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ORIGINAL PAPER

LC-Q-TOF/MS based identification and in silico verification of ACE-inhibitory peptides in Giresun (Turkey) hazelnut cakes

Ahmet Furkan Çağlar[1] · Bilal Çakır[2,3] · İbrahim Gülseren[1,4]

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Abstract

Hazelnut (*Corylus avellana* L.) cakes represent are a rich source of proteins. In an effort to valorize industrially cold-pressed hazelnut cakes, angiotensin-converting enzyme (ACE) inhibitory characteristics of hazelnut protein hydrolysates were evaluated. Trypsin, chymotrypsin and thermolysin hydrolysates of hazelnut protein isolates were fractionated using Fast Protein Liquid Chromatography (FPLC). The hydrolysate fractions were tested for ACE inhibitory characteristics and inhibitor peptide identification was based on Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-Q-TOF/MS). Finally, in vitro ACE inhibitory activity was verified using in silico tools. In all cases, some hydrolysate fractions demonstrated ACE inhibitory characteristics, while inhibitory activity widely varied (7–95%) depending on proteolysis conditions. In ACE inhibitory fractions, 179 different peptides were identified. Their potential inhibitory activity was verified in silico for 22 different peptides generated by thermolysin, 3 for chymotrypsin and 4 for trypsin. While the half maximal inhibitory concentration (IC_{50}) (0.13–4.83 mg ml^{-1}) values were comparable to the previous literature, currently identified sequences were different than ACE inhibitory peptides purified from Asian hazelnuts. The peptides with the highest PeptideRanker scores for each treatment (i.e., SPLAGR, VPHW and PGHF) were studied for their potential ACE binding, Absorption, Distribution, Metabolism, Excretion and Toxicity (ADMET) and circulatory half-life characteristics demonstrating limited pharmokinetic interference, low toxicity as well as comparable short circulation and stronger binding compared to a reference inhibitor peptide (i.e., VPP). Geographical attributes and proteolytic treatments could have a bearing on ACE inhibitory potential of peptides, while hazelnut cakes can be regarded as a valuable resource for ACE inhibitor peptides.

Keywords Hazelnut protein hydrolysates · Hydrolysate fractions · Valorization · LC-Q-TOF/MS · Angiotensin-converting enzyme (ACE) inhibitory activity · Bioactive peptides

Abbreviations

ACE	Angiotensin-converting enzyme
ADMET	Absorption, Distribution, Metabolism, Excretion and Toxicity
FPLC	Fast protein liquid chromatography
IC_{50}	Half maximal inhibitory concentration
LC-Q-TOF/MS	Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry
TNBS	2,4,6-Trinitrobenzenesulfonic acid

Introduction

The World Health Organization (WHO) predicted that heart disease-related mortalities were to become the most common cause of death globally by 2020 [1]. Hypertension can be considered as one of the controllable risk factors associated with myocardial infarction, heart failure and renal failure [2]. Approximately 131 billion USD are being spent on hypertension-lowering drugs in the United States each year [2].

Consequently, anti-hypertensive peptides from animal or plant origin have been commonly investigated [3].

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

1 Department of Food Engineering, Faculty of Engineering and Natural Sciences, İstanbul Sabahattin Zaim University (İZÜ), 34303 Halkalı – Küçükçekmece, İstanbul, Turkey

2 Halal Food R&D Center, İstanbul Sabahattin Zaim University (İZÜ), Halkalı, Küçükçekmece, İstanbul, Turkey

3 Faculty of Pharmacy, Department of Biochemistry, Marmara University, Institute of Health Sciences, Başıbüyük, Maltepe, İstanbul, Turkey

4 İZÜ Food and Agricultural Research Center (GTAUM), Halkalı Campus, 34303 Küçükçekmece, İstanbul, Turkey

Various bioactive peptides have been obtained from food products, such as eggs, rice, spinach, and peas, and demonstrated varying levels of anti-hypertensive activity [4]. Bioactive peptides demonstrate high affinity to tissues with reduced likelihood of side effects compared to the synthetic drugs [1]. Studies on the discovery and utilization of cheaper and safer alternatives are being intensely carried out. Bioavailability of peptides, however, could be limited due to degradation in human body across the gastrointestinal tract. Furthermore in some cases, due to relatively large size or hydrophobicity of peptides, their ability to permeate through epithelium might be negatively affected [5, 6].

