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Research Article

Solubility, Stability and Blood Pressure Lowering-Properties of Fresh and Cured Beef Proteins

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Abstract

This study aimed to investigate the solubility, stability, and blood pressure lowering-properties of fresh and cured beef product (pastirma) proteins. The changes in solubility, oxidation, molecular weights, and surface hydrophobicity of muscle proteins were investigated. Antihypertensive activities of peptic hydrolysates from fresh meat (FM) and pastirma (PS) were also determined using an angiotensin-converting enzyme (ACE) assay. Pastirma showed a higher solubility of sarcoplasmic and myofibrillar protein (3.59 and 6.10mg/ml) than in fresh meat (2.71 and 5.35/ml). Data suggests that the salt-curing process of beef increases the solubility of both sarcoplasmic and myofibrillar proteins. The high solubility in PS proteins referred to the spontaneous proteolyses occurred during the course of the process, which results in a remarkable protein denaturation phenomenon. Data illustrates that SH and free S groups (477 and 413µmol/g) in fresh beef were higher than those in pastirma (116 and 56µmol/g) samples meaning that a protein oxidation-reduction reaction has occurred during the course of processing. Salt as a pro-oxidant in pastirma might have broken the disulfide bridges of the native proteins allowing reactive oxygen species to react in-depth with the hydrophobic amino, which perhaps results in producing thiol and sulfonic-related compounds. Proteins surface hydrophobicity was a 2-fold increase in PS due to protein denaturation meaning that more hydrophobic amino acids were liberated during the course of processing. The SDS-PAGE gel images distinguish the number of changes occurred on protein structure. Myosin heavy chain protein (200kDa), β–galactosidase (117kDa) and glutamic dehydrogenase (55kDa) were present in fresh meat but they have become vanished in the cured beef. The salt-curing process and cemen treatment during the course of manufacturing highly contributed to the inexistence of those proteins. ACE inhibition ratios in FM and PS samples were 85.55 and 77.24%. The antihypertensive activities (IC50) of fresh beef and pastirma were 1.13 and 0.92 mg/ml ($p< 0.05$), respectively. Hydrolysates in pastirma showed a higher antihypertensive activity than in fresh beef. Data of this article suggests that solubility of pastirma protein was increased but the stability decreased, however, pastirma may contain a potential number of constituents that could be utilized in the area of functional food and nutraceuticals.

Keywords: Pastirma; Protein Oxidation; ACE; Bioactive Peptides; Hydrophobicity; Antihypertensive Activity

Abbreviations

ACE: Angiotensin Converting Enzyme; BAPs: Protein-Derived Bioactive Peptides; CVD: Cardiovascular Disease; His-Leu-COOH: Carboxy-Terminal Dipeptide; RAS: Renin-Angiotensin System; FM: Fresh Meat; PBC: Pastirma Before Cemen Covering; PS: Pastirma as a Final Product; GS-ATP: Guba-Straub-Adenosine Triphosphate buffer; WSP: Water Soluble Proteins; EDTA: Ethylenediaminetetraacetic Acid; SDS-PAGE: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; SH: Sulfhydryl Group; Hip–His–Leu: hippuryl-L-histidyl-L-leucine; NaCl: Sodium Chloride; BPB: Chromophore Bromophenol Blue; MHC: Myosin Heavy Chain; kDa: Kilo Daltons; PCH: Protein Concentration of Hydrolysates; IC50: Half Maximum Inhibition Concentration.

Introduction

Dietary proteins of muscle foods are a source of biologically functioning peptides that became well known for their therapeutic properties to prevent lifestyle- or age-related diseases. In

particular, meat bioactive peptides that have antioxidant and antihypertensive activities are generated during processing or cooking. Nowadays, a large number of individuals suffer from chronic diseases such as hypertension, osteoporosis, joint inflammation, diabetes, Alzheimer and cancer due to diet type and lifestyle. There is a remarkable sense by many individuals that more natural methods different from chemical medication should be identified, tested and then applied in the effort to reduce the suffering of those people who are affected by major chronic diseases. Thus, functional and nutraceutical methods are being considered for potential use as natural-based treatments to tackles those diseases. Bioactive peptides are a novel medical alternative and have been the focus of researchers in the last two decades.

