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PHYSIOCHEMICAL PROPERTIES AND ACE INHIBITORY CAPACITY OF HAZELNUT PROTEIN ISOLATE AND HYDROLYSATES

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ABSTRACT

Protein solubility, water holding capacity, fat absorption capacity, emulsion activity index, emulsion stability index, gel formation capacity and Angiotensin-Converting Enzyme (ACE) inhibitory capacity of hazelnut protein isolate (HPI) and its hydrolysate were determined. The results showed that protein solubility had U shape solubility profile with an isoelectric point at pH 4.5-5 and highest value at pH 2 and pH 10. Water holding capacity and fat absorption capacity of HPI was found as 1.95 mL water/g protein and 2.1 g fat/g protein, respectively. Emulsion activity index and emulsion stability index values were found as 44 m^2/g and 46 min at pH 7.0, respectively. Weak gel formation of HPI was seen at 8% and firm gel (LGC) was observed at 12% protein concentration. The concentration of pepsin hydrolysates needed to inhibit 50% of the ACE activity values (IC50) at 0, 30, 60 and 120 min of hydrolysis were 1.47 mg protein/mL, 0.27 mg protein/mL, 0.27 mg protein/mL and 0.26 mg protein/mL respectively. Whereas, IC50 of trypsin hydrolysates at 0, 30, 60 and 120 min were 5.51 mg protein/mL, 0.61 mg protein/mL, 0.56 mg protein/mL and 0.54 mg protein/mL. These results showed that isolates and hydrolysates of hazelnut has valuable functional properties and noted as good candidates for effectively reducing hypertension.

Keywords: Protein solubility, water holding, fat absorption, emulsion activity, gel formation capacity, Angiotensin-Converting Enzyme Inhibition

INTRODUCTION

New life style and longevity expectation of modern societies change nutrition manner of human. Nowadays, peoples and authorized bodies prefer preventive methods instead of treatment to reduce costs of health care. Importance of functional food, with its health promotion, disease risk reduction and reduction in health care costs rises day by day. Among all, functional foods with high content of protein have attracted because of theirs potential effects on supporting health and minimizing disease risk (**Kong *et al*. 2007**). Moreover, some functional properties of protein are used for a processing booster on sensory characteristics or to increase physical behavior of foods during processing and storage (**Ahmedna *et al*. 1999**).
Bioactive peptides can be produced by enzymatic hydrolysis of plant and animal food products, which are normally inactive within the parent protein. Importance of these enzymatically produced peptides generally arises from their nutritional, functional and biological characteristic from self-constituent protein (**Kong *et al.* 2007**). They have functional properties such as reducing blood pressure, strengthening immune system, antimicrobial effect, antioxidant activity, reducing cholesterol, mineral binding activity, binding of bile acids and reducing of blood pressure depending on the amino acid sequence (**Humiski and Aluko 2007; Theodore and Kristinsson 2007**). The specificity of the enzyme, condition of enzymatic reaction and enzyme/substrate ratio have a great influence on functional properties of peptides (**Norris *et al*. 2012; Wu and Ding 2002; Wu *et al*. 2016**).
Antihypertensive effect of several peptides has become prominent for a while now. Angiotensin converting enzyme (ACE) is of primary importance for hypertension by regulation of blood pressure. ACE converts angiotensin I into angiotensin II, a highly potent vasoconstrictor, with simultaneous inactivation of bradykinin, a vasodilatory peptide involved in local flow regulation (**Donkor *et al*. 2007; Erdmann *et al*. 2008; Fujita *et al*. 2000**). ACE inhibitory peptides have been found in many food protein sources (**Otte *et al*. 2007**).
Hazelnut (*Corylus maxima* M. and *Corylus avellana* L.) is a popular tree nut worldwide. Turkey, with 75% of total hazelnut *(Corylus Avellana* L.) production, is the largest producer of the world (**Alasalvar *et al*. 2009**). Hazelnut has been subject of many researches because of its high content of phytochemicals like unsaturated fatty acids (mainly oleic acid) carbohydrate, dietary fiber, vitamins (vitamin E), minerals, phytosterols (mainly β-sitosterol), and antioxidant phenolics contents which play a major role in human nutrition and health (**Alasalvar *et al*. 2003; Oliveira *et al*. 2008**). It is clarified that with 17.4-20.8% of protein content, hazelnut has much more protein than egg or cereals and it is almost same with meat and dried legumes protein content (**Koksal *et al*. 2006).**
The aims of this study were to investigate protein solubility, water holding capacity, fat absorption capacity, emulsion activity, emulsion stability and gel formation capacity of hazelnut protein isolates, produced by isoelectric precipitation method and to determine ACE inhibitory activity of enzymatically hydrolyzed hazelnut protein isolate.

