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The Stability of Food Bioactive Peptides in Blood: An Overview

İbrahim Gülseren[1,2] · **Beyza Vahapoglu**[3]

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Abstract
Food bioactive peptides are keenly investigated due to their potential health promoting bioactivities including antihypertensive, antioxidative, anti-carcinogenic and anti-neurogenerative properties. While various natural resources are studied for their bioactive peptide content, stability of food bioactive peptides in digestive processes and circulation in blood is currently under-investigated. However, half-life of peptides in blood is of critical importance, since the distribution of peptides to specific tissues is a function of the circulatory duration. Here, an attempt was made to predict the potential blood stability of proven food bioactive peptides. Using the sequence information for all the peptides listed on Bioactive Peptides Database (BioPepDB), half-lives of food bioactive peptides in blood were predicted in silico using PLifePred. This analysis encompassed 3074 peptides from 12 different source categories including milk, amphibian, eggs, fish, fungi, etc. In addition, based on the recent work of our group, hazelnut peptides were included in the analysis (n = 179). The majority of food bioactive peptides were found to demonstrate a half-life value of 800–900 s, which was comparable to or lower than circulatory hormones. Only a few exceptional peptides could be anticipated a blood serum half-life > 2500 s. The implications of the current findings on the efficiency of bioactive peptides in food systems have been discussed.

Keywords Bioactive peptides · Cereal proteins · Meat proteins · Hazelnuts · Serum stability

Introduction

Food bioactive peptides are mostly formed through digestion of foods, enzymatic hydrolysis or fermentation. To date, many bioactive peptides have been isolated from different sources including dairy products, meat products, eggs, plants, and marine sources (*i.e.*, fish, algae, seaweed, mollusks) (Hajfathalian et al. 2018; Ghanbari 2019). Bioactive peptides encrypted in milk or other unprocessed foods may not demonstrate their bioactive attributes prior to processing. In processed foods such as yoghurt and cheese, some of these peptides are being released from their native protein, which in turn leads to the manifestation of bioactivity. Bioactive peptides from various food products have demonstrated bioactivities including antimicrobial, antiviral, anticancer, antimutagenic, antihypertensive, antidiabetic, hypolipidemic, opioid, and antithrombotic activity (Kitts and Weiler 2003; Hajfathalian et al. 2018; Maestri et al. 2019). Consequently, bioactive peptides have become an important functional ingredient category due to their positive influence on human health.

Besides the in vitro bioactivity potential of food peptides, their bioaccessibility and bioavailability characteristics have also been intensely studied (Segura-Campos et al. 2011; Hajfathalian et al. 2018; Udenigwe et al. 2021). In this respect, animal and human studies can be considered as the most reliable approaches to assess peptide bioavailability. Due to the time and labor intensive nature of such investigations, simulated in vitro gastrointestinal digestion and absorption studies have also become increasingly popular (Amigo and Hernández-Ledesma 2020; Udenigwe et al. 2021). Since the majority of in vitro digestion studies utilize static digestion models, the scheme is oversimplified and a number of processes or their corresponding kinetics including peristalsis,

✉ İbrahim Gülseren
ibrahim.gulseren@izu.edu.tr

[1] Department of Food Engineering, Faculty of Engineering and Natural Sciences, İstanbul Sabahattin Zaim University (İZÜ), Halkalı – Küçükçekmece, 34303 İstanbul, Turkey

[2] İZÜ Food and Agricultural Research Center (GTAUM), Halkalı Campus, Küçükçekmece, 34303 İstanbul, Turkey

[3] Department of Food Engineering, Faculty of Chemical and Metallurgical Engineering, İstanbul Technical University, Maslak, 34469 İstanbul, Turkey

enzyme secretion, absorption etc. are neglected (Egger et al. 2019).

