Versi Online: http://journal.ipb.ac.id/index.php/jtip
https://doi.org/10.6066/jtip.2023.34.2.200
Hasil Penelitian

J. Teknol. dan Industri Pangan
Vol. 34(2): 200-209 Th. 2023 ISSN: 1979-7788
Terakreditasi kemendikbudristek: 164/E/KPT/2021

Bioactive Peptides from Velvet Bean Tempe: Neutrase-Catalyzed Production in Membrane Reactor

Azis Boing Sitanggang[1)]*, Tsaniyah Ayu Mauliasyam[1)], Nadine Kurniadi[1)], Slamet Budijanto[1)], and Ho-Shing Wu[2)]

[1)] Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, IPB University, Bogor, Indonesia
[2)] Department of Chemical Engineering and Materials Science, Yuan Ze University, Taoyuan, Taiwan

Received August 21st 2023 / Accepted October 28th 2023

ABSTRACT

Velvet beans are potential sources of parent proteins for bioactive peptide production. In this study, a combination of fermentation and neutrase-catalyzed continuous hydrolysis in an enzymatic membrane reactor was performed to produce antioxidative and angiotensin I-converting enzyme inhibitory (ACEi) peptides. The optimum operating conditions were τ = 6 h and [E]/[S] = 7.5%. The resulting permeate, which was a<10-kDa fraction, exhibited antioxidant activity at 0.38 mg ascorbic acid equivalent antioxidant capacity (AEAC)/mL (2,2-diphenyl-1-picrylhydrazyl, DPPH inhibition) and 0.26 mg AEAC/mL (ferric reducing antioxidant power, FRAP), and ACEi activity of 81.02%. Further fractionation of the permeate increased the ACEi activity in which 2-kDa fraction showed the most potent activity (IC_{50} = 0.23 µg protein/mL). The IC_{50} value of the outcome was comparable to those reported in the literature for velvet bean-based peptides. Furthermore, this study suggests that neutrase is a good catalyst candidate for the synthesis of bioactive peptides from velvet beans.

Keywords: *ACE inhibitor, antioxidant, bioactive peptides, neutrase, velvet bean*

INTRODUCTION

Velvet bean (*Mucuna pruriens*), locally known in Indonesia as *kacang koro benguk*, is a local legume commodity found in Java, Bali, Sumatra, North Sulawesi, and Maluku. The bean is commonly utilized as feed. In Indonesia, utilization of velvet beans as food is currently limited to velvet bean tempe (fermented velvet bean) products. However, because of its relatively hard texture, velvet bean tempe is unpopular among consumers. The utilization of velvet beans as protein concentrates or isolates is also unpopular because of their dark color. The underutilization is wasteful, considering that velvet bean is a great protein source, with protein content reaching up to 30% (Kalidass and Mahapatra, 2014). In addition to having high protein content, the protein of velvet bean protein was reported to possess a high portion of hydrophobic amino acid (~30% w/w protein), which is a well-known property of bioactive peptides (Sánchez and Vázquez, 2017; Indrati, 2021). Thus, velvet beans are a potent source of *parent proteins* for the synthesis of bioactive peptides.

Bioactive peptides are specific protein fragments which are categorized based on their biological functions, such as antidiabetic, antihypertensive, antioxidative, and antimicrobial activities (Daliri *et al*., 2018). The peptides can exhibit their bioactivity provided that they are unleashed from their parent proteins. There are various ways to do this: *in vitro* hydrolysis using protease from gastrointestinal tract, microbes, plants, or animals, fermentation with starter or nonstarter cultures, or ripening process. Among these methods, *in vitro* hydrolysis seems to be the most common technique (Daroit and Brandelli, 2021). Nevertheless, a mixed-method approach (*i.e.*, fermentation with *in vitro* hydrolysis) has also been used to enhance the degree of proteolysis, releasing more peptides with smaller molecular weights (Peredo-Lovillo *et al.,* 2022). *In vitro* hydrolysis can be performed in a batch or continuous system. Batch reactions are considered less productive and may result in varied product qualities (Sitanggang *et al.*, 2022). Hence, a continuous system is preferred. That being said, bioactive peptides from velvet bean have been mostly produced in a batch manner (Segura-Campos *et al.*, 2013; Herrera Chalé *et al.,* 2014; Segura-Campos *et al.,* 2014; Chel-Guerrero *et al.*, 2017; Tuz and Campos, 2017; Pratami *et al.,* 2022).

Production of velvet bean-based peptides combining fermentation and continuous *in vitro* hydrolysis has been performed before in our previous study (Sitanggang *et al.*, 2023). The continuous operation was facilitated by an automated enzymatic membrane reactor (EMR) under constant flux operation. A potent angiotensin-I converting enzyme inhibitory

*Corresponding Author: E-mail: boing.lipan@apps.ipb.ac.id

(ACEi) peptide fraction (IC_{50}= 0.60 μg protein/mL) was obtained from this procedure. Long-term production of non-fermented velvet bean-based ACEi peptides has also been demonstrated previously (Kurniadi *et al.*, 2023), however, the obtained permeate fraction showed less potent ACEi activity compared to the one which included fermentation step. That being said, including fermentation has its own downside: longer production time. For instance, the making of velvet bean tempe by Sitanggang *et al.* (2023) took up to six days. In this study, we attempted to shorten the velvet bean tempe fermentation time to three days and employed a different protease, Neutrase®. Neutrase is known to result in a comparable degree of hydrolysis to Alcalase® (He *et al.*, 2012), with the advantage of being able to operate at a lower, neutral pH. Furthermore, neutrase displays a specificity towards hydrophobic amino acids (Damrongsakkul *et al.,* 2008). Hydrophobic residues were commonly known as one of the properties of antioxidative and ACEi peptides (Aluko, 2015a; Aluko, 2015b). This study aimed to determine the optimum operating conditions (*i.e.*, enzyme-to-substrate ratio, residence time) for the production of antioxidative and ACEi peptides from velvet bean tempe using neutrase.