Angiotensin I-converting enzyme (ACE) is a critical enzyme in the renin–angiotensin system. ACE converts the angiotensin I hormone to angiotensin II, which is a vasoconstrictor. It also degrades the vasodilator peptide known as bradykinin. Both mechanisms potentially lead to increasing blood pressure in humans [7, 8], while ACE inhibitory peptides could prevent hypertension in humans.

Hazelnuts (*Corylus avellana* L.) are among the major agricultural products of Turkey, where approximately 80% of the global hazelnut harvest is collected (roughly 550,000 tonnes per year) [9]. The capacity of cold-pressed hazelnut oil production is increasing which generates increasing extents of deoiled hazelnut cake that can be valorized. Although the literature is rich on hazelnut consumption-related allergies including protein-based allergens [10–12], and there are some data on the technical functionality of hazelnut proteins [13, 14], the data on bioactive characteristics of hazelnut proteins [14] and their hydrolysates [15] are limited.

Based on the studies of Liu et al. [16], Asian hazelnut (*Corylus heterophylla* Fisch.) proteins were hydrolyzed using Alcalase and the most intensely ACE inhibitory peptides were reported to be AVKVL, YLVR and TLVGR, which mostly consisted of hydrophobic and basic residues. The latter two were characterized by a C-terminal basic residue (i.e., arginine), which is a common observation for many ACE inhibitory peptides.

In our recent studies, 23 hazelnut proteins were analyzed in silico for their bioactive characteristics and ACE inhibitory activity was predicted to be among the 2 most likely bioactivities in these hydrolysates [17]. Consequently, we conducted a preliminary study on the trypsinolysis of hazelnut protein concentrates which lead to moderate ACE inhibitory activity [18]. Here, enhancement of ACE inhibitory activities of hazelnut protein hydrolysates was targeted using multiple proteases with different catalytic capabilities and FPLC-based fractionation techniques for this commercially critical commodity (Giresun tombul hazelnuts).

Materials and methods

Materials

Cold-press de-oiled hazelnut (*Corylus avellana* L., Giresun, Turkey, "tombul" hazelnuts) cakes were provided by a local company (Neva Foods Ltd., İstanbul, Turkey). All chemicals were purchased from Sigma Chemical Corp.

Manufacture of hazelnut protein isolates

Protein extraction from cold-press de-oiled hazelnut cakes (approx. 50% protein as determined by a Dumas analysis technique) was based on the alkaline extraction-isoelectric precipitation (AE-IP) technique. Protein solubilization was facilitated via the increased surface charges of proteins at basic pH, which was followed by the isoelectric precipitation at low pH as previously described [19].

Proteolytic hydrolysis

Trypsin, chymotrypsin and thermolysin were separately used for enzymatic hydrolysis of hazelnut proteins isolates (approx. 94.8% protein as determined by a Dumas analysis technique). Aqueous dispersions of proteins (20 mg ml^{-1}) were prepared in appropriate 20 mM Tris–HCl buffer solutions. Proteases were added to protein solutions at an enzyme:substrate ratio of 1:100 for trypsin and chymotrypsin (pH 7.4), and 1:50 for thermolysin (pH 8) [20]. The enzymatic process was continued overnight at a mixing rate of 1000 rpm at 37 °C. Immediately afterwards, trypsin and chymotrypsin hydrolysates were inactivated at 95 °C (5 min). For the inhibition of thermolysin, 0.5% formic acid was added to the hydrolysates. For the proteolytic hydrolysates, protein concentration was determined using a Lowry method-based technique [21].

Determination of degree of hydrolysis (% DH)

Degree of hydrolysis was determined based on the 2,4,6-Trinitrobenzenesulfonic acid (TNBS) method [22]. The leucine amino equivalency was determined based on Nielsen et al. [23].

Fractionation of hydrolysates

Hydrolyzed hazelnut proteins were fractionated using AKTA-Pure 25-L1 fast protein liquid chromatography (FPLC) system (GE Healthcare, UK). HiTrap Capto Q anion exchange columns were utilized for fractionation (GE Healthcare). Hydrolysate samples were injected into

the column at a rate of 1 CV min^{-1}. Salt containing 20 mM Tris–HCl buffer (0.6 M NaCl, pH 8.3) was used for the elution of the column-bound compounds. The elution was carried out utilizing a 0–0.6 M NaCl linear gradient over 32 CV. Each and every 2 CV was collected as a separate fraction and numbered sequentially. Prior to Capto Q experiments, a variety of other ion exchange (HiTrap DEAE FF, Capto DEAE, Capto-S) and hydrophobic interaction (HiTrap Phenyl FF, HiTrap Butyl-S FF, HiTrap Octyl FF) columns were tested in pre-trials.

