

PRELIMINARY INVESTIGATIONS *IN VITRO* ACE-INHIBITORY ACTIVITIES OF TRYPTIC PEPTIDES PRODUCED FROM COLD PRESS DEOILED HAZELNUT MEALS

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ABSTRACT

Hazelnut (*Corylus avellana* L.) is a rich source of proteins which are concentrated in deoiled meals during hazelnut oil manufacture. Here, an *in vitro* attempt was made to evaluate the biological functionality of tryptic hazelnut peptides. In our *in silico* investigations, a specific subset of hazelnut proteins was predicted to demonstrate ACE-inhibitory and DPP-IV-inhibitory activities. Consequently, hazelnut protein concentrates were produced from cold press deoiled meals and subjected to tryptic hydrolysis (37°C). The progress of trypsinolysis was monitored using size exclusion chromatography (SEC), fast protein liquid chromatography (FPLC) and Raman spectroscopy. While the protein hydrolyzates had limited ACE-inhibitory activity, after 240 min of trypsinolysis, ACE-inhibitory activity was approximately 40% at a relatively low protein concentration level. Although further clarifications are necessary, current findings demonstrated hazelnut meal could be considered as a valuable source of ACE-inhibitory peptides that can be utilized in the formulation of functional foods and food supplements.

Keywords: Cold press meals; plant protein peptides; common hazelnut (*Corylus avellana* L.); trypsinolysis; ACE-inhibition.

SOĞUK PRES FINDIK POSALARINDAN ÜRETİLMİŞ TRİPTİK PEPTİTLERİN *IN VITRO* ACE İNHİBE EDİCİ AKTİVİTELERİ ÜZERİNE ÖN İNCELEMELER

ÖZ

Fındık (*Corylus avellana* L.) proteince zengin bir kaynaktır ve soğuk pres fındık yağı üretimi sürecinde oluşan fındık posaları proteince de zenginleşmektedir. Bu çalışmada, triptik fındık peptitlerinin biyolojik fonksiyonlarını değerlendirmek için *in vitro* analizlerde bulunulmuştur. Grubumuzun önceki çalışmalarında, *in silico* yöntemlerle fındık peptitlerinin biyoaktivitelerini incelemek için fındık proteinlerinin spesifik bir alt kümesi incelenmiş ve ACE- ve DPP-IV-önleyici aktivitelerin varlığı öngörülmüştür. Bu bulgulara bağlı olarak, soğuk pres yönteminde ortaya çıkan fındık posalarından, fındık proteini konsantreleri üretilmiş ve bu konsantreler triptik hidrolize (37°C) tabi tutulmuştur. Tripsinliz prosesi, boyutsal ayırım kromatografisi (SEC), hızlı protein sıvı kromatografisi (FPLC) ve Raman spektroskopisi kullanılarak takip edilmiştir. Enzimatik muamele görmemiş protein konsantrelerinin sınırlı ACE inhibe edici aktivitesi varken, 240 dakikalık tripsinlizde oluşan peptitlerin ACE-inhibitör aktivitesi, düşük protein konsantrasyonlarında dahi yaklaşık %40 düzeyinde gerçekleşmiştir. Mevcut bulgular, fındık posasının ACE-inhibitör peptitler açısından önemli bir kaynak olarak değerlendirilebileceğini ve fındık posasından hidrolizatlarının fonksiyonel gıdaların ve gıda takviyelerinin formülasyonunda kullanılabileceğini göstermiştir.

Anahtar kelimeler: Soğuk pres posaları; bitki proteini peptitleri; fındık (*Corylus avellana* L.); tripsinliz; ACE-inhibisyonu.

