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ACE-Inhibitory Activity and Structural Properties of Peptide Asp-Lys-Ile-His-Pro [β-CN f(47−51)]. Study of the Peptide Forms Synthesized by Different Methods

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Some of the most potent ACE-inhibitory peptides described in food have a proline at the end of their sequence, a characteristic that can cause problems in the synthesis procedures. In this work, we studied two different preparations of peptide Asp-Lys-Ile-His-Pro (DKIHP), which were obtained by two different synthetic procedures (Boc and Fmoc). The peptide synthesized by the Boc method yielded a unique conformer, containing *trans*-Pro, and significant ACE-inhibitory activity (IC_{50} = 113.18 μM). The chromatographic and NMR data of this active conformer are reported. The peptide synthesized by Fmoc chemistry yielded three conformers, two of them containing *trans*-Pro and a third one containing *cis*-Pro, and showed a lower activity (IC_{50} = 577.92 μM). This was attributed to the presence of conformers with less (or none) activity. We have pointed out the importance of performing structural studies on these type of peptides before testing their ACE-inhibitory activity.

KEYWORDS: ACE-inhibitory peptides; NMR; structure; proline; synthesis

INTRODUCTION

Angiotensin-I-converting enzyme (ACE; EC 3.4.15.1) is a peptidyl-dipeptidase that cleaves dipeptides from the C-terminal end of the substrate and plays an important role in blood pressure regulation. This enzyme converts angiotensin-I to angiotensin-II, a strong vasopressor, and, together with the inactivation of bradykinin, which has hypotensive activity, its action results in an increase of blood pressure (*1*). Therefore, inhibition of ACE is considered a useful therapeutic approach in the treatment of high blood pressure, and ACE-inhibitory drugs, such as captopril, enalapril, or lisinopril, are used as antihypertensive drugs (*2*).

In past years, the food industry has become very interested in the so-called functional foods, that promote individuals' health, and researchers have shown an increased interest in finding functional components in foods. Much research has been done related to bioactive peptides, and some of these studies have been focused on ACE-inhibitory peptides. Oshima et al. (*3*) isolated the first ACE-inhibitory peptide described in food protein. Since then, other peptides with this activity have been found in different foods (*4*−*8*).

Structure and activity studies done with different ACE-inhibitory peptides have indicated that binding to ACE is strongly influenced by the C-terminal tripeptide sequence of the substrate (*9*). Besides, the presence of a proline at the C-terminal end improves ACE-inhibitory activity, probably due to the rigid structure of this residue, which may lock the carboxyl group into a conformation favorable for interacting with the positively charged residue at the active site of the enzyme (*10*). Recent X-ray diffraction studies have given detailed information on the structure of ACE from human sperm and *Drosophila*, as well as the complex formed between the enzyme and its inhibitors lisinopril and captopril (*11*−*13*).

In a previous work, different fractions with strong ACE-inhibitory activity were obtained from Manchego cheese (*7*), a cheese manufactured from ewe's milk. In one of the most active fractions, three peptides were identified. Among these, the peptide DKIHP [β-CN f(47−51)] was a strong candidate for having ACE-inhibitory activity, because it contains a C-terminal proline and a positively charged amino acid within the three C-terminal positions, characteristics of potent ACE-inhibitory peptides (*14*, *15*). To confirm the ACE-inhibitory activity of this peptide, it was necessary to synthesize it. However, it has to be taken into account that peptides containing proline (particularly those containing proline at the C-terminal end) can cause problems during synthesis, resulting in multiple forms, a well-known problem for researchers working in the peptide synthesis field. In some cases, it has not been possible to synthesize some bioactive peptides found in food proteins due to the presence of proline in their structure (*16*).

In this study, the peptide DKIHP was tested for ACE-inhibitory activity and was subjected to structural characterization by MS/MS and NMR. In addition, two preparations of the peptide, synthesized by different methods (using Boc and Fmoc chemistry), were compared for activity and structure.

