer containing sodium borate buffer and the mixture was incubated at 37°C for 30 min. The reaction then was stopped by adding HCl to the samples except for the blank. The hippuric acid liberated by ACE was extracted by adding ethyl acetate to the mixture with vigorous shaking for some time. After centrifugation for 20 min, the ethyl acetate layer was collected; it was then dried at 100°C for 10 min in order to remove residual ethyl acetate. The hippuric acid was dissolved with NaCl solution and its absorption at 228 nm was determined in a spectrophotometer. The concentration of ACE inhibitors required to inhibit 50% of ACE activity was defined as the IC_{50} value. ACE inhibitory activity was calculated as follows [28]:

Inhibition (%) = (C-S) / (B-S) x 100

S: Absorbance of sample;

C: Absorbance of control (buffer for samples);

B: Absorbance of blank.

2.2.5. Statistical Analysis

The data obtained as a result of analysis were evaluated at SigmaPlot 11.0 statistics package program. Tukey multiple comparison test was used to determine differences between groups by applying single factor analysis of variance (ANOVA).

CHAPTER 3

RESULTS AND DISCUSSION

There is a lack of biochemical information on the function of food proteins, especially traditional Turkish food, in particular processed meat products such as pastirma. There is a belief that proteins in pastirma have dozens of nutritional and therapeutic functions [20], and this study was conducted to verify this hypothesis. However, it is unclear how pastirma changes when it is exposed to different treatments such as cooking and digestion. Little information is available regarding the chemical changes of the nature of proteins during the production of pastirma, regardless to the findings reported by Ahhmed et al. [20,51,58,59]. Normally, proteins play major roles in human lives as they are considered to be the most important components of food. Beyond this, they work as bioactive ingredients, such as inhibiting enzymes, or inhibiting reactants that cause unwanted chemical reactions within human cells. Regardless of their source, many proteins and their enzymatic hydrolysates contribute to biological activities. They can act as anti-obesity and anti-diabetic compounds by inhibiting amylase and α-glucosidase. They also possess antimicrobial activities, while other proteins contribute to the reduction of cell inflammation. This study focused on proteins found in Turkish beef and pastirma, and determining their antihypertensive activities. Furthermore, there is a significant public concern regarding Turkish pastirma and its effects on health. Because hypertension or high blood pressure is a disease that affects a large number of Turkish individuals, the study of nutritional alternatives to treat, limit, and or reduce the incidence of such diseases is important. Peptides and hydrolysates derived from meat proteins are known to inhibit ACE, which is believed to be the initial element that contributes to the mechanism of hypertension.

In this study, 3 time point samples were used for analyses of FM, PBC, and PS with respect to bioactivities.

3.1. pH

According to the Turkish Food Codex Meat Products Communiqué (Bulletin no. 2012/74) [60], the pH of pastirma should be a maximum of 6.0. The pH values, which increased as the meat was processed and cured, are shown in Table 3.1.

Table 3.1. pH values of samples

Parameter	Fresh meat		Pastirma before chemen		Pastirma	
pH	5.80^{a}	0.01	5.90^{b}	0	5.91^{b}	0.02

The fresh meat used for pastirma production had a pH of 5.8, which is in the range suggested by Oztan (1999) as the optimal pH of meat to be used for pastirma production (pH 5.4–5.8) [61]. After the fresh meat was salted and pressed (PBC), the pH increased slightly to 5.90, but this increase was not significant ($p > 0.05$). It is suggested that the stability in the pH between FM and PBC is due to the non-existence of lactic acid bacteria. The highest pH was observed in the final product (PS) (5.91 ± 0.02), but this was not a significant increase ($p > 0.05$). The process of curing and salting had no effect on pH. Values are in accordance with the standards, and also in agreement with the data reported by Ahhmed et al. [20]. The pH value tended to increase during pastirma manufacturing. This may be due to proteolysis that results in ammonia and amine production [62]. According to Deniz et al. [63], proteolysis occurs during the processing of raw cured meat product and it is one of the most important biochemical changes. Although it is assumed that microorganisms have a role in proteolysis, endogenous enzymes are primarily responsible for proteolysis in dry cured meat products. It is suggested that the pH values in the tested samples were not changed due to the low level of acidic amino acids generated, or the shifting of amino acids aspartic acid and glutamate to the polar but uncharged amino acids aspartate and glutamine. However, this suggestion assumes the presence of asparagine synthase and glutamine synthase to accomplish the production of aspartate and glutamine.

Surprisingly, in a study conducted by Öz, Kaban, Bar, and Kaya (2017) on the isolation and identification of lactic acid bacteria from pastirma, 106 strains of lactic acid bacteria were isolated from pastirma obtained from 14 different manufacturers [64]. It is clear that the types they used may have contained insignificant amounts of nitrate and/or the cemen used was not effective against microbial growth. The total mesophilic aerobic

bacteria (TMAB) count was lower than 2 log cfu g^{-1} in PS compared to the fresh meat value of 6.70 log cfu g^{-1} ($p < 0.05$). PS samples showed a lower microbial content compared to fresh meat, which was likely due to antimicrobial substances present in cemen, regardless of the salt content [65].

3.2. Protein extraction

Due to its high protein content (16–22%), meat is regarded as a rich source of complete protein. The protein content can be categorized as myofibrillar proteins, sarcoplasmic proteins, and stromal proteins. Myofibrillar or muscle proteins account for 9.5% of protein content, and consist of myosin, actin, tropomyosin, protein M, protein C, α-actinin, and other minor proteins associated with myofibril. In fresh meat, they hold water molecules, but as meat is aged they release moisture. Due to their fibrous structure, high ionic strength buffers such as Guba-Straub-ATP (GS-ATP) are required for their extraction. Sarcoplasmic proteins account for 6% of protein content, and include soluble sarcoplasmic, lysosomal, and mitochondrial enzymes, as well as myoglobin (Mb), hemoglobin, cytochrome and flavor-proteins. These proteins are involved in transportation, degradation, and synthesis, as well as flavoring the meat. These proteins are named "water-soluble proteins" (WSP) because they can be extracted in water or low ionic strength buffers. On the other hand, stroma proteins, which account for 3% of protein content, consist of non-soluble or slightly soluble proteins, including collagen, elastin, reticulin, and others [66].

Improved solubility is obtained by using different solutions, since the molecular weight of muscle proteins vary. For this reason, WSP and GS-ATP solutions were used to assess the protein extractability of 3 samples. In samples of FM, PBC and PS muscles, the extractability of proteins in GS-ATP solution was higher than that of proteins extracted in WSP. Extraction of sarcoplasmic protein was improved in WSP, which is a low-ionic-strength solution, yet myofibrillar protein was extracted in GS-ATP, a high-ionic-strength solution. The main objective of the protein extraction was to quantify, characterize, and determine the degradation process of meat that takes place during pastirma production.

3.2.1. Extractability of WSP

As stated previously, WSP buffer is a low ionic solution. It extracts sarcoplasmic meat proteins and some enzymes that have low molecular weights. These proteins are readily extractable, as they are composed of hydrophilic amino acids, particularly on their surfaces where water molecules can associate. Centrifugation is used to collect and/or isolate additional proteins that are attached to the imidazole molecules in the buffer solution.

It was determined that protein concentrations of WSP extracts from 3 different samples increased significantly from fresh meat to pastirma ($p < 0.05$) (Table 3.2). This increase in the concentration of sarcoplasmic proteins is mediated by protein degradation. In addition, enzymatic hydrolysis results in new peptides and proteins that are less soluble in the low ionic strength solution. Sarcoplasmic protein concentration reached the highest value at the end of the pastirma process. Pastirma samples had the highest protein concentration in WSP solution, while fresh meat had the lowest. This increase was attributed to the degradation of the samples during the pastirma making process. In the study conducted by Ahhmed, the protein concentrations of WSP extracts of FM, PBC and PS, which were produced from different muscle (*Longissimus dorsi*), were determined [9]. In this thesis, it is found that FM, PBC and PS had 2.71, 3.53 and 3.59 mg/ml protein concentration, respectively. Ahhmed found that FM had 3.84 mg/ml while PBC and PS had 2.1 and 4.9 mg/ml protein concentration, respectively. With the exception of the result of PBC, protein concentrations of WSP extracts of FM and PS were lower in the current study than Ahhmed's study. The difference between the results of these studies may be result from the using different muscle while producing pastirma.

Concentration of FM in WSP showed the lowest value compared to PBC and PS. However, the differences in values were statistically insignificant. As previously stated, protein concentration increases during pastirma processing due to proteolysis of muscle proteins. Samples of pastirma before PBC and PS have similarly higher protein concentrations compared to fresh meat. Researchers have suggested that the darker color of the protein extract from PS is due to oxidation of myoglobin (Mb). Mb, which is responsible for the pinkish color of meat, is oxidized to oxymyoglobin. Since the pastirma making process takes 28 days, the oxymyoglobin is further oxidized to

metmyoglobin. The latter is responsible for the dark red color of PS in general and of protein extracts in particular. The primary reason why PS slices are darker than fresh cuts of meat has been reported to be the conversion of iron atoms in Mb to the ferric form during prolonged exposure to the atmosphere [20, 67].

