British Journal of Nutrition (2004), **92**, 357–366
DOI: 10.1079/BJN20041189

Review article

Bioavailability of angiotensin I converting enzyme inhibitory peptides

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(Received 8 January 2004 – Accepted 6 April 2004)

Hypertension or high blood pressure is a significant health problem worldwide. Bioactive peptides that inhibit angiotensin I converting enzyme (ACE) in the cardiovascular system can contribute to the prevention and treatment of hypertension. These ACE inhibitory peptides are derived from many food proteins, especially milk proteins. An ACE inhibitory activity *in vitro* does not always imply an antihypertensive effect *in vivo*. Even if it does, it is very difficult to establish a direct relationship between *in vitro* and *in vivo* activity. This is mainly due to the bioavailability of the ACE inhibitory peptides after oral administration and the fact that peptides may influence blood pressure by mechanisms other than ACE inhibition. To exert an antihypertensive effect after oral ingestion, ACE inhibitory peptides have to reach the cardiovascular system in an active form. Therefore, they need to remain active during digestion by human proteases and be transported through the intestinal wall into the blood. The bioavailability of some ACE inhibitory peptides has been studied. It is also known that (hydroxy)proline-containing peptides are generally resistant to degradation by digestive enzymes. Peptides can be absorbed intact through the intestine by paracellular and transcellular routes, but the potency of the bioactivity after absorption is inversely correlated to chain length. In addition, some strategies are proposed to increase the bioavailability of ACE inhibitory peptides. Further research into the bioavailability of ACE inhibitory peptides will lead to the development of more effective ACE inhibitory peptides and foods.

Bioactive peptides: Hypertension: Gastrointestinal digestion: Intestinal transport

Nutrition science has evolved towards 'nutrition for optimal health' in order to make a further contribution to disease prevention (Diplock *et al.* 1999). In this regard, biologically active peptides have been characterised in a number of natural and modified foods (Korhonen & Pihlanto, 2003). Angiotensin I converting enzyme (ACE) inhibitory peptides are bioactive peptides with potential antihypertensive properties *in vivo*. As hypertension is a significant public health problem worldwide, these ACE inhibitory peptides, as part of a food product or as nutraceutical, may be of functional interest in maintaining a healthy population.

Many studies have focused on the production and isolation of ACE inhibitory peptides from different food proteins (for reviews, see Yamamoto, 1997; FitzGerald & Meisel, 2000; Pihlanto-Leppälä, 2001; Yamamoto *et al.* 2003). In order to reduce blood pressure after oral administration, ACE inhibitory peptides have to reach the bloodstream in an active form. Hence, bioavailability of ACE inhibitory peptides is needed to guarantee bioactivity. This aspect has been considerably less investigated and is discussed in the present review. After a general introduction to ACE inhibitory peptides and their use in the prevention and treatment of hypertension, the disparity between *in vitro* and *in vivo* activity is examined in the light of bioavailability. An overview is given on the available literature on stability of bioactive peptides, particularly ACE inhibitory peptides, against human proteases in the oral delivery route and their intestinal transport. Finally, some future perspectives are introduced, which may lead to more effective ACE inhibitory peptides.

Angiotensin I converting enzyme inhibitory peptides

Biologically active peptides or functional peptides are food-derived peptides that in addition to their nutritional value exert a physiological effect in the body. These bioactive peptides are inactive within the original protein but, once released, function as regulatory compounds with hormone-like activity that is based on the inherent amino acid composition and sequence (Meisel, 1997*b*; Tome, 1998). In this regard, they may present active ingredients

Abbreviations: ACE, angiotensin I converting enzyme; IC_{50}, median inhibitory concentration; SHR, spontaneously hypertensive rat.

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in functional foods and nutraceuticals (Meisel, 1997*a*; Clare & Swaisgood, 2000). Numerous peptides exhibiting various activities have been reported, including opiate, mineral binding, immunomodulatory, ACE inhibitory, anti-thrombotic and antimicrobial peptides (Meisel, 1998; Clare & Swaisgood, 2000; Korhonen & Pihlanto, 2003). Bioactive peptides usually contain two to twenty amino acid residues per molecule. These peptides can be liberated from the parent protein during gastrointestinal digestion in the body or during food processing (Clare & Swaisgood, 2000). Although these exogenic peptides are less active and less specific than their endogenic counterparts, they can be effective after oral administration. They have partial or total resistance to hydrolysis and may either enter peripheral blood intact due to their low molecular size and exert systemic effects, or produce local effects in the gastrointestinal tract (Yoshikawa *et al.* 2000).

Although other animal (as well as plant) proteins contain potential bioactive sequences (Dziuba *et al.* 1999), milk proteins are currently the main source of bioactive peptides. Most major milk proteins have little or no bioactivity in their native state. Proteolytic digestion of milk proteins, however, releases and activates a plethora of bioactive peptides. Some regions in the primary amino acid sequence of milk proteins contain overlapping bioactive peptide sequences, exerting different biological effects. These regions in the multifunctional bioactive peptides are considered as 'strategic zones', which are partially protected from proteolytic breakdown (Meisel & Bockelmann, 1999).