Measurement of ACE inhibitory activity

ACE inhibition in fractions was calculated as a function of the decrease in hippuric acid (HA) in comparison to the control sample and % inhibition values were calculated [24]. Briefly, salt concentrations of peptide fractions in 20 mM Tris–HCl buffer were adjusted to contain approximately 0.6 M NaCl. 1.68 mU of 250 µl ACE and 250 µl peptide fractions prepared in sodium borate buffer (0.1 M, pH 8.3) were mixed and pre-incubated in the thermomixer for 5 min to ensure thermal equilibrium at 37 °C. After the incubation, 3.94 mM HHL (15 µl) was added to the mixture and the reaction was continued for 1 h. Immediately afterwards, 1 M HCl (500 µl) was added to the mixture to stop the reaction. % ACE inhibition was monitored at 228 nm by injecting 10 µl of this mixture directly into the HPLC column (Ascentis C18 Column, 4.6 mm ID × 250 mm, 5 µm particle diameter, Supelco). TFA (0.1%) prepared in 50% methanol was used as the mobile phase. The isocratic flow rate was 0.6 ml per min and 20 mM Tris–HCl buffer containing 0.6 M NaCl was used as the control. In addition, captopril, a well-known ACE inhibitor, was used as a positive control at a concentration level of 0.05 µM.

Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS) analysis of ACE inhibitory fractions

All MS analyses were 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 analyses 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.

In silico analyses

The determined peptide sequences were analyzed in silico for their physicochemical and bioactive characteristics [25–29] including isoelectric point, charge, and toxicity [25], probability of being bioactive [26, 27], the interactions with ACE [28] and ADMET (absorption, distribution, metabolism, excretion and toxicity) properties [29]. In silico verification of peptide bioactivity was based on the PeptideRanker score [27].

Consequently, the primary in silico tool utilized here was PeptideRanker to assess bioactive potential of peptides [27]. The main hypothesis behind the PeptideRanker algorithm is that assessing the general characteristics of bioactive peptides could assist in the elucidation of novel bioactive peptides based on the use of general predictors. Essentially, PeptideRanker score is based on structural homology between peptides. While PepSite2 method depends on the calculation of protein–peptide-binding characteristics from a set of known protein–peptide 3D structures, and distance constraints that were derived from previously characterized peptides [28]. ToxinPred combines machine learning and quantitative matrix modelling approaches and utilizes them along with the toxicity behavior previously characterized toxic and non-toxic sequences [25].

Isoelectric point, charge, and toxicity attributes of the peptides were investigated using the "Batch submission" tool on ToxinPred website (https://webs.iiitd.edu.in/raghava/toxinpred/multi_submit.php) [25]. PeptideRanker score for multiple peptides can be determined simultaneously on

http://distilldeep.ucd.ie/PeptideRanker website [26]. Potential ACE inhibitory activity of the peptides was also verified by BIOPEP "Calculations" tool (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) [27]. Using the appropriate PDB code for human ACE and potentially interacting peptide sequences, peptide-binding sites on ACE surface were determined (http://pepsite.russelllab.org/) [28]. ADMET tool on http://lmmd.ecust.edu.cn/admetsar1/predict/ was utilized to predict the ADMET properties of the potentially bioactive peptides [29].

Using a molecular docking approach, peptide–protein interactions were also studied via HPEPDOCK, which generated docking scores and 3D protein–peptide interaction images [30]. Finally, using PLIFEPRED, half-life of peptides in blood was predicted [31].

Statistical analysis

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

Results and discussion

Determination of degree of hydrolysis (%DH)

The degree of hydrolysis of hazelnut proteins treated with 3 different proteases was determined (Fig. 1). %DH for the reference sample (sodium caseinate) was previously measured to lie within the range of 42–62% depending on the experimental conditions [22]. Current findings were comparable to the previous literature. The %DH values for thermolysin hydrolysates of hazelnut protein isolates (50.4%) were significantly higher than the trypsin- or chymotrypsin-treated samples (26.4 and 21.8%, respectively).

