Chapter 3

Antihypertensive Peptides from Food Proteins

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1. Introduction

Hypertension or elevated blood pressure (BP) is a global health concern, thought to affect up to 30 % of the adult population in developed and developing countries. It is defined by a BP measurement of 140/90 mmHg or above. Hypertension is a major risk factor concomitant with cardiovascular disease (CVD) states such as coronary heart disease, peripheral artery disease and stroke, and kidney disease. Essential hypertension, the most common type of hypertension and to which 90-95% of cases belong, is manifested as an increase in an individual's BP due to an unknown cause. This class of hypertension can be improved with lifestyle choices such as regular exercise, heart-healthy eating, non smoking, reducing sodium intake and reducing the level of stress [1]. For these reasons it is defined as a controllable risk factor of CVD. At present there is a range of synthetic drugs on the market for treatment of hypertension including diuretics, adrenergic inhibitors such as α- and β-blockers, direct vasodilators, calcium channel blockers, angiotensin II (Ang II) receptor blockers and angiotensin converting enzyme (ACE) inhibitors. However, although hypertension can be controlled by pharmacological agents, it represents a major burden on annual global healthcare costs. According to the Centre for Disease Control and Prevention (CDC) [2], it was estimated that hypertension-related costs reached $76.6 billion in the USA in 2010. It is thought that prevention through lifestyle choices and early treatment for individuals with mild hypertension can significantly reduce global health-care costs.

Food proteins contain numerous biologically-active peptides (BAPs). These BAPs can exert positive physiological responses in the body beyond their basic nutritional roles in the provision of nitrogen and essential amino acids. Many bioactivities have been found including peptides with antihypertensive capabilities. This has led to significant research on the discovery and generation of peptides with antihypertensive properties *in vivo.* Food proteins such as the casein and whey protein components of milk, meat, egg, marine and meat proteins have all been found to contain peptides with potential antihypertensive properties within their primary sequences. These peptides may become active when

released through enzymatic/bacterial hydrolysis [3]. The food industry has recognised the potential of these *natural* antihypertensive agents as possible future functional ingredients, aiding in the primary prevention and/or management of hypertension.

2. Hypotensive mechanisms of action

The regulation of BP is complex, involving a variety of intertwining metabolic pathways. By far, the most studied BP control pathways with regard to food-derived peptides involve those shown to inhibit ACE *in vitro*. This enzyme is one of the main regulators of BP and is involved in two main systems, the renin-angiotensin system (RAS) and the kinin-nitric oxide system (KNOS). Inhibition of ACE in these systems leads to dilation of the artery walls or vasodilation and subsequent lowering of BP. However, it is not yet known whether this is the main mechanism followed *in vivo* or whether there are a number of other BP control mechanisms involved [4].

2.1. ACE inhibition

ACE inhibition is an excellent physiological target for clinical hypertensive treatment due to its involvement in two BP related systems, the RAS and the KNOS. The RAS is thought to be one of the predominant pressor systems in BP control. In the RAS the N-terminus of the prohormone angiotensinogen, which is derived from the liver, is cleaved by renal renin to produce the decapeptide angiotensin I (Ang I). ACE then removes the C-terminal dipeptide HL to form Ang II, a potent vasoconstrictory peptide which acts directly on vascular smooth muscle cells. Thus, inhibition of ACE consequentially leads to BP reduction. Ang II binds to AT_1 and AT_2 receptors which are located in peripheral tissues around the body and in the brain. The vasocontriction produced by Ang II is mediated by the AT_1 receptor. [5-7]. In the KNOS, ACE inactivates the vasodilatory peptides bradykinin and kallidin. Kallidin is synthesised from kininogen by kallikrein, and its further action on kallidin leads to the formation of bradykinin among other vasoactive peptides. Bradykinin binds to β-receptors which lead to an eventual increase in intracellular Ca^{2+} level. The binding of bradykinin to β-receptors and the increase in Ca^{2+} stimulates nitric oxide synthase (NOS) to convert L-arginine to nitric oxide (NO), a potent vasodilator. ACE can therefore, indirectly inhibit the production of NO as it hydrolyses bradykinin into inactive fragments [7].

There are a number of widely-used synthetic ACE inhibitors currently on the market that serve as the first line of approach for the treatment of hypertension. Such inhibitors include Captopril, Enalapril and Lisinopril. However, their use is associated with a range of side-effects including cough, skin rashes, hypotension, loss of taste, angiodema reduced renal function and fetal abnormalities [8]. Natural ACE inhibitory peptides from food are not associated with the side-effects brought about by the synthetic drugs. They are not as potent inhibitors of ACE as the synthetic inhibitors which can have IC_{50} values in the nM region. As they inhibit ACE to a lesser extent, this potentially allows for safer levels of bradykinin in the body. Thus, for this reason, ACE-inhibitory peptides have gained interest as potential preventative agents for hypertension control.

ACE-inhibitory peptides have been identified in a range of food proteins including casein, whey, ovalbumin, red algae, wakame, soy, gelatin, chicken muscle, dried bonito, corn, sardines, rapeseed, potato, chick pea, tuna muscle, pea albumin, garlic, wheat germ, sake, porcine haemoglobin and squid. The ACE inhibitory peptides found in different food proteins has been extensively reviewed (for review see [9-12; 3; 131]. Examples of recently reported food protein ACE inhibitory peptide sources include loach (*Misgurnus anguillicaudatus*) [13], pork meat [14], lima bean (*Phaseolus lunatus)* [15], skate skin [16] and boneless chicken leg meat [17]. ACE inhibitory peptides have been generated in a number of different ways. They can be produced naturally during gastrointestinal (GI) digestion by the hydrolytic action of the proteinases pepsin, trypsin, chymotrypsin and by brush border peptidases [18]. Simulated GI digestion has been carried out on a range of protein sources to assess the effect of GI digestion on ACE-inhibitory peptides [19-24]. More commonly, ACE-inhibitory peptides are produced through enzymatic hydrolysis with GI enzymes such as pepsin and trypsin or with enzyme combinations such as Alcalase™ [25]. ACE-inhibitory peptides have also been produced during the fermentation of milk during cheese production. *Lactobacillus* and *Lactococcus lactis* strains have been shown to produce ACE inhibitory peptides. Furthermore, fermented soy products such as soy paste, soy sauce, natto and tempeh have been found to produce ACE-inhibitory peptides [26-29].