ACE plays an important role in the renin–angiotensin system, which regulates arterial blood pressure as well as salt and water balance. In the cardiovascular system, ACE converts angiotensin I to angiotensin II, a potent vasoconstrictor, and degrades bradykinin, a vasodilator (Eriksson *et al.* 2002; Riordan, 2003). Therefore, inhibition of ACE, by ACE inhibitory drugs like captopril and natural ACE inhibitory peptides has been shown to result in an antihypertensive effect in hypertensive human subjects and animals (Takano, 1998; Cushman & Ondetti, 1999). ACE inhibitory peptides derived from casein (casokinins) and whey (lactokinins) have been isolated (FitzGerald & Meisel, 2000; Pihlanto-Leppälä, 2001), in addition to other vegetable and animal source proteins (Yamamoto, 1997; Yamamoto *et al.* 2003). It is only when these ACE inhibitory peptides reach the cardiovascular system in an active form that a blood-pressure-lowering effect is induced.

As for the ACE inhibitory drugs, structure–activity correlations between different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate. Although the precise substrate specificity is not fully understood, ACE appears to prefer substrates or competitive inhibitors containing hydrophobic (aromatic or branched side-chains) amino acid residues at the three C-terminal positions. However, a C-terminal lysine or arginine, with a positive charge on the ϵ-amino group, also seems to contribute substantially to the inhibitory potency. In this regard, it is postulated that the mechanism of ACE inhibition involves inhibitor interaction with an anionic binding site, which is distinct from the catalytic site. Therefore, it is expected that peptide conformation, i.e. the structure adopted in a specific environment, should contribute to ACE inhibitor potency. Due to substrate specificity differences between the two catalytic sites of ACE (Turner & Hooper, 2002), ACE inhibitors may inhibit only one catalytic site. Moskowitz (2003) proposes a model that explains the clinical superiority of hydrophobic ACE inhibitory drugs relative to hydrophilic ones. All ACE inhibitors bind to the C-terminal catalytic site, but only hydrophobic ones bind the occluded N-terminal catalytic site and are therefore better at blocking angiotensin II production. Moreover, this model can explain why hydrophobic ACE inhibitors have specific local benefits, e.g. preventing organ damage, in addition to a reduction in the systemic blood pressure (Moskowitz, 2003). A detailed knowledge of ACE and the conformational behaviour of ACE inhibitory peptides should lead to a better understanding of the mechanism of action of natural ACE inhibitory peptides (FitzGerald & Meisel, 2000).

Hypertension

Hypertension is a sustained increase in blood pressure and is associated with greater risk for CVD. Hypertension is one of the most common chronic medical conditions in the developed world and is rapidly becoming a major problem in developing countries. It is estimated that about 20 % of the world's adult population suffers from hypertension. The prevalence of high blood pressure increases with age, affecting approximately 65 % of the population aged 65–74 years in Western nations (Alper *et al.* 2001; Duprez *et al.* 2002).

Lifestyle modifications and diet therapy are two of the most important tools for effective lowering of blood pressure (Hermansen, 2000). Moreover, even small decreases in blood pressure result in significantly lower risks for CVD (Van der Niepen, 2000). A higher dietary protein intake seems to have favourable influences on the blood pressure in hypertensive individuals (Stamler *et al.* 1996). In addition, the Dietary Approaches to Stop Hypertension trial, a diet rich in fruits, vegetables and low-fat dairy products, is associated with an effective reduction in blood pressure (Harsha *et al.* 1999). Although other mechanisms may play a role, ACE inhibition by bioactive peptides released from food proteins may have caused these antihypertensive effects. Indeed, a number of research reports have demonstrated the antihypertensive effect of ACE inhibitory peptides or foods containing these bioactive compounds in hypertensive patients, even when they received antihypertensive medication (Hata *et al.* 1996; Itakura *et al.* 2001; Pins & Keenan, 2002; Seppo *et al.* 2003). In addition, several studies in spontaneously hypertensive rats (SHR) suggest a significant suppression of the development of hypertension with a diet rich in ACE inhibitory peptides (Yoshii *et al.* 2001; Sipola *et al.* 2002). Overall, this points to the fact that ACE inhibitory peptides, as part of a food product or as nutraceutical, may be of functional interest in both the treatment and the prevention of hypertension.

Compared with ACE inhibitory drugs, food-derived peptides have certain advantages. ACE inhibitory peptides have lower ACE inhibitory activity *in vitro* than the ACE inhibitory drugs, yet do not have the harmful side effects, such as dry cough and angio-oedema, that are associated with synthetically produced drugs (FitzGerald & Meisel, 2000; Riordan, 2003). They also represent a much lower cost to health care. As part of the daily diet, they appear more natural and safe to the consumer. Finally, food-derived ACE inhibitory peptides fit well in the new nutrition concept that emphasises the relationship between nutrition and human health. However, ACE inhibitory drugs have proven their usefulness. Therefore, it is not the intention to completely replace them, but in a number of cases, especially in the prevention of hypertension and as initial treatment in mildly hypertensive individuals, food-derived ACE inhibitory peptides could be applied, while in others they could function as an additional treatment.