MATERIALS AND METHOD

Materials

Velvet bean (*Mucuna pruriens*) was obtained from Surabaya, East Java, Indonesia in June, 2021. Tempe starter culture was purchased from RAPRIMA (Rumah Tempeh Indonesia, Yogyakarta, Indonesia). Neutrase® 0.8 L (EC 3.4.24.28) was from Novozymes A/S (Bagsværd, Denmark). The ultrafiltration (UF) membranes made from polyethersulfone (PES) with different molecular weight cut-offs (MWCOs) of 10-, 5-, 4-, and 2-kDa were purchased from MANN + HUMMEL (Ludwigsburg, Germany).

Design of study

Initially, neutrase filtration was conducted to compare the rejection rates of two membrane MWCOs (*i.e.*, 5- and 10-kDa). The MWCO which could facilitate complete rejection of neutrase was selected for bioactive peptides production using an enzymatic membrane reactor (EMR) (Figure 1). With velvet bean tempe protein concentrate as the substrate, the influences of residence time τ and enzyme-to-substrate ratio *[E]/[S]* were investigated using a one-factor-at-a-time design. Based on the antioxidant and ACEi activity of the accumulated permeate, the optimum operating conditions (*i.e.,* τ, *[E]/[S]*) were determined. The permeate produced under the optimum conditions was subjected to filtration using smaller MWCOs. Each of the obtained fractions' half-maximal inhibitory concentrations (IC_{50}) for ACE was calculated to determine the peptide fraction with the strongest ACEi activity.

Velvet bean fermentation and protein isolation

Fermented velvet bean (or velvet bean tempe) and its protein concentrate were produced as previously described (Sitanggang *et al.*, 2023) with slight modification. The fermentation was conducted for 3 d, instead of 6 d, The protein content of the obtained protein-rich materials was determined with Kjeldahl method, following the Association of Official Analytical Chemists procedures method 960.52 (AOAC, 2012). The protein content of the obtained materials was 55.84% on dry basis, hence classified as flour-rich in protein (Guéguen *et al.,* 2016).

Neutrase filtration

Rejection of neutrase by the membrane was evaluated using two MWCOs (*i.e.*, 5- and 10 kDa). neutrase was diluted in phosphate buffer (1% *v/v*, pH 7.0) and applied to filtration at constant fluxes, thus constant residence times (τ for 5-kDa membrane= 4 h and set flux J_{SV}= 18.17 L/m^2.h, τ for 10-kDa membrane= 3 h and J_{SV}= 24.23 L/m^2.h). By comparing the enzyme's activity before and after filtration, it was possible to assess if neutrase was being rejected. To calculate enzyme's activity, Sigma's non-specific protease activity assay was performed (Cupp-Enyard and Aldrich, 2008). Absorbance was measured using a Genesys 150 UV–Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, US) (Sitanggang *et al.*, 2021b; Kurniadi *et al.*, 2023; Sitanggang *et al.*, 2023).

Production of bioactive peptides using automated EMR

Hydrolysis of velvet bean tempe flour-rich in protein to produce bioactive peptides was carried out in an automated EMR, as described in our previous study (Sitanggang *et al.,* 2023). A schematic overview of the EMR is given in Figure 1. The EMR was constructed in parallel (n= 2) as a means of replication of experimental units. This reactor design facilitated a constant flux (and hence, residence time) filtration, ensuring a continuous operation. The robustness of the developed control design in maintaining specified flux throughout the operation was demonstrated in previous study (Sitanggang *et al.,* 2023). The influences of residence time (*i.e.*, τ = 6, 8, 12 h, corresponding to J_{SV}= 12.12, 9.09, and 6.06 L/m^2.h, respecti-vely) and enzyme-to-substrate ratio (*i.e.*, *[E]/[S]* = 5, 7.5, 10, 15%) was investigated using a one-factor-at-a-time design. The velvet bean tempe protein con-centrate (0.75% w/v) was dissolved in phosphate buffer (pH 7.0). The resulting solution was filtered using Whatman filter paper, Grade 42. Continuous hydrolysis was carried out for 7 h at a

temperature *T* of 50 °C. Permeate sample was taken throughout the 7-h reaction (*i.e.*, at 1, 2, 3, 4, 5, 7 h). The accumulated permeate in the container, obtained during 7-h reaction, was considered as the performance of the reaction in general. Total phenol, protein concentration, antioxidant activity (2,2-diphenyl-1-picrylhydrazyl/DPPH inhibition, FRAP/ferric reducing antioxidant power), ACEi activity of the permeate were determined as performed by Sitanggang *et al.* (2021b); Kurniadi *et al.* (2023); and Sitanggang *et al.* (2023). Based on the antioxidant and ACEi activity, the optimum operating conditions (*i.e., τ*, *[E]/[S]*) were determined. The permeate produced under the optimum conditions was subjected to filtration using smaller MWCOs. Each of the obtained fractions' half-maximal inhibitory concentrations (IC_{50}) for ACE was calculated.