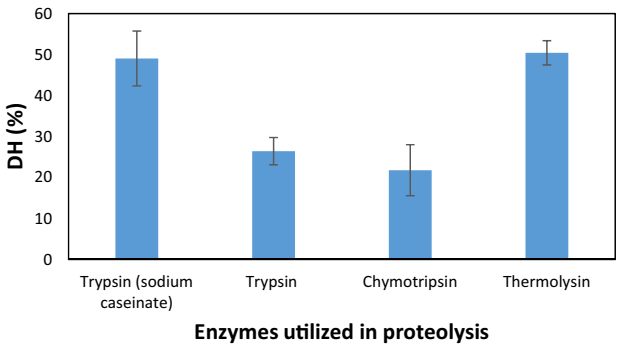

Fig. 1 Degree of hydrolysis (DH%) for hazelnut meal proteins hydrolyzed by various proteases as determined by the TNBS method

These findings can be explained by the relatively higher proteolytic activity of thermolysin towards hazelnut proteins as also predicted by in silico methods [17]. Here, trypsin demonstrated lower proteolytic activity towards hazelnut proteins compared to sodium caseinate. The extent of proteolysis is highly dependent on structural attributes of proteins [32], including the intramolecular distribution of amino acids and relative abundance of secondary structural motifs. In the previous literature, ACE inhibitory activity of hydrolysates were related to hydrolysis duration, demonstrating a direct relationship between ACE inhibitory activity and %DH [18, 19, 33]. Furthermore, ACE inhibitory peptides tend to be relatively shorter compared to other categories of bioactive peptides, such as antimicrobial peptides [34]. Based on Lowry analysis, the approximate protein concentration in the samples was determined to range between 1.20 and 5.80 mg/100 g. However, it must be noted that peptide composition determines ACE inhibitory activity rather than the peptide size. Furthermore, the current literature on, for example, partially fermented milk products, where %DH was relatively low (i.e., 9 to 18%) demonstrated significant ACE inhibition [35].

Fractionation of hydrolysates based on anion exchange chromatography

Hazelnut protein hydrolysates were fractionated using an anion exchange method, prior to which a variety of ion exchange (HiTrap DEAE FF, Capto-Q, Capto DEAE, Capto-S) and hydrophobic interaction (HiTrap Phenyl FF, HiTrap Butyl-S FF, HiTrap Octyl FF) columns were tested in pre-trials (data not shown). Since the binding rate was found to be high, the elution was straightforward and the obtained fractions did not require any pretreatments in the activity tests, Capto-Q was used in the following steps. It is noteworthy that in a variety of recent studies on food bioactive peptides, ACE inhibitory peptides were found to bear charged residues along with hydrophobic residues (for example, [16, 36]). Consequently, ion exchange chromatography was a valid approach to fractionate the hazelnut protein hydrolysates.

First, to test the influence of column volume on fractionation, 2 different sizes of the same column (1 and 5 ml) were examined (Figure S1 of Supplementary Data). As seen in Figure S1, significantly different elution profiles and potentially different hydrolysate compositions were acquired based on the proteolytic enzyme used and analytical column volume. Each and every 2 column volumes (CV) were collected as a separate fraction. Peptides that did not bind to the column were labelled as the "out" fraction. Consequently, 17 different fractions with varying compositions were prepared for each enzymatic treatment (Figure S1).

Measurement of ACE inhibitory activity

Hydrolysate fractions were evaluated in ACE inhibitory activity tests as exemplified on Figure S2. % ACE inhibitory activity values of the fractions that generated significant ACE inhibition (> 0%, $p < 0.05$) were presented in Fig. 2 as a function of both column bed volume and protease utilized. Due to the faster fractionation in the 5 ml column, it was possible to obtain fractions that exhibited ACE inhibition in earlier elution volumes. Consequently, total number of ACE inhibitory fractions in 5 ml column was less than the 1 ml counterpart, while higher inhibitory activities were observed (Fig. 2). In both cases, the number of ACE inhibitory thermolysin fractions was higher than (6 and 4 fractions, respectively, for Fig. 2a and b) that in trypsin or chymotrypsin fractions. The fraction with the highest inhibitory activity was also a thermolysin fraction (E1 (out), 95% inhibition) (Fig. 2b). The majority of the ACE inhibitory fractions contained weakly anionic or cationic peptides, the relatively earlier fractions and "out" fractions, respectively. Similar results were obtained by Liu et al. [16], where catalase was utilized in the hydrolysis of Asian hazelnut proteins. On average, IC_{50} values for the current fractions ranged between 0.13 and 4.83 mg protein ml^{-1} (Table 1), which were mostly comparable to earlier findings of Eroglu and Aksay [15] (0.22–0.29 mg protein ml^{-1} in the hydrolysates vs. 1.29 mg protein ml^{-1} in the protein isolates). Similarly, the IC50 data for Liu et al. [16] corresponded approximately to 0.01–0.13 mg ml^{-1}. While, captopril is a highly potent synthetic ACE inhibitor with a low IC50 value (0004 mg ml^{-1}), various proteolytic hydrolysates of sea cucumber indicated an IC50 value range of approx. 1.7–2.54 mg ml^{-1} [37]. A variety of inhibitor peptides with lower and higher IC50 values were also reviewed in the literature [38].