3.2.2. Extractability of GS-ATP Proteins

Due to the fibrous structure of myofibrillar muscle proteins, their extraction requires highly ionized buffers, such as GS-ATP. Unexpectedly, results of protein content analysis indicate that the protein values fluctuated among the 3 samples, where PBC showed the highest value. Among the 3 different samples, fresh meat exhibited the lowest protein concentration. Results were shown in Table 3.2. Moreover, in the study conducted by Ahhmed worked on pastirma, which is produced from different muscle type, protein concentration value of only PBC (6.36 mg/ml) extracted in GS-ATP was lower than the result of this thesis (7.72 mg/ml). The curing process of meat increases the protein content, as the humidity gets lower. It is possible that extraction of proteins in PS was obstructed by the cemen, causing the higher level of protein in PBC. Moreover, there may have been some proteins bound with phenol compounds derived from cemen, polymerizing the protein and thereby reducing extractability. However, it should be noted that the PS has additional proteins from garlic and fenugreek. The oxygen-based reaction on Mb is a one-way reaction, meaning that if Mb shifts to metmyoglobin, it never returns to Mb. This leads to the proposal that once meat becomes a dark red color, it can never be restored to its original pink color. Another possible explanation of the darker color of pastirma is the red pigment in the red chili pepper in cemen, which is rich in carotenoids [20].

Table 3.2. Protein concentration of Fresh meat, Pastirma before cemen and Pastirma in mg/ml

Parameter	**Fresh meat**		**Pastirma before cemen**		**Pastirma**	
	Means	SEM	Means	SEM	Means	SEM
WSP (mg/ml)	$2,71^{a}$	0,32	$3,53^{ab}$	0,01	$3,59^{b}$	0,09
GS-ATP (mg/ml)	$5,35^{a}$	0,03	$7,72^{b}$	0,01	$6,10^{ab}$	0,19

^{abc} The values indicated by different lowercase letters on the same line show the statistically significant differences between the muscle types ($p < 0.05$)

3.2.3. Protein Separation Using SDS-PAGE

In this thesis, SDS-PAGE images were used for determining the degradation and coagulation of native proteins into smaller or larger compounds, respectively, as a result of meat processing into pastirma. In addition, protein extracts using WSP or GS-ATP were electrophoresed to determine their molecular weights. The effects of the different steps of pastirma processing on the structure of meat proteins were determined by SDS-PAGE gels with 7.5-17.5% gradient. The gel images demonstrate and distinguish the amount of change and denaturation that occurred in proteins during the process of pastirma from their original state in fresh meat.

3.2.3.1. Changes in molecular weights of proteins extracted in WSP

Figure 3.1 shows the changes in protein structure of proteins extracted in WSP compared among all 3 samples. As mentioned earlier, WSP is a low-ionic solution and is effective at extracting Mb, enzymes, and denaturing sarcoplasmic proteins. Proteins extracted from FM, PBC, and PS samples were compared by SDS-PAGE, and 4 different bands disappeared (Figure 3.1) in lanes 1 (FM), 2 (PBC) and 3 (PS). Disappeared protein bands are indicated by red rectangles on the gel images. A small band of myosin heavy chain protein (MHC: 200 kDa) was present in FM (lane 1), while this band was not present in the PBC and PS lanes. Furthermore, glutamic dehydrogenase (55 kDa) was clearly present in the FM sample, but not in the PBC and PS samples. This leads to the suggestion that the curing and salting processes contributed to the degradation of these two proteins in PBC and PS. Interestingly, glyceraldehyde 3-phosphate dehydrogenase (36 kDa) was present in FM, not present in PBC, but present in PS. There is no clear explanation of the behavior of this protein. The most likely hypothesis is that this type of protein is extracted when there is adequate moisture content, as in the case of FM, but was also present in PS because it was present in the cemen. Ahhmed et al. stated that cemen contains garlic and fenugreek, with proteins and peptides having molecular weights ranging from 6.5–66.0 kDa [65]. Aprotinin (6.5 kDa) was also present in FM but was less intense in PBC and PS samples.

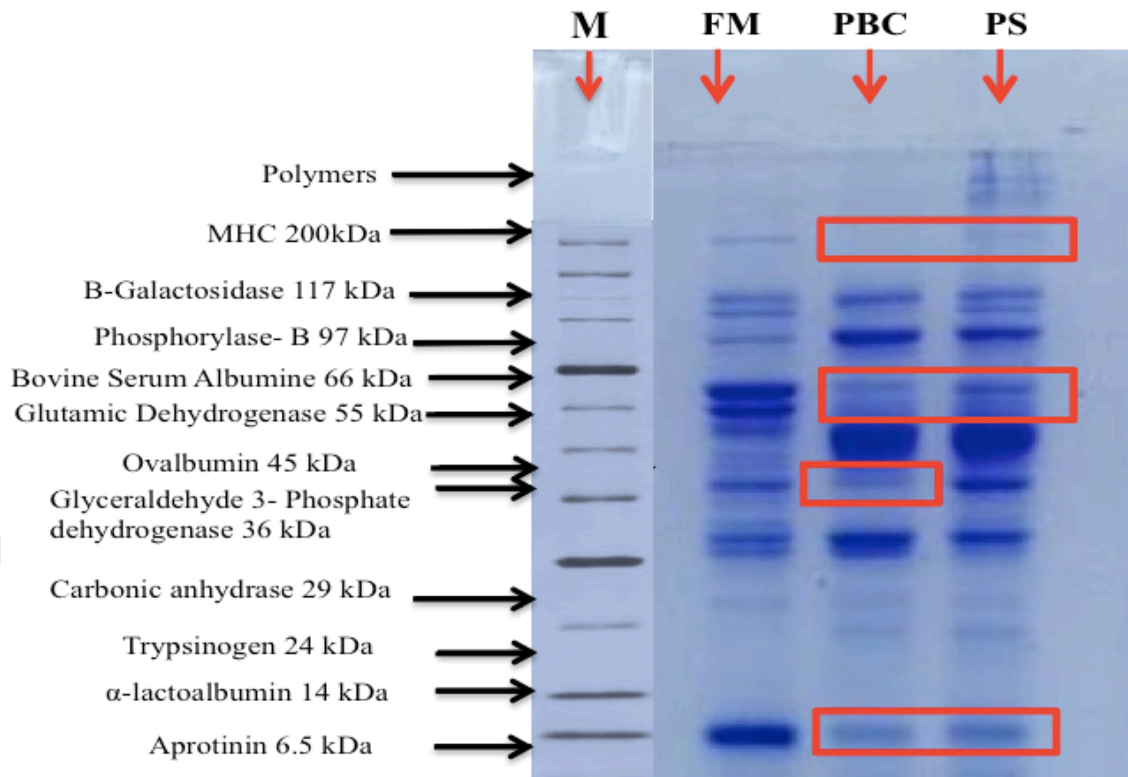

Figure 3.1. SDS-PAGE images of protein fractions of fresh meat, pastirma before cemen covering and pastirma samples dissolved in Water Soluble Protein (WSP). FM: Fresh Meat, PBC: Pastirma before cemen covering, PS: Pastirma

3.2.3.2. Changes in molecular weights of proteins extracted in GS-ATP

GS-ATP solution extracts the myofibrillar proteins that coexist with other proteins within the muscle. Therefore, extracts of samples were separated by molecular weight by using SDS-PAGE. Figure 3.2 demonstrates the changes in protein structure of samples in GS-ATP buffer. In lanes 1 (FM), 2 (PBC), and 3 (PS), the MHC band (200 kDa) vanished in PBC and PS lanes, while there was a band in the FM lane. Moreover, trypsinogen appeared in the PBC lane, while it was very weak in the FM and PS lanes. Likewise, a small band of α-lactalbumin (14 kDa) was present in the PS lane, though it was not present in the FM and PBC lanes. This supports the proposal that the cemen covering process was responsible for this protein being present in PS. The protein content of the cemen might have caused changes in the protein structure of meat during pastirma production. However, due to little information available in this regard, this hypothesis is only supported by the studies conducted by Ahhmed [20, 58, 59, 65]. Ahhmed noted the same phenomenon in the case of pastirma proteins. Notably, aprotinin was not present in PBC, although it was present in FM and PS.

