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Hazelnut peptide fractions preserve their bioactivities beyond industrial manufacture and simulated digestion of hazelnut cocoa cream

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ABSTRACT

Cold press hazelnut cakes represent a concentrated source of proteins that can be industrially exploited. Previously, bioactive attributes of hazelnut protein hydrolysates including antihypertensive and antidiabetic activities were documented. Here, we made an attempt to utilize bioactive hazelnut protein hydrolysates (1 % w/w) in the manufacture of industrial hazelnut cocoa cream and investigate their stability through processing and simulated gastrointestinal digestion. The inclusion of bioactive peptide fractions was a safe practice in the microbiological sense. Proteolysis lowered the potential allergenicity of hazelnut proteins in the cocoa cream products up to about 20 %. *In silico* trypsinolysis predicted partial degradation for 51.8 % of the peptide sequences (i.e., 43/83) that were present in the hydrolysates. However, partial degradation and mixing of degraded vs non-degraded peptides preserved and/or further elevated bioactive attributes in the digested cocoa cream products in terms of Angiotensin converting enzyme (ACE)-inhibitory (up to about 92 %) and antidiabetic activities (between 7.5 and 44.4 %). In most cases, however, antioxidative activity was < 10 %. While simulated *in vitro* digestion potentially influenced the bioactive attributes of protein hydrolysates, the influence of cocoa cream processing and food matrix were relatively limited for hydrolysate fractions and more pronounced for protein isolates. Hazelnut press cakes represent a significant resource for the generation and industrial utilization of bioactive peptides, which could preserve their bioactivity beyond industrial manufacture and digestion and lead to slightly reduced allergenicity.

1. Introduction

Plant protein products are becoming increasingly popular as alternatives to animal proteins (Stone et al., 2015). By-product streams discharged from plant food processing account for a protein concentration of 5–50 % (w/w) of the initial raw materials in many cases (Oreopoulou & Tzia, 2007), which may be further exploited in the manufacture of protein products. For example, in oilseed processing soybean, rapeseed, and canola meals are among the most densely accumulated by-product streams with an initial protein concentration of approx. 36–48 % (w/w) (Yun et al., 2018). Hazelnut meals or cakes contain comparable amounts of protein (35–45 % w/w) (Tatar et al., 2015).

Bioactive peptides are small dietary protein fragments made up mostly of 2–20 amino acid subunits with an average molecular weight of < 3 kDa (Chalamaiah et al., 2019; Sarmadi & Ismail, 2010). Due to their various activities, bioactive peptides derived from dietary proteins have enjoyed recent popularity. Enzymatic hydrolysis, fermentation, and gastrointestinal digestion are among the mechanisms that can be used in bioactive peptide manufacture (Chalamaiah et al., 2018). Bioactive peptides are characterized with various biological activities such as antihypertensive (Sutopo et al., 2020), antioxidative (Cotabarren et al., 2019), antimicrobial (Chandrashekar et al., 2020), antidiabetic (Castañeda-Pérez et al., 2021), and immunomodulatory (Yang et al., 2020) properties. While these beneficial functions largely depend on respective protein sources, processing attributes, sequence and amino acid composition, molecular weight and charge distribution, pH, and chemical treatments (de Castro & Sato, 2015), bioactive peptides have been used to formulate pharmaceuticals that could potentially treat chronic disorders including diabetes and hypertension (Xu et al., 2019).

Foods matrices are being subjected to high temperature and/or pressures, or other processing related stresses during industrial food manufacture. Consequently, bioactive peptides delivered through food

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matrices may be highly susceptible to destabilization during processing or storage, which in turn could alter their bioactive attributes (Udenigwe & Fogliano, 2017). For example, various dairy processing treatments lead to the formation of novel allergenic peptides or to the opposite situations (i.e., reduction of protein related allergenicity) (Fritsché, 2003). Similarly, digestive processes could alter the peptide profile of foods. The absorption of intact peptides, either in isolation or as part of a protein hydrolysate bears a potential for the successful oral usage of bioactive compounds (Chakrabarti et al., 2018).

Recently, a wide range of food-derived bioactive peptides or protein hydrolysates were reported to confer additional health benefits beyond their nutritional roles including antioxidant, ACE inhibitory (*i.e.*, antihypertensive), and antidiabetic activities (Jha et al., 2018; Rao et al., 2012). Antioxidant peptides act by inactivating reactive oxygen species, scavenging free radicals, chelating pro-oxidative transition metals, or boosting the activities of intracellular antioxidant enzymes. Hypertension is one of the key factors in the development of coronary heart disease, stroke, and heart and kidney failure, and it is one of the risk factor that globally lead to the highest fatality rates (Lewington et al., 2002). As a result, ACE-inhibitory peptides produced from natural sources are preferred for the prevention of hypertension. On the other hand, antidiabetic peptides primarily block dipeptidyl peptidase-IV (DPPP-IV) activity (Wang et al., 2019), while various bioactive peptides derived from foods have been shown to perform anti-diabetic properties by suppressing carbohydrases (i.e., alpha-amylase and alpha-glucosidase), increasing pancreatic insulin production, modulating satiety, and lowering glucose absorption rate from the stomach (Yan et al., 2019).

Hazelnut peptides have recently been shown to perform considerable antihypertensive and anti-diabetic activities *in vitro* and *in silico* (Gülseren, 2018; Çağlar et al., 2021A, B). Their industrial implementation remains to be studied. In the current study, hazelnut peptide fractions that demonstrated bioactive attributes such as antihypertensive, antidiabetic, and/or antioxidative properties were used in industrial hazelnut cocoa cream manufacture. The influence of processing treatments and simulated digestion on bioactive attributes were also tested.

2. Materials and methods

2.1. Materials

Cold press cake samples for Giresun tombul hazelnuts (*Corylus avellana* L.) were generously donated by a local manufacturer (Oneva, Neva Gıda ltd., İstanbul, Turkey). Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich (Schnelldorf, Germany), including proteolytic enzymes such as papain (P4762, 10 $U.mg^{-1}$ protein), bromelain (B4882, 3 $U.mg^{-1}$ protein), pepsin (P6887, 3200 $U.mg^{-1}$ protein), ACE (A6778), α-amylase (A0521), DPP-IV (Dipeptidyl Peptidase IV human, D4943), and α-glucosidase (G5003). Pancreatin was acquired from Bio Basic (porcine pancreas pancreatin with a trypsin activity of 625 $U.mg^{-1}$, PB0681).