ACE inhibitory peptides can work in three ways and are classed as inhibitor-type, substrate-type or prodrug-type based on changes in ACE inhibitory activity after hydrolysis of peptides by ACE [30]. Inhibitor-type peptides are ACE inhibitory peptides whose activity is not significantly altered as the peptides are resistant to cleavage by ACE. Substrate-type ACE inhibitors show a decrease in ACE activity due to cleavage by ACE. Prodrug type refers to the conversion to potent ACE inhibitors following hydrolysis of larger peptide fragments by ACE itself. The resulting peptides tend to produce long-lasting hypotensive effects *in vivo* [30]. A prodrug type ACE inhibitor was isolated from a thermolysin-digest of Katsuo-bushi, a Japanese traditional food processed from dried bonito. The study reported an 8-fold increase in ACE-inhibitory activity when the peptide Leu-Lys-Pro-Asn-Met (IC_{50}=2.4 μM) was hydrolyzed by ACE to produce Leu-Lys-Pro [IC_{50}=0.32 μM; 30]. When Leu-Lys-Pro-Asn-Met and Leu-Lys-Pro were orally administered to spontaneously hypertensive rats (SHR), Leu-Lys-Pro-Asn-Met showed a maximal decrease of BP after 4 and 6 h, results which are comparable to that of Captopril inhibition. However, the maximal hypotensive effect of Leu-Lys-Pro was seen at 2 h [30].

Inhibition of ACE is by far the most studied mechanism of BP control with regard to food-derived biologically-active peptides. Most peptides have been found to inhibit ACE to some degree. However, in most cases, it has yet to be answered whether this is the BP mechanism being employed *in vivo*. There are other regulatory pathways of BP control, independent of ACE, that are also potential targets for the action of antihypertensive peptides (see Figure 1 for vasorelaxative peptides and molecules).

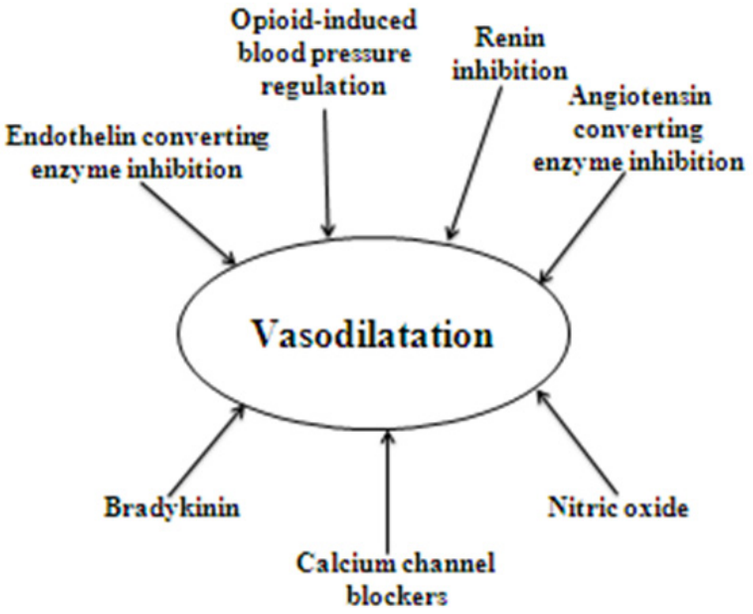

Figure 1. Vasorelaxative peptides and molecules in blood pressure control systems.

2.2. Renin Inhibition

Renin inhibition is another potential target for BP control. It is thought that inhibition of renin could provide a more effective treatment for hypertension it prevents the formation of Ang-I, which can be converted to Ang-II in some cells independent of ACE, by the enzyme chymase [31]. In addition, unlike ACE which acts on a number of substrates in various biochemical pathways, angiotensinogen is the only known substrate of renin. Therefore, renin inhibitors could ensure a higher specificity in antihypertensive treatment compared to ACE inhibitors [31-32]. Food peptides have recently been found to be inhibitors of renin. Peptides from enzymatic flaxseed fractions were found to inhibit both human recombinant renin and ACE. The study concluded that such peptides with the ability to inhibit both ACE and renin may potentially provide better antihypertensive effects *in vivo* in comparison to peptides that only inhibit ACE [33]. A similar outcome was seen in a study carried out by Li & Aluko [34] where fractions of pea protein isolates inhibited both ACE and renin to a high degree with IC_{50} values <25 mM.

2.3. Calcium channel blocking effects

Calcium channel blockers interact with voltage-gated calcium channels (VGCCs) in cardiac muscle and blood vessel walls, reducing intracellular calcium and consequently lowering vasoconstriction. It has been shown in various studies that peptides can have the ability to act as calcium channel blockers. Fifteen synthetic peptides based on Trp-His skeleton analogues were tested for their vasodilatory effects in 1.0 μM phenylephrine-contracted thoracic aortic rings from Sprague-Dawley rats. It was previously reported that Trp-His induced the most potent vasodilation among 67 synthetic di-and tripeptides. The study demonstrated that His-Arg-Trp had an endothelium-independent

vasorelaxative effect in the phenylephrine-contracted thoracic aorta. It was also shown that His-Arg-Trp, at a concentration of 100 μM, caused a significant reduction in intracellular Ca^{2+} concentration. The increase intracellular [Ca^{2+}], brought about by the action of Bay K8644 or Ang II, was significantly inhibited by His-Arg-Trp (>30%). It was proposed that His-Arg-Trp may have supressed extracellular Ca^{2+} influx through voltage-gated L-type Ca^{2+} channels [35]. Another recent study reported a similar result with Trp-His which was also found to block L-type Ca^{2+} channels. Trp-His at 300 μM elicited an intracellular Ca^{2+} reduction of 23 % in 8 week-old male Wistar rat thoracic aortae smooth muscle cells. In addition, the reduction in [Ca^{2+}] brought about by Trp-His was eliminated by verapamil indicating that Trp-His specifically works on L-type Ca^{2+} channels [36].

