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Research Article

Evaluation of Antimicrobial and Antioxidant Activities of Casein-Derived Bioactive Peptides Using Trypsin Enzyme

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In this study, bioactive peptides were produced by enzymatic hydrolysis of casein protein with trypsin (pH = 8). The hydrolysates were analyzed for the degree of hydrolysis (DH) and antioxidant activities, viz. 2, 2′-diphenyl-1picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), and 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay at three temperatures of 40, 50, and 60°C for 4, 5, and 6 hours. Also, the antimicrobial activity of the experimental samples was evaluated using the disk diffusion method. The dilution method was used to evaluate the minimum inhibitory concentrations (MIC) and the minimum bactericidal concentrations (MBC). The antimicrobial activity of the resulting peptides was investigated by forming growth inhibitory region at three concentrations of 2, 2.5, and 3 mg/ml^{-1} on *Escherichia coli*, *Salmonella typhimurium*, *Bacillus cereus*, and *Staphylococcus aureus*. As the degree of hydrolysis increased, more peptides were produced, and antioxidant activity was increased. All of the experiments were conducted with three replicates. The highest degree of hydrolysis (28.44%) and antioxidant properties (DPPH: 76.62%; FRAP: 55 mM Fe(II); ABTS: 84.05%) was at 60°C and four hours ($P < 0.05$). They were compared with the synthetic antioxidant butylated hydroxytoluene (BHT). Peptides had the greatest effect on *S. typhimurium* at a concentration of 3 mg/ml^{-1} but did not affect *S. aureus* ($P < 0.05$). The lowest MIC and MBC were related to *B. cereus* (3.412 and 7.725 μg/ml), respectively, and the highest were related to *S. typhimurium* (8.515 and 8.555 μg/ml). This study examines the antioxidant and antibacterial properties of casein-derived peptides and contrasts them with chemically created ones to see whether these peptides can be substituted by chemical antioxidants and antibiotics. The results showed that the bioactive peptides produced from casein have antioxidant and antibacterial properties and can be recommended for the production, enrichment, and formulation of various food products to increase health.

1. Introduction

Bioactive peptides are released during enzymatic hydrolysis of proteins (digestive digestion, *in vitro*, using protolithic enzymes) as well as during food processing (cooking and fermentation) and are defined as amino acid sequences that in addition to nutritional value have biological functions [1]. Bioactive peptides have been linked to a variety of physiological activities, based on their number, sequence, and properties of the amino acids existing in the peptides that made them suitable products as therapeutic compounds [2]. In recent years, bioactive peptides have been extensively studied because of their potential, and their ability to have beneficial effects on consumers' health. These peptides have antioxidant, antihypertensive, immunity, and antimicrobial activities [3]. Oxidation in food is one of the main causes of degradation and deterioration of food quality, and oxidation of fats is one of the most important reasons for the quality decline. It also causes bad smell and taste and consequently reduces food safety which is due to the formation of secondary and potentially toxic compounds [4]. In addition to food, free radicals are formed in the body because of natural reactions caused by respiration. Different forms of active oxygen are released in the body during these reactions. If they are made in large quantities and are not controlled, they can cause major problems in the body [5]. Oxidative stress

occurs when the production of free radicals reaches a level above the antioxidant capacity of a cell [6]. The defining characteristic of these reactive species is an unpaired electron that can react to various biological molecules, leading to cell damage and disease [7, 8]. Extensive biological, biochemical, and clinical evidence suggests that the oxidative reaction caused by free radicals causes various diseases, food spoilage, and accelerated aging [9]. Diseases such as diabetes, Alzheimer's, hypertension, and Parkinson's are associated with oxidative stress [7, 10]. Because of oxidative stress on various diseases and the potential of antioxidants as a protection against this damage, research into the discovery of new antioxidants has increased, and there is much evidence that confirms the effects of malnutrition and toxicity of antioxidants added to food. In addition, it has been proven that cancer and the risk of liver damage can threaten laboratory animals which are other disadvantages of using fake antioxidants [11, 12]. Therefore, the need for natural antioxidants that are less toxic and more effective is an unavoidable necessity [13]. Recently, compounds resulting from the hydrolysis of proteins have received special attention as oxidative sources. These compounds can inactivate reactive oxygen species and inhibit free radicals [14]. Peptides with unique properties are formed during the enzymatic hydrolysis of proteins, and the hydrolysis process is completely controllable; and less damage is done to the protein substrate, so enzymatic hydrolysis is an ideal strategy to produce peptides [15, 16]. Antibacterial properties are another feature of bioactive peptides [17]. Antimicrobial peptides have a wide range of effects on microorganisms such as fungi, Gram-positive and Gram-negative bacteria, and viruses. One of the unique properties of these peptides is their antimicrobial effect on bacteria which are resistant to various drugs, and these compounds have a slight tendency to increase bacterial resistance. Antibiotic resistance may occur in a variety of ways, such as inhibiting drug-target interaction, changes in drug-receiving sites in the target cell, and separation of the drug from the target cell [18]. Peptides produced by the hydrolysis of animal and plant proteins have shown favorable results on intestinal health, production function, and growth [19]. Most antimicrobial peptides have been extracted from milk and its derivatives so far. Milk is a food, rich in high-quality protein, which due to its high-quality proteins is considered as a standard reference for determining the nutritional value of other food proteins and has many functional, nutritional, and biological properties [20, 21]. In recent years, many studies have been conducted on milk protein-derived peptides that have various biological activities such as regulating immune function, antioxidant activity, lowering blood pressure, antibacterial, and antiviral activity [22]. Cow's milk contains about 3.5 percent protein, of which 80 percent is casein and 20 percent is Whey protein. [23]. Casein peptides may be useful as one of the natural antioxidants in meat processing and help prevent the formation of bad flavor in meat products and improve their shelf life [24]. According to Rossini et al. [25] and Mizuno et al. [26], calcium caseinate peptides may act as a natural antioxidant to stop the oxidation of lipids contained in meat and food [27]. Additionally, casein has been shown to have positive impacts on human health by a number of researches. Some examples of these studies are the ones listed below:

Reducing blood cholesterol [28], anticaries activity of teeth [29], reducing the amount of dental hydroxyapatite dissolution [30], protection against colon cancer [31], reducing the incidence of tumors due to chemicals [31], inhibitory activity against human influenza viruses [32], decrease in demineralization, and increase in remineralization [33]. Therefore, this study looks at the antioxidant and antibacterial characteristics of peptides produced from casein and compares them with chemical ones to see if these peptides may be replaced by chemical antioxidants and antibiotics.