Even beyond absorption, bioactive peptides must remain in the blood for considerably long circulatory durations in order to reach their target tissues. Every peptide has a different half-life in circulation depending on its structural properties such as amino acid composition, hydrophobicity, size, presence of secondary structures, molecular charge and shape (Werle and Bernkop-Schnürch 2006; Mathur et al. 2018; Erak et al. 2018). Depending on their half-lives, highly potent and absorbable peptides may not be able to demonstrate their bioactivities (Werle and Bernkop-Schnürch 2006; Segura-Campos et al. 2011). In therapeutic applications, half-life in blood has the potential to affect the rate of clearance, tissue distribution, and quantity and number of doses that should be administered in each treatment (Mathur et al. 2018). The peptides or hormones that have relatively short plasma half-lives (i.e., 4–20 min for arginine vasopressin) require continuous supplementation (for example, IV infusion) in order to maintain their bioactivities (Treschan et al. 2006). While natural protein or peptide therapeutics can circulate for relatively short durations, various modification strategies have been generated to extend their half-lives (Zaman et al. 2019). Consequently, long-term blood stability of peptides were shown to directly manipulate their bioactivities including antihypertensive attributes (Qin et al. 2015).

Proteolytic degradation in blood is typically investigated via the incubation of peptides in sera or plasma samples. The natural variation in such samples lead to differences in the concentration and activity level of proteases, relevant activators, inhibitors and anticoagulants in the medium (Böttger et al. 2017). These experiments will allow the identification of cleavage sites on peptides. Consequently various stabilization strategies including the incorporation of unnatural or unconventional amino acids, and formation of D-enantiomers or backbone modifications might be considered for therapeutic applications (Böttger et al. 2017).

Based on their structural properties, bioactive peptides may reach the small intestine and blood circulation in intact form or may be degraded by GI proteases and serum peptidases. For example, cytosolic hydrolases present in intestinal epithelium reduce the transport rate of small peptides (Wang and Li, 2017). The major peptide transporters in the human body include PepT1, PepT2, PhT1, and PhT2 (Ashraf et al. 2014). PepT1 was described as the carrier responsible for the uptake of di- and tripeptides in the small intestine, where PepT1 transporter-mediated transport for di- and tri-peptides has been reported for all 400 dipeptides and 8000 tri-peptides, irrespective of their amino acid sequences (Wang et al. 2019). In addition, paracellular transport through intercellular junctions or transcytosis are among the alternative routes of peptide transport (Wang et al. 2019). While paracellular transport could enable the uptake of peptides that are mostly sized between 500–1000 Da, transcytosis is applicable for larger peptides (i.e., > 1000 Da) (Wang and Li 2017). In all cases, the physicochemical properties of peptides show significant effects on peptides' transepithelial transport and their bioavailability (Gleeson et al. 2016).

Several databases and predictive tools have been developed for the characterization of bioactive peptides and their various properties including physicochemical attributes, potential bioactivity, serum stability, molecular interactions and toxicity in silico, and understanding the mechanism of action behind their bioactivities (Petsalaki et al. 2009; Iwaniak et al. 2019). The current study aimed to determine the half-life of verified food bioactive peptides in blood via in silico methods. In addition, the influence of the structural properties of peptides including molecular weight and hydrophobicity on half-life have also been examined.

Materials and Methods

Data Collection

The sequence information for the peptides analyzed in this study was collected from the Bioactive Peptides Database (BioPepDB) website (http://bis.zju.edu.cn/biopepdbr/index.php?p=species) on February 4, 2021 (Li et al. 2018). The sequence data corresponded to 3074 peptides from 12 different food categories. The categories and corresponding number of peptides in each category were summarized on Table 1. The listed peptides were selected based on their

Table 1 The number of food bioactive peptides for each category investigated in the current study

Source	No. of peptides
Amphibian	1033
Bovine	56
Cereal	269
Chicken	150
Egg	65
Fish	271
Fungi	26
Legume	153
Milk	828
Porcine	165
Potato	25
Shrimp	33
Total	3074

The data was collected from the Bioactive Peptide Database (BioPepDB) and only the peptides suitable for PLifePred analysis (≤ 50 amino acids) were considered

suitability for half-life analysis in PLifePred tools (Mathur et al. 2018), which required a peptide length of ≤ 50 residues. In addition, as a specific case study, the sequences for hazelnut peptides (n = 179) listed in a recent paper published by our team were also employed in similar analysis (Çağlar et al. 2021).

