# Bioactive Peptide Tempe Made from *Mucuna pruriens* (L) DC as an Inhibitor of Angiotensin-I-Converting Enzyme (ACE) in a Digestion Simulation

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**ABSTRACT:** *Mucuna pruriens* (L) DC tempe is a food that functions as an inhibitor of the angiotensin-I-converting enzyme (ACE). The purpose of this research was to study the activity of *M. pruriens* tempe peptides during the digestive process *in vitro* with pepsin-pancreatin, and absorption of peptides in the small intestine using the inverted intestinal sac method. Our results show that *M. pruriens* had the highest ACE-inhibiting activity after digestion *in vitro* after fermentation for 72 h (F72). F72 peptide absorption (%) and ACE-inhibitory activity of the absorbed peptides did not significantly differ between the different segments of the small intestine (duodenum, jejunum, and ileum). These results demonstrate that F72 tempe maintains ACE-inhibitory activity in each segment of the small intestine after both digestion and absorption.

**Keywords:** ACE inhibitors, *Mucuna pruriens* (L) DC, peptide absorption, simulated digestion, tempe

## INTRODUCTION

Hypertension results from the renin-angiotensin system, which is responsible for controlling blood pressure. Activation of this system begins when the enzyme rennin enters the bloodstream and produces the peptide angiotensin I. Angiotensin I is channeled into the small vessels in the lungs and is hydrolyzed into the shorter peptide angiotensin II by angiotensin-I-converting enzymes (ACE). Angiotensin II is a vasoconstrictive hormone that can increase aldosterone to cause hypertension. ACE also causes degradation of the vasodilator bradykinin, which has a role in reducing blood pressure (Iwaniak et al., 2014).

ACE inhibitors are common drugs for hypertension that inhibit the effects of angiotensin II. Some ACE inhibitor medications are associated with side effects such as lethargy, headaches, diarrhea, coughing, and nausea (Sangole and Dadkar, 2010). In contrast, ACE inhibitors derived from natural ingredients have fewer side effects than synthetic ACE inhibitors (Kumar et al., 2010). *Mucuna pruriens* (L) DC. is a natural ingredient and a potential ACE inhibitor (Chalé et al., 2014; Tuz and Campos, 2017; Rahayu et al., 2019).

*Koro benguk* [*M. pruriens* (L) DC.] is a local Indonesian legume that shows potential as an antihypertensive agent. Chalé et al. (2014) reported that a <1 kDa peptide fraction of *M. pruriens* generated from enzymatic hydrolysis with pepsin-pancreatin exhibits high inhibitory activity against ACE [half maximal inhibitory concentration ($IC_{50}$) 19.5 μg/mL]. Further, single, short-term administration of low-dose *M. pruriens* hydrolysate reduces systolic and diastolic blood pressure and provides hypotensive effects in rats (Chalé et al., 2014). This is because *M. pruriens* contains hydrophobic amino acids such as Ala, Ile, Leu, and Phe (Tuz and Campos, 2017), and many peptides that contain residues of hydrophobic amino acids (Tyr, Phe, Trp, Ala, Ile, Val, and Met) can act as ACE inhibitors (Iwaniak et al., 2014).

*M. pruriens* consists of 33.8% protein, whereas soybean consists of 46.3% protein (Handajani, 2001). Since the protein content of *M. pruriens* protein is relatively high, it is used as an alternative raw material for tempe in Central Java, Indonesia. Tempe is made through a fermentation process using *Rhizopus oligosporus*. Rizzello et al. (2016) reported that this process can produce ACE-inhibitory peptides through hydrolysis by the protease produced by the microorganisms used in fermentation. Fur-

thermore, Rahayu et al. (2019) showed that the fermentation time of *M. pruriens* tempe affects the ACE-inhibitory activity of the peptides produced, with an optimum duration of 72 h. Jakubczyk et al. (2013) reported that fermentation also influences the inhibitory activity of ACE peptides after *in vitro* digestion using pepsin-pancreatin. However, the ACE-inhibitory activity of *M. pruriens* tempe peptides after digestion has not been fully described. This research aimed to study the inhibitory activity of the ACE peptide tempe of *M. pruriens* (L) DC in the digestive system through *in vitro* digestion with pepsin-pancreatin, and absorption of peptides in a segment of the small intestine using an inverted intestinal sac.