Angiotensin I converting enzyme inhibitory activity *v.* antihypertensive effect

ACE inhibitory activity is usually analysed *in vitro* and implies the determination of the ACE activity by means of synthetic substrates with amino-substituted tri- and dipeptides, such as hippuryl-L-histidyl-L-leucine (Cushman & Cheung, 1971; Nakamura *et al.* 1995), 2-furanacryloyl-L-phenylalanyl-L-glycyl-L-glycine (Holmquist *et al.* 1979; Vermeirssen *et al.* 2002*b*) and dansyltriglycine (Elbl & Wagner, 1991), via radioisotopic, spectrophotometric, fluorometric and chromatographic methods. While ACE inhibitory activity is a marker for a biological response, the demonstration of an antihypertensive effect represents an intermediate endpoint marker for CVD (Diplock *et al.* 1999). Antihypertensive effects can be measured in SHR, which are genetically predisposed to have a high blood pressure (Yigal *et al.* 1998), and in clinical trials with hypertensive patients. As an example of the structure–activity relationship, the ACE inhibitory activity of dipeptides with tyrosine is higher than those with phenylalanine, but less than dipeptides with proline at the C-terminal (Cheung *et al.* 1980). After oral administration in SHR, dipeptides with tyrosine at the C-terminal caused slow but prolonged reduction of the systolic blood pressure compared with dipeptides with phenylalanine at the carboxy-terminus, which produced a more rapid decrease and a shorter duration of action (Suetsuna, 1998).

However, it is difficult to establish a direct relationship between ACE inhibitory activity *in vitro* and antihypertensive activity *in vivo*. First, the bioavailability after oral administration plays a major role. To exert physiological effects *in vivo* after oral ingestion, it is of crucial importance that ACE inhibitory peptides remain active during gastrointestinal digestion and absorption and reach the cardiovascular system. ACE inhibitory peptides may be released and degraded in the human body. Fig. 1 shows the potential barriers in the human body where bioactive peptides can be (in)activated. This will be further discussed later for bioactive peptides. Second, antihypertensive mechanisms other than ACE inhibition may be of interest. In the case of the opiate and ACE inhibitory peptide α-lactorphin, originally derived from bovine α-lactalbumin, the opiate activity causes the dose–dependent reduction in the blood pressure of SHR and normotensive Wistar–Kyoto rats upon subcutaneous administration, as the effect can be reversed by nalaxone, a specific opiate receptor antagonist (Nurminen *et al.* 2000). Single oral administration of an extract from autologous lysate of *Lactobacillus casei* significantly lowers the blood pressure in SHR and long-term oral administration even suppresses the development of hypertension in these animals (Furushiro *et al.* 1990). The active substance in this extract, a polysaccharide–glycopeptide complex, enhances prostaglandin synthesis only after oral intake; this results in a decrease of peripheral vascular resistance and hence an antihypertensive effect (Furushiro *et al.* 1993). Furthermore, oral administration of the *Lactobacillus casei* cell lysate extract to hypertensive patients reduces both the systolic and diastolic blood pressure, in addition to the total cholesterol and fasting plasma glucose level (Nakajima *et al.* 1995). In SHR, two peptides, the ovokinin phenylalanine-arginine-alanine-aspartate-histidine-proline-phenylalanine-leucine and arginine-alanine-aspartate-histidine-proline-phenylalanine (isolated from a pepsin and α-chymotrypsin digest of ovalbumin respectively), exert a dose-dependent vasodilatation in an isolated mesenteric artery that is preconstricted by phenylephrine. The vasorelaxing activity is attributable to the binding to B_1 receptors and the subsequent release of prostaglandin and NO. Following oral administration, arginine-alanine-aspartate-histidine-proline-phenylalanine lowers the blood pressure in SHR (Fujita *et al.* 1995; Matoba *et al.* 1999). An even more potent and long-lasting hypotensive activity is obtained by oral administration of the synthetic analogue arginine-proline-phenylalanine-histidine-proline-phenylalanine (Matoba *et al.* 2001). Pepsin digests of bonito and beef inhibit the endothelin converting enzyme, which produces the potent vasoconstrictor endothelin (Okitsu *et al.* 1995; Maes *et al.* 2004). Furthermore, it has also been suggested that ACE inhibitory peptides may exert an additional antihypertensive effect by inhibition of chymase (Yamamoto *et al.* 1999).

