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Novel bioactive peptides with antihypertensive activity generated from beef muscle during fermentation with *Lactobacillus rhamnosus* FERM P-15120

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Abstract

Background: This study examined the impact of using *Lactobacillus rhamnosus* FERM P-15120 for the generation of bioactive peptides with antihypertensive action from fermented beef sausage.

Methods: The Lactobacillus strain was used for meat fermentation at 35°C for up to 72 hours, and Angiotensin-converting enzyme (ACE)-inhibiting substances were measured to be released during fermentation. Fermented beef sausage extracts were purified and fractionated using OASIS® HLB 35cc (6g) LP extraction cartridge. Further purification and identification of peptide sequences were done using reversed-phased high-performance liquid chromatography (RP-HPLC) and protein sequencer.

Results: A total of 16 peptide sequences from the active fractions were found to be 100% similar to various bovine skeletal muscle proteins. The molecular weights (MW) of all investigated peptides ranged from 423 to 2456, and the lengths of their amino acid sequences ranged from 3 to 23 amino acids. The IC_{50} of peptides with high MW weight and ACE inhibitory activity was determined. NRYWH was discovered to be the most effective peptide, with an IC_{50} value of 0.04 M. When compared to synthetic antihypertensive drugs, this peptide demonstrated good in vivo activity (captopril) due to it reduced systolic blood pressure by -32.76 ± 3.99 mm Hg ($p < 0.05$) in rats with spontaneous hypertension after 8 hours medication.

Conclusion: The findings of this study suggest that naturally occurring peptides in fermented sausage may have therapeutic potential for human health.

Keywords: Lactobacillus, fermented beef sausage, HPLC, ACE

1. Introduction

Traditional meat products such as fermented sausage is popular among consumers due to their distinctive flavor (Wang et al., 2018). Intestinal Lactobacillus and Bifidobacterium are the primary starting cultures utilized for the fermentation of meat (Arihara, 2006). Most fermented sausages and other foodstuffs contain Lactobacillus species, such as *Lactobacillus rhamnosus, L. sakei, L. plantarum*, and/or *L. curvatus. L. pentosus*, *L. alimentarius*, *L. paracasei*, and *L. casei* are additional lactobacilli that could be present in trace amounts. (Talon and Leroy, 2011).

Meat is rich with ingredients having physiological functions, such as bioactive nitrogen compounds, which are inactive in their primary structure in the original meat protein. The proteolytic actions of probiotics like lactic acid bacteria during fermentation can liberate the active form of these chemicals, resulting in free amino acids and small active peptides. (Nalinanon et al., 2011 ; Torres-Fuentes et al., 2011). Antihypertensive, antibacterial, immune-stimulating, antithrombotic, hypocholesterolemic, and antioxidant peptides are some examples of these peptides.

Angiotensin-converting enzyme-inhibiting peptides (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1) have attracted the most study attention. Since ACE transforms inert angiotensin-I into strong vasoconstrictor angiotensin-II, which increases blood pressure. The control of blood pressure is greatly aided by ACE. Therefore, systolic blood pressure should be reduced by medications that stop ACE activity. (Houston, 2002). It is essential for maintaining electrolyte homeostasis and blood pressure management because the ACE converts the inert decapeptide angiotensin I into the strong vasoconstricting octapeptide, angiotensin II. This powerful vasoconstrictor also has a role in the adrenal cortex's production of the sodium-retentive steroid aldosterone and the vasodilator bradykinin's hydrolysis. (Li et al., 2004 ; Meisel et al., 2006).

Although synthetic hypotensive medications like captopril, enalapril, and lasinopril are popular ways to prevent hypertension, causing adverse effects including changes in serum lipid metabolism, skin rashes, a dry cough, and taste problems. (Israili and Hall, 1992).

The goal of this study was to determine the proteolytic activity of *L. rhamnosus* FERM P-15120 as a probiotic starter on beef sausage and producing peptides with antihypertensive activity.