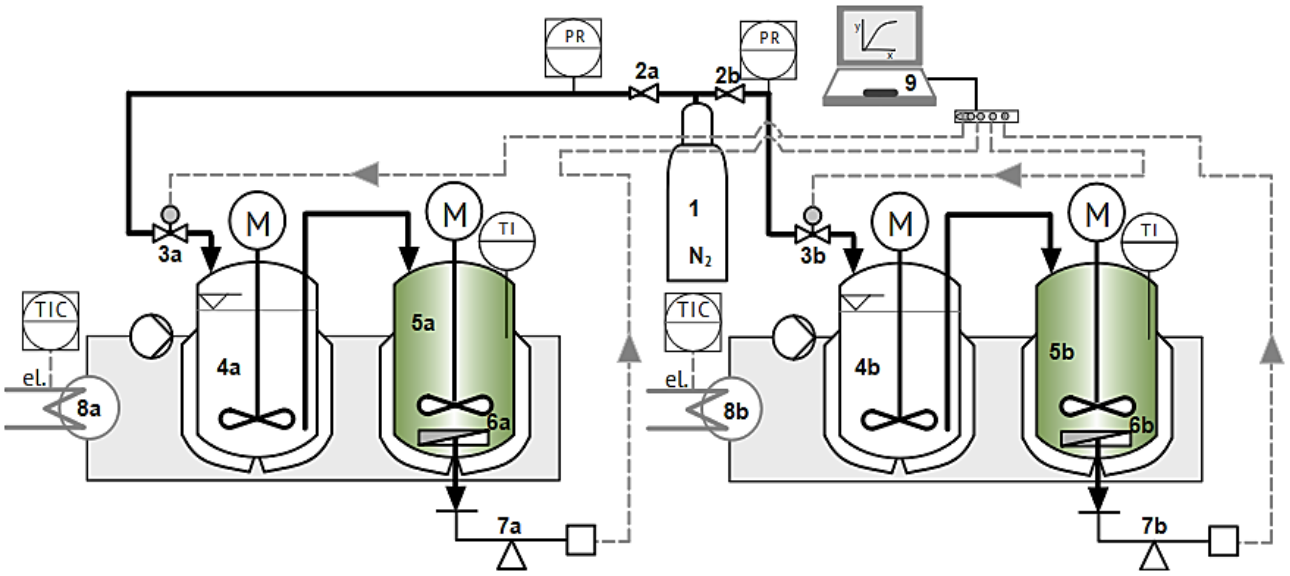
Statistical analysis

All tests for total phenols, protein concentrations, antioxidant activities, and ACEi activities were conducted with two replicates for each EMR. The data were expressed as the mean ± standard deviation of four replicates. Analysis of variance and Duncan's test ($p<0.05$) was performed using SPSS Statistics v24 (IBM, Armonk, US).

RESULTS AND DISCUSSION

Rejection of neutrase by UF membrane

To sustain continual catalytic activity, the membrane's pore size (*i.e.*, MWCO) has to confine enzymes within the reactor (Sitanggang *et al.,* 2022). The MWCO which provided complete rejection is preferable to maintain a constant catalytic activity within the reactor and avoid secondary hydrolysis within the product tank. Both membrane cut-offs (*i.e.*, 5- and 10-kDa) equally achieved complete rejection of neutrase (> 99%) (Figure 2A). This can be attributed to the large size gap between the enzymes (*i.e.*, neutrase ~ 57 kDa) and the membrane (*i.e.*, MWCO 5- and 10-kDa). Nevertheless, this size gap cannot be used as a benchmark for the MWCO selection. Sitanggang *et al.* (2023) reported a complete rejection (> 99%) of Alcalase (EC 3.4.21.62) (~ 27 kDa) using 5-kDa PES membrane, but a significantly lower rejection (88%) with 10-kDa MWCO. Moreover, papain (EC 3.4.22.2) which has a lower molecular weight (~23 kDa) than Alcalase, was completely rejected by both 5- and 10-kDa PES membranes (Sitanggang *et al.,* 2021b). These findings demonstrated the fact that the MWCO values are exclusively determined by molecular weight and do not take the conformation of the molecule into account (Koyuncu *et al.,* 2015). Furthermore, regardless of the set flux J_{SV}, a smaller MWCO naturally led to a higher fouling tendency, as observed in the higher transmembrane pressure (TMP) increase during 5-kDa filtration (Figure 2B). Nevertheless, neither MWCO showed a steep increase. With both MWCOs resulting in complete rejection, the 10-kDa was deemed more suitable for continuous operation, considering its lower fouling tendency.