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INTRODUCTION

Due to the ever increasing global demand for proteins, affordable and high quality protein products are highly sought after. Consequently plant proteins represent a sustainable source of proteins for human diets. Since the conversion efficiency of plant proteins to animal proteins is fairly poor (approximately 15%) (Day, 2013), it makes perfect sense to search for alternative ways to utilize plant proteins in foods and other consumer products. Consequently, one of the research priorities in our group is the valorization of cold press and other oil processing by-products (i.e., deoiled meals of oil fruits and oil seeds) with the aim of generating a variety of economic plant protein products and their corresponding hydrolyzates. Since protein content in oilseeds and oil fruits is relatively high and proteins are further concentrated upon deoiling, they represent a reasonably economic and sustainable resource for protein manufacture.

Hazelnuts (*Corylus avellana* L.) are among the major agricultural products of Turkey, where approximately 80% of the global production is carried out (roughly 550,000 tonnes per year) (Ozdemir and Akinci, 2004), while the other major producers include other Mediterranean countries and western USA. Hazelnuts are rich in oil content (approx. 60%) and known to demonstrate various bioactivities including their cardioprotective effects. This effect is possibly due to the mono- and polyunsaturated fatty acids in the hazelnuts (Parcerisa et al., 1997). Other bioactive compounds of the hazelnuts include polyphenols, tocopherols and squalene (Alasalvar et al., 2006, 2009; Dogan et al. 2007). According to Miraliakbari and Shahidi (2008), phytosterols and phytostanols contribute to the cholesterol lowering and antioxidant properties of hazelnuts.

Industrial production of cold press hazelnut oil is also increasing which means the deoiled meal represents an increasing potential of valorization. According to the data provided by Alphan et al. (1996), it is possible to anticipate up to 45% proteins in the meal. Although there is some data on the technical functionality of hazelnut proteins (Tatar et al., 2015), the data on possible bioactive

characteristics of hazelnut proteins (Aydemir et al., 2014) and their hydrolyzates are truly scarce. Most of the literature related to the hazelnut proteins so far focused heavily on the reduction of food allergies (Ortolani et al., 2000; Vieths et al., 1999; Flinterman et al., 2008). It is noteworthy that the relative digestive stability of hazelnut allergens were found to be lower compared to peanut allergens (Vieths et al., 1999).

Bioactive peptides may form during the processing or digestion of protein-containing foods (Vermeirssen et al., 2004). Furthermore, using proteases, bioactive peptides can be produced by breaking down food proteins *in vitro*. Enzymatic preparation of bioactive peptides are highly advantageous since the use of toxic chemicals and organic solvents can be avoided and the final products are suitable for use in food, cosmetics and pharmaceutical products (Agyei and Danquah, 2011), while the demonstration of a variety of bioactivities including anti-oxidative, anti-carcinogenic, antimicrobial activities as well as ACE-inhibition activities can also be possible.

In our previous studies, an attempt to predict the potential bioactivities of peptides generated from hazelnut proteins was made *in silico* and a specific subset of hazelnut proteins was predicted to primarily demonstrate ACE-inhibitory and DPP-IV-inhibitory activities, while anti-oxidative activity was a much less likely third category (Gülseren, 2018).

Angiotension I-converting enzyme (ACE) converts angiotensin I to angiotensin II, which is a vasoconstrictor. Also, ACE hydrolyzes bradykinin, which is a vasodilator peptide. Consequently, ACE action elevates blood pressure in humans (Vermeirssen et al., 2004; Hartmann and Meisel, 2007). ACE-inhibitory peptides have the capabilities of reducing blood pressure and the likelihood of hypertension (Vermeirssen et al., 2004). Although bioactive peptides are usually not as efficient as synthetic drug molecules, due to the absence of pronounced side effects, they can conveniently be utilized in functional foods or food supplements (Li et al., 2005). Furthermore, it is thought that the

rate of bioactive peptide removal from the body is lower than that of synthetic drugs and the affinity to tissues is higher (Erdmann et al., 2008).

In this study, a further attempt was carried out in order to investigate the ACE-inhibition characteristics of tryptic hazelnut peptides *in vitro*. Due to the promising results presented here, a comprehensive study on the influence of various proteases on bioactivities and allergenic characteristics of hazelnut proteins is currently being executed.