2. Materials and methods

2.1. Preparation of fermented meat

The fermented meat was constructed using fresh beef trim obtained from Japanese markets. Meat was ground and mixed with glucose (10 g/kg) and the *L. rhamnosus* FERM P-15120 strain (isolated from human intestinal tract and identified in Laboratory of Food Function and Safety, School of Veterinary Medicine, Kitasato University, Aomori, Japan (Sameshima et al., 1998)) was inoculated at level (108 CFU/g) then stuffed into glass beaker to make individual pieces of minced meat weighing 200 g. The meat pieces were incubated at fermentation temperature (35 °C). Samples for measuring antioxidant and ACE inhibitory activities were taken from fermented meat at 0, 24, 48, and 72 hours.

2.2. Preparation of meat extract

Specifically, 50 g of fermented meat was homogenized for 5 minutes in an ACE homogenizer with 100 ml of distilled water (DW), centrifuged for 20 minutes at 12000 rpm, and then filtered. In preparation for use, the supernatant was collected and freeze-dried.

2.3. Testing for ACE inhibitory activity

ACE inhibitory activity of fermented samples was measured based on the technique of Cushman and Cheung (1971) with modifications by Arihara et al. (2001). At a wavelength of 228 nm, the solution's absorbance was determined. Inhibitory activity (%) = (C-A) /(C-B) × 100; where A represents the absorbance of the sample reaction, B the absorbance of the blank, and C the absorbance of the control (DW), and where each of these values is specified as an IC_{50} value, that is, the amount of peptide (g protein/mL) necessary to cause 50% ACE inhibition under the given circumstances. Decisions were taken in triplicate.

2.4. Purification and identification of ACE inhibitory peptide

According to Jang and Lee (2005) ACE inhibitory peptides were extracted and identified From fermented meat.

1.4.1. Purification of meat extract

Five grams of freeze-dried fermented meat extract was blended with 100 ml of ethanol then centrifuging the mixture for 20 minutes at 3000 rpm. Following that, the supernatant was collected, filtered, and concentrated. Utilizing a rotary evaporator after the sediment was suspended in 100 ml of ethanol. The concentrated sample was dissolved in DW and then applied to the extraction cartridge for further purification. The fractions obtained were eluted with 50% methanol and then concentrated by the rotary evaporator. The DW was used to dissolve each fraction, and the amount of ACE inhibitory activity was determined.

2.4.2. High-Performance Reversed-Phase Liquid Chromatography

HPLC-RP mode was used to re-fractionate the active fraction obtained using an extraction cartridge. Elution was carried out by flowing solvent A (0.05% formic acid in DW) at a rate of 1 mL/min into solvent B (0.05% formic acid in acetonitrile). The absorbance was calculated at 215 nm. Each fraction's ACE inhibitory activity was assessed. A protein sequencer was utilized to determine the amino acid composition of a protein from the purified, most active portion.

2.4.3. Identifying the purified peptide's amino acid sequences

Utilizing the LCMS-QP8000, the molecular structure of the most active fraction was verified. Then, an automated protein sequencer supplied with an online detection device for PTH-amino-acids was used to sequence it using the Edman degradation method. The protein sequences of the Bos Taurus and the discovered peptides were compared using the fundamental local alignment search tool BLAST. (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4.4. Synthesis of peptides

Script Corporation Gen (Piscataway, USA) synthesized the ACE inhibitory peptides that were found in the fermented meat fractions and employed them for the in vitro inhibition assessment of ACE activity. Analytical LC-MS/MS was used to certify the synthesized peptides' purity. The synthetic peptides were Glu-Ile-Tyr (EIY), Ala-Ala-Gln-Glu-Glu-Tyr-Val-Lys-Arg (AAQEEYVKR), Gln-Ala-Ser-Glu-Gly-Pro-Leu-Lys (QASEGPLK) and Asn-Arg-Tyr-Trp-His (NRYWH).

2.5. Antihypertensive action in spontaneously hypertensive rats

2.5.1. Animal preparation

Six-week-of age male spontaneously hypertensive rats (SHR) were purchased from Charles River Japan Inc. (Yokohama, Japan) and kept in cages in a controlled environment with a 12-hour light-dark cycle at 24°C and 50–60% humidity. The SHR were given a typical laboratory diet, and tap water was always accessible till 12–14 weeks of age (300-340 g body weight).

