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Physiologically Active Peptide Motif in Proteins: Peptide Inhibitors of ACE from the Hydrolysates of Antarctic Krill Muscle Protein

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Abstract
A peptide which inhibits the angiotensin-converting enzyme (ACE) was separated from sequential hydrolysates of defatted Antarctic krill muscle by pepsin and trypsin. The preparation procedure included chromatography on SP-Sephadex C-25, Superose 12, and reverse phase HPLC. The peptide fraction with the ACE-inhibiting activity was nearly pure and the main component was found to be a peptide with the amino acid sequence of Lys-Leu-Lys-Phe-Val showing a half-maximum inhibition concentration (1C50) of 30 μmol/l. A peptide sequence with 66% homology to the present peptide was found in some proteins such as prostaglandin DII reductase, thrombospondin precursor, epidermal growth factor precursor.

Discipline: Food
Additional key words: angiotensin-convering enzyme, anti-hypertension, endothelium differentiation

Introduction

With the progress of microsequencing and cloning techniques of DNA and proteins, the number of proteins for which the amino acid sequence has been determined or estimated from both c-DNA and peptide sequences exceeds 30,000. Thus, the use of a computer for investigations on the homology of amino acid sequences brings a new dimension in the relationship among proteins, for example the observation of a close similarity between egg white lysozyme and α-lactoalbumin. Although the primary structure of both proteins is almost the same, their biological activity is different, the former being an enzyme and the latter being the main component of milk protein without enzyme activity. More recently, peptidyl prolyl-cis-trans isomerase, which may play an important role in intracellular protein folding and be related to the denaturation-renaturation processes of protein, thus to cellular senescence, has been found to be the same protein as cyclophilin, a cyclosporin a-binding protein. Another instance is the presence of the EGF sequence in LDL-receptor, urokinase, and peroxidase.

The functional structure of proteins is represented by subunits, domains, and modules. Recently, the concept of a much smaller functional unit in protein has been introduced, i.e. the "motif" which is a peptide sequence with several amino acids and is responsible for physiological functions such as cellular attachment, intracellular localization, membrane anchoring, and nuclear translocation.

Hypertension is known to be closely related to food components, especially NaCl and protein and the antihypertensive effect of some proteins may be associated with the presence of an antihypertensive peptide motif.

In the human body, the blood pressure is controlled by various regulation systems such as the central nervous system, arterial and cardiopulmonary systems, and hormonal system. Thus, antihyper-

tensive drugs are developed based on the function of these regulation systems. At present, they are classified into (1) diuretics, (2) autonomic ganglion blockers, (3) vasodilators, (4) angiotensin-converting enzyme inhibitors, (5) Ca antagonists, (6) 5-HT_2 receptor blockers based on their action mechanism.

The angiotensin-converting enzyme (ACE) is the enzyme responsible for the production of a hypertensive peptide hormone, angiotensin II, DRVFIHPF, from angiotensin I, DVRYIHPFHL, that is liberated from a glycoprotein with a molecular weight of 57,000 by the action of renin in the blood stream. Accordingly, the substances which affect the renin-angiotensin system are assumed to act as antihypertensive drugs. In fact, chemicals with ACE inhibition such as captopril and rinarapril are used as first choice drugs for hypertensive diseases.

Recently, Ikeda and Yamori[4], and the authors' group[5] have found that the blood pressure level of SHRSP fed with defatted Antarctic krill protein was considerably low compared with that of SHRSP fed with defatted pork, and they remained alive for a longer period of time without exhibiting cerebral arteriosclerosis. Since the intake of an amino acid mixture whose amino acid composition was the same as that of the krill proteins did not depress the blood pressure, it was assumed that the hypotensive effect of krill protein feeding may not be due to the specific amino acid composition, but to the presence of a peptide intermediate degraded during intestinal digestion.

In the present study, ACE-inhibiting peptides were separated from the peptic hydrolysates of defatted krill proteins.

Materials and methods

The angiotensin-converting enzyme was extracted from the acetone powder of rabbit lung (Sigma). ACE activity was determined by measuring the content of the hippuryl group[1,6] or amino groups[2,3] liberated from Hipp-His-Leu (Peptide Institute, Osaka). Porcine pepsin and trypsin were purchased from Sigma, Sp-sephadex and Superose 12 were purchased from Pharmacia, and the RP-HPLC column from Tosoh. Amino acid composition and sequence analysis of the peptides were carried out by using a Hitachi 8500 automatic amino acid analyzer and a solid phase sequencer 477/120A(ABI), respectively. Authentic peptides were synthesized using an ABI-automatic peptide synthesizer.