MATERIALS and METHODS

Production of protein concentrates

Cold press deoiled hazelnut meal samples were generously donated by Oneva (Neva Foods Ltd., İstanbul, Turkey), a local manufacturer of cold press oils. In all cases, the maximum temperature observed by the cold press samples was lower than 40°C. All chemicals used were of reagent grade and purchased from Sigma-Aldrich, unless otherwise stated.

Protein extraction was based on the alkaline extraction-isoelectric precipitation (AE-IP) method. In this technique, firstly, solubilization of protein molecules at basic pH is carried out, which was followed by the isoelectric precipitation at acidic pH values (Boye et al. 2010). Briefly, 50 g of deoiled meal was dispersed in water (1:15, w/v) and the pH of the medium was adjusted to pH 9.5 using 1.0 N NaOH. The dispersions were kept stirred at 500 rpm at ambient temperature (22±1°C) for 2 h to facilitate hydration. Immediately afterwards, the dispersions were centrifuged at 10000×g for 15 min at 4 °C using a CR22N high-speed refrigerated centrifuge (Hitachi Koki Co., Ltd., Tokyo, Japan). The supernatant containing the solubilized proteins was collected and the medium pH was adjusted to pH 4.5 in order to induce isoelectric precipitation. To ensure the completion of protein precipitation, the supernatant was once again centrifuged at identical conditions. The pellet was collected and immediately frozen at -20 °C. Frozen samples were lyophilized using a Teknosem TRS 2/2V freeze drier (Teknosem Corp., İstanbul, Turkey).

In vitro trypsinolysis

Trypsinolysis was carried out based on the previous literature (Gülseren and Corredig, 2013). An aqueous dispersion of the protein concentrate was prepared in 50 mM sodium phosphate buffer (pH 7) at a protein concentration level of 1% by stirring for 1 h. Trypsin was obtained from Sigma Aldrich Corp. (T1426). Tryptic digestion was carried out for 2 h at an enzyme to protein ratio of 1:1000 (w/w) on a thermomixer (MIULAB Thermo Shaker Incubator, 37°C, 1000 rpm). Immediately afterwards, the digest was heated to 95°C and held at this temperature for 5 min. The samples were rapidly cooled using ice. Upon reaching the ambient temperature, the samples were centrifuged for 30 min at 5000×g to remove insoluble aggregates. Prior to chromatographic analysis, all samples were filtered through 0.45 µm PTFE syringe filters (Isolab, Germany).

Size exclusion chromatography (SEC)

In order to monitor the progress of trypsinolysis, size exclusion chromatography was utilized on a Shimadzu LC-20AD HPLC system (Shimadzu Scientific Instruments) which consisted of a pump, thermostated column compartment and photodiode array detector. Immediately after trypsinolysis, the molecular size distribution of the partially hydrolyzed peptides were analyzed using a desalting column (HiTrap™ desalting column, Product No: 17-408-01, GE Healthcare, Uppsala, Sweden). The column equilibration and elution buffer was 50 mM phosphate buffer (pH 7) which contained 0.5 M NaCl. An isocratic flow was utilized (1 ml/min and 40°C). The detection wavelength was 214 nm. As a molecular size reference, a standardized BSA solution was used (Product No: P5619, Sigma-Aldrich Corp).

Fast protein liquid chromatography (FPLC)

Hazelnut proteins and their corresponding peptides were also analyzed using AKTA - Pure FPLC System (GE). In order to facilitate separation, 1 ml HiTrap DEAE FF anion exchange columns (GE) were utilized. Analysis was carried out for 40 column volumes (CV) using 50 mM Tris-HCl buffer as the eluent (pH 8.5), while a NaCl gradient (0-0.6 M) to ensure fractionation. UV detection was carried out at 280 nm.

Raman spectroscopy

Raman spectroscopy was carried out using a Rigaku Progeny X2 instrument. The spectral range was 200 – 2000 1/cm, while the spectral resolution was 7 – 10 1/cm. TE cooled CCD detection was used at a wavelength of 785 nm. BSA reference solution was utilized for comparative purposes.

