STUDY OF ANGIOTENSIN-I CONVERTING ENZYME INHIBITORY PEPTIDES DERIVED FROM *Ficus pumila var. awkeotsang* SEEDS HYDROLYSATE

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ABSTRACT

Hypertension also known as high blood pressure that has associated with multi-metabolic pathways such as the renin-angiotensin-aldosterone system (RAAS). There have been several synthetic ACE inhibitory drugs used clinically as therapeutics for hypertension. However, synthetic drugs showed side effects on human health after long-term use. Therefore, alternative natural ACE inhibitors from food-derived are necessary to develop. The study aimed to examine the activity of angiotensin-I converting enzyme inhibitory peptides derived from *Ficus pumila var. awkeotsang* seeds hydrolysate and to study the efficiency of enzymatic digestion. This study used four enzymes (trypsin, pepsin, thermolysin, and chymotrypsin) to release bioactive peptides from *Ficus pumila var. awkeotsang* seeds that could exhibit an antihypertensive effect. The results showed that *Ficus pumila var. awkeotsang* trypsin hydrolysate has the highest ACEI activity, 90.37%, and the IC_{50} reached 169±3.5 μg/mL. Then, the hydrolysate produced by trypsin has the highest degree of hydrolysis, 4%, after 8 hours of incubation. Hence, *Ficus pumila var. awkeotsang* seeds hydrolysate could develop as an alternative natural product that is responsible for ACE-inhibitory activity.

Keywords: ACE inhibitor; Enzymatic digestion; *Ficus pumila var. awkeotsang*

INTRODUCTION

Angiotensin-converting enzyme (ACE) is one of the Renin-Angiotensin-Aldosteron System components that is responsible for maintaining blood pressure and fluid balance (Boschin et al., 2014), controlling the ACE activity for converting angiotensin I to form Angiotensin II could prevent vasoconstricting blood vessel (Murray and Fitzgerald, 2007). It occurs in the blood circulatory system as well as in the body organs such as the aorta, kidneys, lungs, and brain. Hypertension is one of the cardiovascular diseases that have systolic blood pressure above 130 mmHg and diastolic blood pressure above 80 mmHg (Flack and Adekola, 2020). Indonesia is one of the South East Asia countries that has a high number of hypertension sufferers. Fast food consumption, inactivity, a sedentary lifestyle, smoking, and other factors all contribute to hypertension (Iwaniak et al., 2014). According to the World Health Organization (WHO, 2022), hypertension sufferers are found in low- and middle-income countries, the estimated 1.28 billion adults aged 30-79 years worldwide. Currently, there is a synthetic drug that is used for antihypertensive therapeutic agents such as captopril, lisinopril, and enalapril (Ondetti et al., 1977). However, these kinds of synthetic drugs have many side effects on human health, such as cough, headache, fever, and insomnia (Atkinson and Robertson, 1979). An alternative way to inhibit angiotensin-converting enzyme was necessary to develop from food-derived to produce safe and natural antihypertensive therapeutic.

Ficus pumila var. awkeotsang is one of the indigenous plants of Taiwan that can be grown in mid-to high-altitude mountains (Hu et al., 2022). *F. pumila* is known as jelly fig, and the famous beverage Aiyu jelly was made from *F. pumila* seeds. Besides, *F. pumila* has been characterized by many research, and the results obtained were performed on the database https://www.uniprot.org/. According to the previous study, *F. pumila* also has various biological activities such as anti-inflammatory, antimicrobial, antimutagenic, active phytochemicals, analgesic, antioxidant, analgesic, antiproliferative, and hypoglycemic properties (Revathi et al., 2021) In the previous study, some seeds have antihypertension properties such as soybean (Vallabha and Kaultiku, 2014), winter melon seeds (Priyanto et al., 2015), black cumin (Sutopo et al., 2020), lentil (Garcia-Mora et al., 2015), walnut (Liu et al., 2013; Wang et al., 2014), peanut (White et al., 2014), date seed (Ambigaipalan et al., 2015), hemp seed (Malomo et al., 2015), and others. Therefore, the exploration of biological activity derived from *F. pumila* as an anti hypertensive is also necessary for further study. To examine this activity, the release of bioactive peptides should be conducted. There are two ways to release bioactive peptides: by fermenting foods as they are processed and by hydrolyzing substances with one or more enzymes (Ningrum et al., 2022; Rai et al., 2017).