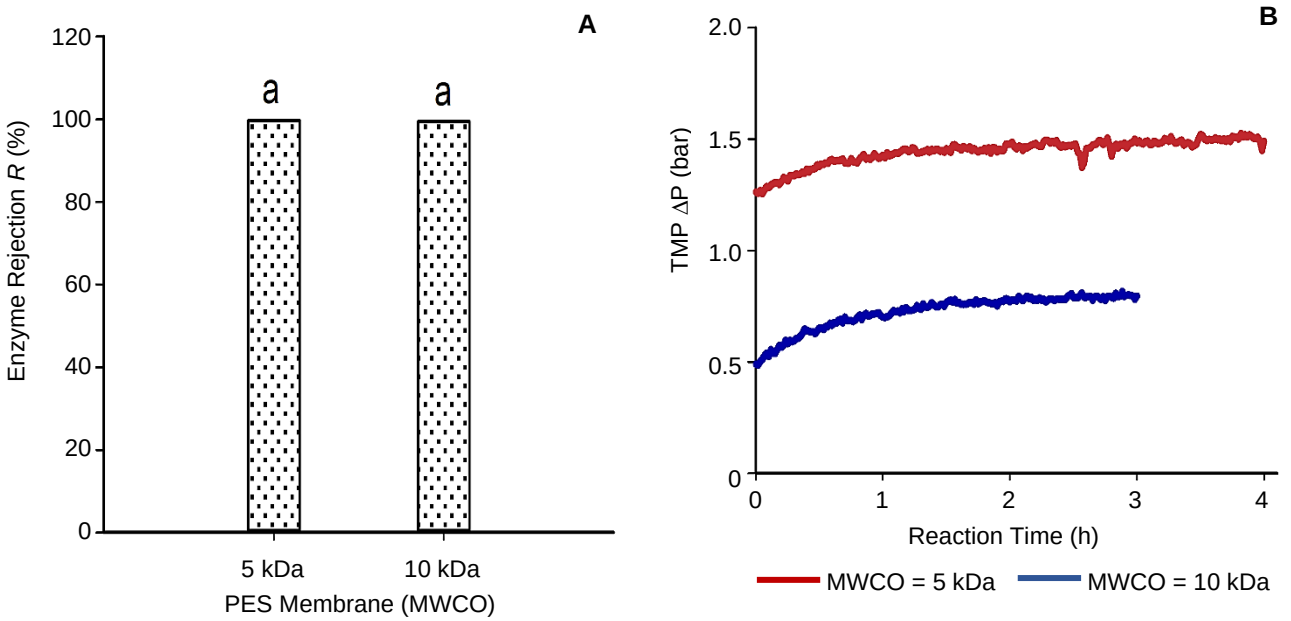


Note: 1= Nitrogen (N_2), 2= pressure reducer, 3= proportional pressure regulator, 4= substrate tank, 5= stirred tank reactor, 6= UF membrane, 7= analytical balance, 8= water bath, 9= PC. M: motor, PR= pressure register, TIC= temperature indicating controller, TI= temperature indicator, el.= electricity

Figure 1. Enzymatic membrane reactor

Influence of residence time

The amount of time the substrate spends within the reactor (*i.e.*, residence time), implies how long the enzyme and protein are in contact with one another. In other words, longer residence times are associated with a higher degree of proteolysis. In spite of that, residence time seemed to have no significant effect on both antioxidant activity and ACEi activity (Table 1). This was presumably due to the degree of proteolysis reaching its maximum level with the available enzyme (*i.e.*, *[E]/[S]* = 10%), resulting in over-degradation of the products. It has been a common agreement that bioactive peptides are small in size (Aluko, 2015a; Daliri *et al.,* 2018; Durand *et al.,* 2021; Sitanggang *et al.,* 2021a), hence a higher degree of hydrolysis is commonly desired. However, over-degradation could lead to the release of free amino acids, instead of peptides.



Note: τ for 5-kDa membrane= 3 h (thus, set flux J_{SV} = 22.73 L/m^2.h) whereas τ for 10-kDa membrane= 2 h (thus, J_{SV}= 34.09 L/m^2.h). Enzyme concentration *[E]*= 1% (*v/v*), pH 7.0. Different letters show a significant difference (p<0.05)

Figure 2. (A) Rejection and (B) filtration behavior of neutrase by 5- and 10-kDa PES membrane