Measurement of ACE inhibitory activity

All the solutions and reactants used in this assay were prepared in 100 mM sodium borate buffer (pH 8.3). ACE-inhibition assay was carried out based on the method described by Sheih et al. (2009) with slight modifications. 200 µl of 5 mM HHL substrate (N-Hippuryl-His-Leu hydrate) was mixed with peptide dispersions prepared by trypsinolysis. The mixture was incubated at 37°C for 10 min. Immediately afterwards, 20 µl of concentrated trypsin solution was added to generate an enzyme concentration of 1.68 mU in the mixture. The incubation was carried out for 0-4 h at 37°C and stopped by the addition of 250 µl HCl (1 M). The final mixture was injected into the HPLC device for the determination of hippuric acid (Sheih et al., 2009). Ascentis C18 Column (4.6 mm ID × 250 mm, Supelco) with a particle diameter of 5 µm was used in the analysis. The mobile phase was composed of 1:1 mixture of ethanol and water by volume containing 1 ml/L TFA. The absorbance was detected at 228 nm. The analysis was carried out at 40°C at a flow rate of 1 ml/min. In order to determine % ACE inhibition, the performance of the peptide samples was compared to the blank sample (i.e.,

water was added instead of peptide dispersion). 0.02% sodium azide was added to all samples and solutions as a bacteriostatic in all cases.

Statistical analysis

Data were presented as the means ± standard deviations from at least triplicate experiments in all cases. The differences between samples were also evaluated based on statistical significance ($P < 0.05$). Statistical significance was determined based on ANOVA tests.

RESULTS and DISCUSSION

Firstly, using a thermomixer, trypsinolysis of hazelnut protein concentrates was carried out at 37°C at an enzyme: substrate ratio of 1:1000. The progress in hydrolysis (0-4 h) was monitored based on size exclusion chromatography (Figure 1). As time passed, the peaks slightly shifted to the right-hand side of the spectrum indicating that there were smaller moieties in the samples due to enzymatic hydrolysis. Since the total number of moles in the system might have increased and some of the peptide bonds (i.e., 214 nm detection) became more detectable, in some cases the total peak area significantly increased as well. Variations in the molar extinction coefficients could also lead to this observation. As trypsinolysis took place, an increasing extent of smaller peptides were observed. Furthermore, UV absorbance values decreased with hydrolysis time as previously observed by De Gobba et al. (2014).

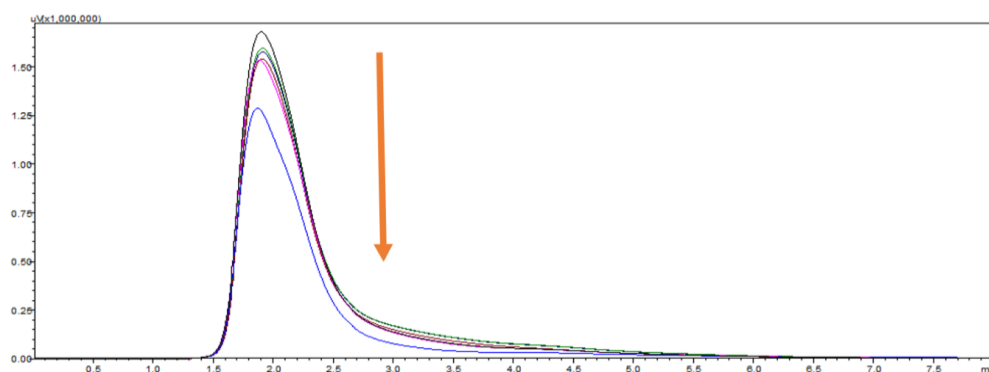


Figure 1. Size exclusion chromatography (SEC) analysis of trypsin treated hazelnut protein concentrates. Time of hydrolysis (0-240 min) increased in the direction of the arrow. Every curve represented a single time point in trypsinolysis (0, 15, 30, 60, 120 and 240 min).

