Waste and Biomass Valorization (2022) 13:3561–3572
https://doi.org/10.1007/s12649-022-01750-9

ORIGINAL PAPER

Industrial Utilization of Bioactive Hazelnut Peptide Fractions in the Manufacture of Functional Hazelnut Paste: ACE-Inhibition and Allergy Suppression

Ayşe Gülden Göksu[1] · Bilal Çakır[2,3] · İbrahim Gülseren[1,3]

Received: 20 July 2021 / Accepted: 6 March 2022 / Published online: 16 March 2022

Abstract

Purpose Press cakes are the primary by-products of cold press oil manufacture and are characterized with significant concentrations of proteins. Recently, our group has investigated the bioactive attributes of peptide fractions generated from hazelnut cakes and demonstrated their angiotensin converting enzyme (ACE)-inhibitory activities in vitro. In the current study, bioactive hazelnut peptide fractions were utilized in industrial settings to generate novel food products.

Methods Hazelnut protein isolates were generated via alkali extraction-isoelectric precipitation (AE-IP) method. Papain, bromelain and pepsin were used in the proteolysis of protein isolates and the hydrolysates were fractionated via fast protein liquid chromatography. In vitro ACE-inhibitory activities of the fractions were tested and their corresponding lyophilizates were added to industrial hazelnut paste (1%). Microbiological analyses of the enriched products were carried out in accordance with the relevant company procedures to ensure safety. Allergenic properties of hazelnut paste products were determined by an indirect ELISA method. Simulated gastrointestinal digestion was carried out and ACE assays were also performed on digested products.

Results ACE inhibitory fractions were characterized with IC_{50} values of approx. 2–27 μg ml^{-1}, respectively. Some of the fractions demonstrated considerable bioactivity (i.e., > 90% ACE-inhibition) after simulated digestion. Intact and further hydrolyzed peptides were predicted to be present in the digested paste and partial hydrolysis of the peptides potentially enhanced ACE-inhibitory attributes. Furthermore, allergic suppression was partially achieved in final products bearing the bromelain hydrolysate fractions (i.e., BRF 10). The interactions of current peptides with ACE and a major hazelnut allergen were investigated using molecular docking. A database (BioPepDB) search indicated that all of the current peptides were novel.

Conclusion Hazelnut cakes represent a resource for ACE-inhibitory and potentially allergy suppressing peptides that can be used in the formulation of functional foods and food supplements.

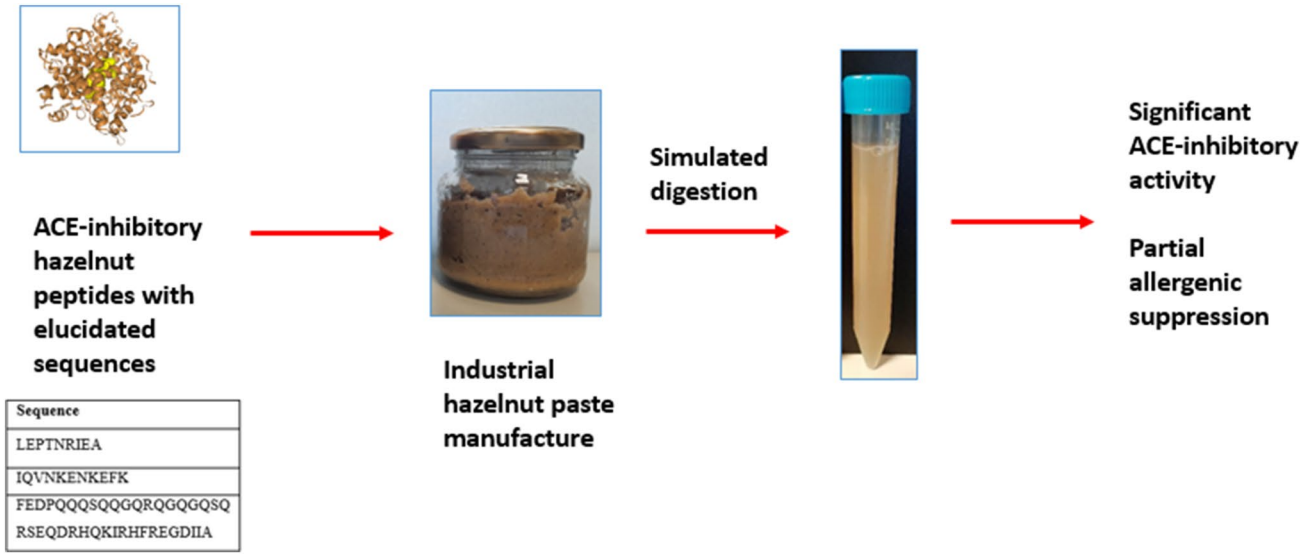
✉ İbrahim Gülseren
ibrahim.gulseren@izu.edu.tr

[1] Department of Food Engineering, İstanbul S. Zaim University (İZÜ), Sabri Ülker R&D Center, Halkalı, Küçükçekmece, İstanbul, Turkey

[2] Halal Food R&D Center, İstanbul S. Zaim University (İZÜ), Sabri Ülker R&D Center, Halkalı, Küçükçekmece, İstanbul, Turkey

[3] İZÜ Food and Agricultural Research Center (GTUAM), Sabri Ülker R&D Center, Halkalı, Küçükçekmece, İstanbul, Turkey

Graphical Abstract



Keywords Hazelnut protein hydrolysates · Peptide fractionation · Bioactive peptides · Molecular docking · Simulated digestion

Statement of Novelty

Press cakes are the primary by-products of cold press oil manufacture and they contain significant amounts of plant proteins. Although there are various plant protein or peptide products on the global market, the literature on the utilization of peptide fractions at relatively high concentrations and in industrial food products is scarce. Here, an attempt was made to utilize bioactive hazelnut peptides in industrially manufactured hazelnut paste. Furthermore, using simulated digestion and allergy suppression assays, physiological potential of such novel food products have been evaluated.

Introduction

Cardiovascular diseases are one of the primary causes of death events worldwide. The World Health Organization reports that an estimated 17.9 million people die each year due to cardiovascular diseases, which account for approximately 31% of all deaths worldwide (https://www.who.int/health-topics/cardiovascular-diseases#tab=tab_1). Hypertension is one of the risk factors that leads to the development of cardiovascular diseases. It is estimated that up to about 1.56 billion people will be affected by hypertension in 2025 [1].

Consequently, various medications including synthetic angiotensin converting enzyme (ACE, EC 3.4.15.1) inhibitors have been widely used for the clinical treatment of hypertension and heart failure in humans [2]. However, these synthetic drugs cause various side effects including diarrhea, allergies, skin rashes, renal dysfunction and in some cases, hypotension [3]. Therefore, the search for natural and safe ACE-inhibitors is a critical area of research.

Bioactive peptides are protein fragments with various biological activities that have been obtained from food proteins by enzymatic hydrolysis or fermentation [4] and are generally inactive in their parent protein. When necessary, bioactive peptides can be metabolized and eliminated from the body, which prevents or minimizes their bio-accumulation and potential toxic side effects [5]. Industrially relevant peptide and protein supplements are estimated to have a market share of more than $40 billion per year [6].

