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Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme

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Numerous casein and whey protein-derived angiotensin-I-converting enzyme (ACE) inhibitory peptides/hydrolysates have been identified. Clinical trials in hypertensive animals and humans show that these peptides/hydrolysates can bring about a significant reduction in hypertension. These peptides/hydrolysates may be classified as functional food ingredients and nutraceuticals due to their ability to provide health benefits i.e. as functional food ingredients in reducing the risk of developing a disease and as nutraceuticals in the prevention/treatment of disease.

Peptides/hydrolysates: Hypertension: Functional foods/nutraceuticals

Introduction

Angiotensin-I-converting enzyme (ACE) is a key enzyme in the regulation of peripheral blood pressure. ACE, a dipeptide-liberating carboxypeptidase (peptidyldipeptide hydrolase, EC 3.4.15.1), classically associated with the renin–angiotensin system, converts angiotensin I into angiotensin II, a highly potent vasoconstrictor molecule (Skeegs *et al.* 1956). Several endogenous peptides such as enkephalins, bradykinin and substance P are inhibitors and competitive substrates for ACE (Maruyama *et al.* 1987*a*; Steve *et al.* 1988). In the kinin kallikrein system, for example, ACE activates bradykinin, a vasodilatory molecule (Erdos, 1975). This enzyme, therefore, plays a key physiological role in the regulation of local levels of several endogenous bioactive peptides. Exogenous ACE inhibitors having an antihypertensive effect *in vivo* were first discovered in snake venom (Ondetti *et al.* 1977). Several food protein sources including fish, gelatin and maize protein contain ACE-inhibitory peptides (for reviews see Ariyoshi, 1993; Meisel, 1993). Milk proteins are also precursors for a range of peptides which inhibit ACE (Meisel, 1993; Takano, 1998; FitzGerald & Meisel, 1999). Casein-derived inhibitors of ACE are known as casokinins (Meisel, 1993), whereas whey-derived inhibitors are known as lactokinins (FitzGerald & Meisel, 1999).

General structural features of milk protein-derived ACE inhibitory peptides

Casokinin sequences have been found in α_{s1}-, β-, and κ-casein, and lactokinins in α-lactalbumin, β-lactoglobulin and bovine serum albumin (Tables 1 and 2). Two strategies have generally been used in the identification and characterisation of such peptides, i.e. isolation of inhibitory peptides from *in vitro* enzymatic digests of milk proteins and chemical synthesis of peptides or peptide analogues having similar structures to those known to inhibit ACE.

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 each of the three C-terminal positions. ACE inhibition studies with dipeptides of varying structure, show that C-terminal tryptophan, tyrosine, phenylalanine or proline residues were most effective in enhancing substrate binding (Cheung *et al.* 1980). All casokinins, i.e. casein-derived ACE inhibitory peptides have proline, lysine or arginine as the C-terminal residue (Table 1). However, the presence of positively charged C-terminal lysine or arginine residues in casokinins, bradykinin and some synthetic inhibitors (Cheung *et al.* 1980) does not fit with the ACE active site model proposed by Ondetti & Cushman (1982). Nevertheless, structure–activity data suggest that the positive charge on the guanidino or ε-amino group of C-terminal arginine and lysine side-chains, respectively, contribute substantially to inhibitory potency. For example, replacement of arginine at the C-terminus of bradykinin results in an essentially inactive analogue (Meisel, 1993). It is postulated that the mechanism of ACE inhibition involves inhibitor interaction with an anionic binding site which is distinct from the catalytic site. Given the above, it is expected that peptide conformation, i.e. the structure adopted in a specific environment, should contribute to ACE inhibitor potency. A detailed knowledge of the

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Table 1. Bovine casein-derived angiotensin-I-converting enzyme (ACE) inhibitory peptides*

