J. Applied Membrane Science & Technology, Vol. 28, No. 3, December 2024, 33–46

Continuous Hydrolysis of Nile Tilapia Skin Collagen using an Enzymatic Membrane Reactor

M. Thuanthong[a], N. Sirinupong[a], T. Sirinupong[a,b], W. Youravong [a,b*]
[a]Center of Excellence in Functional Foods and Gastronomy, Faculty of Agro-Industry, Prince of Songkla University, Songkhla, Thailand, 90110
[b]Membrane Society (Thailand), Prince of Songkla University, Songkhla, Thailand, 90110

Submitted: 30/9/2024. Revised edition: 4/11/2024. Accepted: 5/11/2024. Available online: 12/12/2024

ABSTRACT

The continuous enzymatic membrane reactor (EMR) was developed for producing Angiotensin-I Converting Enzyme (ACE) inhibitory peptides from Nile tilapia skin collagen. The productive condition of EMR and peptide property were studied. The results showed that protein conversion and EMR capacity were remarkably affected by collagen concentration and enzyme to substrate ratio while ACE-inhibitory activity was influenced by only enzyme to substrate ratio. In addition, the proper operating condition of ACE-Inhibitory peptides produced by EMR were Alcalase to substrate concentration ratio of 0.08 at 50 °C, pH 8.0 and permeate flow rate of 0.4 ml min^{-1}. It was evident that the EMR was an effective means for producing small peptides (< 1000 Da) with ACE-inhibitory activity. The permeate fractions containing peptides with molecular weight <1000 Da were homogeneous and also showed relatively high ACE-inhibitory activity.

Keywords: Enzymatic membrane reactor, Nile tilapia, fish-skin collagen, peptides, ACE-inhibitory activity

1.0 INTRODUCTION

Bioactive peptides are current used as functional food ingredients producing from various sources of protein [1]. Fish skin has currently been utilized as a source of collagen for producing bioactive peptides [2-4]. Collagen peptides are one of the most popular food supplement products exhibiting various bioactivities such as antioxidant, antimicrobial, hypotensive [2-8]. Indeed, skin of various fish species can be used to produce such peptides. Among them, Nile tilapia (*Oreochromis niloticus*) is one of the fish species cultured in Thailand with high volume therefore large amount of skin is generated as low- value by product during Nile tilapia processing. Regarding sustainable industry concept, it is necessary to fully utilize and to increase the value of this by product for producing high-value functional food ingredient such as bioactive peptides particularly ACE-Inhibitory peptides. It is known that ACE exerts an important physiological role in controlling blood pressure by catalyzing the conversion of angiotensin-I into angiotensin-II and inactivation of bradykinin [9]. However, synthetic ACE-inhibitors can cause adverse side effects therefore there has been an increasing interest in natural ACE-inhibitory peptides. In addition, many studies have been reported that collagen peptides delivered from fish skin showed high ACE-Inhibitory activity [7,8,10]. This

* Corresponding to: W. Youravong (email: wirote.y@psu.ac.th)
DOI: https://doi.org/10.11113/jamst.v28n3.303

may be related to their high content of proline which promotes the inhibition of ACE, when presents in the C-terminal of the peptides [11]. However, commercial production of bioactive peptides has been limited by the lack of suitable large-scale technology. Since it is conventionally performed in batch hydrolysis reactor which has many disadvantages such as time-consuming process, labor-intensive, using large amount of enzyme and producing non-homogeneous products with varying in molecular weights [12]. To overcome these limitation, the enzymatic membrane reactor (EMR) has been developed [12-15]. Indeed, it is important to note that molecular weight of peptides is one of the main factors which highly relate with their bioactive activities. The advantages of an EMR over batch process are that the peptides with target molecular weight can be separated using appropriate selection of membrane molecular weight cut off and the enzyme can be repeatedly reused [12]. However, flux reduction and decline due to fouling are the critical constraint in membrane applications. Since collagen solution even at low concentration is usually viscous leading to limit the rate of back diffusion of solute or particle moving away from the membrane surface resulting in flux reduction. Therefore, the collagen is pre-hydrolysed prior introducing to EMR [16]. Nonetheless, the other process parameters e.g. transmembrane pressure (TMP) and substrate to enzyme ration are expected to have remarkably influence the EMR performance and stability. This study aimed to employ a continuous EMR to produce ACE-inhibitory peptides derived from Nile tilapia acid soluble collagen (ASC). The effect of collagen concentration and enzyme to substrate ratio on EMR performance as indicated using protein conversion, capacity, productivity and ACE-inhibitory activity of peptides were evaluated. The stability of the EMR system operated with constant permeate flow rates were evaluated using the response of TMP to indicate fouling severity as operation time increased. In addition, the molecular weight distribution and ACE-inhibitory of the obtained peptides were analysed.