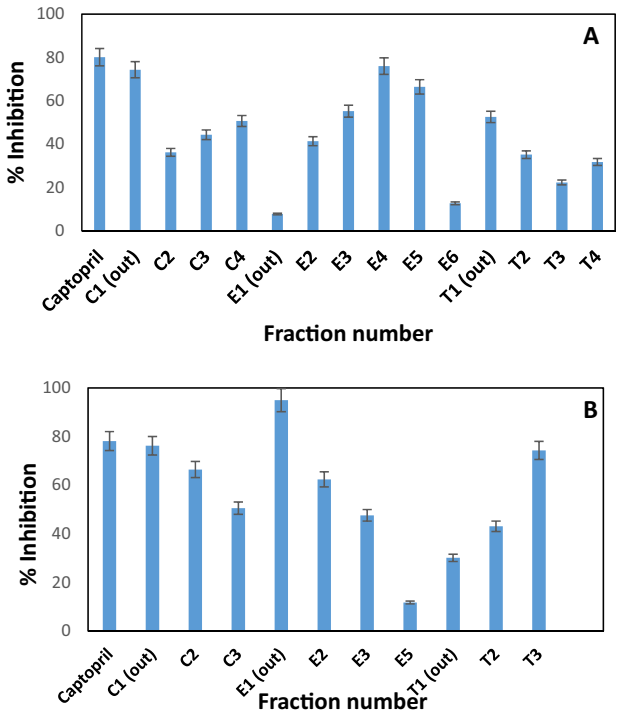

Fig. 2 ACE inhibitory activity (%) of peptide fractions hydrolyzed by various proteases and fractionated by a Capto Q (**a**) 1 ml, or (**b**) 5 ml column connected to an FPLC instrument. *C, E, T* Chymotrypsin, thermolysin and trypsin hydrolysate fractions, respectively. *Out* Unbound fractions

LC-Q-TOF/MS analysis of the hydrolysates

Fractions demonstrating ACE inhibitory activity were collected and analyzed via appropriate LC-Q-TOF/MS analysis techniques. First, in the literature (for example, UniProt database), there are more than 400 proteins that are related to *Corylus avellana* L. and after enzymatic proteolysis, possibly thousands of different peptides formed with various sizes and activity potentials. Here, we made an attempt to clarify the structures of all ionizable peptides. As detailed in the Supplementary Data section, each fraction was characterized with various peptides at different concentrations and since the concentration of each peptide is unknown, it was not possible to calculate an effective molecular weight for the fractions. However, an average molecular weight was calculated for all treatments and listed on Tables S1–S3.

A total of 179 different peptide spectra found in ACE inhibitory fractions and only a single spectrum is shown here as an example (Figure S3). The sequences of all determined peptides are listed in Supplementary Data section (Tables S1–S3) for all the 3 enzyme treatments along with their calculated physicochemical characteristics and bioactive potential based on their PeptideRanker values. The majority of the identified peptides originated from the "out" fractions (147 peptides), whereas 32 belonged to the weakly anionic fractions.

Table 1 IC_{50} (mg/ml) values for the FPLC fractions based on the 5 ml Capto Q column

Fraction	IC_{50} (mg ml^{-1})
C1 (out)	0.19
C2	0.24
C3	0.37
E1 (out)	0.13
E2	0.26
E3	0.41
E5	2.94
T1 (out)	0.82
T2	0.48
T3	0.20

In silico analysis of the identified sequences

As detailed on Tables S1–S3, none of the identified hazelnut peptides were found to be toxic agents in silico [25]. As anticipated, "out" fractions were mostly found to demonstrate higher pI values compared to the later fractions. However, the majority of the peptides (53.6%) had a pI value of ≤ 7, while approx. 61% was characterized by a predicted pI value < 8. A number of high pI peptides were also observed in the column-bound fractions. These findings are of practical interest, since cationic peptides could demonstrate various bioactivities including anti-microbial activity [34]. 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 Tables S1–S3 were different than ACE inhibitory peptides (namely AVKVL, YLVR and TLVGR) purified by Liu et al. [16] from Asian hazelnuts (*Corylus heterophylla* Fisch.).