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MATERIALS AND METHODS

Synthesis of DKIHP. *Method I.* Synthesis was performed at the Peptide Synthesis Unit (University of Barcelona), using Boc chemistry and a manual method. This method included the activation of Boc-aa-OH with TBTU/HOBt/DIEA. This peptide preparation will be referred to as DKIHP-I.

Method II. Synthesis was performed at the protein facility of the Centro de Biología Molecular (CBM, CSIC, Cantoblanco, Madrid), using Fmoc chemistry, with a 431A peptide synthesizer (Applied Biosystems Inc., Überlingen, Germany). Fmoc amino acids were activated with a mixture of HOBt/NMP and DCC/NMP. For histidine, a Fmoc-His(Trt)-OH derivative was used. This peptide preparation will be referred to as DKIHP-II.

RP-HPLC-MS/MS. RP-HPLC analyses were performed on an Agilent HPLC system (Agilent Technologies, Waldbronn, Germany) connected on line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). The HPLC system was equipped with a quaternary gradient pumping system, an in-line degasser, a variable wavelength absorbance detector set at 214 nm, and an automatic injector (all 1100 Series, Agilent Technologies). The column used in these experiments was a 250 mm × 4.6 mm Widepore C_{18} column (Bio-Rad, Richmond, CA). The injection volume was 50 μL.

Solvent A was a mixture of water:TFA (1000:0.37), and B was a mixture of acetonitrile:TFA (1000:0.27). A step gradient system of water and acetonitrile was used as mobile phase: solvent A (100%) for 5 min, then a linear gradient to 10% B in 10 min, from 10% to 30% B in 10 min, and from 30% to 70% B in 5 min. The flow (0.8 mL/min) was split post detector by placing a T-piece (Valco, Houston, TX) connected with a 75 μm ID peek outlet tube of an adjusted length to give approximately 20 μL/min of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulizing and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (*m*/*z*) range 100−1000. About 25 spectra were averaged in the MS analyses, and about 5 spectra were averaged in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 10 000 (i.e., 5% of the total signal), and the precursors ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using Data AnalysisTM (version 3.0; Bruker Daltoniks), the *m*/*z* spectral data were processed and transformed to spectra representing mass values.

NMR Analysis. The peptide was dissolved in D_2O or H_2O:D_2O (80:20) at a concentration of 1−10 mg/mL, and the pH was adjusted to pH 2.5 with DCl. ^{1}H NMR spectra were recorded in a 400 MHz Varian ^{UNITY}INOVA spectrometer (Varian NMR Instruments, Palo Alto, CA). One-dimensional spectra were recorded with 3.7 s acquisition time, 1 s delay time, and water was suppressed using presaturation for 2.5 s. COSY, TOCSY (80 ms mixing time), and NOESY (700 ms mixing time) spectra were obtained with 2048 points in the t_2 dimension, 256 scans in the t_1 dimension, and at least 64 transients. A delay time of 1 s was applied between scans, and the water line was suppressed using presaturation (1.5−2.5 s presaturation time). All spectra were taken at 30 °C, unless stated. Raw data were processed using Varian software.

Determination of ACE-Inhibitory Activity. ACE-inhibitory activity was assayed by the method of Cushman and Cheung (*17*) with some modifications (*7*). Each 20 μL of peptide inhibitor solution was incubated, at 37 °C for 30 min, with 180 μL of 0.1 M potassium phosphate buffer (pH 8.3), containing 5 mM hippuryl-histidyl-leucine and 0.3 M NaCl, and with 10 μL of ACE (5 mU). The reaction was finished by adding 100 μL of 1 M HCl. The hippuric acid formed by the action of ACE was extracted with ethyl acetate, and after removal of ethyl acetate by heat evaporation, the amount of hippuric acid was measured spectrophotometrically at 228 nm. The inhibitory activity was expressed as the amount of peptide needed to inhibit 50% the ACE activity (IC_{50}). The activity was tested in triplicate to five different concentrations.

