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Angiotensin-Converting Enzyme Inhibitory Activity of Kampo Medicines

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Abstract

The angiotensin-converting enzyme (ACE) inhibitory activity of five Kampo medicines (Chujo-to, Dai-saiko-to (TJ-8), Oren-gedoku-to (TJ-15), Toki-shakuyaku-san (TJ-23) and Keishi-bukuryo-gan (TJ-25)) was investigated and the isolation of active components from Chujo-to and TJ-23 was tried.

At 100 μl dose, over 60% inhibition for the ACE activity was observed for the five Kampo medicines. Among these medicines, Chujo-to, TJ-8 and TJ-15 induced stronger inhibition than the others. The ACE inhibitory activity of water extracts from each Chujo-to and TJ-23 was decreased after dialysis. A series of chromatographies, such as reversed ODS stationary phase, Sep-Pak® C_{18} and cation-exchange resin, were carried out by using methanol extracts of these Kampo medicines to identify active components from Chujo-to and TJ-23.

The results were as follows; ① Two ACE inhibitory components which have different physico-chemical properties exist in Chujo-to. ② The inhibitory components are composed of acidic, basic and amphoteric compounds. ③ The inhibitory activity was produced by both conditions in a strong acidic solution and an alkaline one. These results indicate that the several Kampo medicines used for the treatment of hypertensive disorders contain ACE inhibitory components which have different physico-chemical properties.

Introduction

It is well known that the original biological activities of medicinal plants have been lost

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Angiotensinogen

⬇ Renin

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

Angiotensin I

⬇ Angiotensin-Converting Enzyme

Asp-Arg-Val-Tyr-Ile-His-Pro-Phe + His-Leu

Angiotensin II

Fig. 1 Action of angiotensin-converting enzyme.

frequently during the isolating course of the active components. These facts suggest that the biological activities of medicinal plants are derived from physiological and/or biochemical interactions among the plural active components. It has been also considered that the biological activities of Kampo medicines (Japanese herbal medicine) would be exhibited by the same interacting mechanisms. Therefore, the pharmacological activities of Kampo medicines should be analyzed and assessed as an integral response of the active components contained in each prescribed medicinal plant.

Though there are many Kampo medicines which are used for the treatment of hypertensive disorders, the mechanisms of their efficacies have not been elucidated. Angiotensin-converting enzyme (ACE) which plays an important role in the regulation of blood pressure by conversion of the inactive decapeptide angiotensin I to the vasoactive octapeptide angiotensin II, as well as by inactivation of the vasodilator bradykinin, is known to distribute widely in many organs such as the lung, kidney, brain and testis (Fig. 1)[1),2)]. Therefore, several ACE inhibitors have been synthesized and effectively used for the clinical treatment of hypertension. Possibility of relationship between ACE inhibitor-like peptides in foods and their therapeutic effect on hypertension was also reported[3),4)]. It is suggested, therefore, that natural ACE inhibitors exist in medicinal plants and foods.

In the present paper, we describe the ACE inhibitory activity of the five Kampo medicines and the isolation trial of active components from two Kampo medicines.

Materials and Methods

1. Reagents

Hippuryl-L-histidyl-L-leucine (HHL) was obtained from Protein Research Foundation (Osaka, Japan). 2, 4, 6-Trichloro-S-triazine (TT), 1, 4-dioxane, hydrochloric acid and hippuric acid were purchased from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). Kampo medicines were obtained from Tumura & Co., LTD. (Tokyo, Japan).

2. Kampo medicines

Kampo medicines used in these experiments were as follows;

Chujo-to (in 12 g): Toki 1.92 g, Shakuyaku 1.92 g, Keihi 1.44 g, Senkyu 0.96 g, Bukuryo 0.96 g, Sojutu 0.96 g, Botanpi 0.96 g, Kobushi 0.48 g, Jio 0.48 g, Tonin 0.384 g, Kanzo 0.384 g, Oren 0.192 g, Choji 96 mg, Shokyo 96 mg, Ninjin 96 mg, Chinpi 0.672 g.

Dai-saiko-to (TJ-8, in 7.5 g): dried extracts 4.5 g of Saiko 6.0 g, Hange 4.0 g, Ogon 3.0 g, Shakuyaku 3.0 g, Taiso 3.0 g, Kijitu 2.0 g, Shokyo 1.0 g and Daio 1.0 g.

