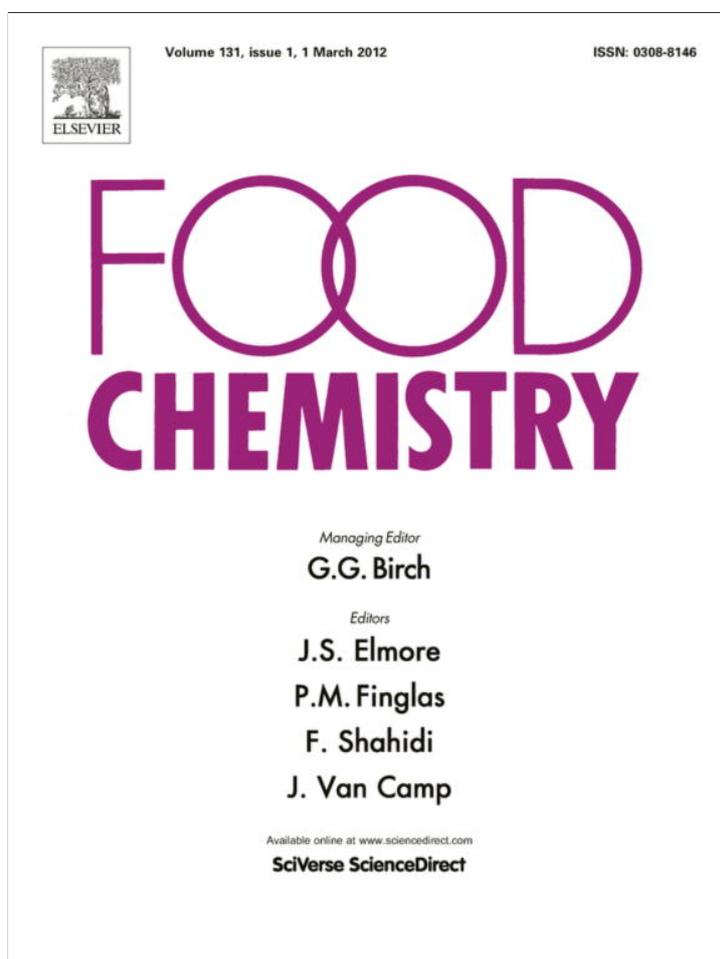


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Short communication

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

Ahmad Asoodeh^{a,c,*}, Mina Memarpour Yazdi^b, JamshidKhan Chamani^b^a Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran^b Department of Biology, Faculty of Sciences, Mashhad Branch, Islamic Azad University, Mashhad, Iran^c Department of Chemistry, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran

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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 F₇ (from papain-trypsin hydrolysate), F₈ (from papain hydrolysate) and F₃ (from trypsin hydrolysate) fractions. The IC₅₀ values were 0.03, 0.155 and 0.23 mg/ml for F₇, F₈ and F₃, respectively. The F₇ 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 F₇ peptide acts as an uncompetitive inhibitor against ACE. The kinetic parameters (K_m , V_{max} , and K_i) for the F₇ peptide were measured and compared to the control.

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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 (Erdős, 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.

* Corresponding author at: Cellular and Molecular Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran. Tel.: +98 9155215590; fax: +98 5118795457.

E-mail address: asoodeh@um.ac.ir (A. Asoodeh).

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 7000g 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 C₁₈ 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 ZnCl₂). 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, & Allaupe, 2010):

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

2.5. IC₅₀ determination of the primary hydrolysates and purified peptides

The IC₅₀ 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 IC₅₀ of the different peptides was determined by plotting the % ACE inhibition against the different concentrations of peptide. The IC₅₀ of the purified peptides was compared with the IC₅₀ 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 F₇ peptide because this peptide showed the most potent ACE inhibition. The kinetics of ACE inhibition in the presence of the F₇ 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 IC₅₀ 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 IC₅₀ 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 (IC₅₀ = 0.054 mg/ml), by chymotrypsin (IC₅₀ = 0.078 mg/ml), by alcalase (IC₅₀ > 0.750 mg/ml), by esperase (IC₅₀ > 0.750 mg/ml) (Chiang, Tsou, Weng, & Tsai, 2008), by pepsin (IC₅₀ = 0.055 mg/ml) and ovalbumin by pepsin and thermolysin (IC₅₀ = 0.045 and 0.083 mg/ml) (Fujita, Yokoyama, & Yoshikawa, 2000). The IC₅₀ values of these egg white proteins varied from 0.045 to 0.750 mg/ml, and the IC₅₀ 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 fractions 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 F₃, F₈ and F₇,