Previous studies have reported that many animal or vegetable proteins were potential sources for bioactive peptides. Milk, egg and red meat proteins have been considered as rich animal resources in the production of bioactive peptides [5, 7, 8]. In addition, bioactive peptides were obtained from seafood [9–11] and vegetable protein resources [12–15]. Peptides with ACE-inhibitory activity are the most intensely studied category among bioactive peptides [16].

Hazelnuts (*Corylus avellana* L.) belong to the genus Corylus of the Betulaceae family. Turkey has produced

approx. 595,000 tons of hazelnuts over the last 2 reported seasons (2017/18 and 2018/19), which roughly corresponded to 60% of the global hazelnut production [17]. Hazelnuts are widely used as a food ingredient, especially in chocolate and confectionery products, as well as as a raw material for the production of edible oil. An approximate composition of hazelnuts correspond to 55–65% fat, 13–15% protein and 5–12% carbohydrates [18]. Consequently, hazelnut meal could contain up to 40% protein.

In recent studies, hazelnut peptides were produced by enzymatic treatments and their biological activities were investigated. Liu et al. [19] treated Asian hazelnut (*Corylus heterophylla* Fisch.) proteins with Alcalase, and demonstrated that ACE-inhibitor peptides in hazelnuts were Ala-Val-Lys-Val-Leu, Tyr-Leu-Val-Arg, and Thr-Leu-Val-Gly-Arg. While the peptides inhibited ACE activity in a non-competitive manner, in vivo data showed that administration of Tyr-Leu-Val-Arg significantly reduced systolic blood pressure in spontaneously hypertensive rats. In the recent studies of our team, proteolytic (trypsin, chymotrypsin, and thermolysis) hydrolysis of hazelnut protein isolates from Giresun (Turkey) tombul hazelnuts (*C. avellana* L.) were shown to generate a variety of ACE-inhibitory fractions [20], where the identified peptides were different than the 3 novel sequences identified by Liu et al. [19].

The current findings have shown that hazelnut cakes/meals can be considered as an important source of ACE-inhibitor peptides that can be utilized in the formulation of functional foods and nutritional supplements. Although there are numerous studies on the production and purification of bioactive peptides in the current literature, the findings on utilization of peptides in industrial products are relatively limited. Commercialization of bioactive peptide-bearing functional foods lead to a number of challenges, including complexities in quality assurance methodology, insufficient data on bioavailability and bioefficacy, and potential bitterness of peptides [21]. In this study, inclusion of bioactive hazelnut peptide fractions in industrial hazelnut paste, evaluation of its ACE-inhibitory activity after simulated in vitro digestion and influence of enzymatic proteolysis on its allergenic attributes have been investigated.

Materials and Methods

Materials

The cold press cake samples for Giresun tombul hazelnuts (*Corylus avellana* L.) used in this study were kindly donated by Oneva (Neva Gıda Ltd., İstanbul, Turkey). Unless otherwise stated, the chemicals and reagents were purchased from Sigma-Aldrich (Schnelldorf, Germany) including the proteolytic enzymes such as papain (P4762), bromelain (B4882), pepsin (P6887), ACE (A6778), and α-amylase (A0521). Pancreatin from porcine pancreas with a trypsin activity of 625 U mg^{-1} was acquired from Bio Basic (PB0681).

Manufacture of Protein Isolates

The manufacture of hazelnut protein isolates was based on an alkali extraction-isoelectric precipitation (AE-IP) method [22]. Hazelnut cakes were mixed with distilled water at a ratio of 1:15 (cake:water, w/v) and the medium pH was adjusted to pH 9.5 using 1 N NaOH. The dispersion was kept stirred at ambient temperature at a stirring rate of 500 rpm for 1 h to ensure protein dissolution. The mixture was centrifuged at 11,000 × *g* at 25 °C (30 min) using a high speed refrigerated centrifuge (Himac CR22N, Hitachi, Japan). The supernatant was collected and its pH value was brought down to 4.5 using 1 N HCl to promote isoelectric precipitation. The dispersion was once again centrifuged under the identical conditions as before. After centrifugation, the precipitated proteins were collected and freeze-dried (TRS 2-2 V, Teknosem, İstanbul, Turkey) to generate powdered protein isolates, which were stored at − 20 °C until further use.

Enzymatic Proteolysis

The enzymatic hydrolysis of the protein isolates was carried out using a thermomixer (MTC-100, MIULAB, China). Papain, bromelain and pepsin were used in the hydrolysis of hazelnut protein isolates. In all cases, enzyme: substrate ratio was 1:100. These concentration ratios corresponded to 1, 0.3 and 32 U ml^{-1}, respectively, in enzyme units for papain, bromelain and pepsin treatments.

In papain treatments, the mixture was prepared in 20 mM sodium phosphate buffer (pH 7.0) and the treatment was continued for 150 min at 1000 rpm and 50 °C. In the case of bromelain, 30 mM sodium acetate buffer (pH 4.5) was used for 150 min at 1000 rpm and 45 °C. Pepsin was was prepared in distilled water, where pH was set to 2.0 using 1 N HCl. The proteolysis was continued for 18 h at 37 °C and 1000 rpm. In all cases, the microcentrifuge tubes containing the reaction mixture was transferred to a 95 °C water bath for 10 min and then in an ice-bath until the samples reached the ambient temperature in order to stop the enzymatic reaction. The sample tubes were centrifuged at 5000 × *g* for 30 min to remove the insoluble matter. Finally, the supernatant was filtered through PVDF syringe filters (0.45 µm) (Millex-HV, Merck-Millipore, Germany) [23]. The filtered hydrolysates were stored at − 80 °C until further use. Degree of hydrolysis (DH%) was determined based on the 2,4,6-Trinitrobenzenesulfonic acid (TNBS) method [24]. The leucine amino equivalency was determined based on Nielsen et al. [25].

Fractionation of Hydrolysates

Using an fast protein liquid chromatography (FPLC) system (ÄKTA pure 25 L, GE Healthcare Life Sciences, Sweden), the enzymatic hydrolysates were fractionated. A variety of columns were tested in pre-trials including HiTrap Capto DEAE, HiTrap Capto Q, HiTrap Capto S and HiTrap DEAE FF columns produced by the same company. Due to a favorable signal intensity in the fractions, weakly anionic HiTrap Capto DEAE (5 ml) columns were used all experiments.

Sample and elution buffers were 20 mM Tris–HCl (pH 8) and 20 mM Tris–HCl + 0.8 M NaCl (pH 8), respectively. The fractions were collected during a 35 CV flow, where a 0–100% linear gradient was administered and absorbance (280 nm) was measured by means of a UV detector. In addition, medium pH, conductivity, column and system pressure values were monitored. For each hydrolysate sample, the "out" fraction containing the materials that were not be bound to the column and 9 fractions consecutively numbered between 2 and 10 were collected. PAF, BRF and PEF refer to fractions of papain, bromelain and pepsin hydrolysates, respectively. The collected fractions were stored at − 20 °C until further use.

ACE-Inhibitory Activity Tests

The in vitro ACE-inhibitory activities of the peptide fractions were determined based on the decomposition rate for Hippuril-His-Leu (HHL) to His-Leu dipeptide and hippuric acid (HA), which normally takes place due to ACE activity [26]. In the presence of ACE-inhibitory agents, a reduction in decomposition rate is anticipated.

