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Research Article/Araştırma Makalesi

Antioxidative activities of hazelnut protein hydrolyzates as predicted by *in silico* analysis techniques

Fındık protein hidrolizatlarının antioksidatif aktivitelerinin in silico analiz teknikleri ile öngörülmesi

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ABSTRACT

Recently, a variety of studies have been carried out on the production and analysis of bioactive peptides. Here, based on *in silico* methods, antioxidative behavior of hazelnut (*Corylus avellana* L.) peptides, which could be prepared by enzymatic treatments with 3 gastrointestinal (GI) and 3 non-GI enzymes were evaluated. On 10/03/2017, UniProt database listed 469 hazelnut proteins. In the current study, a subset (23 ribosomal proteins) of these proteins were examined and the activity of the GI proteases (trypsin, pepsin, chymotrypsin) for the production of antioxidative peptides were compared to non-GI proteases (thermolysin, papain and bromelain). Firstly, potential antioxidative peptide sequences were determined. GI proteases were less effective compared to non-GI proteases in the manufacture of antioxidative peptides. Antioxidative property of peptides, which were obtained by thermolysin or papain treatments, were significantly higher compared to GI proteases. When all 23 proteins were treated 37 antioxidative peptides were formed by thermolysin, while 10 antioxidative peptides were predicted for trypsin. Of the 138 cases studied (23 proteins x 6 proteases), 44 antioxidative peptides were detected (i.e., 1 peptide in approx. 32% of all cases). Based on current findings, hazelnut proteins can be considered a valuable resource for antioxidative peptide manufacture.

Key Words: Plant protein peptides, Common hazelnut (Corylus avellana L.), Ribosomal proteins, *In silico* proteolysis.

ÖZ

Günümüzde biyoaktif peptitlerin üretimi ve analizi ile ilgili olarak birçok çalışma yapılmaktadır. Bu çalışmada, *in silico* yöntemler kullanılarak 3 gastrointestinal (Gİ) ve 3 Gİ olmayan enzim muamelesi ile hazırlanması muhtemel fındık (*Corylus avellana* L.) peptitlerinin antioksidatif davranışları değerlendirilmiştir. 10 Mart 2017 itibariyle, UniProt veri tabanında 469 fındık proteini listelenmiştir. Bu çalışmada, söz konusu proteinlerin bir alt kümesi (23 ribozomal protein) incelenmiştir ve *in silico* proteoliz yöntemleri ile antioksidatif peptitlerin üretimi için gastrointestinal (Gİ) proteazların (tripsin, pepsin, kimotripsin) etkinliği; termolisin, papain ve bromelain gibi Gİ olmayan proteazlarla karşılaştırılmıştır. Elde edilen muhtemel antioksidatif peptit dizilimleri ve bunların sayıları her bir proteaz için belirlenmiştir. Birçok durumda, Gİ proteazlarının antioksidatif peptitlerin üretiminde Gİ olmayan proteazlara oranla daha az etkili olduğu gösterilmiştir. Özellikle termolisin veya papain kullanımı ile elde edilmesi olası peptitlerin antioksidatif özelliği Gİ proteazlara baskındır. Örneğin söz konusu 23 proteinden termolisin kullanımı ile 37 antioksidatif peptit oluşurken tripsin ile toplam 10 antioksidatif peptit oluşması beklenmektedir. 23 protein ve 6 proteaz göz önüne alınarak yürütülen 138 analizin 44 tanesinde (incelenen bütün durumların yaklaşık %32'sinde) antioksidatif peptitlerin oluşma olasılığı saptanmıştır. Mevcut bulgulara dayanarak, fındık proteinlerinin antioksidatif peptitlerin üretimi için değerli bir kaynak olarak kabul edilmesi mümkün görünmektedir.

Anahtar Kelimeler: Bitki proteini peptidleri, Fındık (*Corylus avellana* L.), Ribozomal proteinler, *In silico* proteoliz.