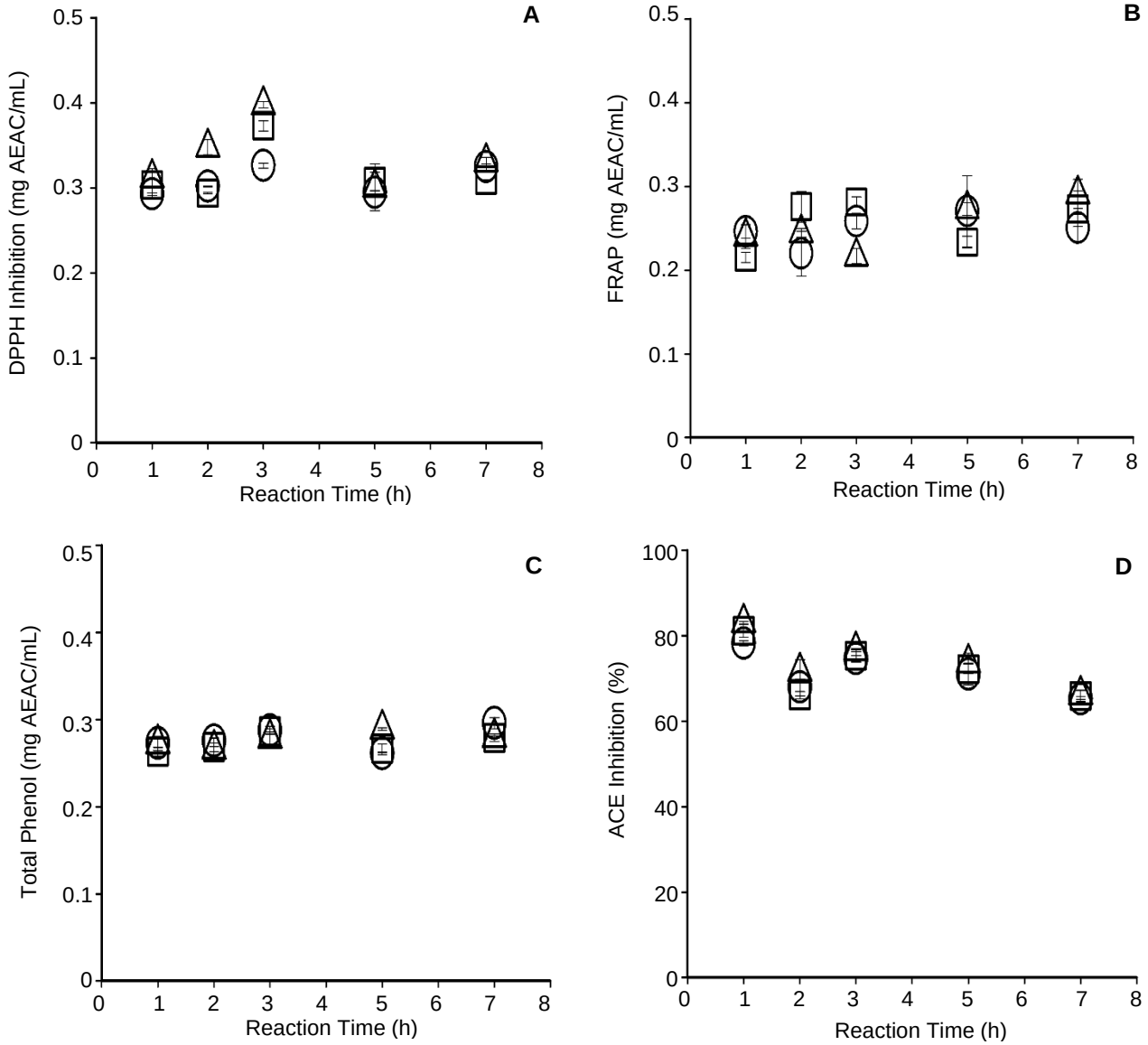
Table 1. Characteristics of the initial substrate and cumulative permeate. Reacting conditions for different treatments are given Figure 3 and Figure 4

Treatment		Antioxidant (mg AEAC/mL)		ACE Inhibition (%)	Protein (mg/mL)	Total Phenol (mg GAE/mL)
		DPPH Assay	FRAP Assay			
Substrate		0.4089±0.0116^{bD}	0.2646± 0.0098^{aA}	70.77±1.17^{aA}	0.3465±0.0103^{aD}	0.3780± 0.0074^{bD}
Permeate, τ	6 h	0.3257±0.0043^{a}	0.2269± 0.0034^{a}	74.98±0.59^{b}	0.1063±0.0032^{b}	0.2702± 0.0033^{a}
	8 h	0.3291±0.0027^{a}	0.2300± 0.0039^{a}	75.72±0.48^{b}	0.1250±0.0054^{c}	0.2698± 0.0037^{a}
	12 h	0.3304±0.0016^{a}	0.2333± 0.0047^{a}	76.07±0.28^{b}	0.1344±0.0029^{d}	0.2738± 0.0040^{a}
Permeate, [E]/[S]	5%	0.3115±0.0040^{A}	0.2131± 0.0023^{A}	73.97±0.35^{B}	0.0938±0.0047^{a}	0.2640± 0.0055^{A}
	7.5%	0.3765±0.0070^{C}	0.2561± 0.0003^{C}	81.02±0.66^{D}	0.0997±0.0027^{a}	0.2776± 0.0019^{A}
	10%	0.3257±0.0043^{B}	0.2269± 0.0034^{B}	74.98±0.59^{C}	0.1063±0.0032^{b}	0.2683± 0.0022^{A}
	15%	0.3202±0.0042^{AB}	0.2169± 0.0046^{A}	75.41±0.34^{C}	0.1231±0.0046^{c}	0.2689± 0.0081^{A}

Note: Data were expressed as means ± standard deviations of four replicates. Different superscript letters in lowercase (a, b, c) show a significant difference between residence time τ treatments while different superscript letters in uppercase show a significant difference between enzyme-to-substrate ratio *[E]/[S]* treatments (p<0.05). AEAC= ascorbic acid equivalent antioxidant capacity, GAE= gallic acid equivalent

The over-degradation, resulting in amino acid products, was presumably the cause of the insignificant difference in bioactivity between the treatments. On one hand, the increase in residence time did lead to an increase in protein content (Table 1), which could be an implication of more peptides being released. On the other hand, the measured value of protein content does not provide information regarding the structure of the molecules (*i.e.*, peptides or amino acids) as the Bradford assay is associated with the presence of amino acids (*i.e.*, arginine, lysine, histidine). The highest protein content recorded in the longest residence time might be a result of an abundance of amino acids in the cumulative permeate.