ACE-activity was tested in a medium that contained approx. 400 mM NaCl. Fifty µl of 6.5 mM HHL and 90 µl of peptide fraction sample prepared in sodium borate (0.1 M, pH 8.3) buffer were mixed and pre-incubated in a thermomixer at 37 °C, 1000 rpm for 10 min. Immediately afterwards, 40 µl of ACE (25 mU ml^{-1}) was added to the mixture and the reaction was continued for 1 h, and immediately afterwards, it was stopped by the addition of 1 M HCl (180 µl) to the mixture.

ACE-activity was assayed using Prominence LC-20A HPLC system (Shimadzu Corporation, Japan), which consisted of a degasser (DGU-20A5R), pump/solvent distributor module (LC-20AD), auto sampler (SIL-20A HT), column furnace (CTO-10AS VP), and a UV–VIS detector (SPD-20A). The method of Sheih et al. [27] was used with appropriate modifications and an ODS-3 C18 column (250 × 4.6 mm, pore size 110 Å, particle size 5 µm, Phenemonex, USA) was utilized in this assay.

ACE inhibition was monitored at 228 nm by injecting 10 µl of samples directly into the column. The mobile phase was 0.1% TFA prepared in 50% methanol and the isocratic flow rate was 1 ml min^{-1}. Twenty mM Tris–HCl + 0.8 M NaCl buffer was used as a negative control, whereas captopril was used as the reference inhibitor. The extent of reduction in HA formation was compared with that of control sample and % inhibition was determined.

Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-Q-TOF/MS) Analysis of ACE-Inhibitory Fractions

All MS analysis was carried out using the default settings of Labmed, Acıbadem University, İstanbul, Turkey, using a Xevo G2-XS QTof (Waters) device. Samples treated with different proteases were incubated with 10 mM DTT at 55 °C for 10 min to ensure the reduction of the peptides. The reduced peptide mixtures were then alkylated with 20 mM iodoacetamide (IAA) in the dark at ambient temperature conditions. Samples were filtered through 30 kDa filters. Peptide concentration of the samples was determined and the samples were taken into vials for LC-Q-TOF/MS analysis at 1 mg per injection.

LC separation was based on an HSS T3 Column (1.8 µm, 2.1 × 150 mm), detection wavelength of 214 nm and a column temperature of 60 °C. A flow rate of 0.2 ml min^{-1} was administered. MS conditions were based on a sample cone voltage of 80 V, source temperature of 120 °C, desolvation temperature of 300 °C and desolvation gas flow rate of 800 L h^{-1}.

Prior to the analyses, the detector and calibration settings were made via MassLynx program specific to the Xevo G2-XS QTof device where the analyzes were performed. The peptide fractions were further fractionated with an acetonitrile gradient (5–35%) in an HSS T3 column based on their hydrophobicity and the separated peptides were analyzed by mass spectrometry upon electrospray ionization. MS analysis was performed for 0.7 s and information was collected about the entire peptide. Afterwards, MS/MS analysis was performed for 0.7 s and the peptide fragmentation and sequence information were obtained. For protein identification, appropriate protein databanks were used for each sample.

Peptides and proteins were identified using ProteinLynx Global Server (PLGS 3.0) software. Analysis was performed using the appropriate databank for each sample type. "False positive rate" was set to 1%. Further details are not presented here to ensure brevity.

Im Silico Analyses and Molecular Docking

Upon the acquisition of MS-data, the determined peptide sequences were analyzed in silico for the prediction of their physicochemical and bioactive characteristics. Firstly, the isoelectric point, charge and toxicity assessment were

carried out based on the work of Gupta et al. [28]. The probability of peptide sequences being bioactive was determined using PeptideRanker [29]. In cases where bioactivity was observed in vitro and these findings were supported by in silico calculations [30], the interactions between the respective peptides and the enzyme they inhibit (i.e., ACE from human) were analyzed based on the method of Zhou et al. [31], which is based on a molecular docking approach (HPEPDOCK). Peptide-allergenic protein interactions were also studied via HPEPDOCK, which generated docking scores and 3D protein-peptide interaction images [31].

Industrial Hazelnut Paste Production and Product Analysis

One ACE-inhibitory fraction (PAF4, BRF10, and PEF6) produced by each and every protease was used in product trials due to their ACE-inhibitory activities and high lyophilization efficiency. Due to relatively large sample requirements, this parameter was adopted as a primary criterion. Industrial manufacture of bioactive peptide bearing hazelnut paste was carried out at FISKOBIRLIK, Giresun, Turkey, where manufacture and product analyses were carried out in accordance with internal company procedures.

Industrial Hazelnut Paste Production

The recipe for bioactive peptide fraction bearing hazelnut paste consisted simply of 70% hazelnuts and 30% sugar. Hazelnuts were roasted and automatically selected for suitability for hazelnut paste manufacture. Freeze-dried peptide fractions were added to the products at a level of 1%. Roasted hazelnuts and granulated crystal sugar were ground in a meat grinder and a powdered sugar mill, respectively. Both ground materials were mixed in the filling chamber and filled in and kept at 300 g glass jars until further use. No further additives or preservatives were used in the formulations.

Microbiological Analysis

To ensure the microbiological stability of the finished products, a variety of microbial tests including total bacterial count (PCA medium, 35 °C, 48 h), yeast-mould count (YGC Agar, 25 °C, 5–7 days), total coliform and *Escherichia coli* count (Compact Dry EC, 35 ± 2 °C, 24 h), *Staphylococcus aureus* count (Compact Dry SA, 35 ± 2 °C, 24 h) and *Salmonella* 25 g. Da count (Compact Dry SA, 35 ± 2 °C, 24 h) were carried out.

Indirect Allergenicity Testing

Indirect allergenicity analyses were performed at LTS Laboratory Services, İstanbul, Turkey, using a commercial ELISA kit (Ridascreen® FAST Hazelnut, R-Biopharm, Germany) in accordance with the procedures of the accredited laboratory (Turkish Accreditation Agency Accreditation No: AB-0351-T). This protocol aims to detect hazelnut proteins in potentially hazelnut bearing samples.

Simulated In Vitro Gastrointestinal Digestion Assays

In order to evaluate the residual ACE-inhibitory activity in digested hazelnut paste, simulated in vitro gastrointestinal digestion tests were performed based on the recommendations of INFOGEST [32].

For the oral stage, 5 g of sample and 3.5 ml of simulated salivary fluid (SSF) were mixed and the mixture was homogenized with a lab blender (EB 8011, Waring, USA). Alpha-amylase solution prepared in SSF was added to the mixture (0.5 ml, 290 U ml^{-1}). Finally, 25 µl of 0.3 M $CaCl_2$ and 975 µl of distilled water were added to this mixture and all reagents were kept stirred for 10 min.

For the gastric stage, 10 ml of oral bolus was mixed with 7.5 ml of simulated gastric fluid (SGF). Pepsin stock solution (1.6 ml, 25,000 U mg^{-1}), 5 µl 0.3 M $CaCl_2$, 0.2 ml 1 M HCl and 695 µl distilled water were added to this mixture. The pH was adjusted to 3.0 and the samples were kept stirred for 2 h.

