J Food Sci Technol (January 2023) 60(1):171–180
https://doi.org/10.1007/s13197-022-05601-2

ORIGINAL ARTICLE

Sequence alterations affect the antidiabetic attributes of hazelnut peptide fractions during the industrial manufacture and simulated digestion of hazelnut paste

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Revised: 26 August 2022 / Accepted: 14 September 2022 / Published online: 26 September 2022

Abstract Press cakes are by-products of cold press oil manufacture and are characterized by significant protein concentrations. Our group has previously demonstrated potential bioactive attributes of hazelnut protein hydrolysates including their antidiabetic activities. Here, an effort was made to utilize DPP-IV (Dipeptidyl peptidase-IV)-inhibitory hazelnut peptides in industrial food manufacture. Hazelnut protein isolates (approx. 95% protein) were obtained via an alkali extraction-isoelectric precipitation method. Papain, bromelain and pepsin were used in the enzymatic hydrolysis and hydrolysates were fractionated via Fast Protein Liquid Chromatography. As a general observation, although fractionation lead to dilution of the samples, fractions were observed to be more bioactive than the total hydrolysates. In vitro antidiabetic activities of the fractions were tested and 3 antidiabetic fractions were added to hazelnut paste. Afterwards simulated gastrointestinal digestion and antidiabetic activity assays were performed. DPP-IV inhibition was the major antidiabetic mechanism in the fractions and digested paste, while some fractions were characterized by comparable IC_{50} values as the positive controls. Alpha-glucosidase inhibition was limited by digestion trials, whereas alpha-amylase inhibition was only slight in the digested paste (< %6). In silico analyses predicted partial degradation of the peptides, whereas the interactions between DPP-IV or alpha-glucosidase and hazelnut peptides were predicted to be significant ($p < 0.05$). Consequently hazelnut press cakes were regarded as a potential source of antidiabetic peptides that can be used in industrial manufacture of functional foods, while food processing conditions or gastrointestinal digestion could largely affect peptide bioactivity.

Keywords Hazelnuts · Antidiabetic peptides · DPP-IV inhibition · Molecular docking · Functional foods

Abbreviations

AE-IP	Alkali extraction-isoelectric precipitation
DEAE	Diethylaminoethyl
DNS	3,5-Dinitrosalicylic acid
DPP-IV	Dipeptidyl peptidase-IV
FPLC	Fast protein liquid chromatography
GLP-1	Glucagon-like peptide-1
IC_{50}	Half maximal inhibitory concentration
LC-Q-TOF/MS	Liquid chromatography quadrupole time-of-flight mass spectrometry
pNA	p-nitroanilide
pNPG	p-nitrophenyl glucopyranoside
PVDF	Polyvinylidene
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated salivary fluid

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13197-022-05601-2.

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Introduction

Diabetes is a group of chronic metabolic disorders or diseases characterized by a persistent increase in blood sugar level. If not treated properly, diabetes can affect various organs and lead to various life-threatening complications. The incidence of diabetes is globally increasing, and the International Diabetes Federation (IDF) estimated that approx. 592 million people could be affected by 2035 (Guariguata et al. 2014). While there are two major types of diabetes, Type 2 diabetes or insulin independent diabetes accounts for 90% of diabetic cases that are characterized by a prolonged increase in blood sugar level due to a defect in insulin secretion (i.e., impaired cellular function) or insulin receptors (i.e., insulin resistance) (Li et al. 2019).

Dipeptidyl peptidase-IV (DPP-IV) inactivates two incretin hormones that increase insulin secretion, glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide. Therefore, inhibition of DPP-IV is a molecular target in the treatment of diabetes. DPP-IV inhibitors may improve insulin levels, lower blood sugar levels, limit hypoglycemia and increases in body weight (Mojica et al. 2016).

Enzymes secreted from various organs such as salivary glands, pancreas and small intestine enable the digestion of proteins and carbohydrates and affect blood sugar levels. Initially, alpha-amylase secreted by the salivary glands and pancreas converts natural substrates such as starch and glycogen into glucose and maltose in food, resulting in an increase in blood sugar level. Alpha-glucosidase is a membrane-bound enzyme found in small intestinal epithelium that catalyzes the separation of glucose from disaccharides, which breaks down starch granules in the small intestine and increases blood glucose levels. Inhibition of this enzyme has been recognized as an effective approach to lower the serum glucose level (Johnson et al. 2011). The guidelines of the American Diabetes Association and the European Society for Diabetes Research recommended the use of alpha-amylase and alpha-glucosidase inhibitors as a potential first-line agent or in combination with other antihyperglycemic drugs.