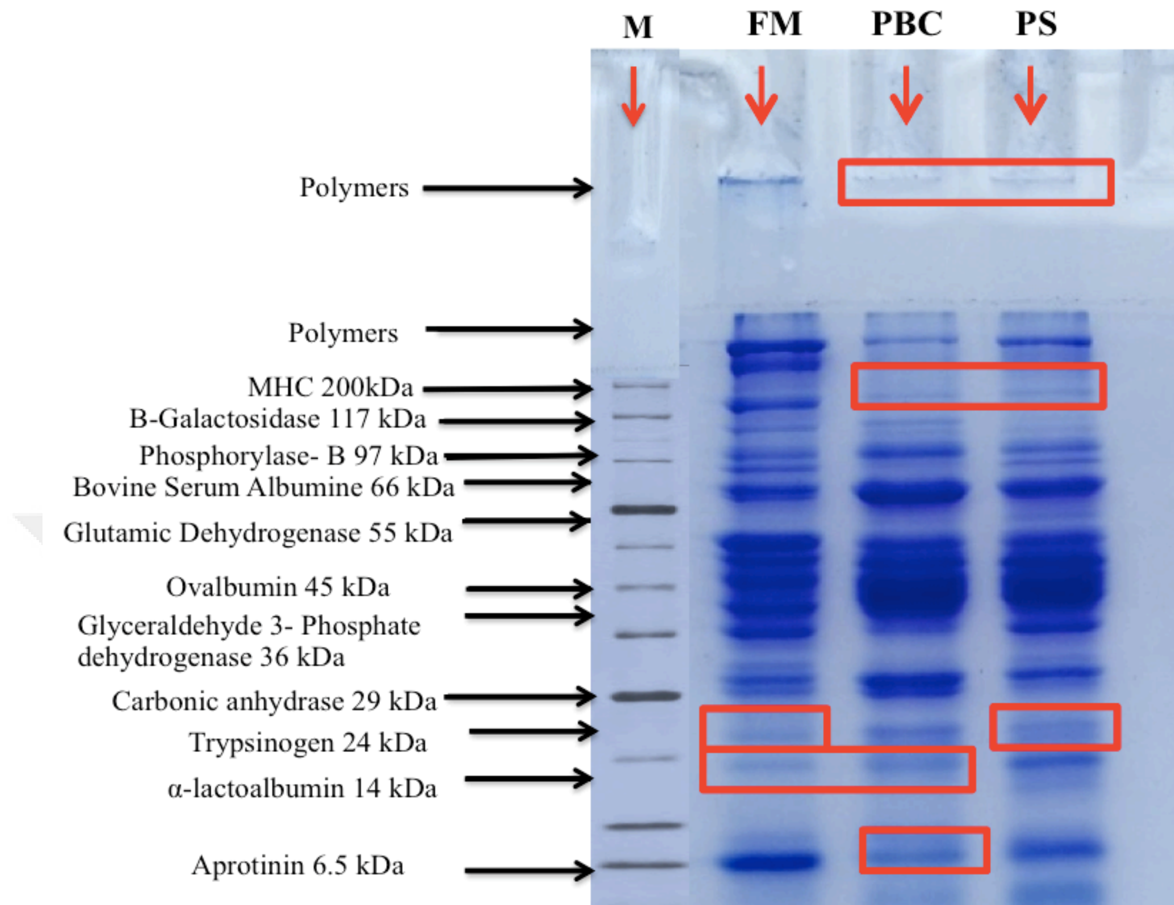

Figure 3.2. SDS-PAGE images of protein fractions of fresh meat, pastirma before cemen covering and pastirma samples dissolved in Guba Straub ATP (GS-ATP). M: Marker, FM: Fresh Meat, PBC: Pastirma before cemen covering, PS: Pastirma

3.3. Biological activity of hydrolysates

Bioactive peptides are liberated by microbial activity or proteolytic enzymes [3]. This research was carried out to examine if meat becomes a biologically active food through gastric and intestinal digestion, and if the considerable amount of constituents in pastirma could be utilized as nutraceuticals for clinical therapies. Identification of ACE inhibitory hydrolysate from both fresh meat and pastirma samples, and determination and comparison of antihypertensive activities of fresh meat and pastirma peptides were the aims of this study. FM, PBC, and PS samples were subjected to enzymatic hydrolysis by pepsin and trypsin to simulate human digestion. Samples were different at the imposed processes, including salting, pressing and cemen covering. Moreover, detection and comparison of the ACE inhibitory activity of peptides was a goal of this study.

Several methods of bioactivity assays were performed on hydrolysates that were obtained by enzymatic hydrolysis to determine if they detected new peptides in the structure of pastirma [32]. Analyses were conducted with hydrolysates of PBC and PS samples to determine the effect of cemen covering on peptides that have ACE inhibitory activity. In order to compare the effect of salting and pressing on ACE activity during pastirma processing, hydrolysates obtained from fresh meat were analyzed. In addition, hydrolysates were diluted to 50%, and analyses were also performed on these diluted solutions (1, 1/2, 1/4, 1/8, 1/16).

3.3.1. Protein concentration of hydrolysates

Protein concentrations were determined separately for non-digested and digested samples of FM, PBC, and PS. As expected, digested samples for all 3 different groups had higher protein concentrations than non-digested samples. Protein digestion involves the breakdown of food proteins to polypeptides, and further to peptides and amino acids. Therefore, protein structure is changed by the digestion process, where long protein chains are broken down by enzymes and smaller protein peptides are generated. The protein concentration values of the hydrolysates were used as an indication of the level of degradation in the pastirma process.

Pastirma production, in general, causes an increase at the amount of protein extracted with the help of processes such as salting, pressing, and cemen covering. The results demonstrate that larger protein chains are broken into smaller peptides due to pastirma-making processes. This resulted in an increase in protein concentration (Table 3.3). On the other hand, some proteins may have defused during the mechanical pressing treatment.

While FM had a concentration of 4.4 mg/ml protein, after digestion with pepsin and trypsin this increased to 15.9 mg/ml. The digestive enzymes cleave proteins to smaller polypeptides that give a higher absorbance spectrophotometrically. As well, protein concentration of PBC increased from 6.5 mg/ml (for non-digested sample) to 18.1 mg/ml with the digestion process. Moreover, PS had 8.0 mg/ml protein concentration for non-digested sample, while digested PS had 16.7 mg/ml protein concentration. This shows that pastirma had twice the amount of proteins as a result of processing, which

allowed the release of bioactive peptides with hydrophobic side chains that were trapped in the native protein structure.

When the non-digested samples were compared to each other, it was clear that protein concentration increased as the meat was cured. Protein degradation arises from treatments such as salting, pressing, and cemen covering. Protein content of fresh meat nearly doubled when it was processed to pastirma (4.4–8.0 mg/ml).

When protein concentrations of digested samples were compared with each other, the low protein concentration in PS showed an unexpected fall. Digested PBC had a higher protein concentration than digested fresh meat sample as expected. However, digested PS had a lower concentration as compared to PBC. The low protein concentration in PS was possible due to the polymerization process and phenolic compounds originated from cemen attached with disjoined proteins from their native structure. It is well known that cemen contains enormous amounts of phytochemicals, as Ahhmed extensively discussed in a recent report [65].

Table 3.3. Protein concentration of digested and non-digested samples in mg/ml

Parameter	Fresh meat		Pastirma before cemen		Pastirma	
	Means	SEM	Means	SEM	Means	SEM
Non-digested	4.4^{a}	0.001	6.5^{b}	0.002	8.0^{c}	0.004
Digested	15.9^{a}	0.003	18.1^{b}	0.029	16.7^{ab}	0.003

^{abc} The values indicated by different lowercase letters on the same line show the statistically significant difference between the muscle types ($p < 0.05$)

3.3.2. SDS-PAGE images analysis of hydrolysates

Hydrolysates from the 3 groups were electrophoresed after dilution with dH_2O. In Figure 3.3, protein bands of pure hydrolysates (100%) and 25% dilutions of hydrolysates (1/4) of FM, PBC and PS, are shown. The high molecular weight bands detected from non-digested samples (Figure 3.1 and Figure 3.2) disappeared after the digestion process. Newly generated proteins at 50 kDa and lower in molecular weight were detected. On the other hand, when the protein bands of pure hydrolysates (100%)

in each sample were compared with the marker, it was clear that fresh meat had more protein with varied molecular weights. As the meat was processed, the pure hydrolysate bands disappeared. When meat turns into pastirma, proteins are broken down into smaller peptides with lower molecular weights. In addition, the effect of the pastirma-making process can be observed in the image. Moreover, pastirma hydrolysate did not express protein bands from 50 kDa to 15 kDa, while the hydrolysates of two other samples had. At the same time, the proteins with lower molecular weights in all samples were similar, despite the fact that density of smaller protein bands varied. Hydrolysates of FM and PBC samples also had similar protein bands with different densities.

Furthermore, when each group was evaluated separately, only a few protein bands were present in the lanes of 25% diluted (1/4) of hydrolysates. In the 25% dilution of FM hydrolysate, 15 and 6.5 kDa proteins were present. In the lanes of 25% dilutions of PBC and PS hydrolysates, none of the protein bands were exhibited. This indicates that all the proteins in this regard were less than 6.5 kDa, thus SDS-PAGE using 30% acrylamide gels did not detect these small protein or polypeptides in terms of molecular weight