Oren-gedoku-to (TJ-15, in 7.5 g): dried extracts 1.5 g of Ogon 3.0 g, Oren 2.0 g, Sanshishi 2.0 g and Obaku 1.5 g.

Toki-shakuyaku-san (TJ-23, in 7.5 g): dried extracts 4.0 g of Shakuyaku 4.0 g, Sojutu 4.0 g, Takusha 4.0 g, Bukuryo 4.0 g, Senkyu 3.0 g and Toki 3.0 g.

Keishi-bukuryo-gan (TJ-25, in 7.5 g): dried extracts 1.75 g of Keihi 3.0 g, Shakuyaku 3.0 g, Tonin 3.0 g, Bukuryo 3.0 g and Botanpi 3.0 g.

3. Water extraction of Kampo medicines

Water extraction solutions were prepared from the Kampo medicines (1 g each) which were stirred with 10 ml of water for 24 hrs at 37°C and filtrated. Each filtrated solution was assayed for ACE inhibitory activity.

4. Preparation of angiotensin-converting enzyme

ACE was prepared from human kidney by the method described previously[5].

5. Assay of angiotensin-converting enzyme inhibitory activity

ACE inhibitory activities of Kampo medicines were assayed by using the following solutions:

Solution A: 0.25 M borate-sodium buffer, pH 8.3, containing 1.25 M NaCl;

Solution B: substrate, 0.0125 M HHL in solution A;

Solution C: 1 M HCl;

Solution D: 0.2 M potassium phosphate buffer, pH 8.3;

Solution E: 0.02 M potassium phosphate buffer, pH 8.3;

Solution F: 3% TT in 1, 4-dioxane.

Each water extract sample (1, 2, 4, 7, 10, 20, 40, 70 or 100 μl) was added to assay tubes and adjusted a total volume to 100 μl with solution E and pre-incubated with 50 μl of ACE solution (in solution E) for 5 min at 37°C. In the experiment, 50 μl of test samples were used for the assay of ACE inhibition.

The enzyme reaction with the test samples was started by the addition of solution B (100 μl), lasted for 30 min at 37°C and terminated by the addition of solution C (50 μl). After the additdon of solution D (3.2 ml), solution F (1.6 ml) was added to the reaction mixture with vigorous stirring for 15 sec and centrifuged for 5 min at 3,000 r.p.m. to remove denatured protein and excess TT reagent. The amount of enzymatic product in the resulting supernatant was determined by its absorbance at 382 nm. The control run was treated identical to the above procedures without the incubation.

6. Definition of enzyme units

One unit (U) of ACE activity was defined as the amount of enzyme which hydrolyzed 1

μmol of HHL to hippuric acid and L-histidyl-L-leucine in 1 min at 37°C under the conditions described above.

7. Dialysis

Water extracts (2 ml) of Chujo-to and Toki-shakuyaku-san (TJ-23) were dialyzed against running water (20 l/hr) for 4 days by using dialysis membranes (pore size 10,000). ACE inhibitory activities of the resulting inner solutions were assessed.

8. Isolation of ACE inhibitory components from Chujo-to and TJ-23

Each 10 g of Chujo-to and TJ-23 was stirred with 100 ml of methanol for 24 hr at 37°C. The methanol extracts were used for the further isolation studies described below.

1) Chromatography on reversed ODS stationary phase

The methanol extracts were subjected to chromatography on a column of reversed ODS stationary phase and the column were eluted with water and 50% methanol.

Each methanol fraction was separated by a concentration gradient method and the methanol was evaporated. The each fraction residue was dissolved in 1 ml of water and the inhibitory activity was measured.

2) Chromatography on Sep-Pak® C_{18}

The methanol extracts were evaporated and the residues were dissolved in 100 ml of water. After adjusting the pH to 4, 7 and 10, 1 ml of each water solution was applied on Sep-Pak® C_{18} cartridge. Elution was carried out with 10 ml of water and methanol, respectively. Methanol fraction was evaporated to dryness, dissolved in 10 ml of water and assayed for ACE inhibitory activity by the method described above.

3) Chromatography on cation-exchange resin

The methanol extracts (10 ml each) of Chujo-to and TJ-23 were subjected to column chromatography on Amberlite IRA-401 and Amberlite IR-120 resins (Orugano Co., Tokyo, Japan). Elution was carried out by step wise gradient system with water (30 ml each) adjusted pH from 2 to 12 with hydrochloric acid and/or ammonium hydroxide. Each eluate was evaporated to driness, dissolved in 10 ml of Solution E and assayed for inhibitory activity of ACE by the method described above.

