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ACE-inhibitory activity of seed storage proteins and hydrolysates from Job's tears (*Coix lacryma-jobi* L.)

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Abstract

Though hypertension is a serious health problem, the inhibition of the angiotensin-I converting enzyme (ACE) provides a means to treat and manage it. In the present work, we investigated the ACE-inhibitory activity of crude proteins and protein hydrolysates from Job's tears (*Coix lacryma-jobi* L.). ACE inhibition of 22.15 ± 0.94% was observed with 400 µg of protein from Job's tears as compared to captopril, a common ACE inhibitor, equaled to 53.7 ± 2.3 nmol captopril equivalent per mg of protein (IC_{50} of captopril towards ACE was 4.8 nmol). The crude proteins from Job's tears were enzymatically hydrolysed for 1, 2, and 3 h (E/S of 1:20 by weight) using commercial proteolytic enzymes including Alcalase, Papain, Pronase, and Trypsin. All hydrolysates exhibited increased ACE-inhibitory activity. The protein hydrolysates (400 µg) prepared using Pronase for 2 h (CLPrH-2h) exhibited the highest inhibitory activity (78.38 ± 0.23% or 190.0 ± 0.5 nmol captopril equiv. per mg protein) and were ~3.5 times more active as compared to the crude proteins. Fractionation of the peptides was performed using semi-preparative reverse-phase high-performance liquid chromatography (RP-HPLC), and all the fractions exhibited ACE-inhibitory activity. The most active fraction was F2 (41.58% inhibition) which was ~7.5 times more active than the crude proteins. These results suggested that seeds from Job's tears could be an interesting source for developing functional foods with antihypertensive properties.

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Introduction

The angiotensin I-converting enzyme (ACE; EC. 3.4.15.1) is a key enzyme for controlling blood pressure in the renin angiotensin system (RAS). It catalyses the transformation of angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), which is produced in the liver to a shorter peptide, angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). Angiotensin II has strong vasoconstriction activity, and inhibits the dilation of the blood vessels, thus causing increased blood pressure (Jang *et al*., 2011). High blood pressure may be prevented by hindering ACE activity. ACE inhibitors such as captopril, lisinopril, and enalapril are often used to treat hypertension. However, synthetic compounds can cause certain side effects such as coughing, skin rashes, loss of taste, haematological effects, and angioneurotic oedema (Je *et al*., 2004). Food-derived antihypertensive peptides such as casein, whey protein, soy, walnut, chicken, and egg have been reported as safer alternatives (Liu *et al*., 2013).

Peptides with ACE-inhibitory activity are present in several animal-derived products; milk and egg are good sources of antihypertensive peptides because they contain high amounts of protein. Moreover, peptides from meat (Lafarga *et al*., 2014) and marine animals (Martínez-Maqueda *et al*., 2012) are being studied as new sources of peptides with antihypertension activity. Recently, plant-derived peptides have attracted interest since plant-based foods are cholesterol-free, present high fibre content, and are often alkalising. Vegetables, fruits, soybeans, tea, and mushrooms are food sources potentially containing ACE-inhibitory peptides. Reports on plant-derived peptides with ACE-inhibitory activity include many sources such as canary seed (*Phalaris canariensis* L.; Urbizo-Reyes *et al*., 2022), broccoli (*Brassica oleracea* L.; Zhang *et al*., 2022), corn distiller soluble (Sharma *et al*., 2022), peony

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(*Paeonia suffruticosa* Andr.; Ye *et al*., 2022), green lentil protein (Rezvankhah *et al*., 2021a; 2021b), *Spirulina* (*Arthrospira platensis* Gomont; Anekthanakul *et al*., 2019), and hazelnut (*Corylus heterophylla* Fisch.; Liu *et al*., 2018).