Bioactive peptides are defined as peptide sequences that demonstrate a beneficial effect on body functions and/or positively affect human health beyond their known nutritional value. They can be released during gastrointestinal digestion, hydrolysis of proteins using proteolytic enzymes, chemical (i.e., acidic or alkaline) hydrolysis, and also during food processing (i.e., cooking, ripening and fermentation) (Abdel-Hamid et al. 2017).

The studies on production and purification of bioactive peptides, and their efficacy in vitro and in vivo have been extensively studied. However, studies on the industrial utilization of bioactive peptides are still relatively limited. For example, in a recent study examining the bioactive properties of fish protein hydrolysates, the inclusion of peptides to commercial fish soup and commercial apple juice samples was studied in order to examine the effect of sterilization and sugar addition treatments on the stability of bioactive peptides (Rivero-Pino et al. 2020). Antidiabetic peptides added to tomato soup and tomato juice samples were found to retain their bioactivity before and after simulated digestion, and before and after heating treatments (Harnedy-Rothwell et al. 2021).

Hazelnut (*Corylus avellena* L.) is a plant belonging to the Betulaceae family of Fagales. "Tombul" hazelnuts grown in Giresun province, Turkey, are accepted among the most important commercial varieties worldwide. Hazelnut meals or cakes constitute the major by-product stream of hazelnut oil production and a low-cost source for the manufacture of protein and peptide products. In recent studies, bioactive peptides produced from hazelnut proteins via proteolytic treatments were investigated for their biological activities. Peptides that demonstrate antidiabetic characteristics are an important category among bioactive peptides. We have recently demonstrated the DPP-IV inhibitory potential of hazelnut peptides, which was potentially attributed to hydrophobic interactions (Çağlar et al. 2021a). Furthermore, the influence of industrial hazelnut paste manufacture on ACE-inhibitory activity of hazelnut peptides were studied (Göksu et al. 2022).

Here, an attempt was made to investigate the influence of hazelnut paste manufacture and simulated digestion on the antidiabetic attributes of hazelnut peptides. Bioactive hazelnut protein hydrolysates with potentially antidiabetic activities were industrially utilized in hazelnut paste manufacture and their stability in food proccessing and after in vitro simulated digestion was tested.

Materials and methods

The cold press cakes for Giresun tombul hazelnuts (*Corylus avellana* L.) used in this study were kindly donated by Oneva (Neva Foods Ltd., İstanbul, Turkey). Unless otherwise stated, the reagents used in the study were purchased from Sigma-Aldrich (Schnelldorf, Germany) including the 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), alpha-amylase (A0521), DPP-IV (Dipeptidyl Peptidase IV human, D4943), alpha-glucosidase (Type I, *Saccharomyces cerevisiae*, G5003). Pancreatin from porcine pancreas with a trypsin activity of 625 $U{\cdot}mg^{-1}$ 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 (Göksu et al. 2022). Afterwards, 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. The protein, moisture and ash contents of the samples were determined according to AOAC Official Methods 920.87 (%N × 6.25), 925.10 and 923.03, respectively (AOAC 2003). In this configuration, while the detection limit was approximately 0.5% for nitrogen determination, this could translate to a detection limit of up to 3.125% in total protein determination.

Enzymatic proteolysis

The enzymatic hydrolysis of the protein isolates was carried out using a thermomixer (MTC-100, MIULAB, China) under agitated conditions (100 rpm). Papain, bromelain and pepsin were used in the hydrolysis of hazelnut protein isolates (Çağlar et al. 2021a). In all cases, enzyme: substrate ratio was 1:100 (w/w). 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 50 °C. In the case of bromelain, the buffer used was 30 mM sodium acetate buffer (pH 4.5) and the treatment was continued for 150 min at 45 °C. Pepsin was was prepared in distilled water, where pH was set to 2 using 1 N HCl solutions. The enzymatic process was continued for 18 h at 37 °C. In all cases, the microcentrifuge tubes containing the reaction mixture was transferred to a 95 °C water bath for 10 min and then to an ice-bath until the samples cooled down to the ambient temperature. The sample tubes were centrifuged at 5000xg for 30 min to remove the insoluble matter. Finally, the supernatant was filtered through polyvinylidene (PVDF) syringe filters (0.45 µm) (Millex-HV, Merck-Millipore, Germany). The filtered hydrolysates were stored at − 80 °C until further use. Degree of hydrolysis (%DH) in the hydrolysates was determined based on Adler-Nissen (1979).

Fractionation of hydrolysates

Using an FPLC system (ÄKTA pure 25 L, GE Healthcare Life Sciences, Sweden), proteolytic hydrolysates were fractionated. Due to a favorable signal intensity in the fractions, weakly anionic HiTrap Capto DEAE (Diethylaminoethyl) (5 ml) columns were used in this procedure. 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 25 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 also monitored. For each hydrolysate sample, the "out" fraction containing the materials that were not 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.