In addition, different ACE inhibition assays, where other substrates and calculations for the median inhibitory concentration (IC_{50}) are used, hamper the comparison of ACE inhibitory activities. In addition, in *in vivo* experiments and clinical trials, different experimental designs (measurement of the mean arterial or systolic and/or diastolic blood pressure; intravenous, subcutaneous or oral administration; other doses) and the use of the SHR model *v.* the hypertensive patient hinder stating an unequivocal effect. Although the IC_{50} of captopril is about 1000-fold lower than that of food-derived ACE inhibitory peptides, there is no discrepancy observed in the antihypertensive effect. Indeed, oral administration of 200 mg/kg body weight dipeptide isolated from garlic results in a reduction of blood pressure in SHR of about 30 mmHg, while captopril at a dose of 10 mg/kg body weight exerts an antihypertensive effect of about 50 mmHg (Suetsuna, 1998). There are no substantial differences in duration of the effect. When compared on molar basis,

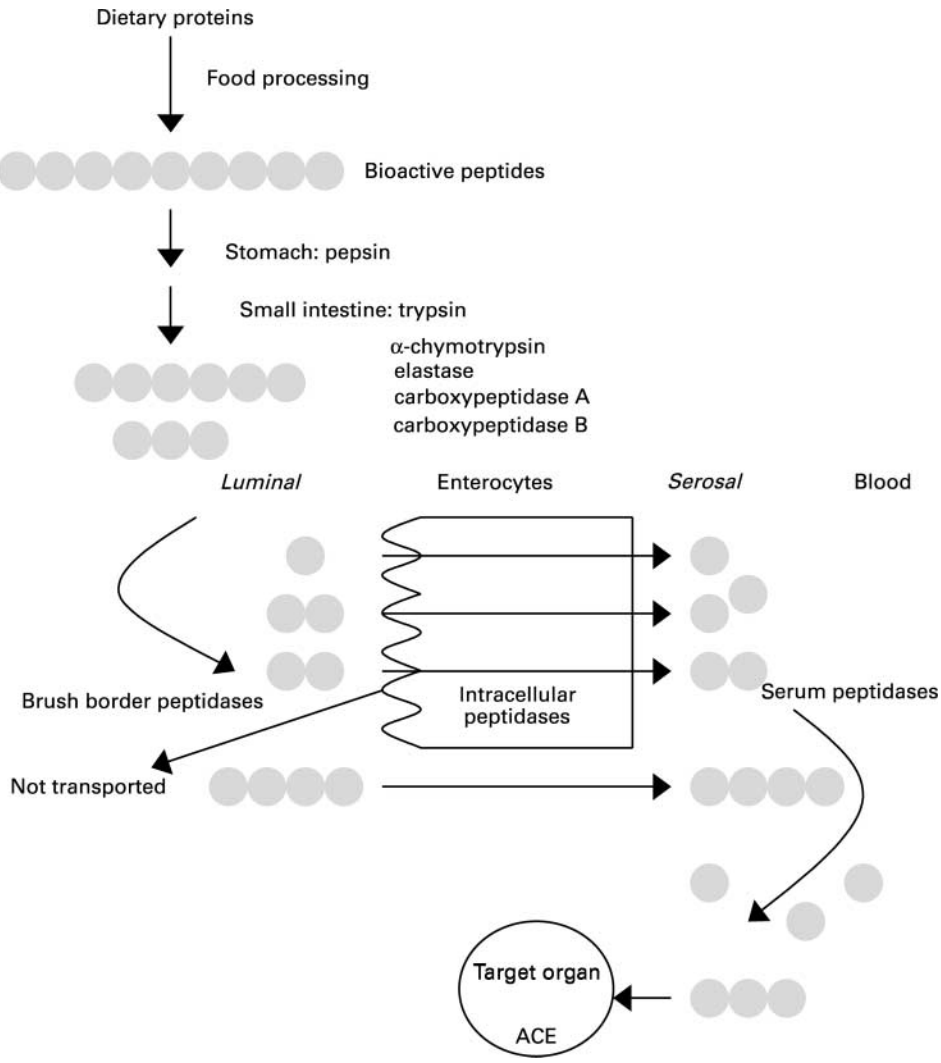

Fig. 1. The potential activation and inactivation of angiotensin I converting enzyme (ACE) inhibitory peptides in the human body during gastrointestinal digestion and absorption, and in the blood.

the antihypertensive activity of leucine-lysine-proline after oral and intravenous administration in SHR amounted to 91 and 20 % of that of captopril respectively, whereas the *in vitro* ACE inhibitory activity was 7·73 % compared with captopril (Fujita & Yoshikawa, 1999). Hence, these findings indicate that compared with antihypertensive drugs, food-derived ACE inhibitory peptides with antihypertensive activity possess higher *in vivo* activity than would be expected from their *in vitro* activity. A sound explanation for this observation cannot be proposed yet, but in addition to the reasons mentioned earlier, it has been suggested that food-derived peptides have higher affinities for tissues and are more slowly eliminated than the synthetic captopril (Fujita & Yoshikawa, 1999). Moreover, it is plausible that the access to the ACE enzyme *in vivo* is limited, so that above a certain affinity substances inhibit ACE *in vivo* to a similar extent. After feeding 100 mg soy-protein hydrolysate/kg body weight to SHR for 1 month, blood pressure decreased by 38 mmHg, compared with 46 mmHg on providing 50 mg captopril/kg body weight (Wu & Ding, 2001). When assessing the ACE activity in serum, aorta and lung of these rats, the soy-protein ACE inhibitory peptides did not show any significant effect compared with the control, whereas captopril greatly enhanced the ACE activity in serum and reduced the ACE activity in the aorta. Although the real causes of these observations remain unknown, these different responses might indicate that food-derived ACE inhibitory peptides and captopril act via different antihypertensive mechanisms.

