

Does the *cis/trans* configuration of peptide bonds in bioactive tripeptides play a role in ACE-I enzyme inhibition?

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Background: The milk casein-derived bioactive tripeptides isoleucine-proline-proline (IPP) and valine-proline-proline (VPP) have been shown to prevent development of hypertension in animal models and to lower blood pressure in moderately hypertensive subjects in most but not all clinical trials. Inhibition of angiotensin-converting enzyme 1 (ACE-1) has been suggested as the explanation for these antihypertensive and beneficial vascular effects. Previously, human umbilical vein endothelial cells (HUVEC) have not been used to test ACE-1 inhibiting properties of casein derived tripeptides in vasculature.

Purpose: We focused on the *cis/trans* configurations of the peptide bonds in proline-containing tripeptides in order to discover whether the different structural properties of these peptides influence their activity in ACE-1 inhibition. We hypothesized that the configuration of proline-containing peptides plays a significant role in enzyme inhibition.

Methods: AutoDock 4.2 docking software was used to predict suitable peptide bond configurations of the tripeptides. Besides modeling studies, we completed ACE-1 activity measurements in vitro using HUVEC cultures.

Results: In HUVEC cells, both IPP and VPP inhibited ACE-1. Based on molecular docking studies, we propose that in ACE-1 inhibition IPP and VPP share a similar *cis* configuration between the first aliphatic (isoleucine or valine) and the second (proline) amino acid residues and more different configurations between two proline residues. In vivo experiments are needed to validate the significance of the present findings.

Keywords: ACE inhibition, Autodock modeling, Ile-Pro-Pro, Val-Pro-Pro, vascular function

Introduction

The milk casein-derived tripeptides isoleucine-proline-proline (IPP) and valine-proline-proline (VPP), formed during fermentation or enzymatic hydrolysis, have been shown to have physiological effects not only on blood pressure-regulating systems in animal models¹ and in humans,² but also on other tissues such as eye and blood vessels.^{3,4} These tripeptides prevent development of hypertension in different animal models⁴⁻⁷ and lower blood pressure in mildly hypertensive humans.⁸⁻¹⁰ They also improve impaired endothelium-dependent vascular relaxation in hypertensive rats.^{2,11-13} Tripeptides inhibit angiotensin-converting enzyme 1 (ACE-1) in vitro;^{6,7,14} tripeptides also inhibit the activity of ACE-1 measured in plasma¹⁵ and serum¹⁶ after peptide feeding. ACE-1 is the main blood pressure-regulating enzyme in the renin-angiotensin system and is therefore a target for antihypertensive therapy. Tripeptides have also been studied with other blood pressure-affecting enzymes in addition to ACE-1³, including prolyl oligopeptidase and arginase-1.^{14,17}

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Interestingly, they preserve endothelial function in vitro¹¹ and potentiate on bradykinin and angiotensin-(1-7) [Ang-(1-7)] induced vasodilatation.¹⁸ Bradykinin and Ang-(1-7) are physiological vasodilators believed to have some interaction with each other via the ACE-2-Mas-receptor axis.^{19,20}

In this study our interest is vascular endothelial ACE-1. We suggest that the antihypertensive effects of the peptides are somehow related to their configuration in ACE-1 inhibition. Some controversies in clinical studies and distinctions between our findings and a few others^{4,5,11–14} led us to suggest that the peptides could exist not only in various conformations but also different configurations in experimental studies, which would explain the varying findings in clinical studies.^{8–10} Differences in the geometrical forms of active site residues and substrates influence binding affinity and specificity of enzyme comprehensively; additionally, because all enzymes are composed of chiral amino acids residues, it is logical that enzyme inhibition depends on their inhibitor's geometric isomers. Normally, while D/L and R/S stereoisomer configuration in the peptide inhibitors is discussed,^{21,22} *cis/trans* configuration isomers in the peptide chain (as shown in Figure 1) are usually neglected. All these suggestions may be relevant to the absorption of peptides that require selective transporters.