4) Analysis by high performance liquid chromatography (HPLC)

ACE inhibitory fractions which were isolated from Chujo-to and TJ-23 previously by cation exchange chromatography were used for further analysis by HPLC. The HPLC analysis system was consisted of Develosil® ODS-5 and Develosil® CN-5 columns (Nomura Chemicals Co., Seto, Japan), Jasco TRI-Rotar® VI pump and Jasco UVIDEC®-100-VI detector (monitoring wave length: 254 nm, Jasco Co., Tokyo, Japan). The mobile phase was water-methanol (90 : 10, v/v) and the flow rate was 1 ml/min.

Results and Discussion

1. ACE inhibitory activity of Kampo medicines

ACE inhibitory activity of five Kampo medicines extracted with water was shown in Fig. 2. Singnificant inhibition was induced by the extracts of all medicines at 7 μl dose except

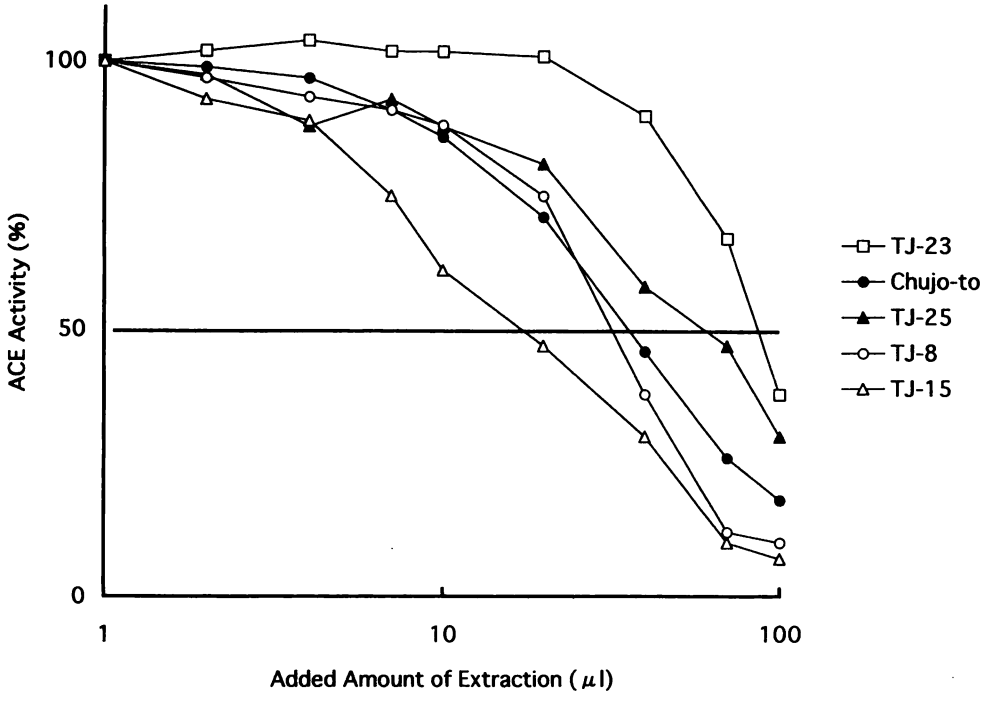


Fig. 2 Effects of five Kampo medicines on ACE activity.

Table 1 Effects of water extracted substances from five Kampo medicines on ACE inhibitory activity

Name of Kampo Medicines	IC_{50} (μl)
Chujo-to	36
Dai-saiko-to (TJ-8)	31
Oren-gedoku-to (TJ-15)	18
Toki-shakuyaku-san (TJ-23)	88
Keishi-bukuryo-gan (TJ-25)	45

IC_{50}: median inhibitory concentration (volume μl) of test samples to inhibit ACE activity

TJ-23 which showed inhibition at 40 μl dose. At 100 μl dose, over 60% inhibition was seen for all of them. The IC_{50} values, the test sample doses required to produce 50% inhibition of ACE activity, were calculated and summarized in Table 1. All Kampo medicines tested were weaker than captopril and enarapril, synthetic ACE inhibitors, in terms of IC_{50} values. Among these medicines, TJ-15, TJ-8 and Chujo-to induced stronger inhibition than the others. The facts that TJ-15 and TJ-8 are clinically used for the treatment of hypertension, suggest that their anti-hypertensive action may be, at least partly, attributable to the inhibitory effect on ACE activity. Figure 3 shows the double-reciprocal plots of ACE activity in the presence of 10 μl of Chujo-to water extract. This result indicates that an inhibition of ACE by Chujo-to is competitive-type.