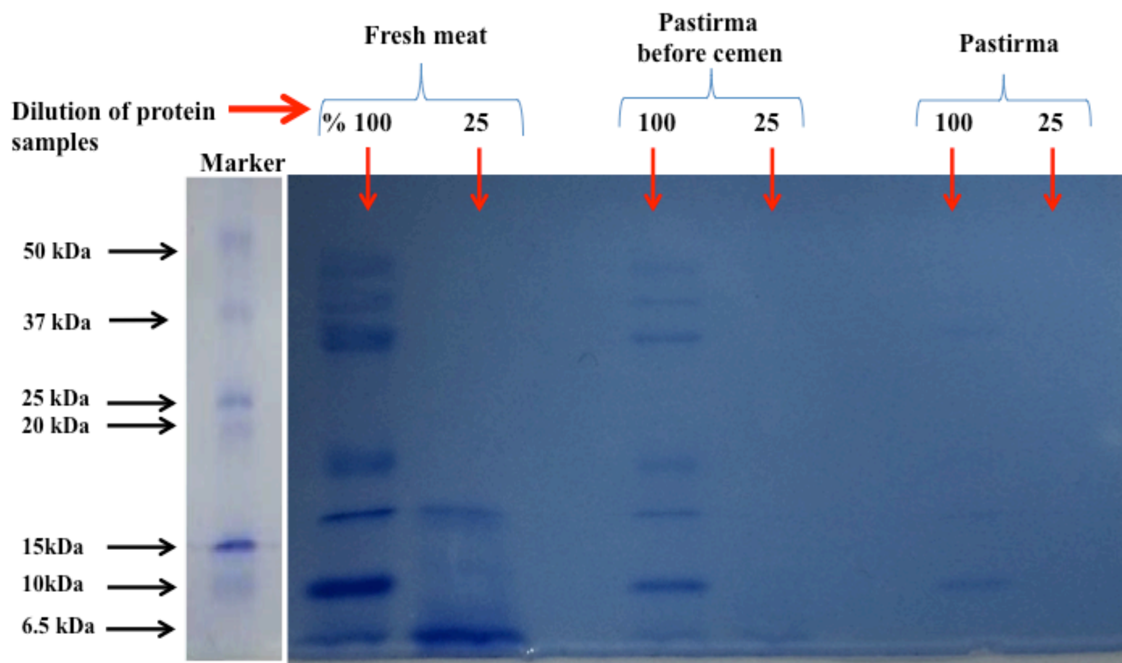

Figure 3.3. SDS-PAGE images of fresh meat, pastirma before cemen covering and pastirma after digestion with pepsin and trypsin enzyme

3.6.3. Antihypertensive Activities (IC_{50})

As stated previously, due to the treatments during pastirma processing, including salting, pressing, and cemen covering, a large number of peptides are generated by means of proteolysis of meat proteins. Many of these peptides are effective at preventing and reducing chronic lifestyle-related diseases such as hypertension. Many of these can lower blood pressure by their strong ACE inhibitory activity. Inhibition of ACE activity was evaluated by determining the absorbance of hippuric acid, which is released as an end product of ACE activity (Inhibition ratio = C – S / C – B x 100) [28]. The lower the absorbance, the lower the production of hippuric acid, which indicates stronger ACE activity. The inhibition of ACE activity in FM, PBC, and PS was calculated as 85.55%, 62.4% and 77.24%, respectively (Figure 3.4, Figure 3.5 and Figure 3.6).

Figure 3.4, Figure 3.5, and Figure 3.6 show the changing percentage values of the inhibition ratio of ACE at different dilutions of FM, PBC, and PS hydrolysates, respectively.

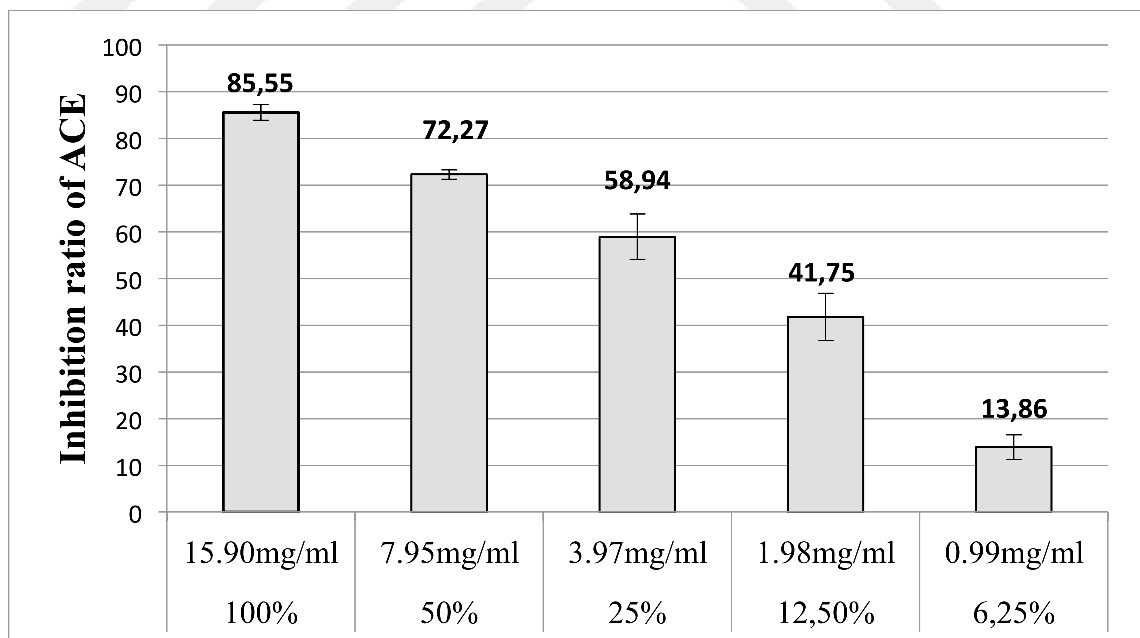

Figure 3.4. Inhibition ratio of hydrolysates in fresh meat samples with varying concentration

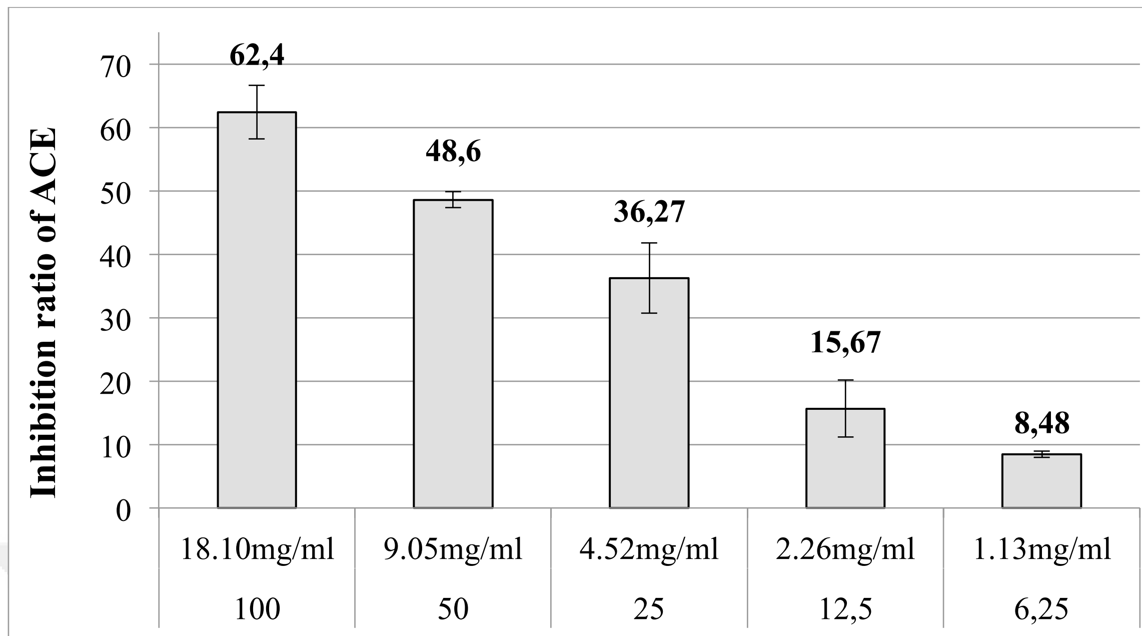

Figure 3.5. Inhibition ratio of hydrolysates in pastirma before cemen samples with varying concentration

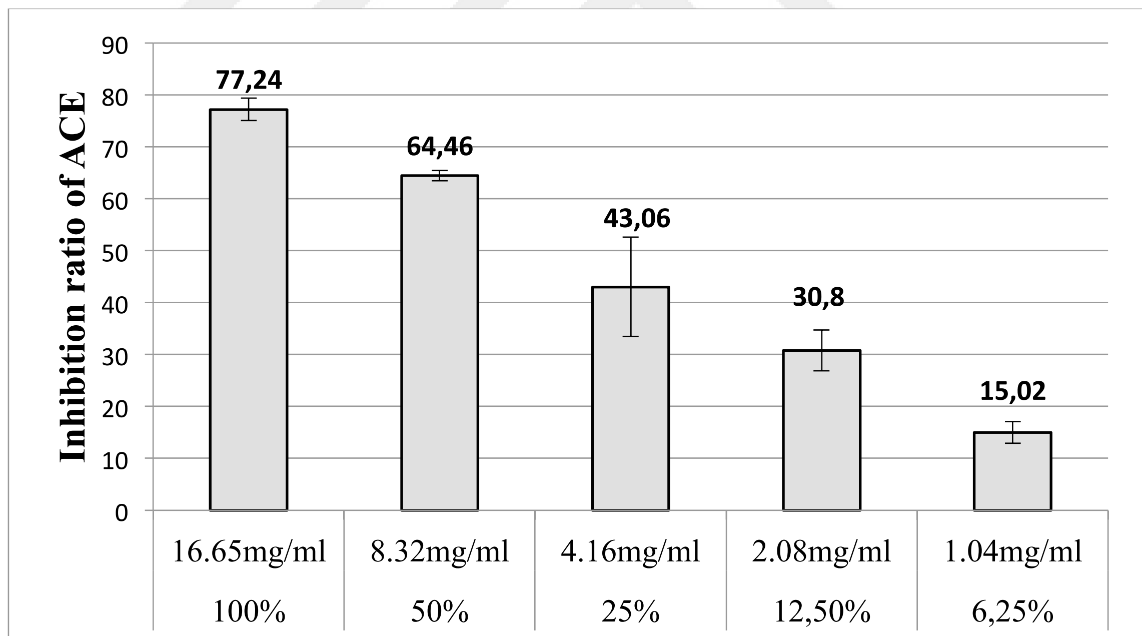

Figure 3.6. Inhibition ratio of hydrolysates in pastirma samples with varying concentration

In order to evaluate ACE activity; the biological IC_{50} value was determined. The definition of IC_{50} is the amount of bioactive peptide component required to inhibit 50% of an enzyme or a radical in an active medium [27]. In this study, the IC_{50} of the different hydrolysates was verified by plotting the ACE inhibition activities against a

variety of concentrations of hydrolysates (100% : 1, 50% : 1/2, 25% : 1/4, 12.5% : 1/8, 6.25% : 1/16).

