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Identification and characterization of antioxidant and antimicrobial peptides from enzymatic hydrolysates of Turkish fermented sausage (sucuk)

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RESEARCH ARTICLE

Abstract

In this study, in order to examine the effect of fermentation on bioactive peptide (BAP) formation, samples were taken from fermented sucuks produced using the traditional method on days 0, 1, 3, 5, and 10, and peptide extractions were obtained. The extracted samples were enzymatically hydrolyzed using two different enzymes (pepsin and trypsin), and the hydrolysates were injected into HPLC and separated into peptide fractions through a column filled with Sephadex G-25 stationary phase. Lyophilized fractions were subjected to LC-MS/MS analysis to determine peptide profiles. According to LC-MS/MS mass spectrometry data of peptide fractions obtained from sucuk samples during the 10-day fermentation, a total of 10 different peptides were detected, including 6 different dipeptides (KD, LK, EL, KP, HL, and IR) and 3 different tripeptides (GPP, GAA, and RHA) with antioxidant activity and 1 tetrapeptide (CIRA) with antimicrobial activity.

Keywords: Bioactive peptide, enzymatic hydrolysis, LC-MS/MS, mass spectrometry, sephadex G25

Introduction

Red meat and its products are important food sources for a healthy and balanced diet because of the amount of protein and essential amino acids they contain. Proteins found in these sources contain many "bioactive peptides" (BAP) with different properties as antimicrobial, antithrombotic, antihypertensive, antioxidative, etc. BAPs are specific protein fragments formed as a result of amino acids linked by peptide bonds (Evren and İnan Çınkır 2019).

Various processes applied to meat and its products can create free radicals, weakening the antioxidant defense system and accelerating the development of lipid oxidation. Oxidation results in bad odor, off-flavor development, color change, loss of nutritional value of the food, and reduced shelf life, and creates compounds that may pose a risk to human health (Xiong *et al.,* 2020). Many researchers have stated that protein hydrolysates with antioxidant, antibacterial, and antihypertensive activities and have the potential to be used in functional food production. BAPs, which can exhibit hormone-like activity and act as regulators when released in the body, can be used in the field of food and pharmacological applications as health-protective components (Kesler *et al.,* 2008). This makes it important to obtain BAPs from various sources. Parameters such as peptide bioactivity in the

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product, selection of protein source, enzyme selection for hydrolysis, the peptide enrichment method, as well as stability of peptides in the food matrix and bioavailability are sensitive decision points that must be coordinated for the purpose (Dullius *et al.*, 2020).

Traditional Turkish sucuk is defined as follows:

> "It is a fermented meat product without heat treatment, with the cross-sectional surface having a mosaic appearance, by chopping the bovine and/or ovine carcass meat and fats, mixing them with flavorings, then filling them into natural or artificial casings and applying fermentation and drying processes under certain conditions" (Anonymous 2019).

Fermented sucuk is a food in high demand in Turkey because it suits the Turkish culture and appeals to the palate (Beşir 2019). Fermented foods are defined as functional foods in which various enzymatic changes and health-beneficial end products occur as a result of fermentation of foods through controlled processes with the participation of beneficial microorganisms. (Akdeniz Oktay and Özbaş 2020). Fermentation is a method of food production and preservation that has been practiced since ancient times (Yüce 2023). It has been reported that BAP formations, which can be defined functionally, are seen in the composition of fermented products, and this formation can occur in two different ways. BAPs occur during fermentation and maturation, as a result of the microorganism's own proteolytic system or through some endogenous proteolytic enzymes (Ay and Şanlı 2018; Frias *et al.*, 2016). BAPs, which are released as a result of proteolysis during fermentation, one of the main steps of production of fermented meat, dairy, and legume products, can improve the functional properties of these products (Yüksel and İnanç 2022).

BAPs are specific protein fragments that have many positive effects on body functions. While some BAPs are found freely in their natural sources, the majority of BAPs are inactive in the main protein structure and are released by digestive enzymes during gastrointestinal transit or as a result of fermentation or ripening during food processing. It has been reported that there are more than 1500 different BAPs in a database called "BIOPEP" (Bhat *et al.*, 2015; Ünal *et al.*, 2018). The first BAP obtained from foods was described by Mellander in 1950 in his study on casein peptides (Ryan *et al.,* 2011). Since then, information about BAPs has constantly increased, especially in recent years, and many peptides that exhibit antithrombotic, antihypertensive, antioxidative, and antibacterial activities, immunomodulation, or mineral utilization-enhancing properties have been reported (Bhat *et al.*, 2015). The activity of BAPs is directly related to the number of amino acids in its structure and the sequence of these amino acids (Daliri *et al.*, 2017). Antioxidant peptides are inactive within the amino acid sequence of the parent protein and become active by enzymatic hydrolysis. These can be functionally added to food systems to reduce oxidative changes and used as nutraceuticals or pharmaceuticals (Wang *et al.*, 2015). The antioxidant properties of some protein hydrolysates have been confirmed in many foods (Wang *et al.*, 2020). For example, Seo *et al.* (2016) found that beef plasma protein hydrolysates added to pork meatballs at a rate of 2% prevented lipid oxidation better than butylated hydroxytoluene (BHT) used at a rate of 0.02%. Bai *et al.* (2017) reported that peptides obtained from marine fish skin showed an inhibitory effect on the lipid oxidation of pork meatballs.

Foodborne BAPs can be spontaneously released from the main protein as a result of food storage or various food processing processes such as fermentation, maturation, and cooking, or they can also be formed as a result of hydrolysis in the gastrointestinal system of the human body. Today, enzymatic or microbial hydrolysis is the most commonly used method for obtaining peptides with the desired specific bioactivity, but methods such as chemical synthesis, recombinant DNA technology, and enzymatic synthesis are also used in the production of BAPs (Montesano *et al.*, 2020; Piovesana *et al.*, 2018).