Hence, further investigation into the antihypertensive effect of food-derived ACE inhibitory peptides is necessary. *In vivo* experiments and clinical trials are necessary to demonstrate their physiological effects. However, evidence of *in vitro* ACE inhibitory activity is a good starting point, because it is based on a biological mechanism. As ACE exhibits several functions in the human body, ACE inhibition may have additional or other implications than antihypertensive effects (Moskowitz, 2002).

Gastrointestinal digestion

Digestion of proteins starts in the stomach by the action of pepsin at acidic pH. In the luminal phase of the small

intestine, the polypeptides are further cleaved by the pancreatic proteases trypsin, α-chymotrypsin, elastase and carboxypeptidase A and B at more alkaline pH. This results in a mixture of oligopeptides and free amino acids, of which oligopeptides constitute a major portion. The free amino acids are absorbed as such into the enterocytes across the brush border membrane via distinct amino acid transport systems. The oligopeptides undergo further hydrolysis by the action of a battery of brush border peptidases, resulting in a mixture largely consisting of free amino acids and di- and tripeptides. The intestinal brush border membrane is particularly rich in aminopeptidase activity, which provides functional complementation to the carboxypeptidases present in the pancreatic juice. Aminopeptidase N and A are the major representatives and cleave N-terminal neutral and anionic amino acids respectively. In addition to these enzymes, the intestinal brush border contains endopeptidase and dipeptidase activity. ACE or dipeptidyl carboxypeptidase cleaves dipeptides from the C-terminus of oligopeptides with proline, phenylalanine or leucine in the ultimate position. Dipeptidyl aminopeptidase IV releases dipeptides from the N-terminus of oligopeptides with proline or alanine in the penultimate position. Hence, the dipeptides released by these enzymes are generally of the X-proline type (Ganong, 1997; Ganapathy & Leibach, 1999).

Proline- and hydroxyproline-containing peptides are generally resistant to degradation by digestive enzymes. Furthermore, tripeptides containing the C-terminal proline-proline are reported to be resistant to proline-specific peptidases (Vanhoof *et al.* 1995; FitzGerald & Meisel, 2000). This stresses the fact that several bioactive peptides that have been shown to exert an *in vivo* effect are isolated from casein and gelatine, as these proteins have a high proline content.

Bioactive peptides have already been characterised from *in vivo* digests. When diets containing bovine casein are given to minipigs, the opiate peptide β-casomorphin-11 and the mineral binding casein phosphopeptide α_{s1}-casein f(66-74) are isolated from small intestinal contents (Meisel & Frister, 1989). In another study, β-casomorphin-7 immunoreactive material was detected in small intestinal contents of adult human subjects after bovine milk ingestion (Svedberg *et al.* 1985). In adult human subjects after milk or yoghurt ingestion, the appearance of bioactive peptides in the stomach, small intestine and blood has been investigated. Many peptides derived from α_{s1}-, β- or κ-caseins have been detected in the stomach, smaller peptides derived from casein and lactoferrin have been recovered from the small intestine. Two long peptides, casein glycomacropeptide, κ-casein f(106-117) and the N-terminal peptide f(1-23) of α_{s1}-casein, are absorbed and detected in plasma (Chabance *et al.* 1998). Hence, casoplatelines (casein glycomacropeptide fragments) are released during gastrointestinal digestion and absorbed intact into the blood, which supports the concept that they exert an anti-thrombotic effect *in vivo*. The peptide phenylalanine-valine-alanine-proline-phenylalanine-proline-glutamate-valine-phenylalanine-glycine-lysine-glutamate, and α_{s1}-casein f(24-35) and f(25-32), are released in the human stomach. The fragments f(25-32) and f(28-35) are also detected in the duodenum. As these contain sequences of ACE inhibitory peptides (Maruyama *et al.* 1987; Karaki *et al.* 1990), they might exert a physiological effect after absorption in the blood. The peptide α_{s1}-casein f(1-23) is the antibacterial peptide isradicin with immunomodulatory capacities. This includes the fragment α_{s1}-casein f(1-9), arginine-proline-lysine-histidine-proline-isoleucine-lysine-histidine-glutamine, which has been shown to have a small antihypertensive effect in SHR (Saito *et al.* 2000).