2. Materials and Methods

2.1. Chemicals and Reagents. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), gallic acid, ethanol, methanol, trichloroacetic acid (TCA), acetate buffer, Mueller–Hinton agar (MHA), butylated hydroxytoluene (BHT), tryptic soy broth (TSB), and Folin–Ciocalteu reagents were provided by Merck Germany Company and Sigma-Aldrich Chemical Co.

2.2. Casein Hydrolysis. To extract the peptide by enzymatic hydrolysis, casein in a concentration (by weight) of 5% in 0.1 M phosphate buffer (pH = 7.4) is dissolved which allowed it to be fully hydrated during continuous stirring for 30 minutes at room temperature. Then, the initial solution of trypsin enzyme in the aforementioned buffer was added to the casein-containing solution (pH = 8), in the ratio of enzyme to protein substrate (w/w) 2%. Afterwards, hydrolysis in the temperature range of 40–60°C was performed for four to six hours in a shaker incubator (Model-8480 VS, South Korea) at 100 rpm, then the enzyme was inactivated by heating to 90°C for 15 min. To remove excess soluble compounds, it was cooled to room temperature at 8000 rpm for 30 minutes, the resulting liquid was frozen at −20°C; and finally, to produce casein peptide powder, the sublimation dryer (freezer dryer) was dried and stored at −20°C until further use [34].

2.3. Determination of Casein Bioactive Peptides (CBP) Concentration and Molecular Weight Distribution. TSK gel 3000 PWXL columns (Tosoh, Japan) coupled with an HPLC system were used to determine the molecular weight distribution of CBP (Agilent 1100, Agilent Technologies Inc., Santa Clara, CA, USA). The mobile phase was acetonitrile in water (1 : 1, v/v) containing TFA (0.1 percent, v/v). At 225 nm, the absorbance was measured with a flow rate of 0.5 mL/min. The molecular weight standards used were bovine serum albumin (BSA, MW: 66,000 Da), cytochrome C (MW: 12,384 Da), bacitracin (MW: 1423 Da), and reduced glutathione (GSH, MW: 307 Da). The protein concentration of lyophilized CBP was determined using the Biuret method [35] with BSA as a reference.

2.4. Determining the Degree of Hydrolysis. The degree of hydrolysis (DH) of casein hydrolyzed by 10% trichloroacetic acid-soluble protein (TCA) was determined by Vaguer et al. [36]. For this purpose, the hydrolyzed casein suspension was combined with trichloroacetic acid (20%) in a volume ratio of 1 : 1, and the resulting mixture was centrifuged at 16,000 rpm for 15 minutes. The amount of protein in the supernatant containing 10% of trichloroacetic acid was determined by the Bradford method, and finally, the degree of hydrolysis was determined using the following equation:

$$DH = \frac{\text{10\% TCA} - \text{soluble protein in the sample}}{\text{Total protein in the sample}} \times 100. \quad (1)$$

2.5. Measurement of Antioxidant Properties

2.5.1. Evaluation of Free Radical Scavenging Activity (DPPH). The inhibition of 2,2′-diphenyl-l-picrylhydrazyl free radical (DPPH) was measured according to the method of Wu et al. [37]. For so doing, 1.5 ml of the sample was mixed with 1.5 ml of 95% ethanol solution of DPPH (0.1 mM), and the resulting solution was kept in the dark for 30 minutes. Its absorption was then read at 517 nm using a spectrophotometer (UNICO 2100). Finally, the percentage of free radical scavenging (DPPH) was calculated according to the following equation. BHT was also measured by the same method as follows:

$$\text{Inhibition of DPPH free radicals} = \frac{\text{sample adsorption} - \text{DPPH adsorption}}{\text{adsorption}} \times 100\ \text{DPPH adsorption}. \quad (2)$$

2.5.2. Measuring the Regenerative Power of Iron III

(1) Preparation of FRAP Solution (Ferric Reducing the Activity of Plasma). Acetate buffer 0.3 M (pH = 3), TPTZ reagent, and 20 mM solution of 6-aqueous iron chloride III in a ratio of 1 : 1 : 10 were mixed and stored in a dark place (this solution should be freshly prepared because TPTZ solution is unstable and sensitive to light [38]).

(2) Drawing a Calibration Curve of the Concentration of iron II Against the Read Adsorption. Standard solutions of ferrous sulfate II were prepared at concentrations of 200 to 2000 micromoles per liter. 30 μl of standard solutions, 900 μl of FRAP solution, and 90 μl of deionized water were mixed inside the test tube, the resulting solution was placed inside a water bath, and after reaching the temperature of 37°C, the absorbance was read at 595 nm [38].

(3) Measuring the Regenerative Power of the Sample. Depending on the inhibitory power of the sample, a solution containing 100 mg of sample in 10 ml of methanol was prepared, and 30 μl of it with 900 μl of FRAP solution and 90 μl of distilled water was placed and mixed in a test tube which was placed in a water bath. It was read at 37°C by absorbance at a wavelength of 595 nm. BHT was also measured by the same method [38].

2.5.3. Measuring Antioxidant Capacity by ABTS Method. ABTS method was used to measure free radicals based on Ak and Gülçin [39] method. To prepare the ABTS solution, 7 mmol of ABTS was poured into a dark container containing 2.45 mmol of potassium persulfate (K2S2O8) and placed for 24 hours in a dark place at room temperature. ABTS is a relatively stable green free radical that is colorless in its nonradical form. In this method, ABTS was added to the radical solution and after a fixed period, the remaining ABTS was measured by spectrophotometry at 134 nm. The amplification range of ABTS solution, as the percentage of adsorption reduction, was calculated. Then, the inhibitory ability of the antioxidant compound was measured using the following equation:

$$\text{ABTS inhibition \%} = \left(\frac{1 - A2}{A1}\right) \times 100, \quad (3)$$

where $A1$ = without ABTS inhibitor solution absorption and $A2$ = residual in the presence of ABTS adsorption inhibitor.

Then, 50 μl of ABTS solution was added to 2950 μl of sodium phosphate buffer, and immediately the tube was placed in the device and its absorption was recorded at 734 nm, then 15 μl of the diluted sample was added to ABTS solution and sodium phosphate buffer. After an immediate mixture, it was placed in the device, and after 5 minutes, its absorption was measured and recorded by the device. BHT was also measured by the same method.