The widespread application of protein-derived bioactive peptides (BAPs) with health-promoting properties in human nutrition is currently limited [1]. However, the first study of bioactive peptides was conducted by Mellander [2], as he found that bioactive peptides obtained from casein contributed to bone development independently of vitamin D in rachitic babies. It was reported that in the last 20 years, interest in this topic has increased as most researchers focused on the production of bioactive peptides from milk proteins [3]. In fact, there are many bioactive peptides function as anti-osteoporosis [4], antidiabetic, antimicrobial, antilipase, and antioxidant activity [1]. Peptides with blood pressure lowering properties were sourced from many food types like fermented meats [5] milk [6,7], oilseed proteins [8], as well as soya, chicken, fish [9], pork, and chicken bones [10,11], but yet not many from beef sources. The discovery of antihypertensive peptides that are involved in the inhibition of ACE has been the most interesting type of research on bioactive peptides. It is a well-established theory that ACE plays an important role in hypertension by regulating blood pressure [12]. One of the most common types of cardiovascular disease (CVD) is hypertension, in which the blood pressure is persistently higher than normal values (systolic pressure 120 mmHg and diastolic pressure 180 mmHg) [13]. Systematically, ACE is a well-characterized Zn_{4+} metallopeptidase that removes the carboxy-terminal dipeptide (His-Leu-COOH) from the decapeptide angiotensin I to generate the potent vasoconstrictor angiotensin II. ACE is a circulating transmembrane dipeptidyl peptidase that is capable of cleaving any angiotensin peptide that is available in the endothelial or soluble form in blood and body fluids. This enzyme plays an important role in the renin-angiotensin system (RAS) and consequently on the regulation of homeostatic pressure of blood. In addition, it catalyzes the transformation of the inactive form of angiotensin I to active angiotensin II, and further in rare cases 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 [13-18]. In addition, hypertension is largely influenced by lifestyle-related habits, including a high intake of sodium or fats, the presence of high stress, lack of sports, being diabetic and or excessive use of medications. CVDs are the most common cause of death globally, killing 17.5 million people per year, also cardiovascular diseases are the leading cause of death as it caused almost 32% of all deaths in women and 27% in men in 2004 [19]. The global prevalence of high blood pressure in adults aged 18 years and over was approximately 22% in 2014 [20]. It was reported that the age-standardized prevalence of hypertension disease was lower in high-income countries (18 and 27% in women and men, respectively) but slightly higher in countries with middle-income (24 and 30% in women and men, respectively) [21]. In Turkey, Age-standardized prevalence % of raised blood pressure, 18+ years, is between 23-25% for both women and men population [21]. Dozens of synthetic antihypertensive drugs with ACE inhibitors have been reported to have side effects such as hypotension, angioedema, skin rashes, dizziness, tiredness, coughing, and headache, as well as heart damage and stroke [14,22].

Alternately, bioactive peptides sourced from functional foods that can be utilized as a viable and novel medical option to reduce hypertension. Muscle foods are rich a source of bioactive peptides as the protein of fish, chicken, beef, and pork and their by-products have been shown to have bioactive peptides, specifically ACE inhibitory and hypocholesterolemic action peptides [10,11,13,23-26,28]. However, there is not sufficient research information regarding the purification of ACE inhibitory peptides from beef and its processed products. Fresh meat and pastirma are the most commonly-consumed meat products in Turkey.

Pastirma is a sort of cured meat which has an attractive exterior and interior appearance, delicious taste, unique smell and muscle-like shape [29], that its production process takes about 4 weeks. The salting, curing and drying procedure during that period of time affects the structure of the proteins and the enzyme mechanism potentially increase the nutritional and sensory values of pastirma [30,31]. Little information is available regarding the nature of chemical changes in proteins during the traditional process of pastirma production as well as the yield of bioactive peptides.

Objectives of this study were to investigate the ACE inhibitory activity of hydrolysates derived from FM, and PS. This research was carried out in an attempt to utilize nutraceutical compounds to substitute the usage of chemical medication that mitigates or lower high blood pressure. Likewise, the chemical changes occurred on muscle proteins during the traditional process of pastirma production were also evaluated.