2.2. Manufacture of protein isolates

The alkali extraction-isoelectric precipitation (AE-IP) method was used to prepare hazelnut protein isolates (Çağlar et al., 2021B). Hazelnut press cakes were mixed 1:15 (cake:water, w/v) with distilled water, and the medium pH was adjusted to pH 9.5 with 1 N NaOH. To ensure protein dissolution, the dispersion was kept stirred at room temperature for 1 h. Centrifugal separation was carried out at 11,000xg for 30 min (25 °C) (Himac CR22N, Hitachi, Japan). To enhance isoelectric precipitation of the solubilized proteins, supernatants were collected and medium pH was lowered to 4.5 using 1 N HCl. Immediately afterwards, centrifugation step was repeated. The precipitated proteins were collected and freeze-dried (TRS 2–2 V, Teknosem, İstanbul, Turkey) to generate lyophilized protein isolates, which were stored at −20 °C until further usage. The protein content of the protein isolates were determined based on the Kjeldahl technique (NMKL, 2003).

2.3. Enzymatic proteolysis

A thermomixer was used to carry out the enzymatic hydrolysis of protein isolates (MTC-100, MIULAB, China) under agitated conditions. Hazelnut protein isolates were hydrolyzed with papain, bromelain, and pepsin. The enzyme:substrate ratio was 1:100 (w/w) in all cases.

The mixture was prepared in 20 mM sodium phosphate buffer (pH 7) for papain treatments, and the reaction was carried out for 150 min at 1000 rpm (50 °C). Bromelain treatments were carried out in 30 mM sodium acetate buffer (pH 4.5) at 1000 rpm (45 °C for 150 min). Pepsin treatments were carried out in distilled water that was pre-set to pH 2 using 1 N HCl. Peptic proteolysis was continued for 18 h at 37 °C and 1000 rpm. To stop the enzymatic reaction, the microcentrifuge tubes holding the reaction mixture were immersed in a 95 °C water bath for 10 min and subsequently cooled in an ice bath. To remove the insoluble materials, the sample tubes were centrifuged at 5,000xg for 30 min (25 °C). Finally, polyvinylidene fluoride (PVDF) syringe filters (0.45 μm) were used to filter the supernatant (Millex-HV, Merck-Millipore, Germany). The hydrolysates were filtered and kept at −80 °C as necessary. The 2,4,6-Trinitrobenzenesulfonic acid (TNBS) method was used to evaluate the % degree of hydrolysis (%DH) (Adler-Nissen, 1979). The method of Nielsen et al. (2001) was used to calculate the leucine amino equivalency.

2.4. Fractionation of hydrolysates

The enzymatic hydrolysates were fractionated using a Fast Protein Liquid Chromatography (FPLC) system (ÄKTA pure 25 L, GE Healthcare Life Sciences, Sweden). In pre-trials, HiTrap Capto DEAE, HiTrap Capto Q, HiTrap Capto S, and HiTrap DEAE FF columns, all produced by the same manufacturer, were examined (data not shown). Weakly anionic HiTrap Capto DEAE (5 ml) columns were utilized in fractionation due to a positive signal intensity in the fractions. Twenty mM Tris-HCl (pH 8.0) and 20 mM Tris-HCl + 0.8 M NaCl (pH 8.0) were used as sample and elution buffers, respectively. The fractions were collected during a 25 column volume (CV) flow, which included a 0–100 percent linear gradient and a UV detector to measure absorbance (280 nm). In addition, medium pH, conductivity, and system pressure were recorded during the runs. The "out" fraction, which contained components that were not bound to the column, and 9 fractions numbered 2 to 10 were collected for each hydrolysate sample and stored at −20 °C until further analysis. In the following sections, fractions of papain, bromelain, and pepsin hydrolysates were referred to as PAF, BRF, and PEF, respectively.

2.5. Industrial hazelnut cream production

Industrial production of bioactive peptide bearing hazelnut cocoa cream was carried out at FİSKOBİRLİK in Giresun, Turkey, where manufacturing and product analysis was based on internal standards of the company. Flake samples accounting for a 180 g powder mix (cocoa, sugar, hazelnut puree containing particles < 40 μm and other powdered ingredients), approx. 10 g solid fat, 10 g liquid oil, 1 g lecithin, and 1 g aroma were kept stirred for 1 h to generate a mixture that contained 16 % (w/w) hazelnuts. Larger quantities were prepared using a similar composition. Following grinding, the conching process was carried out at 45–50 °C for 4 h to ensure the smoothness and creaminess of the final product and remove any residual moisture. Immediately afterwards, the finished mixture was filled into glass jars (350 g). Due to their significant bioactivity and superior lyophilization efficiency, 1 bioactive fraction generated by each protease (namely, PAF4, BRF10, and PEF6), was employed (1 % w/w) in the manufacture of hazelnut cocoa cream. Experimental controls included hazelnut protein isolate bearing control cocoa cream samples (CONTROL) and no peptide or protein added

control cocoa cream (BLANK) samples.

2.6. Analyses on hazelnut cocoa cream

A variety of analyses were carried out on the finalized cocoa cream products to ensure microbial stability, allergenic attributes and bioactivity.

2.6.1. Microbiological analysis

Microbiological analysis was carried out to ensure the microbiological safety of hazelnut cocoa cream 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 based on the internal procedures of the supporting company.

2.6.2. Indirect allergenicity testing

Indirect allergenicity studies were carried out at LTS Laboratory Services in İstanbul, Turkey, using a commercial ELISA kit (Ridascreen® FAST Hazelnut, R-Biopharm, Germany) and following the accredited laboratory's procedures (Turkish Accreditation Agency Accreditation No: AB-0351-T). This approach was chosen to detect hazelnut proteins in samples that may contain hazelnuts. The reduction in the extent of hazelnut detectability was attributed to the presence of an allergy suppressing treatment as previously established in Göksu et al. (2022) for hazelnut paste samples.

2.6.3. Simulated in vitro gastrointestinal digestion assays

Simulated *in vitro* gastrointestinal digestion experiments were performed based on the recommendations of INFOGEST (Minekus et al., 2014) to assess the residual bioactivities in digested hazelnut cocoa cream.

Ten grams of sample was mixed with 7 ml of simulated salivary fluid (SSF) for the oral stage, and the mixture was homogenized with a lab blender (EB 8011, Waring, USA). Alpha-amylase solution prepared in SSF was added to the mixture (1 ml, 290 U. ml^{-1}). Finally, 50 µl of 0.3 M $CaCl_2$ and 1.95 µl of distilled water were added and the mixture was stirred for 10 min.