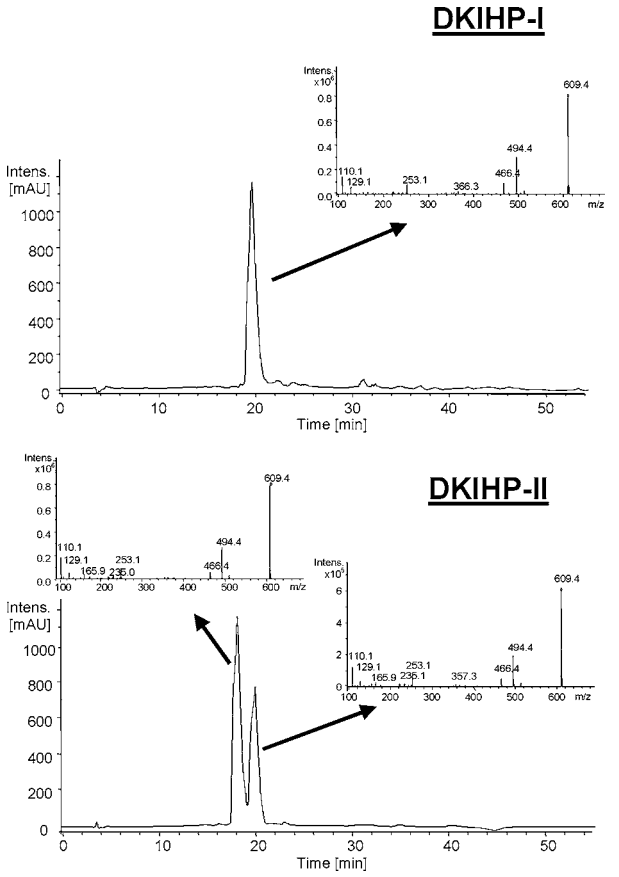

Figure 1. HPLC-MS profiles of DKIHP-I (synthesized by Boc chemistry) and DKIHP-II (synthesized by Fmoc chemistry). HPLC-MS profiles show a single peak for DKIHP-I, and a double peak for DKIHP-II. All of them show a very similar MS pattern.

RESULTS

Characteristics of DKIHP-I. When the peptide was analyzed by HPLC-MS, one single peak was found with *m*/*z* of 609.4 (**Figure 1**). MS/MS analysis confirmed the amino acid sequence of the synthesized peptide (data not shown).

One-dimensional and TOCSY spectra showed a single structural form (**Figure 2**), with chemical shifts and coupling constants as shown in **Table 1**. Results from NOESY spectra (**Figure 2**) showed some interresidue resonances (i.e., H_α(His)−H_δ(Pro)) that were assigned to *trans*-Pro.

Characteristics of DKIHP-II. When this peptide was analyzed by HPLC-MS, two separated peaks, with relative UV-areas around 3:2, were found (**Figure 1**). Both peaks had a *m*/*z* of 609.4, and, upon fragmentation by MS/MS, they showed the same fragmentation profile, equal to the fragmentation pattern found for DKIHP-I. Thus, it was concluded that the two peaks found in this preparation corresponded to the same peptide, probably with different conformations.

NMR analysis of this peptide showed different resonances than those found for DKIHP-I, both in the one-dimensional and TOCSY spectra (**Figure 2**). In the TOCSY spectra, it is clearly observed that the first two amino acids (Asp^1-Lys^2) showed single resonances, similar to those found in DKIHP-I. However, three different signal sets were found for the last three residues (Ile^3-His^4-Pro^5), as compared with the single set found for DKIHP-I (**Figure 2**). This led us to conclude that DKIHP-II contained three structural forms of the peptide, with differences occurring around the three last residues, and probably involving *cis*−*trans*-proline isomerization. NOESY spectra revealed cross-

