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The Function of Renin and the Role of Food-Derived Peptides as Direct Renin Inhibitors

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Abstract

Food proteins contain active peptide fragments encrypted within their structure that can exert beneficial effects on human health above and beyond their expected nutritional value. Among many types of food-derived peptides, peptides with antihypertensive activity have received the most significant attention due to the prevalence of hypertension and its associated complications with pharmacological interventions. One strategy for the selection of potential food-derived antihypertensive peptides is to search for *in vitro* renin inhibitory activity. Thus far, various food protein-derived peptides and protein hydrolysates have shown *in vitro* renin inhibitory capacity. Many of these peptides have induced antihypertensive effects when orally administered to spontaneously hypertensive rats, and also, antihypertensive effects in hypertensive humans have been reported. Indeed, the results indicate that antihypertensive food protein-derived peptides may be acting at the same time via multiple pathways at the protein level as well as at the gene level modulating the renin-angiotensin system. Important knowledge on structure-function parameters of peptides is increasing constantly, which can greatly enhance the production and processing of peptides with high physiological efficacy. By means of novel nutrigenomic approaches, it is possible and, in future, perhaps essential to investigate the impact of peptides on the expression of genes and hence endeavor to optimize the nutritional and health effects delivered by peptides. Novel technologies are available to standardize and stabilize the concentrations of active peptides in the products in down-stream processing. The existing data provide strong potential for developing new added-value products with scientifically approved health effects for consumers. This review provides an overview of food-derived peptides that may mediate the antihypertensive activities through inhibiting renin, one of the key enzymes in renin-angiotensin system, and reviews also the safety and applicability aspects of the these peptides.

Keywords: bioactive peptide, renin inhibition, antihypertensive, peptides

1. Introduction

Cardiovascular diseases (CVD) account approximately one third of the total deaths, totaling ≈17 million annually worldwide [78]. Hypertension is considered one of the key risk factors for the development of CVD such as coronary heart diseases, peripheral artery disease and stroke, and kidney disease. Hypertension is often termed as "silent killer" affecting 1 billion people worldwide and causes up to 9 million deaths every year. In addition to health burden, treatment and prevention of hypertension are also associated with substantial socioeconomic consequences. A range of synthetic drugs, such as direct vasodilators, diuretics, adrenergic inhibitors, and angiotensin converting enzyme inhibitors, are commonly used for the treatment of hypertension [50]. The estimated costs for treating hypertension and related diseases were $156 billion in the USA in 2011 and nearly €110 billion in Europe in 2006. Healthy lifestyle choices and early treatment for individuals with mild hypertension are of high importance for reducing the global healthcare costs [50].

In addition to nutritive value of food proteins, they can have various biological activities either intact or after released during processing or digestion. The active peptide fragments, bioactive peptides, can exert beneficial effects on human health in addition to nutritional value. These fragments can be released from various food proteins by gastrointestinal digestion or food processing. According to the Biopep and BioPD (bioactive peptide database) databases, more than 1200 different bioactive peptides have been recorded. These peptides have 2–20 amino acids and molecular masses of less than 6000 Da. Their bioactivity is mainly determined by their composition and amino acid sequence [17, 56, 64]. Especially, peptides with antihypertensive activity have received the significant attention due to the persistence of hypertension and its associated complications. Inhibition of angiotensin I converting enzyme (ACE) has been the main target of these peptides. ACE plays crucial role through renin-angiotensin system (RAS) in the regulation of blood pressure and electrolyte balance in human body. At present, the correlation between *in vitro* and *in vivo* antihypertensive activities appears to be weak [18, 23]. To develop effective antihypertensive peptides, it is important to understand the complex pathophysiology of hypertension and the potential targets where these bioactive peptides may exert their specific actions. This review provides an overview of food-derived peptides that may mediate the antihypertensive activities through inhibiting renin, one of the key enzymes in RAS.