Materials and Methods

- **Samples:** The meat samples were sourced from 30 months-old male cow (Species: Montofon), this beef breed is widely available in Turkey. Precisely, for each experiment two groups of muscles were prepared based on the treatment and time point of processing: one group was analyzed as FM, while the other group was processed into pastirma, which had two subgroups [pastirma before cemen process (PBC) and (PS)]. Biceps Femoris muscles (48-hour post-mortem) were obtained from the same animals had pH values about 5.6. PBC and PS samples were taken from production [29]. The pastirma was traditionally manufactured in a local producer "Şahin-Melek for meat and meat products in Kayseri province.
- **pH measurements:** pH values of samples were measured by preparing 5 g of MF, PBC, PS that homogenized in 20 ml distilled water by a silent crusher and pH was measured by a pH meter (Mettler Toledo, Switzerland).
- **Protein solubility:** Concentrations of extracted proteins were determined by Biuret method [32]. The absorbance of samples was measured by a spectrophotometer (Agilent, Cary 60 UV-Vis, USA), at 540nm. Proteins were extracted using two different solutions: a low-ionic-strength solution (50 mM imidazole-HCl, pH 6.0, 2 mM (EDTA)) that extracts the water-soluble proteins (WSP) which includes mitochondrial enzymes [29-31], cytochromes, myoglobin, and hemoglobin. While the second solution was a high-ionic-strength solution, the Guba-Straub-Adenosine Triphosphate buffer (GS-ATP), (0.09 M KH_2PO_4, 0.06 M K_2HPO_4, 0.3 M KCl, 1 mM ATP, pH 6.5) [28]. The latter is prepared to extract muscle proteins including heavy protein such as actomyosin complex.
- **Protein electrophoresis:** Molecular weights of native proteins and also digested proteins sourced from FM, PBC and PS were determined by gradient slab gels (7.5-20%) of Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). BIO-RAD 161-0363 marker was used to compare molecular weights of proteins in each sample. Extracted and fractionated proteins were dyed and electrophoresed at 36mA/Gel constant current. Consequently, the gels were dyed with Coomassie Brilliant Blue R-250 and eventually destained to get the gels ready for imaging [10].
- **Determination of protein oxidation:** Total sulfhydryl groups were measured by adding 0.5 ml of myofibrillar proteins extracted in GS-ATP and/or hydrolysate samples to Tris-HCL buffer [mixture of Tris-hydrochloride, sodium dodecyl sulfate (SDS), urea, (EDTA) and H_2O, 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 room temperature, and then they were centrifuged for 10 min, the supernatant checked by a spectrophotometer (UV-1800, UV, Shimadzu) at 412 nm [33,34]. Sulfhydryl (SH) groups were calculated using the following equation: Total SH µmol/g =73.53 x A/P. The extinction coefficient is 73.53; A: sample`s absorbance; P: sample`s protein concentration (mg/ml). Free sulfide molecules were determined by adding 0.2 ml of myofibrillar proteins and or hydrolysates from digested samples to Tris-HCl buffer, and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) buffer [β-mercaptoethanol and DTNB]. The mixture was left at room temperature in a dark place for an hour, thus a cold Trichloro-acetic-acid buffer (50% TCA) was added and the samples were centrifuged for 5 min and a Tris-buffer [SDS, EDTA, urea, Tris-HCl and distilled water] was added too. The absorbance of samples was determined by a spectrophotometer at 412 nm [33,34]. The free sulfhydryl groups were checked using the following equation: SH µmol/g=73.53 x A/P. Finally the free of sulfide molecules µmol S/g **=** µmol total SH/g - µmol free SH/g.

Surface hydrophobicity

Hydrophobicity of proteins was measured by mixing 400 µl of myofibrillar proteins with 80 µl buffer Bromophenol Blue (BPB). Mixtures were left in a dark place for 10 min at room temperature. Eventually, the samples were centrifuged at 6200 rpm/5min, and then 2700 µl of H_2O was added to the supernatant. The absorbance of samples was determined by a spectrophotometer at 495 nm [35] and the following equation was used:

BPB bond (µg/ml) = 200 µg x $(S_{Abs}-C_{Abs})/(C_{Abs})$. S_{Abs}: Absorbance of samples; C_{Abs}: absorbance of control.

Protein digestion process: Peptic hydrolyzation was carried out by separately adding 50 g of FM, PBC, and PS to 130 ml of $D.H_2O$ in which all were blended for 5 min in a food. Then, the mixtures were homogenized and then heated at 70°C for 30 min in a water bath on account of cooking simulation. The samples were cooled down to 40°C, and then pepsin (Sigma–Aldrich, Inc. St. Louis, MO, USA) from porcine gastric mucosa was added at an acidity medium 1.5. After the first peptic digestion, the enzyme was inactivated by boiling the samples for 10 min. In the same manner, trypsin was added but in alkaline medium. Eventually, supernatants of centrifuged samples were then passing through cheesecloth to remove fats and unwanted tissues. Filtrates were fractionated by a cellulose membrane filter (0.45 µm).

ACE inhibitory activity assay

The ACE inhibitory activity was determined by using the method of Cushman and Cheung [36]. This method was partially modified by Katayama., *et al.* [23,27]. 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 the peptide was mixed with Hip–His–Leu (Nacalai Tesque Inc., Kyoto, Japan) as a substrate containing sodium borate buffer and sodium chloride (NaCl) and then pre-incubated at 37°C. ACE of rabbit lung (Sigma–

Aldrich, Co., MO. USA) was added to the mixture and incubated at 37°C for 30 min. The reaction then was stopped and hippuric acid liberated by ACE was extracted by adding ethyl acetate to the mixture with vigorous shaking for a certain period of time. The resultant of centrifugation and ethyl acetate evaporation process was dissolved with NaCl solution and its absorbance was determined at 228 nm in a spectrophotometer. ACE inhibitory activity was expressed as IC_{50} values and the ACE inhibition ratio calculated using the following equation: Inhibition (%) = (C-S)/(B-S) x 100. S: sample`s absorbance of; C: absorbance of control; B: Absorbance of the blank samples.