Job's tears (*Coix lacryma-jobi* L.) are a popular crop in East and Southeast Asian countries (Xi *et al.*, 2016). The seeds contain phenols, flavonoids, polysaccharides, proteins, fibres, vitamins, and oils, and have strong antioxidant, anti-inflammatory, and anti-obesity properties. They can stimulate reproductive hormones, and promote uterine contraction and gut microbiota (Devaraj *et al*., 2020). Recent studies reported that Job's tears exhibit ACE-inhibitory activity and antioxidant properties. *Coix* glutelin hydrolysed using five different enzymes (pepsin, thermolysin, papain, flavourzyme, and α-chymotrypsin) exhibited ACE-inhibitory activity, with the pepsin hydrolysate presenting the highest activity (IC_{50} 52.34 ± 3.71 μg/mL; Yuan *et al.*, 2014). Novel peptides derived from *Coix* glutelin, GAAGGAF, have been reported to present antihypertensive properties (IC_{50} 14.19 µmol/L; Li *et al*., 2017b).

In the present work, we investigated the ACE-inhibitory activity of crude proteins from Job's tears and their hydrolysates prepared by digestion with certain proteases. Fractionation was also conducted using RP-HPLC, and the obtained fractionated peptides were further analysed.

Materials and methods

Crude protein extraction

Seeds from Job's tears were obtained from Sakon Nakhon province, Thailand. The dried seeds (100 g) were washed with tap water, and soaked in distilled water overnight (28 ± 3°C). The imbibed seeds were rinsed, washed twice with distilled water, and homogenised in distilled water using a blender (1:3 w/v). The slurry was stirred for 1 h at 4°C, and then centrifuged (10,000 × *g*, 4°C, 20 min). The sediment was discarded, and the clear supernatant was kept (crude protein). The seed residues were extracted twice with distilled water. After determining the protein content, the supernatant was stored at 4°C for future experiments, or freeze-dried and stored at -20°C.

Protein determination

Proteins were determined following the Lowry method (Lowry *et al*., 1951) using a Modified Lowry Protein Assay Kit (Thermo Scientific, USA). Bovine serum albumin (BSA) was used to construct a standard curve. During the purification process using RP-HPLC, protein measurement was followed by absorbance measurement at 214 nm.

ACE-inhibitory activity assay

The ACE-inhibitory effect of the crude proteins (or protein hydrolysates) was performed using a previously described and modified method (Lee *et al*., 2004; Kokram *et al*., 2016). Briefly, the activity of rabbit lung ACE enzyme (Sigma-Aldrich, USA) was determined using *N*-Hippuryl-Histidyl-Leucine hydrate (HHL) (Sigma-Aldrich, USA) as a substrate; activity toward the substrate was compared to that measured in the presence of inhibitor and test compound. The ACE-inhibitory activity was reported as an inhibition percentage using Eq. 1:

$$\%\text{Inhibition} = \frac{C-(I-IB)}{C} \times 100 \quad \text{(Eq. 1)}$$

where, C = absorbance of the control reaction with no inhibitor, I = absorbance of the reaction with inhibitor, and IB = absorbance of the blank solution (only inhibitor). The results were reported as half-maximal inhibitory concentration (IC_{50}) of ACE activity inhibition. The ACE-inhibitory ability could also be expressed as equivalent to nmol captopril, a standard ACE-inhibitory drug.

Protein hydrolysate preparation

The crude proteins (2 mg/mL) from the seeds of Job's tears were enzymatically hydrolysed using four proteases; Alcalase (Sigma-Aldrich, USA), Papain (Sigma-Aldrich, USA), Pronase (Roche, Germany), and Trypsin (Sigma-Aldrich, USA). The reactions were performed at the recommended conditions for each enzyme using a 1:20 mass ratio of protease enzyme to protein substrate (E/S). The incubation time was 1, 2, or 3 h, after which the enzyme was deactivated in boiling water for 10 min. The protein hydrolysates prepared at 1, 2, and 3 h using Alcalase were named CLAlH-1h, CLAlH-2h, and CLAlH-3h. The protein hydrolysates prepared using Papain were named CLPaH-1h, CLPaH-2h, and CLPaH-3h. The protein hydrolysates prepared using Pronase were named CLPrH-1h, CLPrH-2h, and CLPrH-3h, and the protein hydrolysates prepared

using Trypsin were named CLTrH-1h, CLTrH-2h, and CLTrH-3h. The ACE-inhibitory activity of the protein hydrolysates was tested as described previously, and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted for verifying the end of the hydrolysis.