2. Renin-angiotensin-aldosterone system

In cascade system of blood pressure regulation, the renin-angiotensin-aldosterone system (RAAS) plays a key role. The importance of RAAS in diseases such as hypertension, congestive heart failure, and chronic renal failure has been recognized; moreover, the inhibition of RAAS is an effective way to intervene with the pathogenesis of these disorders [11, 43]. Secretion of renin (EC 3.4.23.15) is the first step in RAAS pathway and, importantly, also the rate-limiting step of the RAAS by converting angiotensinogen (Ang) into inactive decapeptide angiotensin I (Ang I), which is converted at the endothelial surface of blood vessels by the enzyme ACE into angiotensin II (Ang II), the primary effector molecule of the RAAS. Therefore, physiological

total renin activity, measured as plasma renin activity, can reliably indicate the risk of hypertension, and the inhibition of renin activity by natural products can be explored for the management of hypertension. Inhibition of renin could provide a more effective treatment for hypertension as it prevents the formation of Ang-I, which can be converted to angiotensin II (Ang-II), the vasoconstrictor compound, independent of ACE, by the enzyme chymase. In addition, unlike ACE which acts on a number of substrates, angiotensinogen is the only known substrate of renin. ACE inhibitors and AT1 receptor blockers (ARBs) are proven to be effective therapeutic agents in the treatment of CVD. However, both ACE inhibitors and ARBs lead to a substantial compensatory rise of circulating active renin and Ang peptides that may eventually limit their therapeutic potential [24, 67]. Moreover, the increased Ang I can be converted to Ang II by nonACE pathways, mediated by chymase and chymotrypsin-like enzyme. In addition to the side effects of ACE inhibitors, such as cough and angioedema, a meta-analysis of randomized controlled trials in 2010 suggested that ARBs are associated with a modestly increased risk of new cancer diagnosis, although conclusions about the exact risk of cancer associated with each particular drug have not been drawn [65]. Therefore, direct renin inhibition may be an alternative pharmacological approach to RAS inhibition.

The first-generation renin inhibitors were peptide analogs prosegment of renin or substrate analogs of the amino-terminal sequence of angiotensinogen containing the renin cleavage site and were synthesized already more than 30 years ago. The second generation inhibitors were peptidomimetic agents that are dipeptide inhibitors of the active site. However, the clinical use of these renin inhibitors is limited due to poor metabolic stability and oral bioavailability, short duration of action, weak antihypertensive activity, and high cost of synthesis [61, 66]. Pepstatin, a statine-containing hexapeptide, is the first reported renin inhibitor, but the inhibitory activity of pepstatin was remarkably lower against renin than against pepsin [20]. An endogenously expressed renin-binding protein (RnBP) has been reported to inhibit renin activity [68] based on the selective binding mediated by a leucine zipper (f195–216) in RnBP [33]. The primary RnBP sequence in the renin-binding region is a valuable information for designing potent renin-inhibiting peptides that may be identified and released from food proteins using bioinformatic tools. Aliskiren is the only commercial clinically proven synthetic renin inhibitor for managing hypertension; it has been approved for use in Europe and the United States from 2007 [34]. It has been found to be a more effective antihypertensive agent than ACE inhibitors [74], but recent clinical evidence suggests that Aliskiren may be harmful to patients with type 2 diabetes who are at risk of developing cardiovascular and renal diseases [54].

3. Structural characteristics of food protein-derived renin inhibitory peptides

To reduce the time and cost-intensive steps in the peptide discovery with the conventional pathway, it is important to understand the relationship between peptide structure and subsequent bioactivity. By utilizing the knowledge of structure activity relationship putatively, active peptide sequences can be released in a targeted manner. To date, the research has focused in production and characterization of bioactive peptides, and data concerning the structure-activity relationship are still quite limited.