2.4. Opioid peptide vasorelaxive effects

Food-derived peptides have also been found to be sources of opioid like-activities. These peptides bind to opioid receptors to produce morphine-like effects. Natural opioid peptides include endorphins, enkephalins and dynorphins. In humans opioid receptors are found in the nervous, endocrine and immune systems, and in the intestinal tract. These receptors may be involved in various regulatory processes in the body including the regulation of circulation which can affect BP [37; 38]. Nurminen *et al* [39] found an antihypertensive effect on oral administration of the tetrapeptide, α-lactorphin (Tyr-Gly-Leu-Phe), to SHR and to normotensive Wistar Kyoto rats (WKY). Maximum BP reductions were found in SHR, with a decrease of 23 ± 4 and 17 ± 4 mm Hg in systolic BP (SBP) and diastolic BP (DBP), respectively. However, the α-lactophin-induced reduction in BP was not found after administration of the specific opioid receptor antagonist, Naloxone. Therefore, the antihypertensive effect was considered to be a result of interaction with opioid receptors. A follow-up study looked at the effects of α-lactophin along with a second milk-derived peptide β-lactorphin (Tyr-Leu-Leu-Phe) on mesenteric arterial function to demonstrate the regulatory mechanisms of action. It was shown with the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) that α-lactophin produced an endothelium-dependant vasorelaxation, whereas, β-lactorphin also enhanced endothelium-independent vasorelaxation. The study concluded that α-lactophin may stimulate opioid receptors which in turn releases NO causing the vasorelaxative effect [40]. The casein-derived peptide casoxin D (Tyr-Val-Pro-Phe-Pro-Pro-Phe) has also been reported to have an hypotensive effect via opioid receptors. The peptide was found to have an endothelium-dependent relaxation in canine mesenteric artery strips. Anti-opioid and vasorelaxing effects were mediated by the opioid μ-receptor and BK B1-receptor, respectively [41-42]. Furthermore, it has been suggested that opioid-induced BP regulation by such peptides may act upon receptors in the intestinal tract. Interestingly, this would mean that the peptide would not need to be absorbed into the blood stream at the brush border membrane [43]. It could very well be that opioid-mediated reduction in BP may be the principal mechanism for antihypertensive peptides.

2.5. Endothelin-1 and endothelin converting enzyme (ECE) inhibition

The vasoconstrictory peptide endothelin-1 (ET-1) is released from big endothelin-1 (big ET-1) by the action of endothelin-converting enzyme (ECE). ET-1 mediates vasoconstriction via 2 receptors, ETa and. ETb. Both receptors mediate contractions on smooth muscle, but ETb also induces relaxation of endothelial cells by the production of nitric oxide. ET-1 is known to have a greater vasocontrictive effect than Ang II [44; 7]. Endothelial-dependent release of NOS was found to be the mechanism of action for the antihypertensive egg protein derived ovokinin (f2-7) peptide (Arg-Ala-Asp-His-Pro-Phe). Dilation of isolated SHR mesenteric arteries was found to be inhibited by L-NAME but not by indomethacin, demonstrating NO release from the endothelial cells [45]. A later study showed that ovokinin (2–7) modulates a hypotensive effect through interaction via B_2 bradykinin receptors [46].

It has been found that food proteins have the ability to act as inhibitors of ECE. Okitsu *et al* [47] found ECE inhibitory peptides in pepsin digests of beef and bonito pyrolic appendix. Up to 45 and 40 % of ECE activity could be inhibited with the beef and bonito peptides, respectively. A second study showed that the ACE-inhibitory peptide Ala-Leu-Pro-Met-His-Ile-Arg, released through tryptic digestion of bovine β-lactoglobulin, can inhibit the release of ET-1 in cultured porcine aortic endothelial cells (PAECs). At a concentration of 1 mM Ala-Leu-Pro-Met-His-Ile-Arg, ET-1 release was reduced by 29 %. The study concluded that the ET-1 reduction may be due to indirect reduction of ET-release by ACE inhibition through the BK pathway, rather than direct action on ET-1 by the peptide [48]. ACE breaks down BK into inactive fragments in the KNOS. Subsequent accumulation of BK (vasodilator) due to ACE inhibition leads to increased release of the vasodilator NO, and antagonises the release of the ET-1 by endothelial cells.

3. Structure activity relationships

An understanding of the relationship between a peptide and its bioactivity allows for the targeted release of potentially potent peptide sequences. This would eliminate the need for the time-consuming conventional peptide discovery strategy. There is limited knowledge on the structure-activity relationship of hypotensive peptides. To date, the main focus with regard to bioactive peptide research has been on the generation and characterisation of these peptides. ACE inhibition is by far the most widely studied biomarker with regard to antihypertensive effects of bioactive food peptides. ACE can work on a wide range of peptide substrates, and appears to have a broad specificity. Some structural features that influence the binding of a peptide to the ACE active site have been recognised (Table 1). However, potent inhibitory peptides of ACE are generally short sequences, i.e., 2-12 amino acids in length. However, some larger inhibitory sequences have been identified. Studies have indicated that binding to ACE is strongly influenced by the substrate's C-terminal tripeptide sequence. Hydrophobic amino acid residues with aromatic or branched side chains at each of the C-terminal tripeptide positions are common features among potent inhibitors. The presence of hydrophobic Pro residues at one or more positions in the C-terminal tripeptide region seems to positively influence a peptide's ACE inhibitory activity.

Tyr, Phe and Trp residues are also present at the C-terminus of many potent ACE inhibitors, especially with di- and tripeptide inhibitors [9]. It has been suggested that Leu residues may also contribute to ACE inhibition [49]. Furthermore, the positive charge on the side chains of Arg and Lys residues at the C-terminus have been noted to contribute to the ACE inhibitory potential of a peptide [50-51; 9]. An L-configured amino acid at position three at the C-terminus of the inhibitory peptide may be a requirement for potent inhibition. A study showed that the IC_{50} for the tripeptide D-Val-Ala-Pro (2 μM) increased to 550 μM with L-Val-Ala-Pro, yet only a slight increase in IC_{50} was seen for the peptide L-Phe-Val-Ala-Pro (17 μM; Maruyama *et al*., 1987). It is thought that conformation contributes to the ACE inhibitory potential of long-chain peptide inhibitors [3].