The most common method used to obtain BAPs is enzymatic hydrolysis of protein molecules. Most of the known BAPs are produced using different enzyme combinations of animal-derived proteinases such as pepsin, trypsin, alcalase, chymotrypsin, pancreatin, carboxylpeptidase, aminopeptidase, and thermolysin; plant-derived enzymes such as papain and bromelain; or bacterial or fungal enzymes with a wide range of activity conditions (Bhat *et al.*, 2015; Jang and Lee 2005; Liu *et al.*, 2020).

Factors such as being able to easily switch from laboratory to a larger scale, having a shorter reaction time, being in a more easily controlled environment compared to microbial hydrolysis, and allowing specific peptides with the desired bioactivity to be obtained have made the enzymatic hydrolysis method one of the most preferred methods for obtaining BAP today (Chakrabarti *et al.*, 2018). High specificity, mild conditions, and the absence of residual organic solvents and toxic chemicals in final peptide preparations have also positioned enzymatic hydrolysis as the most preferred method for the production of BAPs. However, the high cost of enzymes, low yields, and limited selection of food-grade enzymes have caused the industry to seek alternatives (Ulug *et al.*, 2021).

Elucidating the bioactivities of different BAPs of animal origin is an important research topic that will lead to the

use of these peptides in processed food formulations. It is estimated that traditional Turkish fermented sucuk contains BAP, but there is no study that clearly examines and reveals the amino acid sequences and antioxidant and antimicrobial activities of these BAPs. In this study, it was aimed to examine the effect of sucuk fermentation on the formation of BAP, to obtain the formed BAPs, to investigate their antioxidant and antimicrobial activities under laboratory conditions, and to identify the BAPs obtained using the BIOPEP database.

Materials and Methods

Materials

The fermented sucuk samples to be used in the research were produced at the Meat Pilot Plant of Pamukkale University, Faculty of Engineering, Department of Food Engineering. The spices and salt used in the production of sucuk were supplied from a local herbal shop operating in Denizli; chemicals used in the analysis and production and starter culture were supplied from relevant organizations in analytical purity and with the necessary specifications.

Methods

Production of fermented sucuk

The formulations presented in Table 1 were used for the production of the fermented sucuks. After the matured meat and frozen animal fat were minced in a meat grinder (12 mm diameter plate), spices, starter culture (Baktogard FT 120, lactic acid bacteria, and cocci), and sodium nitrite were added and mixed well in a mixer. The resulting sucuk dough was left for pre-ripening for a day at 4±1°C. After pre-ripening, the sucuk dough was thinned by pulling it through the 3 mm plate of the meat grinder.

After the prepared sucuk dough was filled into 28–32 caliber natural beef intestine casings soaked in 5% lactic acid solution, they were tied with cotton threads and hung, and after showering, the sucuk samples were subjected to fermentation in the ripening cabin (rooms with controlled temperature, humidity, and air flow rate) (Figure 1). On the first day, the ripening program was started so that the temperature was 28±1°C and the relative humidity was 90±2%. The temperature and relative humidity were reduced by two units until the fifth day. The ripening process was terminated at the end of the 10th day by keeping the temperature constant at 10±1°C, and the relative humidity was 80±2% after the fifth day. Air flow was kept between 1.5 and 2 m/s in the first 3 days of ripening and was gradually reduced to 0.5 m/s in the following days.

Physicochemical analyses

Fat, protein, ash, salt, and moisture contents of fermented sucuks were determined following the AOAC methods (A. of O. A. C. AOAC 1990). The protein content of

Table 1. Formulations of fermented sucuk.

Ingredients	(%)
Red meat	74
Animal fat	18
Salt	2
Garlic	1.5
Red pepper (sweet)	1
Red pepper (hot)	0,5
Black pepper	0,4
Cumin	1.5
Allspice	0.8
Sodium nitrite	0.03
Ascorbic acid	0.25
Starter culture	0.02
Total sucuk dough	100

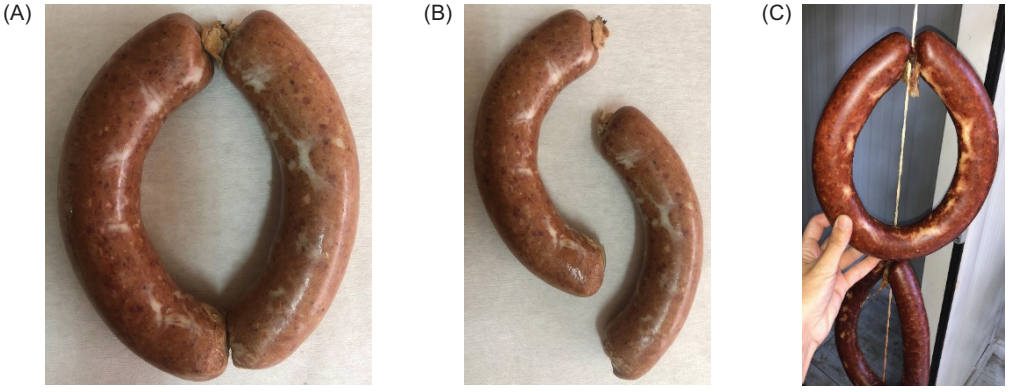

Figure 1. Filled fermented sucuk.

sucuk samples was determined according to the Kjeldahl method by using 6.25 factor to convert nitrogen to protein. The salt content of the samples was determined according to the Mohr's titration method. Ten grams of the sucuk sample was homogenized by adding 100 mL of distilled water, and the pH value of the samples was read by dipping the digital pH meter (HANNA H1 2211 pH/ORP METER) probe, calibrated with the appropriate buffer solution, into the mixture (Aro Aro *et al.*, 2010).

