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Short communication

# Purification and characterisation of angiotensin I converting enzyme inhibitory peptides from lysozyme hydrolysates

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ABSTRACT

Hen egg white lysozyme (HEWL) was hydrolysed with trypsin, papain and a combination of the two. The prepared hydrolysates exhibited ACE inhibitory activity. The hydrolysates were fractionated using ultrafiltration and reverse phase-high performance liquid chromatography (RP-HPLC). Three fractions, which showed the highest ACE inhibitory activities, were purified by RP-HPLC. They were the $F_7$ (from papain–trypsin hydrolysate), $F_8$ (from papain hydrolysate) and $F_3$ (from trypsin hydrolysate) fractions. The $IC_{50}$ values were 0.03, 0.155 and 0.23 mg/ml for $F_7$, $F_8$ and $F_3$, respectively. The $F_7$ fraction was the most potent ACE inhibitor peptide, and was composed of 12 amino acids, Phe-Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg (MW: 1428.6 Da). Lineweaver–Burk plots suggest that the $F_7$ peptide acts as an uncompetitive inhibitor against ACE. The kinetic parameters ($K_m$, $V_{max}$, and $K_i$) for the $F_7$ peptide were measured and compared to the control.



## 1. Introduction

Angiotensin I converting enzyme (ACE or kinase II; EC 3.4.15.1) plays important roles in renin–angiotensin and kallikrein–kinin systems. ACE is a dipeptidyl carboxypeptidase that belongs to the class of zinc metalloproteases, which require zinc and chloride for activation (Lee, Qian, & Kim, 2010). ACE catalyses the conversion of angiotensin I (an inactive decapeptide), by cleaving a dipeptide from the C-terminus, into angiotensin II (a potent vasoconstriction octapeptide) (Quist, Phillips, & Saalia, 2009). Additionally, ACE inactivates bradykinin, which has vasodilator activity in the kallikrein–kinin system and results in an increase in blood pressure (Erdös, 1975). Inhibition of ACE leads to an elevation in the concentration of bradykinin, a decrease in the concentration of angiotensin II and finally a decrease in blood pressure. Therefore, inhibition of ACE is considered to be a useful procedure in the prevention and treatment of hypertension and related diseases (Bougatef et al., 2008). Synthetic ACE inhibitors such as captopril, enalapril, lisinopril and alacepril are remarkably effective at regulating blood pressure and are used as clinical antihypertensive drugs (Ondetti, Rubin, & Cushman, 1997). However, these synthetic drugs have demonstrated diverse side effects, such as allergic reactions, skin rashes, cough, taste disturbances (Bougatef et al., 2008). Therefore, a search to find nontoxic, safer, economical, and innovative ACE inhibitors is required for the control and treatment of high blood pressure.

Hen egg white lysozyme (HEWL) is a natural protein containing 129 amino acids with a total molecular weight of 14.3 kDa. Lysozyme comprises 3.4% of total egg white proteins (Miguel & Aleixandre, 2006). Much research has been done on bioactive peptides derived from HEWL, but no antihypertensive peptides have been derived from it. Therefore, our attention was focused on bioactive peptides with ACE inhibitory activity derived from HEWL hydrolysates and on the determination of the inhibition pattern of the purified peptides from HEWL on ACE activity.

## 2. Materials and methods

### 2.1. Enzymes and chemical reagents

ACE (angiotensin I converting enzyme) from rabbit lung, FAPGG (N-(3-[2-furylacryloyl-Phe-Gly-Gly])), TFA (trifluoroacetic acid), acetonitrile, trypsin (from bovine pancreas) and papain (from paw-paw sap) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). HEWL (hen egg white lysozyme) was purchased from Merck Chemical Co. Analytical and semi-preparative columns were purchased from Macherey Nagel GmbH Co. (St. Neumann Neander, Düren, Germany). Ultrafiltration membranes with a 3 kDa cut off were procured from Millipore (Bedford, MA, USA). All other chemicals used were of analytical grade.

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### 2.2. Preparation of HEWL hydrolysate

To extract the ACE inhibitory peptides from HEWL, enzymatic hydrolysis was performed using two commercial enzymes (trypsin and papain) and a mixture of them. The ratio of protein substrate to each protease was 20:1. At first, HEWL was dissolved (4.2 mg/ml) in Tris–HCl buffer (50 mM, pH 7.5). Each enzyme was dissolved (0.21 mg/ml) in the same buffer separately. The hydrolysis reaction was incubated at 37 °C for 2 h. The enzymatic hydrolysis was stopped by heating in boiling water for 15 min. The hydrolysate was then centrifuged at 7000*g* for 10 min, and the supernatant solution was transferred to fresh tubes for subsequent studies.

### 2.3. Isolation and purification of ACE inhibitory peptides

The hydrolysate derived from HEWL was passed through an ultrafiltration membrane with a cut-off of 3 kDa. The resulting filtrate was fractionated using RP-HPLC on a semi-preparative $C_{18}$ column (10 × 250 mm, manufactured by Macherey–Nagel GmbH & Co. Düren, Germany). The mobile phase includes eluent A, which was composed of 0.1% TFA in distilled water (v/v), and eluent B, which was composed 0.098% TFA in acetonitrile. The elution was conducted using a linear gradient of 5–50% eluent B with a flow rate of 2 ml/min for 45 min. The absorbance of the elution peaks was monitored at 220 nm using a UV detector. Major peaks were collected, lyophilised and evaluated for their ACE inhibitory activity. The fractions, which showed high ACE inhibition activity, were further purified by using the same RP-HPLC conditions except for the use of a gradient of 0.5% eluent B per minute. Then, the collected fractions were lyophilised for use in the ACE inhibitory activity assay.