2.0 MATERIAL and METHOD

2.1 Material

Nile tilapia (*Oreochromis niloticus)* (frozen) fish skin was supplied from the factory, located in Nakhonpanom province, Thailand and shipped with a chilling condition by air to the laboratory at the Faculty of Agro-Industry

2.2 Nile Tilapia Skin Collagen Preparation

The ASC was extracted using the method of Thuanthong *et al*. [17]. The ASC was then diluted with water to achieve a final concentration of 0.7 $g.L^{-1}$. The insoluble skin was removed by filtering with sieve and acetic acid was removed by using a hollow fiber membrane with 300 kDa molecular weight cut off (MWCO) operated using diafiltration mode. Then, the operation was change to batch concentration mode to achieve final concentration of 8.0 $g.L^{-1}$.

2.3 Enzymatic Membrane Reactor (EMR) Setup

All the studies were carried with the EMR that is showed as Figure 1.

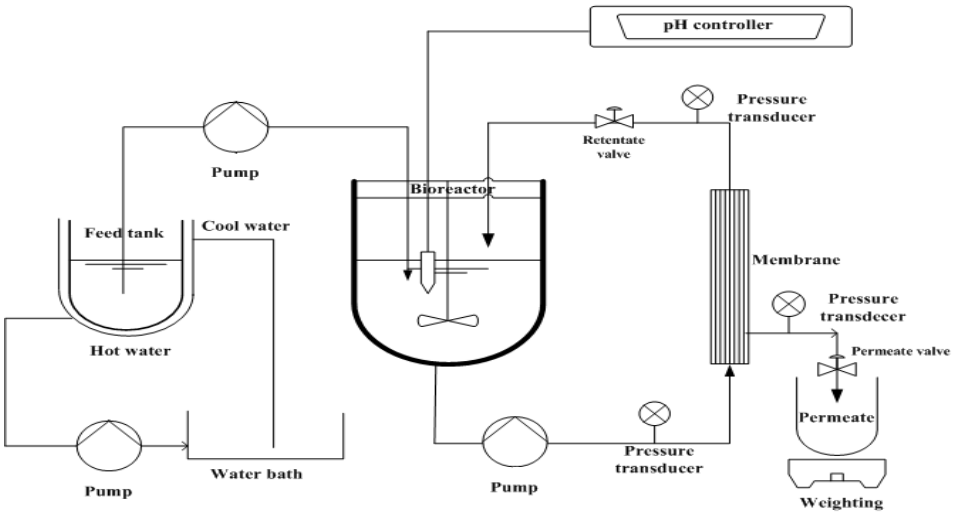


Figure 1 Schematic of the continuous EMR system

The main components included a 3.0 L reactor tank, united with a heat exchanger, coupled a membrane via 3 peristaltic pumps, three pressure transducer, a pH sensor and controller, and two counter pressure valves. The reactor was equipped with a 1 kDa MWCO polysulfone hollow fiber module (13 fibers; 1.0 mm inside diameter; 30 cm lenght; 0.014 m^2 filtering area) (Amersham Biosciences, UK). Pressure transducers (MBS 3000, Danfoss, Denmark, accuracy 1.0 mbar) were used to measure the pressure at the inlet and outlet of the membrane module, and of the permeate side. The transmembrane pressure (TMP) was controlled using a retentate valve, permeate valve and peristaltic pump.

The pre-hydrolyzed feed was used for the continuous EMR operation. Samples of retentate and permeate during EMR operation were periodically taken for analysis. The flow rate of permeate was controlled by peristaltic pump of the permeate. After each run, the membrane was cleaned by flushing with cleaned water followed with 0.5 N NaOH at 50 ˚C for 1 h then rinsed with cleaned water until the pH return to 7.