For the intestinal phase, 11 ml of simulated intestinal fluid (SIF) (68856, Fluka Analytical) was added to 20 ml of gastric mixture. Pancreatin was prepared in SIF solution at a trypsin activity level of 800 U ml^{-1} and was also added to the previous mixture. In addition, 2.5 ml of 160 mM freshly prepared bile, 40 µl of 0.3 M $CaCl_2$ and 0.15 ml of 1 M NaOH and 1.31 ml of distilled water were added to this mixture and medium pH was adjusted to pH 7. The mixture was kept stirred for 2 h. Immediately afterwards, the enzymatic reaction was stopped by transferring the samples to a boiling water-bath (appox. 100 °C) for 15 min. Digested samples were filtered through coarse filter paper and centrifuged at 4000 × *g* (4 °C, 30 min). Finally, the supernatants were passed through a CA syringe filter (0.22 µm) and stored at − 20 °C until further analyses.

The ACE-inhibitory activity of digested samples was measured as detailed above in the "ACE-Inhibitory Activity Tests" section. The ACE-inhibitory activity in the digested hazelnut paste with no hydrolysates was evaluated as an additional control and the sample mean for ACE-inhibitory activity in the digested hazelnut paste was

subtracted from the activity data for peptide bearing samples to ensure that only hydrolysate components accounted for the measured ACE-inhibitory activity.

Statistical Analysis

The data collected in the current investigations were reported as sample means ± standard deviations based on at least duplicate experiments with 2 replications each. Whether differences existed between various treatments were studied based on statistical significance ($p < 0.05$).

Results and Discussion

Fractionation of Peptides and Determination of ACE-Inhibitory Activity

The degree of hydrolysis (%DH) for the hydrolysates prepared using papain, bromelain, or pepsin treatments were determined using the TNBS assay [24]. %DH values were found to be 60.97 ± 0.2% for papain hydrolysates, 46.18 ± 0.1% for bromelain hydrolysates, and 74.33 ± 0.4% for pepsin hydrolysates, respectively. Consequently, pepsin treatment was found to yield the most intense degradation. These values indicated significantly higher degradation than the enzymatic treatments used in our previous studies [20], which in part can be attributed to changes in proteolysis protocols.

Papain, bromelain or pepsin hydrolysates of hazelnut protein isolates were fractionated using an FPLC system and 10 fractions were collected in each case (Fig. 1). The chromatograms were distinctly different in all cases indicating large differences in composition and concentration of peptides per each fraction and hydrolysate. The main reason for the formation of different profiles for each proteolytic treatment is due to the structural and functional differences of the enzymes. Regardless of the enzyme used, temperature, hydrolysis time and enzyme:protein ratio are considered as important factors in the production of peptides with ACE-inhibitory activity [33–35].

Following fractionation, average % ACE inhibitory activity values of enzymatic hydrolyzates and their fractions were measured (Table 1). In most cases, the hydrolyzate samples and the out fractions were characterized with the highest % ACE-inhibitory activity. This finding can in part be attributed to the relatively high concentration of peptides in the unfractionated hydrolysate and unbound materials.

Because of the important roles of ACE in the regulation of blood pressure, its inhibition has been utilized as a method to treat hypertension [36]. The IC_{50} value is the amount of inhibitor required to inhibit 50% of ACE-activity under experimental conditions [37]. Due to their importance, IC_{50} values of the current samples were determined for peptides with the highest % ACE-inhibitory activity in order to compare the findings with the current literature. The IC_{50} for captoril was calculated as 4.9E − 5 μg ml^{-1}, whereas the lowest IC_{50} values were attained for the PAF8, BRF3, PEF3 and PEF6 fractions (6.05, 20.79, 27.42, and 1.97 μg ml^{-1}, respectively). Although the ACE-inhibitory activity of the fractions was not as high as that of the positive control, natural ACE-inhibitor peptides have a potential for being utilized in the prevention of hypertension and in the initial treatment of mildly hypertensive individuals [38]. Since the fractions have demonstrated a wide range of ACE inhibitory activities, the most potent fractions in terms of ACE-activity and ease of manufacture were further collected and utilized in industrial trials.

In many studies, ACE-inhibitor peptides produced from food samples have been investigated as an alternative to synthetic drugs. For example, the IC_{50} value was found to be 25.67 μg ml^{-1}, for WPERPPQIP (1033.42 Da) peptide obtained from walnut proteins [39]. The IC_{50} value of VSGARY (708 Da) which was formed as a result of thermolysin treatment of bitter melon seed protein, was calculates as approx. 6.12 μg ml^{-1} [33]. The present findings were comparable to the results present in the current literature.

LC-Q-TOF/MS Analysis of the Hydrolysates

After ACE-inhibitory activity was carried out, bioactive fractions were collected and sequence analysis was carried out based on LC-Q-TOF/MS. In the current protein databases, for example, in UniProt, more than 400 proteins related to hazelnuts (*Corylus avellana* L.) are listed. Following enzymatic proteolysis, it is reasonable to assume thousands of different peptides formed in each hydrolysate with varying compositions and bioactivities. In the current MS analysis, the sequence information was derived as exhaustively as possible for the ionizable peptides in all fractions of proteolytic hydrolysates. The elucidated sequences and their corresponding molecular weights along with their calculated isolectric points and bioactive potential based on their PeptideRanker values and BIOPEP A and B parameters for ACE-inhibitory activity [30] were listed on Table S1 in Supplementary Data section.

A total of 83 different peptide spectra were elucidated in ACE-inhibitory fractions and the majority of the identified peptides originated from the "out" fractions (53 peptides). While some of these sequences were common with the subsequent fractions, 30 of the identified peptides belonged solely to the weakly anionic fractions (approx. 36%).