In Silico Analyses

Peptides that were ≤ 50 amino acids were evaluated via PLifePred analyses. Their corresponding molecular weights, hydrophobicity values and half-lives in blood were predicted in silico using PLifePred "Batch Submission" tool (https://webs.iiitd.edu.in/raghava/plifepred/batch.php) (Mathur et al. 2018). The distribution of molecular weights or half-lives for each category was plotted either via individual sample numbers (1 to n as listed in Table 1) or as a function of cumulative % frequency. When necessary, single amino acid mutants of a peptide sequence were generated which took into account all possible combinations based on the 20 natural amino acids. Hence, 19 substitutions for each and every residue was analyzed throughout the sequence (i.e., 19 substitutions x N, where N is the total number of residues in a peptide). All mutants were also analyzed via PlifePred. In order to investigate the proteolytic stability of peptides, "Enzyme Action" tool of BIOPEP (Minkiewicz et al. 2019) was utilized.

Statistical Analyses

Pearson's correlation method was performed to determine the bivariate correlation coefficient between half-life and peptide properties including molecular weight or peptide hydrophobicity. All the data were analyzed using IBM SPSS Statistics 27.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

First of all, the number of peptides analyzed in this study for each food category was listed in Table 1. The number of peptides was not equal for all categories. For example, the number of peptides analyzed was 1033 for the amphibian category, whereas it was as low as 25 for the potato peptides. These selections were based on the availability of the sequences at the specified time and database. Since the analysis relied on the current state-of-the-art, all available peptide sequences have been evaluated regardless of their total number.

For the peptides of each and every food category in the dataset, half-life analysis was carried out and the half-life values were plotted in ascending sequence (Fig. 1A). Although the number of peptides was different for all