Obtaining BAPs

Extraction of peptides from sausage samples

Extraction of peptides was performed according to the method described by Mora *et al.* (2015). After the casing of 5 g of fermented sucuk sample was peeled and shredded, it was homogenized in 25 mL of 0.01 N HCl in a stomacher. The mixture was centrifuged at 10000 g for 20 minutes (4±1°C), then three volumes of ethanol were added to the samples, kept at 4±1°C for 20 hours, deproteinized and centrifuged again (Nüve NF 1200R). The mixture was filtered through a Buhner funnel with Whatman No: 4 filter paper and evaporated under vacuum at 45°C (CLS Scientific CLRC-08C). The aqueous fraction of the mixture was freeze-dried and stored at –20°C until use.

Enzymatic hydrolysis

As a combination of the enzymatic hydrolysis methods applied by Pepe *et al.* (2016) and Parrot *et al.* (2003), 0.5 g of lyophilized water-soluble extracts was weighed and dissolved in ultrapure water. The pH was adjusted to 2 with 1 M HCl, and a pepsin solution of 0.1 g pepsin/L was prepared in 0.01 M HCl, and then 1 mL of pepsin was added to the extract for 10 mL of water-soluble extract. The first hydrolysis was completed in 24 hours in a shaking water bath at 37°C. Then, the extracts were kept in a water bath at 90°C for 15 minutes to inactivate the pepsin enzyme. After the extracts were cooled, the pH was adjusted to 8 with 0.1 N HCl, and an enzyme solution of 0.5 g trypsin/L was prepared in pure water, and 0.20 mL trypsin was added for 10 mL of water-soluble extract. The hydrolysis process was completed in 24 hours in a shaking water bath at 37°C, and the extracts were kept in a water bath at 90°C for 15 minutes to inactivate the trypsin enzyme. At the end of the period, the hydrolysates were cooled in an ice water bath and then centrifuged at 5,000 g for 10 minutes. Then, the hydrolysates were dried and dissolved in 100 mg/mL acetate buffer (0.02 M and pH = 4) to obtain lipid fractions and filtered through 0.45 and 0.22 μm filters, respectively, and fractions were collected by injecting into HPLC according to Gallego *et al.* (2018).

Separation of BAPs

Five milliliters of each extract was taken and injected into a column (4.6×250 mm) filled with Sephadex G-25 (Amersham iosciences, Uppsala, Sweden) stationary phase. The separation was performed with the following conditions: mobile phase: 0.01 N tris-HCl buffer solution (pH = 7) at 4°C, flow rate: 1 mL/minute, and detection wavelength: 214, 254, and 280 nm (Shimadzu, Detector: PDA, Column oven temperature: 20 °C). Five milliliter fractions were collected manually by monitoring at 214, 254, and 280 nm. The obtained fractions were lyophilized and dissolved in 2 mL of pure water and stored at –20 °C until further analysis (Gallego *et al.* 2018).

Bioactivity analyses

Determination of antimicrobial activity

Antimicrobial activity of peptide fractions obtained from fermented sucuk samples subjected to enzymatic hydrolysis was determined by modifying the well diffusion method applied by Daoud *et al.* (2005). Microorganisms were incubated in Nutrient Soy Broth at 37°C for 24 hours. A bacterial solution was prepared from bacterial cultures with an absorbance value of 0.5 at 600 nm (density of 106 cfu/mL) in a spectrophotometer (EMC-11-UV). Sterilized nutrient agar (20 mL) was poured into sterile petri dishes, and after the agar solidified, 100 μL of bacterial suspension was planted in the petri dishes by the spreading plate method. The media were left to dry for 5–10 minutes at room temperature. After the drying process, three wells with a diameter of 5 mm were opened on the media with a sterile cork drill, equidistant from each other and from the edges of the petri dish. Peptide fractions were added to the wells (20 μL) and the petri dishes were incubated at 35±1°C for 24 hours.

Antimicrobial activity was measured by evaluating the diameter of the clear growth inhibition zone (formed zones) (Djabou *et al.* 2013).

- **8.0 mm and below:** Peptide fraction does not have sufficient antimicrobial effect on the relevant bacteria (-),
- **8–14 mm:** Moderately active (+),
- **14–20 mm:** Active (++),
- **20 mm and above:** Very active (+++)

Determination of antioxidant activity based on DPPH (1,1-Diphenyl-2-Picrylhydrazyl) radical capture capacity method

DPPH radical scavenging activity, which is an expression of the antioxidant capacity of the isolated peptides, was determined by Wang *et al.* (2003). Peptide fractions (0.1 mL) were taken and added into vials. Five milliliters of DPPH solution with 0.1 mM concentration was added to each vial, mixed by vortex, and incubated for 20 minutes at 27°C in a dark environment. At the end of the period, absorbances were read at 517 nm wavelength. Pure methanol was added as a blank solution, and 0.1 mL of pure

water was added instead of 0.1 mL of peptide fraction as a control solution. DPPH radical scavenging capacity (%) values, which are a measure of the antioxidant capacity of the extracts, were calculated according to the following formula:

$$\text{DPPH Radical Capture Capacity (\%)} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

$A_{control}$: Absorbance of the control sample
A_{sample}: Absorbance of test sample