Renin is a 335-amino acid, glycosylated aspartic protease belonging to pepsin-like family [14, 69]. In contrast to other aspartic proteases such as pepsin, which cleaves a wide variety of substrates, renin specificity is very restricted. The high specificity of renin catalysis is explained by the restricted three-dimensional space of the active site. The C- and N-terminal domains of renin form a deep cleft constructing the active site in which the inhibitors bind [34, 60]. Angiotensinogen is the highly specific physiological substrate of renin, but new renin inhibitors—among which the best known is nonpeptidic Aliskiren—have been developed based on the structural data of the active site [34]. Aliskiren is an orally active renin inhibitor with a very high binding affinity for renin [77], but it is a complicated molecule and thus, drugs simpler in structure and with high bioavailability are desirable in the drug market.

The structure of the active site of renin and the binding of Aliskiren is illustrated in **Figure 1** [58]. It is known that the binding to the catalytic aspartate residues is vital for all the protease inhibitors [9]. The active renin inhibitors seem to presuppose interactions with the aspartate residues of renin (Asp 32 or Asp 215) and the S3sp sub pocket unique for renin. Thus, it has been suggested that any new renin inhibitor should interact with these sites in the active site of renin [58].

Several renin inhibitor peptide sequences have been identified thus far (**Table 1**), however, quite little is known on detailed structure-activity relationship (SAR). Some general characteristics, such as hydrophobicity and molecular size of the peptide fractions, are suggested to correlate with the renin inhibitory activity, but the results are somewhat contradictory [2, 31, 36, 40, 44]. Taken together, the position of amino acid residues in the peptide sequence is more important for the renin inhibition capacity than the actual molecular size or total net charge.

The presence of N-terminal aliphatic (e.g., leucine, isoleucine, valine) and C-terminal bulky amino acid residues (e.g., phenylalanine, tryptophan) has been suggested to contribute to
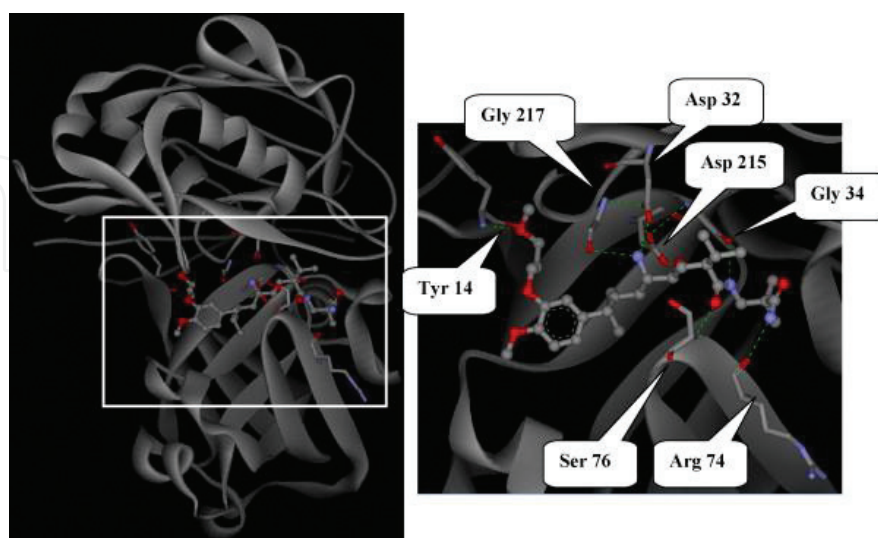

Figure 1. Binding mode of aliskiren as produced from crystallographic data. The protein backbone is shown in ribbons. Residues of the binding site are displayed as gray sticks and aliskiren as ball and sticks. The right panel shows a zoom in the active site and the formed H-bonds with aliskiren [58].