Introduction

Plant proteins represent a sustainable protein source that is both fit for human consumption and reduction of global carbon footprint (Nadathur et al., 2016). Sustainability primarily depends on the poor conversion rate of plant proteins to animal proteins. Once 100 g of plant protein is fed to animals, approximately 15 g animal protein can be manufactured (Day, 2013). Since the global demand for proteins are constantly rising, once again the utilization of alternative protein sources in the manufacture of foods and other commercial products gain importance. In our group, one of the research priorities include production and utilization of plant proteins based on the usage of agricultural and/or industrial byproducts, particularly from cold press deoiled plant meals. Once oil is removed from oilseeds or oil fruits, the protein concentration in the meals significantly increases, hence generating relatively inexpensive byproducts for protein production (Coşkun et al., 2019).

Hazelnut (*Corylus avellana* L.) is one of the most important agricultural products in Turkey (Ozdemir and Akinci 2004) and approximately 80% of the total global harvest is generated. According to TURKSTAT data, hazelnut harvest in 2017/18 accounted for 675.000 metric tons (TÜİK, 2019). Other major hazelnut producers include other Mediterranean countries and the western states of the USA. Based on its high oil content, hazelnut may be considered a high calorie plant food, whereas due its various valuable components, many physiological benefits may occur upon its consumption (Richardson, 1997). A variety of bioactivities including protective effects on cardiovascular system, reduction of cholesterol, antioxidative effects etc. have been reported. The majority of these influences have been attributed to hazelnut oil components (mono- and polyunsaturated fatty acids, especially oleic and linoleic acids) (Parcerisa et al., 1997), polyphenols, tocopherols and squalene (Alasalvar et al., 2006, 2009; Dogan et al., 2007) as well as phytosterols and phytostanol (Miraliakbari and Shadidi, 2008), most of which are oil soluble or dispersible components. The studies on the bioactivities of water soluble or dispersible components including proteins is somewhat limited (Aydemir et al., 2014). Especially since hazelnut kernels contain significant amounts of proteins (10% to 24%) depending on the cultivar (Köksal et al., 2006), biological importance of hazelnut proteins has to be further investigated.

In addition to other valuable oils, the extent of cold press hazelnut oil production is also increasing. Consequently, increasing extents of cold press meals are being produced industrially. The literature on hazelnut proteins has traditionally been focused on allergenic characteristics of these proteins (Vieths et al., 1999; Ortolani et al., 2000; Flinterman et al., 2008). It is noteworthy however that the relative digestive stability of hazelnut allergens was found to be lower than that of peanut allergens (Vieths et al., 1999). A recent study has demonstrated potential antioxidative, anti-carcinogenic and antihypertensive properties of hazelnut protein isolates as well (Aydemir et al., 2014). However, literature on hazelnut protein hydrolysates is largely scarce.

Bioactive peptides are protein hydrolysates that can demonstrate beneficial physiological responses in the human body. These sequences often become active upon *in vitro* or *in vivo* enzymatic hydrolysis (Korhonen and Pihlanto, 2003, 2006), while such activities may not be revealed in the parent molecules. For example, generation of antioxidative responses has been demonstrated in the current literature (Lorenzo et al., 2018). Most of these bioactive molecules are valuable components with high bioactivity at relatively low doses, have the capabilities to interact with specific targets, and demonstrate a variety of therapeutic effects (Agyei and Danquah, 2011). It is possible to mention therapeutic effects such as anti-carcinogenic, anti-oxidative, anti-microbial, anti-HIV, cholesterol or hypotensive, anti-rheumatic

activities among these features (Shahidi and Zhong, 2008). Bioactive peptides can also form during the processing or digestion of protein-containing foods (Korhonen, 2009). In many cases, various proteases were shown to generate bioactive peptides *in vitro* from food proteins (Gibbs et al., 2004; Korhonen and Pihlanto, 2006). As a general rule, hydrolysis of proteins can reduce the content of major protein allergens, while increasing the solubility of peptides by the formation of free amino and carboxyl groups (Sarmadi and Ismail, 2010). Upon the manufacture of bioactive peptides, an improvement in both the technical and biological properties might take place (Karami and Akbari-adergani, 2019).