Statistical analysis

Statistically the results 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).

Results and Discussion

There is a lack of biochemical information on the function of food proteins, especially from traditional Turkish foods such as pastirma. It was not clear how do proteins change in pastirma when it is exposed to different treatments such as cooking and digestion. Furthermore, there is a significant public concern regarding Turkish pastirma and its effects on health. There is a belief that proteins in pastirma have dozens of nutritional and therapeutic activities [31], and this study was conducted to verify this hypothesis. Because high blood pressure is a disease that affects a large number of Turkish individuals, the study of nutritional alternatives to treat, mitigate, and or reduce the incidence of such diseases is important. Peptides and hydrolysates derived from meat proteins are known to inhibit ACE, which is the latter believed to be the initial element that contributes to the mechanism of hypertension.

pH values: According to the Turkish Food Codex Meat Products Communiqué [37], the pH of pastirma should be a maximum of 6.0.

Parameter	Fresh meat		Pastirma before cemen		Pastirma	
	Means	SEM	Means	SEM	Means	SEM
pH values	5.80[a]	0.01	5.90[b]	0.0	5.91[b]	0.02
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

Table 1: pH values and protein concentrations of fresh meat, pastirma before cemen and pastirma samples in mg/ml. All values are reported as the mean ± SEM of four determinations.

[abc]The values indicated by different lowercase letters on the same row show the statistically significant differences between the muscle types ($p < 0.05$)

The pH values of pastirma samples in this study were slightly higher than the value in fresh meat (FM: 5.8, PBC: 5.9 and PS: 5.91). The fresh meat used for pastirma production had a pH of 5.8, which is in the range as the optimal pH of meat to be used for pastirma production. After the fresh meat was salted and pressed PBC, the pH increased slightly to 5.90, but this increase was not significant ($p > 0.05$) (Table 1). The slight increase in pH values suggests that there was no production of lactic acid in pastirma cuts that normally comes from a fermentation process by any spontaneous bacterial invasion. 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 also was not a significant increase ($p > 0.05$). The process of curing and salt adding had no effect on pH values, which are in accordance with standards of the Turkish Food Codex for Meat Products Communiqué [37].

Protein extraction

The solubility of WSP

The main objective of the protein extraction was to quantify, characterize, and determine the degradation process of meat that takes place during pastirma production. As results, protein concentrations of WSP extracts from 3 different samples increased significantly from FM to PS ($p < 0.05$) (Table 1). Sarcoplasmic protein concentration reached the highest value in PS samples while FM had the lowest. Data indicate that protein concentration in FM, PBC, and PS had 2.71, 3.53 and 3.59 mg/ml, respectively. This phenomenon is always predicted as the sarcoplasmic protein tissues get damaged due to the aging process of proteins which results in the liberation of the WSP. This increase in the concentration of sarcoplasmic proteins is mediated by protein degradation. In addition, enzymatic hydrolysis results in new peptides and protein fragments that are less soluble in the low ionic strength solution. This increase was attributed to the degradation of the samples during the pastirma making process. In a previous study, it was suggested that FM had 3.84 mg/ml while PBC and PS had 2.1 and 4.9 mg/ml protein concentration, respectively [29]. Regardless of the PBC, protein concentrations of WSP extracts of FM and PS were lower in the current study than in our previous study. Differences between the results in both studies may refer to the different muscle types used in research.

The Solubility of myofibrillar or GS-ATP proteins

Myofibrillar or muscle proteins account for 9.5% of protein content, and due to their fibrous structure, their extraction requires highly ionized buffers, such as GS-ATP. Ironically, 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 (Table 1). The lower value in FM perhaps stands for the lack of endogenous enzymes' quantity which is always responsible for the liberation of proteins that are trapped within muscle fibers during the extraction process. It was reported in another research paper of beef and its pastirma but carried out on a different muscle type that the protein concentration value of PBC was 6.36 mg/ml, in fact, lower than the values in its counterparts in the current study (7.72 mg/ml) [30]. The difference in that result might refer to muscle type and other inputs such as intrinsic and extrinsic factors. The curing process of meat increases the protein content in the GS-ATP buffer (myofibrillar), as the humidity gets lower, possibly the extraction of proteins in PS was obstructed by the cemen. Moreover, there may have been some proteins bound with phenol compounds derived from cemen, polymerizing the protein into a protein-polyphenol complex that stood as a barrier and thereby reducing extractability.