Protein pattern analysis

Protein patterns of the crude proteins and protein hydrolysates were analysed following the Laemmli method (Laemmli, 1970), where SDS-PAGE was conducted using a 15% resolving gel and a 5% stacking gel. A constant voltage of 100 V/gel was applied, and the gel was stained with Coomassie brilliant blue R-250. It was then destained using a destaining solution (250 mL methanol: 200 mL distilled water: 50 mL acetic acid) until the protein bands were visible.

Fractionation of the Pronase-prepared hydrolysate using RP-HPLC

After Pronase treatment with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$ at 40°C, CLPrH-2h (2 mg/mL) was filtered using a 3 kDa MWCO membrane. Two fractions (MW > 3 kDa and MW < 3 kDa) were subjected to ACE-inhibitory assay. The fraction with the highest activity was further fractionated using a semi-preparative reverse-phase HPLC (1525 Binary HPLC Pump, 2998 Photodiode Array Detector, Waters, Ireland) equipped with a Sunfire^{TM} Prep C_{18} column (5 μm, 10 × 250 mm; Waters, Ireland). The injection volume was 1 mL, and the flow rate was 1.0 mL/min. The mobile phase was 0.1% trifluoroacetic acid (TFA) in DI (solvent A) and 0.1% TFA in acetonitrile (solvent B). The column was equilibrated using solvent A for 2 h, and the gradient was set as follows: 0 - 50 min: 0 - 50% B; 50 - 65 min: 50% B; 65 - 70 min: 50 - 100% B; and 70 - 75 min: 100% B. The detection wavelength was 214 nm, and the peptides were pooled based on their peaks. The samples were freeze-dried and stored at 4°C before analysis, as described in the previous section.

Statistical analysis

The experiments were performed in triplicate, and the results were reported as mean ± standard deviation, and analysed by One-way ANOVA using the SPSS version 18 software (SPSS Inc., Chicago, USA). Significant differences were considered when $p < 0.05$.

Results and discussion

Crude protein extraction of seeds from Job's tear

The water-soluble protein content in the crude protein extract was 4.53 ± 0.06 mg/mL and ~0.82% dry weight of the seeds, as determined by the Lowry method. The residue was extracted again using water, and yielded 30-fold lower protein content; this third extract was discarded to maintain smaller extract volume. The *Coix* seed storage protein comprises albumin, globulin, prolamin (known as coixin), and glutelin as 1.43, 6.20, 44.74, and 37.38% of total protein, respectively (Wang *et al*., 2012). Among the seed storage proteins, prolamin was the major component (~44.74% of the total protein). *Coix* prolamins are classified into four types, α-, β-, γ-, and δ-prolamin, based on their solubility (Igbokwe *et al*., 2021). Among them, the α-prolamin alcohol-soluble storage proteins are the major component. In the present work, alcohol was not used during the extraction process, meaning that the γ-prolamin water-soluble storage proteins were mainly extracted but were not the most abundant proteins in the endosperm of Job's tears. Therefore, the protein content obtained in the present work was relatively low.

Protein pattern of the crude protein and protein hydrolysates

The water-soluble protein fraction of the seeds exhibited a wide range of MW distribution as determined by SDS-PAGE (Figure 1). The crude protein contained protein bands with molecular weights (MW) ranging from 97 to < 14.4 kDa, and major protein bands at < 14.4 kDa, as shown in Figure 1, lane 2. *Coix* γ-prolamin contained protein bands of MW ~27 - 28 kDa, which reacted with γ-zein antiserum, and showed high homology in its gene (Shewry and Halford, 2003). Water solubility was attributed to high histidine content: all γ-prolamins contain ten conserved cysteine residues, and were rich in proline, glutamine, and non-polar amino acids. The α-amylase/Trypsin inhibitors (ATIs, MW ~12 - 16 kDa; Sagu *et al*., 2020) are widely distributed in many seeds and grains, including Job's tears. However, in the present work, the water extract did not contain any ATIs since the band disappeared after digestion with Trypsin (Figure 1D). Most antihypertension activity studies are conducted using short peptides. Therefore, the crude protein fraction from the seeds of Job's tears was independently

hydrolysed using four proteases, Trypsin, Pronase, Alcalase, and Papain at 37, 40, 50 and 60°C for 1, 2, and 3 h. Based on the disappearance of the protein bands, all the tested proteases hydrolysed the seed's proteins almost completely (Figures 1A - 1D), apart from Pronase, which left some small proteins with MW ≤ 14.4 kDa (Figure 1C). This was attributed to the unsuitable E/S ratio as it was not yet optimised.