Ten ml of oral bolus was combined with 7.5 ml of simulated gastric fluid (SGF) for the gastric stage. This mixture consisted of 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. The pH of the samples was adjusted to 3.0, and they were kept stirred for 2 h.

Eleven ml of simulated intestinal fluid (SIF) (68856, Fluka Analytical) was added to 20 ml of gastric mixture in order to prepare the intestinal phase. Pancreatin was prepared in SIF at a specific trypsin activity level (800 $U.ml^{-1}$) and mixed with the initial combination. Freshly made 160 mM bile (2.5 ml), 40 µl 0.3 M $CaCl_2$, 0.15 ml 1 M NaOH, and 1.31 ml distilled water were added to this mixture, and the medium pH was adjusted to pH 7. The final mixture was kept stirred for 2 h. The enzymatic reaction was then stopped by placing the samples in a boiling water bath (approx. 100 °C) for 15 min. The digesta were filtered and centrifuged at 4,000xg for 30 min at 4 °C. Finally, the supernatants were filtered with 0.22 µm cellulose acetate (CA) syringe filters and kept frozen at −20 °C until further analysis.

2.7. ACE-inhibitory activity test

In vitro ACE (Angiotensin converting enzyme)-inhibitory activities of the peptide fractions were measured based on the protocols of Sinha et al. (2007). ACE activity leads to the decomposition of Hippuril-His-Leu (HHL) substrate to His-Leu dipeptide and hippuric acid (HA). Calculation of % ACE inhibition was based on the changes in HA concentration in the presence of peptide treatments.

activity was examined at a specific salt concentration level (400 mM NaCl). Fifty µl of 6.5 mM HHL and 90 µl of peptide fraction sample produced in sodium borate buffer (0.1 M, pH 8.3) were combined and pre-incubated for 10 min using a thermomixer set to 37 °C and 1000 rpm. Following this procedure, 40 µl of ACE (25 $mU.ml^{-1}$) was added to the mixture, and the reaction was maintained for 1 h before being terminated by the addition of 1 M HCl (180 µl).

High performance liquid chromatography (HPLC) unit consisted of a degasser (DGU-20A5R), pump/solvent distributor module (LC20AD), auto sampler (SIL-20A HT), column furnace (CTO-10AS VP), and UV–vis detector (SPD-20A). An ODS-3 C18 column (250 × 4.6 mm, pore size 110 Å, particle size 5 µm, Phenemonex, USA) was utilized and 10 µl samples were injected directly into the column. ACE inhibition was measured at 228 nm (Sheih et al., 2009). The mobile phase was 0.1 % TFA (v/v) in 50 % methanol (v/v), and the isocratic flow rate was 1 ml. min^{-1}. The reference inhibitor was Captopril. This test was administered for all hydrolysate fractions, hazelnut protein isolates and blanks containing no peptides or proteins.

In addition, ACE-inhibitory activity of digested samples was also measured based on this method. For the digestion experiments, ACE-inhibitory activity in the digested hazelnut paste with no hydrolysates was evaluated as an additional control (DIGESTED BLANK) and the sample mean for ACE-inhibitory activity in the digested hazelnut paste control was subtracted from the activity data for peptide bearing samples to ensure that only hydrolysate components accounted for the measured ACE-inhibitory activity. Similar practices were also administered for protein isolate enriched samples (DIGESTED CONTROL) here and in the other bioactivity tests listed in Sections 2.8 and 2.9. Protein isolate related details will not be repeated for brevity. Finally, an enzymatic blank was administered in order to subtract any contributions from digestive enzymes.

In all experiments, the hydrolysates were prepared from 1 % hazelnut protein isolate dispersions (*i.e.*, 10 $mg.ml^{-1}$). However, in order to accurately describe half maximal inhibitory concentration (IC_{50}), several dilutions of the original dispersions were necessary down to approx. $\mu g.ml^{-1}$ levels for all bioactivity assays (*i.e.*, ACE inhibition, antidiabetic activity or antioxidative activity tests). After fractionation, the peptide concentration in every hydrolysate fraction was determined. Since the determination of IC_{50} values required the utilization of multiple samples, the fractions were lyophilized and reconstituted in order to generate 5 different concentrations of the lyophilizate that span a concentration range of 1 log (i.e., M to 10 M, where M represents the concentration of the most dilute sample). After the *in vitro* bioactivity assays for all 5 samples, IC_{50} values were determined. Similar protocols were also adopted for positive controls and 5 different concentrations were prepared from the corresponding stock reference.

2.8. Antidiabetic activity tests

The potential antidiabetic properties of the hazelnut peptide fractions were investigated using several antidiabetic activity tests based on *in vitro* enzyme inhibition (DPP-IV, alpha-glucosidase and alpha-amylase). In addition, these tests were administered for hazelnut protein isolates and blanks containing no peptides or proteins as well.

Furthermore, antidiabetic activity of digested samples was also measured based on this method. For the digestion experiments, antidiabetic activity in the digested hazelnut paste with no hydrolysates was evaluated as an additional control and the sample mean for antidiabetic 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 antidiabetic activity. Similarly, an enzymatic blank was administered in order to subtract any contributions from digestive enzymes.

2.8.1. DPP-IV inhibitory activity test

DPP-IV (Dipeptidyl peptidase IV) inhibitory activities of the peptide

fractions were determined based on the method of Nongonierma et al. (2018) with slight modifications. Fifty µl of fraction samples and 50 µl 0.8 mM substrate (Gly-Pro p-nitroanilide hydrochloride, Gly-Pro-pNA, G0513, Sigma-Aldrich) were mixed and pre-incubated for 10 min at 37 °C in a thermomixer set to an agitation rate of 1,000 rpm. The reaction was initiated by the addition of 100 µl DPP-IV (0.01 $U.ml^{-1}$) to the mixture. After 1 h, the proteolytic reaction was stopped by the addition of 200 µl of 1 M sodium acetate buffer (pH 4). The amount of pNA released from the substrate was measured based on absorbance at 405 nm (SP-3000 nano, Optima, Japan). The reference inhibitor was Diprotin A (Ile-Pro-Ile, I9759, Sigma-Aldrich). All reagents were prepared in 100 mM Tris-HCl buffer solutions (pH 8). % DPP-IV inhibition values were determined by comparing the sample absorbance values to the negative control samples.