To further illustrate the differences between the untreated protein concentrates and trypsin treated samples (4 h), FPLC was utilized (Figure 2). Using an anion exchange column and a linear gradient of NaCl (0-0.6 M) for 40 column volumes (CV), untreated and trypsin treated protein concentrates were analyzed. Figure 2 demonstrated the differences between the two samples clearly pointing out that trypsinolysis took place. In the hydrolyzed sample, a number of new peaks were found to exist. Previously, it was shown that FPLC approach can be utilized in the

fractionation of bioactive peptides and evaluation of their bioactivities including ACE-inhibitory characteristics (Kapel et al., 2006). The values for UV absorbance decreased considerably due to the formation of the new peaks which corresponded to the formation of new peptide fractions. While unbound fractions were detected rapidly prior to the salt gradient, the increasing salt concentrations enabled the elution of various bound fractions.

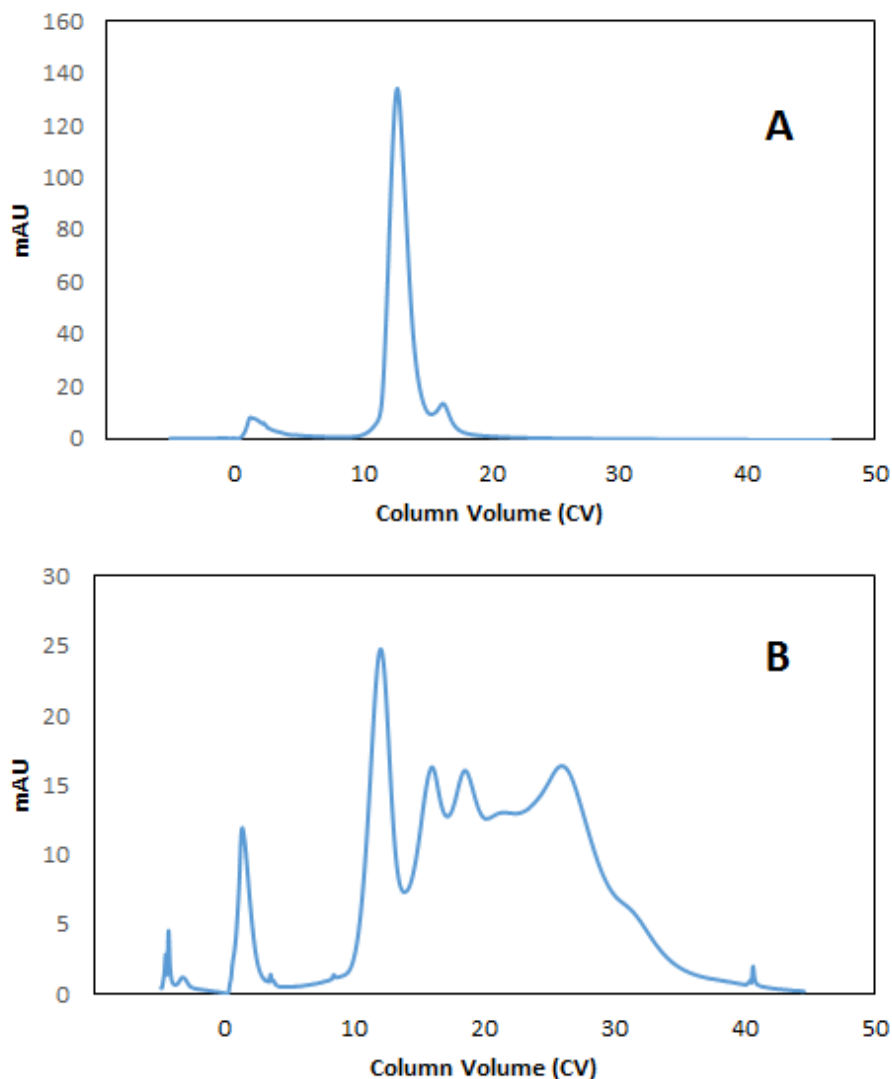


Figure 2. Elution of (A) untreated and (B) trypsin treated hazelnut protein concentrates based on anion exchange separation. Conductivity data was plotted as a function of column volume (0-40 CV).