Protein oxidation

Phenomenally, muscle structure and proteins in PS ran into many turnovers among which are organoleptic, physical, virtual, and chemical and biochemical changes. The protein oxidation that occurs in pastirma has yet to be fully or partially explained. Based on the difference in the thiol group and sulfide molecule content between FM and PS, it is estimated that amount of the denatured proteins in PS was higher by ~ 50-51% than in the native proteins of fresh meat ($p < 0.05$) (Table 2). In other words, the muscle protein in FM was undenatured and maintained their structures. Unlikely, in the PBC and PS, the protein experienced a great denaturation phenomenon due to production processes. In PBC and PS, the reduction in the number of free sulfide molecules perhaps results in the production of compounds such as sulfonic, sulfinic acid salts, and thioesters. Oxidation of proteins and fat in meat products is an important biochemical change. It is crucial because it profoundly deteriorates meat quality traits, leading to a possible commercial reduction in revenue of pastirma or any aged meat products. The rate of protein oxidation depends on many factors such as the protein composition and concentrations, the existence and activities of pro-oxidants and antioxidants, the oxygen partial pressure, the structure and retained water in the meat [38], the method of processing and the conditions in which the meat is stored and cooked [39,40]. Precisely, oxidation reaction influence the protein structure, function and also the flavor of meat products because at the end of its reaction, it generates many cross-linked sulfide groups, thiols and carbonyl groups. 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 [41]. Protein oxidation of meat, which occurs during pastirma production, is determined by monitoring carbonyl formation [42] and sulfhydryl losses [43] 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 a marker for protein oxidation [34]. These carbonyl groups originated from peptide scission, amino acid residue, side chain groups, and carbonyl compounds that are interconnected with proteins [44]. For the sulfhydryl (SH) content in FM and PS was determined using DTNB. The oxidation assay measures SH groups and free sulfide (S) molecules but not sulfide bridges. In other words, protein oxidation causes the disulfide cross-linking [45]. Moreover, these linkages contribute to the formation of gaps between muscle fibers that helps the DTNB to detect free sulfide molecules within muscle filaments [46]. Data of this study suggest 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 disulfide bonds were being created and increased when compared to the FM samples.

Moreover, when the results of SS and SH groups were compared in digested and non-digested samples, it is obvious that non-digested samples had higher values in all 3 groups (Table 2). As digested protein samples are more denatured, they are expected to have higher SS and SH values. However, sulfur molecules in digested samples might be affected by some factors such as thermal treat-

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
FM	477[a]	0.06	413[a]	0.15	316[a]	0.39	311[a]	0.40
PBC	394[ab]	0.06	320[b]	0.07	176[b]	0.09	175[b]	0.09
PS	116[b]	0.05	56[c]	0.08	161[b]	0.16	159[b]	0.16

Table 2: Thiol group and free sulfide molecules isolated from non-digested and digested fresh meat, pastirma before cemen and pastirma hydrolysates µmol per 100g of protein. All values are reported as the mean ± SEM of four determinations.

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

ment, enzyme activity, and the addition of hydrochloric acid (HCl) and sodium hydroxide (NaOH) (pH adjustment for the digestion process). Precisely, higher SH and free SS molecules in fresh meat also mean that DTNB could not have a good opportunity to re-bride the free sulfide into S-S bonds less than that in processed samples. Perhaps that is due to the physiologically trapping phenomena of thiol group inside the protein structure.

Data suggests that the increase in values of proteins oxidation in PS and PBC samples were higher than in FM, which is due to the salt treatment and oxygen exposure during the course of processing. Salt is known as pro-oxidant at levels (500-2500 mg/100g) widely used in processed meat products. Similarly, it is suggested that the pro-oxidant activity of salt in pastirma breaks the bridges inside the native proteins, which allows oxygen radicals to in-depth react with the hydrophobic amino acids and then initiate oxidation [47]. There is a positive correlation between oxidation progression and salt concentration in processed meats and this trend is more apparent in red meat (beef) than in white meat (chicken) [48], which is in agreement with the results of this study. Oxidation of protein in beef contributes to off-flavors in products that are resulted from heme iron content but that also facilitates lipid oxidation [49].

Hydrophobicity

Most of the moisture in the meat is reserved and trapped in muscle proteins in the association of functions between myofibrillar and sarcoplasmic proteins. When protein structures are broken down, hydrophobic clusters are spread out through membranes of sarcoplasmic proteins leading to leakage of water. On account of this, hydrophobicity can be an indicator of the denaturation of proteins, which in turn supports data of SDS-PAGE and protein oxidation. The method used in this study counted on the degree of interaction of the hydrophobic BPB with myofibrillar proteins, and the segregation of free and bound BPB by centrifugation [35]. Since the quantity of bound BPB is an index for protein hydrophobicity, the results indicate that the 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 (<0.01), (Figure 1). A positive correlation between surface hydrophobicity and the increase of temperature has been approved and discussed in previous studies. Chelh., *et al.* [35], who conducted research about surface hydrophobicity of meat proteins had 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 [35]. Also, Santé-Lhoutellier, and others [50] found that heating (45 min, 100°C) increased bound BPB (~ 40 µg) in beef myofibrils. This increase in protein surface hydrophobicity indicates that thermal and curing treatments trigger 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, aging and squeezing perhaps have caused protein denaturation resulting in surface hydrophobicity.