2.8.2. Alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibitory activity of fractions were determined using the protocols presented in Kim et al. (2005), while the enzyme (1 $U.ml^{-1}$) and substrate (35 mM p-nitrophenyl glucopyranoside in methanol, pNPG, N1377, Sigma-Aldrich) concentrations were revised as necessary. Ten µl of alpha-glucosidase (1 $U.ml^{-1}$), 10 µl of hydrolysate sample, 20 µl of substrate, and 500 µl of phosphate buffer were mixed and incubated for 20 min at 37 °C. After the incubation period, the reaction was stopped by adding 2 ml of 0.1 M Na_2CO_3 to the reaction mixture and absorbance measurements were carried out immediately afterwards (405 nm).

2.8.3. Alpha-amylase inhibitory activity test

The method described by Johnson et al. (2011) was used with slight modifications. Fifty µl fraction samples were pre-incubated for 5 min at 20 °C with 100 µl alpha-amylase (2 $U.ml^{-1}$). After incubation, 100 µl of 1 % (w/v) starch (S2004, Sigma-Aldrich) dissolved in sodium phosphate buffer (0.02 M, pH 6.9) and boiled for 15 min was added to the enzyme-hydrolysate mix and the substrate bearing mixture was incubated for 15 min. Finally, using the identical buffer, 100 µl of 1 % (w/v) DNS (3,5-dinitrosalicylic acid, D0550, Sigma-Aldrich) was added to the mixture and the contents were placed in a boiling water bath for 15 min. Immediately afterwards, 350 µl of distilled water was added to the mixture and absorbance measurements were carried out (540 nm).

2.9. Antioxidant activity tests

The potential antioxidant properties of the peptide fractions were investigated using several antioxidative activity tests including ferric iron reducing antioxidant power (FRAP), iron chelating activity and superoxide anion radical scavenging activity assays. In addition, these tests were administered for hazelnut protein isolates and blanks containing no peptides or proteins as well.

Furthermore, antioxidative activity of digested samples was also measured based on this method. For the digestion experiments, antioxidant activity in the digested hazelnut paste with no hydrolysates was evaluated as an additional control and the sample mean for antioxidative 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 antioxidative activity. Similarly, an enzymatic blank was administered in order to subtract any contributions from digestive enzymes.

2.9.1. Determination of iron chelating activity test

The iron (Fe^{2+}) chelating capacity of the current hydrolysates were determined in accordance with the method of Ebrahimzadeh et al. (2009). Firstly, hydrolysate samples (0.5 ml) of various concentrations, 1.6 ml distilled water and 0.05 ml 2 mM $FeCl_2$ were mixed for 30 s. Afterwards, 0.1 ml 5 mM ferrozine was added to the mixture and it was kept incubated for 10 min at the ambient temperature. Absorbance of the Fe^{2+}-ferrozine complex was measured (562 nm) following incubation. In this test, EDTA was used as a positive control and the chelating capacities of the samples were compared to that of EDTA.

2.9.2. Ferric reducing antioxidant power (FRAP) test

The ability of the samples to reduce Fe^{3+} ions was measured according to the method detailed by Zhang et al. (2008) with slight modifications. Firstly, fraction samples (500 µl) were mixed with 1 % (w/v) potassium ferricyanide (500 µl) and heated in a 50 °C water bath for 30 min. Then 10 % (w/v) TCA (500 µl) was added and the mixture was centrifuged at 1,650xg for 10 min. Ferric chloride solution (0.1 % (w/v), 200 µl) was added to the supernatant and the mixture absorbance was monitored at 700 nm for the first 5 min. In this test, Trolox and BHT were used as positive controls.

2.9.3. Superoxide anion radical scavenging activity test

In this test, superoxide anion radical scavenging activities of the current hydrolysates were investigated based on the method of Marklund and Marklund (1974). Firstly, hydrolysate samples (0.2 ml) of various concentrations and 1.8 ml 50 mM phosphate buffer (pH 8.2) were mixed (25 °C, 20 min). After this step, 40 µl 45 mM pyrogallol in 10 mM HCl was added to the mixture. For the first 5 min, absorbance values were measured (320 nm) at ambient temperature. In this test, Trolox and BHT were used as positive controls and the autoxidation inhibitory capacity of the hydrolysates were compared with them.

2.10. Statistical analysis

The results of the current analyses were presented as sample means ± standard deviation based on at least 3 replicates each with two subsamples. Statistical significance was tested using ANOVA at 95 % confidence interval and Tukey's post-hoc test as necessary and appropriate. Sample mean values labelled with the same letter indicate no significant differences between the groups and/or treatments.

3. Results and discussion

3.1. Fractionation of hazelnut peptides

The protein content of the protein isolates were determined based on the Kjeldahl technique. The results demonstrated that hazelnut protein isolates contained approx. 94.8 ± 2.9 % protein (NMKL, 2003). After proteolysis, TNBS assay was utilized to measure the degree of hydrolysis (% DH) for hazelnut protein hydrolysates generated by papain, bromelain, or pepsin treatments (Adler-Nissen, 1979). In this assay, Npb parameter represents the nitrogen content of the peptide bonds of proteins that are in question. In Spellman et al. (2003), specific data for this definition was given as 123.3 mg/g protein for whey protein. In our particular case, the sequences for the peptides present in the hydrolysates were used to calculate Npb, followed by %DH. Based on the sequence information, Npb value was calculated as 111.2 mg/g protein which is reasonably comparable with whey proteins.

% DH values for papain, bromelain, and pepsin hydrolysates were determined to be 61 ± 0.2, 46.2 ± 0.1, and 74.3 ± 0.4 %, respectively. Consequently, pepsin treatment produced the most intense hydrolysis in hazelnut proteins. In some of the earlier studies on enzymatic proteolysis plant proteins, Alcalase and chymotrypsin (Famuwagun et al., 2021) or trypsin treatments (Olusegun & Emmanuel, 2019) were found to generate higher % DH values than pepsin treatments. These differences could be explained by various factors including variations in enzyme: substrate ratios, proteolysis conditions and specificities in enzymatic reactions (Ovissipour et al., 2009; Valencia et al., 2014). It is noteworthy that %DH values attained here were mostly comparable to or higher than the data presented in the above mentioned references.