Determination of peptide profile by LC-MS/MS

Identification of peptide profiles of fractions by LC-MS/MS was done according to the method applied by Gümüş *et al.* (2022). Chromatographic separation was performed using an HPLC Agilent 1260 Infinity series (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, online degasser, and autosampler. A Poroshell 120 EC-C18 column (3.0×100 mm, particle size 2.7 µm) (Agilent Technologies) was used to separate the components. The gradient program applied for the mobile phase system consisting of water containing 0.1% formic acid (A) and acetonitrile (B) was as follows: 0–0.5 min 5% B; 0.5–7 minutes 25% B; 7–16 minutes 50% B; 16–23 minutes 75% B; 23–30 minutes 95% B, and 5% B concentration was used for 30–40 minutes to equilibrate the column. The column temperature was kept at 35°C. The injection volume was set to 10 µL, and the flow rate was 0.4 mL/min. Ionization of chromatographic eluates was performed under the conditions specified below using an Agilent 6550 iFunnel high-resolution total mass spectrometer (QTOF-MS) equipped with an Agilent Dual Jet Flow electrospray ionization (Dual AJS ESI) interface operating in negative ion.

Drying gas flow 14.0 L/min; drying gas temperature 290°C; nebulizer pressure 35 psi; sheath gas temperature 400°C; sheath gas flow (nitrogen) 14 L/min and capillary voltage 1000V.

All scan mass spectra were collected in targeted MS/MS mode using a scan range of 50–3200 m/z to initiate MS/MS data collection. During analysis, the collision energy was set to 20 eV. Integration and data evaluation were performed using MassHunter Workstation software.

Computer prediction of identified peptides

Peptides identified in fermented sucuk samples were subjected to peptide sequence analysis via the PeptiteRanker program internet server, and the identified peptide sequences were matched with peptide sequences showing antioxidant and antibacterial activities in the BIOPEP-UWM database in the program (Minkiewicz *et al.*, 2019).

Statistical analyses

Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) using the SPSS 16.0 (SPSS Inc., Chicago, IL, USA) package program. Differences ($p<0.05$) between fermentation periods of samples were determined by the Duncan's multiple comparison test. This research was performed in two replications, and all analyses were made in duplicate for each replication.

Results and Discussion

Physicochemical properties of fermented sucuks

The change in pH values of fermented sucuk samples measured on the zeroth, first, third, fifth, and tenth days of fermentation is given in Table 2. As can be understood from Table 2, the pH value of the fermented sucuk samples was measured as 5.50 on the day they were produced, and 4.36 on the 10th day when fermentation was terminated. Organic acids, especially lactic acid, are formed in highly acidic fermented sucuks as a result of carbohydrate breakdown during fermentation, and pH values fall below 5.3 (Muguerza *et al.*, 2002). It is thought that the low pH values of the fermented sucuks produced in this study are becaue of the high amount of starter culture used in sucuk production and the rapid development of acidity.

Moisture, salt, ash, protein, and fat contents of fermented sucuk samples measured on the day they were produced are given in Table 3. The average moisture, salt, ash, protein, and fat values of the sucuk samples on the day of production were found to be 47.57%, 2.36%, 5.67%, 16.22%, and 27.42%, respectively. Ince *et al.* (2018), in their study where they examined the chemical properties of eight fermented sucuks offered for sale in supermarkets in Turkey, found that the lowest moisture content of the sucuks was 35.63%, the highest was 51.48%, and the average was 43.27%. The % humidity determined in the study is higher than the average humidity value

Table 2. Variance analysis table of pH values of fermented sucuk samples and fermentation times.

Variance source	Factor	Average
Fermentation Time (Days)	Day 0	5.50±0.09
	Day 1	5.04±0.07
	Day 3	4.86±0.07
	Day 5	4.82±0.07
	Day 10	4.36±0.06

±: Standard deviation0

Table 3. Moisture, salt, ash, protein, and fat contents (%) of fermented sucuk samples measured on the day they were produced.

Variance source	Factor	Average
Chemical composition	% Moisture	47.57±0.66
	% Salt	2.36±0.10
	% Ash	5.67±0.51
	% Protein	16.22±0.76
	% Fat	27.42±3.62

±: Standard deviation.

determined in this study in the literature. Özturunç (2022) determined the salt values of fermented sucuk samples as 2.09% on average, while Demir (2013) found it to be 3.06%. The % salt amount determined in the study is between these two reported values. Öksüztepe *et al.* (2011) examined the quality characteristics of 100 sucuks offered for sale in Elazığ and reported that the ash content was at least 1.70%, at most 8.85%, and on average 5.39%. The % ash amount detected in sucuk samples was found to be higher than the study in question. It is thought that this situation is because of the quality of the meat used in sucuk production and the salt and spice levels. The protein contents of fermented sucuks are between the 14.21 and18.00% values determined by Kaynarca and Gümüş (2020) and the 15.83 and 23.8% values determined by Karakuş (2011), and it was found to be lower than the 28.42% value determined by Ergezer *et al.* (2018). According to the Turkish Food Codex Communiqué on meat, prepared meat mixtures, and meat products, the total meat protein value must be at least 16% by mass. According to this information, sucuk samples comply with the notification with an average protein value of 16.22%. Pehlivanoğlu *et al.* (2015) reported in their study that the % fat content of sucuks was between 16.05 and 40.85%, and the average value was 25.62%. Although the average fat value, determined as 27.42% in the study, was partially higher than the average value of the 30 sucuks in this study, it was determined that it was suitable for first class fermented sucuk values, as it was below 30% in terms of the values reported in the TS 1070 Turkish Sucuk Standard.

Obtaining peptide fractions by high performance liquid chromatography (HPLC)

Five milliliters of each extract obtained by enzymatic hydrolysis was taken and injected into a column (4.6 × 250 mm) filled with Sephadex G-25 (Amersham iosciences, Uppsala, Sweden) stationary phase. Then, the fractions were monitored at 214, 254, and 280 nm and collected as 5 mL in amber glass bottles (Shimadzu, Detector: PDA, 214, 254, 280 nm, flow rate: 1 mL/min, column oven temperature: 20°C). Fraction could only be taken at 254 nm. Chromatograms obtained on days 0, 1, 3, 5, and 10 of fermentation are given in Figure 2.

