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One Peptide Derived from Hen Ovotransferrin as Pro-drug to Inhibit Angiotensin Converting Enzyme

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ABSTRACT

Angiotensin I-converting enzyme (ACE) inhibitory peptide was derived from hen ovotransferrin and identified as Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr. It produced a concentration-dependent inhibition of ACE activity *in vitro* with an IC_{50} value of 102.8 μM. After hydrolysis by ACE, the product (Lys-Val-Arg-Glu-Gly-Thr) has an IC_{50} value of 9.1 μM that was about 11-fold lower of the parent peptide. Thus, Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr can be considered as a pro-drug. Moreover, Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr and Lys-Val-Arg-Glu-Gly-Thr were intravenously administered into spontaneously hypertensive rats (SHR) to monitor the time-course change of systolic blood pressure. We found that Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr and its hydrolyzed product produced the maximal reduction of systolic blood pressure at 40 min and 20 min after injection, respectively. This 20 min delay might be considered as the time required for conversion of prodrug into Lys-Val-Arg-Glu-Gly-Thr, the true ACE inhibitor. In conclusion, the obtained results suggest that Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr works as the pro-drug of Lys-Val-Arg-Glu-Gly-Thr to inhibit ACE activity *in vivo*.

Key words: angiotensin-converting enzyme (ACE) inhibitor, antihypertensive peptide, hen ovotransferrin

INTRODUCTION

The renin-angiotensin system is well known to regulate blood pressure in the circulatory system. Actually, angiotensin I-converting enzyme (ACE) (dipeptidyl carboxypeptidase, EC 3.4.15.1) plays an important role in this renin-angiotensin system. Captopril and enalapril are known as antihypertensive drugs by retarding the catalytic action of ACE. Thus, ACE inhibitors exhibit antihypertensive activity in spontaneously hypertensive rats (SHR) and hypertensive patients[(1)]. Recently, many ACE inhibitory peptides were derived from food such as egg yolks[(2)], fish muscle[(3-5)], wakame[(6)], sour milk[(7)] and dry bonito[(8)].

Ovotransferrin, an iron-binding glycoprotein belonging to the transferrin family protein, was found to contain 13% of the total protein in egg white. Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr isolated from chymotryptic hydrolysates of ovotransferrin has ACE inhibitory activity. Also, it can lower blood pressure in SHR after an intravenous injection. In the present study, using the preincubation method[(3)], Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr was investigated and characterized as a true ACE inhibitor or a pro-drug peptide.

MATERIALS AND METHODS

I. *Chemicals*

Peptide for assay of the ACE inhibitory activity was ordered to prepare using Fmoc amino acid active derivatives from Sigma-Genosys (TX, USA). ACE (EC 3.4.15.1) and hippuryl-histidyl-leucine were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Other reagents used were of analytical grade.

II. *Determination of the Stability of Peptide for ACE*

Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr (0.02 mM) was incubated with 28 mU of ACE at 37°C for 3 hr, and the reaction was stopped by boiling for 10 min, as described previously[(8)]. In order to check the stability of the tested inhibitory peptide for ACE, the reaction was immediately applied to a RP-HPLC system, using a LiChroCART C18 column (4 mm I.D. × 250 mm, a product of Merck, Frankfurter City, Germany) and a mixture of solvent A (0.1% trifluoroacetic acid in water) and B (0.1% trifluoroacetic acid in acetonitrile). A linear gradient of A and B (0 to 67% B) was applied to the column for 18 min at a flow rate of 1 mL/min and the absorbance of the eluate at 214 nm was monitored.

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III. *ACE Inhibitory Activity between Digests with ACE and Purified Peptide*

IC_{50} values (concentration of testing agent required to inhibit 50% of the ACE activity) of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr before and after incubation with ACE were compared. Moreover, fractions due to the digests with ACE applying RP-HPLC system were collected and the sequence of peptides was determined using an Applied Biosystems gas-phase sequencer 492 Protein Sequencer (Applied Biosystems Inc., Foster City, CA, USA). The acetonitrile in fraction was removed using a centrifugal evaporator and the sample was dissolved in water and neutralized by adding an alkaline solution for the assay of ACE inhibitory activity.

