Essential of Proline and Valine Residues in the Peptide Derived from Lactoferrin for Angiotensin Converting Enzyme Inhibition

ARTICLE *in* JOURNAL- CHINESE CHEMICAL SOCIETY TAIPEI · JUNE 2006

Impact Factor: 0.65 · DOI: 10.1002/jccs.200600068

4 AUTHORS, INCLUDING:

Toshiki Enomoto
Ishikawa Prefectural University
66 PUBLICATIONS **324** CITATIONS
SEE PROFILE

Ichiro Nakamura
Hokkaido University
47 PUBLICATIONS **558** CITATIONS
SEE PROFILE

Communication

Essential of Proline and Valine Residues in the Peptide Derived from Lactoferrin for Angiotensin Converting Enzyme Inhibition

Nai-Yuan Lee[a] (李乃元), Juei-Tang Cheng[b]* (鄭瑞棠),
Toshiki Enomoto[c] (榎本俊樹) and Ichiro Nakamura[d] (中村一郎)
[a]*Livestock Research Institute, Tainan 712, Taiwan, R.O.C.*
[b]*Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan 70101, Taiwan, R.O.C.*
[c]*Department of Food Science, Ishikawa Prefectural University, Nonoichi, Ishikawa 9218836, Japan*
[d]*Institute of Animal Experimentation, School of Medicine, Hokkaido University, Sapporo 0608589, Japan*

We synthesized Leu-Arg-Pro-Val-Ala-Ala-Glu, the peptide contained in lactoferrin (Lf), to identify the angiotensin converting enzyme (ACE) inhibition. In an attempt to know the structure-activity relationship of this peptide, we replaced Pro (the third amino acid residues from N-terminal) or Val (the fourth amino acid residues from N-terminal) with Ala (neutral amino acid), Glu (acidic amino acid) or Lys (basic amino acid) to produce six peptides. From the in vitro ACE inhibition (IC_{50}) of these synthesized peptides, the original peptide (Leu-Arg-Pro-Val-Ala-Ala-Glu) showed higher ACE inhibition than the replaced six peptides. Thus, replacement of Pro at the third amino acid residues or Val at the fourth position with Ala, Glu or Lys revealed the ACE inhibition to be lower than the original form of Leu-Arg-Pro-Val-Ala-Ala-Glu. Otherwise, we added one peptide at the C-terminal of Leu-Arg-Pro-Val-Ala-Ala-Glu and found both products with an addition of Val (Leu-Arg-Pro-Val-Ala-Ala-Glu-Val) or Ile (Leu-Arg-Pro-Val-Ala-Ala-Glu-Ile) showing a lower ACE inhibition than the original one. The ACE inhibitions produced by both replaced peptides were without significance. Also, deletion of the last peptide at the C-terminal (Leu-Arg-Pro-Val-Ala-Ala) failed to produce a marked change of ACE inhibition as compared to the original one. These results suggest that Pro and Val are essential in the peptide for inhibition of ACE activity.

Keywords: Synthesized angiotensin-converting enzyme (ACE) inhibitory peptide; Lactoferrin; Proline; Valine; IC_{50} value.

INTRODUCTION

Angiotensin Converting Enzyme (ACE) plays an important role in the regulation of blood pressure because this dipeptidyl carboxypeptidase can raise blood pressure via converting angiotensin I that was produced from angiotensinogen by renin into the potent vasoconstrictor angiotensin II.[1] It has been mentioned that ACE is an unusual zinc-metallopeptidase that can be activated by chloride showing narrow *in vitro* substrate specificity.[2] Some inhibitors of ACE have widely been used in clinics, including captopril, enalapril, lisinopril and others.[3,4] The competitive inhibitors of ACE were obtained from naturally occurring peptides in snake venom.[5,6] These inhibitors possess many Pro residues. Fragments of food proteins, i.e., gelatin,[7] casein[8,9] and Gouda cheese[10] also inhibit ACE and each of these inhibitors has at least one Pro residue. Also, both Pro and Val residues were observed in many ACE inhibitory peptides contained in dried-salted fish,[11] cheese whey protein,[12] bovine skin gelatin[13] and sunflowers.[14]

Moreover, many ACE inhibitory peptides are derived from milk proteins.[15] A peptide of Leu-Arg-Pro-Val-Ala-Ala derived from lactoferrin (Lf) was found to have ACE inhibition in our previous study.[16] In this study, Leu-Arg-Pro-Val-Ala-Ala-Glu was synthesized from Lf and ACE inhibition of this peptide was measured. The present study is designed to know the role of Pro and Val residues in the ACE inhibition of Leu-Arg-Pro-Val-Ala-Ala-Glu; Pro was replaced with Ala (neutral amino acid), Glu (acidic amino acid) or Lys (basic amino acid) at the third amino acid resi-

* Corresponding author. Tel: +886-6-2372706; Fax: +886-6-2386548; E-mail: jtcheng@mail.ncku.edu.tw

due and Val was replaced by Ala, Glu or Lys at the fourth position. Then, the inhibitory activity was compared using in vitro assay of ACE activities.