When the chromatograms obtained on the zeroth, first, third, fifth, and tenth days of fermentation are examined, it is seen that a single clear peak is obtained at the end of the 30 and 45 minute flow times on all days. With this result, it is thought that the column filled with Sephadex G-25 stationary phase is effective in the separation process. As soon as peaks began to appear, the fractions began to be collected in amber glass bottles, and the collection process was terminated when the peak was completed and returned to a straight line.

It is observed that peptide bonds decrease during fermentation. As a result of bacterial fermentation during sausage (sucuk) production, proteins undergo partial degradation, especially with the activity of lactic acid bacteria, and BAPs are formed. Therefore, it is an expected situation that peptide bonds will decrease because of the breakdown of proteins during the formation of BAPs.

Determination of the antimicrobial activity

The antimicrobial activities of the peptide fractions obtained as a result of enzymatic hydrolysis on *Escherichia coli* (ATCC 25922), *Salmonella typhimurium* (ATCC 14028), *Listeria monocytogenes* (ATCC 7644), and *Lactobacillus pentosus* (ATCC 8041) were measured under laboratory conditions and the findings are given in Table 4.

The antimicrobial activities of the fractions were measured by evaluating the diameter of the clear growth inhibition zone (formed zones) (Djabou *et al.*, 2013). According to the table, it was detected that:

- BAP fractions do not have sufficient antimicrobial effects on *E. coli* (ATCC 25922) and *L. monocytogenes* (ATCC 7644) (-),
- It is moderately active (+) on *S. typhimurium* (ATCC 14028),
- On *L. pentosus* (ATCC 8041), Day 0 fractions are moderately active (+), while Day 1, 3, 5, and 10 fractions are active (++).

There are almost no studies examining the antimicrobial activity of BAPs in fermented sucuks. Verma *et al.* (2017) evaluated the antimicrobial activity of the protein hydrolyzate they extracted from pig liver by measuring the inhibition zone (mm) and found that the antimicrobial activity was positively correlated with the enzymatic hydrolysis time corresponding to the degree of hydrolysis, regardless of the enzyme type. As a result of the 6-hour hydrolysis, they determined that trypsin hydrolysates showed the highest antibacterial activity

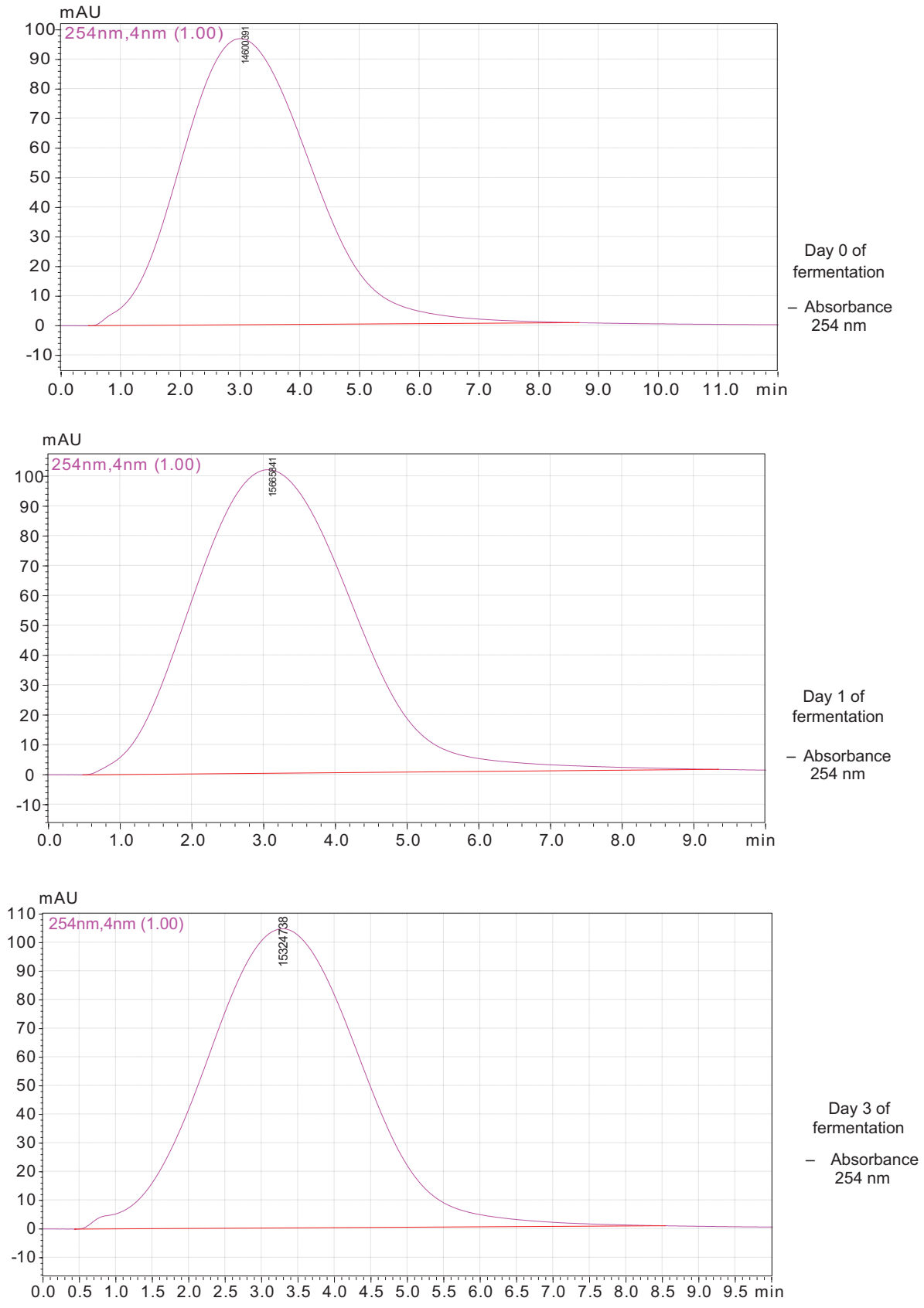

Figure 2. Chromatograms of hydrolysates obtained on days 0, 1, 3, 5, and 10 of fermentation.