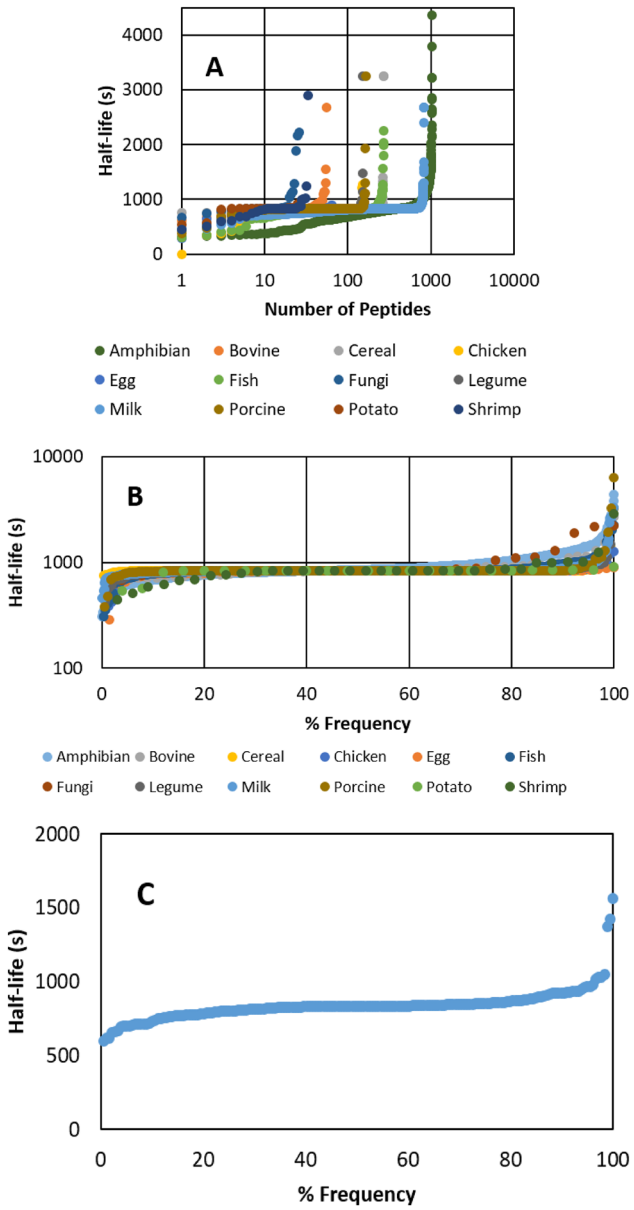

Fig. 1 In silico predicted half-life values for peptides analyzed using PLifePred. **A** Peptides for each category were numbered sequentially from 1 to N in the order of ascending half-life values. **B** Peptides for each category were numbered using cumulative frequency (%) sequentially in the order of ascending half-life values. **C** In silico predicted half-life values (s) for hazelnut peptides (n = 179) analyzed using PLifePred. Peptides were numbered sequentially from 1 to 179 in the order of ascending half-life values

categories, overall trends were comparable. Mostly, peptides with very short half-lives were found to range between, approx. 286–500 s. This group corresponded to only 1.37% of the population. The majority of peptides in all categories demonstrated a half-life of 500–1000 s, while especially around 800 s, a plateau zone was generally observed. The peptides with a half-life of 500–800 s and 800–900 s

corresponded to 14.48% and 66.46%, respectively. The longest half-life peptides with > 900 s corresponded to 17.7%.

In a number of cases, highly stable peptides with a half-life of > 2500 s were observed. Since longer half-lives could imply enhanced probabilities of becoming bioavailable and/or extended circulatory durations to access target tissues in the human body, these long-lived peptides were further listed in Table 2. Some of those peptides, especially shorter ones (i.e., VP, VVPP), could be encountered in multiple sources. VP was found in milk, cereal, chicken, fish, potato and porcine categories, whereas VVPP was encountered in milk and cereal categories. VVVPP peptide from milk was predicted to have a remarkably longer half-life than all food peptides analyzed here (33,594.91 s). This peptide was formed after digestion of β-casein peptides and exhibited ACE-inhibitory activity (Quirós et al. 2009). While most ACE inhibitory drugs have less than a 12 h half-life, a native food peptide that has a 9 h half-life is quite rare (Auron et al. 2011). Another relatively long half-life (6350.81 s) was achieved by a longer porcine peptide (RADTQTYQPYNKDWIKEKIYVLLRRQAQQAGK). These two peptides were removed from Fig. 1 to enhance the clarity of overall trends in various categories.

According to PlifePred, VVVPP is a combination of aliphatic/hydrophobic and conformationally special residues. Hydrophobicity or proline content of this peptide could potentially have a bearing on its extended stability. A brief in silico prediction using the "Enzyme Action" tool of BIOPEP (Minkiewicz et al. 2019) demonstrated that trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and pepsin (EC 3.4.23.1) would not degrade VVVPP peptide.

Based on further PlifePred analysis, when 608 mutants (i.e., 32 residues × 19 conventional amino acids) of RADTQTYQPYNKDWIKEKIYVLLRRQAQQAGK were studied, the highest extent of reduction in half-life was predicted to take place upon a W to V (tryptophan to valine) transition on the 14th residue (6350.81 s to 2231.11 s). Consequently, in this particular case, aromatic to aliphatic/hydrophobic mutation was the cause of this potential observation. Addition of a further Won the 7th, 19th or 20th residues was predicted to set the stability to 12,894.41 s replacing Y residues (tyrosine) in each case possibly due to the increasing size of the aromatic side chain. This point mutation was found to provide higher extent of stability than any other mutation possibility.

In any case, as demonstrated by Fig. 1A, approximately 83% of milk peptides were characterized by a half-life value between 800 and 900 s. In the similar range only approximately 35.2% of the amphibian peptides were predicted. In Fig. 1A, the distribution of amphibian data was possibly the furthest category from the plateau. Although the number of cereal peptides was relatively lower, approximately 95.2% of them had a half-life value between 800–900 s. The plateau was clearer when sequential peptide numbers were replaced by % frequency (Fig. 1B).

