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# Three novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) using digestive proteases

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ABSTRACT

The angiotensin I-converting enzyme (ACE) inhibitory activities of protein hydrolysates prepared from muscle of cuttlefish (*Sepia officinalis*) by treatment with various digestive proteases were investigated. The most active hydrolysate was obtained with the crude protease extract from the hepatopancreas of cuttlefish (64.47 ± 1.0% at 2 mg of dry weight/ml) with a degree of hydrolysis of 8%. By gel filtration on Sephadex G-25 and RP-HPLC on C18 column, three novel peptides with high ACE-inhibitory activity were purified and their molecular masses and amino acid sequences were determined. The three peptides Val-Tyr-Ala-Pro, Val-Ile-Ile-Phe and Met-Ala-Trp with $IC_{50}$ values of 6.1, 8.7 and 16.32 μM, respectively, were novel ACE-inhibitory peptides. Lineweaver–Burk plots suggest that the three purified peptides act as non-competitive inhibitors against ACE. These results suggest that some peptides from cuttlefish could be a beneficial ingredient for nutraceuticals against hypertension.



## 1. Introduction

Hypertension is a major chronic disease. It is defined as a systolic blood pressure above 140 mm Hg and/or a diastolic blood pressure above 90 mm Hg. Hypertension affects up to 30% of the adult population in most countries. However, more than 50% of hypertensive individuals are unaware of their condition (Chockalingam, 2008). It is estimated that 7.6 million premature deaths (about 13.5% of the global total) and 92 million deaths and disability-adjusted life years (DALYS) (6.0% of the global total) are attributable to high blood pressure (Lawes, Horrn, & Rodgers, 2008). Untreated hypertension can lead to stroke, coronary heart disease (CHD), kidney dysfunction, disability, and death. The angiotensin converting enzyme (ACE; EC. 3.4.15.1) plays an important physiological role in the regulation of blood pressure. The enzyme converts an inactive form of the decapeptide, angiotensin I, to a potent vasoconstrictor the octapeptide angiotensin II, and also inactivates bradykinin, a vasodilator (Li, Le, Liu, & Shi, 2005). Therefore, the inhibition of ACE activity is a good target for hypertension. The currently used synthetic drugs for the treatment of hypertension, e.g., captopril, and enalapril have certain side effects (Je, Park, Jung, Park, & Kim, 2005).

In recent years, peptides that can inhibit ACE are receiving special attention since they are considered as non pharmacological alternative for the prevention and control of systemic arterial hypertension (Vermeirssen, Van Camp, & Verstraete, 2004). These peptides, which are inactive within the sequence of the parent protein, are liberated during enzymatic digestion or food processing. Among these bioactive peptides, a variety of angiotensin I-converting enzyme (ACE) inhibitory peptides with various amino acid sequences have been found in hydrolysates from food proteins digested with different proteases under different hydrolysis conditions, such as milk protein (Gobbetti, Ferranti, Smacchi, Goffredi, & Addeo, 2000; Jérôme, Laurent, & Jean-Luc, 2002; Meisel, 1998; Rober, Razaname, Mutter, & Juillerat, 2004), soy-protein (Wu & Ding, 2002), egg protein (Yoshii et al., 2001), fish protein (Fujita & Yoshikawa, 1999; Sugiyama et al., 1991) and porcine muscle protein (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001). In contrast to the many ACE-inhibitory peptides derived from vertebrate muscle, very few studies on ACE-inhibitory peptides from invertebrate muscles have been conducted.

Animal digestives proteases such as pepsin, chymotrypsin and trypsin are frequently used in hydrolysis to produce ACE-inhibitory peptides (Lee, Qian, & Kim, 2010; Wang et al., 2008). Microbial alkaline proteases are also utilized in the production of ACE inhibitors from food proteins such as tilapia (*Oreochromis niloticus*) (Raghavan & Kristinsson, 2009) and whey (Guo, Pan, & Tanokura, 2009). However, few research reports have been published on the utilization of endogenous enzymes from fish and aquatic invertebrates to produce bioactive peptides (Bougatef, Nedjar-Arroume, et al., 2008; Bougatef et al., 2009; Khantaphant & Benjakul, 2008).

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In the present study, three new peptides with high ACE-inhibitory activity were isolated from cuttlefish protein hydrolysates, and their amino acid sequences were determined.

## 2. Materials and methods

### 2.1. Cuttlefish sample preparation

Cuttlefish (*S. officinalis*) was purchased from the fish market of Sfax city, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w) and transported to the research laboratory within 30 min. The mantle was cleaned, deskinned and eviscerated and then stored in sealed plastic bags at −20 °C until used.

### 2.2. Enzymes and chemicals

Angiotensin I-converting enzyme from rabbit lung and the ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Acetonitrile was of HPLC grade. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Purified water was obtained from a Culligan system; the resistivity was approximately 18 MΩ. Other chemicals and reagents used were of analytical grade.

Chymotrypsin and trypsin (from bovine pancreas), and pepsin (from porcine stomach mucosa) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Crude protease extracts from sardinelle viscera (*Sardinella aurita*) (Ben Khaled et al., 2008), smooth hound intestines (*Mustelus mustelus*) (Bougatef, Balti, Jellouli, Triki-Ellouz, & Nasri, 2008), and cuttlefish hepatopancreas (*S. officinalis*) (Balti, Barkia, Bougatef, Ktari, & Nasri, 2009) were prepared in our laboratory.