2.5.2. Animal dosage

The SHR were 12–14 weeks of age 300-340 g body weight (BW). Asn-Arg-Tyr-Trp-His (NRYWH) and Glu-Ile-Tyr (EIY) solutions of both manufactured peptides were dissolved in DW and tested for in vivo antihypertensive action at 1mg/kg BW. As a control, DW and captopril (10 mg/kg BW) were employed. Peptide oral dosages were all 1 mL in volume. Before treatment, the mean SBP of the SHR was 215 mmHg. Using a metal gastric tube, the rats received the samples and the control.

2.5.3. Blood pressure measurement

SHR's systolic blood pressure was assessed using the tail-cuff method using a programmable electro sphygmomanometer. By observing the change in SBP at 0, 2, 4, 6, 8, and 24 hours, the antihypertensive activity of the samples and the control was assessed according to the schedule in Figure 1.

2.6. Statistical analysis

The variation between SBPs before and after management was used to express SBP change. Standard errors and means are used to express data. The Student's t-test was used for the statistical analysis (Daniel, 1987).

3. Results

Figure 2 revealed the ACE inhibitory activity of fermented meat. The mean value of ACE inhibitory activity (%) at zero-day was 37.11 ± 2.98 %. After 24 hrs of fermentation, the ACE inhibitory activity sharply rose with a mean value of 60 ± 1.28, 82.44 ± 0.84, and 93.02 ± 1.07 % after 24, 48 and 72 hours of fermentation, respectively.

Fractionation of muscle protein from fermented meat using RP-HPLC (first HPLC run) with the corresponding ACE-inhibitory activities of these fractions shown in the chromatograph of Figure 3. The highly active fraction was selected from the first run. It was fraction 5 (elution time 2.5–5 min) with ACE inhibitory activity of 82.60 % (Fig. 3). This fraction was further purified using a second run (Fig. 4). Active sub-fractions from fraction 5 were obtained. They were fractions 16, 17, 18, and 21 with ACE inhibitory activities of approximately 56.52, 60.67, 56.88 and 57.72%, respectively (Fig. 4).

The third run was used to further purify these fractions using a

different column and technique (Fig. 5). Another active fractions (F16-3, F17-3, F17-8, F18-3, F18-7, and F21-6) with ACE inhibitory activities of approximately of 22.54, 24.85, 23.54, 30.43, 32.89 and 61.5 %, respectively were obtained. Then, using a fourth run under conditions similar to the run that resulted in a single peptide, these fractions were further purified.

Results obtained in table 1 revealed that a total number of eight peptides were discovered from fermented meat in fractions 16 (2), 17 (3), 18 (2) and 21 (1). The peptides identified from fraction 16 were Glu-Ile-Tyr (EIY) and Ala-Ala-Gln-Glu-Glu-Tyr-Val-Lys-Arg (AAQEEYVKR) with molecular weights of 423.60 and 1093 Da which found to be originated from Titin and Fructose-bisphosphate aldolase A (Bos Taurus), respectively. The peptides identified from fraction 17 were Glu-Glu-Lys-Ala-Arg-Arg-Glu-Glu-Glu-Asp-Ala-Lys-Arg-Arg-Ala-Glu-Asp-Asp-Leu-Lys-Lys Lys-Lys (EEKRREEEDAKRRAEDDLKKKK), Ile-Ala-His-Arg-Ile- Val-Ala-Pro-Gly-Lys-Gly (IAHRIVAPGKG) and Ile-Pro-Val-Gln-Ser-Gln-His-Pro-Ile-Arg-Asn-Gly-Leu-His-His-Lys-Ile (IPVQ SQHPIRNGLHHKI) with molecular weights of 2858.47, 1118.51, and 1860.46 Da which was originated from troponin T fast skeletal muscle type, Fructose-bisphosphate aldolase and Glucose-6-phosphate isomerase (Bos Taurus), respectively. For fraction 18, The peptides identified were Gln-Ala-Ser-Glu-Gly-Pro-Leu-Lys (QASEGPLK) and Lys-Phe-Lys-His-Leu-Lys-Thr-Glu-Ala-Glu-Met-Lys-Ala-Ser-Glu-Asp-Leu-Lys-Lys-His-Gly (KFKHLKTEAE MKASEDLKKHG) with molecular weights of 829.03 and 2456.17 Da which were found to be originated from Glyceraldehyde-3-phosphate dehydrogenase and Myoglobin (Bos Taurus), respectively. There was only one peptide identified from fraction 21 with sequences of Asn-Arg-Tyr-Trp-His (NRYWH) and a molecular weight of 774.30 Da and this peptide was found to be originated from Four and a half LIM domains protein 1. For synthesized peptides, the IC50 was calculated from their dose–response (concentration vs. percentage ACE inhibition) curves. The tri-peptide Glu-Ile-Tyr (EIY) and the peptide Ala-Ala-Gln-Glu-Glu-Tyr-Val-Lys-Arg (AAQEEYVKR) were verified to be an ACE inhibitory peptides with IC50 of 134.6 and 89 µM while the peptide Gln-Ala-Ser-Glu-Gly-Pro-Leu-Lys (QASEGPLK) showed no ACE inhibitory activity. the most powerful peptide was found to be Asn-Arg-Tyr-Trp-His (NRYWH) with an IC50 of 0.04 µM (novel ACE inhibitory peptides).