To investigate whether ACE inhibitory peptides resist gastrointestinal digestion, they are usually subjected *in vitro* to hydrolysis by pepsin, trypsin, α-chymotrypsin or pancreatin. For example, ACE inhibitory wheatgerm hydrolysate and the peptide isoleucine-valine-tyrosine isolated from it are hydrolysed by pepsin, trypsin and α-chymotrypsin alone and in combination. The ACE inhibitory activity of the wheatgerm hydrolysate increases by 27 % after a combined digestion, indicating that any active peptides must be newly produced by action of these proteases, in particular trypsin. The IC_{50} value of isoleucine-valine-tyrosine does not change during digestion, signifying that this peptide would resist *in vivo* gastrointestinal digestion (Matsui *et al.* 1999). We also observed that high ACE inhibitory activity was produced upon *in vitro* gastrointestinal digestion of pea and whey protein (Vermeirssen *et al.* 2003*a*,*b*). Another example of activation of ACE inhibitory peptides during gastrointestinal digestion is as follows. The antihypertensive peptide lysine-valine-leucine-proline-valine-proline-glutamine showed only low ACE inhibitory activity *in vitro*. However, the potent ACE inhibitor lysine-valine-leucine-proline-valine-proline was formed after pancreatic digestion due to the action of carboxypeptidase A. In the same study, an α_{s1}-casein-derived peptide, tyrosine-lysine-valine-proline-glutamine-leucine, with strong ACE inhibitory activity, failed to exert an antihypertensive effect due to pancreatin degradation (Maeno *et al.* 1996).

After the small intestine, the non-digested and/or non-absorbed food peptides enter the large intestine or colon, where they can be metabolised by the intestinal microbiota. Whether ACE inhibitory peptides can still exert an antihypertensive effect after they enter this compartment needs to be investigated.

Intestinal absorption

Peptides consisting of two or three amino acids are absorbed intact across the brush border membrane by a specific peptide transport system. The peptide transporter PepT1 uses a transmembrane electrochemical proton gradient as the driving force and has broad substrate specificity (Yang *et al.* 1999). Small peptides are absorbed more rapidly than free amino acids and their transport is favoured (Webb, 1990). Once inside the enterocyte, these peptides are usually hydrolysed to free amino acids in the cytoplasm by various intracellular peptidases. Among these, aminotripeptidase, which releases the amino acid from the N-terminus of tripeptides, and several dipeptidases with different substrate specificity towards specific amino acids, are present. Here, iminodipeptidase or prolidase is of special interest because of its restricted specificity toward dipeptides of

the X-proline or X-hydroxyproline type. Via specific amino acid transport systems, the amino acids cross the basolateral membrane and enter the portal circulation (Ganapathy & Leibach, 1999).

Transport of intact peptides and proteins from the intestinal lumen into the blood circulation is a unique phenomenon, which differs from the regular process of food digestion and absorption. The concept that significant amounts of small peptides can escape total digestion to amino acids and enter the circulation in intact form is rather new, but is gaining acceptance (Gardner, 1988; Grimble, 2000). Apart from the peptide transporter route, peptides can be absorbed intact across the intestinal mucosa via other mechanisms (Fig. 2) (Gardner, 1998). There is evidence in support of both paracellular and transcellular routes for passage of intact peptides, but there is still debate as to the relative importance of these. Paracellularly, large water-soluble peptides pass via the tight junctions between cells. Highly lipid-soluble peptides appear to be able to diffuse via the transcellular route. Peptides may also enter the enterocytes via endocytosis, which entails membrane binding and vesiculisation of the material (Ziv & Bendayan, 2000). The intestinal basolateral membrane also possesses a peptide transporter, which facilitates the exit of hydrolysis-resistant small peptides from the enterocyte into the portal circulation (Gardner, 1984).

Biologically active peptides generated in the diet can be absorbed intact through the intestine and produce biological effects at the tissue level. However, the potency of the administered peptides decreases as the chain-length increases (Roberts *et al.* 1999). As in infants, the gastrointestinal barrier is not yet completely mature, intact peptides and proteins are much better absorbed in infants than in adults (Walker, 1985). Together with the limited gastrointestinal proteolysis in infants, this explains why milk contains several bioactive proteins and peptides that are thought to have a physiological role in the young.

In this regard, β-casomorphin-immunoreactive material is detected in the plasma of newborn calves after milk intake (Umbach *et al.* 1985). Higher plasma levels of casein glycomacropeptide are found in 5-d-old infants compared with human adults after ingestion of milk (Chabance *et al.* 1995, 1998). In the latter study, it was demonstrated that a peptide containing twenty-four amino acids is absorbed intact into the bloodstream of human adults after milk ingestion (see earlier; Chabance *et al.* 1998). Intestinal transport of β-casomorphins and synthetic analogues is investigated in isolated rabbit ileum mounted in Ussing chambers. While natural β-casomorphins are degraded by the intestinal mucosa and no intact transepithelial passage is detected for these peptides, the synthetic analogue tyrosine-D-alanine-phenyalanine-D-alanine-tyrosine crosses the epithelium intact (Tome *et al.* 1987).