We concentrate herein on tripeptides – namely IPP and VPP – that consist of two prolines and one aliphatic residue. Proline residue has less conformational freedoms in proteins and peptide structures since its side chain is covalently bonded

to the main chain; there is thus the possibility of the proline peptide bond having a broader configuration distribution. The *cis/trans* isomers are with the same amino acid residue content, but in different peptide bond configurations. For most peptide bonds, the *trans* configuration is favored about 1,000 times more than the *cis* configuration. However, in cases where the peptide has a proline residue, the *trans* configuration is only about four times more dominant.²³

Various three-dimensional (3D) structures of the ACE-1 enzyme are currently available in the Protein Data Bank (PDB; <http://www.pdb.org>),²⁴ allowing for detailed knowledge to be obtained about the nature of the active sites and the interactions between inhibitors and enzymes. To elucidate the inhibition of ACE-1 by IPP and VPP tripeptides, we computationally docked the tripeptides in order to relate their configuration to the inhibition of ACE-1. AutoDock docking software (version 4.2; Scripps Research Institute, Jupiter, FL, USA) was used to predict suitable peptide chain configurations of the tripeptides. Here we would like to report the relationship between peptide bond *cis/trans* configuration and the inhibition of ACE-1.

Materials and methods

Preparation of ACE-I enzyme and tripeptide structures for the docking procedure

All molecular dockings were performed with the AutoDock 4.2 program package from AutoDock Tools.²⁵ The 3D structure of the target molecules (enzyme) as well as the ligands (tripeptides) must be known to begin the analysis. The PDB, as of March 2012, was queried for human ACE-1 enzyme structures. The total number was five and it consists of four structures with an inhibitor and only one without the inhibitor. To avoid binding bias, an important criterion was that the starting model was an X-ray structure without any ligand. The initial coordinates for the docking analyses were obtained from the ACE-1 enzyme EC 3.4.15.1 (PDB ID 1O8A), determined to 2.0 Å resolution.²⁶ Generally, a resolution of approximately 2 Å provides an accurate structure, and accordingly, such a structure is suitable for docking. To obtain the initial coordinates for the tripeptides, they were constructed using DeepView – Swiss-PdbViewer (Swiss Institute of Bioinformatics, Lausanne, Switzerland).²⁷ The tripeptide models solvated in TIP3P water and were subjected to conjugate gradient energy minimization calculations using the Amber ff99SB force field in NAMD 2.6 (University of Illinois, Urbana and Champaign, IL, USA); the first 4,000 steps were completed with protein atoms immobilized,

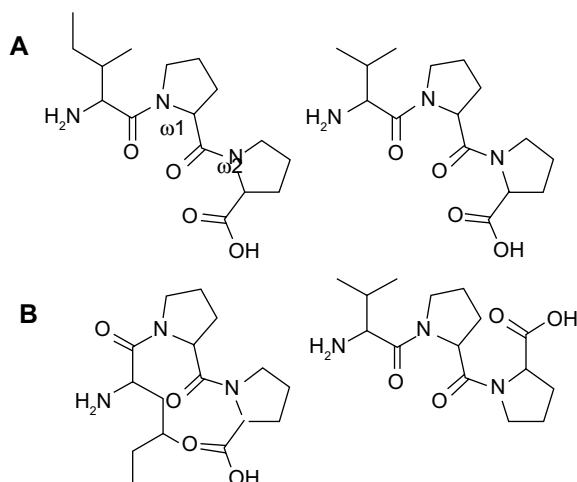


Figure 1 Different *cis/trans* configurations of the peptide bond.

Notes: (A) Peptide bonds values (ω_1 and ω_2) in IPP and VPP are 180° and they are both in *trans* configuration. (B) The first peptide bond (ω_1) in IPP has 0° and the second (ω_2) has 180° values and they are in *cis* and *trans* configuration, respectively. The first peptide bond (ω_1) in VPP has 180° and the second (ω_2) has 0° values and they are in *trans* and *cis* configuration, respectively.