### 2.4. ACE inhibitory activity assay

The ACE inhibitory activity assay was performed based on the method described by Holmquist, Bünning, and Riordan (1979) using FAPGG as the substrate with only slight protocol modifications. In each test sample, the assay mixture was composed of the following components: 22 μl of ACE (50 mU/ml), 50 μl of hydrolysate or peptide (1 mg/ml) and 100 μl of FAPGG (0.5 mM) and 150 μl of ACE buffer (50 mM of Tris–HCl pH 7.5, 0.3 M NaCl and 1 mM $ZnCl_2$). Control sample contained 22 μl of ACE (50 mU/ml), 100 μl of FAPGG and 200 μl of ACE buffer. The reaction was monitored at 340 nm for 60 min. The ACE inhibition was measured by using the comparison of absorbance changes of the test and the control, according to the equation (Lahogue, Réhel, Taupin, Haras, & Allaume, 2010):

$$\text{ACE inhibition } (\%) = [1 - (\Delta A_{\text{inhibitor}}/\Delta A_{\text{control}})] \times 100$$

### 2.5. $IC_{50}$ determination of the primary hydrolysates and purified peptides

The $IC_{50}$ value is defined as the concentration of hydrolysates or peptide that is able to inhibit 50% of the ACE activity. Five different concentrations of inhibitory peptide were selected and evaluated for their % ACE inhibitory activity. The $IC_{50}$ of the different peptides was determined by plotting the % ACE inhibition against the different concentrations of peptide. The $IC_{50}$ of the purified peptides was compared with the $IC_{50}$ of captopril, which was previously reported by Kamath, Niketh, Chandrashekar, and Rajini (2007). Experiments were done in triplicate.

### 2.6. Determination of amino acid sequence of the most active peptide

In order to determine the sequence of the most ACE inhibitor peptide, the sample was desalted using ZipTips [Millipore] and analysed by MALDI TOF–TOF mass spectrometer using a 5800 Proteomics Analyzer [Applied Biosystems at Proteomics International Pty Ltd., Nedlands, Western Australia]. The amino acid sequence was determined by *de novo* sequencing method. MS/MS spectra was analysed using PEAKS Studio Version 4.5 SP2 [Bioinformatics Solutions Inc., Waterloo, Canada].

### 2.7. Determination of the inhibition pattern on ACE activity

To characterise the inhibitory mechanism of purified peptide from HEWL hydrolysates, the experiment was conducted with different concentrations of the FAPGG substrate (0.6, 1.2, 1.8 and 2.4 mM). The other assay conditions were as described in the ACE activity assay section but, the assay was done in the presence of 50 μl of different concentrations of the substrate. The enzyme activities were measured in the absence and in the presence of different concentrations (0.155 and 0.310 mg/ml) of inhibitory peptide. To determine the ACE inhibitory pattern, we used the $F_7$ peptide because this peptide showed the most potent ACE inhibition. The kinetics of ACE inhibition in the presence of the $F_7$ peptide (from trypsin–papain hydrolysate) was determined by Lineweaver–Burk plots. The kinetic parameters of $V_{max}$ and $K_m$ in the absence and in the presence of inhibitory peptide were determined. Additionally, the kinetic parameter of $K_i$ (inhibitor constant for binding of an inhibitor to enzyme–substrate complex) was obtained from the secondary plot (Palmer, 2001).

## 3. Results and discussion

### 3.1. Preparation of HEWL hydrolysate and the assay of ACE inhibition

To isolate ACE inhibitory peptides from HEWL, it was separately hydrolysed using two digestive enzymes, namely trypsin and papain or a combination of the two. All the hydrolysates showed the ACE inhibitory activity. The percent of ACE inhibition of the hydrolysates was observed to be 12% (±1.41), 18% (±2.22) and 31.2% (±3.11) for trypsin, papain and a combination of two enzymes at the concentration of 0.155 mg/ml, respectively, and their $IC_{50}$ values were 0.602 (±0.041), 0.384 (±0.028) and 0.189 (±0.017) mg/ml, respectively. The obtained hydrolysate using trypsin had a higher $IC_{50}$ value than the other hydrolysates, indicating that it has less of an effect on ACE inhibition. The ACE inhibitory activity of the prepared hydrolysates in this research was compared with the hydrolysates from other sources. These sources include hydrolysates obtained from egg white protein by thermolysin ($IC_{50}$ = 0.054 mg/ml), by chymotrypsin ($IC_{50}$ = 0.078 mg/ml), by alcalase ($IC_{50}$ > 0.750 mg/ml), by esperase ($IC_{50}$ > 0.750 mg/ml) (Chiang, Tsou, Weng, & Tsai, 2008), by pepsin ($IC_{50}$ = 0.055 mg/ml) and ovalbumin by pepsin and thermolysin ($IC_{50}$ = 0.045 and 0.083 mg/ml) (Fujita, Yokoyama, & Yoshikawa, 2000). The $IC_{50}$ values of these egg white proteins varied from 0.045 to 0.750 mg/ml, and the $IC_{50}$ values of the hydrolysates obtained in this study are in this range.

### 3.2. Purification of ACE inhibitory peptides from HEWL hydrolysates and the assay of ACE inhibition

The HEWL hydrolysates were fractionated by RP-HPLC, and the major peaks were selected to assay their ACE inhibitory activities. Fig. 1 shows the chromatogram of the RP-HPLC of the different hydrolysates. The fractions were numbered sequentially. All factions were assayed to select the most active fraction. As shown in Table 1, some of the fractions showed ACE inhibitory activity. In Fig. 1, the most active fractions are indicated with an arrow. These peaks, which were derived from hydrolysates of HEWL by trypsin, papain and trypsin–papain, are named $F_3$, $F_8$ and $F_7$,