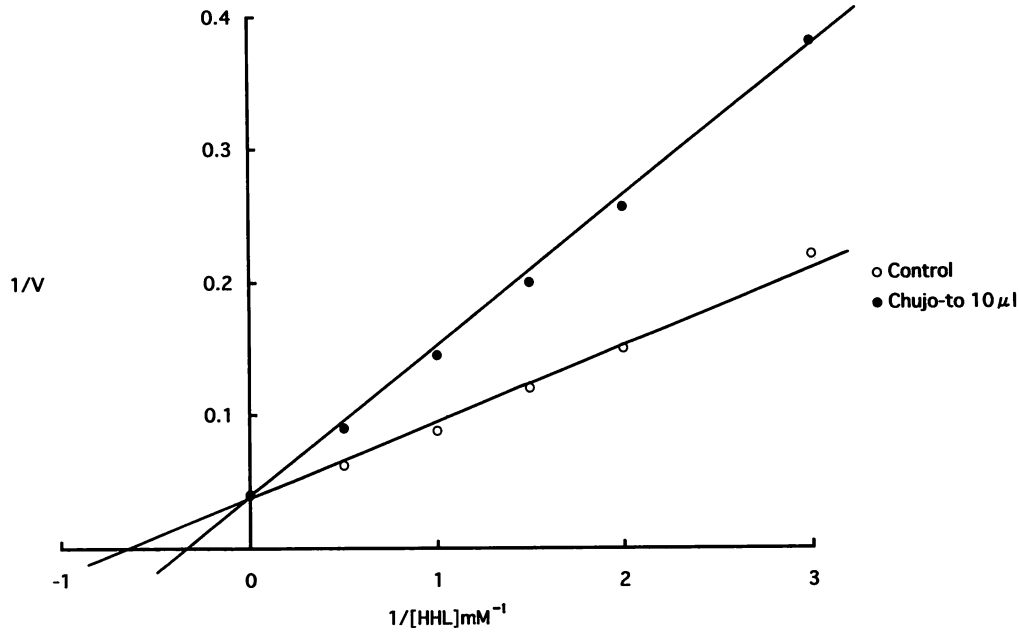


Fig. 3 Lineweaver-Burk plot for inhibition rate of Chujo-to.

2. Effect of dialysis on ACE inhibitory activity of Chujo-to and TJ-23

The ACE inhibitory activity of water extracts of Chujo-to and TJ-23 was dramatically decreased after dialysis against running water (20 l) for 4 days. The pore size of dialysis membrane used in this experiment was cut off substances which were high molecular weights 10,000 and over. These results suggest that the molecular weights of ACE inhibitory components are lower than 10,000.

