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Research Article

Lysis of Proteins Derived from Fresh Beef and Pastirma Generates Miscellaneous Peptides having Angiotensin Converting Enzyme Inhibitory Activity

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Abstract

Hypertension is known to be a major disease that causes advanced and continuous high levels of blood pressure in the human circulation system. The initiative mechanism of action of hypertension starts with an angiotensin converting enzyme (ACE) that exists in two forms (endothelial and soluble in blood). Normally, the disease is treated by chemically prepared medication which may cause health complications. Despite that, the current article attempted to find alternatives to the chemically prepared medications including bioactive compounds (antihypertensive peptides) derived from food products. The aim of this research was to determine the antihypertensive activity of hybrid peptides from muscle proteins of fresh beef and pastirma (dry-cured muscles). The beef and pastirma were digested with pepsin and trypsin to generate miscellaneous but not specific bioactive peptides. The samples were subjected to *in vitro* analyses to evaluate their ACE inhibitory activity using a substrate (HHL) representing angiotensin. Effects of processing and dry-curing treatment, which normally causes a lysis action on the chemical structure of proteins in beef and pastirma were also investigated. As a result, the processing of beef into pastirma degrades the major proteins such as MHC (200kDa) and many enzymes including: β-galactosidase, Phosphorylase B, Lactate Dehyrogenase, Trypsinogene into small peptides. Hydrolysates in fresh beef and pastirma with protein concentration of 5.65 and 6.09mg/ml showed inhibition rates against ACE activity of 83 and 79%, respectively. The biological values (IC50) of antihypertensive activity were 0.68 and 0.78 mg/ml for fresh meat and pastirma, respectively. Proteins in fresh beef have remarkable ACE inhibitory activities, which makes it a potent model for sourcing bioactive peptides to treat hypertensive abnormalities. We suggest that miscellaneous peptides from fresh meat will provide nutraceutical compounds after digestion by intestinal proteases. Hybrid bioactive peptides may differ in potency and duration of action against ACE activity. However, data indicated that it is not necessary to process meat into pastirma to enhance its biological activities. These findings demonstrated the protective effect of bioactive peptides derived from beef demonstrate that they are of significant importance for therapeutic interventions of hypertension-related complications.

Keywords: Antihypertensive Activity; Bioactive Peptides; Pastirma; Protein Molecular Weight; Beef Proteins; ACE Inhibitory Activity

Abbreviations

ACE: Angiotensin Converting Enzyme; 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; IC_{50}: Half Maximum Inhibition Concentration.

Introduction

Hypertension is a major disease that attacks the public with no preference for age due to genetic or lifestyle related reasons. High

blood pressure (HBP) is a serious condition that can lead to: coronary heart disease, heart failure, stroke, kidney failure, and damage to other body organs including the brain and nerve system. The number of adults suffering from hypertension is estimated to increase to 1.6 billion worldwide by 2025 [1]. Global reports indicate that an estimated 1.28 billion adults aged 30-79 years worldwide have hypertension, most (two-thirds) living in low- and middle-income countries. Unfortunately, an estimated 46% of adults with hypertension are unaware that they have the condition. Less than half of adults (42%) with hypertension are diagnosed and treated [2].

Blood pressure is the force of blood pushing against the walls of the arteries as the heart pumps blood around the minute. High blood pressure is usually caused by a biological substance, which is angiotensin converting enzyme (ACE). Hypertension disease has increased in recent decades. As in the following statistics, we have considered meat and pastirma for the antihypertensive peptide model. The global prevalence rate of blood pressure in low, medium and high-income states is about 40, 43 and 21% [3]. ACE is an enzyme produced by the human body that is believed to be the main element in hypertension, which is a Kinase 2-dipeptidyl carboxyl metalopeptidase. In the human body, it exists in two forms (Endothelial and soluble in blood and body fluids) [2,3,9]. that allow itself to come in contact with the substrate that produces Vasoconstrictive effects. ACE was found in horse plasma due to research going back to the 1950s and 1960s [4]. Hypertension is a major cause of premature death worldwide [2].