In this study, bioactive peptides will be released using an enzymatic process. Some of the enzymes have been used to explore novel bioactive peptides that have antihypertensive biological activity with higher inhibiting towards Angiotensin-converting enzyme (ACE) activity (Rai et al., 2017). Several various enzymes are reported to release bioactive peptides such as trypsin, chymotrypsin, thermolysin, pepsin, alcalase, papain, neutrase, and bacterial and fungal protease (Boschin et al., 2014; Ningrum et al., 2022; Priyanto et al., 2015; Sutopo et al., 2020). Each enzyme also has a specific preferential cleavage site. Hence, it could produce a unique sequence of bioactive peptides (Daskaya-Dikmen et al., 2017). Due to the specific preferential cleavage site of an enzyme, the consideration of ratio enzyme substrate, time, and temperature were important for the substrate hydrolysis process (Priyanto et al., 2015). Hence, this study aimed to examine the activity of angiotensin-I converting enzyme inhibitory peptides derived from *Ficus pumila var. awkeotsang* seeds hydrolysate and to study the efficiency of the trypsin digestion process.

MATERIALS AND METHODS

Materials and Chemical Reagents

The main material of this study was *Ficus pumila var. awkeotsang*, which was originally planted in Taiwan. The chemicals reagents such as ACE (EC 3.4.15.1) from rabbit lungs, hippuryl-L-histidyl- L-leucine (HHL), ferulic acid (FA), sodium chloride (NaCl), and sodium hydroxide (NaOH) Trypsin (from bovine pancreas), α-chymotrypsin (from bovine pancreas), thermolysin (from Geobacillus stearothermophilus), pepsin (from porcine gastric mucosa), acetic acid trichloroacetic acid (TCA), were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Boric acid, formic acid (HCO_2H, FA) and acetonitrile (ACN), Sodium dedocyl sulfate (SDS) were purchased from J.T. Barker (Phillipsburg, NJ, USA). The other chemicals were analytical grade.

Protein Preparation From *F. pumila*

Ficus pumila var awkeotsang seeds were crushed using a grinding machine. The process of protein isolation in this study was carried out based on the previous study (Sutopo et al., 2020). After grinding, the smaller size of *Ficus pumila* seeds was then continued with an added 1% SDS. The mixture was subjected to an Ultrasound machine (Branson Digital Sonifer®, Terra Universal Inc., Fullerton, CA, USA) to obtain the optimum protein yield. The setting up of the amplitude was chosen to be 30% for 10 seconds 18 times. The mixture solution was altered using centrifugation at 13,000 rpm for 5 min, and then the protein supernatant was obtained. 20% trichloroacetic acid was added in an acetone ratio of 1:1 (v/v) and then incubated for 12 h at 4 ◦C. The residue

was washed and lyophilized to obtain the protein powder.

F. pumila Enzymatic Digestion

F. pumila seeds contain various types of protein, as shown at https://www.uniprot.org/taxonomy/204231. It is potentially a source of bioactive peptides. One of the methods to release bioactive peptides was enzymatic hydrolysis. *Ficus pumila* protein powder obtained from the previous steps was hydrolysed using four various enzymes such as thermolysin, α-chymotrypsin, trypsin, and pepsin. This step aimed to obtain the smaller size of the peptide, which is due to the smaller peptide having a higher ACE-inhibitory activity. The ratio of enzyme: protein powder is 1:50 (w/w) using a different buffer that is suitable for those enzymes. Thermolysin, α-chymotrypsin, and trypsin used 50 mM ammonium bicarbonate as a buffer and adjusted the pH to 8, while pepsin used 20 mM NaCl at pH 1.3 using 4 M HCl. The mixture solution was incubated to maximize the activity of the enzyme-used substrate (peptide). These enzymes (pepsin, α-chymotrypsin, trypsin) have the optimum condition at 37◦C, then thermolysin enzyme mixture at 60◦C, and the incubation was conducted for 16 h. The reaction was quenched with boiled water for 10 minutes and centrifugated at 4◦C for 15 minutes at 13,000 rpm. The supernatant was subjected to an ultrafiltration membrane (3 kDa MWCO) to separate the residue of an enzyme that was left in the *F. pumila* hydrolysate.