Table 2 Food bioactive peptides with a predicted half-life of > 2500 s presented in the order of descending half-life values

Source	Sequence	Half-Life (s)	Molecular Weight (g/mol)
Milk	VVVPP	33,594.91	509.71
Porcine	RADTQTYQPYNKDWIKEKIYVLLRRQAQQAGK	6350.81	3910.94
Amphibian	SLWETIKNAGKGFIQNLDKIR	4363.91	2432.15
Amphibian	GLWQLIKDKIKDAATGFVTGIQS	3790.51	2490.26
Milk/Cereal/Chicken/Fish/Potato/Porcine	VP	3250.21	214.28
Milk/Cereal	VVPP	3250.21	410.56
Amphibian	GLWQFIKDKFKDAATGLVTGIQS	3223.71	2524.27
Amphibian	SIITMTKEAKLPQSWKQIACRLYNTC	3221.51	3028
Fish	GWKKWFTKGERLSQRHFA	2910.41	2262.87
Shrimp	IPAMEPAARVKRSPGYGGCSPRWACGGYG	2903.21	2995.85
Amphibian	GIWDTIKSMGKVFAGKILQNL	2853.41	2320.13
Amphibian	SIITMTKEAKLPQLWKQIACRLYNTC	2827.81	3054.09
Milk/Bovine	FKCRRWQWRMKKLGAPSITCVRRAF	2684.41	3126.17
Amphibian	SALVGCGTKSYPPKPCFGR	2645.31	1968.58
Amphibian	GLWQFIKDKLKDAATGLVTGIQS	2620.01	2490.26
Amphibian	GLRSKIWLWVLLMIWQESNKFKKM	2607.51	3036.14
Amphibian	SALVGCWTKSWPPKPCFGRG	2579.91	2177.85

The peptides that occur in multiple resources were identified in the Source column

Consequently, a half-life range of 800–900 s was predicted to be typical for most food peptides in our analysis, which covers the majority of food bioactive peptides. For example, a typical half-life value of 840 s would imply a 10 times reduction in circulation (*i.e.*, from 100 to 10%) in less than an hour (approx. 46.5 min). Further reduction to 1% will require approximately 93 min. This time range demonstrates an average circulatory period for the majority of food bioactive peptides.

To test the biological relevance of the findings, the half-lives of peptide hormones secreted in the human body were compiled from the relevant literature (Supplementary Table S1, Table S1) as reference systems for food based bioactive peptides and peptide therapeutics. Although these hormones are instrumental in various systems of the body, in many cases, a 14 min limit was reasonably comparable. However, peptide hormones of significantly higher stability are also known (growth hormone, melatonin, etc.). Furthermore, pharmacokinetic studies on proline-rich antihypertensive tripeptides demonstrated relatively short half-lives of 5 to 20 min in the pig (van der Pijl et al. 2008). A recent review on bioavailability of bioactive peptides reported various investigations on half-life of bioactive peptides, which widely varied between 1.9 min to > 8 h. Meanwhile, the majority of the bioactive food peptides were characterized with generally shorter half-lives in plasma (*i.e.*, approximately 10–60 min) (Xu et al. 2019).

In Supplementary Figure S1 (Figure S1), the molecular weights of all peptides investigated here were shown by % frequency. While the trends were similar for the majority of all cases, correlation analysis indicated mostly weak or non-significant correlations between the peptide molecular weights and their corresponding half-life values (Table 3). The correlation was found to be strongly positive for fungi peptides (r = 0.815) and strongly negative for potato peptides (r = − 0.921). As indicated before, the number of investigated peptide sequences for each category might have a bearing on these findings. When a similar correlation analysis was carried out between half-life values and hydrophobicity, no significant correlations were identified (Table 4).

In the previous literature, gastrointestinal peptide stability was not found to be a strict function of molecular weight (Wang et al. 2019). Similarly, although sequence, charge, and hydrophobicity individually influence gastrointestinal stability of peptides, their overall stability is a function of all these attributes in a collective fashion (Wang et al. 2019). While certain hydrophobic amino acids (*i.e.*, proline and glutamic acid) could potentially render peptides more stable (Savoie et al. 2005), in some cases, highly hydrophobic peptides were found to demonstrate poor gastrointestinal stability (Xie et al. 2015).