Consumers recently do not only care about the cost, access and quality of products, they simply have more attention to nutritional values. Questionable opinions are always brought up on a large-scale diffusion of food supplying potential bioactive components and one of those is the bioavailability of the peptides responsible for some positive physiological effects. Therefore, food proteins have attracted considerable scientific interest in recent years for their functional properties in addition to their nutritional value [5]. Food-derived antihypertensive peptides are crucial components able to influence specific physiological functions in the end, leading to improving body health in hypertensive individuals. People in developed countries are attempting to control hypertension conditions by reducing the sodium and potassium intake and using some inhibitory medication. Because of the local community demand to minimize the risk of hypertension in people at different ages by nutritional alternatives, we attempted to take advantage of the functional properties of fresh beef and pastirma cuts to determine their antihypertensive activities. Meat is not only consumed as a raw material but is transformed into a variety of products to preserve its nutrients and improve sensory traits and also prolong its shelf life. While using a traditional or modern technology, many meat products are being created every year due to research and industrial efforts conducted by researchers across the world. In this study, special attention has been given to the isolation of bioactive peptides with ACE-inhibitory activities from fresh and dry cured beef (M. Longissimus lumborum). There are crucial elements considered when researchers attempt to produce antihypertensive peptides from food products of animal origin, including a variety of protein types, the alteration occurred by gastrointestinal digestion, the bioavailability and absorbance capacity, of physiological bioactivities, and the peptide's potency mechanism and activity against the angiotensin converting enzyme. Angiotensin I-converting enzyme (ACE) plays an important physiological role in altering blood pressure [4]. The greater the ACE activity, the more angiotensin I is converted to angiotensin II, which induces high blood pressure. ACE also negatively impacts the bradykinin hormone (vasodilator) 1-7. Thus, specific inhibitors of ACE (bioactive peptides) are useful to mitigate the physiological activities associated with ACE in the human body. As numerous individuals suffer from such diseases, we hypothesize that other tools rather than chemical and pharmacological medication should be identified in the effort to reduce hypertension diseases. Therefore, potential, functional and nutraceutical compounds should be utilized as a treatment to minimize the number of individuals who suffer from hypertension. In the last two decades, many researchers worldwide have paid considerable attention to the use of certain food constituents, including meats, to prevent the action of ACE in elevating blood pressure. For instance, some peptides have recently been reported to play an important role in controlling the development of hypertension by regulating the rennin-angiotensin system [6]. Bioactive peptides (BAPs) are peptides with important nutritional functions, and they have specific physiological functions that are crucial to organisms [7]. Bioactive peptides are known for their capacity to act on other essential nutrients to provide the body with a more comprehensive nutritional profile [8]. For instance, dry-cured hams contain abundant bioactive peptides with significant potential for the development of functional food [9]. Bioactive peptides are usually regarded as safe due to their enzymatic degradation facilitated by food-grade enzymes, without the need for the adding toxic chemicals during the process [9].

Therefore, the aim of this study was to evaluate peptides that may play a crucial role as nutraceutical antihypertensive compounds. The antihypertensive activity of miscellaneous peptides generated by a peptic hydrolysation *in vitro* derived from was determined (IC_{50}) as to reduce the use of chemical medication that minimizes high blood pressure incidents.

Materials and Methods

Fresh beef and dry-cured meat products (Pastirma)

Meat source: Muscles used in this research were Loin-eye (M. Longissimus lumborum (LLU)) obtained from a fresh beef. The beef was slaughtered and processed at the Sahin company for meat and meat products, Kayseri, Turkey. The animal species belongs to the black Turkish beef, which is a hybrid of Montofon and Holstein. We were quite determined to obtain the pastirma from the carcass of the slaughtered cattle of the fresh beef, which was processed by the same meat frim (Sahin for meat products, Kayseri, Turkey). LLU muscles are sourced from male cattle at the age of 36 months old.

The pH values of the muscles before processing ranged from 5.6. SEM muscles were used 48 h post-mortem. The muscles were subdivided into two groups (fresh= control and pastirma= processed) that were prepared for each experiment [10]. Fresh and processed meat samples were sourced from the same animals, and all fresh samples were kept at −80 °C until experiment. On the other hand, pastirma cuts were manufactured using the traditional method, which presumably lasted for 4–6 weeks prior to the experiment [11]. Meat cuts used for pastirma are basically covered with a coating paste called ``çemen`` (12, 20, 13 and 55% of fenugreek, garlic, red pepper and water, respectively), as described by Kaban and also Akus [12,13].

Samples preparation and digestion process

Samples of each cut type were minced by a meat grinder (Promeat W 2000 Grande, Arnica). Fifty grams of each sample were mixed with 130 ml of distilled water. The mixture was then cooked for 30 min at 70 °C. Cooked samples were digested with pepsin and trypsin (2000: 1) consecutively for 4 hours after adjustment of pH [11]. The samples were boiled to inactivate the proteolytic enzymes and also the endogenous enzymes. The samples were stored for further analyses after being fractionated by a centrifuge (Universal 320R, Hetich, Zentrifuen) and then passed through a cellulose membrane filter (0.45µm) [11]. Yet, for the bioactive properties, the samples were used in its original concentration and further diluted to 4 different levels (100, 50, 25, 12.5, 6.25%).