Abbreviations: IPP, isoleucine-proline-proline; VPP, valine-proline-proline.

followed by 8,000 steps with all atoms released.^{28–30} Only energy-minimized structures of IPP are shown in Figure 2.

Autodock

For the docking, ACE-1 enzyme and all tripeptide structures were dissolved. Gasteiger charges and hydrogens for structures were added. The docking grid was prepared with a grid box created using the maximum points with spacing of 0.375 Å. In accordance with the Catalytic Site Atlas database,³¹ active site residues of ACE-1, His353, Glu384, Lys511, His513, Tyr520, and Tyr523 were selected as flexible residues. These side chains were permitted to be flexible over the docking calculations. All of the torsional bonds in each tripeptide were allowed to be free. Docking procedures included 10 independent runs. Module setting parameters were as follows: a population size of 150; a maximum number of 2.5 million energy evaluations; a maximum of 27,000 generations, a crossover rate of 0.8; and a number rate of 0.02. Based on a root mean square deviation criterion of 1.0 Å, the docking results were clustered.

Human umbilical vein endothelial cell (HUVEC) culture

HUVEC cells are a commonly used cell line in cardiovascular research.^{32,33} Commercial HUVEC (Lonza Inc.,

Walkersville, MD, USA) were cultured in endothelial cell basal medium (Lonza) containing 10% fetal bovine serum, 0.1% recombinant human epidermal growth factor, 0.1% gentamicin sulfate (GA-1000), 0.1% hydrocortisone, and 0.4% bovine brain extract. Cells were cultivated at 37°C with 5% CO₂, and a fresh medium was provided every 1–2 days. Cells used in the experiments were from the passage 6. At the end of the experiments, mycoplasma tests (MycoAlert Mycoplasma Detection Kit; Lonza Inc.) were performed to exclude possible infections of the cells. All mycoplasma tests were negative (data not shown).

ACE-I activity assay

ACE-1 activity was measured using the method devised by Santos et al.³⁴ The assay was based on two reactions: in the first reaction, N-hippuryl-histidine-leucine (HHL) (Sigma-Aldrich, St Louis, MO, USA) was degraded into histidine-leucine (HL) (Sigma-Aldrich) by ACE-1, and in the second reaction HL and ortho-phthaldialdehyde (OPA) (Sigma-Aldrich) formed under the alkaline conditions of a fluorescent complex, which was measured at excitation/emission = 365/495 nm. The assay buffer contained 0.1 M sodium borate and 0.4 M sodium chloride (pH 8.3). HHL (10 µM) and HL (0.05–15 µM) were diluted in the assay buffer. Pure HL was used as the standard. OPA (2%) was diluted in methanol. ACE-1 activity was measured from cell lysate using tripeptide (H-4632/H-4634; Bachem, Bubendorf, Switzerland) concentrations 0.1, 1.0, and 3.3 µM. Purity of tripeptides was >98%, but they were a mixture of *cis/trans* configurations. The reaction was stopped using acid after a 60 minute-long incubation and centrifuged at 3,000 rpm for 10 minutes. Fluorescence was measured using a fluorescence spectrophotometer (Varian Cary Eclipse; Agilent Technologies, Santa Clara, CA, USA) from the supernatant shortly after centrifugation. Captopril (1 nM) was used as a positive control for ACE-1 inhibition.^{35,36} All concentrations reported were the final concentrations in the reaction.

Statistics

The results from enzyme activity measurements are presented as mean ± standard error of mean (SEM), (n=2–4 each concentration from duplicate measurements). Statistical analyses were performed using GraphPad Prism software (version 4.02; GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was used to compare the results. A difference was considered significant if $P < 0.05$.