## MATERIALS AND METHODS

### Materials
*M. pruriens* (L) DC beans were obtained from farmers in Yogyakarta, Indonesia. Fermentation was carried out using "raprima", a commercial tempe innoculum that contains *R. oligosporus*, which was purchased from the Yogyakarta market. ACE from rabbit lungs, pepsin from porcine gastric mucosa (EC. 3.4.23.1), pancreatin from porcine pancreatic mucosa (EC. 232-468-9), and hippuryl-L-histidyne-leucine (HHL) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sprague-Dawley rats (±250 g, male, three months) were purchased from the Inter-University Center, Universitas Gadjah Mada, Yogyakarta, Indonesia.

### Preparation of tempe fermentation
Fermentation of *M. pruriens* tempe was prepared following the methods described by Rahayu et al. (2019) and Puspitojati et al. (2019), based on the traditional methods used in Yogyakarta, Indonesia. To determine the most effective fermentation time for producing high ACE-inhibitory activity, fermentation was carried out for 48 h (F48) and 72 h (F72); there were also not fermented (NF) groups, where raprima was not added. After fermentation, the samples were freeze-dried and made into a powder.

### *In vitro* digestion simulation
*In vitro* digestion simulations were performed based on the method described by Minekus et al. (2014), with a slight modification. Five mg/mL of each sample was brought to pH 3, and pepsin (2,000 U/mL) was added. Reactions were carried out at 37°C for 2 h and analyzed every 30 min. Hydrolysates from the simulation at 120 min were used for a simulation of duodenal digestion. The solution was then brought to pH 7.5 through addition of 2.0 N NaOH, and pancreatin (100 U/mL) was added. Reactions were then incubated for 2 h at 37°C

and samples were taken every 30 min for analysis. The hydrolysis reaction was then stopped by heating to 80°C for 10 min, and the solution was brought to pH 7 with 2 N NaOH. The hydrolysate was centrifuged at 8,000 *g*, 4°C for 10 min, and the supernatant was stored at −25°C until use.

### Peptide absorption with the reverse intestinal sac technique
The reverse intestinal sac technique was performed based on the method described by Amenta et al. (2018), with a slight modification. Sprague-Dawley rats were placed in separate cages (20×20×24 cm) at room temperature in a 12 h dark/light cycle. Before intramuscular injection of ketamine (0.06 mg/g rat) to induce sedation, rats were fasted for 20~24 h but were allowed to drink *ad libitum*. With the head far away from the secant, incisions were made into the abdominal wall, cutting the skin and musculature, proceeding to the right and left sides, and cranially cutting the costae so that the thoracic cavity was opened. The intestine was divided into three segments: the duodenum, jejunum, and ileum. To extract the three segments, the intestine was removed 1 cm from the pylorus up to 5 cm above the ileocecal valve. The U-shaped part of the intestine flanking the pancreas is the duodenum, the jejunum lies between the duodenum and the ileum, and the final segment is the ileum. Each segment was washed with 0.9% NaCl and quickly inverted using a glass rod. Each intestine was then filled with 0.9% NaCl, which is a serosal fluid, and the two end segments were bound. Each intestinal segment was inserted until submerged into tubes containing *M. pruriens* tempe hydrolysate solution (5 mg/mL). As a control, leucine (5 mg/mL) was prepared in the same manner. The tubes were incubated in a water bath shaker at 37°C and administered oxygen (95% $O_2$ and 5% $CO_2$) for 120 min. The amount of peptides absorbed into the reverse intestines were sampled every 20 min for analysis. The inverted intestine was then opened, and the serosal fluid was extracted (centrifuged at 13,500 *g* at 40°C for 15 min) and kept at −25°C until use. Fluid that was not absorbed as mucosal fluid was extracted. All animal experiments were approved by the Medical and Health Research Ethics Committee (Ref. No: KE/0338/EC/2019), Faculty of Medicine, Universitas Gadjah Mada, Yogyakarta, Indonesia.

