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Design of substrate-type ACE inhibitory pentapeptides with an antepenultimate C-terminal proline for efficient release of inhibitory activity

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ABSTRACT

Much research has verified that tripeptides initiated with a branched-chain aliphatic amino acid residue and terminated with a proline have a strong antihypertensive activity *in vivo*. However, it is difficult to release from their precursor proteins that are orally administered. Based on the selectivity of angiotensin I-converting enzyme (ACE) on C-terminal dipeptides in its substrate, six pentapeptides with the same tripeptide IKP (as a model) at N-terminus including IKPVQ, IKPVA, IKPVK, IKPVR, IKPFR, and IKPHL were designed and chemically synthesized. It was shown that all the pentapeptides released IKP after ACE incubation. The release rate ranged from 23% (IKPHL) to 84.6% (IKPVR) as compared to the peptide sample before incubation. The *in vitro* digestion experiment demonstrated that all of the pentapeptides except IKPVA with a retention rate of 80.5% were resistant to pepsin hydrolysis but not to pancreatic hydrolysis. It should be noted that IKP could be released from IKPFR by pancreatin digestion. These results suggest that IKPVA and IKPFR potentially have a great antihypertensive effect *in vivo*. Furthermore, the dipeptides VA and FR described here may be widely used as linkers to help the release of the active peptides with proline at C-terminus from their protein precursors by ACE or gastrointestinal enzymes in human body.

1. Introduction

Angiotensin I-converting enzyme (ACE; EC.3.4.15.1), a zinc metallopeptidase, is widely found in most cells, body fluids, and tissues, in particular the lung tissue with the highest content. ACE plays an important physiological role in the regulation of blood pressure, as it hydrolyzes C-terminal dipeptides by converting inactive angiotensin I (DRVYIHPFHL) to potent vasoconstrictor angiotensin II (DRVYIHPF) as well as by inactivating vasodilator bradykinin (RPPGFSPFR), thereby increasing the blood pressure [1,2]. ACE inhibitory peptides are a class of small peptides and usually composed of 2–11 amino acid residues. These small peptides can bind with ACE and are not easily released from the binding site. In this way, the activity of ACE is inhibited by ACE inhibitory peptide, and the blood pressure is subsequently lowered [3]. For the last four decades, the natural food-derived ACE inhibitory peptides have attracted much attention due to their predominance over synthetic drugs in terms of mildness, safety, reliability, absorbability and no-effect on normal blood pressure, thereby becoming a hot topic for controlling and treating hypertension [4].

Although more than 90% of ACE inhibitory peptides are prepared by enzymatic hydrolysis of food proteins, low yield and high cost hanger their commercialization [5,6]. Recently, microbial fermentation has been used to produce ACE inhibitory peptides. The inhibitory peptide genes were incorporated into the gene of functional proteins [7–11] or synthesized as tandem multimers [12–15] and subsequently expressed in bacteria. After expression, these inhibitory peptides were released from the corresponding precursor proteins or precursor polypeptides by restriction digestion. It was obvious that microbial fermentation has the potential for producing ACE inhibitory peptides at a large scale. In general, the ACE inhibitory peptides selected for designing precursor proteins or polypeptides should be resistant to degradation (or could be decomposed into smaller active fragments) by gastrointestinal enzymes, easy to be released by digestive enzymes in the human body, and have a potent antihypertensive activity *in vivo*. It has been reported that the tripeptides that have a proline at the C-terminus and a branched-chain aliphatic amino acid residue (valine/leucine/isoleucine) at the N-terminus, have a very high ACE inhibitory activity. Furthermore, these tripeptides can reach the target sites in an active form and exhibit remarkable antihypertensive activity *in vivo* (Table 1) [16–24]. However, our previous studies

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Table 1
Tripeptides with proline at C-terminus and with branched-chain aliphatic amino acid residues (Val, Ile, and Leu) at N-terminus.

Peptides	Source	Preparation	IC_{50} (μM)	SHR		References
				Dose (mg/kg)	SBP (mmHg)	
LHP	*Acetes chinensis*	Pepsin	1.6	6	−36[a]	[16]
IQP	*Spirulina platensis*	Alcalase	5.77	10	−27[a]	[17]
IPP	Sour milk	Fermentation	5	1	−15[a]	[18,19]
VPP	Sour milk	Fermentation	9	1.6	−20[a]	[18,19]
LRP	Alpha-zein	Thermolysin	0.27	30	−15	[20]
LSP	Alpha-zein	Thermolysin	1.7			[20]
LQP	Alpha-zein	Thermolysin	1.9			[20]
VRP	Bonito bowels	Autolysis	2.2			[21]
LRP	Bonito bowels	Autolysis	1.0			[21]
IRP	Bonito bowels	Autolysis	1.8			[21]
GEP	*Tricholoma giganteum*	Extraction	3.2	1	−36[a]	[22]
LKP	Dried bonito	Thermolysin	0.32	60	−18[a]	[23]
IKP	Dried bonito	Thermolysin	1.6	10	−20[a]	[24]
IKP		Synthesis	0.68			This study

[a] Changes of systolic blood pressure in SHR at 2 h, 4 h, and 6 h after oral administration, respectively.

showed that the active fragments with proline at the C-terminus are difficult to be released from their precursor polypeptides by gastrointestinal enzymes [14,15].