Nevertheless, the release of bioactive peptides as a result of protein hydrolysis was demonstrated. For instance, the permeate's ACEi was significantly greater than the initial substrate's (p<0.05). Slight fluctuation observed in the ACEi activity throughout the 7-h reaction (Figure 3D) was due to the resulting apparent peptides having varied amino acid compositions and combinations, which further translated into different bioactivities (Kurniadi *et al.,* 2023). Furthermore, comparing the profiles of total phenol and antioxidant activities allows one to see how peptides contribute to antioxidant activity (Figure 3A-C).



Note: *[S]* = 0.75% (*w/v*), *[E]/[S]* = 10%, *T* = 50°C, pH 7.0, 10-kDa PES membrane. Residence time τ = 6, 8, 12 h corresponded to J_{SV} = 12.12, 9.09, and 6.06 L/m^2.h, respectively. ○ τ = 6 h, □ τ = 8 h, △ τ = 12 h

Figure 3. Influence of residence time τ on antioxidant activity [(A) DPPH inhibition, (B) FRAP], (C) total phenol, (D) and ACEi activity during the continuous hydrolysis

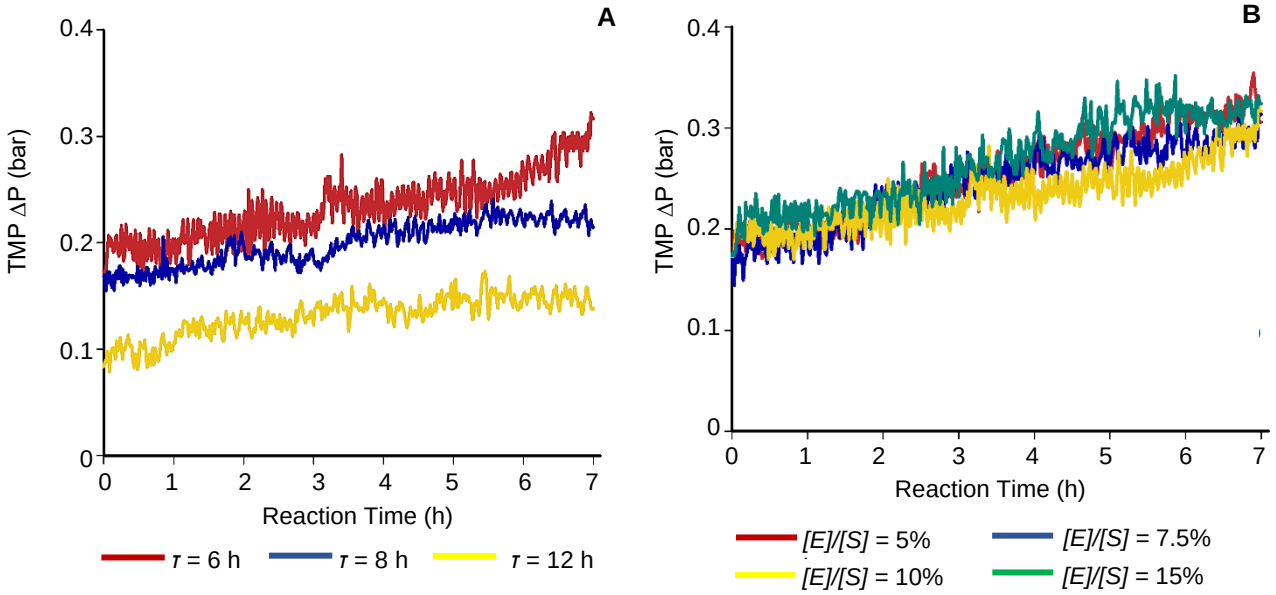
Rejection of the phenolic compounds by the membrane caused a decrease in the antioxidant activity of the permeate, compared to the initial substrate. However, whereas the total phenol concentration remained mostly stable, the antioxidant activity varied slightly. This fluctuation signified that there were peptides that were released through hydrolysis and contributed to the antioxidant activity, along with the phenolic compounds. In previous studies, this tendency was also noticed (Sitanggang *et al.,* 2021b; Kurniadi *et al.,* 2023; Sitanggang *et al.,* 2023).

The influence of residence time on TMP is shown in Figure 4A. The TMP increased more rapidly with the reduction of residence time. Higher flux (*i.e.*, shorter residence time) induced a higher convective transport through the membrane, which increased the membrane's fouling (Sitanggang *et al.,* 2016; Sitanggang *et al.,* 2021b; Sitanggang *et al.,* 2022; Kurniadi *et al.,* 2023; Sitanggang *et al.,* 2023). Considering that there was no discernible difference in bioactivity between residence time treatments and that the highest flux (*i.e.*, shortest residence time) would result in the highest productivity, residence time τ of 6 h was chosen as the optimum treatment.