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. Similarly, antidiabetic activity of digested samples was also measured based on this method. For the digestion experiments, antidiabetic activity in digested hazelnut paste with no hydrolysates was evaluated as an additional control and the sample mean for antidiabetic activity in the digested paste was subtracted from the activity data for peptide bearing samples to ensure that only hydrolysate components accounted for the measured activity. Finally, an enzymatic blank was administered in order to subtract any contributions from digestive enzymes.

DPP-IV inhibitory activity tests

To determine the DPP-IV inhibitory activities of the peptide fractions, the method of Nongonierma et al. (2018) was used. Fifty µl of fraction samples and 50 µl of 0.8 mM substrate (Gly-Pro- p-nitroanilide (pNA) hydrochloride, G0513, Sigma-Aldrich) were mixed and this mixture was pre-incubated at 37 °C in a thermomixer set to 1000 rpm (10 min). At the end of this period, 100 µl of DPP-IV (0.01 $U{\cdot}ml^{-1}$) was added to the mixture and the enzymatic reaction was initiated. Two hundred µl of 1 M sodium acetate (pH 4.0) was added to stop the reaction after 1 h. The amount of pNA released from the substrate was measured based on absorbance measurements (405 nm). Diprotin A (Ile-Pro-Ile, I9759, Sigma-Aldrich) was used as a reference inhibitor. All reagents including the negative controls were prepared in 100 mM Tris–HCl pH 8.0 buffer solution. The % inhibition values of the samples were calculated by comparing the treatments with the negative controls in all enzyme inhibition tests.

Alpha-glucosidase inhibitory activity tests

The inhibitory activity of the fractions on alpha-glucosidase was performed according to the protocol proposed by Kim et al. (2005). Minor changes were carried out regarding enzyme activity (1 $U.ml^{-1}$) and substrate solution concentration (35 mM p-nitrophenyl glucopyranoside, pNPG, N1377,

Sigma-Aldrich) in methanol. Ten µl enzyme (1 U·ml^{-1}), 10 µl sample, 20 µl substrate and 500 µl phosphate buffer were mixed and incubated at 37 °C for 20 min. After the incubation period, 2 ml of 0.1 M Na_2CO_3 was added to the reaction mixture to terminate the reaction. The absorbance of the mixtures was measured at 405 nm.

Alpha-amylase inhibitory activity tests

The method by Johnson et al. (2011) was applied with modifications. Fraction samples (50 µL) were pre-incubated with 100 µl alpha-amylase (2 U·ml^{-1}) for 5 min (20 °C). After incubation, 100 µl of 1% starch dispersion (S2004, Sigma-Aldrich) prepared in 0.02 M sodium phosphate buffer (pH 6.9) was added and the mixture was incubated for 15 min. One hundred µl of 1% DNS (3,5-dinitrosalicylic acid, D0550, Sigma-Aldrich) was also added and the mixture was placed in a boiling water bath for 15 min. Finally, after the addition of 350 µl distilled water, absorbance measurements were performed at 540 nm.

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

All MS analysis was performed on a Xevo G2-XS QTof (Waters) equipment using the default parameters of Labmed, Acıbadem University, İstanbul, Turkey. The experimental details were summarized in Çağlar et al. (2021a).

In silico analyses and molecular docking

Upon the acquisition of MS-data, the determined peptide sequences were analyzed in silico for the prediction of their bioactive characteristics. For example, BIOPEP A parameter was used as an indicator of % frequency of active residues in a given sequence (Minkiewicz et al. 2019). This parameter represents the relative frequency of potentially bioactive (i.e., antidiabetic) residues compared to the total number of residues in each sequence. This information wass calculated based on comparison to the existing data listed in BIOPEP (Minkiewicz et al. 2019). The interactions between the respective peptides and the enzyme they inhibit (i.e., DPP-IV or alpha-glucosidase from human) were analyzed based on a molecular docking approach (Zhou et al., 2018), which generated docking scores and 3D protein-peptide interaction images. When necessary, peptide-DPP-IV interactions were further elucidated using PepSite2 software (Petsalaki et al., 2009). Hydrophobicity and charge characteristics of the peptides were predicted using ToxinPred (Gupta et al. 2013).

Industrial hazelnut paste production and product analysis

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 to hazelnut paste manufacture. Peptide fractions were added to the products at a level of 1% in order to preserve the textural attributes and optimize both the cost and effectiveness, since industrial manufacture requires a significant amount of samples. Roasted hazelnuts and granulated crystal sugar were ground in a meat grinder and a powdered sugar mill, respectively. Both sets of 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 hazelnut paste production. Experimental controls included hazelnut protein isolate bearing control samples and no peptide or protein added control (blank) samples.