### Analysis methods
*Degree of hydrolysis (DH)*: The DH was calculated by determining free amino groups following the method described by Nielsen et al. (2001).
*Peptide fractionation using dialysis*: Peptide fractionation using dialysis was carried out based the methods described by Sadegh Vishkaei et al. (2016) and Ghanbari et al. (2015). Dialysis membranes had molecular weight (MW)

cut off values of 1,000, 3,500, or 14,000 Da. All peptide fractions were freeze-dried and stored at 25°C until use.

### ACE-inhibitory activity

ACE-inhibitory activity was measured based on the method of Cheung et al. (1980) using HHL (8 mM HHL in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid containing 300 mM NaCl at pH 8.3) as a substrate (Puspitojati et al., 2019). ACE activity was calculated at λ=228 nm using the following equation:

$$\text{ACE inhibitory activity (\%)} = \frac{\text{Ac} - \text{As}}{\text{Ac} - \text{Ab}} \times 100$$

where Ac is the absorbance of the control (HHL+ACE), As is the absorbance of the sample (HHL+ACE+sample), and Ab is the absorbance of the blank (HHL+sample).

### Statistical analysis

The data were statistically analyzed using one way ANOVA and SPSS IBM ver. 23 software (IBM Corp., Armonk, NY, USA). Duncan's multiple range test was used to determine differences between treatment groups, and $P<0.05$ considered significant.

## RESULTS AND DISCUSSION

### *In vitro* simulation of digestion

*In vitro* simulation of digestion was carried out by adding pepsin followed by pancreatin, the order in which these enzymes are released during digestion in the body. Then, the DH, peptide content, and ACE-inhibitory activity were analyzed.

### DH

*M. pruriens* tempe F48, F72, and NF showed relatively similar patterns of hydrolysis (Fig. 1A). Hydrolysis was increased a greater amount with pancreatin than with pepsin. Pancreatin is a mixture of the digestive enzymes trypsin, chymotrypsin, and elastase. Therefore, pancreatin contains more enzyme specifications and produces a higher DH than pepsin (Salces, 2015). Lo and Li-Chan (2005) also reported that the DH after digestion with pepsin is lower than that of pancreatin (10% versus 40%, respectively).

The DH was higher for *M. pruriens* tempe NF than for the other groups, possibly because the NF legume still contained long polypeptides so was hydrolyzed to a greater extent. *M. pruriens* tempe F72 yielded the smallest DH value of the experimental groups, likely because the long fermentation would have produced the smallest peptides, thus only allowing a small DH. Jakubczyk et al. (2013) reported a similar finding for pea protein whereby DH was decreased with longer durations of fermentation.

### Peptide hydrolysate concentration

Peptide concentrations during digestion with pepsin-pancreatin showed similar patterns as those for the DH (Fig. 1B). It is reasonable to assume that a higher DH results in greater peptide concentrations. Following pepsin hydrolysis, tempe F72 showed the highest peptide content, probably because its peptides more closely fall within the cutting specifications of pepsin, causing it to be hydrolyzed to a greater extent. The longer fermentation time of tempe F72 compared with tempe F48 probably also resulted in higher peptide concentrations. It is likely that the peptide concentration of *M. pruriens* NF was lower than the others after treatment with pepsin because the hydrolysis that occurs during the fermentation process produces fewer but longer polypeptides rather than a greater number of shorter peptides. However, *M. pruriens* NF had a higher peptide concentration following hydrolysis with pancreatin. This may be because the DH of NF with pancreatin was higher than the DH of tempe F48 and F72.
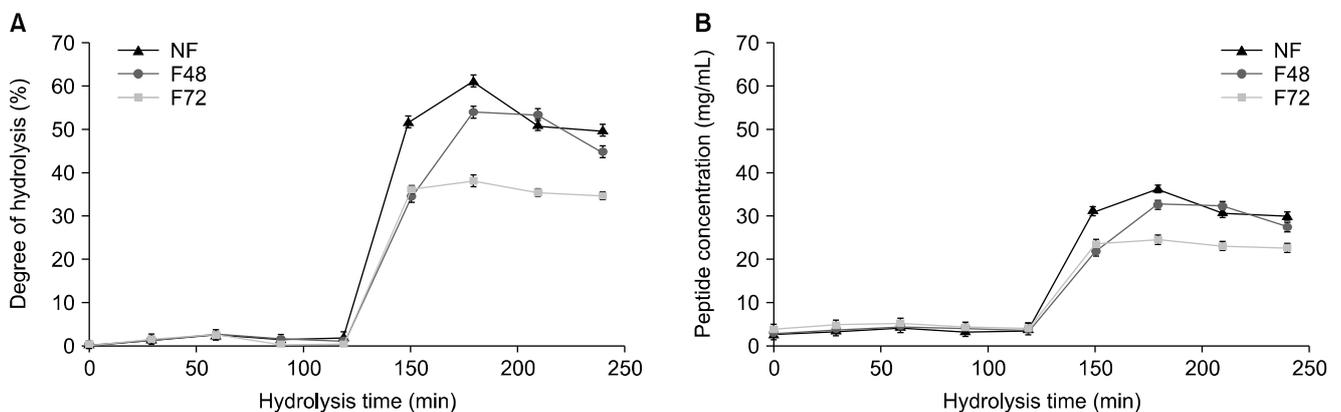