Origin	Treatment	Identified sequences	Renin inhibitory activity *in vitro* IC50	Antihypertensive effects *in vivo*, SHRs	Reference
Bovine serum albumin	Papain, <1 kDa MWCO fraction of the hydrolysate	SLR	1.18 mg/ml 7.29 mM	ΔSBP−32 mmHg after 8 h of oral administration, 200 mg/kg bw nd	[36]
Bovine blood globulins	Papain		1.18 mg/ml	nd	[39]
Bovine fibrinogen	Papain, <1 kDa MWCO fraction of the hydrolysate	YR SLR	32% at 1 mg/ml 8.78 mM 7.29 mM	nd nd nd	[38]
Bovine and porcine hemoglobin, collagen and serum albumin	Papain, pepsin, thermolysin	APPH, IIY, PPL, PPG, PFG, IPP, LPP	15–28% at 1 mg/ml	nd	[37]
Chicken skin protein	Alcalase, pepsin + pancreatin		1.6–2.2 mg/ml	ΔSBP −31.33 mmHg after 6 h of oral administration, 100 mg/kg bw	[52]
Cod muscle proteins	pepsin + trypsin + chymotrypsin RP-HPLC fraction of the hydrolysate		43% at 1 mg/ml 63% at 1 mg/ml	ΔSBP −19 mmHg after 2 h of oral administration, 200 mg/kg bw ΔSBP −40 mmHg after 2 h of oral administration, 30 mg/kg bw	[26]
Kidney bean protein	Alcalase, <1 kDa and 5–10 kDa MWCO fractions of the hydrolysate		40% at 1 mg/ml	nd	[48]
Flaxseed protein	Pepsin, ficin, trypsin, papain, thermolysin, pancreatin, and Alcalase Trypsin-pronase	Cationic, R-rich peptides	1.22–2.81 mg/ml 44.5% at 7.5 mg/ml	nd ΔSBP −17.9 mmHg after 2 h of oral administration 200 mg/kg bw	[71, 73]
Red seaweed (Palmaria palmata) protein	Papain	IRLIIVLMPILMA	42% at 1 mg/ml 3.344 mM	ΔSBP −34 mmHg after 24 h of oral administration, 50 mg/kg bw ΔSBP −33 mmHg after 24 h of oral administration, 3 mg/kg bw	[21, 22]
Hemp seed protein	Pepsin + pancreatin	WYT, SVYT, IPAGV	0.81 mg/ml 0.054 mM (WYT), 0.063 mM (SVYT), 0.093 mM (IPAGV)	ΔSBP −30 mmHg after 8 h of oral administration, 200 mg/kg bw ΔSBP −13.36 mmHg after 4 h of oral administration, 30 mg/kg bw	[29, 30]

Origin	Treatment	Identified sequences	Renin inhibitory activity *in vitro* IC50	Antihypertensive effects *in vivo*, SHRs	Reference
Hemp seed protein	Alcalase, pepsin, papain, pepsin + pancreatin		0.08–0.24 mg/ml	ΔSBP −25.33 mmHg after 4 h of oral administration, 200 mg/kg bw	[44]
Pea protein	Thermolysin, <3kDa MWCO fraction of the hydrolysate		17% at 1 mg/ml	ΔSBP −19 mmHg after 4 h of oral administration, 200 mg/kg bw ΔSBP −29 mmHg after 8 weeks of oral administration to Han:SPRD-*cy*, 0.1% of diet. Renal expression of renin mRNA levels was reduced significantly. ΔSBP −6 mmHg in a 3-week human intervention trial	[41]
African yam bean seed	Alcalase RP-HPLC fraction of the hydrolysate		35% at 1 mg/ml 55% at 1mg/ml		[1]
Rapeseed and canola protein	Alcalase, pepsin, trypsin, pancreatin Alcalase Pepsin + pancreatin	RALP, LY, TF GHS	15.80% at 1 mg/ml 0.968 mM (RALP), 1.868 mM (LY), 3.061 mM (TF) 0.320 mM	ΔSBP −25 mmHg (pepsin) and −34 mmHg (Alcalase) after 4 h of oral administration, 200 mg/kg bw ΔSBP −12 mmHg (TF), −26 mmHg (LY) and −16 mmHg (RALP) after 6 h of oral administration, 30 mg/kg bw ΔSBP −17 mmHg after 6 h of oral administration, 30 mg/kg bw	[2, 30, 31]