MATERIALS AND METHODS

The raw hazelnut was purchased from a local market (Mersin-Turkey). Kjeldahl catalyst, hydrogen peroxide, methyl red, bromcresol green, boric acid, hydrochloric acid, trypsin, phosphoric acid, n-hippuryl-his-leu hydrate powder, hippuric, angiotensin converting enzyme from rabbit lung (ACE-1.0 unit), trifluoroacetic acid, acetonitrile were purchased from Sigma-Aldrich (USA). All the other chemicals were of analytical grade.

Preparation of Defatted Hazelnut Flour

In this work, unshelled, plump raw hazelnut fruit was used in order to obtain protein concentrate. 500 g of hazelnut fruits were rehydrated in 1,000 mL of water for a night. Rehydrated fruits were dried in a drying oven at 30 °C for 6 hours to remove hulls. In order to prevent protein denaturation during grinding process, unshelled raw fruits were freezed in liquid nitrogen. Frozen hazelnut were grinded by laboratory type grinder (IKA-Werke M20, Germany). Ground hazelnut were mixed with petroleum ether and homogenized in blender (Waring, CB15E, USA) for effective fat extraction. Hazelnut meal were mixed at magnetic

stirrer for 6 hours and filtrated using coarse filter paper by vacuum pomp. Filtered hazelnut meal was again mixed with petroleum ether for 6 hours. After filtration, hazelnut meal was dried in an oven at 30 °C for one night in order to remove petroleum ether.

Preparation of Protein Isolates.

Protein isolates were prepared by some modification of method described by Siddeeg *et al*. (2014). Ground and defatted hazelnut flour was suspended in water with the ratio of 1:10 (w/v). pH was adjusted to 9.0 by 1 N NaOH. Suspension was stirred for 1 hour with a magnetic stirrer. After 1 hour, slurry was centrifuged at 9,000 x g for 30 min at 4 °C. Supernatant was collected in a beaker and pellet was again mixed with water in the ratio of 1:5 (w/v) and same extraction procedure was applied. Slurry was again centrifuged at 9.000 x g for 30 min at 4 °C. Supernatant gained from two centrifugations were collected. The pH of supernatant was adjusted to 4.5 with 1 N HCl and stirred for 30 min. Precipitate was separated by centrifuge at 9,000 x g for 30 min at 4 °C and supernatant was discarded. Pellet was suspended with water and pH was adjusted to 7.0 using 1 N NaOH. Dried HPI was obtained from suspension by using of freeze drier (Telstar Cryodos Model: 230, Spain). HPI was stored in a refrigerator at 4 °C until use. Total protein content of the samples were determined by micro-Kjeldhal method (AACC 2000).

Protein Solubility Analysis

Solubility of protein was determined by some modifications of method described by **Carbonaro *et al*. (1997)**. HPI suspension was prepared 1% by water. The pH of suspension was adjusted with HCl and NaOH in a range of 2.0-10.0. The pH adjusted suspensions were stirred in magnetic stirrer for 30 min at room temperature and centrifuged at 4,000 x g for 30 min at 20 °C. Soluble protein concentration in supernatant was determined by Lowry method (Lowry *et al*. 1951).