Determination of ACE-Inhibitory Activity

According to the previous study (Cushman and Cheung, 1971), with some modifications, this study's ACE-inhibitory activity was determined. Hippuryl-L-histidyl-L-leucine (HHL) 2.5 mM was diluted in 30 mL of water, and 10 mL of *F. pumila* hydrolysate that had been dissolved in a 200 mM borate buffer containing 300 mM NaCl was then added (adjusted to pH 8.3). A blank sample (without protein hydrolysate) and positive control (hypertension medication commercial: captopril) were added with the same volume (10 µL) in the other tube in order to compare the outcomes. All samples were pre-incubated at 37°C for 5 min to achieve the best conditions for each enzyme, and then ACE (0.05 mU/L) in 20 L of borate buffer was added (200 mM). For chymotrypsin, trypsin, and other enzymes, the static incubation was carried out at 37°C for 30 min, respectively, for chymotrypsin, trypsin, and pepsin hydrolysate while thermolysin hydrolysate at 60◦C for 30 min. This procedure was carried out in a thermostatic incubator at 200 rpm for 30 minutes. Using 60 mL of 1 M HCl, the incubation process was quenched throughout. Using RP-HPLC and a C18 column, the quantitative study of ACE-I activity from *F. pumila* hydrolysate revealed HHL and HA area as the product hydrolysis of ACE towards the substrate. An isocratic elution of 18% mobile phase A (5% ACN and 0.1% TFA in deionized water) was used to separate the hydrolysate mixture for 25 minutes at a constant flow rate of 1 mL/min. The value of ACE inhibition was calculated using the equation below:

$$\text{ACE Inhibition (\%)} = \left[1 - \left(\frac{\Delta A \text{Inhibitor}}{\Delta A \text{Blank}}\right)\right] \times 100\% \quad \text{.....(1)}$$

Where, ΔA Inhibitor and ΔA Blank were the peak areas of HA in testing and blank samples, respectively.

Measurement of _F. pumila_ Hydrolysate IC_{50}

Five different concentrations of the *F. pumila* hydrolysate that exhibit the highest ACE-I activity were used to calculate the IC_{50}. GraphPad Prism 8.2.1 (GraphPad Software, Inc.) was used to investigate the value of the IC_{50} at five distinct concentrations using a nonlinear regression of the percentage of ACE inhibition produced by various inhibitor concentrations.

Degree of Hydrolysis

The degree of hydrolysis used to determine the ability of enzyme hydrolysis was performed according to a previous report by a previous study (Sutopo et al., 2020). DH was calculated based on the equation:

$$DH\ (\%) = (ht/htot) \times 100\% \quad \text{.....(2)}$$

Where:

ht : The concentration of amino acids released after a specific time

htot : The total amino acid concentration of *Ficus pumila var. awkeotsang protein*

Statistically Analysis

To find the substantially different enzyme treatments, all the data collected for this investigation were statistically analyzed using Minitab 17. The P value for statistical significance was chosen at lower than 0.05. Graphpad 8.2.1 version established the graph and the IC_{50} (GraphPad Software, Inc.).

RESULTS AND DISCUSSION

The natural plant *F. pumila* lives on Taiwan's plains. However, little is known about its properties as a source of protein. However, various kinds of amino acids are the building blocks of proteins that have the potential bioactive peptides in this study. For this reason, it is necessary to process the release of bioactive peptides from their parent protein in the right way. The release of bioactive peptides could be carried out by fermentation and enzymatic hydrolysis. In this study, enzymatic hydrolysis was conducted to release bioactive peptides that have biological activities, especially antihypertensive activity.

There are four enzymes for releasing bioactive peptides: trypsin, chymotrypsin, thermolysin, and pepsin. Each enzyme has a preferential cleavage site to produce bioactive peptides that are responsible for inhibiting the Angiotensin-converting enzyme. According to Kim et al. (1999), the unique sequenced peptide with enhanced ACE activity could be created by the enzyme's distinct favored cleavage site. For instance, trypsin would be hydrolyzed lys and Arg at C-terminal (Kim et al., 1999), Chymotrypsin would be hydrolyzed Tyr, Trp, Phe, Leu, Asn, His, and Met at C-terminal and Ile N-terminal (Jung et al., 2005; Jung et al., 2006), Thermolysin would be hydrolyzed Ala, Leu, Ile, Val, Tyr, Phe in N-terminal (Matsubara, 1966), as well as for the pepsin would be hydrolyzed Phe, Trp, Leu and Tyr at C-terminal (Murray and Fitzgerald, 2007; Sornwatana et al., 2015). It has been hypothesized that hydrophobic amino acids like Try, Phe, Trp, Ala, Ile, Val, and Met, as well as positively charged amino acids like Arg, Lys, and Pro at the C terminal position of the peptides, may be associated with a higher affinity with ACE (Rai et al., 2017). Moreover, that peptide may be superior in terms of ACE-inhibitory activity due to the aliphatic and hydrophobic residues at its N-terminus, a basic Arg residue in the middle, and an aromatic Tyr at the C-terminus (Moayedi et al., 2017). The result obtained in this study about the ACE-I activity exhibited from each enzyme hydrolysate is shown in Figure 1.