Influence of enzyme-to-substrate ratio

Enzyme-to-substrate ratio *[E]/[S]* is one of the most crucial factors to take into account. This relates to the fact that the degree of proteolysis has a positive association with *[E]/[S]*. In other words, *[E]/[S]* influences the size, and thus peptide's bioactivity. In the previous section, [*E]/[S]* = 10% was used in a previous attempt to determine optimum residence time. It was theorized that the insignificant difference in bioactivity between residence time treatments was a result of over-degradation caused by excessive enzymes. The highest antioxidant activity and ACEi activity were obtained with *[E]/[S]* of 7.5% (Table 1). The bioactivity decreased below and above that point. This is due to the low *[E]/[S]* resulting in under-hydrolyzed products (*i.e.*, large protein fragments) and high *[E]/[S]* resulting in over-degraded products (*i.e.*, free amino acids). The antioxidant activity (DPPH method) and ACEi activity obtained from *[E]/[S]* of 10 and 15% treatments were not significantly different, suggesting an over-degradation in *[E]/[S]*= 10% concentration onwards, hence supporting previous theory.

The total phenol, antioxidant activity, and ACEi activity during the course of the 7-h reaction is shown in Figure 5. While the total phenol remained largely constant, there were minor variations in the antioxidant activity and ACEi activity, similar to the influence of residence time. This phenomenon suggests the release of bioactive peptides as a result of enzymatic hydrolysis. Furthermore, the influence of *[E]/[S]* on TMP is shown in Figure 4B. There was a slight increase in TMP with the increase in *[E]/[S]*. The net enzyme charge at certain pH governs the type of electrostatic interaction (*i.e.*, adsorption or repulsion) which occurs between enzyme molecules and membrane surface. At pH 7.0, the charge of neutrase was approximately +4.4 (Kurniadi *et al.,* 2023).



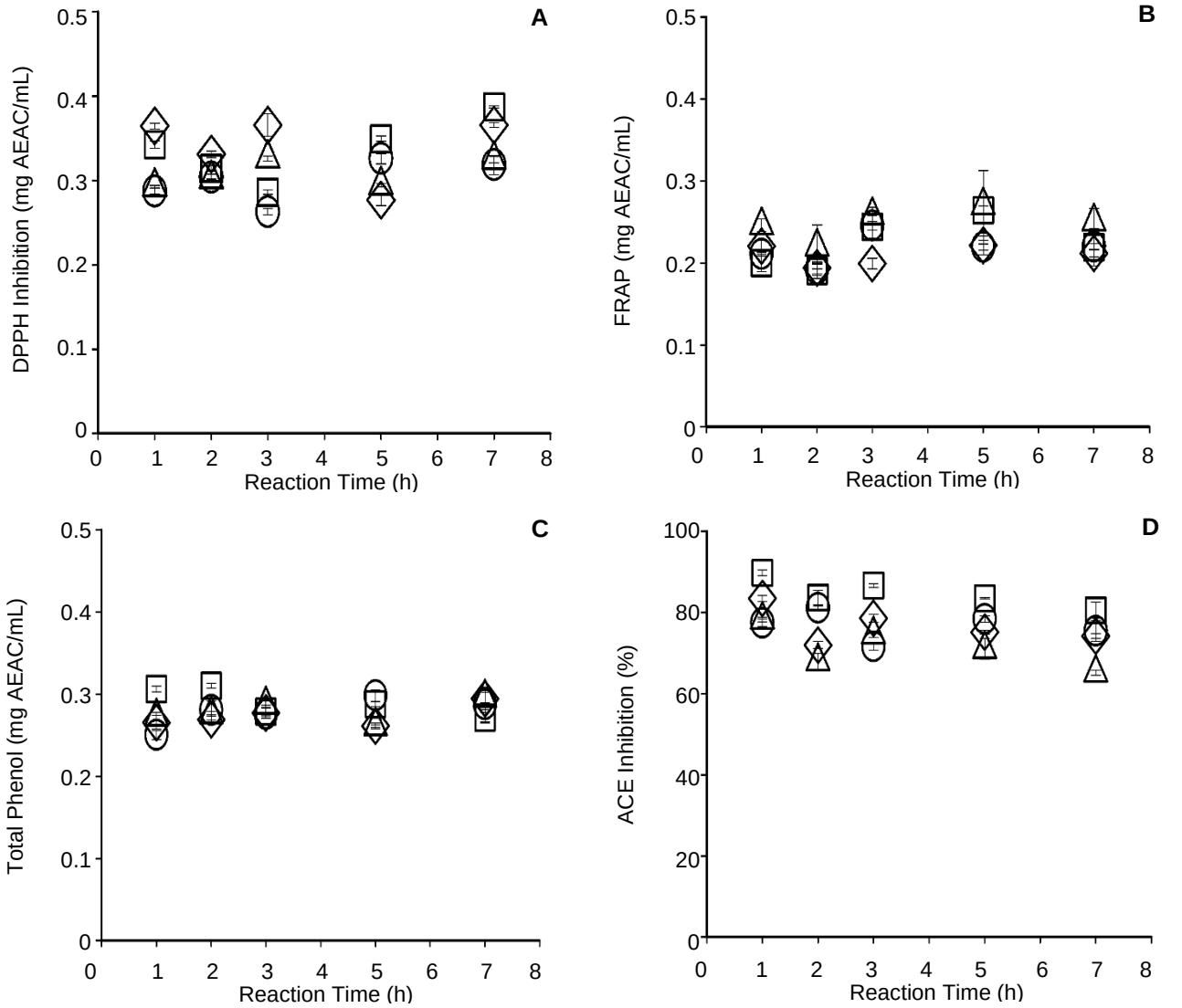
Note: Reacting conditions for different treatments are given in Figure 3 and Figure 4. TMP= Transmembrane pressure