Water holding capacity

Water holding capacity determined by modified method of Boye *et al*. (2010) 0.5 g of HPI was weighed in a pre-weighed centrifuge tube. 3.0 mL of water was added into the tube. After 1 hr of stirring, slurry was centrifuged at 2,000 x g for 30 min at 25 °C to collect the sediment. The centrifuge tube with HPI pellet was weighed. Water holding capacity of HPI was calculated as (M2-M1)/(M1) where M2 was weight of water absorbed HPI sediment. M1 was weight of dry sample and expressed as amount of water absorbed by 1 g of dry isolate.

Fat absorption capacity

Fat absorption capacity was carried out by the method explained above for water holding capacity with some modification **(Boye *et al*. 2010).** 0.5 g of HPI was weighed in a pre-weighed centrifuge tube and mixed with 3.0 mL of sunflower oil. Suspension was stirred for 1hr and centrifuged at 2,000 x g for 30 min at 25°C. The tubes were weighed with pellets after discharge of residual oil if any. The fat absorption capacity was calculated by weight difference of oil absorbed and dry protein isolate and expressed by 1 g of dry isolate.

Emulsion activity and stability

Emulsion activity and emulsion stability were determined according to method of Pearce and Kinsella (1978). 0.1% protein solution was prepared with 0.1 M phosphate buffer (pH 7.0) and stirred 1 hour. 30 mL of protein solution was homogenized with 10 mL of sunflower oil at room temperature using homogenizer (Ultra-Turrax T-50) for 1 min at 20,000 rpm. 0.1 mL of samples were taken at 0 and 10 min after emulsion formation and mixed with 0.1% of sodium dodecyl phosphate (SDS). Absorbance was measured at 500 nm with Perkin Elmer UV/VIS spectrophotometer (Lambda EZ201). 0.1% SDS solution was used as blank. Emulsion activity index (EAI) and emulsion stability index (ESI) were calculated by following equations.

$$EAI = \frac{2.303 \times 2 \times A \times D}{(1-V) \times C \times 10{,}000} \qquad ESI = \frac{A0 \times t}{\Delta A}$$

Where A: Absorbance at 500nm, D: Dilution factor (100), V: Volume fraction of oil (0.25), C: Protein content in beginning (0.1%), A0: Absorbance value of emulsion at 0 min, t: time (min) and ΔA: The absorbance difference between 0 and 10 min

Gel formation

Gel formation was determined by some modifications of method described by Sathe and Salunkhe (1981). Protein solutions were prepared with 0.075 M phosphate buffer (pH 7.6) to obtain 3 mL of protein concentrations increasingly from 6% to 24% in screw cap plastic tubes. Tubes were heated at boiling water for 30 min and then samples were cooled to 4 °C for one night in a refrigerator. Tubes were controlled for gelation. The least gelling protein concentrate (LGC) that firm gel was formed was determined.

Enzymatic hydrolysis for preparing antihypertensive peptides

Protein hydrolysis was carried out according to Wu and Ding (2002) with some modification. Before hydrolysis, 6% of HPI were homogenized for 30 min phosphate buffers at pH 2.0 and pH 7.0 for pepsin and trypsin hydrolysis, respectively. Pepsin and trypsin enzymes were added with 1:20 enzyme substrate (w/w) ratio at 37 °C for 120 min. Samples were taken at the beginning and 30, 60 and 120 min of hydrolysis. All samples were placed in to 85 °C hot water for 10 min to terminate enzymatic activity and centrifuged for 15 min at 4000 x g. Supernatant were used for measuring ACE inhibitory activity.