Results and discussion

Peptic-tryptic hydrolysates of defatted krill protein were prepared according to the following procedures. Muscle of Haxan-defatted krill was separated from the shell and head portions. The krill muscle was homogenized in 5 vol. of a 0.4 M KCl solution, pH 4.0, sequentially using a homogenizer with a blade, and then polytron-type homogenizer. After the pH of the homogenates (suspension) was adjusted to 3.5 with 2N HCl, 1/500 amount of pepsin (W/W) was added to the homogenates, and digested for 4 hr at 37°C. Then trypsin digestion was carried out by the addition of 1/500 amount of trypsin and 0.01 mM $CaCl_2$ for 12 hr at 4°C after the pH of the solution was adjusted to 7.8. The digestion was terminated by heating on boiling water for 10 min. After cooling in cold water, the digests were centrifuged and the supernatant was freeze-dried.

Outline of the separation of an ACE-inhibiting peptide from the freeze-dried sample is shown in Fig. 1. The freeze-dried powder was dissolved in

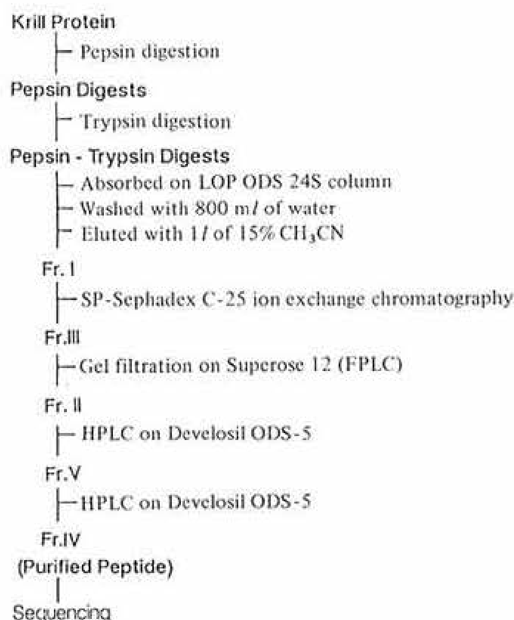
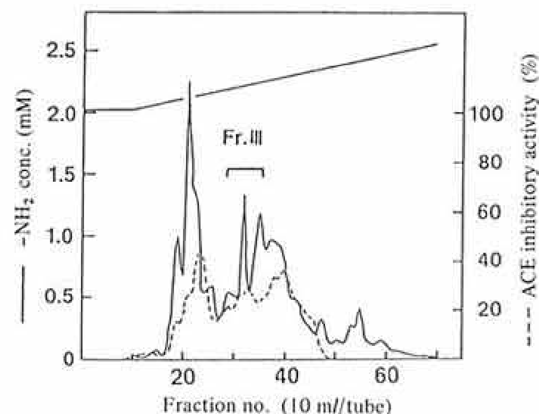


Fig. 1. Isolation procedure of ACE-inhibiting peptides from peptic and tryptic hydrolysates of Antarctic krill muscle

0.05% trifluoroacetic acid (TFA), then the solution was applied in batches to an ODS resin and eluted with 15% acetonitrile in 0.05% TFA. After freeze-drying, the samples were dissolved in 0.02 M $AcONH_4$–AcOH buffer, pH 3.0, then applied to a column of Sp-Sephadex C-50, eluted with a linear pH gradient from 3.0 to 6.0 as shown in Fig. 2.

The ACE-inhibiting activity was eluted as three peaks. In this paper, the fractions showing the second peak (Fr.III) were collected and freeze-dried. The freeze-dried samples were dissolved in 0.2 M



Column: 1.6x15 cm
Eluent: 0.02M $AcONH_4$-AcOH buffer pH 3.0
→ 0.3M $AcONH_4$-AcOH buffer pH 6.0 (linear gradient)
Flow rate: 27 ml/h

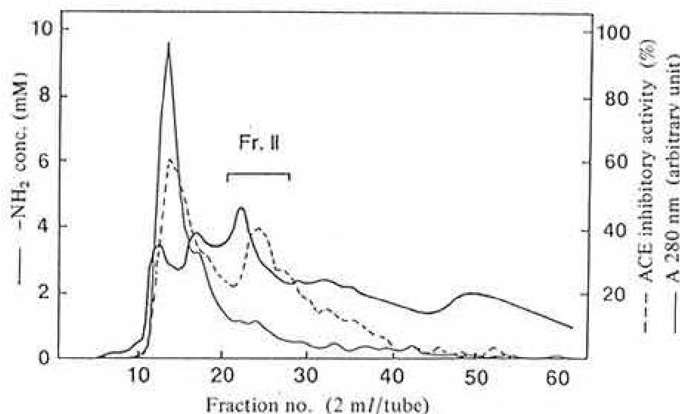
Fig. 2. SP-Sephadex C-25 chromatography of 15% CH_3CN eluted fraction