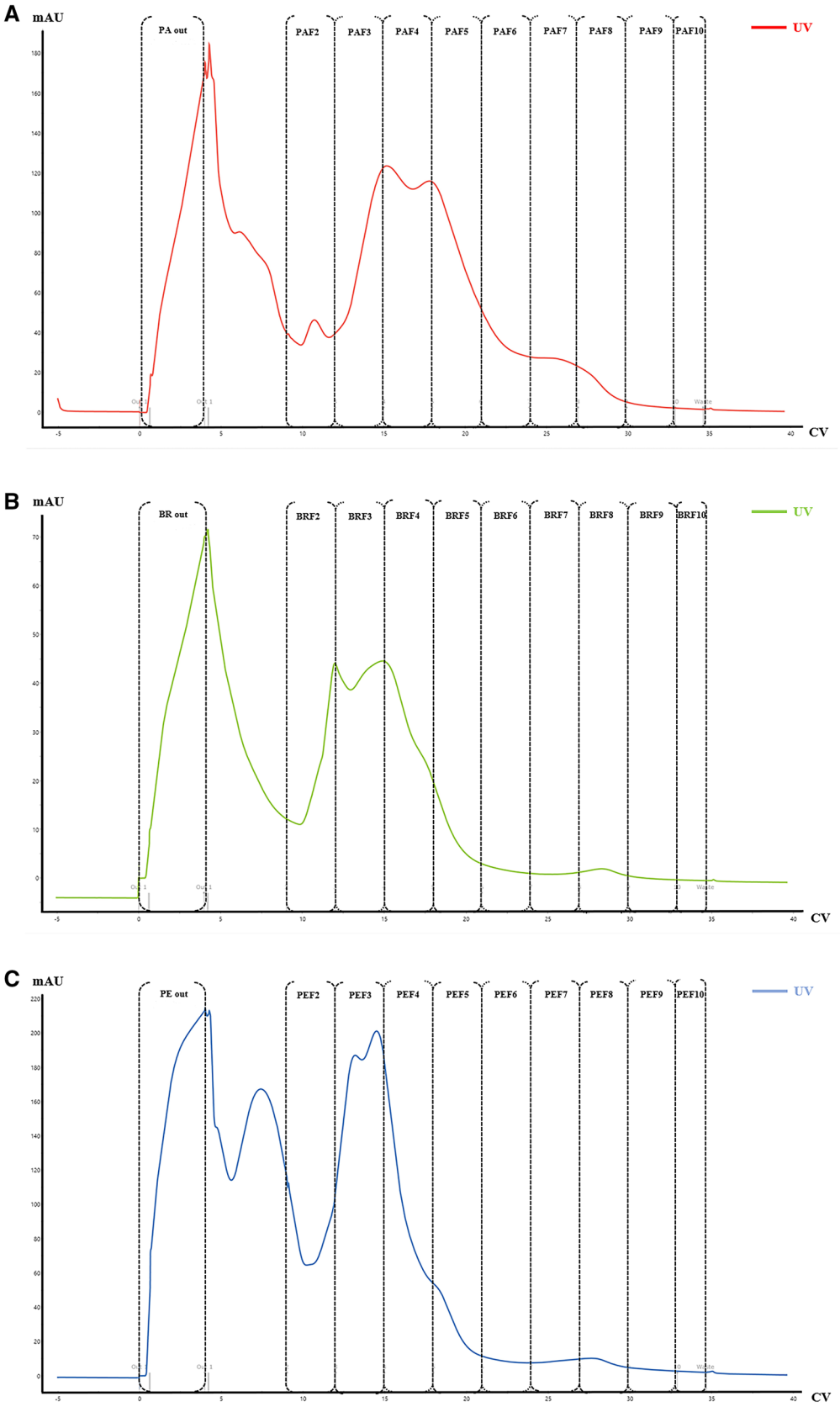


Fig. 1 FPLC chromatograms for **A** papain, **B** bromelain, and **C** pepsin treated hazelnut protein hydrolysates

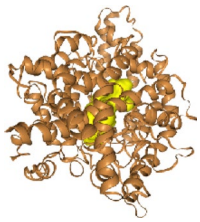
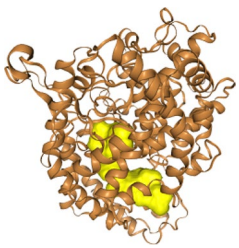
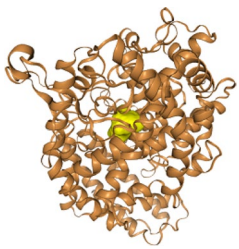
Table 1 Average % ACE-inhibitory activity values of total hazelnut protein hydrolyzate and its corresponding fractions obtained by enzymatic proteolysis treatments

Sample	% ACE inhibitory activity—PAF	% ACE inhibitory activity—BRF	% ACE inhibitory activity—PEF
Total hydrolyzate	98.62 ± 0.2	86.98 ± 0.1	98.47 ± 0.1
Out	93.13 ± 0.1	63.00 ± 0.3	96.42 ± 0.1
F2	21.88 ± 0.1	29.88 ± 0.1	28.79 ± 0.1
F3	23.63 ± 0.1	30.04 ± 0.1	40.97 ± 0.6
F4	18.52 ± 0.1	11.69 ± 0.1	15.77 ± 0.1
F5	6.75 ± 0.1	2.66 ± 0.0	33.95 ± 1.5
F6	0.53 ± 0.1	1.95 ± 0.1	32.52 ± 0.0
F7	15.86 ± 0.1	9.54 ± 0.0	21.45 ± 0.1
F8	22.34 ± 0.1	27.53 ± 0.1	25.81 ± 0.2
F9	21.05 ± 0.0	29.64 ± 0.1	28.74 ± 0.1
F10	4.81 ± 0.0	18.25 ± 0.1	31.18 ± 0.1

BR, PA and PE represent bromelain, papain and pepsin treated hydrolysates, respectively

F fraction

Table 2 Docking scores and visual representation of the predicted protein-peptide interactions between ACE and potentially ACE-inhibitory peptides based on HPEPDOCK analysis

Sequence	Docking parameter (best match)	Visual representation
LEPTNRIEA	− 163.872	
IQVNKENKEFK	− 188.424	
VPP (Reference peptide)	− 97.004	

In Silico Analysis of the Identified Sequences

As detailed on Table S1, none of the identified hazelnut peptides were found to be toxic agents in silico [28]. As anticipated, "out" fractions were mostly found to demonstrate higher pI values compared to the later fractions. However, the majority of the peptides (51) had a pI value of ≤ 7, while approx. 79% was characterized by a predicted pI value < 8. A number of high pI peptides were also observed in the column bound fractions. Since the majority of the current peptides were not listed in peptide databases, some of the current findings pointed out to the identification of novel proteins and/or peptides. All peptides listed on Table S1 were different than the 3 novel ACE-inhibitory peptides (namely AVKVL, YLVR and TLVGR) purified by Liu et al. [40] from Asian hazelnuts (*Corylus heterophylla* Fisch.). Furthermore, Bioactive Peptide Database (BioPepDB) [41] was scanned for the sequences identified in the current study. Currently (13/01/2022), BioPepDB lists 6103 bioactive peptides, mostly based on various food sources. None of the current sequences matched the peptides listed in this database supporting our hypothesis that the current peptides were novel.

Based on PeptideRanker evaluation (Table S1), active fractions were demonstrated to contain both active and inactive peptides. In bromelain treated fractions, 9 different peptides demonstrated a PeptideRanker score of > 0.5. Similarly, 2 papain and 5 pepsin-treated peptides were expected to be bioactive according to their PeptideRanker values. In total, potentially bioactive peptides accounted for approx. 19.3% of the identified peptides (*i.e.*, 16 out of 83). Corresponding ACE-inhibition parameters (A and B) were also listed on Table S1.

The sequences that were verified in silico via PeptideRanker and BIOPEP tools as ACE-inhibitory were studied for their interactions with human ACE using a molecular docking approach [31]. The mechanism of interaction between ACE and the peptide with a PeptideRanker score > 0.5 and the most significant ACE-inhibitory parameters (i.e., A/B ratio based on BIOPEP calculations) were considered for each proteolytic treatment (Table 2). Namely, LEPTNRIEA (from BRF2), and IQVNKENKEFK (from PA out) peptides were analyzed. Since FEDPQQQSQQGQRQGQGQSQRSEQDRHQKIRHFREGDIIA (from PEF2) is a much longer peptide, and BIOPEP analysis predicted that its trypsinolysis would generate a variety of smaller peptides (namely, FEDPQQQSQQGQR-QGQGQSQR-SEQDR-HQK-IRHFR-EGDIIA), this peptide was removed from the molecular docking analysis.

Based on the most probable model, all 3 potentially ACE-inhibitory peptides were predicted to bind to human ACE and yield relatively high docking scores and hence, potentially induce inhibition. For comparative purposes,

ACE-inhibitory VPP peptide [42] was investigated as a reference peptide that has already been exploited in commercial products and its docking score was compared to the current peptides (Table 2). In all cases, hazelnut peptides and VPP were predicted to generate significant binding to human ACE, whereas hazelnut peptides generated higher docking scores than VPP, which demonstrated their potential in ACE-inhibition.