EXPERIMENTAL

Materials

ACE (EC 3.4.15.1), hippuryl-histidyl-leucine and phthaldialdehyde (OPA) were purchased from Sigma Chemical Co., (St. Louis, MO, USA). Other reagents used were of analytical grade.

Synthetic peptides

The synthetic peptides for assay of ACE inhibition were purchased from Sigma-Genosys-Japan (Sapporo, Japan). Ten kinds of the chemically synthesized peptides were compared and the sequence of these peptides was described as follow: Leu-Arg-Pro-Val-Ala-Ala-Glu, Leu-Arg-Ala-Val-Ala-Ala-Glu, Leu-Arg-Glu-Val-Ala-Ala-Glu, Leu-Arg-Lys-Val-Ala-Ala-Glu, Leu-Arg-Pro-Ala-Ala-Ala-Glu, Leu-Arg-Pro-Glu-Ala-Ala-Glu, Leu-Arg-Pro-Lys-Ala-Ala-Glu, Leu-Arg-Pro-Val-Ala-Ala-Glu-Val, Leu-Arg-Pro-Val-Ala-Ala-Glu-Ile and Leu-Arg-Pro-Val-Ala-Ala.

The obtained rude peptides were further purified by RP-HPLC system, using a TSKgel ODS 80TM column (21.5 mm ID × 300 mm, a product of Tosoh, Japan) 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 (5 to 50% B) was applied to the column for 120 min at a flow rate of 4 mL/min and the absorbance of the eluate at 230 nm was monitored. Peptide was collected and the acetonitrile was removed using a rotary evaporator. The sample was then freeze-dried and the lyophilized powder was used to assay the ACE inhibition after the confirmation using TOF-MASS and HPLC.

Measurement of ACE inhibition

These peptides were assayed *in vitro* for the ability to inhibit ACE activity according to the method of the National Food Research Institute (Tsukuba city, Japan) with some modifications as mentioned in a previous report.[17] 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 with 50 μL of testing peptide or vehicle used to dissolve the testing peptide. 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 with 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 peptide 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 the absorbance of samples under the presence of inhibitor or testing peptide, B is the absorbance of blank and C is the absorbance of control. Peptide sample was tested at five concentrations to construct the standard curve for the determination of the IC_{50} value (concentration of inhibitor required to inhibit 50% of the ACE activity). Peptide sample was examined in triplicate. Relative activity of each peptide was calculated from IC_{50} value to show as the percentage of IC_{50} value (4.70 μM) of original peptide (Leu-Arg-Pro-Val-Ala-Ala-Glu).

Data analysis

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

RESULTS

Fig. 1 shows the dose-response curves of original peptide (Leu-Arg-Pro-Val-Ala-Ala-Glu) and one derivative (Leu-Arg-Pro-Val-Ala-Ala) for ACE inhibition. The IC_{50} value was obtained from each curve as indicated in Table 1. Leu-Arg-Pro-Val-Ala-Ala-Glu, the original peptide contained in Lf, showed an IC_{50} value of 4.70 μM. After replacement of Pro with Ala (neutral amino acid), Glu (acidic amino acid) or Lys (basic amino acid), the ACE inhibition

Table 1. Sequence of synthesized peptides and their ACE inhibition (IC_{50} value)

	Peptide	IC_{50} (μM)[a]	Relative Activity
1	L-R-P-V-A-A-E	4.70 ± 0.67	100
2	L-R-**A**-V-A-A-E	296 ± 55.2	1.59
3	L-R-**E**-V-A-A-E	527 ± 91.3	0.89
4	L-R-**K**-V-A-A-E	875 ± 153	0.54
5	L-R-P-**A**-A-A-E	5.27 ± 0.69	89.2
6	L-R-P-**E**-A-A-E	5.36 ± 0.75	87.7
7	L-R-P-**K**-A-A-E	26.1 ± 3.69	18.0
8	L-R-P-V-A-A-E-**V**	6.91 ± 0.88	68.0
9	L-R-P-V-A-A-E-**I**	6.99 ± 0.85	67.3
10	L-R-P-V-A-A	4.14 ± 0.43	114

[a] The concentration of each peptide required to inhibit 50% of ACE activity was indicated as IC_{50} value with mean ± SEM.