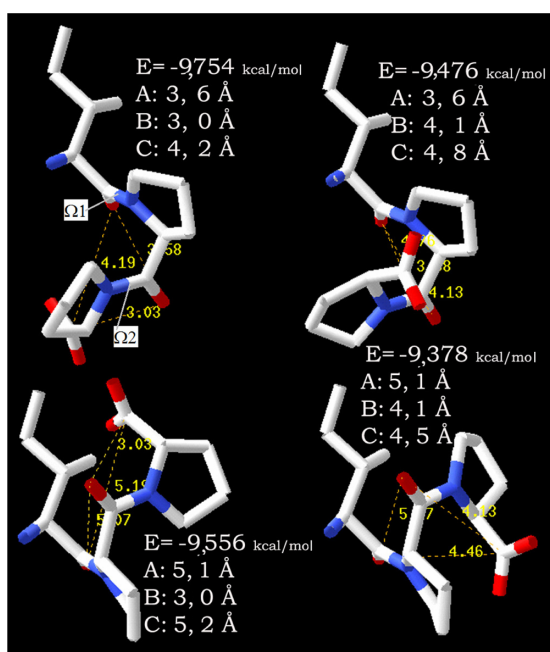


Figure 2 Different *cis/trans* configurations of IPP.

Notes: Different *cis/trans* configurations of the isoleucine-proline-proline are shown in global energy minimum structures. The shortest distances between carbonyl oxygens are provided. Blue color indicates nitrogen and red color oxygen atoms. Different peptide bond configurations of IPP are sorted by energy, from smallest to largest.

Abbreviation: IPP, isoleucine-proline-proline.

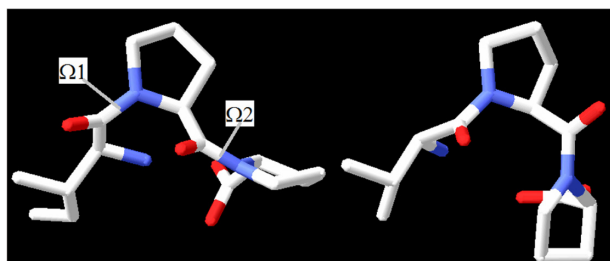


Figure 3 Geometric isomers and omega values IPP and VPP

Notes: Geometric isomers and omega values for IPP were $\omega_1 = -42$ and $\omega_2 = -7$, respectively. Geometric isomers and omega values for VPP were $\omega_1 = -99$ and $\omega_2 = 84$ for VPP, respectively. These values were as predicted in the angiotensin-converting enzyme I.

Abbreviations: IPP, isoleucine-proline-proline; VPP, valine-proline-proline.

Result and discussion

Computed geometric models

Outside a certain enzyme, the tripeptide whirls freely in aqueous solution through rotation of the $N-C_\alpha$ (ϕ , ϕ) and $C_\alpha-C'$ (ψ , ψ) bonds. Since freedom of rotation in the active site becomes restricted through formation of the inhibitor-enzyme complex, these rotations to a large extent become frozen. Peptide inhibitors generally bind enzymes in a single well-defined structure and appropriate interactions. All isomers –including *cis/trans* configuration isomers – get to the energy equilibrium ratio that is dependent on the conditions. Usually, this local structure contains the correct orientations for interactions and is nearly a global minimum energy structure. The interconversion between isomers is a slow process because structure rearrangement in inhibitors requires energy-owing peptide bonds to break,^{37,38} as well as new organizing of enzyme active site residues. According to the Autodock predictions, the primary structures of IPP and VPP are quite different in the active site of the ACE-I enzyme. Their different geometry orientations of carbonyl

oxygens are shown in Figure 3. Carbonyl oxygens are the most potent hydrogen bond acceptors in the common peptide bonds. IPP and VPP share a similar *cis* configuration at the omega bond (ω_1) between the first aliphatic (isoleucine or valine) and the second (proline) amino acid residues and other different configurations between two proline residues. This indicates that particular attention should be paid to this point in the future.