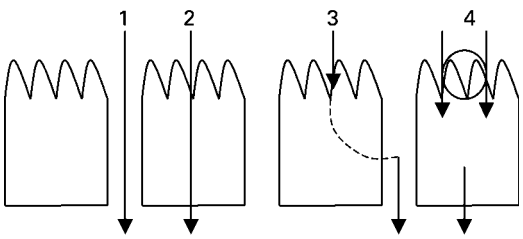

Fig. 2. Mechanisms for intestinal transport of peptides: (1), paracellular; (2), passive diffusion; (3) endocytosis; (4) carrier-mediated transport.

When the intestinal transport mechanism of the antihypertensive peptide valine-proline-proline is studied in a Caco-2 cell monolayer, a small but significant amount of intact valine-proline-proline is transported through the cell monolayer. Despite a flux of $<2\%$ per h in the Caco-2 cell monolayer, the tripeptide has been shown to be present in the abdominal aorta and exerts an antihypertensive effect after oral intake in SHR (Masuda *et al.* 1996). The transport of valine-proline-proline is not mediated by the peptide transporter PepT1 and it has been suggested that paracellular transport is the main mechanism of absorption (Satake *et al.* 2002). In another paper, the transepithelial transport of the oligopeptides bradykinin, β-casomorphin tyrosine-proline-phenylalanine-proline-glycine, ovokinin phenylalanine-arginine-alanine-aspartate-histidine-proline-phenylalanine-leucine, proline-phenylalanine-glycine-lysine and glycine-glycine-tyrosine-arginine was investigated in the Caco-2 model. The susceptibility to the brush border peptidases was one of the factors that decided the transport rate. Bradykinin and glycine-glycine-tyrosine-arginine were hardly degraded, while β-casomorphin and ovokinin were substantially hydrolysed. The transport rate was greatest for glycine-glycine-tyrosine-arginine and smallest for β-casomorphin. Furthermore, the transport mechanism was studied for bradykinin and glycine-glycine-tyrosine-arginine. While adsorptive transcytosis through hydrophobic interaction with the cell membrane was suggested to be involved in the transport of bradykinin, glycine-glycine-tyrosine-arginine was believed to be transported mainly via the paracellular pathway. It seems that molecular size and other structural properties, such as hydrophobicity, determine the major transport route for peptides (Shimizu *et al.* 1997).

The antihypertensive peptide valine-tyrosine, derived originally from sardine muscle, is detected dose-dependently in human plasma and reaches a maximum 2 h after a single oral administration in normotensive human subjects. At the highest dose, more than a 10-fold greater increment in valine-tyrosine plasma concentration is measured compared with the baseline concentration. The low absorption ratios are explained by the fact that most of the administered dipeptide is hydrolysed by membrane-bound peptidases. Therefore, it is suggested that saturation of the peptide transporter enables the entry of excess valine-tyrosine into peripheral blood (Matsui *et al.* 2002*b*).

When the transport of the lactokinin alanine-leucine-proline-methionine-histidine-isoleucine-arginine was studied in a Caco-2 Bbe cell monolayer, this peptide was found to be absorbed at low concentrations (Vermeirssen *et al.* 2002*a*).

Few studies on the transport of ACE inhibitory peptides have been undertaken, although it is important for the bioavailability of these peptides to investigate whether they are transported and what the transport mechanism is. Generally, the Caco-2 cell monolayer is used to investigate intestinal transport. This confluent cell monolayer displays

several properties typical of differentiated intestinal epithelial cells (Wilson *et al.* 1990) and is widely used as a model in transport studies of drugs (Augustijns *et al.* 1998; Boisset *et al.* 2000) and food compounds (Rubio & Seiquer, 2002). Caco-2 cell monolayers are known to be tighter than mammalian intestinal tissues (Boisset *et al.* 2000), while brush border membrane-associated enzyme activities are generally lower (Bolte *et al.* 1998). Species-specific differences in intestinal brush border enzyme activity have been observed (Drucker *et al.* 1997). For example, esterase activities in homogenates prepared from freshly scraped intestinal rat mucosa is up to six times greater than that of pig, the latter being considered more representative for the human small intestine (Augustijns *et al.* 1998). In order to get an overview of the transport possibilities and limitations of a certain compound, it is advisable to apply different absorption models. Therefore, intestinal transport *in vivo* might be more pronounced than observed in the Caco-2 cell monolayer. In addition, during oral consumption of ACE inhibitory peptides, the presence of other food compounds may have important consequences on the susceptibility to peptidase degradation and intestinal transport (Charman *et al.* 1997). While some specific food compounds have been observed to decrease the transepithelial electrical resistance in the Caco-2 cell monolayer (Shimizu, 1999), active transport of sugars and amino acids initiates a solvent drag through the tight junctions in rat intestine, by which oligopeptides may be absorbed (Pappenheimer & Volpp, 1992). This could be an additional advantage of protein hydrolysates containing amino acids compared with pure peptides.

Stability in the blood

Blood contains substantial activities of peptidase enzymes. The half-life of certain peptides in plasma is very short, with an order of magnitude of 1 min (Gardner, 1998). Angiotensin II degradation occurs even within seconds (Moskowitz, 2003).