3. Isolation of ACE inhibitory components from Chujo-to and TJ-23

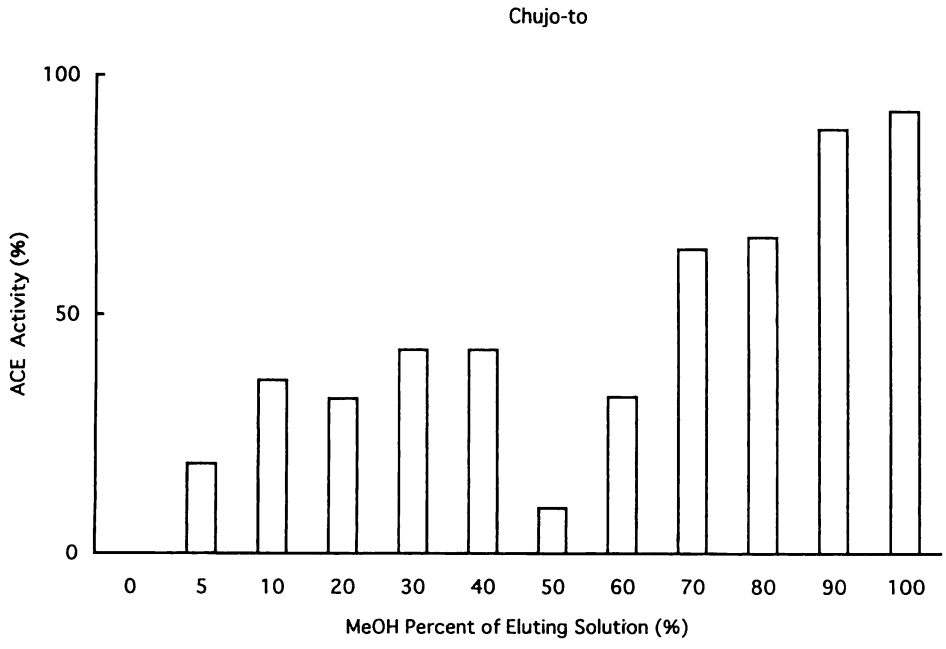
Since an extraction of Kampo medicines with methanol was simple and able to gain relatively large amount of ACE inhibitory active substances in comparison with water using extraction procedure, a series of chromatographies have been carried out by using methanol extracts of Chujo-to and TJ-23.

1) Chromatography on reversed ODS stationary phase

As shown in Fig. 4, a strong inhibition was observed only in water fraction (methanol 0%) for TJ-23 while a strong inhibitory activity was recovered in both fraction of water and 50% methanol for Chujo-to. These results suggest that, at least, two ACE inhibitory components having different physico-chemical properties exist in Chujo-to. Since the inhibitory activity of both medicines was effectively recovered in water extracts, indicating that the main inhibitory component are not absorbable on ODS stationary phase, the inhibitory components are considered to have extremely hydrophilic property.

2) Chromatography on Sep-Pak® C_{18}

Although methanol extract of Chujo-to was applied to Sep-Pak® C_{18} cartridge after adjusting the pH to 4, 7 and 10 and the test samples were eluted with 10 ml of each water and



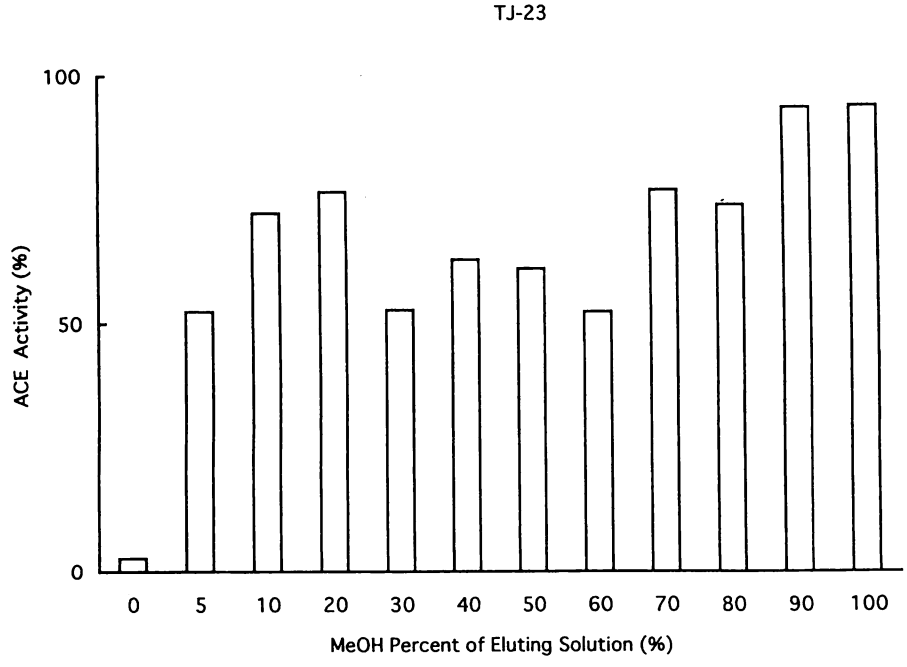


Fig. 4 Effects of fraction in different methanol concentration on ACE inhibitory activity of Chujo-to and TJ-23.