Experimental inhibition models

While in the X-Pro type main chain the peptide bond is more likely to be in the *cis* configuration than in the other type, the actual percentage amount of distribution between *cis/trans* configurations can vary. Additionally, clinical studies and in vitro experiments with somewhat contradictory results raise the possibility that different production procedures might finalize compounds of varying geometric isomers.^{39–41} Therefore, we suggest that the bacterial fermentation or enzymatic treatment of milk casein might produce divergent tripeptides which have different structural distribution (eg, *cis/trans* peptide configuration). The main point is that configuration modifications clearly need more energy than conformation changes.³⁸ A previous study by Counterman and Clemmer⁴² shows that in tryptic-digested peptides less than ten amino acid residues have different *cis/trans* configurations; this study supports our hypothesis.

It has been evident for some time that enzymatic breakdown or bacterial fermentation produces small peptides from milk casein; peptides possess many biological activities⁴³ and are marketed as functional foods in some countries (eg, Japan). We used IPP and VPP for inhibition of ACE-I dose-dependently (Figure 4), and half maximal inhibitory

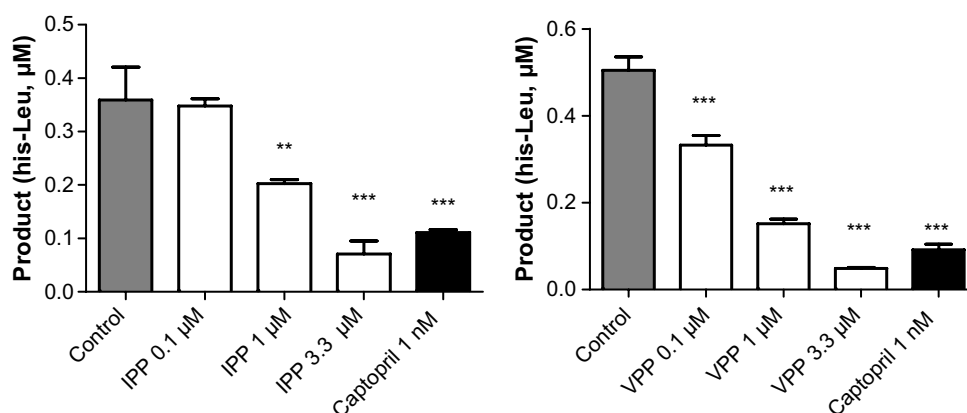


Figure 4 Angiotensin-converting enzyme I activity from HUVEC lysate.

Notes: Angiotensin-converting enzyme I activity from HUVEC lysate incubated for 60 minutes with/without IPP or VPP peptides. Captopril was used as a reference inhibitor. Mean \pm SEM, $n=2-4$, from duplicate measurements. ** $P<0.01$; *** $P<0.001$ versus control.

Abbreviations: HUVEC, human umbilical vein endothelial cells; IPP, isoleucine-proline-proline; VPP, valine-proline-proline; SEM, standard error of mean.