2.6. Investigation of Antimicrobial Effects of Peptides Extracted from Casein

2.6.1. Disk Diffusion. Evaluation of the antimicrobial effects of peptides extracted from casein of different standard strains was prepared by Iran Scientific and Industrial Research Organization. The antibacterial activity of casein-derived peptides was determined according to the 1977 National Committee Clinical Laboratory Standard. For this purpose, *E. coli*, *S. typhimurium*, *B. cereus*, and *S. aureus* were grown for 30 hours in TSB medium at 24°C. Then, 0.1 ml of 0.5 Mac-Farland microbial suspensions (106 CFU/mL; 0.5 McFarland) of each bacterium was spread on the surface of Müller-Hinton agar (MHA). Subsequently, 10 μl of the peptide with different concentrations was obtained at 60°C and 4 hours (2 mg/mL^{-1}, 2.5 mg/mL^{-1}, 3 mg/mL^{-1}) which was spread on the filter paper until it was completely absorbed by it. After placing the discs on the culture medium, the plates were incubated at 37°C for 24 hours. After

incubation, the diameter of the growth inhibition zones around each disc was measured in millimeters by a caliper.

(1) Determination of Minimum Inhibitory Concentration (MIC). The minimum inhibitory concentration was determined using the dilution method in the well. For this purpose, 96-well sterile microplates were used. The stock solution of the extracts was prepared in sterile distilled water, and different concentrations of the extract (40 to 0.156 mg/l) were diluted with the stock solution using the Müller-Hinton broth culture medium. As a result, YGC broth was prepared. Bacteria and yeast were cultured on Nutrient agar, and YGC agar at 37°C one night before the experiment, respectively. Then, McFarland No. 0.5 was used to prepare 106 CFU/ml microbial suspension. After filling the well, the microplates were incubated overnight, after which the turbidity was read by the ELISA reader at 630 nm [40].

(2) Determination of Minimum Lethal Concentration (MBC). From the wells where no turbidity was observed, 5 μl was transferred to a solid culture medium (Müller-Hinton agar and YGC agar) and kept overnight at an appropriate temperature. The first concentration at which no growth was observed was regarded as the minimal lethal concentration (MBC) [41].

2.7. Statistical Analysis. The effect of temperature and time on the degree of hydrolysis and antioxidant properties of casein-extracted peptides was analyzed in a factorial arrangement (3×3) with three replicates. Microbial experiments were conducted using a completely randomized design with three replications in each treatment. The data were analyzed using the general linear model (GLM) of SAS Institute (SAS, 2004) were compared by Duncan's multiple range tests (1955). Differences were considered significant at $P < 0.05$.

3. Results and Discussion

3.1. The Molecular Weight Distribution of CBP. The molecular weight distribution of casein peptides was performed using TSK gel with high-performance liquid chromatography. Casein digestion in 4 hours resulted in the production of high levels of low molecular weight dipeptide and tripeptide. Peptides extracted from casein by the enzymatic method were in the range of 180 to 3000 daltons. Most peptides (61.8%) were di-/tripeptides (MW 230–900 Da), followed by oligopeptides/polypeptides (31.5%) (MW 900 to >5000 Da), and free amino acids (6.7%) (MW 230 Da) (Table 1).

3.2. Determining the Degree of Hydrolysis. The results of the degree of hydrolysis of the trypsin enzyme used in this study are shown in Table 2. The results and their reciprocal effect were significant at five percent ($P < 0.05$). The findings revealed that as time and temperature in the hydrolysis process increased, the degree of hydrolysis increased as well, which was then followed by a decrease.

Table 1: Distribution of molecular weight of obtained casein peptides enzymatic hydrolysis at 60°C for 4 hours.

Molecular weight range (Da)	Peptide fraction (%)
>5000	0.05 ± 0.02
5000–4000	0.19 ± 0.02
4000–3000	2.5 ± 0.19
3000–2000	3.12 ± 0.22
2000–1000	10.19 ± 0.1
1000–900	15.45 ± 0.52
900–230	61.78 ± 0.2
<230	6.7 ± 0.59

The data is presented as mean standard deviation from the duplicate sample's mean values.

The lowest degree of hydrolysis was at 40°C in four hours (18.36%) and the highest degree of hydrolysis was at 60°C in 4 hours (28.44%).

Determining the degree of hydrolysis is one of the most important factors in the study of the properties of hydrolyzed proteins, which indicates the degree of peptide bonds' breakdown, and it is important to control its rate. Enzymatic hydrolysis of proteins is affected by various factors including protein structure, temperature, enzyme-to-protein ratio, enzyme concentration, PH, and many properties of hydrolyzed proteins including the molecular weight of proteins produced and the number of free amino acids. The solubility depends on the degree of hydrolysis as well [42]. Increased hydrolysis with increased temperature and time can be due to increased bioavailable enzyme efficiency which is accompanied by a greater impact of enzyme activity at constant time and hydrolysis of primary proteins. This results in more chain breakage and more amino acids which ultimately leads to the development of smaller peptides [43]. Another reason is that by increasing the duration of hydrolysis, more enzyme activity will take place. Also, peptide bonds are available for a longer period of time and break more. With the breaking of more peptide bonds, we will have higher hydrolysis [44]. The reason for its decrease may have roots in the fact that by an increase in temperature, the proteolytic activity of the enzyme was reduced which results in a reduction of available peptide bonds of the enzyme [45]. Kumar et al. [46] by investigating the effect of Alcalase, alpha-chymotrypsin, and papain on peptides extracted from camel milk casein reported that the degree of hydrolysis increased significantly with increasing the hydrolysis time. The hydrolysis of sodium caseinate was 20%, according to Rao et al. [47], and stated that there was initially a rapid increase in the hydrolysis process, which grew to 7.6% in the first 15 minutes, following an increase to 12% in 30 minutes. It peaked in 120 minutes reaching 20% and then remained relatively stable. Ballatore and his colleagues [48] in 2020 determined the degree of hydrolysis on Whey protein using the enzyme trypsin and found that after trypsin hydrolysis, the highest DH (in percentage) reached was 18 ± 1% and the lowest was 8 ± 1%. Cheison et al. [49] also reported that as the temperature changed from 37°C to 50°C, the amount of DH decreased from 13.71 to 11.67, respectively.

Table 2: Interaction effects of temperature and time on DH (%).

	T_1t_1	T_1t_2	T_1t_3	T_2t_1	T_2t_2	T_2t_3	T_3t_1	T_3t_2	T_3t_3
DH	$18.36^{c} \pm 1.01$	$19.45^{b} \pm 1.57$	$20.50^{a} \pm 1.66$	$25.31^{c} \pm 1.08$	$25.80^{b} \pm 1.29$	$26.38^{a} \pm 1.33$	$28.44^{a} \pm 1.06$	$27.67^{b} \pm 1.36$	$26.64^{c} \pm 1.57$

T_1: 40°C, T_2: 50°C, and T_3: 60°C. t_1: 4 h, t_2: 5 h, and t_3: 6 h. The reaction was carried at pH 8 and 40, 50, and 60°C with times 4, 5, and 6 h. Different letters within each column indicate a significant difference between means ($P < 0.05$).