Table 2 Effects of ACE activity of each water and methanol extraction from Chujo-to at respective pH 4, 7 and 10 using Sep-Pak® C_{18}

Extracting Conditions	ACE Activity (%)
Control (vehicle)	100
pH 4	
Water extraction	12.8
Methanol extraction	30.4
pH 7	
Water extraction	14.3
Methanol extraction	43.4
pH 10	
Water extraction	19.0
Methanol extraction	40.6

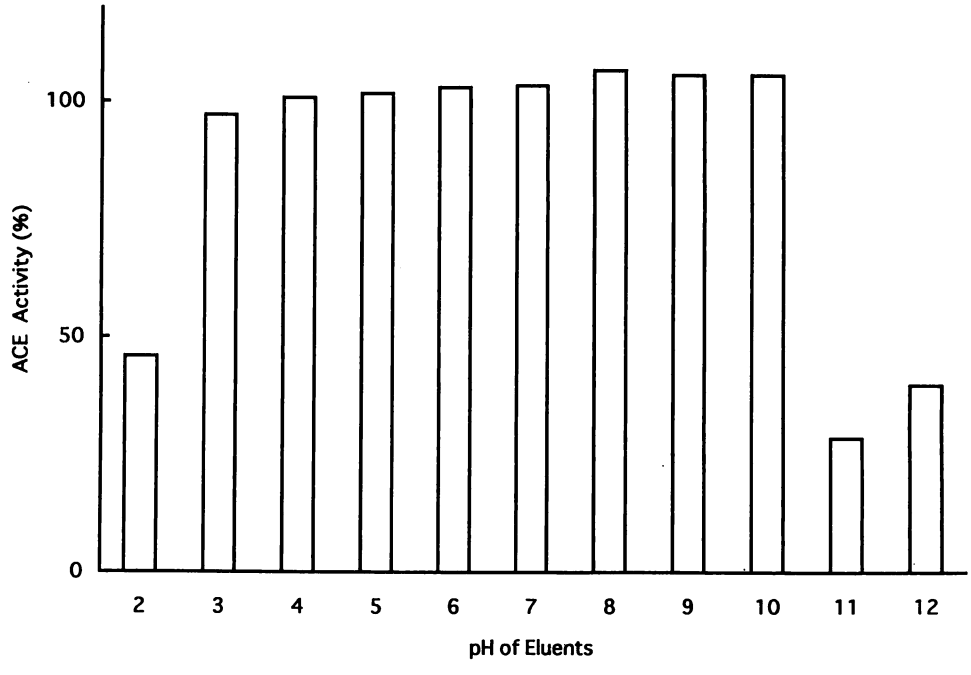


Fig. 5 Elution profile of ACE inhibitory activity by Chujo-to at different pH values using cation-exchange resin column.

methanol, ACE inhibitory activity was recovered no significant difference in neither water fractions nor methanol fractions at any pH (Table 2). The recovered activity in water fraction was much higher than that in methanol fraction. It is assumed, therefore, that the inhibitory components of Chujo-to are mixture of acidic and basic substances or amphoteric compounds.

3) Chromatography on cation-exchange resin

In order to examine the pH characteristics of inhibitory components, methanol extract of Chujo-to applied to cation-exchange column and the column was eluted with water solutions of different pH values from 2 to 12. Figure 5 shows the elution profile of ACE inhibitory activity at different pH values. Inhibitory potencies at pH of 2, 11 and 12 were 54.1%, 71.5% and 60.1%, respectively. Little inhibition was recognized at pH from 3 to 10. Thus the inhibitory active substances of Chujo-to was recovered by a strong acidic and alkaline conditions.

4) Analysis by HPLC

The ACE inhibitory components recovered at pH 2 and pH 11 described above were further analyzed by HPLC. For pH 2 fraction, two peaks at 2 min and 7 min after injection were obtained by chromatography on ODS stationary phase column while chromatography on CN stationary phase column gave more different peaks than that on ODS column did. For pH 11 fraction, although no good separation profiles by chromatographies on ODS and CN columns were observed, the chromatograms were quite different from those of pH 2 fraction. These results suggest that the ACE inhibitory components in fractions pH 2 and pH 11 have different physical and chemical properties. At present, we are trying further analysis of ACE inhibitory components contained in pH 2 and pH 11 fractions by HPLC.

The above results indicate that some Kampo medicines, especially those used for the treatment of hypertensive disorders, contain ACE inhibitory components having different physico-chemical properties. In order to clarify their physico-chemical properties and also mode of action, we are further analyzing ACE inhibitory components by HPLC.

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