Peptide fragment/analogue		Primary structure (one letter code)	IC_{50}† (μ mol/l)	Enzymatic hydrolysis	Peptide synthesis	Reference
α_{S1}-casein	f (23–34)	FFVAPFPEVFGK	77	+	−	Maruyama & Suzuki, 1982; Maruyama *et al.* 1985
	f (23–37)	FFVAP	6	+	−	Maruyama *et al.* 1985
	f (24–27)	FVAP	10	−	+	Maruyama *et al.* 1987*a*
	f (25–27)	VAP	2	−	+	Maruyama *et al.* 1987*a*
	f (27–30)	PFPE	> 1000	−	+	Maruyama *et al.* 1987*a*
	f (28–34)	FPEVFGK	140	+	−	Maruyama *et al.* 1987*a*
	f (32–34)	FGK	160	−	+	Maruyama *et al.* 1987*a*
	f (104–109)	YKVPQL	22	+	−	Maeno *et al.* 1996
	f (142–147)	LAYFYP	65	+	−	Pihlanto-Leppälä *et al.* 1998
	f (143–148)	AYFYPE	106‡	+	−	Yamamoto *et al.* 1994
	f (157–164)	DAYPSGAW	98	+	−	Pihlanto-Leppälä *et al.* 1998
	f (194–199)	TTMPLW	16	+	−	Maruyama *et al.* 1987*b*
	f (197–199)	PLW	36	−	+	Maruyama *et al.* 1987*b*
	f (198–199)	LW	50	−	+	Maruyama *et al.* 1987*b*
β-casein	f (57–64)	SLVLPVPE	39	+	−	Yamamoto *et al.* 1994
	f (60–66)	YPFPGPIP	500	−	+	Meisel & Schlimme, 1994
	f (74–76)	IPP	5	+	−	Nakamura *et al.* 1995
	f (84–86)	VPP	9	+	−	Nakamura *et al.* 1995
	f (108–113)	EMPFPK	423‡	+	−	Pihlanto-Leppälä *et al.* 1998
	f (169–174)	KVLPVP	5	−	+	Maeno *et al.* 1996
	f (169–175)	KVLPVPQ	1000	+	−	Maeno *et al.* 1996
	f (177–179)	AVP	340	−	+	Maruyama *et al.* 1987*a*
	f (177–181)	AVPYP	80	−	+	Maruyama *et al.* 1987*a*
	f (177–183)	AVPYPQR	15	+	−	Maruyama *et al.* 1987*a*
	f (179–181)	PYP	220	−	+	Maruyama *et al.* 1987*a*
	f (181–183)	PQR	>400	+	−	Maruyama *et al.* 1987*a*
	f (193–198)	YQQPVL	280	+	−	Pihlanto-Leppälä *et al.* 1998
	f (193–202)	YQQPVLGPVR	300	−	+	Meisel & Schlimme, 1994
κ-casein	f (25–34)	YIPIQYVLSR	nd	−	+	Chiba & Yoshikawa, 1991
	f (35–41)	YPSYGLNY	nd	−	+	Chiba & Yoshikawa, 1991
	f (58–59)§	YP	720	+	+	Yamamoto *et al.* 1999
	f (108–110)	IPP	5	+	−	Nakamura *et al.* 1995

nd, not determined.
* Details of other casein-derived peptides/or related peptides which inhibit ACE are available within the references used to generate this Table.
† Peptide concentration required to inhibit ACE by 50 %.
‡ IC_{50} value given in μg/ml.
§ This sequence also occurs in α_{S1}-casein f(146–147) and f(159–160) and in β-casein f(114–115).

mechanism of action of ACE and the conformational behaviour of ACE inhibitory peptides should lead to a better understanding of the antihypertensive potential of milk protein-derived peptides.

Physiological effects

ACE is widely distributed in mammalian tissues. It is present in plasma, lung, kidney, heart, skeletal muscle,

Table 2. Whey protein-derived angiotensin-I-converting enzyme (ACE) inhibitory peptides

Peptide fragment/analogue		Primary structure (one letter code)	IC_{50}* (μmol/l)	Enzymatic hydrolysis	Peptide synthesis	Reference
α-lactalbumin	f (50–51)	YG	1523	−	+	Mullally et al. 1996
	f (50–53)	YGLF	733	+	+	Mullally et al. 1996
	f (52–53)	LF	349	−	+	Mullally et al. 1996
	f (105–110)	LAHKAL	621	+	−	Pihlanto-Leppälä *et al.* 1998
β-lactoglobulin	f (9–14)	GLDIQK	580	+	−	Pihlanto-Leppälä *et al.* 1998
	f (15–20)	VAGTWY	1682	+	−	Pihlanto-Leppälä *et al.* 1998
	f (102–103)	YL	122	−	+	Mullally *et al.* 1996
	f (102–105)	YLLF	172	+	+	Mullally *et al.* 1996
	f (104–105)	LF	349	−	+	Mullally *et al.* 1996
	f (142–148)	ALPMHIR	43	+	+	Mullally *et al.* 1997*b*
	f (146–148)	HIR	953	−	+	Mullally *et al.* 1997*b*
	f (146–149)	HIRL	1153	−	+	Mullally *et al.* 1996
	f (147–148)	IR	695	−	+	Mullally *et al.* 1996
	f (148–149)	RL	2439	−	+	Mullally *et al.* 1996
Bovine serum albumin	f (208–216)	ALKAWSVAR	3	−	+	Chiba & Yoshikawa, 1991