2.4 Effect of Collagen Concentration on Protein Conversion, Capacity and ACE-inhibitory Activity

The effect of collagen concentration on the EMR performance was evaluated. The 2.5, 5, 10 or 15 g of collagen samples were dissolved in 1 L of 0.05 mM phosphate-buffered (pH 8) at 50 ºC in a beaker and then introduced into the hydrolysis tank. After the reaction temperature (50 ºC) and pH (8) were stabilized, 200 µL of Alcalase enzyme was added to the hydrolysis tank. The collagen was pre-hydrolyzed for 60 min prior introduced to the EMR unit. During the operation, the pH was controlled at pH 8.0 with 2.0 M sodium hydroxide. The EMR was operated at constant permeate flow rate (0.6 $ml.min^{-1}$), thus the change of TMP with operating time due to membrane fouling was monitored to indicate the stability of the EMR system. The cross flow velocity (CFV) of the feed was also constant at 1.0 $m.s^{-1}$. The reaction mixture was continuously pumped to the membrane while the permeate was continuously separated and collected from the system and the rejected mixture (enzyme and substrate) was recycled back to the hydrolysis tank.

2.5 Effect Of Enzyme Substrate Ratio (R) on Protein Conversion, Capacity and ACE-inhibitory Activity

Most operation in this study was set as mention above except the amount of the Alcalase that was added to the hydrolysis tank to obtain various enzyme to substrate ratios (R) (0.05, 0.06, 0.07, 0.08, 0.09 and 0.10 mg enzyme. mg protein^{-1}). The permeate flow rate was constant at 0.4 ml.min^{-1} thus the initial TMP was set at 1.0 bar and increased with operating time due to fouling.

2.6 EMR Performance Evaluation Conversion

The conversion (X) expresses the ratio protein concentration in the permeate to protein concentration in the reactor [18]. For a continuous EMR system, it can be calculated using the following equation:

$$X(\%) = \frac{C_p}{C_r} \times 100 \quad (1)$$

where C_p and C_r are the protein concentration in the permeate and reactor (mg/ml), respectively.

Capacity

Capacity (C) is defined as the mass of protein produced per unit mass of enzyme per unit time (mg protein.mg enzyme^{-1}. min^{-1}) and is expressed as;

$$C = \frac{X \cdot S_0 \cdot J}{E \cdot V} \quad (2)$$

where S_0 is the total protein in the reactor tank (mg.ml^{-1}), J is the permeate flow rate (ml.min^{-1}), E is the amount of enzyme (mg.ml^{-1}) and V is the substrate volume (ml).

Productivity

Productivity (P) is defined as mass of protein produced per unit mass of enzyme and expressed as:

$$P = \frac{X_t \cdot S_0 \cdot J_t \cdot \mathrm{t}}{E \cdot V} \quad (3)$$

where t is time period (min).

2.7 Chemical, Physical and Bioactivity Analysis

Residual Enzyme Activity

The residual enzyme activity was determined according to the method of Cheison [19] with slight modifications. Within 2 min after adding the enzyme, 1 ml of the mixer were drawn into two test tubes. The blank was a mixture of 1 ml of 5 g.L^{-1} casein and 5 ml of 0.4 mol.L^{-1} trichloroacetic acid (TCA). The reaction was incubated for 10 min at 37 °C in a water bath. The reaction was stopped by adding 5 ml of TCA. The mixture was stood for 30 min at room temperature and then filtered with Whatman No. 4 filter paper. Finally, the absorbance of the filtrate at 680 nm was measured using the Folin colorimetry method to determine TCA-soluble nitrogen. The samples taken from different operating times were also analyzed using this method.

The residual enzyme activity (A) in the reactor was calculated as below:

$$A(\%) = \left(\frac{OD_t - OD_{bt}}{OD_2 - OD_{b2}}\right) \times 100 \quad (4)$$

where OD_t is the OD of TCA-soluble nitrogen obtained from enzyme hydrolysis after t min of reaction, OD_{bt} is the blank reading for reactor content at t min and OD_2 is the OD of reactor enzyme drawn after 2 min after the enzyme was introduced, OD_{bt} is the blank reading for reactor content at 2 min.

Degree of Hydrolysis (DH)

The DH was analyzed by relating the increase in the concentration of the

liberated α amino groups (α-NH_2) using the o-phthaldehyde (OPA) method according to Nielsen and Nielsen (2001) [20]. The initial concentration of the α-NH_2 groups in the collagen solution was determined just before adding the Alcalase enzyme. The DH for the EMR system was calculated using the equation as shown below:

$$DH_{EMR}(\%) = \frac{([NH_2]_p \; x \; V_p)+([NH_2]_r \; x \; V_r)}{h_{tot}} \quad (5)$$

where $[NH_2]_p$ is the concentration of NH_2 in the permeate (mg.ml $^{-1}$), $[NH_2]_r$ is the concentration of NH_2 in retentate (mg.ml $^{-1}$), h_{tot} is the concentration of NH_2 of collagen, (V_r) is the volume of retentate (ml), and (V_p) is the volume of permeate (ml).