Based on PeptideRanker evaluation (Tables S1-S3), active fractions were demonstrated to contain both active (i.e., ACE inhibitory) and inactive peptides. In thermolysin-treated fractions, 22 different peptides demonstrated a PeptideRanker score of > 0.5. Similarly, 3 chymotryptic and 4 tryptic peptides were expected to be bioactive according to their PeptideRanker values. In total, potentially bioactive peptides accounted for approx. 16.2% of the identified peptides (i.e., 29 out of 179). Similar to our earlier findings [17], non-gastrointestinal thermolysin was found to be more efficient in the generation of ACE inhibitory peptides from hazelnut proteins compared to trypsin and chymotrypsin, both in terms of the number of inhibitory peptides generated (Tables S1–S3), and the extent of in vitro ACE inhibition (%) (Fig. 2).

In the next set of investigations, in silico verified ACE inhibitory peptides were studied for their interactions with human ACE based on Pepsite2 methodology [28]. The mechanism of interaction between ACE and the peptide with highest PeptideRanker score for each proteolytic treatment was examined and a sample figure was presented (Figure S4). Namely SPLAGR, VPHW and PGHF peptides were analyzed. PeptideRanker is a respectable tool that has been utilized as a prediction tool in a number of studies on the evaluation of bioactive food peptides (for example, [36, 39]). Table 2 summarizes the amino acids in these 3 peptides that could potentially interact with ACE. In all cases, the binding models were shown to be statistically significant and their potential ACE inhibitory activity was also verified by BIOPEP "Calculations" tool [27] (data not shown).

Based on the most probable model, SPLAGR tryptic peptide was predicted to yield 16 potential binding points (i.e., amino acids) on the ACE molecule (Table 2) and hence, potentially induce inhibition. Since human ACE consists of 1306 amino acids, the interaction between a certain peptide and ACE molecule being limited to only specific 16 amino acids may be classified as a relatively "specific" interaction. For comparative purposes, ACE inhibitory VPP peptide [40] was investigated as a reference and predicted to bind 14 different amino acids on ACE molecule. IPP and VPP are possibly the most intensely investigated bioactive food peptides. They are lactotripeptides generated from milk casein. They have been studied in many clinical studies in addition to in vitro experiments [41–43]. Furthermore, some commercial products (i.e., food supplements) that include these peptides in their formulations are currently available. These peptides could also form in situ in partially fermented dairy products. Consequently, they are valuable food components that can be regarded as a reference point for novel food peptides. In that sense, we have utilized VPP as a reference molecule for in silico predictions. Among the potentially VPP bound amino acids, 11 of them were predicted to be common with hazelnut peptides listed on Table 2, while 3 of them (bold on Table 2) were specific to VPP. Similarly, chymotryptic VPHW and thermolysin generated PGHF could interact with 10 and 11 amino acids, respectively, of ACE, potentially leading to inhibition (Table 2).

For the peptides listed on Table 2 and their potential binding sites on ACE, a brief in silico analysis was carried out to determine pI values and molecular charges (Table 3). In these calculations, ACE amino acids were treated as an intact peptide. The findings demonstrated that VPP and its corresponding binding sites had similar pI and differences

Table 2 List of the active amino acids on current sequences that potentially interact with ACE and comparison of the potential binding sites

Sequence	Active amino acids	*p* value	Potentially bound amino acids on the ACE molecule
SPLAGR	Pro-2 Leu-3 Ala-4 Gly-5 Arg-6	0.0001473	Trp279, Gln281, His353, Ala354, His383, Glu384, Glu 411, Asp415, Phe457, Phe460, Lys511, His513, Tyr520, Tyr523, Ser526, Phe527
VPHW	Val-1 Pro-2 His-3 Trp-4	3.959E-05	Gln281, His353, Ala354, His383, Glu411, Asp415, Lys511, His513, Tyr520, Tyr523
PGHF	Pro-1 Gly-2 His-3 Phe-4	2.263E-05	Gln281, His353, His383, Glu384, His387, Glu411, Phe457, Lys511, His513, Tyr520, Tyr523
VPP	Val-1 Pro-2 Pro-3	2.951E-06	Gln281, His353, His383, **His387, His410**, Glu411, **Ala412**, Asp415, Phe457, His513, Tyr520, Tyr523, Ser526, Phe527

VPP peptide was included as a positive control

Table 3 pI values and corresponding charge pairs for the bioactive peptides that potentially interact with ACE and their potential binding sites on the ACE molecule