Table 1. Food protein-derived renin inhibitory peptides and antihypertensive effects *in vivo*.

higher renin inhibitory activity of dipeptides [71]. For example, dipeptides Leu-Tyr, Ile-Trp, and Thr-Phe have been reported to inhibit renin activity with IC50 values of 1.8, 2.3, and 3.7 mM, respectively [30]. The structures of these peptides mostly agree with the characteristics proposed to contribute to renin inhibition. The importance of C-terminal bulky hydrophobic amino acid residue was also observed by changing the position of amino acids residues from Thr-Phe to Phe-Thr, which resulted to substantial decrease in renin inhibition [30, 71]. However, highly hydrophilic peptides, such as Gly-His-Ser, have also been reported to inhibit renin with IC50 value of 1.09 mM [31]. Also, a cationic tetrapeptide Arg-Ala-Leu-Pro and a 13-amino acid residue, Ile-Arg-Leu-Ile-Ile-Val-Leu-Met-Pro-Ile-Leu-Met-Ala, have also shown rather high renin inhibitory potency [22, 30]. The highest renin inhibitory activity among the reported food protein-derived peptides thus far is 0.054 mM for Trp-Tyr-Thr produced from hemp seed protein [27]). Taken together, more research is needed to gain more knowledge on detailed SAR for designing potential renin inhibitory peptide sequences as physiological antihypertensive agents.

Quantitative computational tools are increasingly applied in medicinal and pharmaceutical drug discovery. At present, the relationship of peptide structure and bioactivity, especially the enzyme inhibitors of ACE are known in some extent. The knowledge of the active peptide sequences enables utilization of quantitative structure-activity relationship modeling (QSAR) for evaluating the crucial physicochemical features of the peptide for the effective bioactivity. A small number of QSAR studies have been carried out on ACE-inhibitory peptides [59, 66] however, no studies have been carried out with seeking potential renin inhibitory peptides.

4. Bioavailability

To induce health effects *in vivo*, peptides need to reach the physiological target organs in intact and active conformation. Considering the renin inhibition, there are three main barriers and hydrolytic threats on the way to the *in vivo* outcome: the digestive proteinases in the gastrointestinal tract, enzymes in the site of absorption, and serum peptidases in the circulation. Thus far, the published data concerning the bioavailability of the peptides, which have shown *in vitro* renin inhibitory activity, are very limited. This makes it very difficult to predict the *in vivo* antihypertensive effect of the *in vitro* renin inhibitory peptides. However, some structural characteristics have been shown to correlate with the bioavailability of, e.g., ACE-inhibitory peptides. These general peptidic characteristics can be considered with renin inhibitory peptides as well.

At first, after oral ingestion bioactive peptides need to resist the hydrolytic actions in stomach by pepsin and pancreatic peptidases, including trypsin, elastase, and chymotrypsin, and further, carboxypeptidases in the small intestine. Several different methods have been applied to model the gastrointestinal digestion *in vitro*. Most of the methods not only concern utilization of commercial porcine enzyme mixtures (e.g., Refs. [42, 46, 75]) but also human digestive liquids have been utilized [19, 49]. Due to the variation in the methods, the comparison of the results across the studies is difficult and thus, a harmonization of the various *in vitro* methods

would be important. A consensus for a static process to model the digestion of plant secondary metabolites has been constructed based on *in vivo* data [3]. Indeed, the future research should focus more on the *in vivo* bioavailability of the peptides and based on the correlation with *in vivo* data, a harmonized *in vitro* method could be proposed.