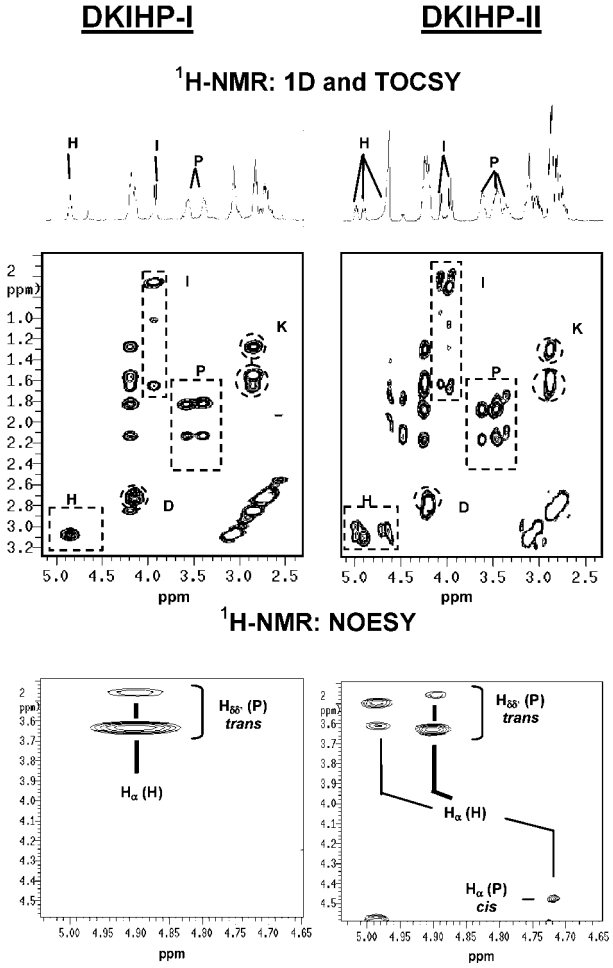

Figure 2. NMR spectra of DKIHP-I (synthesized by Boc chemistry) and DKIHP-II (synthesized by Fmoc chemistry). One-dimensional (1D) and TOCSY spectra show, for DKIHP-I, single resonances for all residues. The same spectra show, for DKIHP-II, single resonances for D and K and multiple resonances for I, H, and P, as a consequence of the presence of different conformers. NOESY spectra show, for DKIHP-I, a single set of resonances, characteristic of *trans*-Pro and, for DKIHP-II, three sets of resonances, assigned to one *cis*-Pro and two *trans*-Pro forms. The thick line represents the resonances belonging to the most active form of the peptide. Spectra were obtained from a D_2O solution, at acidic pH and 30 °C.

peaks characteristic of both *trans*-Pro (i.e., H_α(His)−H_δ(Pro)) and *cis*-Pro (i.e., H_α(His)−H_α(Pro)). Two of the structural forms contained *trans*-Pro, while the third one was a conformer with *cis*-Pro (**Figure 2**). The three forms were almost equally populated (1:1:0.8) and also very stable, as changes in temperature and pH effects were small (**Figure 3**). The presence of two different *trans*-Pro forms indicated the presence of at least one conformer different from the *cis*−*trans*-Pro isomers. We could not determine the structural differences between the two forms that carried *trans*-Pro, but some coupling constants associated to His[4] and Ile[3] differed between the two *trans*-Pro forms. One of the *trans*-Pro forms in DKIHP-II had similar NMR parameters to DKIHP-I, thus indicating the same structure. The other *trans*-Pro form differed from it, showing larger coupling constants ($^3J_{NH-H\alpha}$ = 8.5 Hz for Ile[3], and $^3J_{H\alpha-H\beta}$ = 9 Hz for His[4]), when compared to that of DKIHP-I (**Table 1**).

ACE-Inhibitory Activity. The ACE-inhibitory activity was checked in DKIHP-I and DKIHP-II. The reciprocal of inhibition activity was plotted against the reciprocal of the peptide concentration, and a linear regression was used to determine the IC_{50} value. DKIHP-I, containing only one conformer, showed an IC_{50} value of 113.18 μM. In contrast, DKIHP-II, containing three conformers, presented an IC_{50} value of 577.92 μM, about one-fifth the inhibitory activity found for the peptide DKIHP-I. This represents a poor activity as compared not only to DKIHP-I, but also to other peptides that have a proline at the C-terminal end found in food (*18*).