Sequence	pI value	Corresponding charge	pI value for potentially bound ACE amino acids	Corresponding charge
SPLAGR	10.11	1	5.76	− 0.5
VPHW	7.1	0.5	6.29	0.5
PGHF	7.1	0.5	5.76	− 0.5
VPP	5.88	–	6.17	0.5

ACE inhibitory VPP peptide was included here as a positive control

Table 4 Molecular docking scores for the most probable ACE-inhibitor for each and every protease treatment along with the VPP peptide vs human ACE

Rank	Docking score			
	VPP	VPHW	PGHF	SPLAGR
1	− 96.288	− 202.233	− 192.080	− 179.023
2	− 95.176	− 197.225	− 192.059	− 178.591
3	− 92.827	− 197.045	− 190.518	− 177.795
4	− 91.325	− 196.942	− 190.407	− 177.455
5	− 91.268	− 196.027	− 190.313	− 174.295
6	− 90.473	− 194.653	− 188.750	− 174.087
7	− 90.455	− 193.983	− 187.640	− 172.810
8	− 89.591	− 193.341	− 187.408	− 172.625
9	− 89.312	− 192.846	− 187.271	− 172.512
10	− 89.203	− 192.718	− 185.796	− 171.870

in molecular charges were small, consequently, the electrostatic interactions were relatively less influential. In the current case, the differences between pI and charge values for the 3 peptides and their potential binding sites on ACE were more pronounced, especially for the tryptic SPLAGR peptide (Table 3). Consequently, in the current samples, electrostatic interactions may have a stronger influence on the ACE inhibitory activity.

Molecular docking

Using a molecular docking approach, peptide–protein interactions were also studied via HPEPDOCK, which generated docking scores and 3D protein–peptide interaction images [30]. The docking scores for the interactions between the current potentially bioactive peptides and human ACE were summarized on Table 4. In HPEPDOCK, the first model energy scores are the best out of the energy scores obtained from 100 different models related to Protein–Peptide interactions. VPP peptide achieved an energy score of − 96.288 in HPEPDOCK, while VPHW, PGHF and SPLAGR peptides were assigned energy scores of − 202.333, − 192.080 and − 179.023, respectively. Based on these docking scores, the current peptides were predicted to demonstrate a stronger interaction with ACE compared to the VPP peptide. In addition, Figure S5 demonstrated the regions of interaction for all cases, which were visibly different in all cases.

ADMET analysis and half-life of the peptides

ADMET properties of the most probable ACE inhibitory peptides listed on Table 2 were studied in silico [29]. In terms of absorption characteristics (Table 5), tryptic SPLAGR, chymotryptic VPHW, and thermolysin generated PGHF peptides could not penetrate the blood–brain barrier (BBB) or demonstrate Caco-2 permeability. However, VPHW and PGHF could be absorbed in the human intestinal absorption (HIA) system.

The major peptide transporters expressed in Caco-2 include HPT1 and PepT1 and it was shown that with alteration to the cell model or origin, the level of expression could be tailored [44]. Following oral administration, the major peptide transporters in the human body include PepT1,

Table 5 Critical ADMET parameters for the most probable ACE-inhibitor for each and every protease treatment

		SPLAGR	VPHW	PGHF
Absorption	Blood–Brain barrier	–	–	–
	Human intestinal absorption	–	+	+
	Caco-2 permeability	–	–	–
Metabolism	CYP450 2C9 substrate	Non-substrate	Non-substrate	Non-substrate
	CYP450 2D6 substrate	Non-substrate	Non-substrate	Non-substrate
	CYP450 3A4 substrate	Non-substrate	Substrate	Non-substrate
	CYP450 1A2 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 2C9 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 2D6 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 2C19 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 3A4 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
Toxicity	Carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens

PepT2, PhT1, and PhT2 [45]. While Pept1 is a low-affinity, high-capacity transporter that is predominantly expressed in the intestine, Pept2 is a high-affinity, but low-capacity transporter that has a broader tissue distribution predominantly in the kidneys. Pept1 was described as the carrier responsible for the uptake of di- and tripeptides in the small intestine. Previously some ACE inhibitors were shown to be transported via Pept1, whereas the specificities of certain peptides could affect their transportation characteristics with Pept1 or Pept2 [45]. In addition, since the 2 of 3 peptides studied here include a histidine residue (H), PhT1 or PhT2 might also be relevant for their uptake.