3.3. Trap Diphenylpicrylhydrazyl (DPPH). The results and their reciprocal effect were significant at five percent ($P < 0.05$). This finding showed that the amount of antioxidant activity increased with increasing time and temperature of hydrolysis to 4 hours and 60°C. Following this, its amount at 60°C and five hours decreased and they were better than BHT in terms of their ability to inhibit free radicals. DPPH free radical scavenging had also been reduced. However, the lowest amount of antioxidant properties was recorded at 40°C and 4 hours ($P < 0.05$). Table 3 shows the effect of enzymatic hydrolysis of casein using trypsin on the production of bioactive peptides with the ability to inhibit anionic radical DPPH.

This discrepancy in outcomes may be caused by the rate at which the protein is being hydrolyzed, the higher impact of the enzyme on the protein, and the composition of the generated amino acid sequence, which has a significant impact on the level of antioxidant activity. Further development of the hydrolysis process reduces the ability to inhibit free radical scavenging of DPPH due to the complete hydrolysis of peptides and the accumulation of smaller peptides and amino acids leading to complete release and high availability of hydrophilic amino acids [43]. Researchers found that the DPPH activity of camel milk casein hydrolyzes increased significantly and then decreased ($P < 0.05$) as the hydrolysis time progressed, creating a positive relationship between hydrolysis time and DPPH activity [50]. Numerous experiments have shown that different substrates can be hydrolyzed by proteases and have antioxidant effects. Casein isolated from fungal proteases, for example, was confirmed to have antioxidant properties by Neto et al. [51]. Mao et al. [52] also registered increased DPPH activity of hydrolyzed buffalo milk protein as the hydrolysis process progressed up to 7 hours. Khantaphant et al. [53] reported that by hydrolyzing the muscle protein of a fish using the enzymes flavourzyme and Alcalase, increasing the degree of hydrolysis also improved the activity of the radical inhibitor DPPH, all of which are in line with our results.

3.4. Measurement of Iron Regenerative Power III (FRAP). Table 4 shows the simultaneous effect of two variables of time and temperature on the FRAP strength of casein hydrolyzed proteins. This experiment showed a positive relationship between the time of hydrolysis and the amount of FRAP and increasing the time and temperature of hydrolysis up to four hours and temperature of 60°C, FRAP levels rose and then fell ($P < 0.05$). Peptides extracted from casein performed better than BHT in terms of their ability to inhibit free radicals.

The FRAP method is based on the reduction of the combination of TPTZ (F2, 4, 6- tripyridyl-s- triazine) with iron chloride FeCl 3. 6H2O which is almost colorless and slightly brown. After reducing antioxidants, this chemical substance forms aqueous iron complexes [54]. When antioxidants are present in the environment, the intensity of the blue color in the environment is higher and by measuring the intensity of the color, the antioxidant capacity can be understood [55]. Studies have shown that proteases can hydrolyze a variety of substrates and produce an antioxidant effect. Pepsin, for example, can effectively hydrolyze Whey proteins and casein from goat milk [56]. Studies have also shown after hydrolysis by a microbial protease isolated from *Bacillus* sp., caseinate derived from sheep milk had antioxidant activities ranging from 69.3–83.4% for DPPH and 70.8–83.3% for iron chelation [57]. The findings of this study match that of Peng et al. [58], who used the Alcalase enzyme to hydrolyze Whey protein. Kumar et al. [46], who studied camel milk peptides reported similar findings. Additionally, the outcomes of a study by Zhang et al. [59] that focused on the extraction of peptides from soybean using the Alcalase enzyme were similar to our own.

3.5. ABTS (2,2′-Azino-Bis (3-Ethylbenzothiazoline-6-sulfonic Acid). Table 5 shows the percentage of radical inhibition of ABTS on casein-derived peptides. The results showed a significant increase in the antioxidant activity of GBP peptides. In general, increasing the degree of hydrolysis also caused an increase in the radical inhibition of ABTS ($P < 0.05$) and performed better than BHT in terms of their ability to inhibit free radicals.

The results of various studies indicate that the antioxidant activity of peptides is significantly dependent on the type of enzyme, protein source, degree of hydrolysis, and process conditions. These results are in line with the results of Wang and Xiong [60] who examined the effect of the degree of hydrolysis of potato protein on the free radical scavenging of ABTS. Unlike the DPPH, the ABTS did not show this tendency, which claimed to decrease as the degree of hydrolysis rose at 60°C and 5 h. You et al. [43] conducted a study on Tian fish peptides which supports these findings. They attributed these findings to the increased release of hydrophobic amino acids and peptides. Also in 2015, Irshad et al. evaluated the antioxidant activity of bioactive peptides generated from bovine casein hydrolyzed with trypsin and pepsin at various time points. Their findings demonstrated that these peptides have antioxidant properties and the 1-kDa fraction had stronger antioxidant activity than the 10-kDa fraction [61].

Table 3: Interaction effects of temperature (T) and time (t) on DPPH (%).

	T_1t_1	T_1t_2	T_1t_3	T_2t_1	T_2t_2	T_2t_3	T_3t_1	T_3t_2	T_3t_3
DPPH	$52.17^{c} \pm 1.61$	$53.77^{b} \pm 1.29$	$54.76^{a} \pm 1.36$	$60.62^{c} \pm 1.57$	$63.50^{b} \pm 1.29$	$66.76^{a} \pm 1.43$	$76.62^{a} \pm 1.12$	$74.59^{b} \pm 1.42$	$72.80^{c} \pm 1.0$
BHT	$48.76^{c} \pm 1.01$	$51.69^{b} \pm 1.11$	$53.27^{a} \pm 1.24$	$57.63^{c} \pm 1.32$	$59.88^{b} \pm 1.14$	$63.67^{a} \pm 1.23$	$73.53^{a} \pm 1.08$	$71.49^{b} \pm 1.37$	$69.54^{c} \pm 1.09$

T_1: 40°C, T_2: 50°C, and T_3: 60°C. t_1: 4 h, t_2: 5 h, and t_3: 6 h. The reaction was carried at pH 8 and 40, 50, and 60°C with times 4, 5, and 6 h. Different letters within each column indicate a significant difference between means ($P < 0.05$).

Table 4: Interaction effects of temperature (T) and time (t) on FRAP (mM Fe(II)).