**Fig. 1.** Degree of hydrolysis (A) and peptide concentration (B) of *Mucuna pruriens* tempe in a simulation of digestion with pepsin-pancreatin. ▲, not fermented *M. pruriens* (NF); ●, 48 h fermented *M. pruriens* (F48); ■, 72 h fermented *M. pruriens* (F72). Data averages three replications.
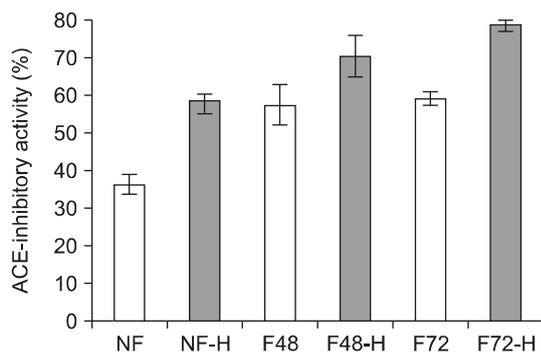
**Fig. 2.** Angiotensin-I-converting enzyme (ACE)-inhibitory activity (%) of *Mucuna pruriens* tempe after simulation of digestion. NF, non-fermented *M. pruriens* tempe; NF-H, hydrolysate of non-fermented *M. pruriens* tempe; F48, 48 h *M. pruriens* tempe fermentation; F48-H, hydrolysate of 48 h *M. pruriens* tempe fermentation; F72, 72 h *M. pruriens* tempe fermentation; F72-H, hydrolysate of 72 h *M. pruriens* tempe fermentation. Data averages three replications.

### ACE-inhibitory activity

Compared with respective pre-digestion states, the hydrolysates of tempe F48, F72, and NF after digestion showed increased ACE-inhibitory activity (Fig. 2). The greatest increase in ACE-inhibitory activity was observed for *M. pruriens* NF. NF had a higher DH and higher peptide concentrations, so the increase of ACE-inhibitory activity was greater than those of the other tempe *M. pruriens* groups. However, overall tempe 72 showed the highest ACE-inhibitory activity of the experimental groups. This is likely because the fermentation and digestion processes produce more peptides with low molecular weights, therefore the ACE-inhibitory activity is higher. Rahayu et al. (2019) reported that *M. pruriens* tempe fermented for 72 h possesses the highest percentage of peptides with MW < 1 kDa.

### Peptide fractionation with dialysis

The data above show that a higher DH (resulting in shorter the peptides) corresponds with higher ACE-inhibitory activity. We therefore performed fractionation based on the molecular weights using dialysis membranes. The re-

**Table 1.** Composition of peptide fractions by molecular weight, ACE-inhibitory activity, and peptide hydrolysate fraction of tempe F72 *Mucuna pruriens* (L) DC

| Fraction | Percentage of peptide (%) | ACE-inhibitory activity (%) |
|---|---|---|
| >14 kDa | 2.39 | 51.82±0.36[a] |
| 3.5~14 kDa | 2.53 | 52.86±2.57[a] |
| 1~3.5 kDa | 5.53 | 66.14±0.73[b] |
| <1 kDa | 89.53 | 79.43±3.35[c] |