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

Simulated in vitro gastrointestinal digestion assays

Simulated in vitro gastrointestinal digestion tests were performed to evaluate the residual DPP-IV-inhibitory activity in the digested hazelnut paste products based on the recommendations of INFOGEST (Minekus et al. 2014).

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, 20 ml of oral bolus was mixed with 15 ml of simulated gastric fluid (SGF). Pepsin stock solution (3.2 ml, 25,000 U·mg^{-1}), 10 µl 0.3 M $CaCl_2$, 0.4 ml 1 M HCl and 1.39 ml distilled water were added to this mixture. Medium 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) (68,856, 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 (approx. 100 °C) for 15 min. Digested samples were filtered through coarse filter paper and centrifuged at 4000xg (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 (i.e., antidiabetic activity tests).

Statistical analysis

The results of the current analyses were presented as sample means ± standard deviation based on at least 3 replicates each with two sub-samples. 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.

Results and discussion

Compositional analysis

The protein, moisture and ash contents of the cold press hazelnut cake and hazelnut protein isolate were reported on Table 1. While the protein concentration for the cake was approx. 50%, the protein isolate was as high as 94.8% in protein content. Moisture and ash contents of the isolates were minimal.

Fractionation of hazelnut peptides

The degree of hydrolysis (% DH) for the hydrolysates prepared by papain, bromelain, or pepsin treatments were determined using the TNBS assay. % DH values were found to be approx. 61 ± 0.2 for papain, 46.2 ± 0.1 for bromelain, and 74.3 ± 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 (Çağlar et al. 2021b), which in part can be attributed to different proteolytic enyzmes utilized, their specific sites of hydrolysis, variations in enzyme and substrate combinations, and chosen proteolysis.

Table 1 Moisture, ash, protein contents (%) of hazelnut press cake and hazelnut protein isolate

Component	Hazelnut press cake	Hazelnut protein isolate
Moisture (%)	8.94 ± 0.1	3.44 ± 0.3
Ash (%)	6.42 ± 1.4	1.18 ± 2.4
Protein (%)	50.02 ± 0.4	94.81 ± 2.9

Papain, bromelain or pepsin hydrolysates of hazelnut protein isolates were fractionated using an FPLC system and 10 fractions were collected in each case. The chromatograms were distinctly different in all cases indicating large differences in composition and concentration of peptides per each fraction and hydrolysate (Göksu et al. 2022). In all cases, the peptide concentration in the fractions demonstrated roughly a normal distribution, whereas the peptide concentrations ranged between 0 and 0.2 $mg.ml^{-1}$ (Table S1, Supplementary Material section). Antidiabetic activity tests were performed in order to establish the effects of these differences in chromatograms on different activity categories.

Antidiabetic activity tests and LC-Q-TOF/MS analysis of the antidiabetic fractions

Following fractionation, antidiabetic activity tests were carried out in order to determine DPP-IV, alpha-glucosidase, or alpha-amylase inhibitory activities of the fractions.

After antidiabetic activity tests were carried out, antidiabetic 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 have been listed. Following enzymatic proteolysis, it is reasonable to assume thousands of different peptides were present in each hydrolysate at varying concentratios and bioactivity potentials. In the current MS analysis, the sequence information was derived as exhaustively as possible for the ionizable peptides in all bioactive fractions. The corresponding sequences were listed on Table S2 (Supplementary Material section). The sequence data will be utilized in the comparison of in silico versus in vitro data in the following sections.

DPP-IV inhibitory activity assay

Average % DPP-IV inhibitory activity values of 3 different enzymatic hydrolysates and their corresponding FPLC fractions were measured (Table 2). In most cases, the hydrolysate samples and their fractions were characterized with measurable % DPP-IV inhibitory activity, although their corresponding peptide concentrations were relatively low (Table S1). Increasing elution volume decreased peptide concentration in the fractions, while bioactive potential and concentration of peptides simultaneously affected the overall activity. We would like to emphasize that the bioactive attributes were observed throughout various

Table 2 Average % DPP-IV -inhibitory activity values of total hazelnut protein hydrolysate and its corresponding fractions obtained by enzymatic proteolysis treatments