	T_1t_1	T_1t_2	T_1t_3	T_2t_1	T_2t_2	T_2t_3	T_3t_1	T_3t_2	T_3t_3
FRAP	$27.33^{c} \pm 1.13$	$32^{b} \pm 1.25$	$34.66^{a} \pm 1.12$	$35.33^{c} \pm 1.01$	$37^{b} \pm 1.08$	$40.33^{a} \pm 1.06$	$55^{a} \pm 1.12$	$51^{b} \pm 1.20$	$48.33^{c} \pm 1.10$
BHT	$21.22^{c} \pm 0.98$	$25.57^{b} \pm 1.21$	$29.45^{a} \pm 1.1$	$32.48^{c} \pm 1.43$	$34.85^{b} \pm 1.32$	$38.45^{a} \pm 1.1$	$52.08^{a} \pm 1.18$	$50.63^{b} \pm 0.97$	$43.72^{c} \pm 1.19$

T_1: 40°C, T_2: 50°C, and T_3: 60°C. t_1: 4 h, t_2: 5 h, and t_3: 6 h. The reaction was carried at pH 8 and 40, 50, and 60°C with times 4, 5, and 6 h. Different letters within each column indicate a significant difference between means ($P < 0.05$).

Table 5: Interaction effects of temperature (T) and time (t) on ABTS (%).

	T_1t_1	T_1t_2	T_1t_3	T_2t_1	T_2t_2	T_2t_3	T_3t_1	T_3t_2	T_3t_3
ABTS	$62.57^{c} \pm 1.6$	$67.22^{b} \pm 0.7$	$69.37^{a} \pm 0.4$	$71.53^{c} \pm 0.5$	$74.21^{b} \pm 0.9$	$78.45^{a} \pm 0.3$	$84.05^{a} \pm 0.3$	$82.14^{b} \pm 1.1$	$80.85^{c} \pm 0.8$
BHT	$55.67^{c} \pm 0.7$	$61.55^{b} \pm 0.5$	$62.86^{a} \pm 1.2$	$67.63^{c} \pm 1.1$	$69.58^{b} \pm 0.8$	$72.56^{a} \pm 0.6$	$76.05^{a} \pm 0.7$	$75.31^{b} \pm 0.9$	$73.44^{c} \pm 0.6$

T_1: 40°C, T_2: 50°C, and T_3: 60°C. t_1: 4 h, t_2: 5 h, and t_3: 6 h. The reaction was carried at pH 8 and 40, 50, and 60°C with times 4, 5, and 6 h. Different letters within each column indicate a significant difference between means ($P < 0.05$).

3.6. Antimicrobial Activity

3.6.1. Disk Diffusion. Antibacterial peptides usually consist of 12–50 amino acids and have cationic and amphipathic properties that have a molecular weight of 5–10 kDa. Despite the various structures (alpha-helix and beta-sheet and annular and long-chain), the presence of a positive charge in many peptides enables them to attach to bacterial membranes. This binding is essential for antimicrobial properties, after which the peptides must cross the membrane barrier to reach target sites within the cell. Due to the antibacterial power of hydrolyzed proteins, bioactive peptides can be more effective on the cytoplasmic membrane of bacteria. Most peptides can exert their antimicrobial effect by creating pores in the membrane and interfering with the passage of food and ions. Accumulation of peptides in the cytoplasmic membrane leads to leakage of cytoplasmic contents and eventually bacterial death [62]. It is argued that binding to enterotoxins and preventing bacterial cells from binding to surfaces can also be effective in antimicrobial properties. In addition, bioactive peptides deprive the nutrients needed for bacterial survival and growth and thus exert their antimicrobial effect [63]. The results showed that casein-derived peptides could be effective against all bacteria except *S. aureus* (Table 6). The greatest effect of these peptides was on éclairs with a concentration of 3 mg/mL^{-1} ($P < 0.05$). Bioactive peptides produced by enzymatic hydrolysis of sunflower seeds by digestive enzymes against *S. aureus*, *Listeria monocytogenes,* and *B. cereus* by disk diffusion method have been reported by Taha et al. [64], which reported antimicrobial activity of kappa casein against *E.coli* and attributed it to the high content of general hydrophobic amino acids because antibacterial peptides can inhibit DNA and RNA synthesis by binding to intracellular molecules once they enter the cytoplasm [65]. Cheddar cheese peptides were found to have antibacterial activity against three bacteria, *S. aureus*, *E. coli*, and *B. cereus*, according to Pritchard et al. [66]. In this study, peptides with an approximate size of 13 kDa had the greatest antimicrobial effect on *B. cereus* and the least effect on *S. aureus*.

3.6.2. MIC and MBC Determination. Determination of antimicrobial activity by determining the minimum inhibitory concentration (MIC) is one of the most widely used methods and has been accepted by most researchers as a criterion for determining antimicrobial activity [67]. The minimum lethal concentration of CBP on 4 pathogenic bacteria is shown in Table 7 and is compared with several standard antibiotics.

The *B. cereus* bacteria had the lowest MIC and MBC, and *S. typhimurium* had the highest, but *S. aureus* was not affected. This peptide had a considerably stronger inhibitory effect on *B. cereus* growth than the antibiotics vancomycin and gentamicin, but the antibiotic ampicillin had a greater inhibitory effect on *B. cereus* growth than the CBP peptide. When compared to antibiotics such as ampicillin and gentamicin, this peptide had a stronger inhibitory effect on *E. coli* growth. In a study by Ilić et al. [68], the MIC of ADP1, ADP2, and ADP3 peptides against *E.coli* were 2–4, 1, and 4 μg/ml, respectively, which was lower than the MIC of the peptide used in our study. However, the lethality of the ADP3 peptide on *E.coli* was almost equal to the lethality of the peptide used in this study. The effect of these peptides in inhibiting the growth of *S. aureus* was much less than that of the CBP peptide. Also, the inhibition of the ADP2 peptide on *S. typhimurium* was much lower than the CBP peptide [69]. In another study, the MIC of melittin against *E. coli* was 16 micrograms per milliliter, which was almost twice the MIC of the CBP peptide against this bacterium. This means

Table 6: The antibacterial circle diameters of casein hydrolytes released by different concentration.

Strain concentration (mg/mL^{-1})	*E.coli* (mm)	*B. cereus* (mm)	*S. aureus* (mm)	*S. typhimurium* (mm)
2	$32.73^{b} \pm 1.45$	$29.43^{b} \pm 1.06$	—	$29.12^{b} \pm 1.28$
2.5	$35.87^{a} \pm 2.17$	$33.13^{a} \pm 1.81$	—	$37.91^{a} \pm 2.21$
3	$36.71^{a} \pm 2.73$	$32.23^{a} \pm 1.11$	—	$39.67^{a} \pm 3.11$

Table 7: Mean values of MIC and MBC for CBP peptide compared to commercial antibiotics against three pathogenic bacteria.