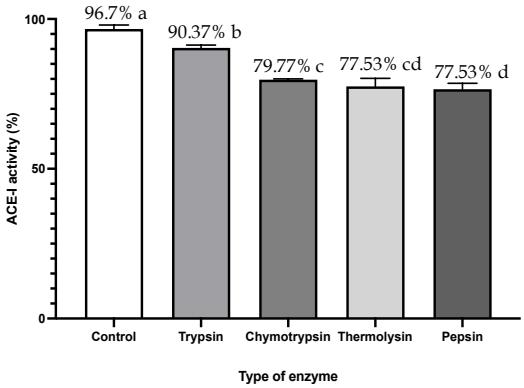

Figure 1. ACE-I Activity of *F. pumila* Hydrolysates

Figure 1 shows that trypsin hydrolysate had the highest activity in inhibiting angiotensin-converting enzyme activity. 90.37% among the other hydrolysates, followed by chymotrypsin, thermolysin, and pepsin hydrolysates, 79.77%, 77.53%, and 76.57%, respectively. Among the *F.pumila*, hydrolysate has a significant difference in ACE-I activity (p-value <0.05). Captopril was used as a control due to this drug's therapy has been used for long-term antihypertension. The ACE inhibitory activity was affected by the characteristics of the bioactive peptide, such as amino acid type and the length of the sequenced peptide. According to this study (Natesh et al., 2003), the bioactive peptide

involved 3-12 amino acids, and the longer chain of bioactive peptide would exhibit lower inhibition activity because some of the amino acids couldn't bind to the active site of ACE. Captopril can significantly inhibit ACE activity because five hydrogen bonds of captopril have been bonded to the ACE active site such as His 353, Glu 384, Lys 511, His 513, and Tyr 520 (Ningrum et al., 2022).

In this study, The IC_{50} value is an inhibitor that could inhibit 50 percent of the ACE activity. The higher ACE-I activity would be shown on the lower IC_{50} value. Due to *F. pumila* trypsin hydrolysate showing the highest ACE-I activity, it was continued to determine the IC_{50} value using several concentrations: 0.5, 0.75, 1, 1.5, and 2 mg. Based on the results obtained, it could be seen that the IC_{50} value is 169.5±3.5 µg/mL in Figure 2.

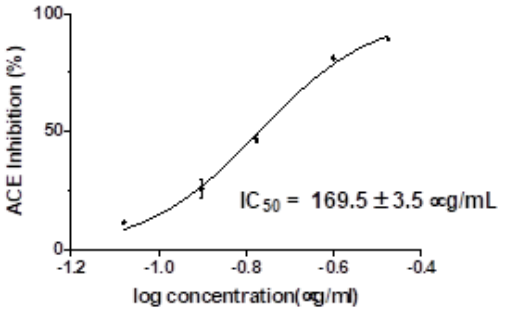

Figure 2. IC_{50} of *F. pumila* Trypsin Hydrolysate

Based on several studies, ACE-I inhibitory bioactive peptides derived from various seeds also exhibited ACE-Inhibitory activity, such as soybean thermolysin hydrolysate with an IC_{50} value of 51.8 µg/mL black cumin chymotrpsin hydrolysate 34.4 µg/mL (Sutopo et al., 2020), walnut proteinase hydrolysate 25.67 µg/mL (Wang et al., 2014), date alcalse hydrolysate 530 µg/mL (Ambigaipalan et al., 2015), peanut alcalse hydrolysate µg/mL (White et al., 2014), hemp seed hydrolyzed by combination of enzymes (alcalase, pepsin, papin and pepsin-pancreatin) with an IC_{50} value of 16-228 µg/mL (Malomo et al., 2015). Those various IC_{50} derived from some seeds that hydrolyzed different enzymes are associated with the N-terminal positions of peptides with aromatic residues (such as Tyr, Phe, Trp, His, or Pro) or hydrophobic residues (such as Ile, Val, or Leu) have a significant ACEI activity (Daskaya-Dikmen et al., 2017). The *F. pumila* trypsin hydrolysate has the highest ACE-inhibitory activity due to the fact that it might contain those sequenced peptides. This process is desired to continue to the purification stage to obtain bioactive sequences peptides that are more specific in inhibiting ACE performance.