Table 1. ¹H NMR Chemical Shift Assignments and Coupling Constants of the Active Form of the Synthetic Peptide DKIHP (i.e., DKIHP-I), at pH 2.5, 30 °C (NH_{bb}, Backbone NH; NA, Not Applicable (N-Terminal End or Imino Acid))

	¹H chemical shifts, δ				
residue	NH_{bb}	H_α	$H_{\beta\beta'}$	H_{other}	coupling constant
Asp[1] (D)	NA	4.20	2.78		
Lys[2] (K)	8.53	4.23	1.67	$H_{\gamma\gamma'}$: 1.26	
				$H_{\delta\delta'}$: 1.53	
				$H_{\epsilon\epsilon'}$: 2.88	
Ile[3] (I)	8.03	3.97	1.69	$H_{\gamma\gamma'}$: 1.28	$^3J_{NH-H\alpha}$: 7 Hz
				1.04	
				$H_{\gamma/\delta(CH3)}$: 0.73	
His[4] (H)	8.23	4.90	3.14	ring H_2: 7.22	$^3J_{NH-H\alpha}$: 8 Hz
			3.09	ring H_4: 8.48	$^3J_{H\alpha-H\beta}$: 7 Hz
Pro[5] (P)	NA	4.25	2.18	$H_{\gamma\gamma'}$: 1.88	
				$H_{\delta\delta'}$: 3.62	
				3.46	

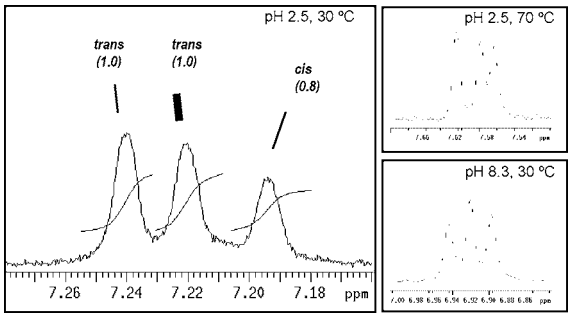

Figure 3. Histidine ring H_2 spectral region of DKIHP-II (synthesized by Fmoc chemistry), showing three resolved resonances for H_2, assigned to the *cis*-Pro and the two *trans*-Pro forms of the peptide, stable at different pH's and temperatures. ¹H NMR spectra taken at: pH 2.5, 30 °C; pH 2.5, 70 °C; and pH 8.3, 30 °C. The thick line represents the most active form of the peptide. Numbers in brackets show the relative areas.

DISCUSSION

In this study, it was shown that DKIHP, a fragment of β-casein found in Manchego cheese (*7*), has ACE-inhibiting activity. In addition, the *trans*-Pro conformer that constituted the only form found in DKIHP-I showed a significant activity, which demonstrates that this structural form of the peptide is active. This supports previous research that has pointed out that peptides containing *trans*-Pro were better substrates for ACE than those carrying *cis*-Pro (*19*, *20*). The chromatographic and NMR results of this active form of DKIHP are described as reference data for further research.

On the other hand, DKIHP-II showed smaller ACE-inhibitory activity, which suggests that, among the three structural forms found, there must be less active or inactive conformers. Among the three conformers, one of them showed characteristics identical to those of DKIHP-I, and constituted about one-third of the total amount of peptide, which suggests that the DKIHP-I conformer might be responsible for the overall activity found in this preparation, the two other conformers found in DKIHP-