Measurement of ACE-inhibitory Activity

The ACE-inhibitory activity was analyzed using the method according to Cushman and Cheung (1971) [21] with slight modification. The 50 µl of sample solution with 50 µl of ACE solution (2.5 mU.ml^{-1}) was pre-incubated at 37˚C for 10 min and the mixture was incubated with 50 ml of substrate (3 mM Hip-His-Leu (HHL)) in 100 mM borate buffer containing 0.3 M NaCl at pH 8.3) for 60 min at the same temperature. The reaction was terminated by addition of 0.5 M HCl (200 µl). The resulting hippuric acid was extracted with 1.5 ml of ethyl acetate. After centrifugation (4000×g, 15 min), 1 ml of the supernatant was transferred into a test tube, and evaporated at room temperature for 4 h under vacuum pressure. The hippuric acid was dissolved in 3.0 ml of distilled water, and the absorbance was determined at 228 nm using a spectrophotometer (Genesys 10 UV-VIS Series, Thermo Scientific, USA). The inhibition activity (IA) was calculated using the following equation:

$$IA\ (\%) = \frac{(A_a - A_b)}{(A_a - A_c)} \times 100 \quad (6)$$

where A_a is the absorbance with ACE and HHL without the sample, A_b is the absorbance with ACE, HHL and the sample; and A_c is the absorbance with HHL without ACE and the sample. The IC_{50} (half maximal inhibitory concentration) value is defined as the concentration of inhibitor that could inhibit 50% of the ACE activity.

Size Exclusion Chromatography

Molecular weight distribution of the collagen hydrolysate from the EMR was determined using a size exclusion chromatography with a Sephadex G-15 column (1.6×50 cm) monitoring at 220 nm. A 1.0 ml of collagen hydrolysate (1 g.L^{-1}) dissolved in distilled was eluted by distilled water at 0.2 ml.min^{-1} at room temperature. The column was calibrated using Gluthathion oxidize (MW 612.64 Da), HHL (MW 429 Da), Glutathione reduce (MW 307.32 Da) and Gly-Tyr (MW 238.2 Da). The evolution volume (ml) of blue dextran was used to verify the void volume of the column.

2.8 Statistical Analysis

Factorial in complete randomized design (CRD) was used for the statistical analysis. Data was subjected to analysis of variance (ANOVA). Mean comparisons were carried out by Duncan's multiple range test at a significant level $p < 0.05$.

3.0 RESULTS AND DISCUSSION

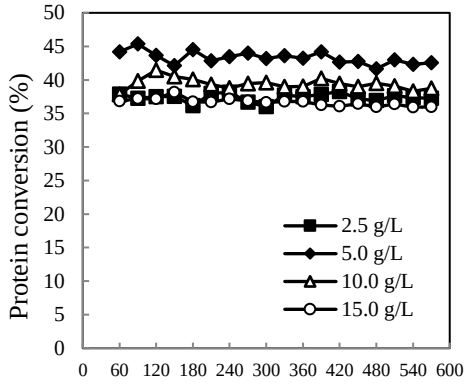
3.1 Effect of Alcalase to substrate ratio on EMR performance

The performance of the EMR is basically affected conversion rate and

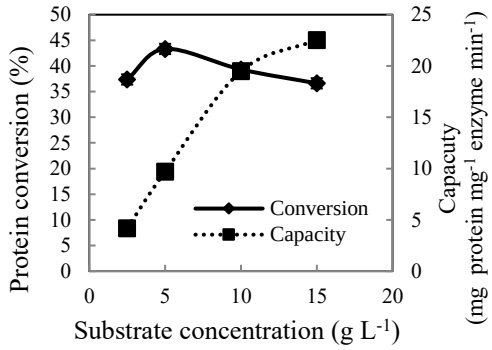
separation ability relating to process parameters such as substrate concentration, enzyme concentration, pH, temperature, permeate flux. Since high molecular weight collagen was used as substrate in this study, its viscosity and prone to fouling possibly limit the EMR performance. Thus, the effect of collagen concentration on the EMR performance was investigated.