Bacteria	MBC (μg/ml)		MIC (μg/ml)		
	CBP		Gentamicin	Ampicillin	Vancomycin
E. coli	4.21 ± 0.22	8.515 ± 0.15	≥16	≥32	—
B. cereus	3.412 ± 0.31	7.725 ± 0.57	≥16	0.5–2	≥16
S. aureus	—	—	—	—	—
S. typhimurium	8.515 ± 0.41	8.555 ± 0.09	16 ≤	2–8	—

that the inhibitory power of the CBP peptide against *S. typhimurium* was twice that of the melittin peptide. The reason for the lower inhibitory power of melittin peptide against *S. typhimurium* is the presence of an outer membrane in Gram-negative bacteria which limits the penetration of hydrophobic components into the lipopolysaccharide layer [70].

4. Conclusion

One of the most important goals of hydrolyzed protein production is to make the best use of the protein part of food by reducing its size to improve its digestion, absorption, and nutritional value. The results of this study showed that bioactive peptides obtained from enzymatic hydrolysis of casein, with functional, antioxidative, and microbial properties, have a high potential for production, enrichment, and formulation of end products which results in increasing the level of public health. Also, due to the significant enhancement in bacterial resistance to synthetic antibiotics and according to the results of this study, hydrolyzed casein proteins can be used as natural compounds with the ability to inhibit the activity of pathogenic bacteria.

Data Availability

The data used to support the findings of this study are available upon request.

Disclosure

This article is a section of a Ph.D. thesis. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Raziyeh Mokhtari (RM) designed the experiment, ran a statistical analysis, and wrote the manuscript; Mansour Rezaei (MR) and Mohammad Kazemifard (MK) assisted in the laboratory part of the project; and Essa Dirandeh (ED) helped in writing the manuscript and revised it.

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References

[1] J. E. Aguilar-Toalá, L. Santiago-López, C. M. Peres et al., "Assessment of multifunctional activity of bioactive peptides derived from fermented milk by specific Lactobacillus plantarum strains," *Journal of Dairy Science*, vol. 100, no. 1, pp. 65–75, 2017.

[2] D. Agyei and M. K. Danquah, "Rethinking food-derived bioactive peptides for antimicrobial and immunomodulatory activities," *Trends in Food Science & Technology*, vol. 23, no. 2, pp. 62–69, 2012.

[3] C. L. Bevins and N. H. Salzman, "Paneth cells, antimicrobial peptides and maintenance of intestinal homeostasis," *Nature Reviews Microbiology*, vol. 9, no. 5, pp. 356–368, 2011.

[4] A. G. Samaranayaka, E. C. Li-Chan, and E. C. Y. Li-Chan, "Food-derived peptidic antioxidants: a review of their production, assessment, and potential applications," *Journal of Functional Foods*, vol. 3, no. 4, pp. 229–254, 2011.

[5] B. H. Sarmadi and A. Ismail, "Antioxidative peptides from food proteins: a review," *Peptides*, vol. 31, no. 10, pp. 1949–1956, 2010.

[6] A. M. Pisoschi and A. Pop, "The role of antioxidants in the chemistry of oxidative, stress: a review," *European Journal of Medicinal Chemistry*, vol. 97, pp. 55–74, 2015.

[7] P. Ghezzi, V. Jaquet, F. Marcucci, and H. H. H. W. Schmidt, "The oxidative stress theory of disease: levels of evidence and epistemological aspects," *British Journal of Pharmacology*, vol. 174, no. 12, pp. 1784–1796, 2017.

[8] K. Kandola, A. Bowman, and M. A. Birch-Machin, "Oxidative stress–a key emerging impact factor in health, ageing, lifestyle and aesthetics," *International Journal of Cosmetic Science*, vol. 37, pp. 1–8, 2015.

[9] B. Halliwell and J. M. C. Gutteridge, "Antioxidant defences: endogenous and diet derived," *Free radicals in biology and medicine*, vol. 4, pp. 79–186, 2007.

[10] H. Cichoż-Lach and A. Michalak, "Oxidative stress as a crucial factor in liver diseases," *World Journal of Gastroenterology*, vol. 20, p. 8082, 2014.

[11] J. J. Gao, K. Igalashi, and M. Nukina, "Radical scavenging activity of phenylpropanoid glycosides in Caryopteris incana," *Bioscience Biotechnology & Biochemistry*, vol. 63, no. 6, pp. 983–988, 1999.

[12] G. M. Williams, M. J. Iatropoulos, and J. Whysner, "Safety assessment of butylated hydroxyanisole and butylated hydroxytoluene as antioxidant food additives," *Food and Chemical Toxicology*, vol. 37, no. 9-10, pp. 1027–1038, 1999.

[13] C. G. Rizzello, D. Tagliazucchi, E. Babini, G. Sefora Rutella, D. L. Taneyo Saa, and A. Gianotti, "Bioactive peptides from vegetable food matrices: research trends and novel biotechnologies for synthesis and recovery," *Journal of Functional Foods*, vol. 27, pp. 549–569, 2016.

[14] P. Vijayabaskar and V. Shiyamala, "Antioxidant properties of seaweed polyphenol from Turbinariaornata (Turner) J. Agardh, 1848," *Asian Pacific Journal of Tropical Biomedicine*, vol. 2, no. 1, pp. S90–S98, 2012.

[15] M. K. Danquah and D. Agyei, "Pharmaceutical applications of bioactive peptides," *OA Biotechnology*, vol. 1, no. 2, pp. 1–7, 2012.

[16] O. Power, P. Jakeman, and R. J. FitzGerald, "Antioxidative peptides: enzymatic production, in vitro and in vivo antioxidant activity and potential applications of milk-derived antioxidative peptides," *Amino Acids*, vol. 44, no. 3, pp. 797–820, 2013.

[17] E. F. Haney and R. E. W. Hancock, "Peptide design for antimicrobial and immunomodulatory applications," *Biopolymers*, vol. 100, no. 6, pp. 572–583, 2013.

[18] J.-S. Seo, Y. S. Ju, W.-C. Lee et al., "The transcriptional landscape and mutational profile of lung adenocarcinoma," *Genome Research*, vol. 22, no. 11, pp. 2109–2119, 2012.

[19] J. McCalla, T. Waugh, and E. Lohry, "Protein hydrolysates/peptides in animal nutrition," *Protein hydrolysates in biotechnology*, pp. 179–190, 2008.