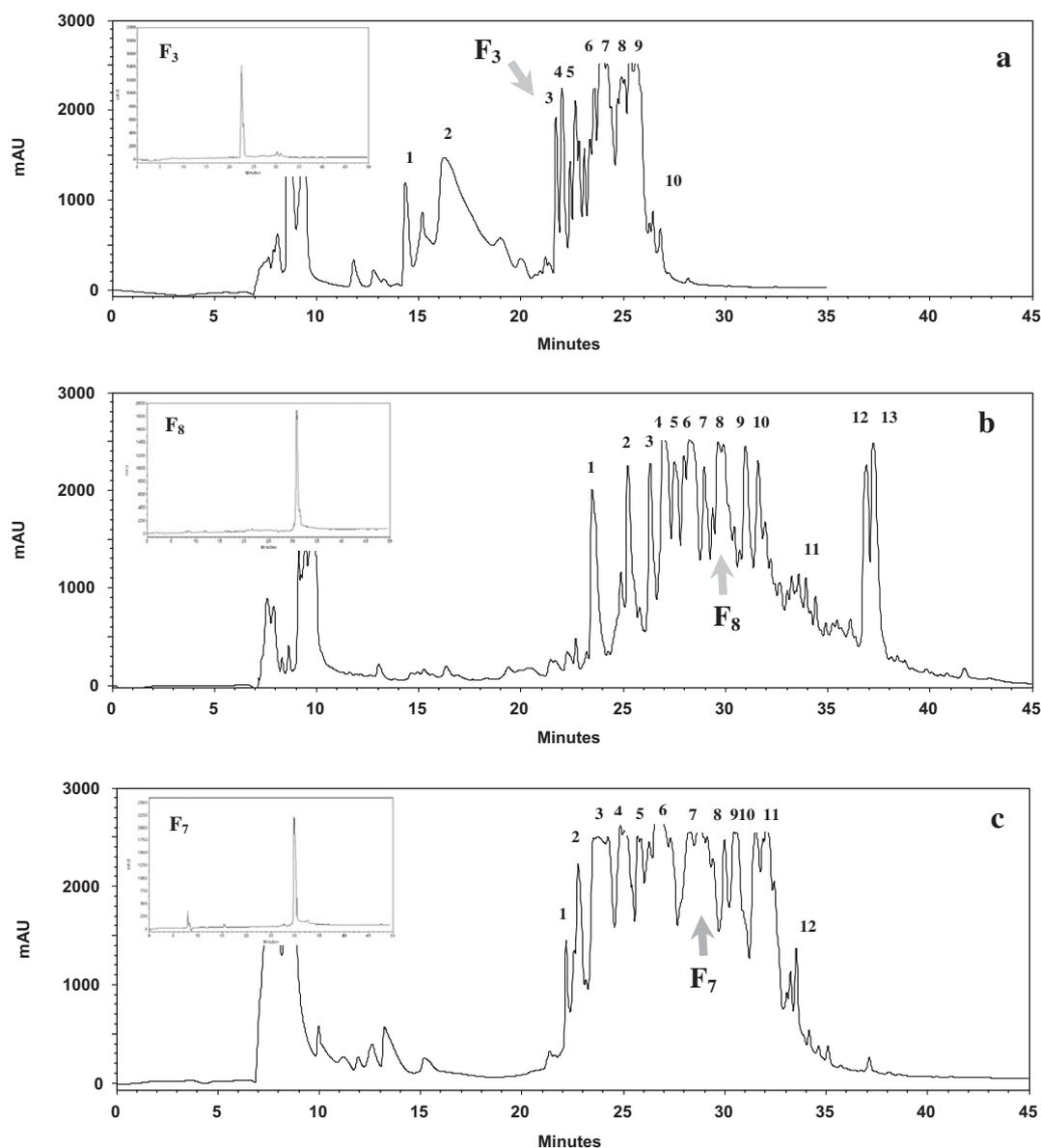


Fig. 1. Chromatograms of RP-HPLC for HEWL hydrolysed by trypsin (a), by papain (b) and by a combine of the two enzymes (c). The inset of each figure indicates the purity of the labelled fraction.