The peptides are exposed to peptidolytic digestion also on the brush border membrane of the intestine. There are number of peptidases with varying specificities bound on the intestinal epithelial cells. It has been suggested that dipeptide and tripeptide tend to resist the gastric and duodenal digestion and also the hydrolytic action of peptidases at the brush border membrane. These small peptides can be absorbed by active transcellular transport or by passive process [63]. To study the absorption *in vitro*, the monolayer of intestinal cell lines, such as Caco-2 cells, simulating intestinal epithelium, is commonly utilized. Clinical data concerning the bioavailability of bioactive peptides are very restricted; however, ACE-inhibitory lactotripeptides, Ile-Pro-Pro and Val-Pro-Pro, have been detected in human and animal circulatory system after oral ingestion [25].

5. Effects of food protein-derived renin inhibitory peptides *in vitro* and *in vivo*

The most widely utilized method for assessing the renin inhibitory potential *in vitro* is a fluorometric assay utilizing a human recombinant renin (Cayman Chemical, MI, USA). Recent data indicate that some food protein-derived hydrolysates and peptides possess *in vitro* renin inhibitory activity. Inhibiting activity against human recombinant renin has been reported, for instance, for hemp seed, pea, bovine blood, and chicken skin protein-derived hydrolysates produced by various food grade proteases (**Table 1**).

Among the protein hydrolysates, the highest renin inhibitory activities have been reported for hemp seed protein hydrolysates with IC$_{50}$ values of 0.08–0.81 mg/ml [27, 44]. These activities are at the same level with the synthetic renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe [22]. Alcalase has yielded to very active renin-inhibiting hydrolysates (e.g., Refs. [2, 30]), and also pancreatin and papain have produced high renin inhibitory activity (e.g., Refs. [21, 27]). Papain has also exhibited good prospects *in silico* in releasing renin inhibitory peptides from, for instance, bovine fibrinogen [37]. Moreover, simulated food protein hydrolysis with gastrointestinal enzymes has also resulted in products with renin inhibitory activities [27, 51]. Taken together, the efficiency of the protease to release renin inhibitory peptides seems to depend on the parent protein matrix. Thus, *in silico* tools are recommended to be utilized prior to the *in vitro* experiments to predict the efficacy of proteases with the particular protein matrixes.

Spontaneously hypertensive rats (SHR) are widely used animal model to assess the antihypertensive effects by *in vivo* experiments. This animal model is applied in short- and long-term manners, for example, to study the antihypertensive effects of milk protein-derived peptides [18, 23]. Recently, food protein-derived renin-inhibiting peptides and protein hydrolysates have induced antihypertensive effects when orally administered to spontaneously

hypertensive rats. Decreases in SBP by 19–33 mmHg have been reported for instance, for enzymatic hydrolysates of chicken skin, red seaweed (*Palmaria palmata*), hemp seed, and pea protein (**Table 1**). Generally, the purified renin inhibitory peptides and RP-HPLC fractions have exerted the antihypertensive activities at lower dosage (30 mg/kg bw) compared to the crude protein hydrolysates and membrane-filtrated fractions, which has shown similar antihypertensive effects with 100–200 mg/kg bw (**Table 1**). Hydrolysates and peptides have shown dual inhibition against renin and ACE, or modulation capacity on the RAAS gene expression. Thus, the antihypertensive effects are not solely due to the renin inhibition (e.g., Ref. [41]). For example, egg-derived pentapeptide RVPSL has been recently shown to decrease renin mRNA expression in the kidney of SHRs with a dosage of 50 mg/kg bw administered daily for 4 weeks [79]. Also, weakly active renin-inhibiting peptides have been shown to display physiological antihypertensive activity. A weakly active pea protein hydrolysate (19% renin inhibition at 1 mg/ml) exhibited SBP lowering effects in SHRs and in a kidney disease rat model and was found to downregulate renal expression of renin mRNA in the rat model (**Table 1**). Also, the pea protein hydrolysate showed antihypertensive effects in hypertensive humans in a 3-week intervention trial (**Table 1**). This indicates that antihypertensive food protein-derived peptides may be acting at the same time via multiple pathways at the protein level as well as at the gene level modulating the RAAS.