Changes in SBP and SHR for both the synthetic peptides Glu-Ile-Tyr (EIY) and Asn-Arg-Tyr-Trp-His (NRYWH) following a single oral treatment at a concentration of 1 mg/kg BW are shown in figure (6). As a control, distilled water and captopril were used. Controlled SBP during the 24-hour monitoring period, SHR was not changed significantly with mean values of 0, 1.3 ± 0.51, 1.47 ± 0.71, 1.43 ± 0.83, 0.77 ± 0.31 and 0.38 ± 1.11 mm Hg at 0, 2, 4, 6, 8 and 24 hours, respectively after administration. The maximal antihypertensive activity was significantly observed in the captopril group ($P < 0.01$) and mean values of change were 0, -12.44 ± 2.92, -21.6 ± 2.46, -28.33 ± 5.64, -37.44 ± 4.72 and -7.11 ± 2.82 mm Hg at 0, 2, 4, 6, 8 and 24 hours, respectively after administration. SBP of SHR after administration of 1 mg/kg BW of tri-peptide Glu-Ile-Tyr (EIY) was significantly reduced compared to the control group (distilled water) between 4 h to 24 h of observation ($P < 0.05$ and 0.01). The reduction in SBP was 0, -3.76 ± 1.81, -7.18 ± 1.52, -12.38 ± 2.85, -20.580 ± 2.76 and -7.47 ± 2.52 mm Hg at 0, 2, 4, 6, 8 and 24 hours, respectively after administration. The change in SBP after administration of NRYWH peptide was 0, -10.40 ± 1.74, -12.54 ± 1.46, -23.79 ± 3.46, -32.76 ± 3.99 and -11.1 ± 2.08 mm Hg at 0, 2, 4, 6, 8 and 24 hours, respectively after administration.

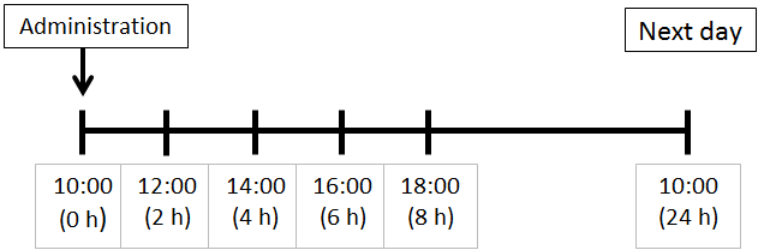

Fig. 1. Schedule showing the time of measuring SBP of SHR after fermented meat extract and peptides administration.

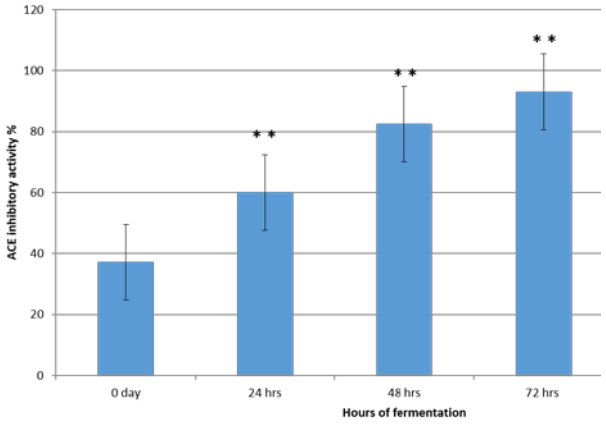

Fig. 2. ACE inhibitory activity of fermented meat at various times of fermentation. Results are mean ± SEM values from three experiments. Significant difference from zero-day samples (non-fermented meat) at each time: *$P < 0.05$, **$P < 0.01$.