In this study, trypsin activity to hydrolyze protein from *F. pumila* was examined. The degree of hydrolysis was measured by incubating the *F. pumila* protein extract as a substrate with trypsin for 16 hours. To determine the hydrolysis degree of trypsin, an L-leucine regression curve was made at five various concentrations (0.2; 0.5; 1; 1.5; 2; and 2.5 mM in 1% SDS), as shown in Figure 3. The regression formula obtained was used as a reference to determine the hydrolysis degree of trypsin.

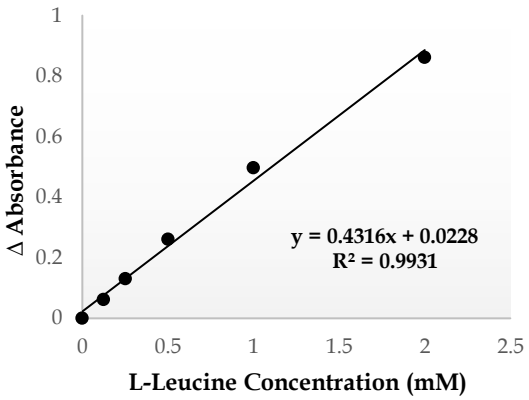

Figure 3. Standard Curve of L-leucine

The degree of hydrolysis measurement was carried out every 4 hours, incubating during a total time of incubation of 16 hours, as shown in Figure 4. The results obtained revealed that the highest degree of trypsin hydrolysis is after incubating 8 hours with a DH value of 4%. This value explains that in the 8th hour, trypsin has the highest activity in binding with the crude protein extract of *F. pumila* in order to release bioactive peptides. The statistical analysis also showed significantly different values between the 8th hour and with others.

According to the previous study by Sutopo et al. (2020), the DH value has correlated with the ACE-I activity value, where the higher the DH value is produced, the more potent the bioactive peptide from the substrate.

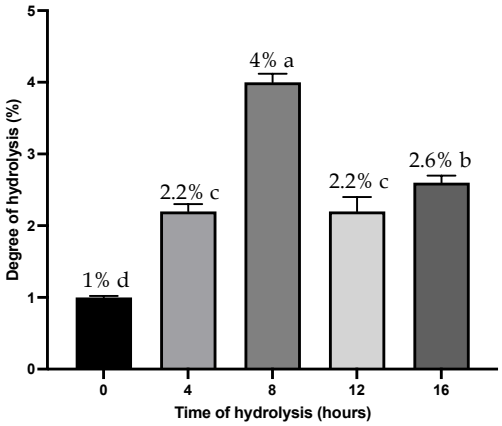

Figure 4. Degree of Hydrolysis Trypsin to Crude Protein Extract of *F. pumila*

As can be seen in Figure 4, The enzyme makes the most of the high substrate concentration to create the enzyme-substrate conformation, which results in a shorter sequence of bioactive peptides at the end of the reaction. These reactions happen due to the altered incubation period and temperature. The optimization of this process is necessary to obtain the highest efficiency of hydrolysis. Based on the previous study by Cheison et al. (2011), trypsin has optimally hydrolyzed conditions at 37^{o}C and pH 7.8. Besides, A specific pH range can influence the degree of substrate and enzyme dissociation and encourage enzyme binding to substrates (Sun et al., 2019). In the previous study, the degree of hydrolysis that has the highest value (9.5%) after 10 hours was examined. The value is higher than our study; it's due to the amount of peptide in the seed and the epidermis structure of the seed.

CONCLUSION

Ficus pumila var. awkeotsang trypsin hydrolysate has the highest ACEI activity, 90.37%, and the IC50 reached 169±3.5 µg/mL. Then, the hydrolysate produced by trypsin has the highest degree of hydrolysis, 4%, after 8 hours of incubation. This value showed a significant difference from other times of hydrolysis (p-value <0.05). Hence, *Ficus pumila var. awkeotsang* seeds hydrolysate could develop as an alternative natural product that is responsible for ACE-inhibitory activity.

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