Protein concentration

The filtrates were used as the extracted protein solutions, in which their concentrations were determined using the biuret method [14]. Absorbance of samples was measured with a spectrophotometer (UV-1800, UV spectrophotometer, Shimadzu) at 540 nm. The protein concentrations were calculated according to the following formula: Protein Concentration (mg/ml) = (abs of sample-abs of blind) *20 [15-18].

pH values

The pH values of the aqueous solutions of extracted proteins and meat cuts were measured to determine the qualities of the meat and protein solutions. The pH value of samples was measured by preparing 5g of both samples, which were homogenized in 20 ml distilled water by a silent crusher. The samples were rested for 5 min and pH values were measured using a pH meter (HM-30R Model HM-30R pH Meter) [16,19]. The samples prepared for pH measurements were taken from 4 manufacturing points; fresh meat, meat treated with salt followed by washing with water, meat treated with salt followed by washing in water and pressed to cause strains on meat tissue to release more moisture, and finally the pastirma in its complete product. In fact, during the pastirma making course, there are a few steps that the manufacturer should implement including washing the excessive salt, pressing by loading extra weight to reduce the internal moisture content.

Determination of the antihypertensive activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung [20], with slight modifications as described in a previous publication [21,22]. 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 beef and pastirma hydrolysates (6 µl) were mixed with 50µl of 7.6 mM Hip–His–Leu (Nacalai Tesque Inc., Kyoto, Japan) as substrate containing 100 mM sodium borate buffer (pH 8.3) and 608 mM NaCl and then pre-incubated at 37oC for 5 min. The reaction was initiated by the addition 20µl of 60 milli-unit/ml rabbit lung ACE (Sigma–Aldrich, Co., MO. USA) in a buffer containing 0.25 M sodium borate buffer (pH 8.3) and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 554µl of 0.1 N HCl to the samples except for the blank (554µl 0.1 N HCl were added before the pre-incubation). The samples were dried up in a nitrogen evaporator for a proper time [19]. The hippuric acid liberated by ACE was extracted by adding 1.5 ml of ethyl acetate to the mixture with vigorous shaking for 2 min. After centrifugation at 3000 rpm for 20 min, 1 ml of the ethyl acetate layer was collected; it was then dried at 100oC for 10 min. The hippuric acid was dissolved with 1 ml 1 M NaCl and its absorption at 228 nm was determined by a spectrophotometer. ACE inhibitory activity was calculated as follows: Inhibition % = C-S/C-B * 100

- C: Absorbance of control (buffer for samples).
- S: Absorbance of sample.
- B: Absorbance of blank (hydrochloric acid was added before ACE).

Lysis evaluation of proteins chemical structure by SDS-PAGE

Proteins were extracted in different buffers from low ionic strength buffer (WSP), and high ionic strength buffer `` Guba Straub-adenosine triphosphate buffer`` (GS-ATP) [16,23]. The WSP buffer contained 50 mM imidazole-HCl and 2 mM EDTA (pH 6). In contrast, the GS-ATP buffer contained 0.09 M KH2PO4, 0.06 M K2HPO4, 0.3 M KCl, and 1 mM ATP (pH 6.5). The contents were homogenized in a polytron homogenizer (Kinematica Co., Littau, Switzerland) at setting 4, then centrifuged at 12,000 rpm for 30 min at 4 °C (Himac CR 20E). The supernatants were recovered and passed through filter paper (No. 5A, Advantec Toyo K. Ltd., Tokyo, Japan) [19,24-26]. Extractions of proteins from fresh meat and pastirma cuts were electrophoresed on gradient gels of Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) to evaluate the impact of processing on the proteins' chemical structure. Electrophoresis on gels is conducted at 20 mA/gel as described (Laemmli, 1970) in which the gels were stained with Coomassie Brilliant Blue. SDS-PAGE analysis was carried out with slight modification according to the method previously reported by Katayama and Erwanto [6,27].

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

The healthy substances in food are not sufficient, yet it is important to remember that some certain components still are not clear if they make a positive physiological contribution. In addition, if the contribution of those compounds is positive to the physiological functions, then the scale of impact also remains unknown. Addressing the bioactive peptides, including the ACE inhibitory peptides derived from meat, their actual source has been extensively studied by many researchers from around the world. There are tremendous peptides sourced from different meat muscles and other foods that positively inhibit the ACE activity (antihypertensive) [19,21,28-30].

As an attempt to fully engage in the development of food products containing ingredients which are presumed to promote health, such as peptides to minimize some diseases. Current food research requires that the health benefit attributed to a meat component be derived from its "nutritive value".