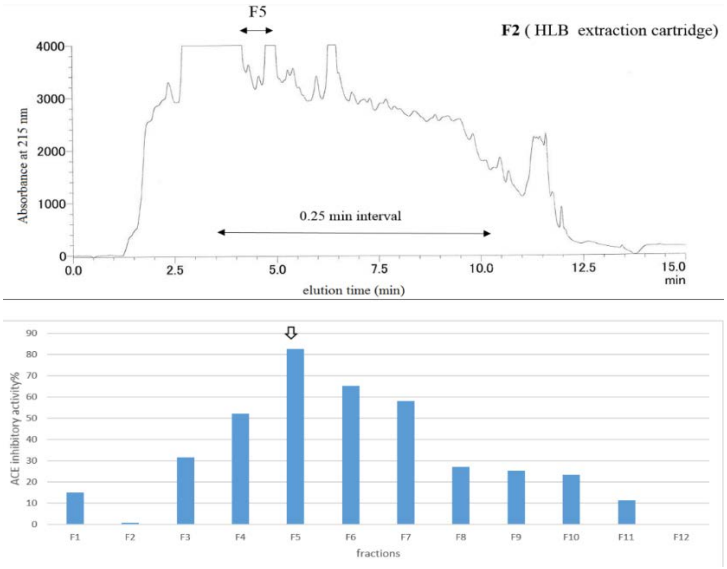

Fig. 3. Fractionation of muscle protein from fermented meat using reversed-phase high-performance liquid chromatography (RP-HPLC) (first HPLC run) with the corresponding ACE-inhibitory activities of these fractions. Fractions were obtained, and their ACE-inhibitory potential was tested. Elution time for fraction 5 was 2.5 to 5 minutes.

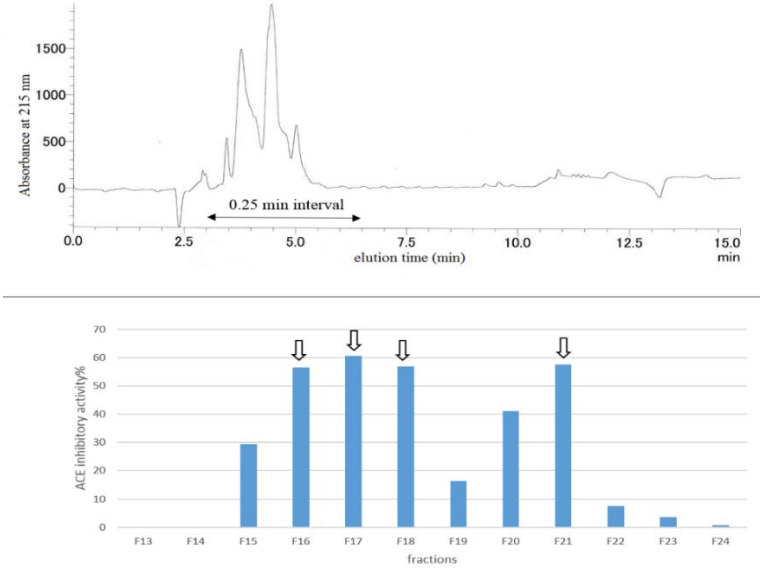

Figure 4. Fractionation of muscle protein from fermented meat using RP-HPLC (second HPLC run) with the corresponding ACE-inhibitory activities of these fractions.