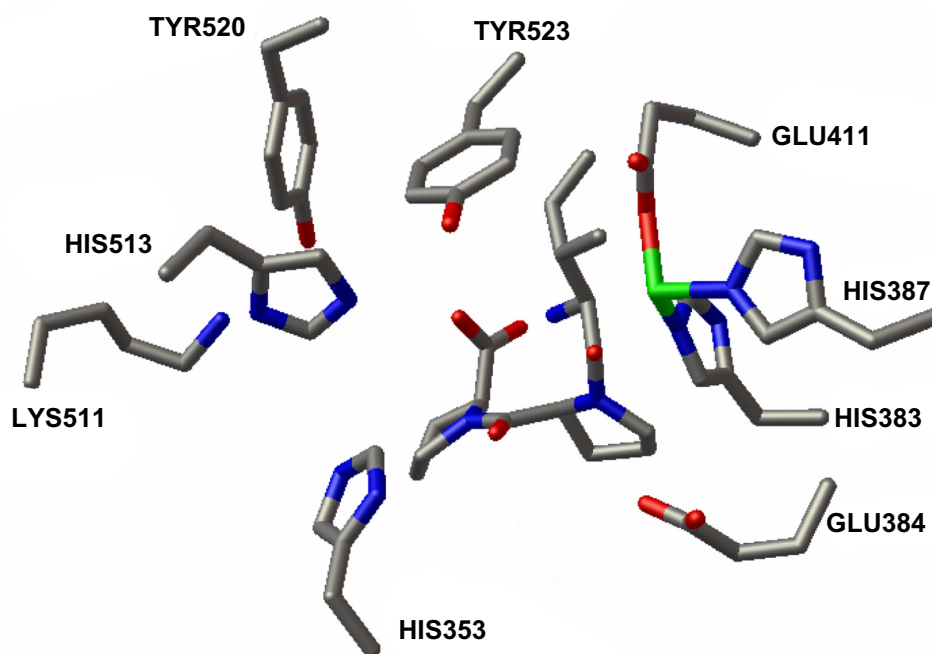


Figure 5 Tripeptide IPP in active site of ACE-1.

Notes: Tripeptide IPP with omega values of $\omega_1 = -42$ and $\omega_2 = -7$ in active site of ACE-1. Autodock flexible residues are labeled, as are zinc (green) and its two histidine (383, 387) and glutamine (384) ligand residues.

Abbreviations: IPP, isoleucine-proline-proline; ACE-1, angiotensin-converting enzyme 1.

concentration (IC_{50}) for IPP and VPP were $1.89 \pm 0.28 \mu M$ and $0.48 \pm 0.38 \mu M$, respectively. The highest IPP and VPP concentration applied ($3.3 \mu M$) induced similar ACE-1 inhibition to captopril ($1 nM$). These results are compatible with our own previous results and those of another study by Foltz et al.⁴⁴ It was also proved that there is a direct link between dipeptide stability and ACE-1 inhibition, as modeled by the quantitative structure–activity relationship method.⁴⁵

We have previously tested tripeptides produced by two different manufacturing processes using a hypertensive rat model. Unfortunately, the peptide powders were only half from bacterial fermentation and split by proline specific endoprotease. Thus the real significance of processing procedures remained unsolved.⁴⁶ Molecular structure aspects are not the only important factor in the evaluation of inhibition of geometric isomers of tripeptides. Pharmacokinetics may also be affected; the absorption, metabolism, and clearance of the peptides may be modified by isomerization. Thus, the individual isomers of the tripeptide may be metabolized at different rates and transformed to another isomer entity. Furthermore, to become a functional food product, it may be necessary to both improve the structural properties of a peptide-like product and to search for optimal additives for a special peptide; improvements in peptide solubility, chemical stability in biological matrices, or metabolic stability will be the next subject of experiments.

Conclusion

Our hypothesis that *cis/trans* configuration variability in peptide bonding may play a significant role in enzyme inhibition was tested by computing molecular modeling and by experimental work with two casein-derived tripeptides (IPP, VPP). Based on our Autodock data, we conclude that the *cis* configuration of the first peptide bond on the IPP and VPP tripeptides is a more potent geometric form for ACE-1 inhibition. Individual configuration of the peptides produced in different ways should be clarified before drawing strict conclusions from in vitro and in vivo experiments. Small peptides may, under appropriate conditions, fold to different geometric forms; therefore, it would be valuable to control the main chain geometry during the peptide manufacturing process. Additionally, the interactions of *cis/trans* configurations can in general be assumed to be geometrically driven at the active site. This means that one configuration binds with higher affinity than the other upon interacting with an enzyme (Figure 5).

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Disclosure

The authors report no conflicts of interest in this work.

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