In order to confirm the data obtained from HPLC-SEC and FPLC analyses, Raman spectroscopy was utilized in the analysis of identical samples (Figure 3). BSA was added as a reference protein to this measurement. Firstly, at all levels of trypsinolysis, the main features in the spectrum were Amide I and III bands and C-C bands. In most cases while the duration of enzymatic treatment increased, the intensity of these bands became more intense possibly indicating that amide bands were more available for detection due to hydrolysis of the proteins.

Amide I band is generally characterized by C-O bond stretching and N-H bond bending in peptides and proteins. In addition, its intensity is

a measure of the presence of α -helical proteins. Increase in the intensity of this band could point out to the fact that during trypsinolysis α -helical content increased for the current samples. Meanwhile Amide III band is characterized by C-N bond stretching and N-H bond bending (Li-Chan, 1996; Herrero et al., 2008a, b). Based on the changes in Amide III band, it might be possible to hypothesize that structural changes including secondary and tertiary level took place due to enzymatic hydrolysis. This could possibly be influential on the biological and technical functionality of the proteins as well, such as their water and/or oil binding, solubility, and hydrophilicity/hydrophobicity characteristics (Herrero et al., 2008a,b).

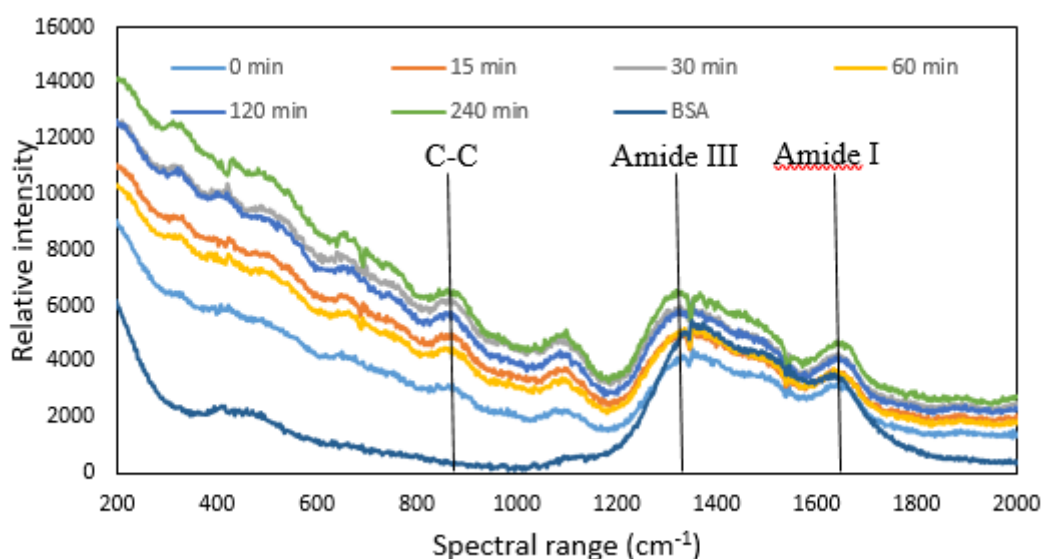


Figure 3. Raman spectroscopy analysis of trypsin treated hazelnut protein concentrates. Enzymatic digestion was carried out at 37°C at an enzyme: substrate ratio of 1:1000 (w/w). In order to enhance the reproducibility, a thermomixer was utilized (1000 rpm). Every curve represented a single time point in trypsinolysis (0, 15, 30, 60, 120 and 240 min). Reference sample was BSA with no trypsin treatment.