Sample	% DPP-IV Inhibitory Activity		
	PAF	BRF	PEF
Total hydrolysate	–	3.37 ± 4.1	8.19 ± 3.6
Out	3.37 ± 0.4	5.30 ± 2.3	3.37 ± 0.4
F2	16.05 ± 1.6	10.91 ± 9.3	2.89 ± 2.5
F3	10.27 ± 5.2	–	6.90 ± 5.0
F4	2.89 ± 4.8	4.50 ± 1.1	3.53 ± 0.2
F5	2.73 ± 1.4	4.33 ± 0.9	4.65 ± 3.2
F6	5.78 ± 1.1	5.14 ± 3.9	7.38 ± 7.9
F7	9.47 ± 0.4	9.95 ± 1.6	5.30 ± 0.9
F8	12.84 ± 0.2	11.56 ± 0.7	6.10 ± 2.1
F9	14.61 ± 3.6	17.01 ± 10.7	13.64 ± 4.1
F10	13.80 ± 1.6	16.21 ± 0.9	16.37 ± 2.9

PA, BR, and PE represent papain, bromelain and pepsin treated hydrolysates, respectively. F: Fraction

fractions, which might be more advantageous in cost compared to further purified counterparts.

In the case of papain treatments, the total (i.e., unfractionated) hydrolysate demonstrated no significant activity, whereas the out fraction was characterized by relatively low activity. Similar observations were made for bromelain and pepsin total hydrolysates, which were also characterized with low activity. While papain fractions demonstrated varying percentages of DPP-IV inhibitory activity, PAF2, PAF9 and PAF10 showed the highest activities. In the case of bromelain fractions, relatively later (i.e., strongly anionic) BRF9, BRF10 and BRF8 fractions were characterized by the highest DPP-IV inhibitory activities. In the case of pepsin, all samples including the total hydrolysate and its fractions were characterized with DPP-IV inhibitory activities. Once again, mostly later fractions (i.e., PEF10, PEF9 and PEF6) demonstrated the highest extent of inhibitory activity. As a general observation, although fractionation lead to dilution of the samples, fractions were observed to be more bioactive than the total hydrolysates.

In order to determine the relative effectiveness of the samples, their IC_{50} values were compared to the positive control Diprotin A (i.e., IPI). The IC_{50} value for Diprotin A was found to be approx. 9.76 $\mu g.ml^{-1}$ (28.59 µM). Here, IC_{50} values for the most influential fractions included 5.58 $\mu g.ml^{-1}$ for PAF2 fraction, and 0.07 $\mu g.ml^{-1}$ for the BRF9 fraction, respectively. In the previous studies, peptides from rapeseed napin were characterized by IC_{50} values of approx. 135.7 (PAGPF), 52.2 (IPQVS), and 78.5 µM (ELHQEEPL), respectively (Xu et al. 2019). Consequently the current hydrolysates performed favorably compared to the positive controls and well-characterized plant peptides in the literature.

Structure-bioactivity relationship studies have revealed that the presence and location of certain amino acids might affect DPP-IV inhibitory action. The presence of Ala, Gly, Ile, Leu, Pro, Met, Glu, and Val, especially at the N-terminal position, contributes significantly to their DPP-IV inhibitory activity (Nong and Hsu 2021). For example, the two well-known competitive inhibitors of DPP-IV, IPI and VPV, feature a Pro residue in their sequences (Nongonierma et al. 2019). Many DPP-IV inhibitory peptides contain hydrophilic amino acids such threonine, histidine, glutamine, serine, lysine, and arginine (Nongonierma and FitzGerald 2019). While the exact function of these hydrophilic amino acids remains unknown, the existence of hydrophobic pockets in the active site of DPP-IV have been thought to play a key role in enzyme inhibition. Hydrophobic residues at the C-terminal sequences have also been detected in a number of DPP-IV inhibitory peptides, while their DPP-IV inhibitory activity is much smaller than the N-terminal counterparts (Nong et al. 2020). The relative importance of interaction mechanisms for the current hydrolysate fractions remain to be further investigated in the following sections, while the majority of the peptides (61 in 80) listed in Table S1 were slightly hydrophobic as predicted by their negative GRAVY values (0 to − 0.78) (Gupta et al. 2013).

Alpha-glucosidase inhibitory activity assay

Firstly, papain, bromelain and pepsin hydrolysates were shown to generate inhibitory activities of approx. 20.4 ± 0.1, 15 ± 0.1, and 11.7 ± 0.1%, respectively. The relatively less anionic out fractions were characterized by 17.6 ± 0.1, 12.3 ± 0.1, and 9.7 ± 0.1% inhibitory activities for papain, bromelain and pepsin hydrolysates, respectively. Average % alpha-glucosidase inhibitory activity values of hydrolysate fractions (F2-F10) were summarized on Fig. 1. All fractions were found to demonstrate varying degrees of alpha-glucosidase inhibitory activity (5.9 to 34.6%). BRF10, PAF10 and PAF9 showed the highest activity among all fractions. PAF9 and PAF10 were characterized by IC_{50} values 0.657 and 0.001 $\mu g.ml^{-1}$, respectively. In the previous literature, three novel alpha-glucosidase inhibitory peptides from soy, namely LLPLPVLK, SWLRL, and WLRL, were characterized by IC_{50} values of approx. 212, 123, and 95 $\mu g.ml^{-1}$, respectively (Wang et al. 2019). The presence of hydroxyl or basic amino acids at the N-terminal of 43 alpha-glucosidase inhibitory peptides was reported to be responsible for their efficacy (Ibrahim et al. 2018). The presence of proline, alanine and methionine at the C-terminal potentially enhanced their inhibitory activities. Alternatively, shorter peptides from almonds (namely Trp-His and Trp-Ser), demonstrated IC_{50}