The sample was assayed *in vitro* for the ability to inhibit ACE activity according to the previous method[9]. In brief, 100 μL of 4.7 mM hippuryl-L-histidyl-L-leucine/300 mM NaCl/400 mM phosphate buffer solution (pH 8.5) was added using 50 μL of testing sample or vehicle used to dissolve the testing sample. Then, 100 μL (2.5 mU) of ACE/distilled water was mixed with the above substrate solution to initiate the reaction that was carried out by incubation in a water bath at 37 ± 1°C under shaking for 60 min. Finally, 1.5 mL of 0.3 M sodium hydroxide was added to terminate the reaction. The formed histidyl-leucine was then labeled by 100 μL of 2% phthaldialdehyde/methanol at room temperature for 10 min and the reaction was terminated by 200 μL of 3 M HCl. The formed fluorescence compound was diluted using distilled water to 250 times and the fluorescence intensity was then estimated by a spectrofluorometer (EX340, EM455; Hitachi, F-3000). Substrate with distilled water only was used as the blank, while the mixture without testing sample but the same volume of distilled water was treated as control. The inhibitory ratio (%) of ACE was calculated as (C - A)/(C - B) × 100%, where A is absorbance of the testing sample, B is absorbance of the blank and C is absorbance of the control. Sample was tested at five concentrations to obtain the standard curve for the determination of the IC_{50} value. Sample was tested in triplicate.

IV. *Determination of Antihypertensive Activity in Hypertensive Rats*

Effects on the systolic blood pressure were determined by intravenous injection of testing peptide into male spontaneous hypertensive rats (SHR) (obtained form the Animal Center of National Science Council, Taipei, Taiwan) that were in an air-conditioned room (25 ± 1°C) having a 12:12 light-dark cycle (light on at 06:00). Food and water were available ad libitum. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, as well as the guidelines of the Animal Welfare Act. The body weight of rats used was between 410 to 550 g and the solution of testing peptide prepared at desired concentration was injected at a ratio of 1 mL/kg of body weight. The blood pressure was measured by tail-cuff method using the MK-2000 blood pressure meter (Muromachi Kikai, Tokyo, Japan) as described previously[10]. Systolic blood pressure was then calculated from four measurements of one animal at the desired times.

The testing peptide was dissolved in physiological salt solution for intravenous administration. After injection of testing peptide into tail vein of SHR, blood pressures were measured at the desired intervals (10, 20, 40, 60 and 90 min) and the time before injection (0 min).

V. *Data Analysis*

Data are expressed as the mean ± SEM for the number (n) of IC_{50} testing in each group indicated in the table and figures. Repeated measures analysis of variance (ANOVA) was used to analyze the changes of blood pressure and other parameters. The Dunnett range post-hoc comparisons were used to determine the source of significant differences where appropriate. A $p < 0.05$ was considered statistically significant.

RESULTS

I. *Characterization of ACE Inhibitory Peptide by Preincubation Method*

A HPLC analysis of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr (0.02 mM) after the incubation with ACE (28 mU) showed that this peptide was totally converted by ACE into Lys-Val-Arg-Glu-Gly-Thr and Thr-Tyr (Figure 1B) as compared with that of before the incubation with ACE, which shows Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr only (Figure 1A). Thus, Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr was digested totally by ACE in this preincubation method. Then, the IC_{50} values of this peptide were also determined before and after preincubation with ACE. The inhibitory activity of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr was intensified about six times from IC_{50} = 102.8 μM before the incubation with ACE to IC_{50} = 17.2 μM after the incubation. However, preincubation with ACE did not alter the inhibitory activity of Lys-Val-Arg-Glu-Gly-Thr (Table 1).