The objective of this study is to design pentapeptides (with an antepenultimate C-terminal proline) that is not only resistant to digestion by gastrointestinal enzymes but also can be easily released from precursor by ACE, thus exerting a potent antihypertensive effect *in vivo*. Based on the selectivity of ACE on the C-terminal dipeptides of its substrates, we designed six pentapeptides which have a proline at an antepenultimate C-terminal position in this study. Subsequently, the stability of the pentapeptides to ACE and gastrointestinal enzymes were evaluated using RP-HPLC–MS/MS.

2. Materials and methods

2.1. Materials

Angiotensin I-converting enzyme (EC 3.4.15.1) from rabbit lung, pepsin (E.C. 3.4.23.1; ≥2500 units/mg protein) from hog stomach, and pancreatin from hog pancreas were purchased from Sigma. Pancreatin is a mix of different peptidases such as trypsin, alpha-chymotrypsin, elastase and carboxypeptidases A and B. Hippuryl-histidyl-leucine (HHL) was obtained from the peptide Institute (Osaka, Japan).

2.2. Peptide synthesis

Six pentapeptides IKPVQ, IKPVA, IKPVK, IKPVR, IKPFR and IKPHL were chemically synthesized to evaluate their stability to ACE and resistance to simulated gastrointestinal digestion. Additionally, the other four synthetic peptides IKP, IKPV, IKPF and IKPH were used for the determination of ACE inhibitory activities (IC_{50}). All synthetic peptides were prepared by GenScript Corporation (Nan Jin, China). The purity (all above 99%) and sequence of these peptides were verified by analytical reverse phase (RP)-HPLC–MS/MS.

2.3. Stability of the peptides to ACE

The stability of the designed pentapeptides against ACE was assessed *in vitro* according to a previously described method [25] with some modifications. Briefly, 100 μL of the 1.5 mM peptide solution was treated with 40 μL of ACE (12 mU) and 160 μL of 0.1 M borate (pH 8.3) for 3 h at 37 °C. The reaction was stopped by heating at 95 °C for 10 min in a water bath, followed by cooling to room temperature. Subsequently, the reaction mixtures were centrifuged at 10,000 × *g* for 20 min and the supernatants were applied to an RP-HPLC–MS/MS system. Also, the ACE inhibitory activities were compared before and after incubation.

2.4. Stability of the peptides to gastrointestinal enzymes

The evaluation of the stability of the designed pentapeptides to gastrointestinal enzymes was conducted employing the method of Alting et al. [26] with some modifications. Aqueous solutions (1 mM) of the peptides were hydrolyzed with pepsin (enzyme/substrate ratio of 1:100, w/w) for 4 h at 37 °C and pH 2.0, and with pancreatin (enzyme/substrate ratio of 1:50, w/w) for 4 h at 37 °C and pH 7.5, respectively. The reaction was stopped by heating at 95 °C for 10 min in a water bath, followed by cooling to room temperature. Then the reaction mixtures were centrifuged at 10,000 × *g* for 20 min, and the supernatants were used for ACE inhibitory activity determination and RP-HPLC–MS/MS analysis.

2.5. Assay of ACE inhibitory activity

ACE inhibitory activity of the peptides was measured *in vitro* following the spectrophotometric assay described by Cushman and Cheung [27] with some modifications [14]. Triplicate tests were performed for each sample. The concentration of each peptide required to inhibit 50% of ACE activity was defined as the IC_{50} value.

2.6. Analysis by online RP-HPLC–MS/MS

Identification of the peptides produced in the hydrolysates of pancreatic digestion and ACE incubation of the synthetic peptides was performed on a Waters HPLC system (Waters Corporation, Milford, MA) which was online connected to a matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF-TOF MS) (Waters Corporation, Milford, MA). The column used in these experiments was an XBridge™ C18 column (4.6 mm × 150 mm i.d., 5 μm particle size, Waters). Solvent A was 0.65 mL/L of trifluoroacetic acid (TFA) in milli-Q water, and solvent B was 0.5 mL/L TFA in acetonitrile. Peptides were eluted with different linear gradients of solvent B in A according to the separation condition of different samples. MALDI-TOF analysis was done in a mass charge (*m*/*z*) range of 100–1000. Automatically, main ions were selected for the subsequent TOF/TOF analysis. The collision energies were set at 5 eV and 20 eV for MS and MS/MS, respectively. The spectra were interpreted using the peptide sequencing module of the MassLynx 4.1 software.