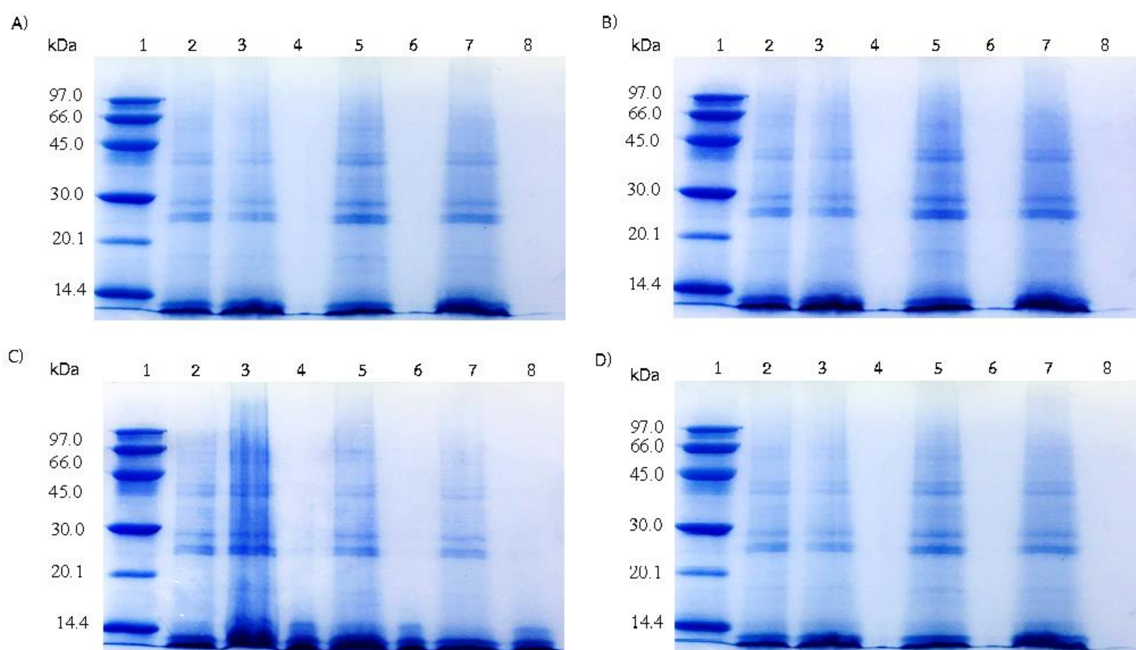

Figure 1. 15% SDS-PAGE of protein hydrolysates derived from Job's tears under various conditions. 1: MW marker; 2: crude protein extract; 3, 4: control enzyme and test reaction at 1 h; 5, 6: control enzyme and test reaction at 2 h; 7, 8: control enzyme and test reaction at 3 h; and A - D: Alcalase, Papain, Pronase, and Trypsin, respectively. Protein loaded at 20 µg/well.

ACE-inhibitory activity of the crude protein and protein hydrolysates

The crude proteins (400 µg) presented a 22.15 ± 0.94% ACE-inhibitory activity (which equaled 53.7 ± 2.3 nmol captopril equiv./mg protein), and a IC_{50} of 0.39 mg/mL (Table 1). The IC_{50} of standard captopril towards ACE is 4.8 nmol; thus 1 mg of the crude proteins exhibited an IC_{50} value ~11.1 times higher than that of captopril.