In other studies we compared FM and PS from different beef muscles [29,30], the reports stated that the hydrophobicity increased by 45% and 16.5% in pastirma made from *M. latissimus dorsi* and *M. semimembranosus,* respectively. A significant difference achieved in 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 expressed 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.

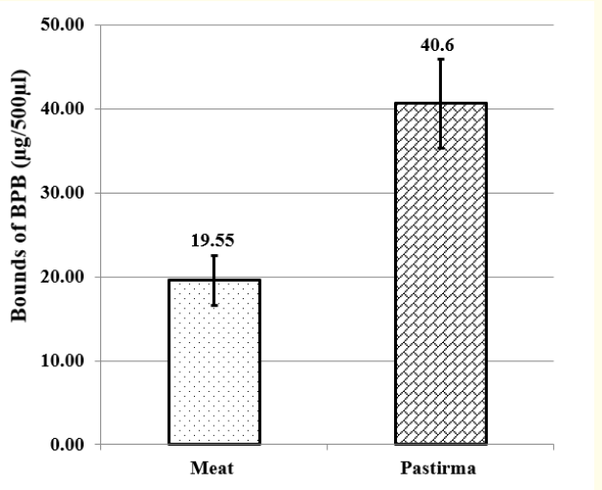

Figure 1: Effect of pastirma manufacturing process on the muscle proteins surface hydrophobicity, measured by the amount of BPB bound to proteins. All values are reported as the mean ± SEM of four determinations. Values of fresh meat and pastirma differ significantly ($p < 0.05$).

SDS-PAGE of proteins extracted in WSP

The gel images demonstrate and distinguish the number of changes and denaturation occurred on protein structure during the process of pastirma from their original state in fresh meat. Figure 3.1 shows that there were 4 different bands disappeared (Figure 2-A) 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 undetectable in the PBC and PS lanes. GS-ATP solution extracts the myofibrillar proteins that coexist with other proteins within the muscle. The MHC band (200 kDa) and β–galactosidase (117kDa) vanished in PBC (lanes 2) and PS (lane 3) unlike what is present in FM sample (lane 1) (Figure 2-B). In SDS-PAGE data, the band represents glutamic dehydrogenase (55 kDa) was clearly present in the FM sample, but not in the PBC nor in PS samples. The difference in existence and inexistence of those bands leads to suggest that those two proteins are salt and curing-sensitive proteins as they were completely de-

Figure 2: SDS-PAGE patterns show MW of proteins and their fractions in fresh meat, pastirma before cemen covering and pastirma samples, where are extracted in water soluble protein buffer (A), and in Guba Straub ATP (GS-ATP) (B). FM: Fresh Meat, PBC: Pastirma before cemen covering, PS: Pastirma. Dotty boxes show the disappeared proteins bands and the normal boxes highlight the newly developed protein bands.

graded in PBC and PS. Interestingly, a band represents glyceraldehyde 3-phosphate dehydrogenase (36 kDa) was present in FM, not present in PBC, but present in PS. There is no clear explanation of such phenomenal mechanism. The most possible hypothesis is that this type of protein gets extracted when there is adequate moisture content, as FM contains natural moisture and in PS the cemen has moderate humidity. In published research, we stated that cemen contains garlic and fenugreek, with proteins and peptides having molecular weights ranging from 6.5–66.0 kDa [51]. In addition, the SDS-PAGE data of myofibrillar proteins extracted in GS-ATP showed that trypsinogen appeared in the PBC lane, while it was expressed in small amounts in 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 covering process with cemen was responsible for the expression of this protein in PS. The protein content of the cemen might have caused protein-protein interaction and changes in the protein structure of meat during pastirma production. This hypothesis is also proposed and supported in earlier published papers about pastirma manufacturing and physicochemical changes [52]. At the loading samples place on the gels, there was a polymer in FM lane but was not detected in PBC and PS lanes. The aforementioned further approves that the course of processing metabolized some of the myofibrillar proteins, in which resulted in the production of bioactive peptides.