respectively (Fig. 1a–c). Each F_3 , F_8 and F_7 fraction was further purified to yield a single peak using the second run of C_{18} RP-HPLC (inset of Fig. 1a–c). The purified F_3 , F_8 and F_7 fractions at concentration of 0.155 mg/ml have 34% (± 2.27), 50% (± 3.11) and 92% (± 4.24) ACE inhibition activity, respectively. The % ACE inhibition activity for all fractions is nearly threefold higher than their unpurified hydrolysates.

3.3. IC_{50} values of the purified peptides and amino acid sequencing

To determine the IC_{50} of the purified peptides, the percent ACE inhibition was obtained for different concentrations of peptide. Then, the IC_{50} was evaluated by plotting the curves of % ACE inhibition against the different peptide concentrations. The IC_{50} values were 0.03 (± 0.008), 0.155 (± 0.02) and 0.23 (± 0.025) mg/ml for F_7 , F_8 and F_3 fractions, respectively. The F_7 peptide has the lowest IC_{50} , which shows it is the most effective inhibitor of ACE activity. Thus, the F_7 peptide was elected for sequencing. The amino acid composition of the F_7 ACE inhibitor peptide was identified as Phe-Glu-

Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg (MW: 1428.6 Da) (Fig. 2a and b). This 12-amino acid sequence corresponds to fragment 34–45 of hen egg white lysozyme (HEWL).

Some antihypertensive peptides derived from egg-white proteins have been identified, for example: IVF, YAEERYPIL and RAD-HPFL (Miguel, Alexandre, Ramos, & López-Fandiño, 2006; Miguel et al., 2007); YRGGLEPINF and ESIINF (Miguel et al., 2007) and RVPSL (Liu et al., 2010). Although, the isolated peptide is longer than these ACE inhibitory peptides, some other peptides have been reported from bonito meat and fruiting body of *Pleurotus cornucopiae*, which had 8–17 amino acid residues (Hasan et al., 2006; Jang et al., 2011). To the best of our knowledge, this report is the first that introduces a new ACE-inhibitory peptide from HEWL hydrolysates.

The IC_{50} value of the F_7 peptide (FESNFNTQATNR, IC_{50} = 0.03 mg/ml, or 21 μ M) was compared with other peptides purified from the hydrolysates of ovalbumin (NIFYCP, IC_{50} = 15.00 μ M and RVPSL, IC_{50} = 20 μ M) (Fujita et al., 2000; Liu et al., 2010); egg white (RAD-HPFL, IC_{50} = 6.2 μ M; IVF, IC_{50} = 33.11 μ M and YAEERYPIL,

Table 1

The ACE inhibitory activity of the obtained fractions from HEWL hydrolysates by trypsin, papain and a combination of the two.

Fraction number ^a	ACE inhibitory activity (%)		
	Trypsin	Papain	Trypsin and papain
F ₁	ND	3.21	ND
F ₂	5.22	ND	6.77
F₃	9.31	3.54	10.4
F ₄	4.13	ND	ND
F ₅	3.02	ND	1.71
F ₆	ND	5.81	8.31
F₇	1.14	1.20	34.25
F₈	4.03	14.92	8.19
F ₉	5.72	7.73	5.55
F ₁₀	ND	ND	ND
F ₁₁	–	6.19	7.22
F ₁₂	–	ND	ND
F ₁₃	–	3.07	–

ND: none detected activity.

^a A total of 10 fractions was collected for trypsin digestion and 13 and 12 fractions collected for papain and trypsin–papain, respectively. The most active fractions and their ACE inhibition activity (%) were shown as bolded.