N-Terminus----------C-Terminus		
Hydrophobic residues	2-12 amino acids in length Peptide conformation important for longer peptides	C-terminal tripeptide Bulky hydrophobic residues Aromatic or branched side chains Proline at one or more positions Positively charged residues in position two, Arg, Lys Tyr, Phe, Trp, Leu L-configured residue in position three

Table 1. Some structural features of potent angiotensin converting enzyme (ACE) inhibitory peptides.

Both domains of ACE (C- and N-domains) contain an active site containing the sequence His-Glu-XX-His. These active sites are located within the cleft of the two domains, and are protected by an N-terminal 'lid'. This 'lid' blocks access of large polypeptides to the active site. This is thought to explain why small peptides are more effective in inhibiting ACE. In addition, ACE inhibition may include inhibitor interaction with subsites on the enzyme that are not generally occupied by substrates or with an anionic inhibitor binding site that is different for the catalytic site of the enzyme. With the catalytic sites of ACE having different conformational requirements, this could indicate that for a more complete inhibition of ACE, there may be a need to use a variety of peptide inhibitors each with slightly different conformational features [52-53].

Quantitative computational tools are increasingly been applied in medicinal and pharmaceutical drug discovery. Recently it has been acknowledged that such models could be adapted to food-derived bioactive peptide sequences. Quantitative structure-activity relationship modelling (QSAR) and substrate docking can be used as an effective tool to assess *in silico* numerous peptide structures for their bioactivity potential. Thus, this work allows for a molecular understanding of peptide structure and bioactivity. QSAR studies are

based on the relationship between chemical structure of ligands and receptors, and biological activity. Physicochemical variables or descriptor variables of a ligand such as steric properties, hydrophobicity and electronic properties, molecular mass and shape are used to quantitatively correlate the ligand's chemical structure with bioactivity [54]. A small number of QSAR studies have been carried out on ACE-inhibitory peptides. The structure-activity relationship of di-and tri-peptides using partial least square analysis (PLS) QSAR was assessed by constructing a database of known ACE-inhibitory peptides. Using a 3-z scale descriptor approach, two models were developed for the amino acid components of the peptide datasets. The dipeptide model had a predictive power of 71.1 % while the tripeptide model had a predictive power of 43.4 %. The dipeptide model indicated that amino acids with bulky and hydrophobic side chains were favoured by ACE while the tripeptide model suggested that C-terminal aromatic residues, positively charged residues in position two and hydrophobic residues at the amino terminus were preferred [55]. Another study by the same authors used a 5-z scale model to assess peptides of 4-10 amino acids in length. The study concluded that the tetrapeptide residue at the C-terminus has a large influence on the potency of peptide's 4-10 amino acids in length [56].

Substrate docking involves the docking of molecules (ligands) to a receptor or into a protein target such as an enzyme. All possible docking or binding conformations are assessed for their binding affinity to a molecule, and their potential as high affinity binding ligands is estimated by use of a scoring function. An integrated QSAR and Artificial Neural Network (ANN) approach was used to assess the ACE-inhibitory potential of 58 dipeptides present in the sequence of defatted wheat germ protein. The model was used to investigate preferred structural characteristics of ACE-inhibitory dipeptides and following this, appropriate proteases were successfully selected to produce the dipeptides predicted to be potent inhibitors by the QSAR-ANN model. The QSAR model predicted that the C-terminal of the peptide had principal importance on ACE inhibitory activity, with hydrophobic C-terminal residues being essential for high potency. Furthermore, proteins with a high abundance of hydrophobic residues were considered to be good substrates for the production of potent ACE inhibitory peptides [57]. Recently, the ability of docking to predict ACE inhibitory dipeptide sequences was assessed using the molecular docking program AutoDock Vina. All potential dipeptides and phospho-dipeptides were docked and scored. Phospho-dipeptides were predicted by the program to be good inhibitors of ACE. However, the experimentally determined IC_{50} results for selected phospho-dipeptides did not correlate and the study concluded that phospho-dipeptides may not be potent inhibitors of ACE *in vivo*. Furthermore, LIGPLOT analysis, a program to plot schematic diagrams of protein-ligand interactions, carried out on two newly identified ACE inhibitory dipeptides Asp-Trp and Trp-Pro (ACE IC_{50} values of 258 and 217 μM, respectively) interestingly showed no zinc interaction with the ACE active site [58].

4. Peptide bioavailability

The potential antihypertensive effect of a peptide depends on the peptides ability to reach their target organ intact and in an active form. However, there are several barriers which lie

in the way of this outcome. Antihypertensive peptides must be resistant to digestive proteinases and peptidases; they must be able to be transported through the bush border membrane intact and must be resistant to serum peptidases. With regard to ACE inhibition, while there have been many studies focusing on the production, isolation and characterisation of ACE-inhibitory peptides, to date little attention has been placed on their bioavailability. It is therefore difficult to determine the relationship between *in vitro* ACE-inhibitory activity and an *in vivo* hypotensive effect. This is made even more difficult with the utilisation of several different *in vitro* assays and assay conditions for the determination of ACE-inhibition [59]. Furthermore, variations in *in vivo* experimental design such as administration by intravenous subcutaneous or oral administration, and the use of animal models or hypertensive patients, all hinder the ability to compare results among different studies [60].