All 3 hydrolysates were fractionated using an FPLC system supplied with an anion exchange column and 10 fractions were recovered in each case. All chromatograms were significantly different demonstrating

significant changes in peptide content and concentration per fraction and hydrolysate (Göksu et al., 2022). The corresponding peptide concentrations of all fractions were summarized on Supplementary Data section Fig. 1 (Figure S1). As further detailed in the following sections, necessary bioactivity tests were carried out for these fractions. One bioactive fraction (PAF4, BRF10, and PEF6) was collected from each hydrolysate, and used in hazelnut cocoa cream manufacture due to their significant bioactivities including ACE, and DPP-IV-inhibitory and antioxidative activities and high lyophilization efficiency. Since industrial trials require significant amounts of lyophilizates, the criteria to select fractions was based on both bioactive potential and freeze drying efficiency.

3.2. Bioactive attributes of the fraction

3.2.1. ACE-inhibitory activity of the fractions

As previously demonstrated in our previous studies (Çağlar et al., 2021A), hazelnut protein hydrolysates and their fractions were characterized with varying degrees of ACE-inhibitory activities (Göksu et al., 2022). The IC_{50} for Captoril was calculated as $4.9x\ 10^{-5}$ $\mu g.ml^{-1}$, while ACE inhibitory fractions were characterized with IC_{50} values of approx. 2–27 $\mu g.ml^{-1}$

These activities corresponded to varying levels of ACE-inhibition in papain (4–23 %), bromelain (1–30 %) and pepsin (15–41 %) hydrolysate fractions. Based on ACE-inhibitory activity data (Göksu et al., 2022), the majority of the pepsin fractions demonstrated pronounced activity. Pepsin is a protease which is characterized by a wide range of cleavage sites, including peptide bonds with aromatic or carboxylic amino acids. Pepsin will primarily cleave the C-terminal Phe and Leu amino acids, which are critical residues for the ACE-inhibitory capacity of peptides (Murray and FitzGerald, 2007; Sornwatana et al., 2015).

In the previous studies, the hydrolysates of the longan seed protein were separated into 4 ultrafiltration fractions, where the most ACE-inhibitory fraction (<3 kDa) was characterized with an IC_{50} of 250 $\mu g.ml^{-1}$ (Nuchprapha et al., 2020). Similarly, various ACE-inhibitory fractions were separated from protein hydrolysates of cottonseed (IC_{50}: 0.792 $mg.ml^{-1}$) (Gao et al., 2019), Qula casein (IC_{50}: 8.75 $\mu g.ml^{-1}$) (Lin et al., 2017), gram flour (IC_{50}: 11.4 $\mu g.ml^{-1}$) (Bhaskar et al., 2019) and macroalgae *Ulva rigida* (IC_{50}: 0.095 $mg.ml^{-1}$). In the present study, unpurified hydrolysates were utilized (i.e., hydrolysate fractions) and these fractions were shown to generate varying extents of activity in various bioactivity categories. While purified peptides may perform better than the hydrolysates, they are fairly expensive to prepare and industrially utilize. We believe that the current investigations provide useful information on the industrial exploitation of protein hydrolysates and their fractions.

3.2.2. Antidiabetic activity of the fractions

DPP-IV and alpha-glucosidase inhibitory activity data for the fractions were shown on Fig. 1. In the alpha-amylase inhibitory activity test, only PAF2 and BRF2 fractions lead to measurable activity (<8%) (data not shown). In the DPP-IV inhibitory activity test, the IC_{50} value for Diprotin A was 9.76 $\mu g.ml^{-1}$ (28.59 μM), while the IC_{50} values for the PAF2 and BRF9 fractions were calculated as 5.58 $\mu g.ml^{-1}$ and 0.07 $\mu g.ml^{-1}$, respectively. IC_{50} values for alpha-glucosidase inhibitory activity were calculated as 0.001 $\mu g.ml^{-1}$ for PAF10 and 0.657 $\mu g.ml^{-1}$ for PAF9. Consequently, considerable activity was observed in both assays. In the previous literature, for example, RVPSLM peptide isolated from egg white demonstrated the intense inhibitory activity against alpha-glucosidase (IC_{50}: 23.07 $\mu mol.L^{-1}$), while it was not equally influential against alpha-amylase (Yu et al., 2011). *Moringa oleifera* seed hydrolysate and fractions demonstrated concentration-dependent inhibition of alpha-amylase and alpha-glucosidase respectively, where IC_{50} values ranged between 0.172 and 1.312×10^{-3} $\mu g.ml^{-1}$.

3.2.3. Antioxidant activity of the fractions

Iron chelation activity, FRAP activity, and superoxide anion sequestration activity tests were performed on hydrolysate fractions in order to determine their antioxidative potential. Iron chelation and FRAP activity of the fractions were demonstrated in Fig. 2. The IC_{50} value for EDTA (i.e., positive control) was 61.82 $\mu g.ml^{-1}$ in the iron chelation activity test, while it was as low as 1.96 $\mu g.ml^{-1}$ for PEF6 and 19.40 $\mu g.ml^{-1}$ for PAF3, respectively. Similarly, IC_{50} value for the positive controls were calculated 9.64 $\mu g.ml^{-1}$ and 10.63 $\mu g.ml^{-1}$ for BHT and Trolox, respectively. Meanwhile IC_{50} values of 35.19 $\mu g.ml^{-1}$, and 24.91 $\mu g.ml^{-1}$ were attained for PAF4 and PAF5, respectively. The activities of PAF2, BRF2, BRF3, PEF2 and PEF3 fractions were relatively high (>20 %) in the superoxide anion sequestration activity test, while BRF4, PEF7, PEF8 and PEF9 showed a lower extent of (<4%) activity (data not shown).

3.3. Manufacture of functional hazelnut cocoa cream and its simulated digestion

Lyophilized fractions and hazelnut protein isolate were added to hazelnut cocoa cream based on the procedures of the supporting