[20] M. J. Playne, L. E. Bennett, and G. W. Smithers, "Functional dairy foods and ingredients," *Australian Journal of Dairy Technology*, vol. 58, no. 3, pp. 242–264, 2003.

[21] G. D. Miller, J. K. Jarvis, and L. D. McBean, *Handbook of Dairy Foods and Nutrition*, CRC Press, Boca Raton, Florida, 2006.

[22] H. Meisel, "Multifunctional peptides encrypted in milk proteins," *BioFactors*, vol. 21, no. 1-4, pp. 55–61, 2004.

[23] D. D. Kitts and K. Weiler, "Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery," *Current Pharmaceutical Design*, vol. 9, no. 16, pp. 1309–1323, 2003.

[24] S. Sakanaka, Y. Tachibana, N. Ishihara, and L. R. Juneja, "Antioxidant properties of casein calcium peptides and their effects on lipid oxidation in beef homogenates," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 2, pp. 464–468, 2005.

[25] K. Rossini, C. P. Z. Norena, F. Cladera-Olivera, and A. Brandelli, "Casein peptides with inhibitory activity on lipid oxidation in beef homogenates and mechanically deboned poultry meat," *LWT-food Science and Technology*, vol. 42, no. 4, pp. 862–867, 2009.

[26] S. Mizuno, K. Matsuura, T. Gotou et al., "Antihypertensive effect of casein hydrolysate in a placebo-controlled study in subjects with high-normal blood pressure and mild hypertension," *British Journal of Nutrition*, vol. 94, no. 1, pp. 84–91, 2005.

[27] E. N. Frankel, "Review. Recent advances in lipid oxidation," *Journal of the Science of Food and Agriculture*, vol. 54, no. 4, pp. 495–511, 1991.

[28] S. Tonstad, K. Smerud, and L. Høie, "A comparison of the effects of 2 doses of soy protein or casein on serum lipids, serum lipoproteins, and plasma total homocysteine in hypercholesterolemic subjects," *The American Journal of Clinical Nutrition*, vol. 76, no. 1, pp. 78–84, 2002.

[29] P. Schüpbach, J. R. Neeser, M. Golliard, M. Rouvet, and B. Guggenheim, "Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci," *Journal of Dental Research*, vol. 75, no. 10, pp. 1779–1788, 1996.

[30] M. A. J. S. Van Boekel, C. N. J. M. Weerens, A. Holstra, C. E. Scheidtweiler, and G. Alink, "Antimutagenic effects of casein and its digestion products," *Food and Chemical Toxicology*, vol. 31, no. 10, pp. 731–737, 1993.

[31] G. H. McIntosh, Y. H. A. Wang, and P. J. Royle, "A diet containing chickpeas and wheat offers less protection against colon tumors than a casein and wheat diet in dimethylhydrazine-treated rats," *Journal of Nutrition*, vol. 128, no. 5, pp. 804–809, 1998.

[32] Y. Kawasaki, H. Isoda, H. Shinmoto et al., "Inhibition by κ-casein glycomacropeptide and lactoferrin of influenza virus hemagglutination," *Bioscience Biotechnology & Biochemistry*, vol. 57, no. 7, pp. 1214-1215, 1993.

[33] M. Moezizadeh and S. Moayedi, "Anticariogenic effect of amorphous calcium phosphate stabilized by casein phosphopeptid: a review article Research," *Journal of Biological Sciences*, vol. 4, no. 1, pp. 132–136, 2009.

[34] L. Chen, J. Chen, J. Ren, and M. Zhao, "Modifications of soy protein isolates using combined extrusion pre-treatment and controlled enzymatic hydrolysis for improved emulsifying properties," *Food Hydrocolloids*, vol. 25, no. 5, pp. 887–897, 2011.

[35] A. G. Gornall, C. J. Bardawill, and M. M. David, "Determination of serum proteins by meansof thebiuret reaction," *Journal of Biological Chemistry*, vol. 177, no. 2, pp. 751–766, 1949.

[36] B. Vazgecer, H. Ulu, and A. Oztan, "Microbiological and chemical qualities of chicken dönerkebab retailed on the Turkish restaurants," *Food Control*, vol. 15, no. 4, pp. 261–264, 2004.

[37] H. C. Wu, H. M. Chen, and C. Y. Shiau, "Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus)," *Food Research International*, vol. 36, no. 9-10, pp. 949–957, 2003.

[38] I. F. Benzie, J. Strain, and J. John, "The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay," *Analytical Biochemistry*, vol. 239, no. 1, pp. 70–76, 1996.

[39] T. Ak and İ. Gülçin, "Antioxidant and radical scavenging properties of curcumin," *Chemico-Biological Interactions*, vol. 174, no. 1, pp. 27–37, 2008.

[40] National Committee for Clinical Laboratory Standards, *"Performance Standards for Antimicrobial Disk Susceptibility Tests"*, 6th Edition, Approved Standard M2-A6 (M100-S7). National Committee for Clinical Laboratory Standards, Wayne, PA, USA, 1997.

[41] F. Oroojalian, R. Kasra-Kermanshahi, M. Azizi, and M. R. Bassami, "Phytochemical composition of the essential

oils from three Apiaceae species and their antibacterial effects on food-borne pathogens," *Food Chemistry*, vol. 120, no. 3, pp. 765–770, 2010.

[42] R. Šližytė, E. Daukšas, E. Falch, I. Storrø, and T. Rustad, "Characteristics of protein fractions generated from hydrolysed cod (*Gadus morhua*) by-products," *Process Biochemistry (Oxford, United Kingdom)*, vol. 40, no. 6, pp. 2021–2033, 2005.

[43] L. You, M. Zhao, J. M. Regenstein, and J. Ren, "Changes in the antioxidant activity of loach(*Misgurnus anguillicaudatus*) protein hydrolysates during a simulated gastrointestinal digestion," *Food Chemistry*, vol. 120, no. 3, pp. 810–816, 2010.

[44] T. A. Aderinola, T. N. Fagbemi, V. N. Enujiugha, A. M. Alashi, and R. E. Aluko, "Amino acid composition and antioxidant properties of Moringa oleifera seed protein isolate and enzymatic hydrolysates," *Heliyon*, vol. 4, no. 10, Article ID e00877, 2018.

[45] M. Ovissipour, R. Safari, M. Ali et al., "Use of hydrolysates from Yellowfin tuna *Thunnus albacares* fisheries by-product as a nitrogen source for bacteria growth media," *International Aquatic Research*, vol. 1, no. 1, pp. 73–77, 2009.