II. *Antihypertensive Activity of Testing Peptide In Vivo*

The antihypertensive effects of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr, the original peptide, and Lys-Val-Arg-Glu-Gly-Thr, the product (active form) after preincubation with ACE, were determined in SHR. As shown in Figure 2, Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr exerted a dose-dependent (from 1 to 1000 pmol/kg, i.v.) antihypertensive activity by an intravenous injection. Similar antihypertensive action of Lys-Val-Arg-Glu-Gly-Thr was also obtained at the same dose range (Figure 2). However, as shown in Figure 3, the maximal decrease of systolic blood pressure (-10.3% = -21.1 ± 4.1 mmHg) by Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr

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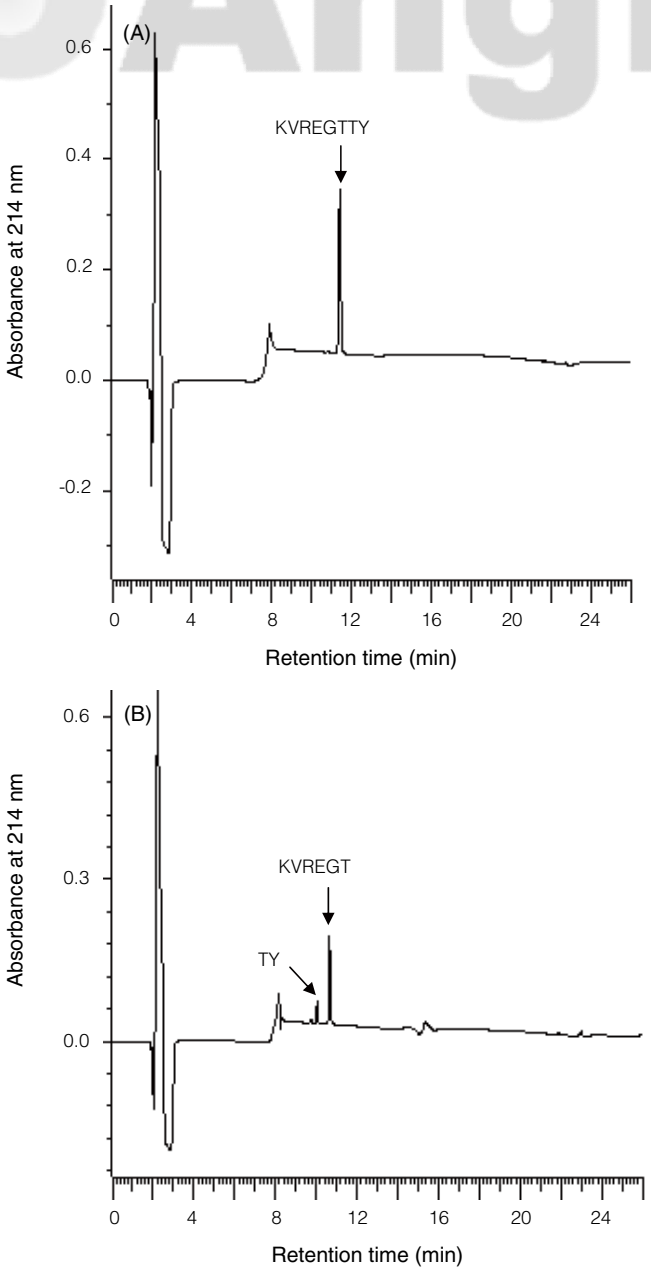

Figure 1. Stability of KVREGTTY during the incubation with ACE. RP-HPLC analysis of reaction mixtures before and after incubation with ACE on a LiChroCART C18 column (4 mm I.D. × 250 mm). Chromatograms (A) before preincubation and (B) after preincubation with ACE. Peptide (0.02 mM) was incubated with 28 mU ACE (37°C, 3 hr).