Protein concentrations of the hydrolysates (PCH) were determined separately, and as expected, digested samples derived from 3 different processing points had higher protein concentrations than in non-digested samples (Table 3). Therefore, the protein structure is changed by the digestion process, where longer protein chains are broken down into smaller polypeptides, peptides and amino acids are generated. The PCH values were used as an indication of the level of protein digestibility. On the other hand, some proteins may have defused during the mechanical pressing treatment during pastirma process. While FM had a concentration of 4.4 mg/ml protein, after digestion with pepsin and trypsin the concentration increased to 15.9 mg/ml, perhaps due to the newly generated peptides causing a higher absorbance. Similarly, the protein concentration of PBC increased from 6.5 mg/ml in the non-digested to 18.1 mg/ml in PCH. Moreover, PS had 8.0 mg/ml protein concentration for the non-digested sample, while PCH of PS had 16.7 mg/ml (Table 3). 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.

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

Table 3: Protein concentration of digested and non-digested samples in mg/ml. All values are reported as the mean ± SEM of four determinations.

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

The digestive enzymes cleave proteins to smaller polypeptides that give a higher absorbance spectrophotometrically. When the non-digested samples were compared to each other, it was clear that protein concentration increased as the meat was cured. Pro-

tein degradation arises from treatments such as salting, pressing, and cemen covering. The protein content of fresh meat nearly doubled when it was processed to pastirma (4.4–8.0 mg/ml) (Table 3). The results demonstrate that pastirma production, in general, causes an increase in the protein quantity, as larger protein chains are broken into smaller peptides due to pastirma-making processes. While PCH was compared with each other, the low protein concentration in PS showed an unexpected fall. PCH of PBC had higher values than PCH of FM and PCH of PS. The low PCH in PS was not statistically different from both FM and PBC, however, the low extractability was possibly 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 extensively discussed in a recent report [51].

Hydrolysates from FM, PBC and PS were electrophoresed as original hydrolysates (no dilution) and after being diluted in $D.H_2O$ 25% dilutions (Data not shown). Newly generated proteins at 50 kDa and lower in molecular weights were observed. On the other hand, when the protein bands of original hydrolysates (1/1) PBC and PS exhibited a lower number of bands than in FM. When meat turns into pastirma, proteins are broken down into smaller peptides with lower molecular weights. Precisely, PS 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, nevertheless, the density of smaller protein bands varied. Furthermore, when each group only a few protein bands were present in the lanes of diluted (25%: 1/4) of hydrolysates. In the dilution of FM hydrolysate (25%), 15 and 6.5 kDa proteins were present. Unlikely, in the lanes of PBC and PS hydrolysates, none of the protein bands were exhibited. The aforementioned 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 proteins or polypeptides in a range of less than 6.5kDa.

Antihypertensive activity

Bioactive peptides are liberated by microbial activity or proteolytic enzymes [16]. ACE inhibitory or suppression from hydrolysate of both fresh meat and pastirma samples were determined and the activities were expressed in IC_{50} values (Figure 4). FM, PBC, and PS samples were subjected to enzymatic hydrolysis by pepsin and trypsin to simulate human digestion. Several methods of bioactivity assays were performed on hydrolysates that were obtained by enzymatic hydrolysis to determine if they are capable to detect new peptides in the structure of pastirma. 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 are also performed on these diluted solutions (1, 1/2, 1/4, 1/8, 1/16) (Figure 3). The inhibition of ACE activity in FM, PBC, and PS was calculated as 85.55%, 62.4% and 77.24%, respectively (Figure 3-a). 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). There was a gradual decrease in the ACE inhibition ratio as the concentration of hydrolysates decreased. Hydrolyzed proteins from fresh meat and pastirma showed 1.13 and 0.92 mg/ml (Figure 3-b) IC_{50} values, respectively. This demonstrates that meat had a slightly higher IC_{50} value than pastirma, which means a low antihypertensive effect.

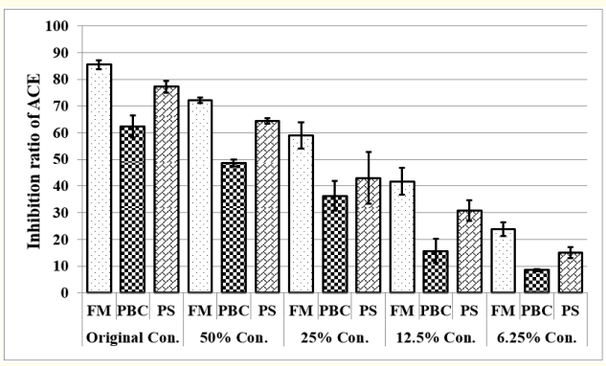

Figure 3: Inhibition ratio of ACE by hydrolysates isolated from fresh meat, pastirma before cemen and pastirma samples with varying concentration [100% protein: original hydrolysates; 50% con.: diluted hydrolysates (1/2); 25% con,: diluted hydrolysates (1/4); 12.5%: diluted hydrolysates (1/8); 6.25: diluted hydrolysates (1/16)]. All values are reported as the mean ± SEM of four determinations.