(IC_{50}) of these modified peptides was markedly decreased because the IC_{50} value was significantly raised as shown in Table 1. In these modified peptides, the amino acid residue for ACE inhibition was Pro > Ala > Glu > Lys. Similar results were observed in the replacement of Val at the 4th amino acid residues from the N-terminal; the Val was replaced with Ala, Glu or Lys, the IC_{50} value was markedly raised as shown in Table 1. Therefore, replacement with neutral amino acid (Ala) shows more efficacy in ACE inhibition than acidic amino acid (Glu) while replacement with basic amino acid (Lys) was the weakest. Otherwise, as shown in Table 1, addition of one amino acid into the original peptide to be Leu-Arg-Pro-Val-Ala-Ala-Glu-Val or Leu-Arg-Pro-Val-Ala-Ala-Glu-Ile showed a lower ACE inhibition than the original one (Leu-Arg-Pro-Val-Ala-Ala-Glu). But the ACE inhibitions produced by both peptides were without significance (Table 1). Also, deletion of the last peptide to be Leu-Arg-Pro-Val-Ala-Ala failed to produce a marked change of ACE inhibition as compared to the original one (Leu-Arg-Pro-Val-Ala-Ala-Glu) in Table 1.

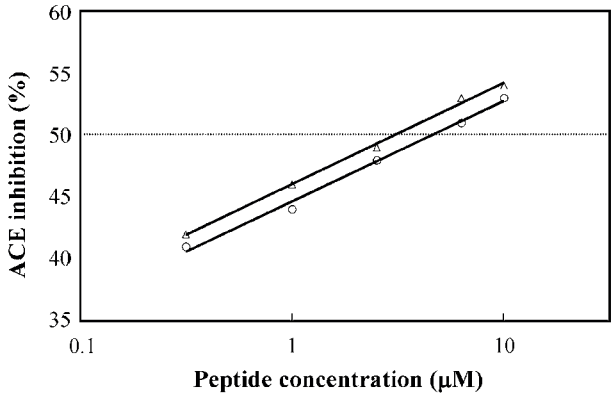

Fig. 1. The dose-response curve of peptides for ACE inhibition. The original peptide (o: LRPVAAE) and derivative (Δ: LRPVAA) was tested at five concentrations, respectively. The inhibition of ACE (%) was obtained as described in the Experimental section. No statistical difference ($p > 0.05$) was observed between the two peptides for ACE inhibition at each concentration ($n = 3$).

DISCUSSION

Actually, the ACE inhibition by Leu-Arg-Pro-Val-Ala-Ala-Glu at IC_{50} value was about 77% of that produced by 31 nM of captopril, the well-known ACE inhibitor.[18] A hypothetical model for the interaction between ACE and two peptides is shown in Fig. 2. In the structure-activity relationship, replacement of Leu-Arg-Pro-Val-Ala-Ala-Glu at Pro (the third amino acid residues from N-terminal) or Val (the fourth amino acid residues from the N-terminal) with Ala (neutral amino acid), Glu (acidic amino acid) or Lys (basic amino acid) showed the reduction of ACE inhibition as compared IC_{50} value with the replaced six peptides (Table 1). The requirement of Pro and Val residues in this

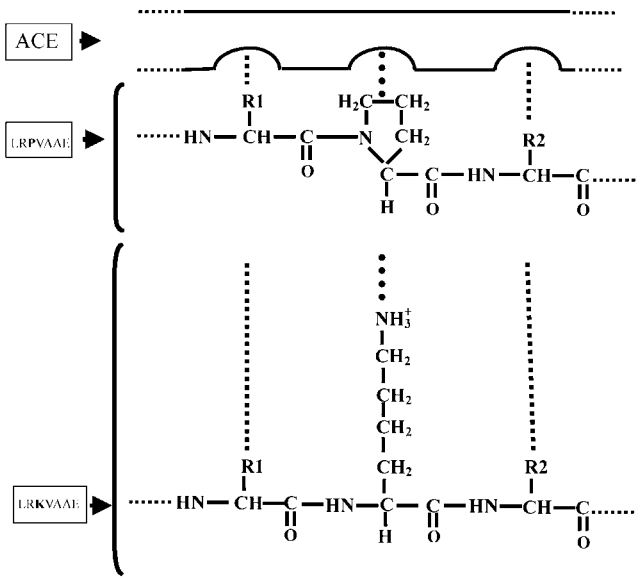

Fig. 2. Hypothetical model of the binding of peptide at the active site of ACE. The original peptide (LRPVAAE) or the less effective modified peptide (LRKVAAE) interacted with ACE, respectively.

peptide for ACE inhibition can thus be considered. Also, replacement with neutral amino acid (Ala) shows more efficacy in ACE inhibition than acidic amino acid (Glu) while replacement with basic amino acid (Lys) was the weakest.