Figure 2 (a) shows protein conversion of the EMR as varying collagen concentration. The results revealed that protein conversions of all collagen concentrations were almost constant with operation time. Please note that collagen samples were pre-hydrolysed prior performing EMR, thus large amount of low molecular weight peptides possibly presented [16]. The average of protein conversions is also presented in Figure 2 (b). Its conversion using 5 g.L^{-1} of collagen was the highest while the use of higher collagen concentration resulted significant lower conversion ($p<0.05$). It was indeed earlier reported that the protein conversion decreased with increasing in substrate concentration [22-26]. The decreasing of conversion at concentration of collagen >5 g/L was probably explained by separation ability due to the impact of fouling and concentration polarization. Knowing that both fouling and concentration polarization can change membrane permeability resulting in lower protein concentration in the permeate [24].

Nevertheless, it is also important to consider a degree of hydrolysis which is the impact of substrate concentration and Alcalase. Figure 3 shows the DH in EMR of each collagen concentration. It appeared that the DH of all concentrations were significantly difference ($p<0.05$). The highest DH was obtained when collagen concentration at 5.0 g.L^{-1} was used. The DH decreased when the concentrations used were higher or lower than 5.0 g.L^{-1}. These results were probably due to a lower enzyme to substrate ratio at a higher substrate concentration [26]. It was also probably due to the presenting of irreversible endoprotease inhibitor in which the active enzyme in the reaction mixer could be bonded irreversibly [27]. The DH consequently decreased when the enzyme was not fully utilized. Moreover, the decreasing of DH at higher collagen concentration was probably affected by the decreasing of residual Alcalase activity. Thus, the protein conversion curve downward tendency could be attributed to one or more of the following phenomena.



(a)



(b)

Figure 2 (a) Changes in protein conversion as varying operation time and (b) average protein conversion and capacity asvarying protein concentration operating condition; permeate flow rate of 0.6 ml/min, initial TMP 1.0 bar, CFV 0.5 m s^{-1}, hydrolyzed at pH 8 and 50 °C)

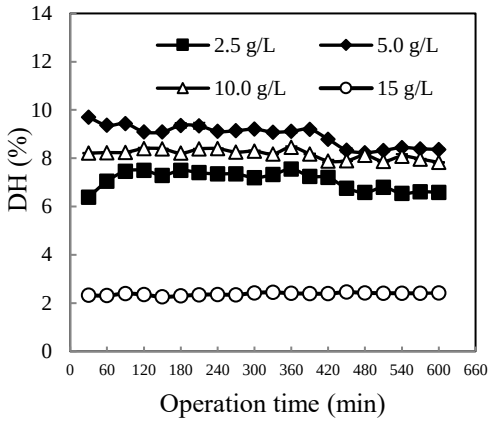


Figure 3 Degrees of hydrolysis (DH) with operating time as varying substrate concentrations (permeate flow at 0.6 ml min^{-1}, CFV 0.5 m s^{-1}, hydrolysed at pH 8, 50 °C)

Firstly, membrane operating factor (concentration polarization, fouling and, residual Alcalase activity affected by flow shear) and secondly, enzymatic hydrolysis factor which is overabundant substrate or irreversible endoprotease inhibitor.

The capacity was also used to indicate the EMR performance and it was defined as mass of product per unit time per unit mass of enzyme. From Figure 2(b), the result showed that the capacity remarkably increased with collagen concentration. It was obviously limited by the lack of enzyme when high concentrations of substrate were used. According to equation 2, when the amount of enzyme (E) was fixed, the increase in substrate concentration (S_o) remarkably resulted in an increase in capacity while the protein conversion increased. Higher collagen concentration could increase capacity but it also caused more severe fouling. Therefore, collagen concentration higher than 5 $g.L^{-1}$ was not recommended for this case.

The ACE-Inhibitory activity can be presented by IC_{50} value indicating the amount of hydrolysate used to inhibit 50% of the initial ACE activity. The IC_{50} value of each hydrolysate obtained as varying substrate concentration are shown in Table 1.