Previous studies have also indicated that there was a positive correlation between the molecular weights of various therapeutic peptides and their half-lives, especially when they were associated, conjugated or fused with albumin. These assemblies were not subject to renal clearance and remained in circulation longer (Sleep et al. 2013). However, the majority of food bioactive peptides analyzed in this study had considerably lower molecular weights or half-lives than serum albumin (approx. 66.5 kDa and 19 days, respectively) (Sleep et al. 2013).

The influence of hydrophobicity on peptide stability was studied in a number of investigations. Highly hydrophobic casein peptide fractions demonstrated enhanced in vitro bioavailability (Wang et al. 2019), while opposite results were obtained by Lee (2002). Wang et al. (2019) reviewed that intestinal transport of peptides generally decreased with the magnitude of positive or negative

Table 3 Correlation analysis between the half-life values of the peptides investigated here and their corresponding molecular weights

	Amphibian	Bovine	Cereal	Chicken	Egg	Fish	Fungi	Legume	Milk	Potato	Porcine	Shrimp
Pearson Correlation	0.150**	− 0.024	0.092	− 0.127	− 0.429**	0.158**	0.815**	− 0.060	0.033	− 0.921**	0.305**	− 0.048
p value	0.000	0.859	0.132	0.119	0.000	0.009	0.000	0.460	0.344	0.000	0.000	0.790

**Significant, significance at which means difference as shown by analysis of variance ($p < 0.01$)

Table 4 Correlation analysis between the half-life values of the peptides investigated here and their corresponding hydrophobicity values

	Amphibian	Bovine	Cereal	Chicken	Egg	Fish	Fungi	Legume	Milk	Potato	Porcine	Shrimp
Pearson correlation	− 0.238**	− 0.292*	0.039	0.090	0.190	− 0.064	0.017	0.054	− 0.085*	0.123	− 0.021	− 0.033
p value	0.000	0.029	0.519	0.270	0.130	0.294	0.936	0.504	0.015	0.558	0.786	0.854

**Significant, significance at which means difference as shown by analysis of variance ($p < 0.05$)

*Significant, significance at which means difference as shown by analysis of variance ($p < 0.01$)

charges, while in most cases; cationic peptides were less likely to be transported when compared with anionic or neutral counterparts. In some studies, however, especially short (i.e., di- and tripeptides) cationic peptides with antimicrobial characteristics demonstrated superior permeation potential (Flaten et al. 2011).

Finally, as a case study, recently published data from our group (Çağlar et al. 2021) was also added to the current analysis. Hazelnut peptides are characterized with antihypertensive (i.e., ACE-inhibitory) characteristics. In Fig. 1C, hazelnut peptides were numbered in ascending order of half-life values and their half-life values were plotted as a function of % frequency. Approximately 62% of hazelnut peptides were characterized by a half-life value between 800 and 900 s and only 7 of them (3.91%) were predicted to have a half-life longer than 1000 s. Consequently, the hazelnut dataset also yielded comparable data as the majority of food categories investigated in this paper.

Conclusion

Blood stability of bioactive peptides is a topic of critical importance, which has not been reviewed in detail for food bioactive peptides. In the current study, half-life of food bioactive peptides from various origins was investigated and the majority of food bioactive peptides have been predicted to demonstrate a half-life of 800–900 s. Moreover, only a small fraction of food bioactive peptides was predicted to enjoy significantly longer half-lives without any modifications to their natural structures.

Although the current study focused only on in silico analyses, especially in pharmaceutical sciences, a great deal of research has been carried out on the circulatory stability of active peptides (Jenssen and Aspmo 2008) and the methodologies to extend these durations. Consequently, the predictive power of the current algorithms such as PlifePred is reasonably high. As Table S1 demonstrated, even in vivo or clinical experiments on half-life are characterized by a certain extent of variation on half-life values. In any case, we believe that evaluation of half-life should be considered towards prediction of a peptide`s biological activity, which in turn will enhance the data on peptide bioavailability.

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Data Availability The data studied here are available online in the corresponding databases.

Code availability Not applicable.

Declarations

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical Approval No approval of research ethics committees was required to accomplish the goals of this study.

Research Involving Human and Animal Rights This article does not contain any studies involved with animal or human subjects.

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