In this study, *in silico* analysis methods were used to predict the potential bioactivity of peptides that could be formed by enzymatic treatment of ribosomal hazelnut proteins with GI or non-GI proteases. Potential ACE-inhibitory and DPP-IV inhibitory activities of hazelnut peptides were recently published in a previous study from our group (Gülseren, 2018) as well as *in vitro* findings on ACE-inhibitory acitivity (Gülseren et al., 2019). Here, potential antioxidative properties of hazelnut peptides are being further investigated. These studies will collectively enable the manufacture of value added hazelnut components bearing a natural antioxidant identity and without inducing any side effects, in addition to their ACE-inhibitory and DPP-IV inhibitory potential.

Due to the presence of hundreds of hazelnut proteins listed in the currently existing protein databases, a relatively small subset of these proteins (ribosomal proteins) has been investigated in the current work. This specific subset corresponds to approximately 5% of the known hazelnut proteins. Although the results do not represent all hazelnut proteins, these efforts have contributed to the supply of funds that is currently supporting our lab group to work on bioactive hazelnut peptides in detail. Currently *in vitro* studies are underway in our labs and the effect of proteolysis by various proteases on both the generation of bioactive peptides and reduction of allergenic properties are keenly investigated.

Material and Methods

Sequences of the ribosomal hazelnut proteins

Sequences of ribosomal hazelnut proteins were obtained from the UniprotKB database (http://www.uniprot.org) on March 10, 2017. Ribosomal proteins represent 23 (about 5% of all hazelnut proteins) out of 469 hazelnut proteins whose sequence was known at that time. Table 1 listed the basic data of these proteins such as the names of each ribosomal

protein, their corresponding database accession numbers, and their total number of amino acids. Due to the time and labor intense nature of the current analyses, this preliminary study was conducted with a limited subset rather than all hazelnut proteins.

In silico proteolysis and the analysis of bioactive sequences

For the *in silico* proteolysis of ribosomal hazelnut proteins, “enzyme action” tool (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) of the BIOPEP web-server was utilized (Minkiewicz et al., 2008). BIOPEP predicts fragments to be produced from each amino acid sequence, based on the general proteolytic characteristics of each enzyme. As a result, for sequences input to the system, BIOPEP calculates a set of outputs containing the peptides formed as a consequence of proteolysis (Minkiewicz et al., 2008). Some of the additional references explaining the BIOPEP database and certain research findings based on BIOPEP can be located elsewhere (Minkiewicz et al., 2011; Iwaniak et al., 2005; Iwaniak and Dziuba, 2011).

Proteolytic simulations were performed separately using 6 different proteases for each protein listed on Table 1. These enzymes were 3 gastrointestinal (trypsin, chymotrypsin and pepsin) and 3 non-gastrointestinal proteases (thermolysin, bromelain and papain) (Udenigwe

et al., 2013; Gülseren, 2018). Commercial forms of all of these proteases are currently available on the market. For each enzyme, the individual sequences formed from each protein were analyzed individually for their potential bioactive properties using the "profiles of biological activity" tool (Minkiewicz et al., 2008). The BIOPEP database also catalogs the previously identified bioactive peptide sequences. Thus, peptides formed during the simulations were compared to the existing peptide data using sequence-based similarities.

Results and Discussion

The names of the ribosomal hazelnut proteins examined in this study, the total number of amino acid number that they contain and the access numbers for the protein databases were summarized in Table 1. The amino acid numbers in the proteins range from 25 to 286 and the average number of amino acids in the ribosomal proteins is calculated as 133.3. In addition, the amino acid sequences of these proteins were downloaded from the Uniprot database and utilized in further analyses.