Determination of ACE Inhibitory Activity

ACE inhibitory effect of hazelnut protein hydrolysates were determined according to method defined by **Wu and Ding (2002)** with some modifications. ACE normally catalyzes N-hippuryl-l-histidyl-l-leucine (HHL) to form hippuric acid (HA), but formation of HA is inhibited by hazelnut protein hydrolysates samples (called as inhibitor). This method was based upon to determine ACE inhibition by using hazelnut hydrolysates. Analyses were performed by addition of 40 µL of 2.0 mU ACE solution into mixture of 40 µL hazelnut protein hydrolysate and 200 µL of a 2.0 mM HHL substrate solution. All solutions were prepared by 100 mM NaCl-borate buffer at pH 8.3. After incubation at 37 °C for 1 hour, the reaction was terminated by adding 340 µL of 1 M HCl. Samples were filtered by using 0.45 µm filters (Millipore, Bedford, MA). Water was used instead of hazelnut protein hydrolysates at control samples, in which it was assumed that HHL was catalyzed for 100% of HA formation.
A Shimadzu 20AD (Japan) HPLC system consisting of a diode array detector was used, and HA was detected at 228 nm. Samples (10 µL) were analyzed by Inertsil ODS 4 (4.6 x 150 mm 5µm) column. Elution was achieved with two solvent system: (A) 0.05% TFA in water and (B) 0.05% TFA in acetonitrile with linear gradient from 5 to 60% B in 10 min. This was followed by isocratic elution for 2 min at a level of 60% B and reduced to 5% B in 1 min. It was followed an isocratic elution for 4 min at this level. Flow rate was constantly 1 mL/min. HA standard curve was prepared from 0.02 mM to 1 mM concentrations with 100 mM NaCl-borate buffer at pH 8.3.

ACE inhibition (%INH) was found using following formula:

$$\%\text{INH} = \frac{(M^{control} - M^{inhinitor})}{M^{control}} x100$$

$M^{inhibitor}$: HA value of inhibitory sample (mM)
$M^{control}$: HA value of control sample (mM)

The necessary concentration of inhibitor required to inhibit 50% of the ACE activity (IC_{50} values) were measured by diluting inhibitor with 2-150X dilution factor.

Statistical analysis

All analyses were done at least in triplicate**.** All results were analyzed by one way analysis of variance (ANOVA) using SPSS. If differences of mean values were significant ($p < 0.05$), differences were evaluated by Tukey's test

RESULTS AND DISCUSSION

Protein Isolate

The fat content of hazelnut was reduced from 62% to 6% by fat defatting before alkaline extraction of protein. In order to ensure effective fat extraction and prevent protein denaturation, hazelnut fruits was firstly grinded after frozen by liquid nitrogen for 30 sec. Final protein and water content of the protein isolate was calculated as 89.71% and 0.8%, respectively after alkaline extraction, isoelectric precipitation and freeze drying applications. These results were compatible with several reports. Defatted hazelnut flour protein content on a dry base was found as 91% (**Tatar *et al*. 2015**). **Aydemir *et al*. (2014)** reported that protein content of HPI was 94.2%. **Saetae *et al*. (2011)** also reported that the yield, moisture content and total protein content of the HPI were 12.6%, 3.43% and 89.95%, respectively. Even if high protein content of HPI is not the only selection criteria for the food additive, it is one of the basic properties that can provide enough amount for human consumption.

Protein Solubility

Protein solubility represents very significant functional property because of its effect on some important properties like emulsification. In this study, protein

solubility of HPI at different pH gave U shape solubility profile (Figure 1). According to figure, minimum HPI solubility was determined in the pH range of 4.5-5 which was around isoelectric point. Solubility of HPI increased substantially at acidic and alkaline pH. At pH 2 and pH 10, solubility of protein was reached 79.12% and 86.87% respectively. According to Yuliana *et al*. (2014), at alkaline and acidic region proteins are charged as negatively or positively and keep apart from each other because of the electrostatic repulsive force. This causes to increase the solubility of proteins.