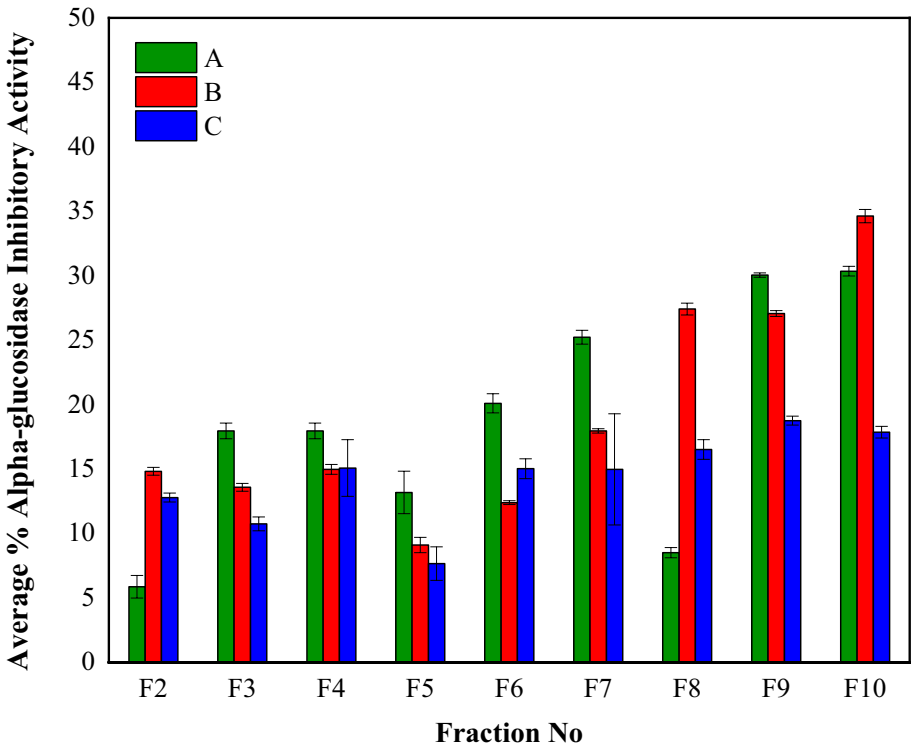

Fig. 1 % Alpha-glucosidase inhibitory activities of hydrolysate fractions obtained from **(a)** papain, **(b)** bromelain, **(c)** pepsin treated hazelnut protein isolate

values were < 45 µmol.L^{-1} in all cases, while simulated gastrointestinal digestion elevated the IC_{50} value of Trp-Ser (Gu et al. 2020). The majority of the peptides listed in Table 3 were slightly hydrophobic (10 in 11) and charged (8 in 11) as predicted by their negative GRAVY values (− 0.04 to − 0.54) and overall charge (− 1 to − 3), respectively. The relative importance of various compositional elements are further discussed in the following sections, while the IC_{50} values of the current fractions compared favorably with the previous findings.

Alpha-amylase inhibitory activity assay

No measurable alpha-amylase inhibitory activity was observed for papain hydrolysates. Similarly, none of the pepsin hydrolysates or fractions demonstrated significant inhibitory activity. However, bromelain hydrolysate and BRF2 fraction were characterized with low inhibitory activities (1.73 ± 0.1% and 4.6 ± 0.1%, respectively).