(1 nmol/kg, i.v.) was occurred 40 min after an intravenous injection. But, maximal decrease of systolic blood pressure (-11.6 % = -23.7 ± 2.3 mmHg) by Lys-Val-Arg-Glu-Gly-Thr at same dose was observed 20 min after similar treatment. Moreover, both at 10 and 100 pmol/kg (i.v.), Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr decreased the systolic blood pressure in SHR after 40 min of intravenous injection. Also, Lys-Val-Arg-Glu-Gly-Thr produced the blood pressure lowering action after 20 min of same treatments (data not shown).

Table 1. ACE inhibitory activity of the peptide derived from ovotransferrin. Comparison of IC_{50} values before and after incubation with ACE

Peptide	IC_{50}[a] (μM)	
	Before incubation with ACE	After incubation with ACE
KVREGTTY	102.8 ± 11.9	17.2 ± 2.9
KVREGT	9.1 ± 1.8	7.6 ± 1.1

[a]IC_{50}: The concentration of each peptide required to inhibit 50% of ACE activity. IC_{50} values were expressed as mean ± SEM.

DISCUSSION

ACE inhibitory peptides can be classified into three groups, depending on their interaction with ACE[3,11,12]. The first group is the true inhibitor, which inhibitory activity is not altered by preincubation with ACE. The second group comprises the substrates for ACE, which converts substrate into inactive peptide, resulting in extensively reduced activity of the peptide by incubation with ACE. The third group is called pro-drug like inhibitory peptides. They are also the substrates for ACE, but they are converted by this enzyme into true inhibitors, resulting in an increase of inhibitory activity after the preincubation with ACE. According to the previous reports[3,5,8,13], only true inhibitor and pro-drug like inhibitory peptide have the ability to lower blood pressure. To distinguish true inhibitor from the substrate, peptides were preincubated with ACE before the measurement of ACE inhibitory activity. Basically, IC_{50} values of the true inhibitors are not affected by preincubation with ACE, whereas substrates for ACE are changed by preincubation with ACE. We found that Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr is a pro-drug type ACE inhibitor because preincubation with ACE of this peptide intensified the ACE inhibitory activity about six times from IC_{50} value 102.8 to 17.2 μM (Table 1). This is also confirmed by HPLC analysis of reaction mixture after preincubation showing that ACE converts Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr to Lys-Val-Arg-Glu-Gly-Thr (Figure 1). This result indicates that Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr is hydrolyzed by ACE into Lys-Val-Arg-Glu-Gly-Thr, the true ACE inhibitory peptide because IC_{50} values for this peptide before and after preincubation with ACE were found to be almost unchanged (Table 1). The active peptide (Lys-Val-Arg-Glu-Gly-Thr) showed an IC_{50} value of 7.6 μM that was lower than that of 9 μM produced by Val-Pro-Pro[14] which exhibited antihypertensive activity[7]. However, the IC_{50} value of Lys-Val-Arg-Glu-Gly-Thr is markedly higher than that of captopril (22 nM), one of ACE inhibitors[3].

In order to assure of this hypothesis, peptides were investigated in animals. Both of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr and Lys-Val-Arg-Glu-Gly-Thr caused an antihypertensive activity after intravenous injection into SHR. Similar dose-dependent antihypertensive activity after an intravenous injection of two peptides from 1 to 1000