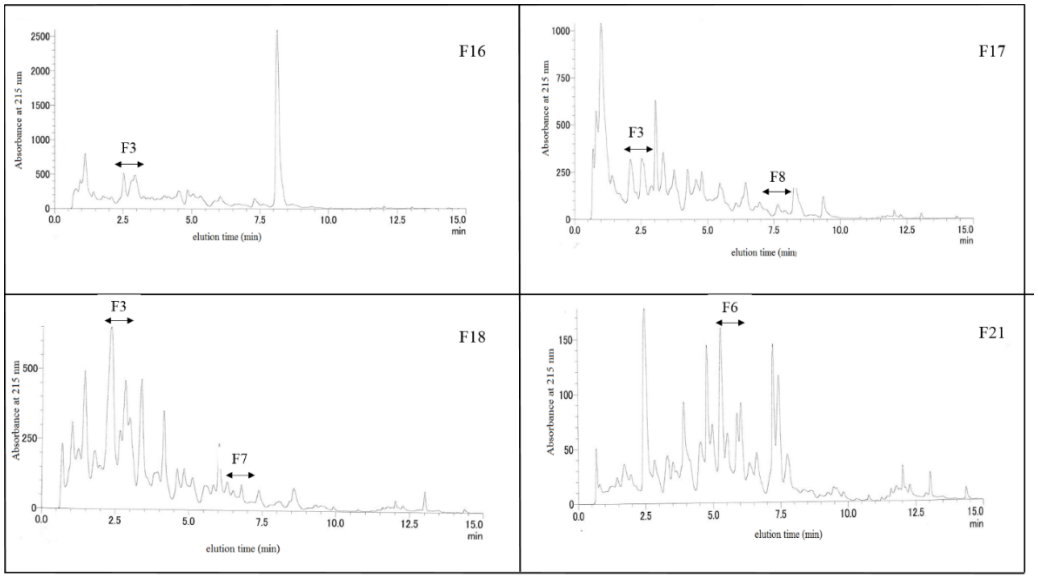

Fig. 5. Fractionation of muscle protein from fermented meat using RP-HPLC (third HPLC run).

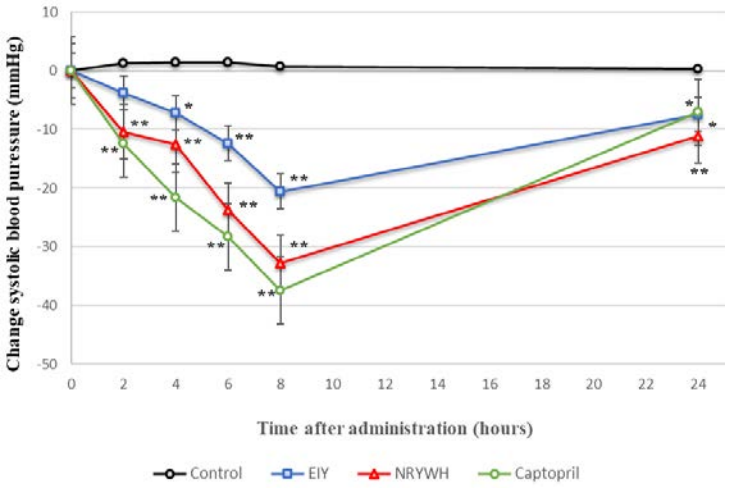

Fig. 6. Antihypertensive activities of two synthesized peptides from fermented meat in SHR by oral administration. Distilled water was used as a control. Significant difference from control at each time: *P < 0.05, **P < 0.01.

Table 1: Identification of peptides from the highly active fractions purified from fermented meat.

Fraction	Peptide No.	Start	End	Length	Estimated MW	Sequence	Parent protein (Bos taurus)
16	1	4395	4397	34340	423.60	E.I.Y	Titin
16	2	323	331	364	1093	A.AQEEYVK.R	Fructose-bisphosphate aldolase A
17	3	136	158	271	2858.47	E.EKRREEEDAKR RAEDDLKKK.K	troponin T fast skeletal muscle type
17	4	19	29	364	1118.51	I.AHRIVAPGK.G	Fructose-bisphosphate aldolase
17	5	408	424	557	1860.46	I.PVQSQHPIRNGL HHK.I	Glucose-6-phosphate isomerase
18	6	262	269	333	829.03	QASEGPLK	Glyceraldehyde-3-phosphate dehydrogenase
18	7	46	66	154	2456.17	K.FKHLKTEAEMK ASEDLKKH.G	Myoglobin
21	8	58	62	194	774.30	N.RYW.H	Four and a half LIM domains protein 1