Figure 4. Filtration behavior of continuous hydrolysis with variation of (A) residence time τ and (B) enzyme-to-substrate ratio *[E]/[S]*

In effect, there could be electrostatic interactions toward adsorption between the positively charged enzyme molecules and the negatively charged PES membrane (de La Torre *et al.*, 2009). This caused a higher fouling tendency, hence explaining the increase in TMP to maintain a certain level of flux. Nevertheless, the contrast in charges was presumably not high enough to cause significant membrane fouling, as implied by the slight differences in TMP between *[E]/[S]* treatments. Overall, with the highest bioactivity, *[E]/[S]* of 7.5% was chosen as the optimum treatment.

ACE IC_{50} of different peptide fractions

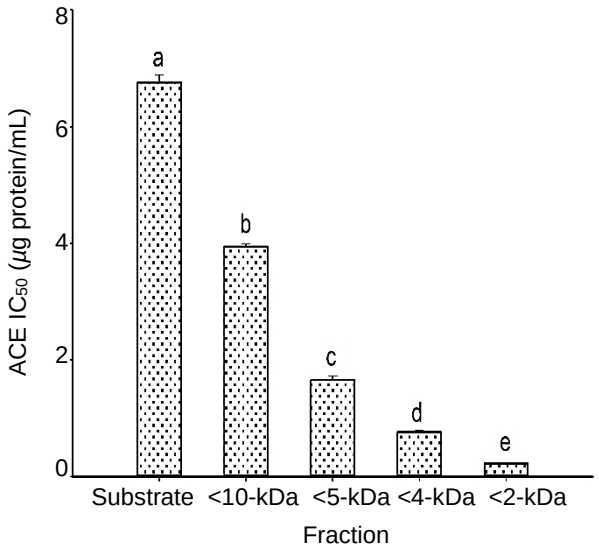
A significant increase in ACEi activity (*i.e.*, low IC_{50}) was observed by employing lower MWCOs to the permeate produced under optimum conditions (Figure 6). This trend has also been reported in various studies (Herrera Chalé *et al.*, 2014; Tuz and Campos, 2017; Yao *et al.*, 2019; Sitanggang *et al.*, 2021b; Kurniadi *et al.,* 2023; Sitanggang *et al.,* 2023). The strongest ACEi activity was found in the lowest fraction (<2 kDa), measuring 0.23 μg protein/mL. This value was lower than other reported values for velvet bean peptides (Herrera Chalé *et al.,* 2014; Segura-Campos *et al.,* 2014; Chel-Guerrero *et al.,* 2017; Rizkaprilisa *et al.,* 2020; Pratami *et al.,* 2022; Kurniadi *et al.,* 2023; Sitanggang *et al.,* 2023) with the exception of Tuz and Campos (2017) who reported IC_{50} value as low as 0.9×10^{-3} μg protein/mL from < 1-kDa fraction.



Note: *[S]* = 0.75% (*w/v*), *τ* = 6 h, *T* = 50°C, pH 7.0, 10-kDa PES membrane. ○ *[E]/[S]* = 5%, □ *[E]/[S]* = 7.5%, △ *[E]/[S]* = 10%, ◇ *[E]/[S]* = 15%

Figure 5. Influence of enzyme-to-substrate ratio *[E]/[S]* on antioxidant activity [(A) DPPH inhibition, (B) FRAP], (C) total phenol, (D) and ACEi activity during the continuous hydrolysis

These findings suggest that small size leads to more potent ACEi activity of the peptides. Notably, a more potent ACEi activity was obtained, compared to a similar study which employed a 6-day velvet bean fermentation process and Alcalase as the catalyst (Sitanggang *et al.,* 2023). This further suggests that degree of hydrolysis aside, neutrase presumably has more desirable specificity to produce ACEi peptides from velvet bean compared to Alcalase, as also found by Pratami *et al.* (2022). Regardless, other than our previous studies (Sitanggang *et al.,* 2021b; Kurniadi *et al.*, 2023; Sitanggang *et al.,* 2023), velvet bean-based peptides were produced batch-wise. Compared to a batch operation, the continuous mode demonstrated in this study had higher productivity.



Note: Different letters show a significant difference (p<0.05)

Figure 6. The half-maximal inhibitory concentration (IC_{50}) of the substrate (velvet bean tempe flour-rich in protein) and four peptide fractions (< 10-kDa, < 5-kDa, < 4-kDa, and < 2-kDa)

CONCLUSIONS

Bioactive peptides, especially antioxidative and ACEi peptides, were produced through a series of 3-day fermentation and continued with *in vitro* continuous hydrolysis using neutrase in an EMR. The optimum operating conditions for the continuous hydrolysis were τ = 6 h and *[E]/[S]* = 7.5%. A significant increase in ACEi activity (*i.e.*, low IC_{50}) was observed at lower MWCOs. This suggests that in the case of velvet bean-based ACEi peptides, small sizes lead to more potent bioactivity. Conclusively, the continuous system for the synthesis of bioactive peptides is anticipated to improve the commercialization of velvet bean-based bioactive peptides.

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