Bioactive peptides when taken orally may be inactived by several digestive proteinases and peptidases including pepsin in the stomach, and the pancreatic enzymes trypsin, elastase, α-chymotrypsin and carboxypeptidase A and B in the small intestine. A number of studies have been carried out investigating ACE-inhibitory peptides and their ability to resist gastrointestinal digestion by these enzymes. These studies involve simulating the gastrointestinal process by sequential hydrolysis of ACE inhibitory peptides with pepsin and Pancreatin™, each concluding the importance of gastrointestinal digestion analysis in the ACE inhibitory activity of the peptide [61-66; 49; 21; 20] . It has been noted that certain protein/peptide structures are resistant to gastrointestinal digestion due to the composition and position of amino acids in their primary chains. The rate of hydrolysis of a peptide is also dependent on the peptide's amino acid composition. Peptides containing Pro and hydroxy Pro residues have been found to be resistant to hydrolysis. Furthermore, glycosylated peptides and peptides which have undergone changes during food processing such as during the formation of Maillard reaction products have been shown to be resistant to GI tract enzyme cleavage [67]. Once the peptides reach the brush border membrane of the large intestine, they may also be subjected to further cleavage by a variety of membrane anchored epithelial cell intestinal peptidases. These include a number of aminotripeptidases and several dipeptidases, each with varying specificities [60]. However, it has been found that certain free amino acids released during gastrointestinal breakdown may in turn serve as inhibitors of the brush border membrane dipeptidases, Moreover, it has been reported that during gastrointestinal proteolysis at the brush border membrane, the large variety and high concentration of peptides present would exceed the apparent *V*max for hydrolysis, allowing for safe passage of many di- and tripeptides through the membrane wall. Absorption through the membrane is possible for both di- and tripeptides with the help of a peptide transporter termed PepT1. PepT1 operates as an electrogenic proton/peptide symporter having wide substrate specificity [67]. There is an increasing body of research that shows the presence of the lactotripeptides (LTPs) Ile-Pro-Pro and Val-Pro-Pro in human and animal circulatory systems after oral administration, suggesting the resistance of these peptides to gastrointestinal degradation and their absorption intact across the brush border membrane [68-72]. However, it has been suggested that intestinal absorption of Val-Pro-Pro

may operate via paracellular transport, rather than with the help of PepT1 [73]. Larger Pro-rich peptides have also been found to be transported intact across the brush border membrane. A study found that the ACE-inhibitory and antihypertensive peptide Leu-His-Leu-Pro-Leu-Pro, β-casein (f133-138), was resistant to gastrointestinal digestion. However, this peptide was hydrolysed to the pentapeptide His-Leu-Pro-Leu-Pro by cellular peptidases before transportation across the intestinal epithelium. The study concluded by use of a Caco-2 monolayer model that the likely mechanism of transport was via paracellular passive diffusion [74]. An earlier study quantifying ACE-inhibitory peptides in human plasma found the pentapeptide to be present in human plasma after oral administration which demonstrates the ability of the peptide to be absorbed through the human brush border membrane [75].

Absorption of peptides across the brush border membrane can be studied by Caco-2 cell monolayers, the representative model for human intestinal epithelial cell barrier. The intestinal transport of pea and whey ACE inhibitory peptides was also studied using a Caco-2 monolayer. It was found that only minor ACE inhibitory activity crossed the Caco-2 cell monolayer in 1 h. However, it was concluded that the extent of ACE inhibitory peptides that may be transported *in vivo* would be higher, as the Caco-2 model is tighter than intestinal mammalian tissue [76]. The transepithelial transport of oligopeptides across the intestinal wall was assessed using a Caco-2 cell monolayer [133]. The study showed that the hydrolysis of peptides by brush-border peptidases is the rate-limiting step for the transepithelial transport of oligopeptides (≥4 residues in length). Bradykinin and Gly-Gly-Tyr-Arg, which were found to be resistant to cellular peptidases, were investigated for their apical-to-basolateral transport mechanism. Bradykinin and its analogues were found to be transported by the intracellular pathway, probably the adsorptive transcytosis. The transport rate was found to be dependent on the hydrophobic properties of the peptides. Gly-Gly-Tyr-Arg was suggested to be transported mainly via the paracellular pathway [133]. Foltz *et al*., [77] devised a predictive *in silico* amino acid clustering model for dipeptides which can predict a dipeptide's ability to withstand small intestinal digestion. Dipeptides (220 in total) were tested for small intestinal stability by simulated digestion and their relative stability (% of initial dipeptide concentration) was plotted against time. Using the area under the curve (AUC) approach, the contribution of N- and C-terminal amino acids were calculated, based on the average AUC of all peptides containing the amino acid of interest. Data clustering allowed for ranking of the N- and C-terminal amino acid residues and they were grouped by their average AUC values. Correlations with experimentally measured stability allowed for classification of dipeptides as intestinally 'stable', 'neutral' or 'instable' using the clustering model.

Following absorption of a peptide into the blood stream, it may undergo hydrolysis by serum peptidases. The ACE inhibitory peptide may need to be able to with-stand hydrolysis in order to reach their target organs intact and yield their antihypertensive effect. It has been suggested that potent ACE inhibitors may be produced in circulation by the action of serum peptidases on less potent inhibitors of ACE and by the action of ACE itself. These peptides have been referred to pro-drug type inhibitors of ACE [78; 60].

Thus, the bioavailability of ACE-inhibitory peptides is essential for their activity. Several approaches to aid in peptide delivery are been considered. Peptides may be chemically modified in order to reduce the rate of enzymatic degradation and to increase bioavailability while also in some cases enhancing bioactivity. The half-life of unmodified peptides in the blood is in most cases very short. They also generally have poor bioavailability in tissues and organs, limiting their ability as preventative therapeutic agents [79; 80]. Modifications such as end changes, glycosylation, alkylation, and conformational changes to amino acids within the peptide may therefore have potential for ACE inhibitory peptides [80; 81]. These approaches have already been adapted to opioid peptides [81]. There is significant scope for these modifications to also be applied to ACE inhibitory and antihypertensive peptides. Encapsulation via nanoparticles and liposomes is also a strategy previously employed for opioid peptides that has possibility for adaption to for ACE inhibitory peptides. These approaches may aid in the passage of a peptide through the GI tract and may enhance the plasma half-life of the peptides. Furthermore, there is potential for bioactive peptides to be produced by microorganisms through genetic engineering to be delivered to target organs *in situ* [60]. Lastly, there is also the possibility to cross-link BAP to protein transduction domains that have been found to be able to cross biological membranes thus promoting peptide and protein delivery into cells [60; 82]. Morris *et al* [82] also devised a similar strategy using the peptide transporter PepT-1 to carry target peptides into cells.