acetic acid and applied to a gel filtration column of Superose 12 on FPLC as shown in Fig. 3. The ACE-inhibiting activity was eluted as two main peaks. The active fraction with low molecular weight (Fr.II) was collected and freeze-dried. The freeze-dried sample was dissolved in 0.05% TFA containing 10% acetonitrile, and applied to a Develosil ODS-5 column followed by linear gradient elution from 10–20% acetonitrile in 0.05% TFA, as shown in Fig. 4. Many peptide peaks detected by 210 nm were present, but only four fractions showed an ACE-inhibiting activity. The latest peak fraction with the activity (indicated as Fr.V in Fig. 4) was collected and concentrated under a nitrogen stream. Then, the concentrate was applied for isocratic HPLC chromatography on Develosil ODS-5. The HPLC chromatogram showed one peak with the ACE-inhibiting activity, suggesting that a peptide with ACE inhibition had been isolated.

The results of the amino acid analysis and the sequence analysis are shown in Tables 1 and 2. The amino acid composition after 6N HCl hydrolysis of the peptide at 110°C under vacuum indicated the presence of Lys, Leu, Val, and Phe in the ratio of 2:1:1:1.

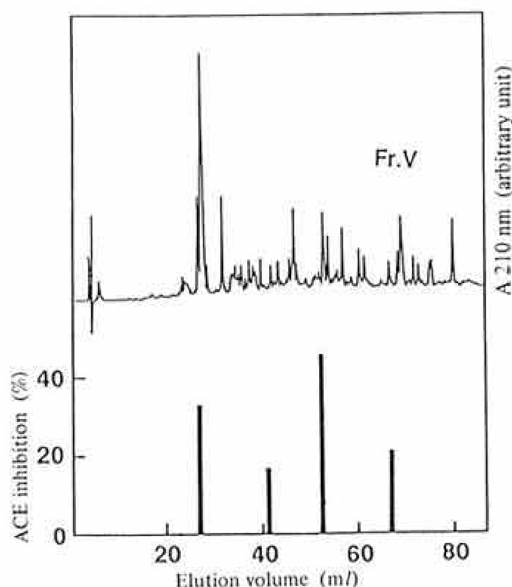
The amino acid sequence from the N-terminal estimated with a peptide sequencer was Lys-Leu-Lys-Phe-Val, indicating a good coincidence between the amino acid composition and the peptide sequence.

The half-maximum inhibition concentration (IC_{50}) of the isolated peptide was 30 μmol/l. A peptide



Column: 1.0x30 cm, Eluent: 0.2M AcOH, Flow rate: 0.1 ml/min

Fig. 3. Gel filtration chromatography of the active fraction from SP-Sephadex chromatography on Superose 12



Column: 0.46x25 cm
Eluent: 10%-20% CH_3CN in 0.05% TFA (linear gradient)
Flow rate: 1.0 m*l*/min

Fig. 4. High performance liquid chromatography of the active fraction derived from the active fraction eluted on Develosil ODS-5

Table 1. Amino acid composition of the isolated peptide

Amino acid		Nearest integral
	mol/mol	
Valine	0.92	1
Leucine	0.87	1
Phenylalanine	0.78	1
Lysine	1.87	2

Table 2. Amino acid sequence of the isolated peptide

Cycle of sequencing	Amino acid recovered
1	Lysine
2	Leucine
3	Lysine
4	Phenylalanine
5	Valine

with the same sequence of Lys-Leu-Lys-Phe-Val was synthesized with a peptide synthesizer.

The synthetic peptide, the sequence of which was confirmed to be Lys-Leu-Lys-Phe-Val by sequence analysis, also showed an IC_{50} value of 30 μmol/*l*. This peptide showed that a homologous sequence was present in more than 20 proteins including prostaglandin D_2 reductase, lipoamido acetyltransferase, pyruvate dehydrogenase, sucrose synthetase, cytochrome oxidase, protein kinase C, plasma kalikrein, T cell receptor α-chain precursor, and tropomyosin α-chain, etc. Since the present peptide was isolated from krill muscle proteins, it is suggested that the peptide was derived from tropomyosin of the krill muscle.

Recently, it has been reported that the local concentration of angiotensin II near the angioendothelium was closely related to cell growth or differentiation of the endothelium in the process of repair of damaged blood vessels and that the presence of the angiotensin-converting enzyme inhibitor prevented the occurrence of hypertrophic changes[7]. This finding may pave the way for the elucidation of the role of angiotensin II.

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