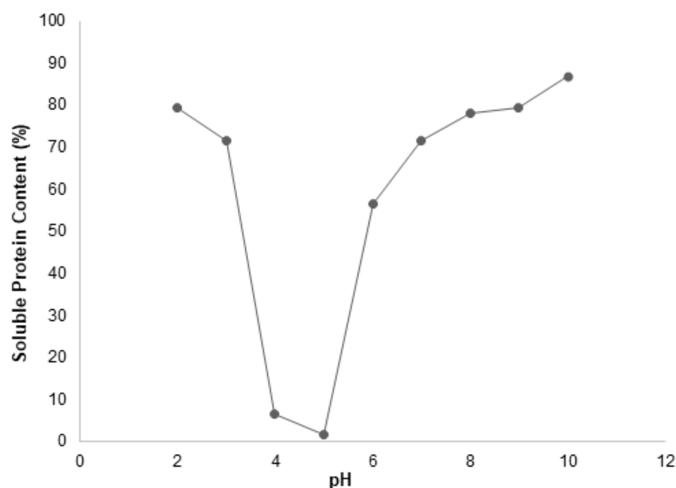

Figure 1 Hazelnut protein solubility

This U shape profile of HPI solubility was rather similar with those of other nuts and seed proteins. Almost all nuts and seed protein solubility profile gave minimum around pH range of 4-6 (**Adiamo *et al*. 2016; Aydemir *et al*. 2014; Saetae *et al*. 2011; Siddeeg *et al*. 2014; Tatar *et al*. 2015; Yuliana *et al*. 2014**). The solubility of food protein is especially important when this protein is used as an ingredient of a food product. While some acidic food needs additive protein soluble at low pH, there may be needed soluble protein at alkaline conditions. (**Adiamo *et al*. 2016**).

Water holding capacity

Water holding capacity is the factor of protein structure, amino acid composition, surface polarity and especially aromatic surface hydrophobicity of protein (**Saetae *et al*. 2011**). In the current study, water holding capacity of HPI was found as 1.95 mL/g protein and this result was reasonable with some studies on nut proteins. Water holding capacity of physic nut protein concentrate, cashew nut protein isolate, chilean hazelnut protein concentrate, peanut protein concentrate, cashew nut proteins were found as 3.22 g water/g , 2.20 mL water/g, 3.20 g water/g, 1 mL water/g, 1.45 mL/g respectively (**Neto *et al*. 2001; Ogunwolu *et al*. 2009; Saetae *et al*. 2011**).

Fat absorption capacity

Fat absorption capacity is another significant functional property of food protein. Although water holding capacity is related with polarity of protein, non-polar amino acid chain of protein can give more fat absorption capacity because of hydrophobic interaction between amino acid and lipid hydrocarbon chain. (**Saetae *et al*. 2011**). Fat absorption capacity of HPI was found as 2.1 g fat/g protein. Tatar *et al*. (2015) reported that higher fat absorption capacity improve emulsifying capacity of food products. Fat absorption capacity result of HPI was comparable with that of other food protein. Fat absorption capacity of physic nut protein concentrate, native Brazil nut Kernel, Cashew nut protein isolate were found as 1.86 mL oil/g protein, 1.41 mL/g protein, 4.42 mL/g, respectively (**Ramos and Bora 2005; Saetae *et al*. 2011**)

Emulsifying activity and stability

Emulsifying activity index (EAI) and emulsifying stability index (ESI) of HPI at pH 7 were found as 44 m^2/g and 46 min, respectively. Polar, non-polar and non-charged polar structures of proteins together have both hydrophobic and hydrophilic surfactant effect. Being polar and nonpolar side chains in a protein improves stability of various emulsions composed of oil and water together in food systems (**Yuliana *et al*. 2014).** Although there has been made various studies on EAI and ESI, percentage (%) has been usually used as a unit. Also, studies has been done various pH regions. This made difficult to compare the studies. However, the researches show that at isoelectric region, proteins have low emulsifying activity and it increases dramatically with increasing pH. The observed EAI value of HPI was comparable with that of the Cashew nut protein isolate measured at different pH. EAI values of cashew nut protein at pH 4 and pH 12 were 24.8 m^2/g and 57.3 m^2/g, respectively (**Ogunwolu *et al*. 2009).** According to **Boye *et al*. (2010)**, EAI values of some pulse proteins (lentils and chickpeas protein concentrate) obtained by isoelectric precipitation were between 4.6 m^2/g and 5.6 m^2/g. ESI values were between 17.8 min and 19.7 min.

Gel formation Capacity

Gel formation capacity of protein is one of the most significant factors that define textural properties of food. In the current study, weak gel formation of HPI was seen at 8% and firm gel (LGC) was observed in a protein concentration of 12% (Table 1).