IC₅₀ = 4.7 μM) (Miguel & Aleixandre, 2006); α-lactalbumin (WLAHK, IC₅₀ = 77.00 μM) (Pihlanto-Leppälä, Koskinen, Phlola, Tupasela, & Korhonen, 2000) and milk (β-lactoglobulin) (IPA, IC₅₀ = 141.0 μM and ALPM, IC₅₀ = 928.0 μM) (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Murakami et al., 2004).

Finally, our identified peptide from HEWL hydrolysate exhibited more potent ACE inhibitory activity in comparison to some reports. The IC₅₀ of the F₇ peptide in comparison with captopril as positive control is high. The previously reported IC₅₀ value of captopril was 1.7 ng/ml (Kamath et al., 2007). Therefore, the inhibition potential of the F₇ peptide is less than captopril.

3.4. The inhibition pattern of the most active peptide on the ACE

The ACE inhibition pattern of purified peptide explains how it binds to ACE and inhibit its activity. The ACE inhibition pattern of the F₇ purified peptide from HEWL hydrolysate by a combination of trypsin and papain was quantitated by Lineweaver–Burk

plots. The result of the kinetics study showed that its ACE inhibition pattern is uncompetitive (Fig. 3a), which means that it binds only to the ACE–substrate complex and not to the free enzyme (Palmer, 2001). The ACE inhibition pattern of the ACE inhibitory peptides probably is related to the structure of the inhibitory peptides. The study of the structure–activity relationships demonstrated that the presence of a positive charge on the guanidine or ε-amino group of the C-terminal Arg and Lys side-chains, respectively, has a key role in the binding to ACE (Cheung et al., 1980). Therefore, the positive charge of Arg residue at the C-termini of the F₇ peptide plays a critical role on ACE inhibition.

Although most of the isolated peptides have the competitive ACE inhibition pattern, some of the isolated peptides showed a noncompetitive or uncompetitive ACE inhibition pattern (Li, Le, Shi, & Shrestha, 2004). Our results in the absence or presence of various concentrations of the F₇ peptide showed that the slope of a Lineweaver–Burk plot is not altered by the presence of the F₇ peptide, but both intercepts are changed (Fig. 3a). The value of K_m in the absence of the F₇ peptide was 0.369 mM, and its values in the presence of two different concentrations 0.155 and 0.310 mg/ml of the F₇ peptide were 0.137 and 0.080 mM, respectively. Furthermore, the values of V_{max} in the absence and presence of two different concentrations 0.155 and 0.310 mg/ml of the F₇ peptide were 0.006, 0.002 and 0.001 (mM/min)⁻¹, respectively. Inhibitor constant K_i is determined from the intercept on the [I₀] axis of secondary plot of 1/V_{max} or 1/K_m against [I₀], and resulted K_i was to be 0.082 mg/ml (V_{max} and K_m are the value of V_{max} and K_m in the presence of the F₇ peptide) (Fig. 3b).

4. Conclusion

In summary, we isolated and characterised ACE inhibitory peptides from HEWL hydrolysates using various digestive enzymes. The most effective peptide for ACE inhibition was the F₇ peptide (FESNFNTQATNR, MW: 1428.6 Da) with 0.03 mg/ml of IC₅₀ value and it was evaluated as an uncompetitive inhibitor against ACE. The F₇ peptide in this study showed higher potency of ACE inhibition than some of the purified peptides from other sources. *In vitro* digestion of this peptide by gastrointestinal tract proteases might generate a number of potent ACE inhibitory peptide fragments,