* Peptide concentration required to inhibit ACE by 50 %.

pancreas, spleen, placenta, arteries, testes, uterus and brain. It is also present as a brush border membrane-bound enzyme on epithelial cells of human jejunum (Ondetti & Cushman, 1982; Steve *et al.* 1988).

A number of studies in spontaneously hypertensive rats (SHR) have demonstrated an antihypertensive effect following intraveneous and oral ingestion of casein-derived ACE inhibitory peptides. These peptides correspond to tryptic (Maruyama *et al.* 1987*b*) and *Lactobacillus helveticus* protease (Yamamoto *et al.* 1994, 1999) digests of α_{s1}-, β- and κ-casein. Oral ingestion of a tryptic digest of whole casein gave an antihypertensive effect in SHR (Karaki *et al.* 1990). A study in normotensive and mildly hypertensive human volunteers reported that twice daily ingestion of 10 g of a tryptic digest of casein for 4 weeks had an antihypertensive effect (Sekiya *et al.* 1992). A placebo-controlled study in hypertensive humans definitively demonstrated a significant reduction in blood pressure following daily ingestion of 95 ml of Calpis sour milk (Hata *et al.* 1996). Milk fermented with Calpis sour milk starter (*L. helveticus* and *Saccharomyces cerevisiae*) contains highly potent tripeptide inhibitors of ACE, i.e. Val-Pro-Pro (β-casein f(84–86)) and Ile-Pro-Pro (β-casein f(74–76)) and κ-casein f(108–110)), (Nakamura *et al.* 1995). It is worth noting that the ingested dose of these ACE inhibitory peptides was in the range of only 1·2–1·6 mg.

ACE inhibitory peptides can be produced during the manufacture of a range of dairy products. Meisel *et al.* (1997) demonstrated that secondary proteolysis during cheese ripening leads to the production of ACE inhibitory peptides. The ACE inhibitory activity in cheese was mainly associated with the low-molecular-weight peptide fraction. It was also demonstrated that low levels of proteolysis, in for example, fresh cheese, quarg, yoghurt and protein hydrolysate supplemented sports nutrition products, were associated with low ACE inhibitory index values (Meisel *et al.* 1997). Rokka *et al.* (1997) demonstrated the presence of ACE inhibitory peptides (i.e. β-casein f(177–183) and f(193–202)) in UHT milk prefermented with *Lactobacillus casei* ssp. *rhamnosus* and subsequently digested with pepsin and trypsin. Recently, Mullally *et al.* (1997*a*) showed that endoproteinase (trypsin, chymotrypsin, elastase and pepsin) digests of whey protein concentrate and fractions enriched in α-lactalbumin and β-lactoglobulin could inhibit ACE *in vitro*. Furthermore, a tryptic fragment of β-lactoglobulin (Ala-Leu-Pro-Met-His-Ile-Arg, f(142–148)) was identified having an ACE $IC_{50} = 42{\cdot}6\ \mu mol/l$ (Mullally *et al.* 1997*b*). Pihlanto-Leppälä *et al.* (1998) identified several casein (α_{s1}- and β-casein) and whey (α-lactalbumin and β-lactoglobulin) derived ACE inhibitory peptides following digestion of cheese whey and isoelectric casein with pepsin and trypsin. No animal or human studies are as yet available on the antihypertensive potential of whey protein-derived hydrolysates/peptides. However, it has been reported that food-derived ACE inhibitory peptides with IC_{50} values in the range 100–500 μmol/l can be of nutritive/physiological importance in that they could be active following oral administration (Sekiya *et al.* 1992). The majority of the peptides listed in Tables 1 and 2 have ACE inhibitory potencies within this range. It is noteworthy that Yamamoto *et al.* (1999) demonstrated that Tyr-Pro, having an ACE IC_{50} value of 720 μmol/l, could mediate a significant hypotensive effect in SHR. Tyr-Pro, which can arise from α_{s1}-casein f(146–147) and f(159–160), β-casein f(114–115) and κ-casein f(58–59), was found in skim milk fermented with *Lactobacillus helveticus* CPN4. The Tyr-Pro dipeptide may result from the hydrolytic action of starter derived post-proline dipeptidyl aminopeptidase (PPDA) activity on casein peptides. PPDA releases amino acyl proline moieties from the N-terminus of peptides (Bouchier *et al.* 1999).