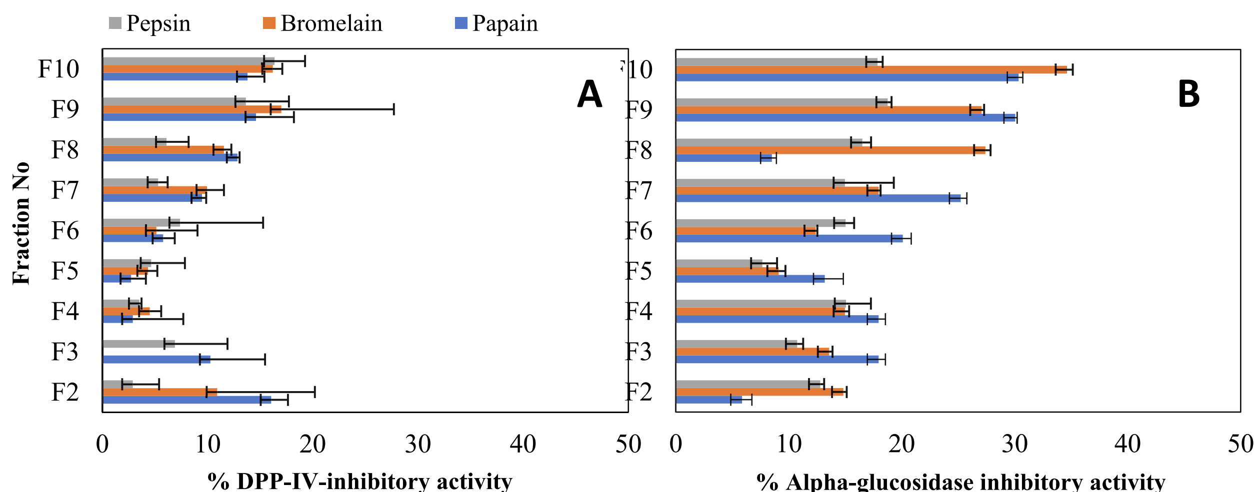

Fig. 1. **(A)** % DPP-IV-inhibitory, or **(B)** alpha-glucosidase inhibitory activities of hydrolysate fractions obtained from papain, bromelain, pepsin treated hazelnut protein isolate (1 % w/v). Enzyme to substrate ratio was 1:100 (w/w). Papain treatments were carried out at pH 7 for 150 min (50 °C). Bromelain treatments took place at pH 4.5 (45 °C for 150 min). Pepsin treatments were carried out in distilled water that was pre-set to pH 2 using 1 N HCl for 18 h at 37 °C.

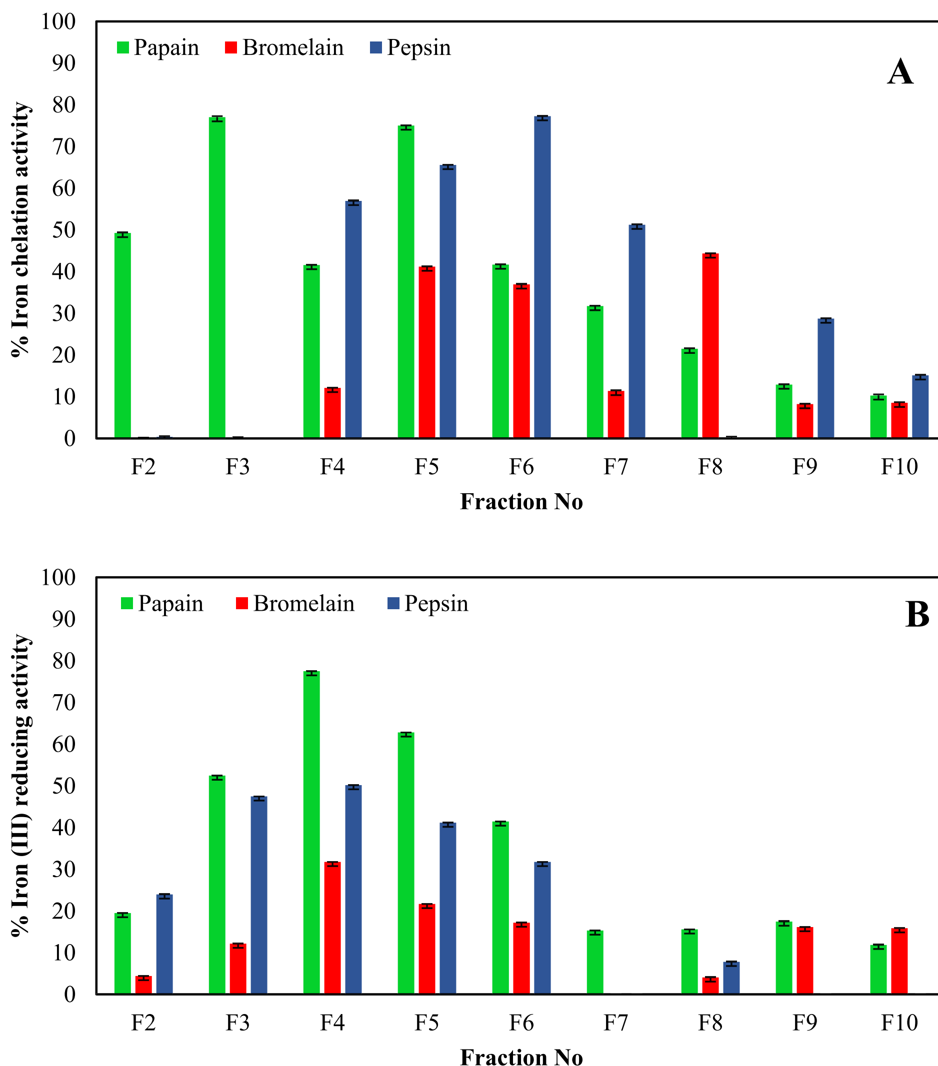

Fig. 2. **(A)** % Iron chelation, or **(B)** iron (III) reducing activities of hydrolysate fractions obtained from papain, bromelain, and pepsin treated hazelnut protein isolate (1 % w/v). Enzyme to substrate ratio was 1:100 (w/w). Papain treatments were carried out at pH 7 for 150 min (50 °C). Bromelain treatments took place at pH 4.5 (45 °C for 150 min). Pepsin treatments were carried out in distilled water that was pre-set to pH 2 using 1 N HCl for 18 h at 37 °C.

company. Firstly, 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 (Anon, 2011). Hence, inclusion of lyophilized bioactive peptide fractions or hazelnut protein isolate was a safe food manufacturing practice.

Immediately afterwards, simulated *in vitro* gastrointestinal digestion tests were conducted to examine the bioactive attributes of the digested hazelnut cocoa cream. These results were summarized in the following sections.