In the previous literature, Garza et al. (2017) reported that Phe, Leu, Pro and Gly residues were required for alpha-amylase inhibition. Similarly, Arise et al. (2016) suggested

Table 3 Alpha-glucosidase inhibitory activities of hazelnut peptides and their corresponding BIOPEP parameters

Peptide No	Fraction	Sequence	Activity	A
1	BRF2	LEPTNRIEA	alpha-glucosidase inhibitor	0.1111
2	PA-OUT	ADIYTEQVGR	alpha-glucosidase inhibitor	0.1000
3	BR-OUT	NAFQISREEA	alpha-glucosidase inhibitor	0.1000
4	BR-OUT	MAISDMPESTG	alpha-glucosidase inhibitor	0.0909
5	BR-OUT	NVEVQAPSSVK	alpha-glucosidase inhibitor	0.0909
6	PAF6	DTDILAAFRVTPQPGVPPEEAGAA VAAESSTGTWTTVWTDGLTSLDR	alpha-glucosidase inhibitor	0.0851
7	PAF2	ESFNVEHGDIIR	alpha-glucosidase inhibitor	0.0833
8	BR-OUT	FQISREEARRLK	alpha-glucosidase inhibitor	0.0833
9	BR-OUT	LSVPNLYVWLCMFY	alpha-glucosidase inhibitor	0.0714
10	BRF5	CPETFEDPQQQSQQG	alpha-glucosidase inhibitor	0.0667
11	PAF4 & PAF5	AISDMPESTGTTATTATMPHGGSDLR	alpha-glucosidase inhibitor	0.0385

that alpha-amylase bound to peptides containing cationic residues such as Lys (K) or branched chain residues such as Phe (F), Tyr (Y), and Trp (W). In addition, aromatic-aromatic interactions in the catalytic region appeared to be critical for alpha-amylase inhibition (Ngoh et al. 2017). Although some cationic fractions were present in the current hydrolysates, no significant alpha-amylase inhibitors were observed. The above mentioned 4 amino acids formed only about 10.5% of all amino acids in the sequences elucidated in this study as listed on Table S2. Consequently the current peptides were assumed to be relatively deficient in these amino acids, which was coherent with the previous literature.

In silico analysis of the identified sequences

The antidiabetic potential (i.e., DPP-IV, alpha-glucosidase and alpha-amylase inhibitory) of the hazelnut peptides was predicted based on BIOPEP analyses (Minkiewicz et al. 2019) and the corresponding data were listed on Table S2 and Table 3. A total of 80 and 11 different peptide spectra were elucidated in DPP-IV (Table S2) and alpha-glucosidase-inhibitory (Table 3) fractions, respectively. The majority of the identified peptides originated solely from the "out" fractions (56 peptides), 50 of which were potentially DPP-IV inhibitory. Current sequences were not predicted to generate any alpha-amylase inhibitory activity, which in turn was coherent with in vitro findings.

Among DPP-IV inhibitory peptides, 4 sequences generated a BIOPEP A value of ≥ 0.9 (Table S2) and were utilized in molecular docking studies. BIOPEP A value for alpha-glucosidase inhibitor peptides were significantly lower. Consequently, only the peptide with the highest score was considered for molecular docking and it was safely assumed that DPP-IV inhibition was the dominant antidiabetic mechanism. This assumption was coherent with the current in vitro data (Table 2).

The docking scores for DPP-IV inhibitory peptides were listed on Table 4. All 4 DPP-IV inhibitory peptides generated higher docking scores than the positive control (i.e., Diprotin-A), while the highest score was attained by VSLLHTNNYA (− 231.391). The highest BIOPEP A Parameter peptide among alpha-glucosidase inhibitory peptides (namely, LEPTNRIEA) attained a docking score of − 162.882. Both peptides were further analyzed for their interactions with the corresponding enzymes (Table S3, Supplementary Material section). Based on GRAVY values, intereacting residues of the VSLLHTNNYA peptide was found to be slightly polar (0.92), whereas the same value for LEPTNRIEA represented moderately apolar residues (− 1.67) possibly indicating different mehanisms in enzyme inhibition (DPP-IV vs alpha-glucosidase). Note that as detailed in the corresponding section, overall hydrophobicity of all Table S2 peptides were less pronounced than that of the interacting residues. Consequently, partial digestive degradation of the peptides could significantly alter antidiabetic characteristics, which, in turn, was further studied.

Table 4 Docking scores of the predicted protein-peptide interactions between DPP-IV from human and listed hazelnut peptides with a BIOPEP A score of ≥ 0.9. IPI was the positive control

Peptide No	Sequence	Docking Parameter (Best match)
Positive control	IPI (Diprotin A)	− 114.062
1	QVLTIPQNFAVA	− 228.503
2	SPQTETKASVG	− 164.985
3	QVLTIPQNFA	− 211.339
4	VSLLHTNNYA	− 231.391

Simulated gastrointestinal digestion of antidiabetic fraction bearing hazelnut paste and determination of residual activities

Functional hazelnut products bearing bioactive peptide fractions were generated under industrial settings. Previous data demonstrated that inclusion of bioactive peptide fractions in hazelnut paste was a safe practice in the microbiological sense (Göksu et al. 2022). Here, their antidiabetic attributes were determined after simulated in vitro gastrointestinal digestion. Firstly, fractions demonstrating significant DPP-IV and alpha-glucosidase inhibitory activities and high lyophilization efficiency were prepared at relatively large quantities for industrial trials (data not shown). Consequently, the lyophilizates were added to hazelnut paste based on the procedures of the supporting company.