First, oligopeptides with an ACE inhibitory activity *in vitro* need to resist ACE to exert an antihypertensive effect *in vivo*. In this regard, the ACE inhibitory peptides can be classified into three groups: the inhibitor type, of which the IC_{50} values are not affected by preincubation with ACE; the substrate type, peptides that are hydrolysed by ACE to give peptides with a weaker activity; the pro-drug-type inhibitor, peptides that are converted to true inhibitors by ACE or other gastrointestinal proteases. Only peptides belonging to the inhibitor or pro-drug type exert antihypertensive activities after oral administration in SHR (Fujita *et al.* 2000).

After intravenous administration to SHR of the ACE inhibitory peptide isoleucine-valine-tyrosine, isolated from wheatgerm hydrolysate, the tripeptide is metabolised by the action of aminopeptidase in plasma to form a subsequent ACE inhibitor, valine-tyrosine. Valine-tyrosine exerts an acute depressor effect in SHR and the blood pressure returns to the normal state about 5 min after injection. On the other hand, after injection of isoleucine-valine-tyrosine, the reduction in blood pressure is much stronger and is held for 15 min. Therefore, it is suggested that the intake of isoleucine-valine-tyrosine as a physiologically functional food would serve in lowering blood pressure by the combined action of itself and its metabolite after absorption (Matsui *et al.* 2000). Valine-tyrosine has been administered orally to mild hypertensive subjects. Although the peptide is present in plasma and the absorption is comparable with that observed in normotensive subjects (see earlier), no marked decrease in blood pressure was seen, suggesting that the peptide exerts no acute hypotensive effect (Matsui *et al.* 2002*a*).

Future perspectives

Several food products contain ACE inhibitory peptides that are known to reach the cardiovascular system and exert an antihypertensive effect. Further research into the bioavailability and the structure–activity can improve the isolation and production of effective ACE inhibitory peptides.

Despite their susceptibility to metabolism and sometimes low membrane permeability, peptides have been recognised as important therapeutic compounds and therefore several approaches have been designed to increase the oral delivery of peptides. First, peptides can be chemically modified to increase their oral delivery (Brayden & O'Mahony, 1998; Dooley & Houghten, 1999; Mayo, 2000). Second, ACE inhibitory peptides can be produced by genetic engineering in a micro-organism and subsequently delivered *in situ*. For example, oligonucleotides encoding the ACE inhibitory peptides isoleucine-tyrosine and valine-lysine-tyrosine are chemically synthesised and designed to be multimerised due to isoschizomer sites. The cloned gene, named *ap3*, is multimerised up to six times in a plasmid and expressed as a fusion protein in *Escherichia coli*, which is easily purified. The digest of AP3 by chymotrypsin has an IC_{50} value of 18·53 μM. Therefore, potent ACE inhibitory peptides may be released upon oral ingestion of AP3 (Oh *et al.* 2002). Several expression vector systems are commercially available to yield substantial amounts of designed proteins. Most of them, however, make the subsequent purification of the artificial protein necessary because the expressed protein resides in the host cell, as described by Oh *et al.* (2002). In the same way as therapeutic compounds are delivered *in situ* by genetically engineered micro-organisms (Krüger *et al.* 2002; Steidler, 2002), ACE inhibitory propeptides could be delivered to the small intestine, where they could be expressed and exported outside the cell, cleaved into active peptides by the action of proteases and/or peptidases and finally transported into the blood stream. A generally recognised as safe (GRAS) micro-organism that is able to survive the gastrointestinal transit and has additional probiotic properties could be selected. This bacterium should be genetically engineered to express upon induction the ACE inhibitory propeptide, coupled to a signal peptide to direct the translocation of the protein through the cell membrane. An inducible promoter should increase ACE inhibitory peptide release in the small intestine and prevent the expression of the polypeptide in the large intestine, where it is of no use and could eventually lead to the formation of harmful N-compounds. When further research has guaranteed the safety of GM

organisms, a functional food based on this concept might have great value, because it not only would enhance the oral delivery of ACE inhibitory peptides, but it would also enable the combination of different functional effects in one food. In addition to micro-organisms, genetic engineering may also be applied to plants and animals, where modified proteins, rich in ACE inhibitory peptide sequences, could be obtained.

The bioavailability of ACE inhibitory peptides may also be increased by cross-linking the target peptide to protein transduction domains or by means of specific peptide carriers like Pep-1 that are able to deliver biologically active proteins and peptides into mammalian cells (Morris *et al.* 2001). Peptide permeation may also be achieved by chemical enhancers and surfactant-like agents, provided the tissue recovers *in vivo* after temporary exposure. Receptor targeting and mucoadhesion represent some other strategies that may increase the delivery of peptides (Brayden & O'Mahony, 1998).

Conclusion

The antihypertensive effect of ACE inhibitory peptides is strongly influenced by their bioavailability, which is predominantly determined by the resistance to peptidase degradation and intestinal absorption. This should be taken into account when developing food products containing ACE inhibitory peptides.

Acknowledgement

This work was supported by a PhD grant (aspirant) for V. V. from the Fund for Scientific Research – Flanders (Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen).

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