Simulated Gastrointestinal Digestion of Bioactive Peptide Bearing Hazelnut Paste and Determination of Residual ACE-Inhibitory Activities

Fractions demonstrating significant ACE-inhibitory activity and high lyophilization efficiency were prepared at relatively large quantities to be utilized in industrial trials. Consequently, they were added to hazelnut paste based on the procedures of the supporting company. Firstly, bioactive peptide fraction bearing hazelnut paste was manufactured under industrial settings and to ensure their microbiological safety, a variety of microbiological tests were carried out. The microbiological tests demonstrated that there was no significant depreciation in the microbial quality of the products upon bioactive peptide inclusion (data not shown) and the findings were coherent with the internal procedures and domestic regulations [43]. Hence, inclusion of bioactive peptide lyophilizates was a safe food manufacture practice.

Immediately afterwards, simulated in vitro gastrointestinal digestion tests were conducted to examine the ACE-inhibitory properties of the digested hazelnut paste. The results of the ACE-inhibitory activity tests were summarized on Table 3. First of all, captopril, the positive control, demonstrated significant ACE-inhibitory activity in the hazelnut paste matrix. In this experimental set, digestion of hazelnut paste with no hydrolysates was utilized as an additional control. The ACE-inhibitory activity in the digested hazelnut paste with no hydolysates was minimal (< 4%, in all cases). The sample mean was approx. $2.7 \pm 0.9\%$. However, the sample mean was subtracted from the activity data for peptide bearing samples to ensure that only hydrolysate components accounted for the measured ACE-inhibitory activity.

Table 3 % ACE-inhibitory activity values of bioactive peptide fraction bearing (1%) hazelnut paste products after in vitro simulated gastrointestinal digestion

Added fraction	Average % ACE-inhibitory activity
PAF4	94.21 ± 0.2
BRF10	94.84 ± 0.4
PEF6	94.82 ± 0.3
Captopril	95.26 ± 0.4

Captopril (1%) was used as a positive control

Hence, the influence of digestion on the ACE-inhibitory activity of the control hazelnut paste was relatively small, but was taken into account in Table 3.

ACE-inhibitory activities of the PAF4, BRF10, and PEF6 fractions (1%) were measured and found to be similar to the positive control group (Table 3). This observation may be due to solubility characteristics of peptides vs captopril in the matrix and other molecular interactions. Ingredients used in product formulations and manufacturing processes have the potential to affect ACE-inhibitory activity of proteins and peptides [44, 45].

It is noteworthy that while the majority of ACE-inhibitory activity should be attributed to the intact peptides, it is still likely that some of the peptides present in the PAF4, BRF10 or PEF6 fractions could have been hydrolyzed by the digestive enzymes in these trials. Consequently, the eventual ACE-inhibitory activity could be a summation of the activities of intact and hydrolyzed peptides. Although the current data still warrants that a significant extent of bioactivity is preserved after the digestion of industrially manufactured hazelnut paste, it was necessary to analyze the potential influence of peptide hydrolysis on the overall ACE-inhibitory activity of digested hazelnut paste.

Therefore, in silico trypsinolysis (BIOPEP, EC 3.4.21.4) was utilized as a rapid prediction tool to investigate this hypothesis. All of the current peptides with a length of ≤ 10 amino acids were tested for this purpose ($n = 33$). Consequently, 22 of these 33 peptides were predicted to remain intact, whereas 11 peptides (i.e., 33.3%) were predicted to undergo hydrolysis at least at 1 spot. Only 2 sequences were characterized by 2 different cleavage steps (FMRWRDRFL, EDRRTDFA). Since these results represent tripsinolysis only, complete enzymatic digestion process could account for a higher extent of hydrolysis.

Based on these findings, current peptides present in the digested paste could be anticipated to undergo a significant extent of enzymatic proteolysis, which in turn could affect their overall composition and ACE-inhibitory characteristics. Basically, intact and further hydrolyzed peptides co-existed in the digested paste and partial hydrolysis of the peptides potentially enhanced ACE-inhibitory attributes.

Influence of Proteolysis on Suppression of Allergenicity

Using an ELISA assay, the presence of hazelnut allergen proteins was tested in peptide bearing hazelnut paste. Since hazelnuts were heavily added (i.e., 70%) to the paste, the measured concentration of hazelnuts was very high in the control sample (567,500 mg kg^{-1}). While the other 2 fractions were not significantly influential on the results, fraction named as BRF10 was characterized by a significant reduction (i.e., approx. 7172 times reduction) in the detection of

Table 4 Docking scores of the predicted protein-peptide interactions between Major pollen allergen Cor a 1 and BRF peptides based on HPEPDOCK analysis

Peptide No.	Sequence	Docking parameter (best match)
1	LYVPHWNLNA	– 205.276
2	NQLDENPRHFY	– 186.998
3	QIESWDHNDQQFQCAG	– 175.719
4	NNVFSGFDA	– 170.401
5	IRALPDDVLA	– 161.997
6	EDRRTDFA	– 137.841
7	MAISDMPESTG	– 162.337
8	CPETFEDPQQQSQQG	– 142.093
9	LEPTNRIEA	– 157.912

allergenic hazelnut proteins. While the nature of this finding and also whether it would translate to actual results in vivo need to be clarified, the interactions between hazelnut allergens and peptides generated herein might inhibit the detection of allergens. These findings definitely warrant further investigations, while a brief effort was made here to study the interactions between one of the major hazelnut allergens (Major pollen allergen Cor a 1) [46] and BRF peptides (Tables 4 and S2). The molecular docking results demonstrated significant interactions between BRF peptides and Cor a 1 potentially elucidating the suppression mechanism (Table 4). Schematic predictions were demonstrated on Table S2. While potential suppression or alleviation of allergenic responses by peptide materials remain a major area of study for future investigations, some literature on allergy treating or preventing peptides have been previously published [47].

Conclusion

In this study, innovative and value-added peptide fractions with potential antihypertensive properties were produced from cold pressed hazelnut cake (*Corylus avellana* L.) and utilized in the manufacture of industrial hazelnut paste.

Unlike synthetic drug molecules, peptide fractions obtained from food are usually a mixture of peptides as in the current study, which in turn could largely affect their bioactivity. Microbiological analysis demonstrated no harmful effects upon the inclusion of freeze-dried peptide fractions in hazelnut paste. Furthermore, as a result of the allergenic activity tests, the allergic suppression was observed to take place in some of the samples. In addition, bioactive peptide bearing digested hazelnut paste was characterized with a significant extent of ACE-inhibitory activity at relatively high peptide concentrations (1%). Sensory, textural and rheological properties of final products deserve further evaluation, while further in vivo and/or clinical tests remain to be critical.

Current findings demonstrated that hazelnut cakes/meals have a great potential as a natural source of bioactive peptides. While preliminary steps have been taken for the commercialization of bioactive peptide fractions in food processing, long-term use of the such peptides in other consumer products such as cosmetics, pharmaceutical products and biomedical applications need further refinement.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12649-022-01750-9.

Acknowledgements Hazelnut cakes were donated by Neva Foods. The authors would like to thank Prof. Dr. Ahmet Tarık Baykal and Ms. Emel Akgün for their technical support at Labmed.

Funding This study was funded by a Grant from TÜBİTAK 1001 Program, Turkey (Grant No. 217O063).