Aliquots from tryptic digests studied in the SEC, FPLC and Raman experiments were collected and further investigated in the ACE-inhibition assays (Figure 4). Although small, the untreated protein concentrate demonstrated a limited extent of ACE-inhibitory activity (7.6%). With the increasing extent of hydrolysis (Figures 1), the extent of ACE-inhibitory activity also increased

and reached to about 40% inhibition after 4 h of trypsinolysis (Figure 4). These findings were coherent with our previous findings since most of the ACE-inhibitory peptides were predicted to be di- or tripeptides (Gülseren, 2018), for which becoming dominant in the system might require extended treatments. In the literature, Teh et al. (2016) demonstrated that ACE-inhibitory

activities of hemp protein hydrolysates treated with various proteases generally increased with enzymatic treatment durations up to 4 h. Current data were coherent with these earlier findings, whereas the extent of increase in ACE-inhibitory activities of pinto bean protein hydrolysates were found to be limited beyond 1 h when treated with subtilisins (Garcia-Mora et al., 2015).

All the treatments induced statistically significant differences in ACE inhibition compared to the controls ($P < 0.05$). At the same level, all the treatments also demonstrated significant

differences from each other with the exception of 30 and 60 min samples, which were statistically similar. However, these samples differed from both other treatments and control samples as well ($P < 0.05$). Based on the current results, hazelnut meal could be considered as a valuable source of ACE-inhibitory peptides, and hazelnut consumption could lead to cardioprotective effects due to the combined effects of both its oil and protein fractions. Meanwhile the potential influence of hazelnut proteins and peptides on other bioactivities remain largely unexplored.

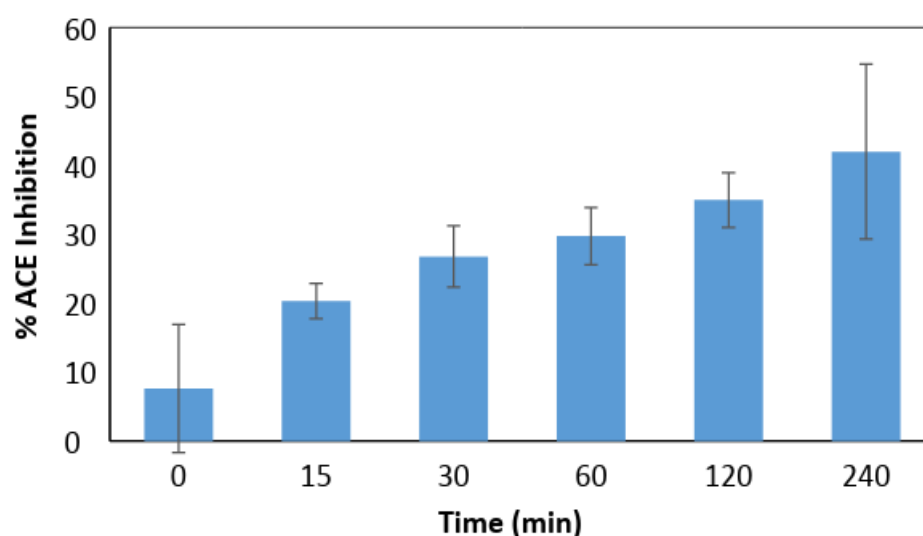


Figure 4. % ACE inhibitory activity of tryptic peptides from the hazelnut protein concentrates as a function of time. % Inhibition was calculated in comparison to a positive control where ACE activity was taken as 100%.

CONCLUSION

In this preliminary report, *in vitro* studies were carried out using hazelnut protein concentrates and a modest extent of ACE-inhibitory activities were detected for all aliquots withdrawn from the trypsinolysis experiments. % ACE-inhibition increased with the extent of trypsin treatment. While studies on other bioactivities remain unexplored so far, we are currently studying the influence of protein isolation methodologies, protein concentrations, and various proteases in order to fully elucidate the bioactive potential of

hazelnut peptides as well as identifying the cardioprotective proteins and peptides from the hazelnuts. Another concern is the reduction of allergenic effects during the enzymatic treatments, which could further enhance the chances of utilizing hazelnut peptides in food formulations. Circulatory system diseases are the primary cause of all death events in Turkey (approx. 40%) (Anonymous, 2015) and many other countries globally, which practically implies the cardioprotective potential of hazelnuts has to be fully exploited.

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