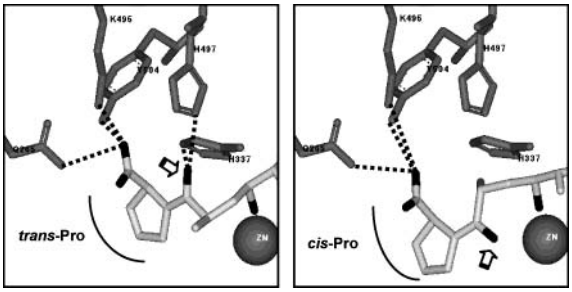

Figure 4. Models showing the interactions between a peptide inhibitor, with *trans*- and *cis*-proline at the C-terminal end (light gray), and the active site of the angiotensin-I-converting enzyme (dark gray). Model modified from the crystal coordinates of *Drosophila* angiotensin-I-converting enzyme bound to lisinopril (Protein Data Bank, PDB code 1J36) (*11*), modeled with DS Viewer Pro 5.0 (Accelrys). H-bonds are labeled with dashed lines. Carboxyl groups (CO and COO^-) are labeled in black. The change of *trans*-Pro to *cis*-Pro results in the displacement of the CO group of the previous residue of the inhibitor (labeled with an arrow) to the opposite side of the peptide chain, losing its original H-bonding with the enzyme.

II probably being inactive forms. Some authors have reported the presence of multiple peaks in RP-HPLC upon running dipeptides containing L-Pro (*21*, *22*). Enalapril, a potent ACE inhibitor, that contains a C-terminal proline, has shown multiple signal sets using HPLC, NMR, and CE (*23*, *24*), and these signals were assigned to *cis*−*trans*-Pro isomer forms. However, DKIHP-II showed the presence of other conformers different from the classical *cis*−*trans*-Pro forms, likely to be enantiomers. It has to be pointed out that the procedure chosen for peptide synthesis was a determining factor in obtaining the right conformation of DKIHP.

Kim et al. (*11*) have modeled the structure of the *Drosophila* ACE active site bound to lisinopril, an inhibitor that contains a −KP dipeptide at the C-terminal end of the molecule. These authors have suggested that residues K495, Q265, and Y504 of the enzyme interact with the COO^- of the proline end group of lisinopril while residues H497 and H337 interact with the CO group of the previous residue (lysine). The change of a *trans*- to a *cis*-form of proline can cause significant changes in the inhibitor structure and its interactions with the active site (**Figure 4**). The carboxyl groups of both the *trans*-Pro and the previous residue lie on the same side of the peptide chain. However, if proline is in the *cis*-configuration, these two groups are forced to move to opposite sides of the chain. This could lead to the loss of interactions with the active site and, in consequence, to a decreased (if any) binding to the enzyme and inhibitory activity.

Many ACE-inhibitory peptides have a proline in their sequence and are studied by using synthetic peptides. Sometimes, two peptides with an identical sequence have shown very different ACE-inhibitory activity. For instance, the proline-containing peptide AVPYPQR showed values as different as 15 and 274 μM depending on whether the ACE-inhibitory activity was checked by Karaki et al. (*25*) or by Pihlanto-Leppäla et al. (*26*), respectively. In addition, some purified peptides identified in several fractions from Manchego cheese with potent ACE-inhibitory activity (*7*), or peptides produced in fermented milk (*27*), have shown very poor activity when they were synthesized. These differences could arise from the presence of less active conformers in the synthetic preparations. The purity and sequence of peptides are usually checked by HPLC-MS or HPLC-MS/MS, methods that are not designed to separate conformers. Therefore, it would be useful to include an analytical technique, such as NMR, able to detect conformational changes. Moreover, it has to be considered that those synthetic peptides showing strong in vitro ACE-inhibitory activity are further tested in vivo. For this purpose, it is important to ensure the right conformation of the peptide before the assays are carried on.

ABBREVIATIONS USED

ACE, angiotensin-converting enzyme; β-CN, β-casein; Boc, *tert*-butyloxycarbonyl; Fmoc, fluoren-9-ylmethoxycarbonyl; MS, mass spectrometry; NMR, nuclear magnetic resonance; COSY, correlation spectroscopy; NOESY, nuclear overhauser enhancement spectroscopy; RP-HPLC, reversed phase high performance liquid chromatography; TFA, trifluoracetic acid; IC_{50}, inhibitor concentration for 50% enzyme inactivation; UV, ultraviolet.

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