Data Availability This manuscript has data included as electronic supplementary material.

Declarations

Conflict of interest The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Kearney, P.M., Whelton, M., Reynolds, K., Muntner, P., Whelton, P.K., He, J.: Global burden of hypertension: analysis of worldwide data. Lancet **365**(9455), 217–223 (2010). https://doi.org/10.1016/S0140-6736(05)17741-1
2. Boye, J.I., Roufik, S., Pesta, N., Barbana, C.: Angiotensin I-converting enzyme inhibitory properties and SDS-PAGE of red lentil protein hydrolysates. LWT-Food Sci. Technol. **43**(6), 987–991 (2010). https://doi.org/10.1016/j.lwt.2010.01.014
3. Tenenbaum, A., Grossman, E., Shemesh, J., Fisman, E.Z., Nosrati, I., Motro, M.: Intermediate but not low doses of aspirin can suppress angiotensin-converting enzyme inhibitor-induced cough. Am. J. Hypertens. **13**(7), 776–782 (2000). https://doi.org/10.1016/S0895-7061(00)00268-5
4. Abdel-Hamid, M., Otte, J., De Gobba, C., Osman, A., Hamad, E.: Angiotensin I converting enzyme inhibitory activity and antioxidant capacity of bioactive peptides derived from enzymatic hydrolysis of buffalo milk proteins. Int. Dairy J. **66**, 91–98 (2017). https://doi.org/10.1016/j.idairyj.2016.11.006
5. Sun, X., Chakrabarti, S., Fang, J., Yin, Y., Wu, J.: Low-molecular-weight fractions of alcalase hydrolyzed egg ovomucin extract exert anti-inflammatory activity in human dermal fibroblasts through the inhibition of tumor necrosis factor–mediated nuclear factor κB pathway. Nutr. Res. **36**(7), 648–657 (2016). https://doi.org/10.1016/j.nutres.2016.03.006
6. Lemes, A.C., Sala, L., Ores, J.D.C., Braga, A.R.C., Egea, M.B., Fernandes, K.F.: A review of the latest advances in encrypted bioactive peptides from protein-rich waste. Int. J. Mol. Sci. **17**(6), 950 (2016). https://doi.org/10.3390/ijms17060950

7. Otte, J., Shalaby, S.M., Zakora, M., Nielsen, M.S.: Fractionation and identification of ACE-inhibitory peptides from α-lactalbumin and β-casein produced by thermolysin-catalysed hydrolysis. Int. Dairy J. **17**(12), 1460–1472 (2007). https://doi.org/10.1016/j.idairyj.2007.04.008
8. Balakrishnan, B., Prasad, B., Rai, A.K., Velappan, S.P., Subbanna, M.N., Narayan, B.: *In vitro* antioxidant and antibacterial properties of hydrolysed proteins of delimed tannery fleshings: comparison of acid hydrolysis and fermentation methods. Biodegradation **22**(2), 287–295 (2011). https://doi.org/10.1007/s10532-010-9398-0
9. Harnedy, P.A., FitzGerald, R.J.: Bioactive peptides from marine processing waste and shellfish: a review. J. Funct. Foods **4**(1), 6–24 (2012). https://doi.org/10.1016/j.jff.2011.09.001
10. Ahn, C.B., Lee, K.H., Je, J.Y.: Enzymatic production of bioactive protein hydrolysates from tuna liver: effects of enzymes and molecular weight on bioactivity. Int. J. Food Sci. Technol. **45**(3), 562–568 (2010). https://doi.org/10.1111/j.1365-2621.2009.02166.x
11. Toopcham, T., Mes, J.J., Wichers, H.J., Roytrakul, S., Yongsawatdigul, J.: Bioavailability of angiotensin I-converting enzyme (ACE) inhibitory peptides derived from *Virgibacillus halodenitrificans* SK1-3-7 proteinases hydrolyzed tilapia muscle proteins. Food Chem. **220**, 190–197 (2017). https://doi.org/10.1079/BJN20041189
12. Siow, H.L., Gan, C.Y.: Extraction of antioxidative and antihypertensive bioactive peptides from *Parkia speciosa* seeds. Food Chem. **141**(4), 3435–3442 (2013). https://doi.org/10.1016/j.foodchem.2013.06.030
13. Garcia-Mora, P., Peñas, E., Frías, J., Gomez, R., Martinez-Villaluenga, C.: High-pressure improves enzymatic proteolysis and the release of peptides with angiotensin I converting enzyme inhibitory and antioxidant activities from lentil proteins. Food Chem. **171**, 224–232 (2015). https://doi.org/10.1016/j.foodchem.2014.08.116
14. Yan, Q.J., Huang, L.H., Sun, Q., Jiang, Z.Q., Wu, X.: Isolation, identification and synthesis of four novel antioxidant peptides from rice residue protein hydrolyzed by multiple proteases. Food Chem. **179**, 290–295 (2015). https://doi.org/10.1016/j.foodchem.2015.01.137
15. Xie, N., Huang, J., Li, B., Cheng, J., Wang, Z., Yin, J., Yan, X.: Affinity purification and characterisation of zinc chelating peptides from rapeseed protein hydrolysates: possible contribution of characteristic amino acid residues. Food Chem. **173**, 210–217 (2015). https://doi.org/10.1016/j.foodchem.2014.10.030
16. Iwaniak, A., Minkiewicz, P., Darewicz, M.: Food-originating ACE inhibitors, including antihypertensive peptides, as preventive food components in blood pressure reduction. Compr. Rev. Food Sci. Food Saf. **13**(2), 114–134 (2014). https://doi.org/10.1111/1541-4337.12051
17. Turkish Dept. of Agriculture and Forestry: Agricultural product markets: hazelnuts (In Turkish). (2021). https://arastirma.tarimorman.gov.tr/tepge/Belgeler/PDF%20Tar%C4%B1m%20%C3%9Cr%C3%BCnleri%20Piyasalar%C4%B1/2021-Ocak%20Tar%C4%B1m%20%C3%9Cr%C3%BCnleri%20Raporu/F%C4%B1nd%C4%B1k,%20Ocak-2021%20Tar%C4%B1m%20%C3%9Cr%C3%BCnleri%20Piyasa%20Raporu.pdf
18. Turkish Food Composition Database: "Hazelnut, blanched" (2021). http://www.turkomp.gov.tr/food-findik-ic-beyazlatilmis-407, Accessed on 28 Jun 2021
19. Liu, C., Fang, L., Min, W., Liu, J., Li, H.: Exploration of the molecular interactions between angiotensin-I-converting enzyme (ACE) and the inhibitory peptides derived from hazelnut (*Corylus heterophylla* Fisch.). Food Chem. **245**, 471–480 (2018). https://doi.org/10.1016/j.foodchem.2017.10.095
20. Çağlar, A.F., Çakır, B., Gülseren, İ: LC-Q-TOF/MS based identification and *in silico* verification of ACE-inhibitory peptides in Giresun (Turkey) hazelnut cakes. Eur. Food Res. Technol. **247**(5), 1189–1198 (2021). https://doi.org/10.1007/s00217-021-03700-6
21. Li-Chan, E.C.: Bioactive peptides and protein hydrolysates: research trends and challenges for application as nutraceuticals and functional food ingredients. Curr. Opin. Food Sci. **1**, 28–37 (2015). https://doi.org/10.1016/j.cofs.2014.09.005
22. Coşkun, Ö., Çakır, B., Vahapoğlu, B., Gülseren, İ: Influence of extraction conditions on structural and functional characteristics of black cumin protein concentrates and ACE inhibition in their hydrolyzates. J. Food Meas. Charact. **13**(3), 2328–2338 (2019). https://doi.org/10.1007/s11694-019-00152-1
23. Gülseren, İ, Corredig, M.: Storage stability and physical characteristics of tea-polyphenol-bearing nanoliposomes prepared with milk fat globule membrane phospholipids. J. Agric. Food Chem. **61**(13), 3242–3251 (2013). https://doi.org/10.1021/jf3045439
24. Adler-Nissen, J.: Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J. Agric. Food Chem. **27**(6), 1256–1262 (1979). https://doi.org/10.1021/jf60226a042
25. Nielsen, P.M., Petersen, D., Dambmann, C.: Improved method for determining food protein degree of hydrolysis. J. Food Sci. **66**(5), 642–646 (2001). https://doi.org/10.1111/j.1365-2621.2001.tb04614.x
26. Sinha, R., Radha, C., Prakash, J., Kaul, P.: Whey protein hydrolysate: functional properties, nutritional quality and utilization in beverage formulation. Food Chem. **101**(4), 1484–1491 (2007). https://doi.org/10.1016/j.foodchem.2006.04.021
27. Sheih, I.C., Fang, T.J., Wu, T.K.: Isolation and characterization of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide from the algae protein waste. Food Chem. **115**(1), 279–284 (2009). https://doi.org/10.1016/j.foodchem.2008.12.019
28. Gupta, S., Kapoor, P., Chaudhary, K., Gautam, A., Kumar, R., Raghava, G.P.: Open source drug discovery consortium.: in silico approach for predicting toxicity of peptides and proteins. PLoS ONE **8**(9), e73957 (2013). https://doi.org/10.1371/journal.pone.0073957
29. Minkiewicz, P., Iwaniak, A., Darewicz, M.: BIOPEP-UWM database of bioactive peptides: current opportunities. Int. J. Mol. Sci. **20**, 5978 (2019). https://doi.org/10.3390/ijms20235978
30. Mooney, C., Haslam, N.J., Pollastri, G., Shields, D.C.: Towards the improved discovery and design of functional peptides: common features of diverse classes permit generalized prediction of bioactivity. PLoS ONE **7**(10), e45012 (2012). https://doi.org/10.1371/journal.pone.0045012
31. Zhou, P., Jin, B., Li, H., Huang, S.Y.: HPEPDOCK: a web server for blind peptide–protein docking based on a hierarchical algorithm. Nucleic Acids Res. **46**(W1), W443–W450 (2018). https://doi.org/10.1093/nar/gky357
32. Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al.: A standardised static *in vitro* digestion method suitable for food–an international consensus. Food Funct. **5**(6), 1113–1124 (2014). https://doi.org/10.1039/C3FO60702J
33. Priyanto, A.D., Doerksen, R.J., Chang, C.I., Sung, W.C., Widjanarko, S.B., Kusnadi, J., et al.: Screening, discovery, and characterization of angiotensin-I converting enzyme inhibitory peptides derived from proteolytic hydrolysate of bitter melon seed proteins. J. Proteom. **128**, 424–435 (2015). https://doi.org/10.1016/j.jprot.2015.08.018
34. García, M.C., Endermann, J., Gonzalez-Garcia, E., Marina, M.L.: HPLC-Q-TOF-MS identification of antioxidant and antihypertensive peptides recovered from cherry (*Prunus cerasus* L.) subproducts. J. Agric. Food Chem. **63**(5), 1514–1520 (2015). https://doi.org/10.1021/jf505037p