Similar findings have been reported by several researchers. Weak and firm gel were formed at 6% and 8% concentrations at green lentil concentrate (**Boye *et al*. 2010**). Cashew nut protein isolate LGC was found as 13.5% (**Ogunwolu *et al*. 2009**). **Sharma *et al*. (2010)** found same hazelnut protein LGC as current study. In same study, almond, Brazil nut, cashew, macadamia, pine nut, pistachio, Spanish peanut, Virginia peanut, and soybean LGSs were determined as 6%, 8%, 8%, 20%, 12%, 10%, 14%, 14%, 16%, respectively. Gel strength of proteins increases according as increment of protein concentrate. In food processing using proteins with small LGS means better gelation capacity of protein to form gel (**Boye *et al*. 2010).**

Table 1 Gelling behavior of HPI concentrates

HPI concentration (%)	Gelling Behavior
2	(-)
4	(-)
6	(-)
8	(±)
10	(±)
12	(+)
14	(+)
16	(+)

(-): No gel; (±): Weak gel; (+): Firm gel (LGC)

ACE Inhibitory Effect of Hazelnut Protein and Peptides

Samples taken at 0 min, 30 min, 60 min and 120 min samples of pepsin hydrolysis were called as P0, P30, P60, P120, respectively, and those of trypsin hydrolysis were called as T0, T30, T60 and T120 respectively.

%INH of pepsin hydrolysates and also P0 had almost 95% ACE inhibitory activity. Although trypsin hydrolysates (T30, T60 and T120) has about 92% ACE inhibitory effect, %INH of T0 was around 80%. The change of ACE inhibitory activity versus hydrolysis time during pepsin and trypsin hydrolysis were shown Figure 2.

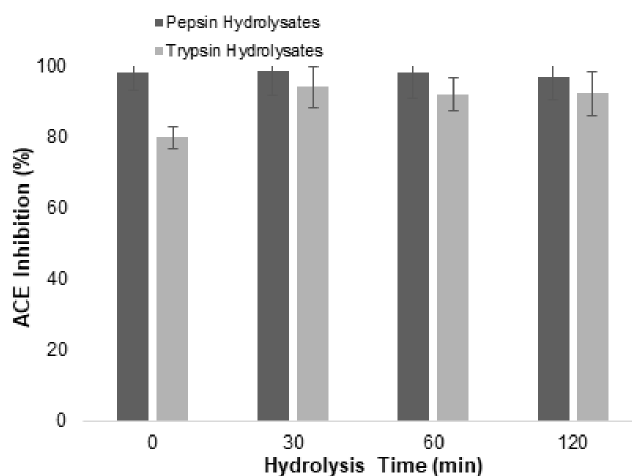

Figure 2 ACE inhibitory activity of hydrolysates

%INH of non- hydrolyzed samples (T0 and P0), prepared with pH 2 and pH 7.5 buffers, were different from each other (p<.0001). This could be the effect of pH on ACE inhibition. At one previous work, ACE inhibitory activity of non-hydrolyzed samples at pH 7.5 resulted in 70-80% of %INH value (**Aydemir *et al*. 2014).** This result is compatible with the current study. Moreover, %INH value of both trypsin and pepsin hydrolysate gave maximum value at first 60 min. Numerous researchers have addressed that 60 min of hydrolysis is enough for ACE inhibitory activity of peptides and increasing hydrolyzing time has no effect on inhibitory activity (**Cha and Park 2005).**

In pepsin hydrolyses, samples with 24.81-29.03 mg/mL protein contents gave ACE inhibition level between 96.93 and 98.42%. However, in trypsin hydrolyses,

ACE inhibition changed between 79.82 and 92.11% with 27.43-30.79 mg/mL of protein concentrate.