Hydrolyzed proteins from fresh meat and pastirma showed 1.13 and 0.92 mg/ml (Figure 3.7) IC_{50} values, respectively. This demonstrates that meat had a slightly higher IC_{50} value than pastirma, which means a low antihypertensive effect. In other words, *in vitro* assay of the pastirma protein hydrolysates indicates that they had more nutraceuticals that lower hypertension than fresh meat protein hydrolysates.

Referring to the previous argument, there are many food sources of ACE inhibitory/antihypertensive peptides, including milk, cheese, yogurt, plants, and meat [68]. For example, Nakamura et al. (1995) reported that sour milk has two antihypertensive peptides that have IC_{50} values of 9.0 and 5.0 μM, respectively [69]. Moreover, research conducted by Kajimoto et al. (2002) [70] and Ong et al. (2007) [71] determined that yogurt and cheddar cheese has IC_{50} values of 9.0 μM and 13.0 μM, respectively. Furthermore, many researchers have studied meat proteins as a source of biopeptides, as well as proteins in dairy products. In addition, fish, cereal, and bean products are considered to be potential sources of antihypertensive peptides. Because meat is known to be a rich source of proteins, many studies have been conducted on the bioactivity of meat proteins. For example, tuna fish protein showed ACE inhibitory activity with an IC_{50} value of 11.28 μM [72], while porcine skeletal muscle protein had an IC_{50} value of 34 μg/ml [73]. Beef is also a source of ACE inhibitory peptides due to its rich protein content. Similarly, our team conducted a study of meat and pastirma, but using different muscle (*Longissimus dorsi*) [9]. In this study, our team found that meat has a higher ACE inhibitory activity. From this work, it was found that it is not necessary to process meat to evaluate its biological values and bioactivities of its proteins. The first study to identify antihypertensive peptide from beef hydrolysate was conducted by Jang et al. (2005), who reported that beef has a hexapeptide with the amino acid sequence VLAQYK (Val-Leu-Ala-Gln-Tyr-Lys); this peptide had an IC_{50} value of 32.06 μM [42].

There have been few studies on the bioactivity of dry cured meat products. Escudero et al. (2013) isolated the peptide AAATP (Ala-Ala-Ala-Thr-Pro), which had an inhibitory activity of 100 μM, from Spanish dry cured ham, which is a dried cured meat product produced from porcine [48]. Like Spanish dry cured ham, pastirma is also a dry cured

meat product; however, they differ in terms of the meat source. Furthermore, there are few studies of the bioactivity of Turkish pastirma present in the literature. One conducted by Ahhmed (2015) showed that beef meat and pastirma have IC_{50} values of 0.68 and 0.78 mg/ml, respectively [9], in contrast to the study conducted by Ahhmed et al. (2015), where they stated that fresh meat is higher in activity than pastirma. Yet the values in that study differ from the values of this study. In another study, Deniz et al. (2016) demonstrated that pastirma showed an ACE inhibitory activity higher than 86% [19]. As mentioned previously, the current research showed pastirma had a higher inhibitory activity of 77.24%. Because studies about the content of ACE inhibitory peptides in pastirma are rare, the results of this study were also evaluated by taking into consideration other meat sources. When compared to other studies with beef [20], pork [28, 29], and chicken meat [33], pastirma has a competitive IC_{50} value. Jang et al. (2008) found 4 ACE inhibitory peptides separated from beef hydrolysates with IC_{50} values of 0.117, 0.0643, 0.0529, and 0.0505 mg/ml, respectively [43]. The IC_{50} values determined in this study strongly indicate that hydrolysates are very effective in inhibiting ACE activity. In contrast, the sample used in the current study seems more effective than the samples used in the study conducted by Jang et al., because the volume of the samples they used were 16-fold higher than the volume of sample used in the current study. In addition, Jang et al. highly purified their samples by ultrafiltration and gel filtration, and used HPLC to fractionate and sequence the amino acid chain of the peptide with the highest ACE inhibitory activity.

Additionally, Arihara et al. (2001) found two peptides from pork with IC_{50} of 945.5 and 549.0 μM [21]. Moreover, Iroyukifujita et al. (2000) studied chicken, which is another meat source for ACE inhibitory peptides. They demonstrated that chicken has inhibitory peptides against ACE with an IC_{50} of 0.045 mg/ml [74]. Therefore, Table 3.4 shows the studies conducted on different types of meat products in order to determine the antihypertensive effect. Calculated IC_{50} values of meat muscles were also shown in the Table 3.4. When the IC_{50} value of inhibitory peptides, which are separated from meat sources, are compared, it is clear that beef and pastirma hydrolysates have peptides with very strong antihypertensive effect. This study showed that pastirma is a source of antihypertensive bioactive peptides with an IC_{50} value of 0.92 mg/ml. This value is very competitive when compared with other foods in the literature.

Table 3.4. Some examples of IC_{50} values of different types of muscle

Source	IC_{50} value	Reference
Sour milk	9.0 µM	Nakamura and others (1995)
Yogurt	9.0 µM	Kajimoto and others (2002)
Cheddar cheese	13.0 µM	Ong and others (2007)
Tuna fish	11.28 µM	Lee and others (2010)
Porcine skeletal muscle	34 µg/ml	Katayama and others (2003)
Beef	32.06 µM	Jang and others (2005)
Spanish dry cured ham	100 µM	Escudero and others (2013)
Pork	945.5 µM	Arihara and others (2001)
Chicken	0.045 mg/ml	Iroyukifujita and others (2000)
Pork	6.1 µM	Muguruma and others (2009)
Beef	0.68 mg/ml	Ahhmed and others (2015)
Pastirma	0.78 mg/ml	Ahhmed and others (2015)

Unpredictably, the IC_{50} value of PBC was determined to be 4.07 mg/ml. In other words, the ACE inhibitory activity of PBC was 4 times greater than that of FM and PS. This means PBC has very low antihypertensive effect compared with the other samples. This fluctuation in the results was not expected. It might be due to the assay of ACE inhibition activity for the PBC sample was conducted at a time different from the FM and PS. This irrelevant result might have been caused by different experimental conditions, such as the concentration of substrate, activity of ACE, origin of ACE, and other variables. Because the enzyme used for the experiment was very expensive, the experiment could not be repeated. Considering the data from this work, it is strongly suggested that fresh and/or pastirma contain a considerable and enormous amount of potentially anti-ACE active peptides. Regardless to the processing; beef may contribute to minimize the risk of high blood pressure disease when consumed in a moderate amount, and regardless of its salt amount; pastirma and its hydrolysate still exhibit a very potent ACE inhibitory activity.

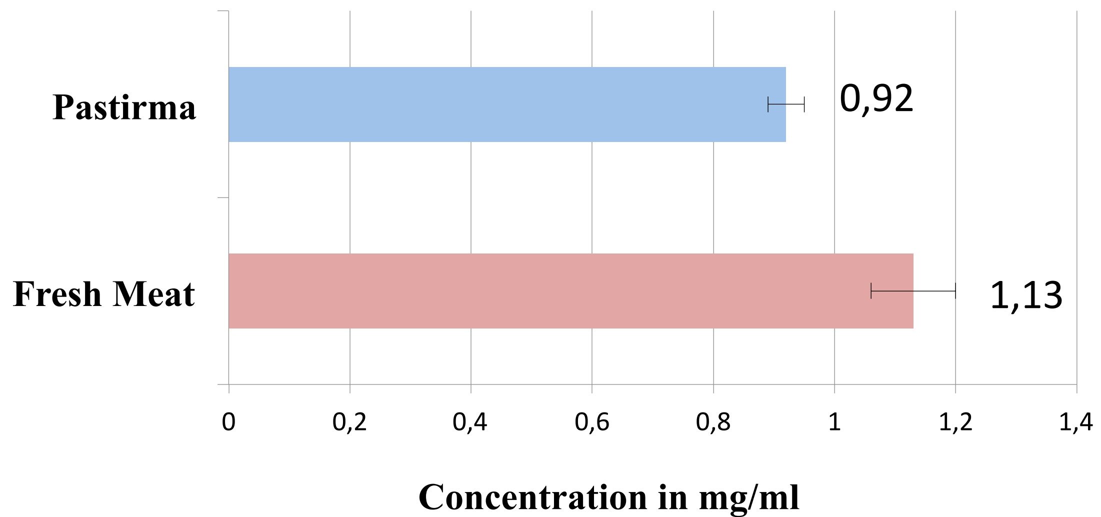

Figure 3.7. IC_{50} values of hydrolysates from fresh meat and pastirma of *Biceps femoris* muscles sourced from beef