35. Chiang, W.D., Tsou, M.J., Tsai, Z.Y., Tsai, T.C.: Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. Food Chem. **98**(4), 725–732 (2006). https://doi.org/10.1016/j.foodchem.2005.06.038
36. Coppey, L.J., Davidson, E.P., Rinehart, T.W., Gellett, J.S., Oltman, C.L., Lund, D.D., Yorek, M.A.: ACE inhibitor or angiotensin II receptor antagonist attenuates diabetic neuropathy in streptozotocin-induced diabetic rats. Diabetes **55**(2), 341–348 (2006). https://doi.org/10.2337/diabetes.55.02.06.db05-0885
37. Thewissen, B.G., Pauly, A., Celus, I., Brijs, K., Delcour, J.A.: 2011 Inhibition of angiotensin I-converting enzyme by wheat gliadin hydrolysates. Food Chem. **127**(4), 1653–1658 (2011). https://doi.org/10.1016/j.foodchem.2010.11.171
38. Li, X.X., Han, L.J., Chen, L.J.: *In vitro* antioxidant activity of protein hydrolysates prepared from corn gluten meal. J. Sci. Food Agric. **88**(9), 1660–1666 (2008). https://doi.org/10.1002/jsfa.3264
39. Liu, M., Du, M., Zhang, Y., Xu, W., Wang, C., Wang, K., Zhang, L.: Purification and identification of an ACE inhibitory peptide from walnut protein. J. Agric. Food Chem. **61**(17), 4097–4100 (2013). https://doi.org/10.1021/jf4001378
40. Liu, C., Yu, Y., Liu, F., You, L.: Purification and molecular docking study of Angiotensin-I converting enzyme (ACE) inhibitory peptide from alcalase hydrolysate of hazelnut (*Corylus heterophylla* Fisch) protein. Food Nutr Sci **10**(11), 1374–1387 (2019). https://doi.org/10.4236/fns.2019.1011098
41. Li, Q., Zhang, C., Chen, H., Xue, J., Guo, X., Liang, M., Chen, M.: BioPepDB: an integrated data platform for food-derived bioactive peptides. Int J Food Sci Nutr **69**(8), 963–968 (2018). https://doi.org/10.1080/09637486.2018.1446916
42. Yamamoto, N., Akino, A., Takano, T.: Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. J. Dairy Sci. **77**(4), 917–922 (1994). https://doi.org/10.3168/jds.S0022-0302(94)77026-0
43. Anon. Regulation on Turkish Food Codex microbiological criteria, Law of Authorization: 5996. Official Gazette of Publication: 29.12.2011–28157 (2011)
44. Akıllıoğlu, H.G., Karakaya, S.: Effects of heat treatment and *in vitro* digestion on the angiotensin converting enzyme inhibitory activity of some legume species. Eur. Food Res. Technol. **229**(6), 915–921 (2009). https://doi.org/10.1007/s00217-009-1133-x
45. da Costa, E.L., da Rocha Gontijo, J.A., Netto, F.M.: Effect of heat and enzymatic treatment on the antihypertensive activity of whey protein hydrolysates. Int. Dairy J. **17**(6), 632–640 (2007). https://doi.org/10.1016/j.idairyj.2006.09.003
46. Pastorello, E.A., Vieths, S., Pravettoni, V., Farioli, L., Trambaioli, C., Fortunato, D., et al.: Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. J. Allergy Clin. Immunol. **109**(3), 563–570 (2002). https://doi.org/10.1067/mai.2002.121946
47. Hafner, R.P., Laidler, P., Larche, M.: Peptides for desensibilization against allergens. EP 2083856 B1 (2010)