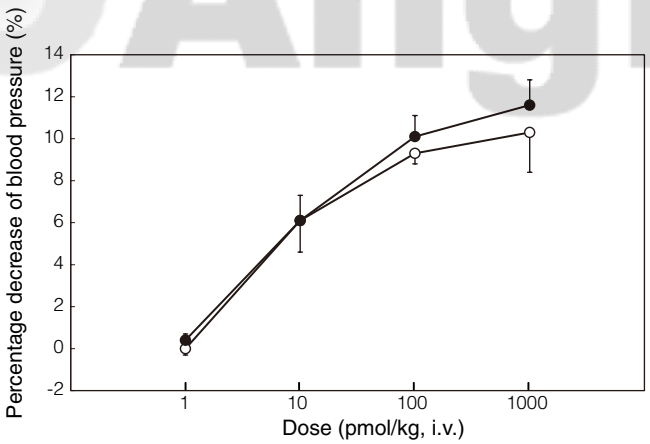

Figure 2. Decrease of systolic blood pressure by KVREGTTY (○) or KVREGT (●) after an intravenous injection into hypertensive rats (SHR). Peptides dissolved in saline were intravenously administered at the dose from 1 to 1000 pmol/kg. Percentage changes of the systolic blood pressure were expressed as means ± SEM in each point (n = 8). The percentage change was calculated from the original systolic blood pressure (205 ± 3 mmHg) in SHR. No statistical difference (P > 0.05) was observed between two peptides to produce maximal effect at each dosing.

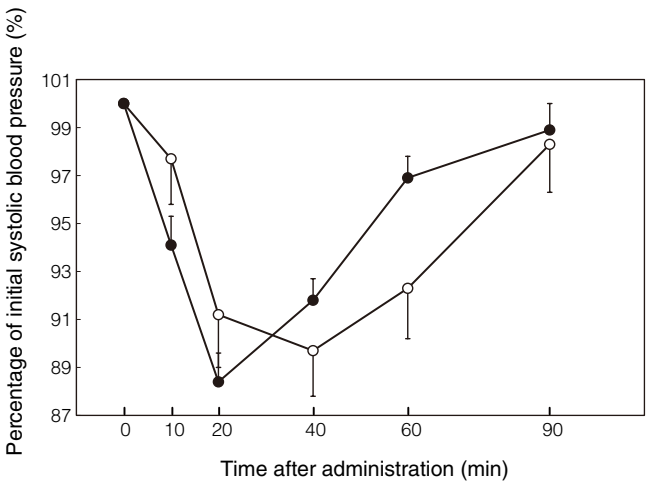

Figure 3. Antihypertensive activity of KVREGTTY (○) or KVREGT (●) after an intravenous injection into hypertensive rats (SHR). Both peptides dissolved in saline were administered at a dose of 1 nmol/kg. Percentage changes of the systolic blood pressure from time zero were expressed as means ± SEM in each point (n = 8).

pmol/kg (i.v.) was observed (Figure 2). It seems reliable to consider that the same peptide is responsible for the decrease of systolic blood pressure after injection. Figure 3 showed the time-course of systolic blood pressure change in SHR induced by two peptides (1 nmol/kg, i.v.), Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr and Lys-Val-Arg-Glu-Gly-Thr. Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr showed the maximal decrease of systolic blood pressure at 40 min after injection, while Lys-Val-Arg-Glu-Gly-Thr produced the maximal effect at 20 min after injection (Figure 3). Moreover, both peptides at 10 or 100 pmol/kg (i.v.) administered into SHR produced a similar time-course change of systolic blood pressure as that of 1 nmol/ml/kg (i.v.) (data not shown). This 20 min delay for the reduction of systolic blood pressure observed might be considered as the time required for conversion of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr by ACE into the true inhibitor of Lys-Val-Arg-Glu-Gly-Thr in SHR. Thus, a conversion of Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr by ACE into the true inhibitor of Lys-Val-Arg-Glu-Gly-Thr was identified *in vivo*. Altogether, Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr can be considered as a pro-drug type of ACE inhibitory peptide. However, it should be noticed that the endogenous situation is more complicated because other peptidases may also participate in the degradation of peptides. A direct analysis of the ACE activity in serum of SHR modified by the testing peptide will be helpful. However, the employed method for analysis of ACE activity was available for *in vitro* assay only. Thus, more studies are needed to clarify the detailed mechanism of this action in human subjects in the future.

In conclusion, the obtained results indicate that Lys-Val-Arg-Glu-Gly-Thr-Thr-Tyr works as a pro-drug type ACE inhibitory peptide because it was converted by hydrolysis with ACE into Lys-Val-Arg-Glu-Gly-Thr, exhibiting a 11-fold augmentation in ACE inhibitory activity.

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