After the enzymatic hydrolysis of the proteins, most protein hydrolysates exhibited a significant increase in their ACE-inhibitory activity (28 – 78% inhibition) except CLPaH-1h (21.27 ± 0.08% inhibition), which exhibited decreased inhibition as compared to the crude protein (22.15 ± 0.94% inhibition) (Table 1). Even though no significant difference was observed between the protein hydrolysates prepared using protease, CLAlH, CLPaH, and CLTrH exhibited increased ACE-inhibitory activity based on incubation times. CLPrH produced after 2 h of treatment exhibited the highest ACE inhibition. The captopril equivalent values (nmol/mg protein) corresponding to ACE-inhibition increased while the IC_{50} values decreased (Table 1).

Based on statistical analysis, no significant difference was observed between the different protease types. The highest activity against ACE was obtained from protein hydrolysates prepared using Pronase (CLPrH). Moreover, CLPrH exhibited the highest captopril equivalent value (nmol/mg protein), which increased to 190.0 ± 0.5, and the lowest IC_{50} value (0.11 mg/mL), which was about 3.5 times higher than that of the crude proteins. Pronase is a non-specific protease with a broad specificity for proteolysis. Hydrolysis incubation time is an important parameter affecting the release of bioactive peptides from their parent proteins. The *Coix* glutelin hydrolysate prepared using pepsin acted as an ACE inhibitor with an IC_{50} of 52.34 ± 3.71 µg/mL (Yuan *et al*., 2014). In the present work, the *Coix* prolamin hydrolysate prepared using Pronase exhibited an almost two-fold higher ACE inhibition with an IC_{50} of 0.11 ± 0.00 mg/mL. We also performed a 3 h single enzymatic hydrolysis of seeds from Job's tears using

four proteases. The degree of hydrolysis (DH) usually increases with increased hydrolysis time, and ACE-inhibitory activity increases with %DH. We confirmed that ACE-inhibitory activity increased with longer hydrolysis time, except for the protein hydrolysate prepared using Pronase. Therefore, each protein hydrolysate preparation should be optimised (time, E/S ratios). According to Rezvankhah *et al.* (2021a; 2021b), a protease combination or sequential hydrolysis using more than one type of protease may lead to higher DH values when compared with single hydrolysis.

Table 1. ACE-inhibitory activities of the crude protein and its hydrolysates extracted from Job's tears as compared to the ACE inhibitor drug, captopril.

Job's tears	ACE inhibition[a] (%)	Captopril equiv.[b] (nmol/mg prot.)	IC_{50} (mg/mL)
Crude protein	22.15 ± 0.94	53.7 ± 2.3	0.39
CLAlH[c]			
CLAlH-1h	33.62 ± 0.07[d]	81.5 ± 2.0[d]	0.26[e]
CLAlH-2h	55.47 ± 1.35[d]	134.5 ± 3.3[d]	0.16[d]
CLAlH-3h	56.80 ± 0.47[d]	137.7 ± 1.1[d]	0.15[d]
CLPaH			
CLPaH-1h	21.27 ± 0.08	51.6 ± 2.0	0.40
CLPaH-2h	28.01 ± 0.42[d]	67.9 ± 1.0[d]	0.31
CLPaH-3h	51.28 ± 0.15[d]	124.3 ± 0.4[d]	0.17[d]
CLPrH			
CLPrH-1h	46.61 ± 0.47[d]	113.0 ± 1.1[d]	0.18[d]
CLPrH-2h	78.38 ± 0.23[d]	190.0 ± 0.5[d]	0.11[d]
CLPrH-3h	45.97 ± 2.80[d]	111.5 ± 6.8[d]	0.19[d]
CLTrH			
CLTrH-1h	30.28 ± 0.12[d]	73.4 ± 0.3[d]	0.28[d]
CLTrH-2h	54.05 ± 0.12[d]	131.0 ± 0.3[d]	0.16[d]
CLTrH-3h	64.25 ± 0.12[d]	155.8 ± 0.3[d]	0.13[d]

[a]400 µg protein used in assay; [b]IC_{50} of captopril equal to 4.8 nmol; [c]hydrolysate prepared by Alcalase (CLAlH), Papain (CLPaH), Pronase (CLPrH), and Trypsin (CLTrH) (E/S ratio, 1:20 by mass) in suitable conditions recommended for each enzyme for 1, 2, and 3 h; [d] $p \leq 0.001$; and [e] $p \leq 0.01$ *vs*. crude protein.