Table 1 IC_{50} value of ACE-inhibitory activities of the permeate from different substrate concentration

Substrate concentration ($g.L^{-1}$)	**IC_{50} ($mg.ml^{-1}$)**
2.5	0.6419 ± 0.0580^a
5.0	0.6172 ± 0.0600^a
10.0	0.6512 ± 0.0089^a
15.0	0.6788 ± 0.0250^a

Same letters in the same column present no statistical differences ($P < 0.05$)

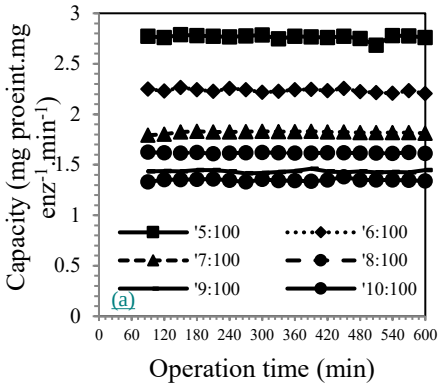
It was found that increasing in substrate concentration tend to increase in IC_{50}. According to the results of protein conversion, capacity and IC_{50} collagen at concentration of 5 $g.L^{-1}$ was used for further study.

To increase the EMR performance, the influence of the ratio of enzyme to substrate (E/S) on EMR performance was studied. The protein conversion and capacity obtained from various ratio of E/S are shown in Figure 4.

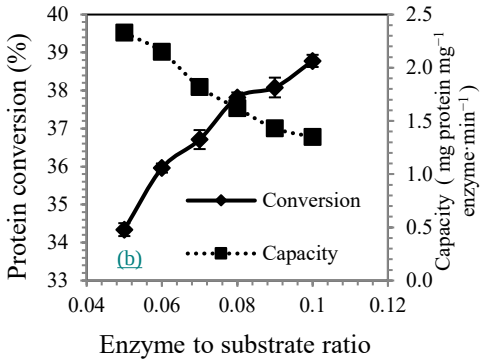
The conversion results showed the similar trend with those found during studying of the collagen concentration effect. Indeed, capacity values of all E/S ratios were stable throughout the EMR operation. These could be due to the use of low permeate flow, low TMP and low CFV already mentioned earlier.

Meanwhile, the protein conversion tended to increase as E/S increased. This result suggested that an increase in enzyme activity could enhance cleavable collagen in the permeate leading to increase in protein conversion. Similar result was previously found in continuous hydrolysis of modified wheat gluten using an EMR. These authors reported

that the protein conversion increased with increasing in E/S ratio but the increasing rate slightly slow down when the E/S ratio was higher than 0.03 [18]. On the contrary, the higher E/S ratio also caused to significantly decline of capacity since the higher amount of enzyme was used as suggested by equation 2. Indeed, it was difficult to select the suitable E/S ratio because the higher capacity was influenced by higher amount of enzyme used.



(a)



(b)

Figure 4 Changes of protein conversion with time as varying E/S ratio (a) and average protein conversion and capacity (10 h of operation) (b) (Operating condition; permeate flow rate of 0.4 ml.min^{-1}, initial TMP 1.0 bar, CFV 0.5 m.s^{-1}, hydrolyzed at pH 8 and 50 °C

Interestingly, the IC_{50} shown in Table 2 were significantly impacted by E/S ratios. In addition, the use of E/S ratio of 0.08 could provide the highest ACE-inhibitory (shows with the lowest IC_{50}). Thus, E/S ratio of 0.08 was recommended to produce collagen hydrolysate because it could provide both the high protein conversion and the highest ACE-inhibitory activity. It is worthy to note that the IC_{50} values obtained from this crude hydrolysate was higher than those purified peptides derived from pacific cod and skate skin [3,10]. One of the reasons is due to the impact of purification technique.

The residual enzymatic activity of Alcalase in the reactor and permeate were also determined. The effect of collagen concentration on the residual Alcalase activity is presented in Figure 5. It was found that Alcalase activities in the reactor rapidly decreased by approximately 20 % within the first 1 h of operation excepting those of collagen concentration of 15 g.L^{-1} (decreased by approximately 50 %).