Table 1. Randomly assigned order numbers, accession numbers for protein databases and number of amino acids in ribosomal proteins of the common hazelnut

Çizelge 1. Rastgele verilen protein sıra numaraları sayıları, protein veri tabanları için erişim kodları ve söz konusu ribozomal fındık proteinlerindeki amino asit sayısı

Order Number *Sıra numarası*	Name of the protein *Proteinin ismi*	Total number of amino acids *Toplam amino asit sayısı*	UniProtKB Accession Number UniProtKB erişim kodu
1	Ribosomal protein small 3 (Fragment)	25	Q9TGB0
2	Ribosomal protein S19	92	A0A1I9RG92
3	Ribosomal protein L23	93	A0A1I9RG90
4	Ribosomal protein S12	124	A0A1I9RG93
5	Ribosomal protein S7	155	A0A1I9RG73
6	Ribosomal protein L32	56	A0A1I9RG76
7	Ribosomal protein S18	101	A0A1I9RG53
8	Ribosomal protein L2	286	A0A1I9RG91
9	Ribosomal protein S16	65	A0A1I9RG19
10	Ribosomal protein L14	122	A0A1I9RG65
11	Ribosomal protein S4	201	A0A1I9RG35
12	Ribosomal protein L16	136	A0A1I9RG66
13	Ribosomal protein S14	100	A0A1I9RG31
14	Ribosomal protein S3	219	A0A1I9RG67
15	Ribosomal protein S11	138	A0A1I9RG62
16	Ribosomal protein L2	287	A0A1I9RG70
17	Ribosomal protein L33	73	A0A1I9RG52
18	Ribosomal protein S2	241	A0A1I9RG25
19	Ribosomal protein L36	42	A0A1I9RG63
20	Ribosomal protein L22	169	A0A1I9RG68
21	Ribosomal protein S15	90	A0A1I9RG85
22	Ribosomal protein S8	134	A0A1I9RG64
23	Ribosomal protein L20	117	A0A1I9RG54

Based on the BIOPEP data, 23 different proteins were subjected to *in silico* proteolysis one-by-one and a total of 6 different proteases were utilized in this process (Minkiewicz et al., 2008). Consequently, a total of 138 different cases were investigated (23 proteins x 6 proteases). These proteases included 3 gastrointestinal (GI) enzymes (pepsin, trypsin and

chymotrypsin) as well as 3 non-GI enzymes (bromelain, papain and termolysin). Since non-GI enzymes have also been demonstrated to be instrumental in the production of bioactive peptides (Gobbetti et al., 2002), these 3 enzymes have also been included in this study. In each and every case, enzymatic treatments generated multiple peptides from all proteins studied here (Table 1).

Peptide sequences with two or more amino acids were recorded. Since the bioactive performances of free amino acids are well-characterized in the literature, single amino acid sequences were not taken into account, while their overall content was also negligible (data not shown). All the two or more amino acid containing peptides were further analyzed using *in silico* tools and their antioxidative characteristics were investigated.

First of all, using the "profiles of biological activity" tool on the BIOPEP database (Minkiewicz et al., 2008), the potential bioactivities of all peptides were analyzed (Tables 2-3; Figures 1-3). In total, among the 138 cases investigated, 44 cases were predicted (i.e., 31.9% of all cases) to generate bioactive peptides with antioxidative activities. That means, since each proteolytic event generated multiple peptides, among the peptides generated in each case, at least one peptide was of potentially antioxidative nature in approx. 32% of the cases. Consequently, the number of antioxidative peptides generated and the total number of amino acids in these peptides were determined for all the 138 different cases. The distribution of these results based on the type of protease used was listed on Figure 1 and the behavior of GI vs. non-GI proteases was compared.