5. *Ex-vivo* and *in vivo* animal studies

The first step employed to determine if a peptide is hypotensive is to conduct trials with small animals such as SHRs, the accepted model for human essential hypertension. A bioactive peptide can only be referred to as 'antihypertensive' after a significant decrease in BP is observed in trials with SHR. There have been many studies carried out in animals to elucidate whether food-derived ACE-inhibitory peptides can lead to an antihypertensive effect *in vivo.* Antihypertensive peptides from milk, egg, animal (including meat and marine animals), plant and macroalgae have recently been reviewed [4; 25; 83-86].

Ile-Pro-Pro (β-casein f74-76; κ-casein f108-110) and Val-Pro-Pro (β-casein f84-86) were among the first dietary peptides found to have a hypotensive effect in SHR. The peptides were first isolated from milk fermented with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Ameal S) and their ACE-inhibitory IC_{50} values were obtained (Val-Pro-Pro and Ile-Pro-Pro having IC_{50}s of 9 and 5 μM, respectively [87]). The antihypertensive effect was first demonstrated when SHR were administered with a single oral dose of the LTPs which resulted in a significant decrease SBP between 6 to 8 h after administration [88]. Thereafter, several studies have been conducted to further characterise the *in vivo* effect of the LTPs. Their long-term effects (12-20 weeks) of administration have been assessed [89-93]. Administration of the peptides via a peptide supplement and via a sour milk drink to SHR resulted in a decrease in SBP of 12 and 17 mm Hg, respectively, compared to the control (water) after 12 weeks [90]. Endothelial function protective effects of Ile-Pro-Pro and Val-Pro-Pro were investigated using isolated SHR mesenteric arteries stored in solutions of

Krebs containing Ile-Pro-Pro and Val-Pro-Pro (1 mM), when mounted in an organ bath. Vascular reactivity measurements demonstrated better preservation of endothelium-dependent relaxation in arteries stored with the LTPs compared to controls [94]. Their bioactive effect in double transgenic rats (dTGR) harbouring human renin and angiotensinogen genes was also assessed. These transgenic rats develop malignant hypertension, cardiac hypertrophy, renal damage, and endothelial dysfunction due to increased Ang II formation. A decrease of 19 mm Hg in SBP was seen in rats administered with fermented milk supplemented with the peptides (Ile-Pro-Pro (1.8 mg/100 ml) and Val-Pro-Pro (1.8 mg/100 ml) compared to the control group Thus, it was concluded that the supplemented fermented milk product can aid in preventing the development of malignant hypertension. There was no effect on BP reported from a group receiving the peptides dissolved in water, despite the higher intake level of peptides. The authors concluded that the reported antihypertensive effect of the fermented milk product can not be explained solely by the Ile-Pro-Pro and Val-Pro-Pro supplements and suggested that a combination of factors such as calcium and potassium content, and less sodium may have contributed to the observed hypotensive effect [95].

Other rat models, such as the normotensive WKY rat, have been used to evaluate the effect of food peptides on arterial BP. However, a significant hypotensive effect is not always observed in WKY. Single oral administration of Ameal S containing the LTPs decreased BP from 6 to 8 h after administration in SHR. However, no change in SBP was observed in normotensive WKYs [88]. Similarly, the ACE inhibitory peptide Leu-Arg-Pro-Val-Ala-Ala from bovine lactoferrin was found to have a significant antihypertensive effect in SHR but no change in BP was found when the peptide was administered via intravenous injection to WKY rat [96]. Thus, the hypotensive effect of some food-derived peptides may be specific to the hypertensive state of the animal. The effect of fermented milk with LTPs on BP and vascular function in salt-loaded type II diabetic Goto–Kakizaki rats has also been assessed. GK rats are characterized by impaired glucose-induced insulin secretion, abnormal glucose regulation, insulin resistance and polyuria. They are normotensive but when on a high-salt diet can develop hypertension. The study showed a significant decrease in BP and enhanced endothelium-dependent relaxation of mesenteric arteries [97].

There are wide variations in BP responses from different food proteins. These variations may be due to the different food sources themselves but also may be due to differences in experimental models such as the type of animal used, the dosage of peptide required for a significant decrease in BP, duration of administration and administration route, i.e., oral versus intravenous administration. In general, it has been found that peptides administered intravenously have a higher decrease in BP than peptides administered orally. This may be due to lower bioavailability of these peptides in the blood stream as transport of the peptides across the brush border membrane in an intact state may not be possible. Hydrolysis or partial hydrolysis of the peptides by GI and serum enzymes may lead to inactive or less active hypotensive peptide forms. Thus, bioavailability studies are essential to assess the antihypertensive potential of a peptide [3].

Furthermore, it must be noted that although dietary peptides have lower ACE IC_{50} *in vitro* in comparison to the synthetic ACE drug inhibitor Captopril (IC_{50} in nM range), in most cases they display higher *in vivo* hypotensive effects than are expected with respect to their *in vitro* results. It has been suggested that this may be due to a higher affinity of dietary peptides to the tissues and a slower elimination in comparison to Captopril. Moreover, it is possible that several BP mechanisms of action are being employed [30; 98]. It was demonstrated that neither the egg-protein derived peptide ovokinin (2-7) (Arg-Ala-Asp-His-Pro-Phe) or Arg-Pro-Leu-Lys-Pro-Trp, the most potent derivative obtained from the structural modification of ovokinin,, inhibit ACE *in vitro*. IC_{50} values obtained were >1000 μmol/L, despite having a significant effect on BP when orally administered to SHR [99]. Thus, it must be acknowledged that *in vitro* ACE inhibitory determination may not be the best approach to assess the potential of a peptide as an antihypertensive agent. In a study by da Costa *et al* [100] it was found that the most potent *in vitro* ACE inhibitory peptides from whey did not have a significant effect on BP when orally administered to SHR. However, whey peptides with relatively low *in vitro* ACE-inhibitory activity in comparison achieved significant reductions in BP.