3.4. Protein oxidation

Before being consumed, meat and meat products undergo a series of processes that can impair the quality of the finished meat products [75]. Likewise, in the case of pastirma, beef muscles experience a series of processes and treatments that last for a month. Because of the length of time during the process, muscle structure and proteins encounter many physicochemical changes [20]. Additionally, it is thought that more biochemical and biological reactions that occur, including lipid and protein oxidation. The protein oxidation that occurs in the dry cured beef product pastirma has yet to be fully or partially explained. Light- or heavy-oxidation of proteins and fat in meat products have become crucial issues, which are caused by processing, packaging, and distribution of meat products. They are important because they profoundly deteriorate meat product quality, leading to a commercial reduction in revenue. Ageing, curing, and packaging in transparent materials are frequently implemented in the manufacturing of meat products. While some aspects of the process are understood, many others remain unclear, particularly those related to the formation of complex compounds in real processes such as pastirma during the course of manufacturing. The rate of oxidation depends on the amino acid and protein composition and concentrations, the activities of prooxidants and antioxidants, the oxygen partial pressure, the structure and retained water in the meat [76], the method of processing (grinding, packaging), and the conditions in which the meat is stored (temperature, lighting) and cooked (method,

temperature, and duration) [77, 78] Prooxidants are compounds that accelerate oxidation, or facilitate the reaction with less use of energy. An antioxidant is any compound that fully or partially inhibits the oxidation process. In general, protein oxidation has been the focus due to its high impact on protein function and flavor/off-flavor formation in meat products as it generates aldehydes and ketone products at the end of its reaction. Impact of protein oxidation on meat quality has incited interest of researchers due to its increased effect on off-flavor formation in products with limited fat content. Compounds such as carbonyls and semi-aldehydes have been suggested to be of major importance in regards to oxidation of proteins that contain methionine, lysine, arginine, or proline in meat products. The oxidation of protein has been proposed to be the major sulfur oxidation product that gives rise to off-flavor. Semi aldehydes were detected in all the tested meat products, which indicated that lysine, arginine, and proline were degraded through oxidation [75].

Muscles of pastirma are normally placed in a curing mixture (crystallized salt 1000 g + 15 g nitrate/kg of meat) at room temperature to be salted on each side for ~ 24 h. After curing, though the muscles are washed thoroughly using fresh water to remove excess salt from the surface, the salt remaining in the final product approaches a level of 5-8%. It is suggested that the increase in oxidation values of proteins in processed PS and PBC samples, which were higher than in FM, is due to the salt treatment and oxygen exposure during the course of processing. Salt is a known prooxidant at levels commonly used in processed meats (0.5-2.5%). A prooxidant is an ingredient or additive that accelerates oxidation of fats or oils resulting in rancidity [79]. Similarly, it is suggested that the prooxidant activity of salt in pastirma breaks the bridges inside the native proteins, which allows oxygen radicals to react with the hydrophobic amino acids and then initiate oxidation. Oxidation increases with salt concentration in this range [79]. This trend is more apparent in red meat (beef) than in white meat (chicken) [80], which is in agreement with the results of this study. Oxidation of frozen raw beef is higher than for chicken due to heme iron content. Heme iron facilitates lipid oxidation producing peroxides, which are enzymatically decomposed by catalase, producing a variety of compounds, some of which contribute off-flavors [81].

Protein oxidation of meat, which occurs during pastirma production, is determined by monitoring carbonyl formation [82] and sulfhydryl losses [83] in myofibrillar protein.

Since protein oxidation results in carbonyl formation (aldehyde, ketone, carboxylic acid, acid halide, acid anhydride, ester, lactone, amide and lactam), the protein-bound carbonyl content is commonly used as marker for protein oxidation [84]. These carbonyl groups originated from peptide scission, amino acid residue side chain groups, and carbonyl compounds that are complexed with proteins [85]. To measure carbonyl content, there are many assays including spectrophotometric DNPH assay (2,4-dinitrophenylhydrazone), enzyme-linked immunosorbent assay, slot blotting, one-dimensional or two-dimensional electrophoresis, and Western blot immunoassay. Moreover, Sulfhydryls (thiol) from cysteine residues are sensitive to oxidation by almost all forms of reactive oxygen species [86]. The reduction in the amount of sulfhydryls provides an additional evaluation of the extent of protein oxidation as related to pastirma production process. In this research, amount of thiol groups is determined in order to assess the protein oxidation during meat turns into pastırma. For the sulfhydryl analysis, total sulfhydryl content of muscles was determined using 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB).

The sulfhydryl groups and sulfide molecules detected were reported as μmol per 100 g of protein. Results are shown in Table 3.4 for both digested and non-digested hydrolysates of 3 different samples.

Table 3.5. Thiol group and free sulfide molecules isolated from non-digested and digested fresh meat, pastirma before cemen and pastirma hydrolysates

Source	**Non-Digested**				**Digested**			
	S-H (μmol/100g)		**S (μmol/100g)**		**S-H (μmol/100g)**		**S (μmol/100g)**	
	Means	SEM	Means	SEM	Means	SEM	Means	SEM
Meat	477^{a}	0.06	413^{a}	0.15	316^{a}	0.39	311^{a}	0.40
Pastirma before cemen	394^{ab}	0.06	320^{b}	0.07	176^{b}	0.09	175^{b}	0.09
Pastirma	116^{b}	0.05	56^{c}	0.08	161^{b}	0.16	159^{b}	0.16

^{abc}The values indicated by different lowercase letters in the same column show that the statistically significant difference between the muscle types ($p < 0.05$)

The oxidation assay measures SH groups and free sulfide molecules but not sulfide bridges. In other words, protein oxidation causes the disulfide cross-linking [87]. Moreover these linkages contribute to the formation of gaps between muscle fibers that

helps the DTNB in muscle in order to detect free sulfide molecules. In the meantime others have suggested the opposite, meaning that the oxidation can be estimated by the number of disulfide bonds. Nonetheless, in using DTNB, both theories are correct in the estimate of oxidation occurring within the protein structure by the free sulfide or total sulfide bonds. Data of this experiment suggests that there was a reduction in the SH groups in all samples, as every sulfide molecule was coupled with another sulfide molecule by DTNB. In addition, the data of pastirma indicates that the free sulfide molecules were reduced, as more disuldife bonds were being created and increased as compared to the FM samples.

Based on the difference in the thiol group and sulfide molecule content between fresh meat and pastirma, it is estimated that amount of the denatured proteins as the effect of the pastirma making process is ~ 75-86% as compared to the native proteins in fresh meat ($p < 0.05$).

According to the results shown in Table 3.4, non-digested FM has 477 μmol/100 g free thiol group (SH), while non-digested PBC and PS has 394 and 116 μmol/100 g, respectively. The numbers of thiol groups in FM are higher than the numbers in the counterpart samples, PBC and PS; it means that FM has the native protein structure. In other words, the muscle protein in FM was undenatured and maintained their structure. Therefore, the amount of thiol group after using DTNB increases. Unlikely, in the PBC and PS the protein experienced a great denaturation phenomenon due to production processes. As SH reduced and donated the H group through a reduction reaction. This hypothesis was also supported by the number of free sulfide molecules as FM samples apparently expressed a higher amount of sulfide molecules. However, protein degradation, which occurs during process in PBC and PS, contributed to the reduction in amount of free sulfide molecules resulting in producing sulfonics, sulfinic acid salts, disulfides and thioesters. Afterwards, this gives a great space for DTNB to react with sulfide molecules again in order to be dervitized for spectrophotometric detection.

Moreover, when the results of SS and SH groups were compared in digested and non-digested, it is obvious that non-digested samples had higher values for all 3 groups. In fact, since digested protein samples are more denatured, they are expected to have higher SS and SH values. However, sulfur molecules in digested samples might be pressured by some treatments such as thermal treatment, enzyme activity, and addition

of HCl and NaOH in order to regulate pH during the digestion process. Precisely, higher SH and free SS molecules in fresh meat also means that DTNB could not have a good opportunity re-bride the free sulfide into S-S bonds. This was possibly due to the thiol group trapped inside the protein structure.

According to the results of the protein oxidation experiments, a hypothetical chart, which shows the oxidation process of muscle proteins as a function of time, was prepared. In figure 3.8, it is shown that protein quality decreases by time. Also, number of free sulfide molecules and free thiol groups are tending to increase until the half of the active stage of chain propagation and then they decrease by time. Moreover, at first stage number of disulfide bridges decreases while it is increases after active stage of chain propagation. Furthermore, number of carcinogenics and organic acids tends to increase by time during oxidation.