Simulated in vitro gastrointestinal digestion assays were conducted to examine the residual antidiabetic attributes of the digested hazelnut paste. The results of DPP-IV, alpha-amylase, and alpha-glucosidase inhibitory activity tests were summarized on Table 5. PAF4, BFR10, and PEF6 fractions demonstrated varying extents of bioactivity in the functional hazelnut paste samples. For example, PAF4 fraction was characterized with an increase of 15% in DPP-IV inhibitory activity. BRF10 fraction caused a 21% increase in DPP-IV inhibitory activity, while PEF6 fraction lead to a 10% increase. While in isolation PAF4 fraction generated negligible alpha-amylase inhibitory activities, its influence after being incorparated into the food matrix was slightly more pronounced. These findings can be attributed in part to processing induced changes in peptide structures. PAF4, BRF10 or PEF6 demonstrated no significant inhibition towards alpha-glucosidase activity. Possibly due to the influence of manufacturing, digestive processes, and extent of dilution, alpha-glucosidase inhibitory potential was not

Table 5 % DPP-IV-inhibitory, alpha-amylase inhibitory, alpha-glucosidase inhibitory activity values of bioactive peptide fraction or hazelnut protein isolate bearing (1%) hazelnut paste products after in vitro simulated gastrointestinal digestion

Added fraction	Average % DPP-IV -inhibitory activity	Average % Alpha-amylase inhibitory activity	Average % Alpha-glucosidase inhibitory activity
Hazelnut protein isolates	31.62 ± 14.3	5.72 ± 0.1	–
PAF4	15.09 ± 6.4	4.20 ± 0.0	–
BRF10	20.87 ± 3.6	–	–
PEF6	10.28 ± 7.3	–	–

preserved in the digested paste. This result was independent of the fraction number (i.e., anionic characteristics). In a similar fashion, protein isolates were also characterized by significant antidaibetic (mostly, DPP-IV inhibitory) activity. Consequently, DPP-IV-inhibitory activity was the dominant antidiabetic activity in the digested formulations. In our previous work, digestion of functional hazelnut paste bearing similar hydrolysate fractions were found to enhance ACE-inhibitory (i.e., antihypertensive) activity (Göksu et al. 2022). In vitro findings were coherent with in silico proteolysis data, which predicted partial degradation of peptides and appearance of highly bioactive moeities upon digestion. Here, the results were highly dependent on the bioactivity type which might require preservation of original sequences throughout manufacture, storage and digestion. While hydrophobicity and electrostatic characteristics could potentially be influential for DPP-IV and alpha-glucosidase inhibitory potential of the peptides, respectively, digestive and/or process induced alteration could affect antidiabetic attributes.

Conclusion

A growing body of evidence supports the use of functional foods and bioactive peptides for the management and treatment of diabetes. Although bioactive peptide literature is rapidly expanding in food science, the utilization of bioactive peptides in industrial food processing remains limited. Our previous investigations predicted that ACE-inhibitory characteristics of hazelnut peptides could be positively affected by simulated digestion, while in this study; the results were highly specific for each antidiabetic activity category.

The most effective mechanism among the antidiabetic mode of action was DPP-IV inhibitory activity prior to processing and digestion. Similarly, after simulated gastrointestinal digestion, various fractions demonstrated DPP-IV inhibitory activity, while some fractions were characterized by comparable IC_{50} values as the positive controls. Alpha-glucosidase inhibition was limited by processing and digestion treatments, whereas slight alpha-amylase inhibition was observed in the digested paste. Consequently, DPP-IV inhibitory activity was still the dominant antidiabetic mechanism after digestion and the findings demonstrated that food processing conditions and/or gastrointestinal digestion could largely affect peptide bioactivity.

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. The industrial manufacture was possible due to the support of FİSKOBİRLİK, Giresun, Turkey.

Author contributions AGG: Methodology; Validation; Formal analysis; Investigation; Data Curation; Writing—Original Draft; Visualization. BÇ: Conceptualization; Methodology; Formal analysis; Supervision; Funding acquisition. İG Conceptualization; Methodology; Resources; Data Curation; Writing—Review & Editing; Supervision; Project administration; Funding acquisition.

Funding This study was funded by a grant from The Scientific and Technological Research Council of Turkey (TÜBİTAK) 1001 Program, Turkey (Grant No. 217O063).

Data availability Some of the manuscript data is available as an electronic supplementary document.

Declarations

Conflict of interest All the authors that thay have no conflict of interest.

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