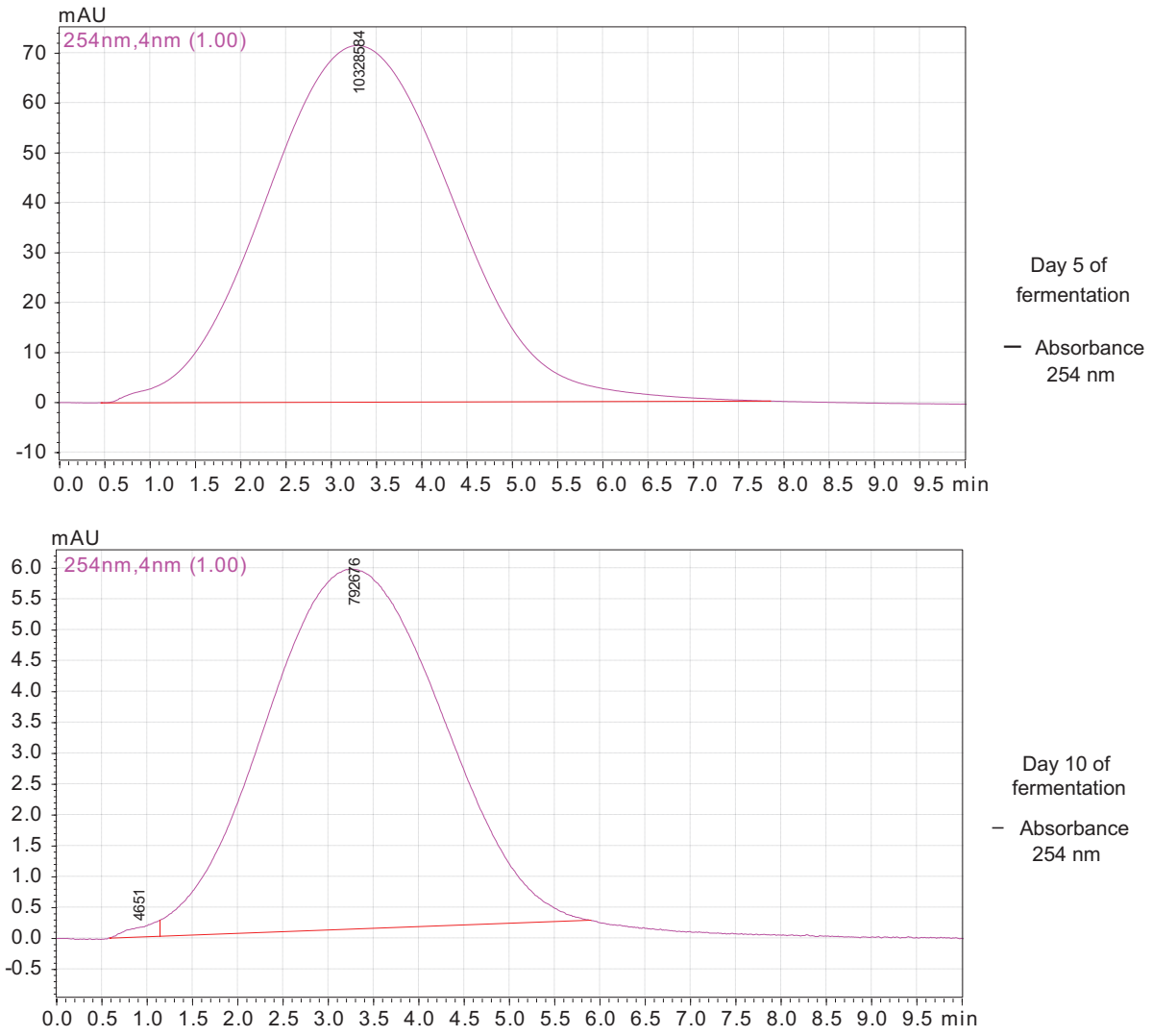

Figure 2. Continued.

Table 4. Diameters (mm) of the clear growth inhibition zone (zones) formed by peptide fractions on the medium.

Types of bacteria	Day 0	Day 1	Day 3	Day 5	Day 10
Escherichia coli	6	5	5	7	7
Salmonella typhimurium	12	10	13	10	10
Listeria monocytogenes	no zone	no zone	no zone	no zone	no zone
Lactobacillus pentosus	13	15	15	14	15

against *L. monocytogenes* (23.39±0.31 mm), it has a moderate antimicrobial effect against *Staphylococcus aureus* (18.35±0.65 mm) and *E. coli* (18.17±0.36 mm), and it showed the least antimicrobial effect against *Bacillus cereus* (16.79±0.35 mm). In this study, it was determined that hydrolysates did not have sufficient antimicrobial effects on *E. coli* and *L. monocytogenes* bacteria.