Addition of one amino acid into the original peptide with Val or Ile beside it to Glu from the C-terminal was performed because the modified peptide was observed in different animal species; Leu-Arg-Pro-Val-Ala-Ala-Glu-Val in Lf of human beings was the same as that in horse while Leu-Arg-Pro-Val-Ala-Ala-Glu-Ile in Lf was observed in cow, water buffalo, camel, pig and goat. Both modified peptides show a higher IC_{50} value than the original one as indicated in Table 1. Thus, addition of amino acid into the original peptide reduced the ACE inhibition. Otherwise, deletion of the last amino acid residue at the C-terminal (Glu) to be Leu-Arg-Pro-Val-Ala-Ala failed to produce a marked change of ACE inhibition. Actually, this modified peptide (Leu-Arg-Pro-Val-Ala-Ala) was the same as the original peptide observed in animal species as mentioned above and it showed antihypertensive activity in spontaneously hypertensive rats as described in our previous study.[14] However, this structure-activity relationship for peptides to inhibit ACE in animals will be investigated in the future.

The obtained results suggest that Pro and Val residues are essential for the production of ACE inhibition. This is consistent with the previous report that Pro or aromatic amino acid residues existing at the C-terminal is suitable for a peptide binding to ACE.[19] However, the important role of Val residue at an internal position has not been mentioned and it needs more investigation in the future.

Received October 28, 2005.

REFERENCES

1. Yang, H. Y.; Erdos, E. G.; Levin, Y. *Biochim. Biophys. Acta.* **1970**, *214*, 374.
2. Erdos, E. G.; Skidgel, R. A. **1987**, *56*, 345.
3. Brown, N. J.; Vaughan, D. E. *Circulation* **1998**, *97*, 1411.
4. Raia, J. J.; Barone, J. A.; Byerly, W. G.; Lacy, C. R. *DICP* **1990**, *24*, 506.
5. Ferreira, S. H.; Bartelt, D. C.; Greene, L. J. *Biochemistry* **1970**, *9*, 2583.
6. Ondetti, M. A.; Williams, N. J.; Sabo, E. F.; Pluscec, J.; Weaver, E. R.; Kocy, O. *Biochemistry* **1971**, *10*, 4033.
7. Oshima, G.; Shimabukuro, H.; Nagasawa, K. *Biochim. Biophys. Acta*. **1979**, *566*, 128.
8. Maruyama, S.; Mitachi, S.; Tanaka, H.; Tomizuka, N.; Suzuki, H. *Agric. Biol. Chem*. **1987**, *51*, 1581.
9. Kohmura, M.; Nio, N.; Ariyoshi, Y. *Agric. Biol. Chem*. **1990**, *54*, 835.
10. Saito, T.; Nakamura, T.; Kitazawa, H.; Kawai, Y.; Itoh, T. *J. Dairy Sci*. **2000**, *83*, 1434.
11. Astawan, M.; Wahyuni, M.; Yasuhara, T.; Yamada, K.; Tadokoro, T.; Maekawa, A. *Biosci. Biotech. Biochem*. **1995**, *59*, 425.
12. Abubakar, A.; Saito, T.; Kitazawa, H.; Kawai, Y.; Itoh, T. *J. Dairy Sci*. **1998**, *81*, 3131.
13. Kim, S. K.; Byun, H. G.; Park, P. J.; Shahidi, F. *J. Agric. Food Chem*. **2001**, *49*, 2992.
14. Megias, C.; del Mar Yust, M.; Pedroche, J.; Lquari, H.; Giron-Calle, J.; Alaiz, M.; Millan, F.; Vioque, J. *J. Agric. Food Chem*. **2004**, *52*, 1928.
15. Yamamoto, N.; Takano, T. *Nahrung* **1999**, *43*, 159.
16. Lee, N. Y.; Cheng, J. T.; Enomoto, T.; Nakamura, I. *Chin. J. Physiol*, in press.
17. Cushman, D. W.; Cheung, H. S. *Biochem. Pharmacol*. **1971**, *20*, 1637.
18. Greene, A. S.; Amaral, S. L. *Curr. Hypertens. Rep*. **2002**, *4*, 56.
19. Cheung, H. S.; Wang, F. L.; Ondetti, M. A.; Sabo, E. F.; Cushman, D. W. *J. Biol. Chem*. **1980**, *255*, 401.