In order to compare ACE inhibition level with literature, IC_{50} value was determined by diluting protein extracts with rates of 2 and 150X dilution factor. Calculated IC_{50} values of samples taken at 0, 30, 60 and 120 min of pepsin hydrolysis were 1.47 mg protein/mL, 0.27 mg protein/mL, 0.27 mg protein/mL and 0.26 mg protein/mL, respectively. The IC_{50} values of samples during of trypsin hydrolysis were calculated in a similar method. The IC_{50} values of 0, 30, 60 and 120 min samples of trypsin hydrolysis were 5.51 mg protein/mL, 0.61 mg protein/mL, 0.56 mg protein/mL and 0.54 mg protein/mL, respectively. Changes of IC_{50} throughout at both hydrolysis have been given at Figure 3.

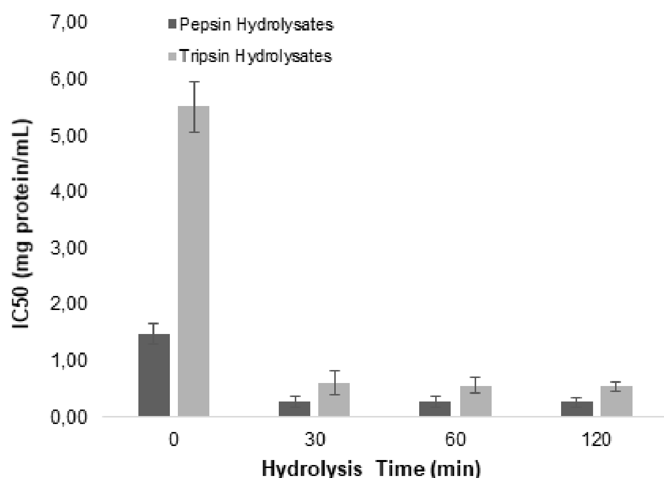

Figure 3 IC50 of hydrolysates

The IC_{50} values of samples of pepsin and trypsin hydrolysis had significantly decreased after 30 min of hydrolysis. These scale-down of IC_{50} value suggest an apparent increasing of ACE inhibitory activity. IC_{50} value of P0 showed almost 4 times more antihypertensive activity than that of T0. This difference has been considered as the reason of acidic hydrolysis of pH 2 sample. IC_{50} of P30 was 0.27 mg protein/mL, which was 5.44 fold lower than that of the non-hydrolyzed sample P0 where after 30 min of hydrolysis there was no significant changes in IC_{50}. IC_{50} of T30 was 9.03 fold lower than that of T0. T30, T60 and T120 showed quite similar IC_{50} values (0.61, 0.56 and 0.54 mg protein/mL respectively). There were not too much researches about ACE activities of hazelnut protein. According to **Eroglu and Aksay (2017)**, IC_{50} values of pepsin hydrolysate of hazelnut were 1.29 mg protein/mL, 0.25 mg protein/mL and 0.22 mg protein/mL at 0, 30 and 60 min, respectively. These results were agreeable with present study. Previous reports have also shown that IC_{50} of food proteins decreased after hydrolysis with several enzymes. IC_{50} of albumin, casein, soy protein isolate, wheat gluten and zein were 8.93, 8.45, 7.57, 9.54 and 7.96 mg/mL before hydrolysis. While after hydrolysis with SS103 protease IC_{50} values were 2.35, 1.82, 0.14, 2.89 and 1.38 mg/mL, respectively **(Cha and Park 2005).** IC_{50} of hydrolyzed corn gluten, soy, legumin, soy milk with alfa amylase, pepsin, pancreatin, alcalase enzymes were measured as 0.18, 0.34, 0.18, 0.28 and 0,88 mg protein/mL, respectively **(van der Ven *et al*. 2002; Wu and Ding 2002; Yust *et al*. 2003)**.

CONCLUSION

The present study was undertaken to characterize protein solubility, water holding capacity, fat absorption capacity, emulsion activity, gel formation capacity and ACE inhibitory capacity of hazelnut protein isolate and its hydrolysate. HPI's most functional properties were comparable with soybean, lupin, almond, Brazil nut, cashew, peanut, and lupin protein isolates. ACE inhibitory activity of hazelnut protein improved with both pepsin and trypsin hydrolyzing. According to these results, hazelnut protein isolates and hydrolysates could offer a valuable source of a protein ingredient in food systems.

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