Determination of antioxidant activity

In the determination of antioxidant activities of peptide fractions obtained from fermented sucuk samples subjected to enzymatic hydrolysis on the zeroth, first, third, fifth, and tenth days of fermentation, as stated in the section "Determination of Antioxidant Activity Based on DPPH (1,1-Diphenyl-2-Picrylhydrazyl) Radical Capture Capacity Method", the DPPH (1,1-Diphenyl-2-Picrylhydrazyl) radical scavenging capacity method was used. The findings obtained are given in Table 5.

In the study, it was found that the peptide fractions obtained on different days of fermentation did not exhibit very strong antioxidant activity, and there was no statistical difference between the antioxidant activities of the fractions obtained on days 0 and 10 of fermentation.

Table 5. Variance analysis table of antiradical activity (ARA) values (%) of peptide fractions.

Variance source	Factor	Average
Fermentation time (Days)	Day 0	16.13±0.03^{bc}
	Day 1	16.04±0.11^{ab}
	Day 3	16.05±0.08^{ab}
	Day 5	15.99±0.10^{a}
	Day 10	16.18±0.04^{c}

The difference between the values shown with different lowercase letters in the same column in the table is statistically significant ($p<0.05$). ±: Standard deviation.

It was determined that the highest antioxidant activity was reached on the tenth day (16.18±0.04%), and the antioxidant activities of the fractions of days 0 and 5 and days 5 and 10 were statistically different. In the study carried out to identify peptides with antioxidant and antihypertensive capacity from dry fermented camel sucuks produced by inoculating different starter cultures and applying different ripening times, it has been determined that the maturation process causes an increase in antioxidant and antihypertensive capacity and that fractions belonging to peptides with a molecular weight below 3 kDa show the highest bioactivity (Mejri *et al.,* 2017). Luan *et al.* (2021), in their study investigating the effect of using *Lactobacillus plantarum* as a starter culture in traditional Chinese fermented sucuk on antioxidant activity, determined that the scavenging capacity of DPPH free radical increased to 75.14%. In the study, it was interpreted that mechanical disintegration increased becaue of the blade being attached to the head of the meat grinder during the filling of the sucuk dough into the intestines, and this caused excessive degradation of proteins and therefore peptide loss.

Identification of peptide profile by LC-MS/MS and computer prediction of identified peptides

Mass spectrometry is one of the leading methods used in identifying peptides and determining their amino acid sequences (Kartal *et al.*, 2023). Peptide identification of the fractions obtained on days 0, 1, 3, 5, and 10 of fermentation was made by LC-MS/MS, and the bioactivities of the peptide sequences determined in mass spectroscopy were scanned using the scanning function of the BIOPEP database. As a result of in silico hydrolysis of actin, collagen, and myosin from meat proteins with pepsin and trypsin enzymes, dipeptides were predominantly formed. Findings obtained from the BIOPEP database are given in Table 6.

According to LC-MS/MS mass spectrometry data of peptide fractions obtained from sucuk samples during 10 days of fermentation, a total of 10 different peptides were identified, including 6 different dipeptides (KD, LK, EL, KP, HL, and IR) and 3 different tripeptides (GPP, GAA, and RHA) with antioxidant activity and 1 tetrapeptide (CIRA) with antimicrobial activity. It was determined that the antimicrobial peptide was formed on the third day of fermentation. Kumari *et al.* (2022) reported that two antioxidant peptides (AIPPHPYP and IAEVFLITDPK) were identified in the Malaysian fermented fish product pekasam (Loma fish/Thynnichthys thynnoides); the naturally fermented fish product budu produced in Iran contains LDDPVFIH and VAAGRTDAGVH antioxidant peptides. They also reported that fermented meat sauce produced in Japan had high DPPH radical scavenging activity and the presence of QYP, an antioxidant tripeptide. Sun *et al.* (2022) reported that they successfully isolated and identified six antioxidant peptide sequences (GEHGDSSVPVWSGVN, HLDYYLGK, HLTPWIGK, DTYIRQPW, WDDMEKIWHH, and MYPGIAD) from duck liver protein hydrolyzate using alcalase. Vaštag *et al.* (2010) found that the antioxidant activity in protein extracts from fermented sucuks was two or three times higher in the final product than in the initial sucuk mixture. Korhonen and Pihlanto (2003) reported that antioxidant peptides generally have short sequences (2–20 amino acids long) and their molecular masses are in the range of approximately 400–3000 Da. When the chromatograms obtained by LC-MS/MS mass spectrometry and the antioxidant peptides identified using the BIOPEP database were examined in the study, it is seen that they mostly consist of two amino acids and their molecular masses are less than 3000 Da. Although peptides are generally more effective as antioxidants, free amino acids can also contribute to the bioactivity profile. Meat and fermented sucuks are important sources of free amino acids with antioxidant activity (Bou *et al.* 2016).

When the bioactive properties of antioxidant peptides identified using the BIOPEP database were examined in the study, it was determined that there was one tetrapeptide (CIRA) with antimicrobial activity. Peptides hydrolyzed by commercial enzymes from bovine sarcoplasmic protein were defined as GLSDGEWQ, GFHI, DFHING, and FHG by Arihara (2006), and the antimicrobial properties of these peptides were investigated.

Conclusions

BAPs obtained using meat proteins have extraordinary potential to perform functional activities that can benefit human health in the form of formulated functional food products. Although meat is a great source of protein, the number of meat-based functional peptide food products on the market is quite limited. Beyond basic laboratory identification and production by discovery, their use is greater for conducting research studies on BAPs.

Table 6. Bioactivity results of peptides detected by LC-MS/MS from the fractions of the zeroth, first, third, fifth, and tenth days of fermentation according to the BIOPEP database.