4. Discussion

Beef meat is a good source of protein which is extremely responsible for the nutritive value and having functional ingredients. among the most vital ingredients is the bioactive peptides. The liberation of these peptides is achieved by the enzymatic proteolysis during meat fermentation producing small active peptides and free amino acids (Nalinanon et al., 2011). In this study, *L. rhamnosus* FERM P-15120 was utilized for meat fermentation and production of ACE inhibitory peptide from fermented beef sausage. ACE inhibitory activity of fermented meat increases substantially with the progress of fermentation. The endogenous proteases' degradation products produced during the zero-day before fermentation may be responsible for the sausages' ACE inhibitory activities (Sentandreu et al., 2002). The boost of ACE inhibitory activity with the progress of fermentation may be attributed to the growth of lactobacillus bacteria in fermented meat which led to a higher rate of proteolysis either by increasing the activity of its microbial proteases or by lowering the pH of the meat matrix and hence raising the level of naturally occurring muscle endogenous proteases (Khan et al., 2011). Castellano et al. (2013) reported that Lactobacillus species used for meat fermentation were able to create functional peptides with remarkable ACE inhibitory activity.

The Blast and UniProt databases searches of all the peptide sequences listed in Table 1 revealed 100% homology with the proteins found in bovine skeletal muscle. All peptides characterized from fermented meat in this study measured differences in relative molecular mass between 423.60 and 2456.17 Da and amino acid sequences between 3 and 23 amino acids in length. Peptide composition, sequence, size, and conformation all influence biological activity (Matsui and Matsumoto, 2006). The current results are in agreement with the concept that Pro, Lys, or aromatic amino acid residues are found in the majority of naturally occurring ACE inhibitory peptides (Suetsuna and Nakano, 2000). López et al. (2015) found 36 peptides from Argentinean fermented sausages with molecular mass values between 1000–2100 Da and found to originate from α-actin, myoglobin, and creatine kinase M-type of sarcoplasmic and myofibrillar proteins.

Even though natural proteases involved in sensory characteristics mostly produce meat peptides, the presence of microorganisms causes a heavy production of amino acids and small bioactive peptides that aid in fermentation and the formation of taste compounds and/or precursors. (Mora et al., 2011 ; López et al., 2015). As an activity indication, the pure meat extract concentration (IC_{50}) needed to achieve 50% inhibition of ACE was employed. Smaller values of this indicator, which was represented as µg protein/mL, indicated stronger ACE inhibitory power. (Chalé et al., 2014). Nearly similar results were obtained by Yokoyama et al. (1992). While the higher results were obtained by Vasdev and Stuckless (2010). The control SBP during the 24 hours of observation, SHR did not change significantly. In contrast, SBP decreased following administration of the two synthesized peptides, with findings that were almost identical to captopril and notably different from the control group. The captopril group showed significantly higher levels of antihypertensive action. Usually, the effects of medications on blood pressure can be more significant than peptides from food (Kuramoto et al., 2013). The penta-peptide Asn-

Arg-Tyr-Trp-His (NRYWH) showed the highest similar antihypertensive activity in comparison with the drug. This peptide also showed a similar pattern of SBP reduction after administration with a significant difference from 2 to 24 hours to the control group.

The greatest decrease of SBP in SHR occurred 8 hours after dosing, and throughout the duration of 24 hours, both quantities of purified fermented meat extract and both generated peptides had essentially identical effects. This demonstrates that ACE inhibitory peptides, likely including a genuine inhibitor type and pro-drug type, were found in purified fermented meat extract. The development of hypertension in these rats resembles that in people. (Fitzgerald et al., 2004). The trend of declining SBP values in our research resembles to Escudero et al. (2013) while Mora et al. (2015) found that the greatest decrease in SBP happened after eight hours of administration of 10 mg / Kg with 12 mm Hg. The peptides produced in the current study from fermented beef meat have not yet been recognized as antihypertensive peptides made from other protein hydrolysates. This makes these peptides new antihypertensive peptides.

Conclusion

This study demonstrated that the lactic acid bacteria's fermentation of beef protein can produce bioactive peptides with an antihypertensive impact. It has been established that the naturally occurring peptides had an antihypertensive impact and caused the SBP to decline in in vivo tests using SHR. A new market for healthier meat products could be opened up by the utilization of the bioactive peptides produced by *L. rhamnosus* FERM P-15120.

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