The antihypertensive potential of milk protein-derived peptides is dependent on the ability of these peptides to reach their target site without being degraded and as a consequence inactivated by the action of intestinal or plasma peptidases. Inhibition of ACE in lung, vascular, kidney and brain tissue by captopril, a drug commonly used in the control of blood pressure, is thought to be central to the antihypertensive effect (Velletri & Bean, 1982; Unger *et al.* 1985). Resistance to peptidase degradation may be a prerequisite for an antihypertensive effect during the oral ingestion and the intravenous infusion of ACE inhibitory hydrolysates/peptides. For example, α_{s1}-casein f(23–27), a potent ACE inhibitor *in vitro*, was shown to have no hypotensive effect *in vivo* (Maruyama *et al.* 1987*b*). A similar situation has been shown by Maeno *et al.* (1996) in the case of α_{s1}-casein f(104–109). The presence of Val-Pro-Pro and Ile-Pro-Pro in heat-treated solubilised aortal fractions of SHR fed on Calpis sour milk demonstrates the resistance of these peptides to intestinal and circulatory peptidases in addition to the absorption of these peptides from the intestine (Masuda *et al.* 1996). Proline-containing peptides are generally resistant to degradation by digestive enzymes (Kim *et al.* 1972; Adibi & Kim, 1981). Furthermore, tripeptides containing C-terminal Pro-Pro are reported to be resistant to proline specific peptidases (Yoshimoto *et al.* 1978; Mock *et al.* 1990). It is interesting that the tryptic peptide, β-lactoglobulin f(142–148), was resistant to further degradation by pepsin and chymotrypsin (Mullally *et al.* 1997*b*). On the other hand, peptide degradation or fragmentation may result in more potent ACE inhibitory activities. For example, removal of C-terminal glutamine from β-casein f(169–175) increased the *in vitro* ACE inhibitory potency from 1000 to 5 μmol/l, however, both β-casein f(169–174) and f(169–175) had strong antihypertensive activities in SHRs (Maeno *et al.* 1996). These results emphasise the necessity of performing *in vivo* studies in all cases.

Several casein and whey protein-derived ACE inhibitory peptides having other biological activities have been reported. Albutensin A, bovine serum albumin f(208–216), displays ileum contracting and relaxing activities in addition to ACE inhibitory activity (Chiba & Yoshikawa, 1991). The casein-derived opioid peptide, β-casomorphin 7 also inhibits ACE (Meisel & Schlimme, 1994). Recently, it was demonstrated that β-lactorphin, the whey protein-derived opioid peptide could inhibit ACE (Mullally *et al.* 1996). This multifunctional bioactivity nature of milk protein peptides merits further research in terms of the general nutritive/physiological consequences of milk protein ingestion.

The majority of milk protein-derived peptides reported to date do not have ACE inhibitory potencies (Tables 1 and 2) approaching that of captopril ($IC_{50} = 0{\cdot}006$ μmol/l). However, being naturally derived these peptides would be expected not to have the side-effects associated with synthetically produced drugs used in the control of hypertension, i.e. cough and alterations in serum lipid metabolism (Ames, 1983; Seseko & Kaneko, 1985; Nakamura, 1987).

Conclusion

Casokinins and lactokinins represent a group of bioactive peptides that have significant potential as naturally-derived agents for the prevention/control of blood pressure and related diseases. The widespread use of these peptides in functional foods/nutraceuticals requires ongoing studies, including extended clinical trials, to demonstrate their long-term efficacy and safety.

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