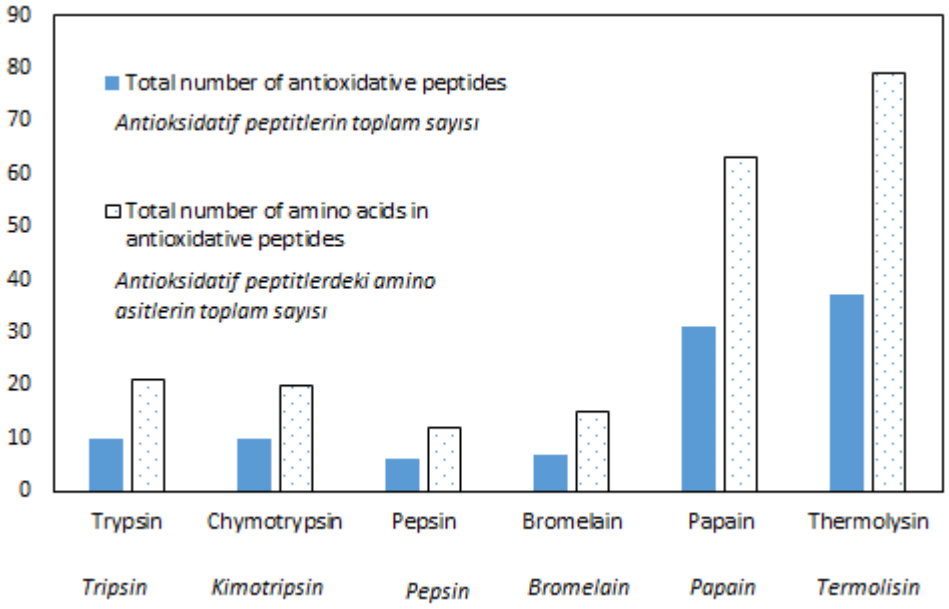

Figure 1. Distribution of antioxidative peptides resulting from *in silico* proteolysis of 23 different ribosomal hazelnut proteins and their total number of amino acids by proteolytic enzymes

Şekil 1. 23 farklı ribosomal fındık proteininin in silico proteolizinden ortaya çıkan antioksidatif peptitlerin ve bu peptitlerin içerdiği amino asit sayılarının proteolitik enzimlere bağlı değişimi

GI proteases were found to generate a lesser extent of antioxidative peptides compared to the non-GI proteases (Figure 1). For the 3 GI proteases, ≤ 10 antioxidative peptides (i.e., in total for 23 different cases) were generated. The total number of amino acids in these peptides were ≤ 21. Non-GI proteases, especially papain and termolysin, generated a larger extent of antioxidative peptides compared to GI proteases. While 31 antioxidative peptides were located in papain hydrolyzates, thermolysin hydrolyzates contained 37 antioxidative peptides, which contained a total of 63 and 79 amino acids, respectively. Thus, the average number of amino acids per peptide was calculated as 2.13 and 2.03, respectively. Since the average number of amino acids were approximately equal to 2, dipeptides were dominant in most cases. Figure 2 detailed

the case where the highest concentration of antioxidative peptides formed. When protein 23 was treated with thermolysin, five different antioxidative peptides, each of which was a dipeptide, were generated. Therefore, there was a potential to generate a 10 amino acid sequence from this 117 amino acid protein that potentially performed antioxidative effect. This identification was based on comparison to the literature data listed on the BIOPEP database. BIOPEP generally identifies certain peptides in the presence of previous literature data and it is possible to analyze any known sequence using BIOPEP tools. Antioxidative peptides that occur in some other proteins also included tripeptides, but none of the 138 cases examined had antioxidative sequences longer than 3 amino acids after proteolytic treatments. In all cases only FKK, IKK, IRW, LHR, LLR and RHN antioxidative tripeptides were formed and only IKK was observed more than once among these peptides. IKK, for example, can be identified as an antioxidative peptide from prawn muscle (*Penaeus japonicus*) based on BIOPEP data, which can also be encountered in hazelnut protein hydrolyzates. Tables 2 and 3 also show the most frequently occurring antioxidative peptides in all cases. The most commonly encountered antioxidative peptide was the IR dipeptide (Table 2). The distribution of such peptides to the 6 proteases was shown on Table 3. It is understood that some antioxidative peptides, especially IR, could be formed by more than one protease.