Data are expressed as mean±SD of three replications. Different letters (a–c) in the same column show statistical differences ($P<0.05$).

sults show that 89.53% of peptides have a MW<1 kDa and highest ACE-inhibitory activity (Table 1). This suggests that peptides of tempe F72 with high MW were hydrolyzed during digestion by pepsin-pancreatin to produce a greater number of peptides with a low molecular weight. This result is similar to that reported by Chalé et al. (2014) in which peptides<1 kDa had the highest ACE-inhibitory activity due to containing more hydrophobic amino acids. In addition, peptides with low MW more easily bind to the active site of ACE than peptides with a higher molecular weights (Pina and Roque, 2009; García-Mora et al., 2017).

### Peptide absorption using reverse intestinal sacs

*Peptide absorption (%)*: Absorption of *M. pruriens* tempe F72 peptide hydrolysates did not significantly differ between the duodenum, jejunum, and ileum (Table 2). Absorption of leucine (the control) also showed no significant difference between the three small bowel segments. This shows that absorption of hydrolysate peptides of tempe F72 is relatively similar to leucine absorption in the small intestine. This high degree of absorption may be caused by a high percentage of small peptide fractions of <1 kDa after digestion, as smaller peptides are more easily absorbed in each segment of the small intestine. Lee (2002) reported that particle absorption in the intestine increases with decreased particle size, therefore peptides

**Table 2.** Absorption of peptide hydrolysates of tempe F72 *Mucuna pruriens* (L) DC and leucine in the duodenum, jejunum, and ileum segments

| Segment of small intestine | Leucine[1] | | Hydrolysate peptide of tempe F72 *M. pruriens* | |
|---|---|---|---|---|
| | Peptide concentration (mg/mL) | Absorption (%) | Peptide concentration (mg/mL) | Absorption (%) |
| Initial | 2.01±0.03[b] | NA | 1.58±0.02[c] | NA |
| Duodenum | 1.79±0.18[a] | 32 | 1.28±0.03[a] | 31 |
| Jejunum | 1.81±0.12[a] | 32 | 1.44±0.07[b] | 35 |
| Ileum | 2.02±0.06[b] | 36 | 1.41±0.45[b] | 34 |

[1]Leucine as a control.
NA, Not available.
Data are expressed as mean±SD of three replications.
Different letters (a–c) in the same column show statistical differences ($P<0.05$).

**Table 3.** Inhibitory activity of ACE by peptides absorbed in the duodenum, jejunum, and ileum segments

| Segment of small intestine | Inhibitory activity of ACE (%) | IC$_{50}$ (mg/mL) |
|---|---|---|
| Duodenum | 78.12±3.68[a] | 0.32±0.01[ns] |
| Jejunum | 85.67±1.84[b] | 0.29±0.006 |
| Ileum | 87.76±0.36[b] | 0.28±0.001 |

Data are expressed as mean±SD of three replications. Different letters (a,b) in the same column show statistical differences (*P*<0.05).
[ns]Not significant.

of low MW are more easily absorbed in each segment of the small intestine.

***ACE-inhibitory activity of the peptides absorbed:*** The ACE-inhibitory activity of the peptides absorbed was lowest in the duodenum compared with the other small intestine segments (Table 3). The IC$_{50}$ values for peptide absorption in the jejunum and ileum did not differ significantly, whereas there was a small increase in IC$_{50}$ value for peptides absorbed in the duodenum. This suggests that the concentration of peptides absorbed through the duodenum, jejunum, and ileum can inhibit 50% of the same ACE as peptides outside the intestin. Thus, *M. pruriens* peptides can potentially inhibit the activity of ACE in blood vessels to reduce blood pressure since peptides absorbed in the small intestine do not show reduced ACE-inhibitory activity. Therefore, ACE-inhibitory activity of *M. pruriens* tempe does not decrease after digestion in a simulation of digestion. Although further study is needed, our results show that tempe F72 is an attractive natural candidate for the treatment of hypertension.

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## AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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