6. Human studies

The majority of the clinical trials regarding the antihypertensive effects of milk-derived peptides to date have been carried out on the LTPs, Ile-Pro-Pro and Val-Pro-Pro. Although some conflicting results exist, the majority of these trials have reported a significant decrease in BP. Their effect on office BP has been well documented (for reviews see 101; 3; 25). It is essential that the BP of test subjects is evaluated in comparison to placebo values and not to baseline values of the test product. As with test products, placebo groups have been found to often decrease in BP over the test period [101]. Furthermore, the 24-h ambulatory BP monitoring (ABPM), of patients BP is thought to be a more reliable method for evaluation of BP as it reduces the 'white coat effect'. Recent clinical trials with LTPs have employed ABPM [102-104; 91]. ABPM was used to assess the effects of LTP administration on dipper (where BP decreases at night time) and non-dipper (where BP does not decrease at night time) hypertensive subjects. Non-dippers are thought to have a higher cardiac vascular risk and BP monitoring in the morning and night can help predict cardiac events such as stroke and myocardial infarction. Twelve patients received a fermented milk product containing Ile-Pro-Pro (1.52 mg) and Val-Pro-Pro (2.53 mg) daily for 4 weeks. The study reported a significant reduction in night-time and early-morning SBP for nondipper subjects but not for dipper subjects [105]. A range of hemodynamic parameters was recently evaluated for 52 human subjects with high-normal BP or first-degree hypertension. These included office BP and ABPM, stress-induced BP increase and cardiac output-related parameters. Subjects were treated with LTPs (3 mg/day) for 6 weeks. The study reported a reduction in office SBP as well as an improvement in pulse wave velocity (an instrumental biomarker for vascular rigidity), stroke volume and stroke volume index (markers of cardiac flow) and acceleration and velocity index (markers of cardiac contractility). No effect on ABPM and BP reaction to stress was observed [106]. LTPs have also been reported to reduce arterial stiffness in

humans. In a double-blind parallel group intervention study, 89 hypertensive subjects received daily milk containing a low dose of 5 mg/day of Ile-Pro-Pro and Val-Pro-Pro for 12 weeks and a dose of 50 mg/day for the following 12 weeks. Arterial stiffness, measured by the augmentation index (AI), decreased in the peptide group by -1.53% compared to 1.20% in the placebo group at the end of the second intervention period [107]. A similar result was seen in a study by Nakamura *et al* [108]. Twelve hypertensive subjects were administered four tablets containing Val-Pro-Pro (2.05 mg) and Ile-Pro-Pro (1.13 mg) daily for 9 weeks and were monitored for various hemodynamic parameters. A significant reduction in AI as well as peripheral SBP and DBP along with central SBP (cSBP) was observed. Furthermore, it has been suggested that LTPs may also have a positive effect on vascular endothelial function in subjects with stage-I hypertension [109] and may improve arterial compliance in postmenopausal women [110].

Other hypotensive peptides and food preparations used in human trials include peptides from casein [111], whey [112], dried bonito [113], fermented milk containing gamma-aminobutyric acid (GABA; 114) sardine muscle [115] and wakame (*Undaria pinnatifida;* 116). Recently, a number of meta-analyses on antihypertensive peptides have been carried out. Pripp *et al* [117] performed a meta-analysis on antihypertensive peptides from milk and fish proteins which included 15 human trials. A pooled decrease in SBP of -5.13 mm Hg and a decrease of -2.42 mm Hg for DBP were found. A similar result was found with a meta-analysis of 12 trials with LTPs, (623 participants in total) when pooled data in forest plots found a decrease of -4.8 mm Hg and 2.2 mm Hg in SBP and DBP, respectively. The observed hypotensive effects also seemed to be greater in hypertensive patients than in patients with pre-hypertension [118]. Another meta-analysis carried out on data from LTPs trials interestingly found that the effect of LTPs on BP was more evident in Asian subjects (SBP = -6.93 mm Hg; DBP=-3.98 mm Hg) than in Caucasians (SBP=-1.17 mm Hg; DBP = -0.52 mm Hg). The study also found that the LTP-induced hypotensive effects were not related to subject age, baseline BP value, administered dose or length of treatment [119]. Conflicting results however, were reported in a recent meta-analysis by Usinger *et al* [132]. Data from 15 controlled trials (1232 subjects in total) that observed the effect of fermented milk or similar products produced by *Lactobacilli* fermentation of milk proteins were used in the meta-analysis. The study reported a pooled decrease in SBP of just -2.45 mm Hg and found no significant decrease for pooled DBP data. Furthermore, the authors stated that the included studies were of variable quality and when excluding the studies with a high risk of bias no significant decrease in SBP or DBP were found.

7. Hypotensive peptides as functional food ingredients

The main considerations which need to be taken into account in the utilisation of antihypertensive peptides as functional ingredients in food products include characterisation of their organoleptic and physicochemical properties. In the first instance, establishment of the optimal method for peptide release via protein hydrolysis is required. Industrial scale processing of BAPs requires that peptides be able to withstand

and retain their bioactivity during multi-step processing including pasteurisation, homogenisation, pressure-driven membrane-based processing such as ultrafiltration and nanofiltration, dehydration by spray-drying or freeze-drying, and peptides must also be stable during long-term storage. Little data exists on the effects of different processing techniques on BAPs in food products. Dehydration via spray drying has been found to produce changes in peptide confirmation, a reduction in amino acid content and may also lead to non-enzymatic browning reactions [43]. The industrial-scale production of a casein hydrolysate containing the antihypertensive peptides Arg-Tyr-Leu-Gly-Tyr (α_{s1}-CN f90-94) and Ala-Tyr-Phe-Tyr-Pro-Glu-Leu (α_{s1}-CN f143-149) and the stability of the hydrolysate incorporated in a yoghurt to processing conditions, i.e. drying, homogenisation and pasteurisation, and to storage at 4°C was recently investigated. The study showed the hydrolysate to be stable after processing as both *in vitro* ACE-inhibitory activity and the *in vivo* antihypertensive properties in SHR were maintained. Analysis by reverse phase-high pressure liquid chromatography-mass spectrometry (RP-HPLC-MS) showed that the integrity of the antihypertensive peptides was also maintained during storage at 4 °C for 1 month [120]. Similar studies are required for other antihypertensive peptides in order to evaluate the optimal processing conditions required for retention of bioactivity.