### 2.3. Production of cuttlefish muscle protein hydrolysates (CMPHs)

Cuttlefish (*S. officinalis*) muscle (500 g), in 1000 ml distilled water, was first minced, using a grinder (Moulinex Charlotte HV3, France), and then cooked at 90 °C for 20 min to inactivate endogenous enzymes. The cooked muscle sample was then homogenized in a Moulinex® blender for about 2 min. The pH of the mixture was adjusted to the optimum activity value for each enzyme. The hydrolysis was performed at the same concentrations of enzyme and substrate ($E/S$ = 3 U/mg) to compare hydrolytic efficiencies. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of 4 N NaOH solution. After the required digestion time the reaction was stopped by heating the solution at 80 °C during 20 min to inactivate the enzyme. The cuttlefish muscle protein hydrolysates were then centrifuged at 5000*g* for 20 min to separate insoluble and soluble fractions. Finally, the soluble phase was freeze-dried using freeze-dryer (Bioblock Scientific Christ ALPHA 1-2, IllK-rich-Cedex, France) and stored at −18 °C for further use. Hydrolysis conditions for the preparation of CMPHs are summarized in Table 1.

### 2.4. Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken ($h$) to the total number of peptide bonds in the substrate studied ($h_{tot}$), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen, 1986) as given below.

$$\mathrm{DH}\ (\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times \mathrm{Nb}}{\mathrm{MP}} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

**Table 1**
Hydrolysis conditions, DH and $IC_{50}$ values of cuttlefish muscle protein hydrolysates obtained with various digestives proteases treatment.

| Enzyme | Optimum conditions | | DH (%) | $IC_{50}$ (mg of dry weight/ml) |
|---|---|---|---|---|
| | Temperature (°C) | pH | | |
| Trypsin | 37 | 8.0 | 7.0 | 2.14 ± 0.05 |
| Chymotrypsin | 37 | 7.5 | 7.0 | 2.31 ± 0.09 |
| Sardinelle proteases | 45 | 8.0 | 10.0 | 1.58 ± 0.20 |
| Cuttlefish proteases | 50 | 8.0 | 8.0 | 1.19 ± 0.17 |
| Smooth hound proteases | 50 | 8.0 | 11.0 | 1.47 ± 0.08 |

$E/S$: 3 U/mg of protein.

where $B$ is the amount of base consumed (ml) to keep the pH constant during the reaction. Nb is the normality of the base, MP is the mass (g) of protein ($N \times 6.25$), and $\alpha$ is the average degree of dissociation of the $\alpha$-$NH_2$ groups released during hydrolysis expressed as:

$$\alpha = \frac{10^{\mathrm{pH}-\mathrm{p}K}}{1 + 10^{\mathrm{pH}-\mathrm{p}K}}$$

where pH and p$K$ are the values at which the proteolysis was conducted. The total number of peptide bonds ($h_{tot}$) in a fish protein concentrate was assumed to be 8.6 meq/g (Adler-Nissen, 1986).

### 2.5. Determination of the ACE inhibition activity

The ACE inhibition activity was assayed as reported by Nakamura et al. (1995). For each assay, 80 μl of CMPH at different concentrations was added to 200 μl of 5 mM hippuryl-L-histidyl-L-leucine (HHL), and then preincubated for 3 min at 37 °C. CMPH and HHL were prepared in 100 mM borate buffer, pH 8.3, containing 300 mM NaCl. The reaction was then initiated by adding 20 μl of 0.1 U/ml ACE from rabbit lung prepared in the same buffer and incubated for 30 min at 37 °C. The enzyme reaction was stopped by the addition of 250 μl of 1 M HCl. The released hippuric acid (HA) was quantified by RP-HPLC on a Vydac $C_{18}$ column connected to a system composed of a Waters TM 600 automated gradient controller pump module, a WaterWisp 717 automatic sampling device and a Waters 996 photodiode array detector. The sample was then eluted using an acetonitrile/trifluoroacetic acid (1000:1, v/v) gradient from 0% to 28% and from 28% to 47% for 50 and 20 min, respectively. The elution profile was monitored at 228 nm. Spectral and chromatographic data were stored on a NEC image 446 computer. Millennium software was used to acquire, analyze and plot chromatographic data. The average value from three determinations at each concentration was used to calculate the ACE inhibition rate as follows:

$$\text{ACE inhibition}\ (\%) = \left[\frac{B-A}{B-C}\right] \times 100$$

where $A$ is the absorbance of HA generated in the presence of ACE inhibitor component, $B$ the absorbance of HA generated without ACE inhibitors and $C$ is the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay).

The IC50 value was defined as the concentration of inhibitor required to reduce the hippuric acid peak by 50% (indicating 50% inhibition of ACE).

### 2.6. Isolation and purification of ACE-inhibitory peptides

The lyophilized hydrolysate (1 g), with a DH of 8%, obtained by treatment with crude protease extract from cuttlefish hepatopancreas, was suspended in 5 ml of distilled water, then separated