Alteration in pH values

Table 1 also shows the pH values for fresh meat and pastirma, measured by mincing 5g of meat and homogenized into 20ml of water. As a result, the pH of the pastirma samples has not changed much during the process when compared to fresh meat values. The pH value of the dry cured meat, around 5.7-6.2, is believed to be very convenient for activities of some endogenous enzymes (amino peptidases) during the course of processing. In general, the pastirma-making process had no negative effect on the pH values, with only a slight increase among all samples compared to fresh samples [17]. The insignificant increase might have been associated with the degradation of some proteins and enzymes, as indicated by SDS-PAGE. The pH values indicate that the acidity of the pastirma was not significantly different from the value of the fresh-cut meat [16]. The only decrease was absorbed in samples that were in samples treated with salt followed by washing with water only (pH 5.68). The salted samples, washed and expressed, showed a negligible increase compared to the fresh samples but still lower than the pastirma samples (pH 5.9).

Parameter	Sample type extracted in H_2O			
	Fresh meat	Salted and washed meat	Salted, washed and pressed meat	Pastirma
pH	5.83±0.09	5.6±0.01	5.9±0.15	6.2±0.05
Electric conductivity	78	77	73	71

Table 1: pH and electric conductivity values of fresh meat and dry-cured product at different stages of pastirma making process.

Changes in protein concentrations

Measurement of protein concentration and the residue of amino acids is one of the molecular biomarkers of food nutritional quality. Data from the current research showed in general that the pastirma-making process had caused a significant increase ($p < 0.5$) in the proteins extracted by all three solutions (WSP, GS-ATP and H_2O) when compared to the fresh beef values. The increase in protein implies that the salting and curing process had a significant but nutritionally beneficial effect (Table 2). GS-ATP buffer showed that the lowest concentration in the samples was treated with salt, washed with water and pressed. Meanwhile, the samples treated with salt and washed with water only and extracted in WSP and H_2O, exhibited the lowest protein concentrations when compared with the concentration with other treatments. Results suggest that some proteins were defused with mechanical pressing treatment. It is clear that pastirma resorted much of the proteins and also contributed in the protein's solubility and lysis. The restoration of some proteins due to the peptide generation might be due to some endogenous enzymes as time goes by. Enzymatic production of bioactive and antioxidative peptides most likely occurred during pastirma-making process. Meat and meat products have been described as a very good source of angiotensin I converting enzyme (ACEI)-inhibitory peptides. The generation of bioactive peptides can occur through the action of endogenous muscular enzymes during processing, gastrointestinal digestion, or by using commercial enzymes in the laboratory or industry under controlled conditions [31]. This process might lead to the generation

Buffer	Fresh meat	Salted and washed meat	Salted, washed and pressed meat	Pastirma
WSP	3.84 ± 0.1	1.38	2.1	4.96 ± 0.15
GS-ATP	7.0 ± 0.18	9.5	6.36	9.28 ± 0.22
H_2O	6.18±0.05	4.15	4.29	10.58 ± 0.25

Table 2: Protein concentration of fresh meat and dry-cured product at different stages of pastirma making process.

of new biologically-active peptides that could provide multifunctional remedies for some cultural diet-related diseases [10].

ACE inhibitory activity (inhibition ratio)

As we have stated in previous reports, food scientists and nutritionists have a responsibility to conduct fruitful research to promote a healthy society. Precise bioactive peptides inhibit ACE actions and have physiological functions as nutraceuticals for treating hypertension [11]. Recently, tremendous food peptides that work positively against ACE have been identified but the inhibitory mechanisms of these peptides within the human body have not completely been reported. However, there are few publications in relation to this research hypothesis. But again, the main differences are the meat type and product specificity and the impact of treatment. This research emphasizes on fresh beef and pastirma that are widely consumed in Turkey, the Middle East and other parts of the world. Pastirma was chosen because, locally and internationally, there is very little information in a few publications (2019) about its bioactive peptides, especially antihypertensive substances. Most of the research was carried out on chicken, game meat porcine and fish [6,32,33], but the processed bovine was neglected to date (to the best of our knowledge).

As a result, hydrolysates of fresh beef and pastirma showed a remarkable inhibition activity against ACE force. The original samples (with no dilution) of hydrolysates in fresh beef and pastirma that had a protein concentration of 5.65 and 6.09mg/ml, showed an inhibition rate against ACE activity valued as 83 and 79%, respectively (Figure 1 and 2). In both figures the ACE inhibitory activity reduces gradually as the concentration of the hydrolysates declined due to the dilution process (Figure 1 and 2). The results indicate that fresh meat would contribute to ACE inhibition effectively even though the concentration of its hydrolysates was less than in pastirma samples. This leads to suggest that the nutritional and therapeutic value of fresh beef might be better than processed beef.