1 MTRIKRGYIA RRRRTK**IR**LF ASSFRGAHSR LTRTITQQK**I R**ALFSAYRDR
51 GRQKRDFRR**L W**ITRINAVIR GKGIYYNYNR LIHNLYKRQL LLNRKILAQI
101 AILNRNC**LY**M ISNEI**LK**

Figure 2. Antioxidative peptide sequences (shown in bold) released from ribosomal protein No.23 by the thermolysin treatment. Note that in this specific case (protein No. 23 and thermolysin), all the antioxidative sequences in the hydrolysate were dipeptides

Şekil 2. Termolizin muamelesi ile 23 numaralı ribozomal proteinden ortaya çıkan antioksidatif peptit sekansları (kalın olarak gösterilmiştir). Bu özel durumda (protein No. 23 ve thermolizin muamelesi), hidrolizattaki tüm antioksidatif dizileri dipeptit yapıdadır

Table 2. The sequences and total number of occurrence for the most frequently formed 5 antioxidative peptides in this study

Çizelge 2. Bu çalışmada en sık oluşan 5 antioksidatif peptit için amino asit sekansları ve bu peptitlerin çalışma boyunca ortaya çıkma sıklığı

Peptit sequence *Peptit sekansı*	Total number of occurrence *Toplam oluşma sıklığı*
IR	34
IY	10
LK	14
EL	9
LY	8

Table 3. Sequences of antioxidative peptides predicted to occur multiple times in this study in all cases and the distribution of these peptides based on their corresponding proteases. The symbol X indicates the number of times the corresponding peptide has been formed

Çizelge 3. Bu çalışmada birden çok kez ortaya çıkacağı öngörülen antioksidatif peptitlerin sekansları ve bu peptidlerin proteazlara göre dağılımı. X sembolü, söz konusu peptitin ortaya çıkma sayısını gösterir

Trypsin *Tripsin*	Chymotrypsin *Kimotripsin*	Pepsin *Pepsin*	Bromelain *Bromelain*	Thermolysin *Termolizin*	Papain *Papain*
8X IR	4X EL	4X EL	3X LK	2X AH	15X IR
	3X IY			3X IKK	3X IY
				11X IR	2X LH
				3X IY	3X LK
				7X LK	5X LY
				2X LW	
				3X LY	

Finally, the distribution of the total number of amino acids contained by the antioxidative peptides resulting from the treatment of 23 proteins with different proteases is presented on Figure 3. The relatively low levels of antioxidative amino acids generated by trypsin (Figure 3A) were elevated in papain treatments (Figure 3B) and reached their highest value in thermolysin treatments (Figure 3C). It is understood, however, that the behavior of each protein was specific and that the relationship between these 3 proteases does not follow a certain trend between different proteins.