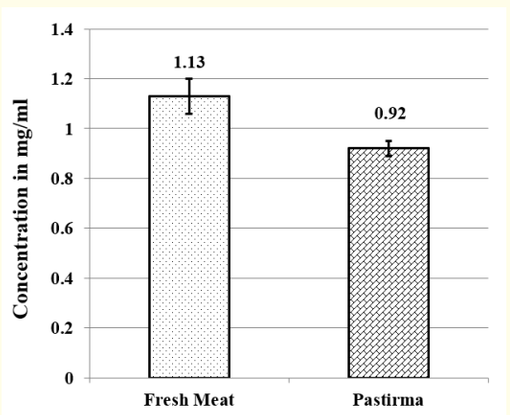

Figure 4: IC_{50} values of hydrolysates from fresh meat and pastirma of *Biceps femoris* muscles sourced from beef (mg/ml). All values are reported as the mean ± SEM of four determinations. Values of fresh meat and pastirma differ significantly ($p < 0.05$).

Due to the treatments during pastirma processing, a large number of peptides are generated by means of the spontaneous decomposition of proteins. Dozens of those peptides are effective at preventing and reducing chronic hypertension that is normally determined by an ACE inhibitory activity assay. This determines residues of hippuric acid, which is released as an end product of ACE activity. The lower the substrate-ACE reaction occurred, the stronger the ACE inhibitor activity (peptide kinetics). In other words, *in vitro* assay of the pastirma protein hydrolysates indicates that they had more nutraceutical potentiality that lowers hypertension than in 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 [53]. The first study to identify antihypertensive peptide from beef hydrolysate was conducted by Jang and Lee [54], 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. There have been few studies on the bioactivity of dry-cured meat products, where they could isolate a peptide AAATP (Ala-Ala-Ala-Thr-Pro), which had an inhibitory activity of 100µM, from Spanish dry-cured ham, which is a dried cured meat product produced from porcine [55]. Likewise, in a previous study, it was suggested that beef meat and pastirma have IC50 values of 0.68 and 0.78 mg/ml, respectively [56], where they stated that fresh meat had higher activity than in pastirma. In another study conducted by Deniz., *et al.* [57] demonstrated that pastirma showed an ACE inhibitory activity higher than 86%. When compared to other studies carried out on beef [31], pork [10,58], and chicken meat [59], pastirma has a competitive IC50 value. Jang, Jo, Kang and Lee [60], found 4 ACE inhibitory peptides separated from beef hydrolysates with IC50 values of 0.117, 0.0643, 0.0529, and 0.0505 mg/ml, respectively. In contrast, the sample used in the current study seems more effective than the samples used in the study conducted by Jang., *et al.* [60], because the volume of the samples they used was 16-fold higher than the volume of sample used in the current study. This study showed that pastirma is a potential source of antihypertensive bioactive peptides with an IC_{50} value of 0.92 mg/ml. It is strongly suggested that fresh beef and/or pastirma contain a considerable and enormous amount of potentially anti-ACE active peptides. Peptides sourced from fresh beef and aged muscles could be encapsulated and offered as prodrug peptides as alternatives to the chemical medications. Regardless to the processing beef may contribute to minimizing the risk of high blood pressure disease when consumed in a moderate amount, and leaving aside its salt amount, pastirma and its hydrolysate still exhibit a very potent ACE inhibitory activity. Protein hydrolysates fresh beef and pastirma (*biceps femoris* muscles) retardate ACE activity whereas stops the progression of hypertension disease.

Limitations and Future Research Direction

Due to the fund limitations and expense of enzymes, more specific analyses were not carried out, however further experiments are necessary to evaluate the by-products of protein oxidation using chromatographic methods. More bioactive properties are considered for investigation such as antioxidant, antiobesity and antidiabetic activities.

Conclusion

The results showed that protein concentrations were increased when fresh meat is processed into pastirma and covered with cemen. It was obvious that the digestion process augmented protein concentration and promoted the generation of bioactive peptides. Spontaneous protein proteolysis occurred during the process had contributed to the reduction in the number of thiol groups, in the aged meat (PBC and PS), while proteins in FM maintained their natural structure. Inhibition ratios against ACE in fresh beef, pastirma before cemen covering and pastirma were varied 85.55, 62.4, and 77.24%, respectively. These results demonstrate that fresh beef 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 nourished a substantial number of peptides that have therapeutic roles, some of which have strong ACE inhibitory activity.

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Conflict of Interest

The authors confirm that this article content has no conflict of interest.

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