Fractionation of the prolamin hydrolysate prepared using Pronase

CLPrH-2h (78.38 ± 0.23% inhibition) was further fractionated by filtration through a 3 kDa MWCO membrane. The filtrate fraction exhibiting a MW < 3 kDa exhibited higher ACE inhibition (73.68 ± 0.30%), and was further fractionated using RP-HPLC. The fraction with a MW < 3 kDa had the highest captopril equivalent value (238.2 ± 1.0 nmol/mg protein, IC_{50} = 0.088 mg/mL), which was ~4.4 times higher as compared to its crude proteins. Li *et al.* (2017b) reported that small molecular *Coix* prolamin peptides presented essential immunomodulatory and ACE-inhibitory activities which significantly lowered the systolic blood pressure (SBP) of spontaneously hypertensive rats (SHR).

The peptides were separated into seven sub-fractions (F1 - F7) using RP-HPLC (Figure 2), and each sub-fraction presented ACE-inhibitory activity, as shown in Table 2. F1 exhibited a captopril equivalent value (238.0 ± 4.0 nmol/mg protein) similar to the < 3 kDa fraction, whereas F2, F3, and F4 exhibited higher values (402.7, 315.0, and 380.0 nmol/mg protein, respectively). F5, F6, and F7 presented lower captopril equivalent values (205.0, 205.0, and 197.0 nmol/mg protein, respectively). F2 exhibited the highest captopril equivalent value (402.7 ± 4.7 nmol/mg protein), which was ~7.5 times the value obtained from crude proteins. The lowest

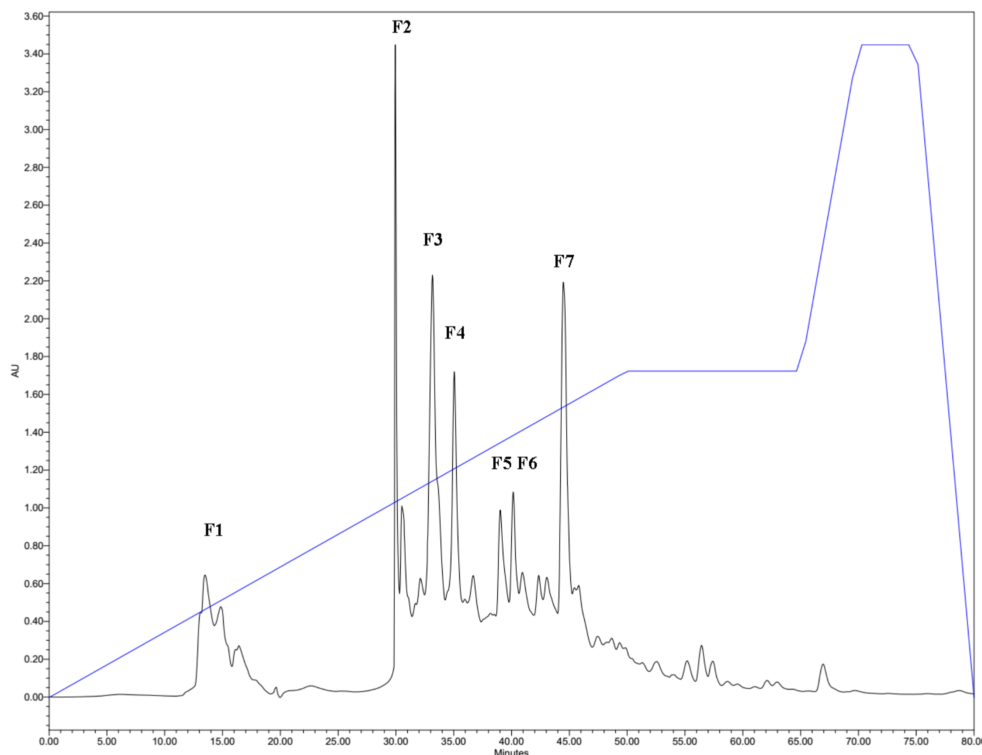

Figure 2. RP-HPLC chromatogram of the Pronase-hydrolysed protein hydrolysates F1 - F7 obtained from Job's tears.