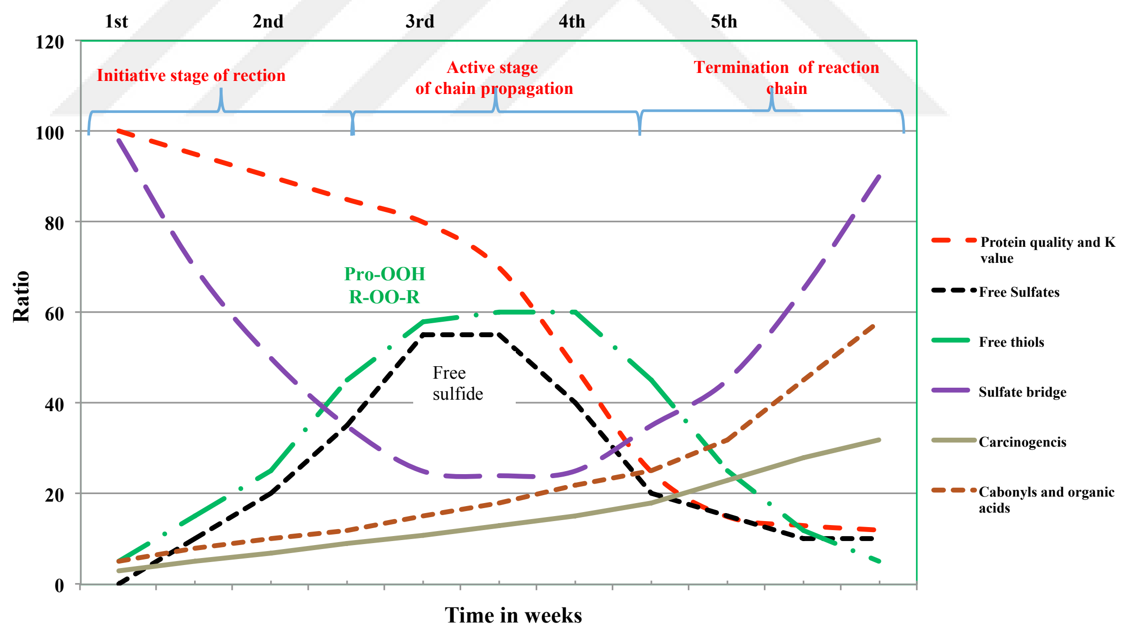

Figure 3.8. Hypothetical oxidation process of muscle proteins as a function of time and other factors

3.5. Hydrophobicity

As in all foods, the major nutrient in meat is water. Moreover, most of the water in muscle is reserved in myofibrillar proteins. When protein structures are broken down,

hydrophobic clusters are spread out by sarcoplasmic proteins. On account of this, hydrophobicity can be an indicator of denaturation of proteins, which in turn to support data of SDS-PAGE and protein oxidation.

The method used in this study depended on the degree of interaction of the hydrophobic chromophore bromophenol blue (BPB) with myofibrillar proteins, and the separation of free and bound BPB by centrifugation [55]. Since the quantity of bound BPB is an index for protein hydrophobicity, the results indicate that increase in the amount of BPB from 19.55 μg/500 μl to 40.6 μg/500 μl demonstrate a statistically significant rise in hydrophobicity. The difference in bound BPB in meat and pastirma samples is shown in Figure 3.8.

A positive correlation between surface hydrophobicity and increasing temperature has been shown by previous studies. Chelh et al. (2006), who conducted research about surface hydrophobicity of meat proteins, and found ~ 100 μg of bound BPB in pork myofibrils after heating (60 min, 70°C), with the amount of bound BPB being ~ 3 times higher at 70°C than at 30°C [55]. Also Santé-Lhoutellier et. al. (2008) found that heating (45 min, 100°C) increased bound BPB (~ 40 μg) in beef myofibrils [88]. This increase in protein surface hydrophobicity indicates that thermal treatment trigggers dispersibility of the myofibrillar proteins and liberates hydrophobic clusters. Although pastirma is not exposed to thermal treatment in terms of processing, other processing steps such as salting, ageing and squeezing may cause protein denaturation resulting surface hydrophobicity. Studies have been mostly based on the increase of surface hydrophobicity with increasing temperatures and heating times. As there is very little work regarding the correlation between hydrophobicity and other treatments, there is not much information about surface hydrophobicity of pastirma.

Ahhmed et al. (2013 and 2014) conducted comparative studies of FM and PS of *M. latissimus dorsi* and *M. semimembranosus* of beef [20, 58]. They found that the hydrophobicity or fluorescence intensity increased by 45% and 16.5% in pastirma made from *M. latissimus dorsi* and *M. semimembranosus,* respectively. This was a significant difference compared to the data of the current study, where pastirma showed a 107% increase in surface hydrophobicity. However, this can be attributed to many factors such as method, time of experiment, muscle type, and units used to express data. It is

possible that hydrophobicity is an indication of protein degradation that occurs as an effect of processing, which is further supported by the SDS-PAGE and protein oxidation data.

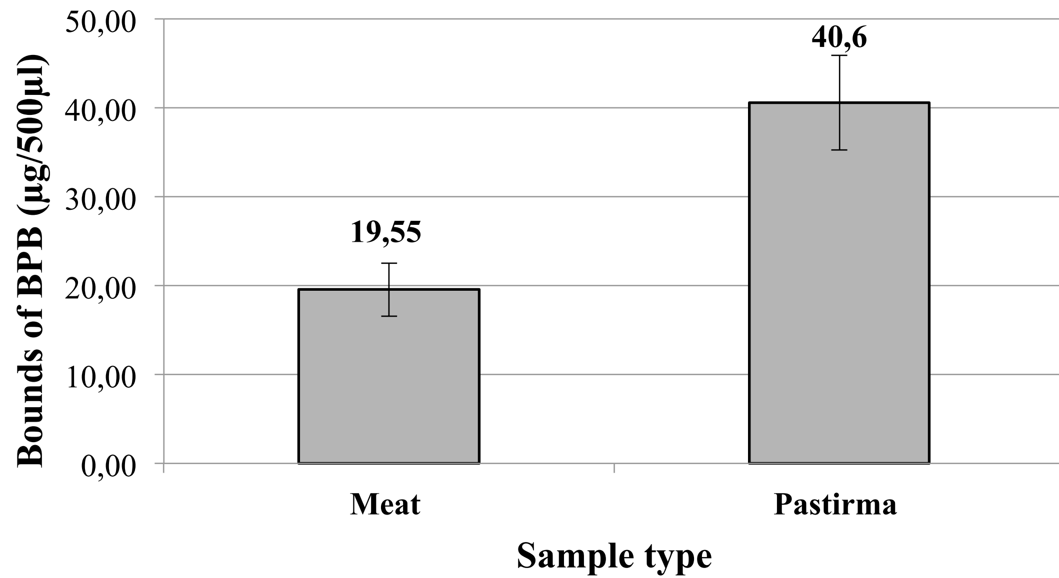

Figure 3.9. Bounds of BPB in meat and pastirma samples

CHAPTER 4

CONCLUSIONS

The utilization of traditional meat products as functional foods has been an area of interest in recent years because they are considered to be preservative- and chemical-free foods with more health options. In scientific studies, many physiologically active compounds including bioactive peptides have been identified in traditional foods. To be more precise, recently foods have been considered as an alternative to use of synthetic drugs because of their effects on lifestyle-related diseases. By this trend, in near future it may be possible to offer food with simple structure in order to minimize the pain of hypertensive subjects from some chronic diseases.

Because it is easier to prevent diseases than to treat them, scientists attach great importance to elucidating the causes of hypertension. There are many factors that contribute to this disease, including stress, obesity, alcohol consumption, diet, inactivity, and smoking. Pharmaceuticals are most commonly used to reduce the incidence of hypertension. However, medicines have many side effects that may cause other diseases and complications. On the other hand, the benefits of the bioactivities of food compounds outweigh their side effects. Based on this idea, this research was carried out in order to focus on the bioactivity of meat and, in particular, meat product hydrolysates, which are present in traditional Turkish pastirma and its counterpart, fresh beef.

Meat and meat products both have an important role in the diet of humans, and they provide the protein, minerals, and vitamins necessary for a balanced diet. As well as being a protein source, meat provides protein superior to other protein sources. Proteins consist of 20 different amino acids, 8 of which are essential amino acids, which cannot be synthesized in the body and therefore must be provided from food with complete proteins. The quality of protein in a food is measured in different ways, however the most common way is to determine the ratio of available amino acids in food compared

with needs. It is well understood that the ratio of meat in this respect is about 0.95 and very high compared with other foods.

In this study, meat and the meat product pastirma were chosen because they have abundant and qualified protein content, particularly since they are highly consumed in Turkey. It was found that protein concentration of non-digested fresh meat was 4.4 mg/ml, whereas pastirma had 8.0 mg/ml protein concentration. Also, non-digested PBC had 6.5 mg/ml protein concentration. As fresh meat is processed into pastirma, protein concentration is increased due to the pastirma producing steps. Proteins are broken into smaller peptides and enzymes. These novel compounds cause the increase in the protein concentration. In addition, the results show that covering the meat with cemen leads to an increase in protein concentration. On the other hand, among the digested samples of FM, PBC, and PS, PBC had the highest protein concentration. Digested FM and PS had protein concentrations of 15.9 and 16.7 mg/ml, respectively, whereas PBC had 18.1 mg/ml. When digested and non-digested samples were compared, it was clear that digestion augmented the protein concentration. With the effect of physical and chemical treatments of the human digestive system, newly produced peptides, it is clear that enzymes and bioactive peptides cause the increase in protein concentration.

The results of these experiments helped to evaluate the changes in the protein structure associated with the pastirma-making process. Ahhmed (2014) mentioned that if meat is dry cured, there must be many biochemical and biological changes accruing during processing [58]. These changes take place on protein and fat structures, with some proteins providing peptides that may have biological benefit. Additionally, SDS-PAGE analysis of hydrolysates also supported the changes in protein structure during the pastirma making process. When the acrylamide gels were compared, it is clear that the pure hydrolysate bands disappeared during meat processing. Therefore, there were not any protein bands of pastirma hydrolysate from 50 kDa to 15 kDa, while the hydrolysates of FM and PBC had protein bands in this range. This infers that proteins are broken down into smaller peptides with lower molecular weights while pastirma production.