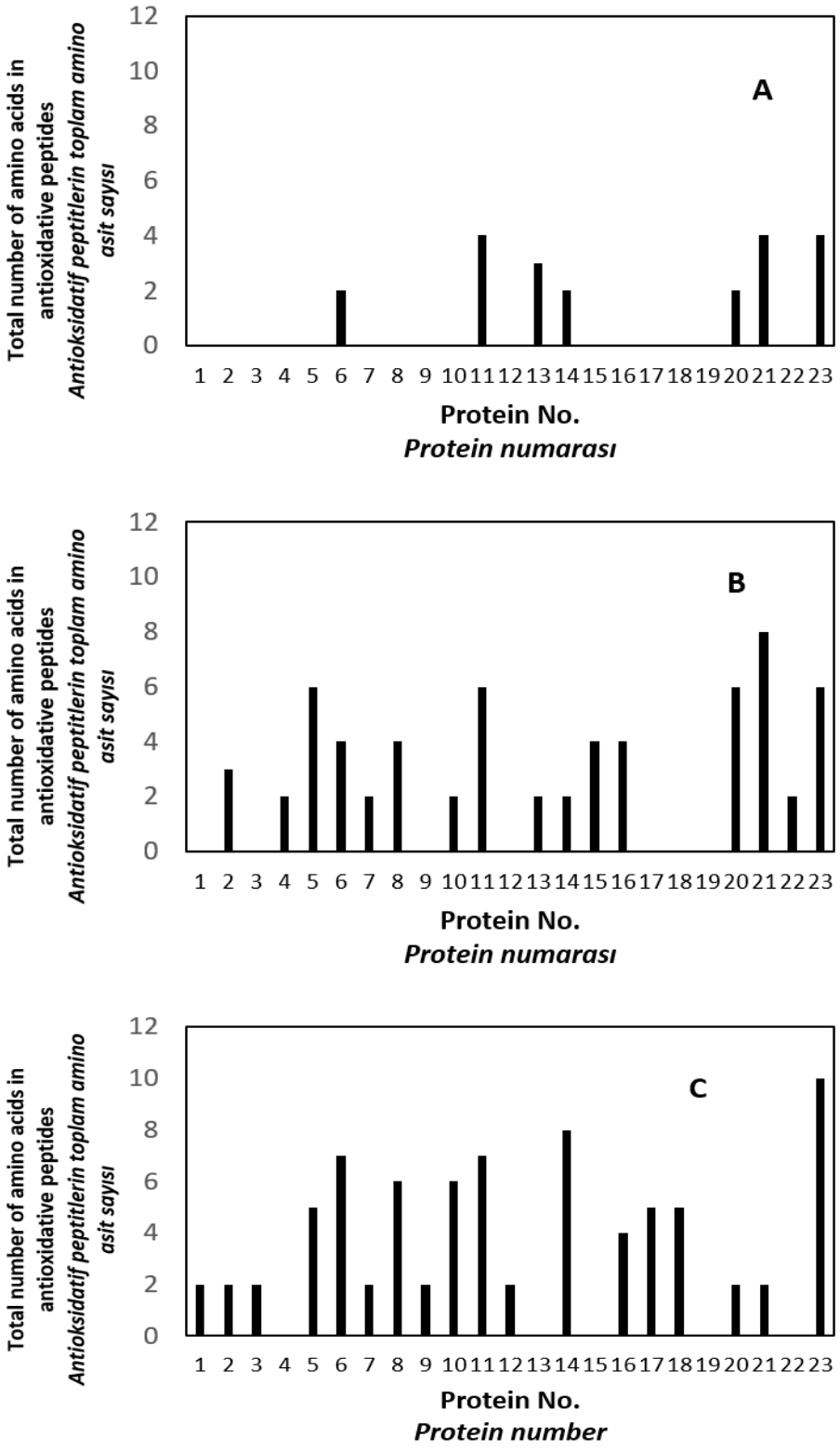

Figure 3. Distribution of the total number of amino acids in antioxidative peptides resulting from *in silico* proteolysis of 23 different ribosomal hazelnut proteins based on (A) trypsin, (B) papain, and (C) thermolysin treatments

Şekil 3. (A) Tripsin, (B) papain ve (C) termolizin muamelelerine bağlı olarak 23 farklı ribozomal fındık proteininin in silico proteolizinden kaynaklanan antioksidatif peptitlerde toplam amino asit sayısının proteinlere dağılımı

Conclusion

In this study, the potential bioactivity of peptides generated by hydrolysis of ribosomal hazelnut proteins was investigated. Generally, non-GI proteases (especially thermolysin and papain) were found to be more effective than GI proteases in the generation of antioxidative sequences.

Based on our group's previous (Gülseren, 2018; Gülseren et al., 2019) and present findings, hazelnut protein hydrolysates have shown potential to demonstrate ACE- and DPP-IV-inhibitory activity and antioxidative effects. In this context, hazelnut proteins and deoiled hazelnut meals may be considered as sources suitable for producing bioactive peptides. In our laboratories, work on *in vitro* proteolysis of hazelnut proteins is currently being carried out on the bioactivity and allergenic properties of all hazelnut proteins.

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