Table 3 shows the relative activity between meat and pastirma against ACE action. Results indicate that meat had the highest relative activity against ACE by 100% when compared to the activity of pastirma at the same dilution point (25%). However, in general, meat showed higher relative activity than the pastirma sample at all dilution points except the case at 6.25%, which pastirma was much higher than fresh meat. From data, the authors claim that water hydrolysis was much more effective on fresh meat proteins than on pastirma proteins, which led to generating valuable miscellaneous bioactive peptides.

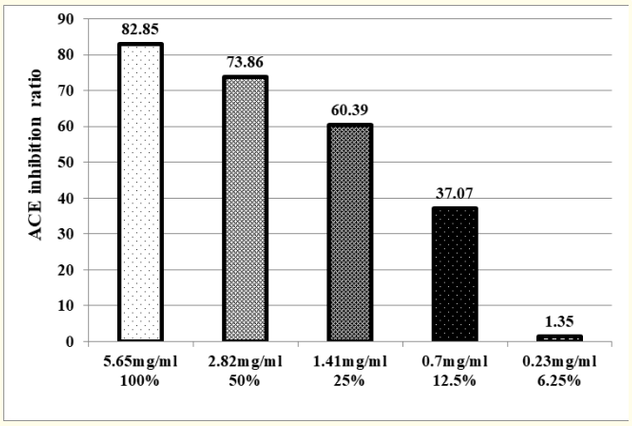

Figure 1: ACE inhibition ratio of fresh meat hydrolysates.

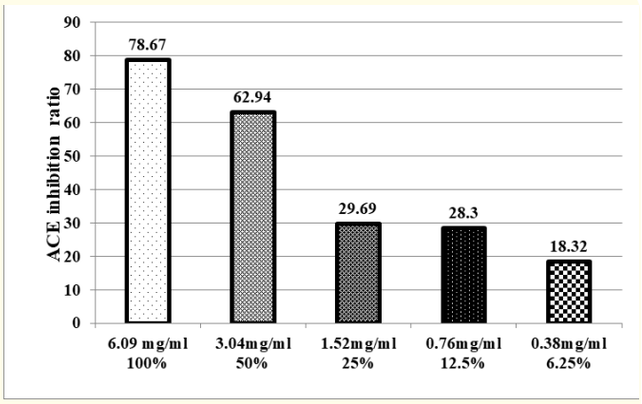

Figure 2: ACE inhibition ratio of pastirma hydrolysates.

Parameter	Hydrolysates concentration of Meat (%)					Hydrolysates concentration of Pastirma (%)				
	100	50	25	12.5	6.25	100	50	25	12.5	6.25
Peptide content mg/ml	5.65	2.82	1.41	0.7	0.23	6.09	3.04	1.52	0.76	0.38
ACE inhibition %	82.85	73.86	60.39	37.07	1.35	78.67	62.94	29.69	28.30	18.32
Percentage of relative activity in meat vs pastirma	5.31 Up	17.34 Up	101 Up	31 Up	1257 Down	--------	-------	--------	--------	-------

Table 3: Numerical values of ACE inhibitory ratio of meat and pastirma hydrolysates and their relative activity.

IC_{50} values as antihypertensive indicator

The number of adults suffering from hypertension is estimated to increase to 1.6 billion worldwide by 2025 [1]. To reduce and tackle this issue, this research was conducted aiming to use meat as a model of bio-active peptides source, which are effective at preventing and reducing hypertension disease. Chemically-based medications may have harmful side effects on individuals who suffer from hypertension. Functional food rich in proteins or biologically active peptides like meat may be widely implemented in lowering blood pressure in persons with essential hypertension, possibly by preventing an underlying cause of the disease. The IC_{50} of miscellaneous bioactive peptides (Hydrolyzed proteins) from

beef and pastirma showed 0.68 and 0.78 mg/ml, respectively (Figure 3). Clearly, this also demonstrates that meat proteins might lead to better nutraceutical therapy that minimizes health problems and might aid in finding the most effective approaches for meeting the needs of all hypertension patients. The results present a strong suggestion that fresh beef is perhaps richer than pastirma in peptides that may provide physiologically functional peptides, thus lowering blood pressure. Ironically, data indicates that pastirma may not be considered as a functional food like fresh meat for two possible reasons

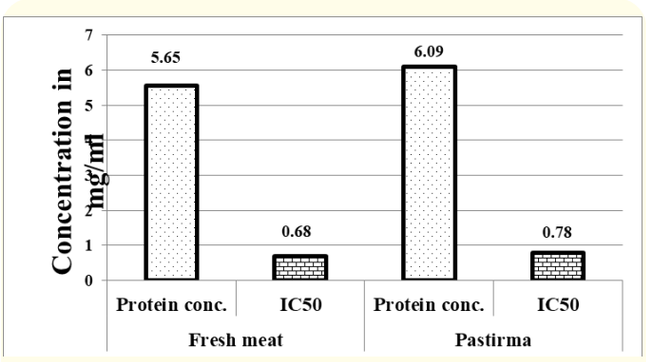

Figure 3: ACE inhibitory activity expressed in IC50 vs Protein conc. of fresh and pastirma made from M. *Longissimus lumborum* (LLU).