Because possible antihypertensive effects of the pastirma samples would be apparent after consumption, the pastirma samples were enzymatically hydrolyzed with pepsin

and trypsin to simulate the human digestive system, and subjected to ACE inhibition analysis. The total ACE inhibitory activity from small proteins or peptides found in the structure of pastirma hydrolysate was expressed as IC_{50}. Also, in order to compare the hypertensive inhibitory effects of pastirma with meat and pastirma before cemen, the other samples were also exposed to the same digestion and inhibition analysis process.

Fresh meat, pastirma before cemen covering and pastirma showed inhibition ratios against ACE of 85.55%, 62.4% and 77.24%, respectively. These results demonstrate that meat and pastirma metabolized in the intestinal tract are sources of antihypertensive peptides. The study demonstrates that both meat and pastirma have not only nutritional utility but also nutraceutical value, because proteolysis on meat generated a substantial number of peptides that have therapeutic roles, some of which have strong ACE inhibitory activity. Hydrolyzed proteins from beef and pastirma had IC_{50} values of 1.13 and 0.92 mg/ml, respectively. Apparently, this demonstrates that pastirma proteins have better nutraceutical therapy that minimizes hypertension with no impact due to salt content, but it does not mean that consuming pastirma is particularly healthy. This study attempts to show that pastirma has bioactive peptides.

Unexpectedly, the IC_{50} value of pastirma before cemen-covering was 4.07 mg/ml. This indicates that PBC has 4 times less antihypertensive effect than that of FM and PS. For the PBC sample, the experiment was performed on a different day than the other samples. This unexpected result might have been caused by different experimental conditions such as the concentration of substrate, origin of ACE, and other variables. PBC was only added to the research in order to compare the effect of cemen on meat protein structure and to determine the variations that occur during processing stages.

Results of the protein oxidation experiment showed that as meat was processed into pastirma, oxidation occurred. The results demonstrate that non-digested FM has 477 μmol/100 g free thiol groups (SH), while non-digested PBC and PS has 394 and 116 μmol/100 g, respectively. The reduction in thiol groups while meat aged from fresh state to pastirma indicates that the muscle proteins in FM were denatured. In other words, the muscle protein in FM was undenatured and maintained their structure, while protein degradation occurring during the process in PBC and PS contributed to the reduction in amount of thiol groups.

Denaturation of myofibrils by pastirma production such as curing, oxidation, and pressure was monitored by determination of surface hydrophobicity. The results show that there was a 2-fold increase in protein hydrophobicity between fresh meat and pastirma. The difference in binding of BPB of meat and pastirma samples indicates the extent of protein denaturation during pastirma processing. Furthermore, the results of hydrophobicity support the results of SDS-PAGE and protein oxidation.

Therefore, the results of this thesis, which determined that the ACE inhibitory peptides in pastirma hydrolysates have therapeutic effects, have a great importance in the literature. This research is one of the rare studies that identify bioactive peptides from Turkish traditional pastirma.

In conclusion, it is recommended that food researchers focus on ACE inhibitory peptide sources, development of their bioactivity, and introducing these functional foods to the general population. Since chemical drugs have many side effects, consuming functional foods is a healthy alternative for treating lifestyle-related diseases such as hypertension. On the other hand, since the effect of peptides in these foods is not as strong as the effect of chemical drugs, these foods cannot replace medications in hypertension. However, they may be used for the prevention of hypertension. Furthermore, these model peptides sourced from food could be encapsulated and sold as prodrug peptides as alternatives to the chemical medications used to treat hypertension.

The results of this study exhibited that meat might become a biologically active food through gastric and intestinal digestion. This research provided evidence that pastirma contains a considerable number of constituents that could be utilized as a source of functional ingredients and nutraceuticals such as peptides, which could be used for clinical therapies. The results signified the value of dietary alternatives to chemicals to treat hypertensive diseases. Moreover, the results of the experiments demonstrate that during production of pastirma protein structure changes.

4.1. Suggestions

In conclusion, the inferences and interpretations that can be made in the context of the findings of the study are summarized below:

- The results support that fresh meat has remarkable antihypertensive activities. Moreover, the peptides resulting from protein degradation that occurs during pastirma production process exhibit ACE inhibitory activity. Pastirma was shown to have antihypertensive activity (IC_{50} = 0.92 mg/ml).

- Although pastirma has a high level of salt content, it can still provide significant ACE inhibitory activity. However, encouraging patients with high blood pressure to eat pastirma is not practical because of the high salt content. Thus, these model peptides could be encapsulated and sold as prodrug peptides as alternatives to chemical medications against hypertension.

- Pastirma can be presented to individuals as a product with antihypertensive effect. However, the antihypertensive peptides should be isolated from the structure of pastirma using RP-HPLC.

4.2. Problems and Limitations

- HPLC could not be used in order to isolate bioactive peptides because of its heavy schedule. If it had been used, more experiments on collected fractions would be conducted. This required more time and money to spend.
- The low budget of the project was another limitation for this thesis. The enzyme used for the inhibition experiment is very expensive. Because it was used for 3 different samples and their replicates, a major part of the budget was spent for the enzyme.
- Further isolation experiments including using animals, especially rats, as models could not be performed due to low budget of the project. A bigger budget was required for the rats and experiment materials of *in vivo* study.
- Another limitation was the lack of the chromatography instrument and its software for amino acid sequencing.

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APPENDIX

Appendix 1. pH Raw Data

Sample	pH value
FM 1	5.78
FM 2	5.82
FM 3	5.81
PBC 1	5.90
PBC 2	5.90
PBC 3	5.92
PS 1	5.89
PS 2	5.96
PS 3	5.90

Appendix 2. Water-Soluble Protein Concentration Raw Data

Sample	Absorbance 1	Absorbance 2	Absorbance 3
Control	0.0578	0.0588	
FM	0.2267	0.1765	0.18
PBC	0.2335	0.2367	0.2362
PS	0.2366	0.2311	0.2466

Appendix 3. GS-ATP Protein Concentration Raw Data

Sample	Absorbance 1	Absorbance 2	Absorbance 3
Control	0.0578	0.0588	
FM	0.3233	0.3293	0.3253
PBC	0.4430	0.4449	0.4457
PS	0.3834	0.3539	0.3538

Appendix 4. Hydrolysates' Protein Concentration Raw Data

Sample	Absorbance 1	Absorbance 2	Absorbance 3
Control	0.0603	0.0610	
Digested FM	0.8498	0.8566	0.8506
Digested PBC	1.0260	0.9720	0.9250
Digested PS	0.8873	0.8936	0.8992
Non-digested FM	0.2799	0.2863	0.2847
Non-digested PBC	0.3820	0.3860	0.3900
Non-digested PS	0.4546	0.4676	0.4701

Appendix 5. Hydrolysates' Angiotensin Converting Enzyme (ACE) Inhibition Raw Data

Sample	Absorbance (%100)	Absorbance (%50)	Absorbance (%25)	Absorbance (%12.5)	Absorbance (%6.25)
Control	0.0398				
Control	0.0377				
Digested FM	0.1181	0.2432	0.2959	0.4171	0.5616
Digested FM	0.1496	0.2286	0.3656	0.4889	0.5989
Digested FM	0.1567				
Digested PBC	0.341	0,4011	0,4592	0,6157	0,7039
Digested PBC	0.2801	0.4191	0.5395	0.6808	0.6966
Digested PS	0.2209	0.2984	0.3754	0.5030	0.6580
Digested PS	0.2102	0.2846	0.5119	0.5588	0.6282
Digested PS	0.1707				

Appendix 6. Raw Data of SH group

Sample	Absorbance 1	Absorbance 2	Absorbance 3
Digested FM	0.516	0.786	0.755
Digested PBC	0.443	0.393	0.469
Digested PS	0.439	0.347	0.317
Non-digested FM	0.287	0.285	0.297
Non-digested PBC	0.346	0.360	0.342
Non-digested PS	0.119	0.141	0.126

Appendix 7. Raw Data of free sulfide molecules

Sample	Absorbance 1	Absorbance 2	Absorbance 3
Digested FM	0.015	0.007	0.018
Digested PBC	0.004	0.007	0.002
Digested PS	0.009	0.002	0.004
Non-digested FM	0.158	0.159	0.103
Non-digested PBC	0.066	0.065	0.068
Non-digested PS	0.075	0.067	0.060

Appendix 8. Raw Data of bounds of BPB

Sample	Sample 1	Sample 2	Sample 3
Fresh meat	21.7	23.31	13.66
Pastirma	30.75	49.04	42.01

CURRICULUM VITAE

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SELECTED PUBLICATIONS

1. Ahhmed, A., Özer, N., Özcan, C., Çam, M., Sakata, R., Muguruma, M., Yetim, H., 2015. Meat therapy for hypertension: hybrid hydrolysate as ace inhibitory compounds. In proceeding of the 19th International Conference of FFC - Functional and Medical Foods, Bioactive Compounds and Biomarkers (P 108-111). 17Th -18th Nov., Kobe University, Kobe, Japan. (Oral presentation)

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