3.4. Indirect allergy suppressing attributes

The amount of detectable allergens in the samples was calculated using an appropriate standard and % allergy suppressing attributes were determined (Table 1). In bioactive fraction bearing hazelnut cocoa cream, a slight extent of allergenic suppression was observed for all samples, while no suppression took place in the hazelnut cocoa cream sample containing untreated hazelnut protein isolate. Consequently, enzymatic activity lowered the potential allergenicity of hazelnut proteins and furthermore, possibly reduced allergenic response due to interactions between the peptides and allergenic hazelnut proteins. In the literature, for example, hydrolysis of several peanut proteins were found to lower their ability to bind IgE (Cabanillas et al., 2012). Endoprotease treatments reduced IgE-binding ability of soluble proteins in roasted peanuts more effectively than exoprotease treatments. Pretreatments and choice of proteases were influential on allergenic response (Cabanillas et al., 2012), which was coherent with the current

Table 1
% Allergenic suppression in bioactive peptide fractions or hazelnut protein isolate bearing (1 % w/w) hazelnut cocoa cream products in comparison to the reference sample. Standard deviation was < 5 % of the sample mean in all cases. N/A (*i.e.*, not applicable) indicates that the treatment did not lead to allergenic suppression. In such cases, statistical significance was not tested. Fractions of papain, bromelain, and pepsin hydrolysates were referred to as PAF, BRF, and PEF, respectively. Fraction numbers were also added to these descriptions. Different superscript letters indicate significant differences at $p < 0.05$ level.

Added fraction	% Allergenic suppression
Hazelnut protein isolates	N/A
PAF4	19.62 ± 0.1^{a}
BRF10	13.33 ± 0.4^{b}
PEF6	17.91 ± 0.5^{c}

findings.

3.5. Bioactive attributes of the digested samples

3.5.1. ACE-inhibitory activity of the digesta

The bioactive fraction or hazelnut protein isolate bearing cocoa cream samples were subjected to simulated *in vitro* gastrointestinal digestion assays and ACE-inhibitory activity tests were carried out using digested hazelnut cocoa cream (Table 2). Firstly, all samples including hazelnut protein isolate, PAF4, PEF6 and BRF10 fractions demonstrated significant ACE-inhibitory activity in hazelnut cocoa cream.

While the food matrix and especially conching treatments could demonstrate a negative effect on ACE inhibitory potential of the peptides, their activities potentially increased by partial degradation induced by digestion. In previous studies, ACE inhibitory peptides were found to be unaffected by heat treatments and maintained significant ACE inhibitory activity after simulated *in vitro* digestion (Escudero et al., 2014). ACE inhibitor peptides are generally short-chain peptides containing 2–12 amino acids, and crystallography studies showed that relatively larger peptides could not bind to the active sites of ACE (Natesh et al., 2003). The probability of smaller peptides to be further digested is potentially lower than larger peptides. Matsui, Li, and Osajima (1999) reported that most ACE inhibitor peptides are short peptides containing only 2 to 9 amino acids. In our previous studies, at least 256 different hazelnut peptides were reported in various proteolytic digests of hazelnut proteins (Çağlar et al., 2021A). Among these peptides, 83 of them were generated using the 3 proteolytic enzymes utilized here (Supplementary Material section, Table S1). Based on *in silico* trypsinolysis (Minkiewicz et al., 2019), the gastrointestinal stability of hazelnut peptides listed on Table S1 were investigated. Consequently, 51.8 % of the listed peptides were found to be degraded by trypsin at least at one site. Further digestion (pepsin, chymotrypsin etc.) could lead to further degradation. Therefore, while some of these peptides could be further degraded by simulated digestion, some of them would remain intact. A mixture of intact and digested peptides were potentially present in the digesta. Consequently, these mixtures enhanced ACE-inhibitory activity of the digested hazelnut cocoa cream. These predictions are potentially applicable to the findings on other bioactivity categories as well (*i.e.*, antidiabetic activity). It is noteworthy that recent studies pointed out to synergism in multiple peptide bearing protein hydrolysates, which sometimes could be lost in purified peptides (Li et al. 2019). We also tested whether the increase in inhibitory activity was due to peptides from the added fractions and/or peptides formed *in situ* during the digestion of hazelnut proteins contained in the cream using a blank sample with no hazelnut protein isolate or fraction content. The inhibitory activity of the hazelnut protein isolate bearing sample was pronounced, while the blanks demonstrated a limited extent of activity (i.e., <2%). In any case, the contribution due to the digestion of the blank sample was subtracted from the activity data, and measured increase in inhibitory activity was solely due to peptides generated from the added fractions and/or protein isolates (Table 2). The digests from the hazelnut cocoa cream enriched with the fractions of the different hydrolysates obtained showed the same ACE inhibitory activity as the digests of the cream of cocoa enriched with the protein isolate. In the case of this bioactivity, it would not be convenient to obtain hydrolysates and their fractionation.

Table 2
% ACE-inhibitory activity values of bioactive peptide fractions or hazelnut protein isolate bearing (1 % w/w) hazelnut cocoa cream products after *in vitro* simulated gastrointestinal digestion. Fractions of papain, bromelain, and pepsin hydrolysates were referred to as PAF, BRF, and PEF, respectively. Fraction numbers were also added to these descriptions. Different superscript letters indicate significant differences at $p < 0.05$ level.

Added fraction	Average % ACE-inhibitory activity
Hazelnut protein isolates	90.31 ± 0.21^{a}
PAF4	91.26 ± 0.28^{b}
BRF10	91.39 ± 0.17^{b}
PEF6	91.68 ± 0.20^{b}

3.5.1.1. Antidiabetic activity of the digesta.

The results of the DPP-IV, alpha-amylase, and alpha-glucosidase inhibitory activity tests performed on the digested samples were listed on Table 3. Hazelnut protein isolate fortification did not lead to significant DPP-IV inhibitory activity in the hazelnut cocoa cream products. PAF4, BRF10 and PEF6 fractions caused an increase in DPP-IV inhibitory activity of approx. 35, 27, and 22 %, respectively, compared to the negative controls. Meanwhile hazelnut protein isolate, PAF4, BRF10 and PEF6 fractions caused an increase in alpha-amylase inhibitory activity of approx. 9, 7, 13 and 44 %, respectively. However, hazelnut protein isolate, PAF4, or BRF10 fractions did not demonstrate any alpha-glucosidase inhibitory activity in digested hazelnut cocoa cream. PEF6 fraction lead to an approx. 11 % increase in alpha-glucosidase inhibitory activity.

Based on these findings, DPP-IV inhibitory activity was enhanced in the final products. While the inhibitory activity of the fractions were negligible for alpha-amylase, digested cocoa cream products were characterized by measurable alpha-amylase inhibitory activity. In the case of alpha-glucosidase, while PAF4 and BRF10 demonstrated inhibitory activity, which was not the case for the product whereas for PEF6, inhibitory activity was reduced from 16 to 11 %. Consequently, as discussed in the previous section, further degradation of the peptides present in the hydrolysates clearly influenced their antidiabetic potential.