- Processed meat, especially pastirma, puts a strain on the digestive system due to the high amounts of acidic and alkalic bile needed during its metabolic process. We checked that pastirma took double the volume of HCL and NaOH as fresh meat required during pH adjustment for peptic hydrolysis in the laboratory.
- The hydrolysates in pastirma showed a lower IC_{50} value than in fresh meat. The lower the IC_{50} value, the higher the ACE inhibitory activity. This leads to suggest that fresh meat had a higher activity than pastirma against ACE actions.

An obvious conclusion was suggested that it is not necessary to dry-cure beef to enrich its biological activities. We rather encourage people to consume fresh meat instead of the processed meat products.

This research aimed to support health claims on a broad-based scientific criterion that addresses the extensive links between health and nutrition or health and meat. Increasing interest in the relationship between the diet, nutrients and health has contributed to the development of new directions in research focused on the determination of the actions of specific compounds on physiological functions in living organisms [34]. Individuals who suffer from hypertension disease would be more consistent and appreciate it if we could replace medication drugs with nutraceuticals with more appropriate functions. The concept here is to find nutritive alternatives for medications to treat hypertensive people, a treatment based on natural nutrients rather than chemicals.

Values of IC50 in this study reflect the importance of empowering hypertensive people in quality versus quantity of eating habits and in offering natural nutritional treatment options rather than medical alternatives. Results of the current research are anticipated to contribute to the value of meat products and the nutritional quality of beef, meanwhile disapproving the theory that meat harms the human body. Thus, data evidently addresses the potentiality of beef and pastirma in terms of the nutraceutical value that affects positively on meat-eating individuals. In fact, not only the dry-cured meat products, but also fermented and even game meat products are quite good sources of nutraceuticals (Takeda, 2017, Takeda 2020).

Results on miscellaneous peptides from this work may considerably bring more therapeutic information for the global meat scientists' society, and for the meat consumers in particular. Bioactive peptides can be used as an alternative to synthetic com pounds due to their insignificant side effects and high potential for use in the functional food market [35]. Data analysis of this study concluded that both meat and pastirma have a physiological therapeutic effect, because proteolysis of meat muscle generated a substantial number of peptides that have nutrafunctional (nutritional functions) roles and some of which have strong ACE inhibitory activity. However, there are a few intrinsic and extrinsic factors that we should consider in making the bioactive research statement of beef hydrolysates, which is the effect of age and on the genome of humans [36]. Both factors highly contribute to the positiveness and negativity of utilizing any functional food loaded with bioactive peptides like meat products. There are a few challenges that might face the application of bioactive peptides, including the digestive impact once they are ingested by patients. Other moderate challenges are cost-related production [37], standardizing clinical trial protocols [38] and processing conditions (heat and pH treatment) [39]. Moreover, it is very challenging when the objective is to generate specific peptide sequences that are able to exert certain activity [31].

SDS-PAGE patterns of protein lysis in the undigested samples

SDS-PAGE pattern (gradients 7.5-17.5%) of protein extracts in WSP and GS-ATP buffers indicated that most muscle proteins were metabolized to new, smaller molecules, including peptides (Figure 4). Lane 3 represents meat treated with salt, washed with water and pressed, exhibiting a very small number of protein bands, which might be due to the pressing process. The gels confirmed with evidence that water-soluble proteins are quite difficult to extract from dry cured meats. Considering that an amino acid has an average molecular weight of 110 Da (40), the molecular weight of bioactive peptides generally varies between 0.2–2 kDa. However, peptides with longer amino acid sequences have also been reported to be bioactive [41]. Furthermore, according to previous reports, ACE inhibitory peptides are usually composed of 2–12 amino acids [24] and those having hydrophobic amino acids in the primary amino acid sequence have good inhibitory activity [42].