It has been shown that heat treatments and mechanical damage can reduce peptides bioactivity. As a result of changes in protein structure, the profile of peptides released may differ as digestive enzymes may be capable of digesting these regions of the protein that were previously inaccessible to the enzyme. This has been previously shown to be the case for whey protein [121-122]. Furthermore, ACE inhibitory peptides from whey protein isolates (WPI) pretreated at 65 °C were shown to have greater inhibitory activity than peptides from WPI pretreated at 95 °C. This result can be explained by the formation of whey aggregates [100].

Optimisation of the hydrolytic process should also be considered when planning to up-scale the production of BAPs. Bioactive peptides may be produced by enzymatic or microbial hydrolysis. However, it is thought that enzymatic hydrolysis is more suited for food-grade BAP production over microbial fermentation [123]. Enzyme immobilisation offers several advantages over the addition of soluble enzymes directly to the product. They can be recycled and the use of immobilized enzymes potentially avoids the generation of interfering metabolite products due to autolysis of the enzymes. Furthermore, protein hydrolysis using immobilized enzymes can also be carried out in milder more controlled conditions and does not need to be inactivated by heat or acidification, which may be damaging for the product [124; 123]. The use of membrane bioreactors may be a substitute for the development of functional materials from food proteins. This system integrates a reaction vessel with a membrane separation system allowing for the recycling of the enzyme, separation, fractionation and/or concentration of the bioactive compound. The use of membrane bioreactors for the development of functional materials from sea-food processing wastes has been recently reviewed [125].

BAP fractionation and enrichment steps include membrane processing incorporating ultrafiltration and liquid chromatography, ion exchange, gel filtration and reverse phase matrices. Electro-membrane filtration (EMF), a combination of conventional membrane filtration and electrophoresis, may be a consideration for industrial scale isolation of BAPs. EMF is more selective than conventional membrane filtration (ultrafiltration) and is less costly than chromatography [123].

As mentioned earlier, a large portion of antihypertensive peptides are of low molecular weight and many contain hydrophobic residues, attributes which have been classically associated with bitterness in foods. Hence, this is notably an obstacle that must be resolved during the processing of antihypertensive food products. A number of strategies have been applied with the aim of debittering protein hydrolysates including absorption of bitter peptides on activated carbon, selective extraction with alcohols and chromatographic removal using different matrices. Peptidase-mediated debittering has also been applied. This involves the concomitant or sequential incubation of the protein hydrolysates with exopeptidases, with priority cleavage at hydrophobic residues [126]. However, these debittering strategies may lead to the loss of some amino acid residues from hydrolysates. As bioactivity relies greatly on peptide sequence, these debittering methods may not therefore be suitable for debittering of BAPs including antihypertensive peptides, as hydrolysis may result in loss of activity. Changes in peptide structure may also have implications for absorption at the brush border membrane. Therefore, enzymatic debittering strategies need to be approached on a case-by-case basis.

The widespread commercialisation of antihypertensive food products is dependent on the availability of scientific data from *in vivo* animal and human models that positively demonstrates their contribution in reducing BP. Furthermore, legislation which governs health claims in relation to functional foods needs to be taken into account. In Japan, the FOSHU (food for specified health use) licensing system was put in place whereby foods claiming health benefits must first be approved by the system before been allowed to be put on the market [127]. Since then a number of antihypertensive products currently on the market in Japan have been granted FOSHU approval. 'Ameal-S' which is manufactured by Calpis Co., Ltd. is a fermented sour milk containing the LTPs Ile-Pro-Pro and Val-Pro-Pro. The soft drink Casein DP 'Peptio' manufactured by Kanebo Co., Ltd. contains the antihypertensive peptide Phe-Phe-Val-Ala-Pro-Phe-Pro-Gln-Val-Phe-Gly-Phe (α_{s1}-casein f23–34) and is also FOSHU approved. There has been a new European Regulation on nutrition and health claims in the EU since 2007 (Regulation 1924/2006). Advised by the European Commission (EC), the European Food Safety Authority (EFSA) reviews evidence of health claims made by food companies. Interestingly, EFSA has not allowed/approved any peptide related hypotensive claim to date [128-129]. For both the C12-peptide and the bonito protein-derived peptide Leu-Lys-Pro-Asn-Met, it was concluded that a cause and effect relationship has not been established between the consumption of the peptides and maintenance of normal BP [128-129]. In the US, the Food and Drug Administration (FDA) assesses the scientific evidence for health claims under the 1990 Nutrition Labelling and Education Act [130] and the 1994 Dietary Supplement Health and Education Act [131].

Therefore, industrial manufacturers of functional food products need to provide a significant amount of scientific evidence that satisfies the legislative governing body in the specific market region before any new food products can be put on the market claiming to have hypotensive effects.

8. Conclusion

Antihypertensive peptides have major potential as functional ingredients aiding in the prevention and management of hypertension. Although these peptides have been found to be less potent than antihypertensive synthetic drugs, as part of the daily diet they could play an important part as natural and safe BP control agents. Further detailed mechanistic studies on food protein-derived antihypertensive peptides must be carried out to elucidate the BP mechanism(s) involved. With regard to ACE-inhibitory peptides, a better understanding of the interactions involved in the binding of peptides to the active site of ACE is required such that more effective food peptide-based inhibitors of ACE can be discovered. The use of bioinformatics and *in silico* methods for identification of potential bioactive sequences may allow for more substrates to be assessed in a shorter time scale. Cost effective production methods including enrichment, isolation and purification procedures must first be considered and ease of scalability must be achieved. Before advancement of functional hypotensive products onto the market, moreover, the physicochemical, technofunctional and sensory properties must be considered prior to production of new antihypertensive food products.

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