6. Production of food protein-derived renin inhibitory peptides

A general challenge is how to process the protein hydrolysates further into peptide products with high yield and biological efficacy. Careful choice of suitable enzymes and conditions such as temperature, hydrolysis time, degree of hydrolysis, and enzyme-substrate ratio are crucial for production of peptides with targeted bioactivities and functional properties. Hydrolysis process is recommended to be performed as a continuous process rather than traditional batch process to reduce the enzyme consumption and increase the efficacy [45, 76]. One advantage of enzymatic hydrolysis process is the feasibility in pilot and industrial scale production [6, 7, 28].

To enhance the bioactivity, the active peptides should be concentrated after protein hydrolysis. Size, net charge, and hydrophobicity of the peptides have an important role to select the most suitable techniques to enrich the active peptides. The commonly used techniques include ultrafiltration membranes and chromatographic techniques to obtain an uniform product with the desired range of molecular mass (e.g. [15]). For example, ultrafiltration with 1 kDa membrane has been utilized to concentrate renin inhibitory peptides from rapeseed protein hydrolysate into permeate [36, 38, 48]. In addition to separation based on molecular size, ultrafiltration can be applied to separate peptides according to the net charge. This electrodialysis-ultrafiltration can be utilized to separate anionic, cationic, and neutral peptides of corresponding size range [5, 16, 17]. Large-scale chromatographic methods, used in sugar recovery and wastewater treatments, have been used to enrich peptides from hydrolysates and to separate off ineffective peptides or further undesirable components of the hydrolysates, such as colors, abnormal flavors, and/or salts [10]. Large-scale food-grade processing protocols for designed peptides fractions are needed for further development. Understanding the

structural characteristics of peptides with targeted bioactivity and exploitation of these characteristics is a crucial requirement for this approach.

7. Safety aspects of peptides

The term food allergy refers to an immune response directed toward food and affects approximately 8% of children and 1–2% of adults, and its frequency is increasing [35]. Most allergens reacting with IgE antibodies are proteins found in peanuts, soybeans, tree nuts, milk, egg, fish, crustaceans, and wheat [53, 70].

European Food Safety Authority (EFSA) encourages the use of *in silico* tools for initial prediction of potential allergens from food proteins [8]. Although the toxicity and the allergenicity of food products must be assessed also *in vitro* and *in vivo*, the *in silico* tools can be also used to predict the toxicity of peptides [29]. The available bioinformatics-based allergen prediction tools consist of two groups. The first group is based on searches for sequence similarities following the Codex alimentarius guidelines produced by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), which states that a "protein is potentially allergenic if it either has an identity of over six contiguous amino acids or a minimum of 35% sequence similarity when compared to known allergens" [13]. The second group utilizes databases aiming to identify conserved, allergenicity-related linear motifs [13]. AlgPred (http://www.imtech.res.in/raghava/algpred/) integrates different approaches by means to predict the allergenicity of proteins [62].

After ingestion of food, proteins are naturally hydrolyzed in the gastrointestinal digestion. The digestion often produces peptides with low MW and free amino acids, which are transported across the intestine wall [57]. Highly hydrolyzed proteins and peptides with low MW are not generally toxic and are known to be less allergenic than the native proteins and are widely used in the formulation of hypoallergenic infant foods [32]. However, toxic peptides have been identified from plant as well as animal origin, and they can result in acute, physiological effects, and death. Toxic peptides are usually rich in residues like Asn, Cysm His, and Pro, whereas nontoxic peptides contain dominantly residues Ala, Arg, Gln, Ile, Leu, Lys, Met, Phe, Thr, and Val. [53].

Altogether, the *in silico* assessment of toxicity is not enough, and *in vivo* studies in animal models should be carried out before human consumption. The *in vivo* assessment of the toxicity of food products must be carried out following the guidelines proposed by international authorities. Large quantities of scientific evidence and tests need to be carried out on vertebrate models and cell lines, or unicellular microbial species [47]. Multiple peptide toxicity studies have been carried out in animal models to date [12, 57].