In terms of metabolic characteristics (Table 5), these three peptides were mostly not substrates or inhibitors for Cytochrome P450 (CYP450) enzymes. The only exception was that chymotryptic VPHW could potentially serve as a substrate for CYP450 3A4 enzyme. Cytochrome P450 (CYP 450) is a hemeprotein that plays a key role in the metabolism of drugs and other xenobiotics [46]. As indicated in the previous literature, there might always be a certain likelihood that small peptides or peptide drugs might result in CYP inhibition which in turn could lead to drug–drug interactions and side effects [47]. Based on these information, current findings could imply a slight potential to generate interactions with other xenobiotics.

Finally, none of the peptides were classified as carcinogens by the admetSAR system [29]. Based on these findings, the hydrolysates generated from hazelnut protein isolates are well-tolerable, potentially non-toxic, non-carcinogenic components which do not or minimally affect the usual metabolism or the pharmokinetics of medicinal components. Meanwhile, intestinal uptake seems to be possible in the human body.

In addition to ADMET characteristics, potential lifespan of the bioactive peptides is also critical in the circulatory system. Hence, the half-life of these three peptides in blood was predicted in silico via PLIFEPRED [31]. Based on the predictions, the half-lives of the current peptides and the reference were found to be approx. 834, 981, 834 and 837 s for PGHF, SPLAGR, VPHW and VPP, respectively. Essentially, the half-life of all 4 peptides was reasonably comparable and they can all be classified as relatively short-lived peptides.

Discussion

To summarize the findings, in the current work, a variety of hazelnut protein hydrolysate fractions bearing weakly anionic and cationic sequences were generated and analyzed for ACE inhibitory potential. SPLAGR peptide listed on Table 2 consisted of 50% hydrophobic, 16.67% basic and 33.33% neutral (i.e., 2 in 6) residues. For VPHW, the sequence was characterized by 75% hydrophobic and 25% basic (i.e., 1 in 4) residues. For PGHF, 50% of the residues in the sequence were hydrophobic, and 25% each were basic and neutral residues, respectively. Consequently, while all three were considerably hydrophobic, there was some positive charge in all cases, which suggested electrostatics could potentially influence ACE inhibitory activity.

Hydrophobic characteristics and/or positive charge of C-terminal amino acid is highly influential on the activities of small ACE inhibitory peptides (i.e., $\leq$6 amino acids), whereas possibly due to the steric effects, C-terminal is less influential for larger peptides [48]. Previously, positively charged C-terminal lysine or arginine residues in casein based peptides were shown to contribute to ACE inhibition [49]. The three small peptides studied here were characterized by C-terminal arginine, tryptophan and phenylalanine residues. Two of these residues are potentially non-charged and non-polar residues, while one has polar and basic characteristics (arginine). The data acquired by Liu et al. [16] on Asian hazelnuts also demonstrated that ACE inhibitory peptides mostly consisted of hydrophobic and basic residues. Here, while anion exchange was utilized, the presence of apolar and/or basic residue bearing peptides primarily enhanced the ACE inhibition potential.

The findings pointed out to the low toxicity, non-carcinogenicity and short circulation attributes of hazelnut peptides along with their potentially strong binding with human ACE. The effectiveness (IC50) of these peptides was comparable to various foods resources, while relatively short circulation durations remain to be a major problem for many unmodified food peptides including the current hydrolysates.

Conclusion

Based on simple aqueous extraction techniques, hazelnut protein isolates can be generated in a fashion that can be scaled up to industrial settings. Current investigations predicted that hazelnut protein hydrolysates were well-tolerable, potentially non-toxic, and non-carcinogenic. The peptides identified here were not identical to the peptides generated from Asian hazelnuts [16, 50].

Although purification of bioactive peptides generates high-activity products, here, we have effectively demonstrated that multiple fractions in the enzymatic hydrolysates possessed ACE inhibitory characteristics. In terms of manufacture costs in commercial applications, such as functional foods and food supplements, we think that less purified fractions or in some cases, a total hydrolysate is more likely to be exploited. While further work on synthesis of the currently identified peptides and their re-evaluation is inarguably necessary, MS-based identification of hazelnut peptides could lead the path to the design of functional agents that can be utilized in functional foods, food supplements and pharmaceutical formulations. Unless stabilized through

encapsulation, molecular complexation, conjugation or other means of stabilization including molecular modification, many food bioactive peptides could bear various digestive and/or circulatory stresses in the human body, which in turn could reduce their effectiveness. This problem remains to be an under-investigated issue in the food bioactive peptides including that of hazelnuts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Compliance with ethics requirements This article does not contain any studies with human or animal subjects.

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