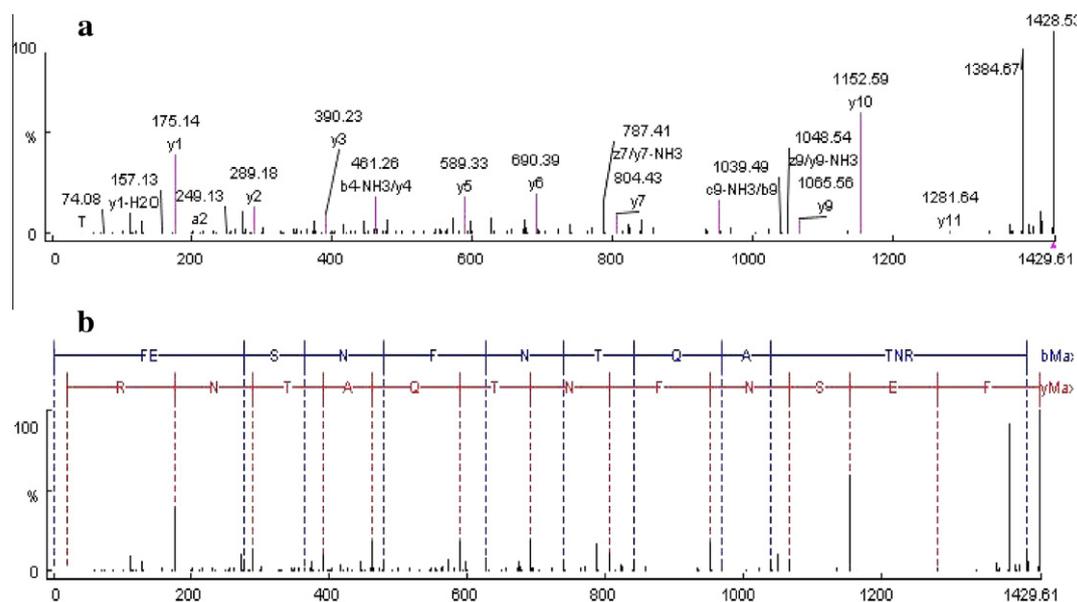


Fig. 2. Identification of the molecular mass and amino acid sequence of the F₇ peptide using MALDI–TOF (Matrix-assisted laser desorption/ionisation–Time Of Flight) spectrometer. (a) MS/MS spectra of the F₇ peptide, and (b) the interpretation of the obtained spectra.

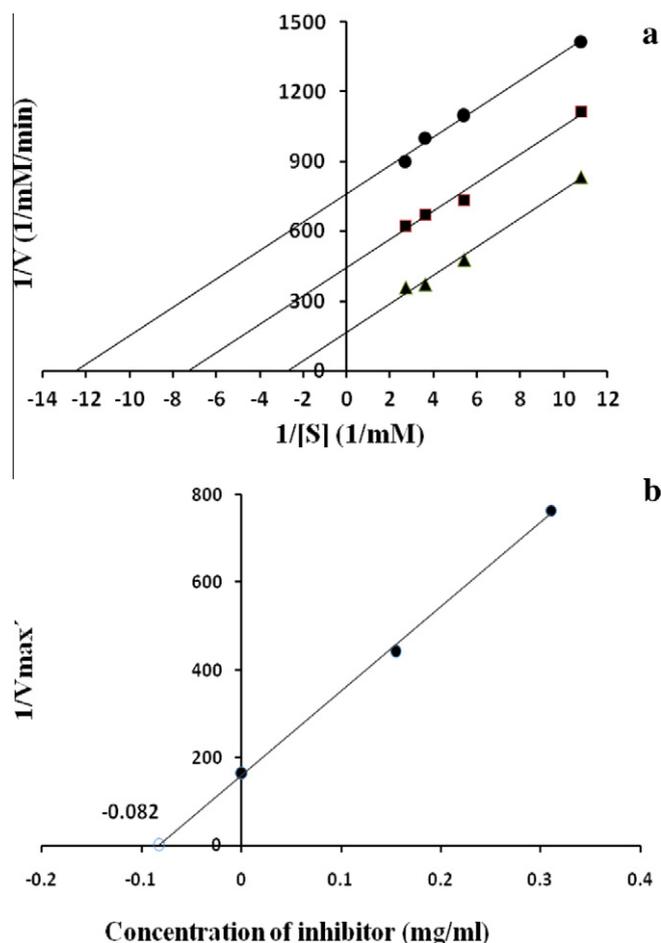


Fig. 3. (a) The Lineweaver–Burk plot for determination of the inhibition pattern of the F₇ peptide on ACE. ACE inhibitory activity was determined in the absence or presence of the F₇ peptide (▲, control; ■, 0.155 mg/ml; ●, 0.310 mg/ml). The Lineweaver–Burk plots show the effect of uncompetitive inhibition. (b) The secondary plot for uncompetitive inhibition.

which may have implications for cardiovascular disease prevention, including hypertension.

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