[46] D. Kumar, M. K. Chatli, R. Singh, N. Mehta, and P. Kumar, "Enzymatic hydrolysis of camel milk casein and its antioxidant properties," *Dairy Science & Technology*, vol. 96, no. 3, pp. 391–404, 2016.

[47] P. S. Rao, A. Mayur, N. B. Harisha, R. Bajaj, and B. Mann, "Comparison of OPA and pH stat methods for measurement of degree of hydrolysis of alcalase and flavourzyme digested casein," *Indian Journal of Dairy Science*, vol. 71, pp. 107–109, 2018.

[48] M. B. Ballatore, M. D. R. Bettiol, N. L. Vanden Braber et al., "Antioxidant and cytoprotective effect of peptides produced by hydrolysis of whey protein concentrate with trypsin," *Food Chemistry*, vol. 319, Article ID 126472, 2020.

[49] S. C. Cheison, E. Leeb, J. Toro-Sierra, and U. Kulozik, "Influence of hydrolysis temperature and pH on the selective hydrolysis of whey proteins by trypsin and potential recovery of native alpha-lactalbumin," *International Dairy Journal*, vol. 21, no. 3, pp. 166–171, 2011.

[50] R. He, A. T. Girgih, S. A. Malomo, X. Ju, and R. E. Aluko, "Antioxidant activities of enzymatic rapeseed protein hydrolysates and the membrane ultrafiltration fractions," *Journal of Functional Foods*, vol. 5, no. 1, pp. 219–227, 2013.

[51] Y. A. A. H. Neto, J. C. Rosa, and H. Cabral, "Peptides with antioxidant properties identified from casein, whey, and egg albumin hydrolysates generated by two novel fungal proteases," *Preparative Biochemistry & Biotechnology*, vol. 49, no. 7, pp. 639–648, 2019.

[52] X.-Y. Mao, X. Cheng, X. Wang, and S. J. Wu, "Free-radical-scavenging and anti-inflammatory effect of yak milk casein before and after enzymatic hydrolysis," *Food Chemistry*, vol. 126, no. 2, pp. 484–490, 2011.

[53] S. Khantaphant, S. Benjakul, and H. Kishimura, "Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases," *Process Biochemistry (Oxford, United Kingdom)*, vol. 46, no. 1, pp. 318–327, 2011.

[54] A. Bougatef, N. Nedjar-Arroume, L. Manni et al., "Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (Sardinella aurita) by-products proteins," *Food Chemistry*, vol. 118, no. 3, pp. 559–565, 2010.

[55] T. Z. Liu, N. Chin, M. D. Kiser, and W. N Bigler, "Specific spectrophotometry of ascorbic acid in serum or plasma by use of ascorbate oxidase," *Clinical Chemistry*, vol. 28, no. 11, pp. 2225–2228, 1982.

[56] A. S. Ahmed, T. El-Bassiony, L. M. Elmalt, and H. R. Ibrahim, "Identification of potent antioxidant bioactive peptides from goat milk proteins," *Food Research International*, vol. 74, pp. 80–88, 2015.

[57] A. P. F. Corrêa, D. J. Daroit, J. Coelho et al., "Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease," *Journal of the Science of Food and Agriculture*, vol. 91, no. 12, pp. 2247–2254, 2011.

[58] X. Peng, Y. L. Xiong, and B. Kong, "Antioxidant activity of peptide fractions from whey protein hydrolysates as measured by electron spin resonance," *Food Chemistry*, vol. 113, no. 1, pp. 196–201, 2009.

[59] Q. Zhang, X. Tong, Y. Li et al., "Purification and characterization of antioxidant peptides from Alcalase-hydrolyzed soybean (Glycine max L.) hydrolysate and their cytoprotective effects in human intestinal Caco-2 cells," *Journal of Agricultural and Food Chemistry*, vol. 67, no. 20, pp. 5772–5781, 2019.

[60] L. L. Wang and Y. L. Xiong, "Inhibition of lipid oxidation in cooked beef patties by hydrolyzed potato protein is related to its reducing and radical scavenging ability," *Journal of Agricultural and Food Chemistry*, vol. 53, no. 23, pp. 9186–9192, 2005.

[61] I. Irshad, A. Kanekanian, A. Peters, and T. Masud, "Antioxidant activity of bioactive peptides derived from bovine casein hydrolysate fractions," *Journal of Food Science & Technology*, vol. 52, no. 1, pp. 231–239, 2015.

[62] Y. Shai, "From innate immunity to de-novo designed antimicrobial peptides," *Current Pharmaceutical Design*, vol. 8, no. 9, pp. 715–725, 2002.

[63] J. Théolier, I. Fliss, J. Jean, and R. Hammami, "Antimicrobial peptides of dairy proteins: from fundamental to applications," *Food Reviews International*, vol. 30, no. 2, pp. 134–154, 2014.

[64] F. S. Taha, S. S. Mohamed, S. M. Wagdy, and G. F. Mohamed, "Antioxidant and antimicrobialactivities of enzymatic hydrolysis products from sunflower protein isolate," *World Applied ScienceJournal*, vol. 21, pp. 5–651, 2013.

[65] X. Cheng, X. Tang, Q. Wang, and X. Y. Mao, "Antibacterial effect and hydrophobicity of yak κ-casein hydrolysate and its fractions," *International Dairy Journal*, vol. 31, no. 2, pp. 111–116, 2013.

[66] S. R. Pritchard, M. Phillips, and K. Kailasapathy, "Identification of bioactive peptides in commercial Cheddar cheese," *Food Research International*, vol. 43, no. 5, pp. 1545–1548, 2010.

[67] R. J. W. Lambert, "Susceptibility testing: inoculum size dependency of inhibition using the Colworth MIC technique," *Journal of Applied Microbiology*, vol. 89, no. 2, pp. 275–279, 2000.

[68] N. Ilić, M. Novković, F. Guida et al., "Selective antimicrobial activity and mode of action of adepantins, glycine-rich peptide antibiotics based on anuran antimicrobial peptide sequences," *Biochimica et Biophysica Acta (BBA) - Biomembranes*, vol. 1828, pp. 1004–1012, 2013.

[69] F. Shahidi and Y. Zhong, "Bioactive peptides," *Journal of AOAC International*, vol. 91, no. 4, pp. 914–931, 2008.

[70] A. Ebbensgaard, H. Mordhorst, M. T. Overgaard, C. G. Nielsen, F. M. Aarestrup, and E. B. Hansen, "Comparative evaluation of the antimicrobial activity of different antimicrobial peptides against a range of pathogenic bacteria," *PLoS One*, vol. 10, no. 12, Article ID e0144611, 2015.