Table 2. ACE-inhibitory activities of the hydrolysates obtained from Job's tears after fractionation using RP-HPLC.

Job's tears	ACE inhibition[a] (%)	Captopril equiv.[b] (nmol/mg prot.)	IC_{50} (mg/mL)
Crude protein	22.15 ± 0.94	53.7 ± 2.3	0.39
CLPrH-2h	78.38 ± 0.23[c]	190.0 ± 0.5[c]	0.11[c]
CLPrH-2h (< 3 kDa)	73.68 ± 0.30[c]	238.0 ± 1.0[c]	0.088[c]
RP-HPLC			
F1	24.51 ± 0.44[d]	238.0 ± 4.0[c]	0.088[c]
F2	41.53 ± 0.49[c]	402.7 ± 4.7[c]	0.052[c]
F3	32.48 ± 0.78[c]	315.0 ± 8.0[c]	0.066[c]
F4	36.05 ± 0.42[c]	350.0 ± 4.0[c]	0.060[c]
F5	21.17 ± 1.11	205.0 ± 11.0[c]	0.102[c]
F6	21.13 ± 0.75	205.0 ± 7.0[c]	0.102[c]
F7	20.28 ± 0.40	197.0 ± 4.0[c]	0.106[c]

[a]30 and 20 µg protein used in assay of CLPrH (< 3 kDa) and RP-HPLC fractions, respectively; [b]IC_{50} of captopril equal to 4.8 nmol; [c] $p \leq 0.001$; and [d] $p \leq 0.01$ *vs*. crude protein.

IC_{50} value of F2 was 0.052 mg/mL (Table 2). Our results indicated that the ACE-inhibitory activity of CLPrH-2h-F2 was comparable to the pepsin-prepared glutelin hydrolysate (IC_{50} of 52.34 ± 3.71 µg/mL; Yuan *et al.*, 2014). However, according to Chen *et al.* (2020), *Coix* prolamin peptides prepared by pepsin hydrolysis for 2 h, and separated using a DEAE Sepharose Fast Flow column and a Sephadex G-10 column followed by RP-HPLC exhibited higher ACE-inhibitory activities as compared to our results. Further analysis of the novel peptides from each protein hydrolysate that act as ACE inhibitors is needed in order to identify the relationship between their structure and function, as well as their inhibitory

mechanisms. Further peptide synthesis is required to confirm ACE-inhibitory activity, with IC_{50} values reported in µmol/L for comparison with other antihypertensive peptides, such as the pepsin-prepared GAAGGAF from *Coix* glutelin (IC_{50} = 14.19 ± 0.89 µmol/L; Li *et al.,* 2017a). Chen *et al.* (2020) reported the isolation of a novel peptide (VDMF) from *Coix* prolamin exhibiting ACE-inhibitory activity that influences the gene expression of the RAS signaling pathway. Moreover, future work should include further *in vivo* studies on blood pressure as well as the development of nutraceuticals and pharmaceuticals using active compounds deriving from the seeds of Job's tears.

Conclusion

The crude protein and prolamin fractions from the seeds of Job's tears exhibited ACE-inhibitory activity, whereas enzymatic protein hydrolysates exhibited a higher activity. The protein hydrolysate prepared using Pronase (2 h) presented the highest activity, and was further fractionated into seven sub-fractions using RP-HPLC. Among them, F1, F2, F3, and F4 exhibited higher ACE-inhibitory activity as compared to the crude protein. F2 presented the highest captopril equivalent value of 402.7 ± 4.7 nmol/mg protein (IC_{50} = 0.052 mg/mL), which was ~7.5 times higher as compared to that of the crude protein. The present work demonstrated that the storage protein derived from the seeds of Job's tears could be promising as a functional food ingredient for lowering high blood pressure.

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