8. Application

Intensive research on bioactive peptides being carried out around the world has already led to the introduction of a wide range of commercial products. The bioactive peptides offer an

exciting opportunity in the area of the development of novel functional foods which in turn could contribute to the prevention and management of certain diseases, such as hypertension, type 2 diabetes, or obesity, and more broadly metabolic syndrome. The functional foods or food ingredients containing milk-derived bioactive peptides, such as the fermented milk Calpis, are already in the market [55]. The claims related to peptides are hypotensive properties, aiding mineral absorption, improving athletic performance, and reducing stress. Since 1991, the Ministry of Health and Welfare in Japan has awarded the status of *Food of Specific Health Use* (FOSHU) to foods with scientifically validated health claims. Since then, antihypertensive peptides, such as Val-Pro-Pro, Ile-Pro-Pro, Val-Tyr, have obtained FOSHU approval [55]. In Europe, applications for nutrition and health claims are submitted to the European Food Safety Authority (EFSA) under Regulation 1924/2006 and are evaluated by Dietetic Products, Nutrition and Allergies (NDA) panel of scientific experts [4]. There are three categories of health claims as defined by EU legislation. Article 13.1 claims are defined as new function or emerging science claims. Recently, the aspects concerning the scientific information needed for the use of a health claim in the functional food product labeling and marketing should include the scientific evidence on the beneficial effects of the product. The characterization of food components with *in vitro* and animal models is needed but they are not sufficient to substantiate the biological functionality in humans. Human studies to investigate the effects of food or food components on reliable markers, such as blood pressure and oxidative damage, are essential. There is still a lot of confusion within the food industry as to what evidence is required with the EU. Regarding the applications already processed, the Commission of European Communities has not yet authorized any claims relating to the effect of bioactive peptides in foods.

9. Conclusions

There is no doubt that the hydrolysis of proteins gives rise to diversity of peptides, some of them displaying remarkable functionalities relevant to human health. The research should encourage the industry to invest more in the added-value products with scientific evidence of health benefits. To this end, novel technologies are available to standardize and stabilize the concentrations of active peptides in the products by means of chromatographic, membrane separation techniques, and encapsulation. Important structure-function parameters of peptides are increasing constantly, which can greatly enhance the production and processing of peptides. With improved understanding of the structure-activity relationship, we may be able to design targeted enzyme hydrolysis strategies to release these peptides.

According to Foltz et al. [25], it appears that it is only valid to propose efficacy once the peptide exhibits reasonable proteolytic stability and physiologically relevant absorption, distribution, metabolism, and excretion profiles. In this field, more in-depth topics include the stability of the biological activity of peptides, during processing as well as *in vivo* in the body before being absorbed and transported to the target site. Greater understanding of the biological fate of peptides and the site of action will allow delivery or an effective dose and formulation of the

peptides to ensure that they reach their target sites. Moreover, we need to gain better understanding of the relationship between these *in vitro* activities and, especially, long-term health benefits in humans and establish appropriate biomarkers of biological efficacy. For example, the extent of the antihypertensive effects has been suggested to depend on the nature of delivery system, dose, study duration, genetic background of the subjects, and stages of hypertension (reviewed in [72]). Furthermore, molecular studies are needed to assess the mechanisms by which bioactive peptides exert their activities in the body. To this end, it may be necessary to employ proteomic and metabolomic methods. By means of these novel nutrigenomic approaches, it is possible and, in future, perhaps essential to investigate the impact of peptides on the expression of genes and hence, endeavor to optimize the nutritional and health effects delivered by peptides.

The safety of all novel peptides intended for food or pharmaceutical uses should be tested in accordance with international and national food safety regulations. In cases of products intended to be marketed in the EU member states, the novel food legislation has to be observed. Other challenges with dietary bioactive peptides are posed by health claims, which in the EU countries are strictly regulated and require science-based documentation before approval by the European Commission. At present, there are worldwide efforts to harmonize these regulations so as to develop fair global food marketing and protect consumers against false or misleading product information.

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