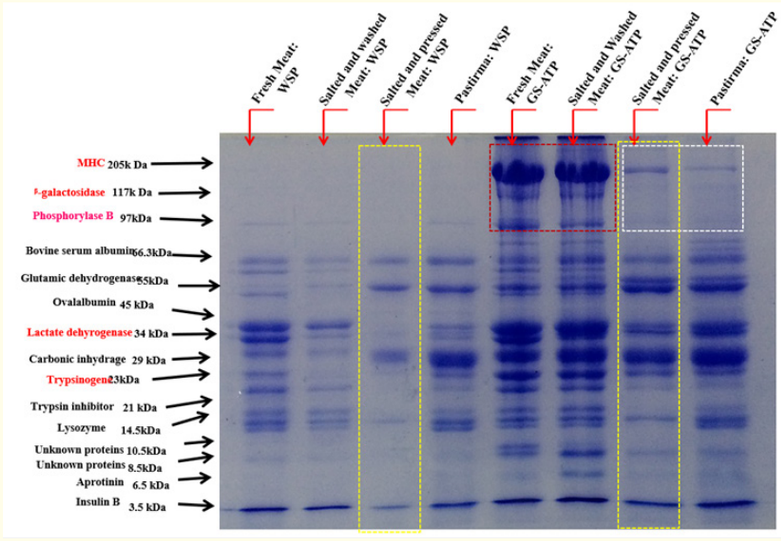

Figure 4: SDS-PAGE patterns show the protein lysis and molecular weight changes in undigested samples extracted in low ionic strength buffer WAS and high ionic strength buffer GS-ATP.

In samples extracted in WSP buffer in both meat types, the proteins ranged from 10-6.5kDa have not been observed. However, in the pastirma samples (lane 4) the extractability was much improved, which is in harmony with previous findings [19]. In contrast, the MHC protein band in the GS-ATP sample was clear in both fresh meat cuts and meat had salt treatment and washed with water (Lane 5 and 6), with another band representing a subunit of MHC (lanes 5 and 6). In addition, there are likely to be several bands of different molecular weights. Obviously, major proteins were degraded, such as MHC (200kDa) and many enzymes including: β-galactosidase, Phosphorylase B, Lactate Dehyrogenase, Trypsinogene and that is because of the pastirma making process. In contrast, the MHC bands were absent in the meat treated and pressed samples and also pastirma samples (lane 7 and 8), suggesting a degradation of the muscle proteins had occurred during the pastirma-making process. Likewise, in fermented meat products, it was reported that the expression levels of those substances were consistent, but the number or type of the amino acids would be diverse, which resulted in differing molecular weights of the substances. Hereby, it is believed that the digested meat products in this study had specific peptide sequences and characteristic bioactivities, including ACE inhibitory activity [43]. The same authors have conducted research on the implementation of lactic acid bacteria in dry cured meat products. They have reported that the inherent lactic acid bacteria in dry-cured meat products without bacterial starter is important for ACE inhibitory and antioxidant activities of the products. Additionally, the game meat is food that is believed potentially to offer high bioactivities, particularly antihypertensive forces [44].

Other bands, possibly representing enzymes, were present in the GS-ATP and WSP extracts (lanes 1, 5 and 6), but absent from the pastirma samples (phosphorylase B 97kDa). Furthermore, separation of the same samples in different buffers suggest more degradation occurred on low-molecular-weight proteins 10.5 and 8.5-kDa or probably into smaller peptides and possibly functional compounds. These results thus demonstrate that certain proteins were degraded by enzymes activated during or after the processing. Thus, the traditional pastirma-making process results in the degradation of many proteins into peptides, which might then be obtainable to treat some diet-related diseases [17]. The angiotensin I-converting enzyme (ACE) inhibitory activity and the antioxidant activities of meat and meat products have featured in many studies as potential bioactivities that can support human physiological functions [29]. However, as we suggested previously, further biochemical and physiological studies are needed to confirm this hypothesis [11].

SDS-PAGE patterns of protein lysis in the digested samples

The ACE inhibitory hydrolysates and peptides of fresh beef and pastirma were digested with pepsin and followed with trypsin, and were centrifuged and fractionated with a cellulose membrane (45μm). This sieving process allows only peptides with molecular weights less than 50kDa. Usually, the hydrolysis of proteins by trypsin results in bioactive peptides that inhibit angiotensin I converting enzyme (antihypertensive activity) and DPP-IV (glucose regulation) and exhibited antioxidant activity [45].

In the current study, the samples after being cooked were digested by pepsin and trypsin consecutively were further subjected to an electrophoresis process. Necessary treatments of meat before consumption such as cooking could facilitate the later generation of bioactive peptides due to denatured proteins being more susceptible to being hydrolysed by the enzymes of the intestinal tract [31].

Figure 5 illustrates that lane 1 had more bands above 29kDa when compared with other lanes including the non-digested pastirma (lane 4). In the SDS-PAGE analysis of dry-cured meat products, almost all bands were observed below 66.4 kDa [29]. In the current study, the fractionation process removed dozens of proteins and peptides. Lane 2 showed fewer bands than in meat and pastirma (lane 1 and 4) and that is due to the enzymatic degradation that occurred by pepsin, and also that happened to the pastirma pepsin digest (lane 5). Surprisingly, pepsin did not have much effect on the pastirma as the bands in lanes 4 and 5 remain consistent and similar. Obviously, there was a chemical cleavage and crack in the amino acid sequence, but it is possible that protein degradation was not very significant.