It is noteworthy that based on Table 3 data, hazelnut protein isolate addition did not generate comparable antidiabetic activity, while it was influential on ACE-inhibition (Table 2). Consequently, similar processing and digestion protocols lead to pronounced differences in different bioactivity categories.

3.5.1.2. Antioxidant activity of the digesta.

Iron chelation, FRAP, and superoxide anion sequestration activity tests were performed on digested products after simulated gastrointestinal digestion in order to determine the antioxidative potential of the current hydrolysates.

After the gastrointestinal digestion assays, antioxidant activities of the samples were reevaluated (Table 4). PEF6, PAF4 and BRF10 fractions lead to an increased iron chelation activity of approximately 41 %, 12 % and 1 % respectively, in the digested hazelnut cocoa cream compared to the controls. However, PAF4, BRF10, PEF6 fractions yielded a low extent of increase in iron (III) ion reducing activity of approximately 4 %, 2 % and 3 %, respectively. While the PAF4 fraction had no effect on the superoxide anion scavenging activity, the BRF10

Table 3
% DPP-IV-inhibitory, alpha-amylase inhibitory, alpha-glucosidase inhibitory activity values of bioactive peptide fractions or hazelnut protein isolate bearing (1 % w/w) hazelnut cocoa cream products after *in vitro* simulated gastrointestinal digestion ($n = 3$). N.D. (i.e., not detected) indicates that the treatment did not lead to measurable bioactivity. In such cases, statistical significance was not tested. Fractions of papain, bromelain, and pepsin hydrolysates were referred to as PAF, BRF, and PEF, respectively. Fraction numbers were also added to these descriptions. Different superscript letters indicate significant differences at $p < 0.05$ level.

Added fraction	Average % DPP-IV inhibitory Activity	Average % Alpha-amylase inhibitory activity	Average % Alpha-glucosidase inhibitory activity
Hazelnut protein isolates	N.D.	9.88 ± 0.02^{a}	N.D.
PAF4	35.00 ± 10.23^{b}	7.45 ± 0.01^{b}	N.D.
BRF10	27.93 ± 2.91^{ab}	13.83 ± 0.13^{c}	N.D.
PEF6	22.15 ± 11.62^{a}	44.41 ± 0.04^{d}	11.04 ± 0.03

Table 4
% Iron chelation, iron (III) reducing, superoxide anion sequestration activity values of bioactive peptide fractions or hazelnut protein isolate bearing (1 % w/w) hazelnut cocoa cream products after *in vitro* simulated gastrointestinal digestion (n = 3). N.D. (i.e., not detected) indicates that the treatment did not lead to measurable bioactivity. In such cases, statistical significance was not tested. Fractions of papain, bromelain, and pepsin hydrolysates were referred to as PAF, BRF, and PEF, respectively. Fraction numbers were also added to these descriptions. Different superscript letters indicate significant differences at $p < 0.05$ level.

Added fraction	Average % iron chelation activity	Average % iron (III) reducing activity	Average % superoxide anion sequestration activity
Hazelnut protein isolates	N.D.	4.23 ± 0.0^{a}	N.D.
PAF4	12.77 ± 0.0^{a}	4.16 ± 0.0^{a}	N.D.
BRF10	1.54 ± 0.0^{b}	2.5 ± 0.0^{a}	2.27 ± 0.1^{a}
PEF6	41.54 ± 0.7^{c}	3.33 ± 0.0^{a}	1.94 ± 0.1^{a}

and PEF6 fractions caused a low extent of increase in this category. While the hazelnut protein isolate had no effect on the iron chelation activity and superoxide anion scavenging activity in the hazelnut cocoa cream, the iron (III) ion reducing activity was increased by 4 %. Consequently, processing and digestion conditions lead to significant changes in the antioxidative potential of hazelnut protein hydrolysates, mostly causing a certain extent of reduction in antioxidative activity.

These findings may be due to the action mechanism of the individual antioxidative tests or molecular interactions due to the multi-component nature of the digested products. In the iron chelation test, for example, there was a significant decrease in the activity of the PAF4 and PEF6 fractions in the digested product, whereas in the iron (III) reduction and superoxide anion retention tests, the fractions demonstrated minimal activity. Antioxidative peptides can generally be relatively longer than some of the other bioactive peptide categories including ACE-inhibitory peptides and their antioxidative activity could decrease with further proteolysis. In this study, the hydrolysates were industrially utilized on an "as is" basis and no attempt was made to further stabilize them. Chen and Li (2012) made similar observations in their work suggesting that the antioxidant activity of peptides reduced during gastric digestion. After simulated gastrointestinal digestion, the antioxidant peptide AEEEYPDL was observed to split into smaller peptides AEEEY and PDL and lose its antioxidant properties (Gallego et al., 2018). To summarize the findings, while the ACE-inhibitory and antidiabetic attributes were partly enhanced due to processing and digestion, antioxidative attributes were mostly attenuated.

4. Conclusion

In the current literature, a great deal of emphasis is given to the manufacture, isolation and characterization of bioactive peptides. However, industrial implementation of bioactive peptides has been much less explored. In this study, we made an attempt to investigate the influence of industrial processing and simulated digestion on the bioactive attributes of hazelnut protein hydrolysates.

Based on *in silico* proteolysis, the peptides present in the hydrolysates could potentially be further degraded, which in turn would lead to the co-existence of intact and partly degraded peptides in the digesta. Consequently, partial hydrolysis did not necessarily increase the ACE-inhibitory activities since they were mostly equivalent to that of digests of cocoa cream enriched bearing the protein isolate. However, in most cases, ACE-inhibitory and antidiabetic attributes were preserved in the digested hazelnut cocoa cream products, while antioxidative attributes were considerably reduced. Molecular weight could be a major factor responsible for these observations. As anticipated food processing, dilution in the food matrix, digestion, and interactions with other food ingredients have an impact on bioactive attributes of all food bioactive components. However, the influence of hazelnut cocoa cream processing was relatively limited. Based on our current findings, hazelnut press cakes represented a significant resource for the generation of protein and bioactive peptide products and their industrial utilization. Although peptide sequences in the hydrolysates could be partially degraded during digestive processes, the collective bioactivity of the peptides present in the hydrolysates could be preserved beyond industrial manufacture and digestion. Consequently, these approaches could enable the design of functional foods with slightly reduced allergenicity.

CRediT authorship contribution statement

Ayşe Gülden Göksu: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Bilal Çakır:** Conceptualization, Methodology, Formal analysis, Supervision, Funding acquisition. **İbrahim Gülseren:** Conceptualization, Methodology, Resources, Data curation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2022.111865.

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