Fermentation time (Days)	Protein-enzyme	Peptide sequence	BAP sequence	Antimicrobial activity	Antioxidant activity
Day 0	Actin-Pepsin	PENTMHA		–	–
		MLRQKDY	KD	–	+
		GKLRKDDPL	KD	–	+
	Collagen-Pepsin	MGPRGPPGA	GPP	–	+
	Collagen-Trypsin	GPSGDRGPR		–	–
		GAAGIPGGKGEK	GAA	–	+
		GERGLPGVAGSVGEPGPLGIAGPPGAR	GPP	–	+
	Myosin-Pepsin	NFDVTGY		–	–
		GMNVMEF		–	–
		KTKKGMF		–	–
		QAQMKDY	KD	–	+
		QDMRQRHA	RHH	–	+
	Myosin-Trypsin	QQQLSALK	LK	–	+
		RHAEQER	RHA	–	+
		CIIPNHEK		–	–
		KTTLQVDTLNTELAAER	EL	–	+
		QIVSNLEKK		–	–
		QRHATALEELSEQLEQAK	EL, RHA	–	+
		LDGETTDLQDQIAELQAQIDELKIQVAK	EL, LK	–	+
Day 1	Actin-Pepsin	PENTMHA		–	–
		HNEVSKI		–	–
		DRDHSGTL		–	–
	Collagen-Pepsin	GKPGERGI	KP	–	+
	Myosin-Pepsin	NFDVTGY		–	–
		LKDVEVL	KD, LK	–	+
		GMNVMEF		–	–
		QAQMKDY	KD	–	+
		QREGIEW		–	–
		KKMEEEIL		–	–
	Myosin-Trypsin	YAEERDR		–	–
		IVFQEFR		–	–
		DEIFAQSK		–	–
Day 3	Actin-Pepsin	PENTMHA		–	–
		HNEVSKI		–	–
		DRDHSGTL		–	–
	Actin-Trypsin	LDHLAEK	HL	–	+
		LAKPERGK	KP	–	+
	Collagen-Pepsin	PGERGRVG		–	–
		RGFPGTPGL		–	–
		STGETCIRA	CIRA	+	–
			IR	–	+
		RGHNGLDGL		–	–
		SFVDTRTL		–	–
	Collagen-Trypsin	GPSGDRGPR		–	–
	Myosin-Pepsin	NFDVTGY		–	–

(*continues*)

Table 6. Continued.

Fermentation time (Days)	Protein-enzyme	Peptide sequence	BAP sequence	Antimicrobial activity	Antioxidant activity
		QAKDEEL	EL, KD	–	+
		LKDVEVL	KD, LK	–	+
		GMNVMEF		–	–
		GEHLKSDL	HL, LK	–	+
		QAQMKDY	KD	–	+
		QREGIEW		–	–
	Myosin-Trypsin	QQQLSALK	LK	–	+
		DEIFAQSK		–	–
		YAEERDR		–	–
		IVFQEFR		–	–
Day 5	Actin-Pepsin	RLSNRPA		–	–
		PENTMHA		–	–
		HNEVSKI		–	–
		DRDHSGTL		–	–
	Actin-Trypsin	LDHLAEK	HL	–	+
	Collagen-Pepsin	GKPGERGI	KP	–	+
		STGETCIRA	CIRA	+	–
			IR	–	+
		RGHNGLDGL		–	–
		SFVDTRTL		–	–
	Myosin-Pepsin	NFDVTGY		–	–
		QAKDEEL	EL, KD	–	+
		LKDVEVL	KD, LK	–	+
		GMNVMEF		–	–
		QAQMKDY	KD	–	+
		QREGIEW		–	–
		KKMEEEIL		–	–
Day 10	Actin-Pepsin	DQVMASF		–	–
		RLSNRPA		–	–
		PENTMHA		–	–
	Collagen-Pepsin	STGETCIRA	CIRA, IR	+	+
		RGHNGLDGL		–	–
		SFVDTRTL		–	–
		GKPGERGI	KP	–	+
	Collagen-Trypsin	GERGLPGVAGSVGEPGPLGIAGPPGAR	GPP	–	+
	Myosin-Pepsin	NFDVTGY		–	–
		QAKDEEL	EL, KD	–	+
		LKDVEVL	KD, LK	–	+
		KKMEEEIL		–	–
	Myosin-Trypsin	YAEERDR		–	–
		IVFQEFR		–	–
		DEIFAQSK		–	–

One-letter abbreviations of amino acids; A: Alanine, R: Arginine, N: Asparagine, D: Aspartic acid, C: Cysteine, E: Glutamic acid, Q: Glutamine, G: Glycine, H: Histidine, I: Isoleucine, L: Leucine, K: Lysine, M: Methionine, F: Phenylalanine, P: Proline, S: Serine, T: Threonine, W: Tryptophan, Y: Tyrosine, V: Valine.

Bioactive peptides with positive effects on health can be obtained from many sources by using the type of microorganisms and fermentation conditions under optimum conditions in the fermentation process, which is a way of obtaining bioactive peptides. It is known that the amount of BAPs in fermented meat products is higher in meat products with a longer ripening process. The 10-day fermentation period we applied in this study did not allow us to clearly see the release phase of BAPs. For this reason, it is thought that other studies in which the fermentation period is planned to be longer will be more useful.

In the study, as a result of the identification of peptide sequences determined by LC-MS/MS using the BIOPEP database, it was observed that BAPs that were mostly ACE inhibitors were encountered. Among BAPs derived from meat proteins, ACE inhibitory peptides have been most extensively investigated. More studies are needed to identify BAPs, characterize the peptide composition, confirm their beneficial effects, and use them on an industrial scale.

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Author Contributions

All authors contributed equally to this article.

Conflict of Interest

All the authors declare that they have no conflict of interest.

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