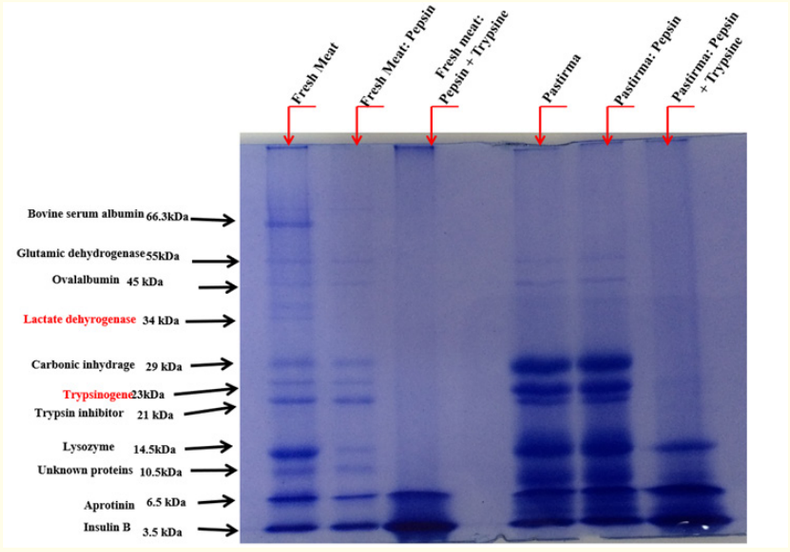

Figure 5: SDS-PAGE patterns show the protein lysis and molecular weight changes in digested samples extracted in low ionic strength buffer WAS and high ionic strength buffer GS-ATP. The samples were digested by pepsin and trypsin.

Conversely, the addition of trypsin as a second step had a great effect on both meat and pastirma samples, as observed in lane 3 and 6 (Figure 5). Samples digested with both enzymes in both meat types showed that the proteins having molecular weights ranging from 29-6.5 kDa were totally degraded. It was suggested in other reports that exogenous enzymes are used in the meat industry to produce restructured products (transglutaminase), to obtain bioactive peptides (peptides with antioxidant, antihypertensive and gastrointestinal activity) [45]. To sum up, the pattern of the fresh samples showed stronger and clearer bands compared to the samples of pastirma. This difference in intensity might be due to the genetic reason and the volume of protein expression from its gene. The images suggest that the proteins in both samples might have been metabolized into some functional peptides. Therefore, not only meat products but also meat being mixed with other functional foods like whey peptides can be a useful ingredient to enhance meat functional properties and the quality of meat products [46]. As there was no need to further treat meat to enhance its biological activities, this finding is in accordance with a previous report, which stated that the *in vitro* result indicates that fresh beef may generate nutraceutical peptides that lower hypertension more than the peptides generated by treated beef [47]. Thus, this research suggests that the fresh beef would offer better ACE inhibitory activities than the processed meat dose.

Observation about hybrid peptides in fresh beef and pastirma

Hybrid peptides can be classified based on their origin with muscle proteins and possible interactions into three categories, such as the following:

- Class I miscellaneous peptides are those that show weak interactions (low potency, slow action) that may exist in pastirma.
- Class II miscellaneous peptides are those that show moderate interactions (low potency but fast action, or vice versa) that may exist in fresh beef.
- Class III miscellaneous peptides are those that show strong interactions (high potency and fast action) that we could not approve if they exist in samples of this research due to the limitation of the research fund.

Conclusion

Results *in vitro* of this work suggest that meat proteins once metabolized in the gastrointestinal tract may exhibit bioactive effects. Precisely, they become functional substances inhibiting the ACE action of the mechanism. Biologically active substances derived from different fresh and processed meats must be carefully considered, and the environment of each digestive method should also be determined. Major proteins were degraded, such as MHC (200kDa) and many enzymes including: β-galactosidase, Phosphorylase B, Lactate Dehyrogenase, Trypsinogene and that is because of the

pastirma making process. Fresh beef and pastirma proteins have remarkable antihypertensive activities, and the research assumes that it is not necessary to process meat to enhance its biological activities. We rather encourage people to consume fresh meat instead of the dry-cured meat products. Miscellaneous peptides from fresh meat will provide nutraceutical compounds after digestion by intestinal proteases and cooking at 70°C/30 min have simulated muscle proteins to release peptides with antihypertensive activity. However, hybrid bioactive peptides may differ in potency and duration of action against ACE activity. In summary, fresh beef and pastirma proteins are good sources of bioactive peptides with angiotensin-I converting enzyme inhibitory activity and *in Vitro* showed considerable antihypertensive effects.

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Conflict of Interest

The authors declare that the research was conducted with no conflict of interest.

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