Table 2 IC_{50} value of ACE-inhibitory activities of the permeate as varying enzyme to substrate ratio

E to S ratio	IC_{50} (mg.ml^{-1})
0.05	0.5503±0.0094^{b}
0.06	0.5042 ±0.1423^{a}
0.07	0.5597±0.0497^{b}
0.08	0.3075±0.0264^{e}
0.09	0.5951±0.0464^{c}
0.10	0.6370±0.0973^{d}

Same letters in the same column present no statistical different ($P < 0.05$)

Figure 5 also shows the Alcalase activity as affected by the ratios of E/S. The Alcalase activity of the high E/S ratio (0.07-0.10) slightly decreased by approximately 20 % along the operating time while it slightly decreased to around 70 and 60 % for 0.06 and 0.05 of E/S ratios, respectively. The decrease of Alcalase activity was possibly due to several reasons. Firstly, it may be affected by the presence of irreversible endoprotease inhibitor in the substrate

that has mentioned earlier. Secondly, the enzyme was forced to leak through the membrane by the effect of flow rate or velocity which may be attributed to the effect of shear forces. They have reported that Papain enzyme could pass through both 10 kDa MWCO ultrafiltration membrane and 0.14 µm microfiltration [28]. However, there was no Alcalase enzyme in permeate in this study (data not shown). Since low velocity (0.5 m s^{-1}) was used and Alcalase molecular weight (45 kDa) is too large to pass through the 1 kDa MWCO membrane. Finally, the enzyme activity also declined by the temperature used with long operation time (10 h). These factors led to decrease Alcalase activity in the EMR.

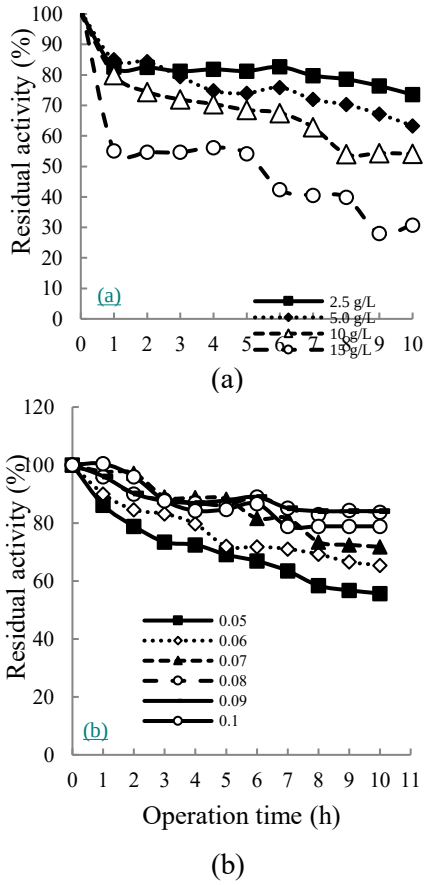


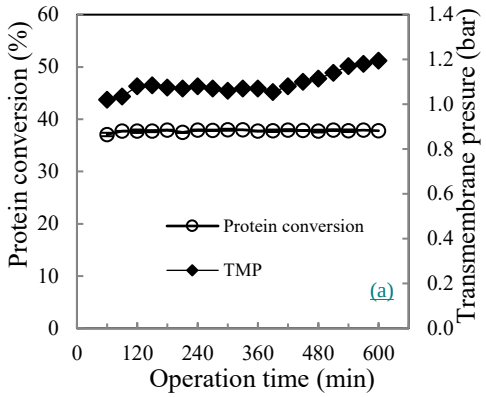
Figure 5 Effect of substrate concentration (a) and enzyme to substrate ratio (b) on residual activity of Alcalase in EMR (substrate concentration 5 g L-1, permeate flow 0.4 ml min-1, CFV 0.5 m s-1, hydrolyzed at pH 8 and 50 °C)

Stability of EMR

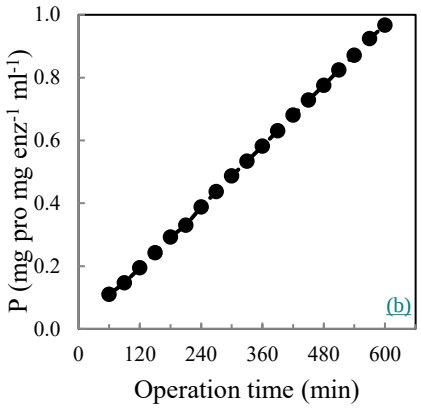
The EMR performance possibly declines during EMR operation due to several factors such as decreasing of enzyme activity, thermal degradation, increasing of insoluble materials and product inhibition [18, 23, 24]. Therefore, the stability of EMR was investigated at substrate concentration of 5.0 $g.L^{-1}$, E/S ratio of 0.08 and permeate flow rate of 0.4 $ml.min^{-1}$.

The changes of conversion were continuously evaluated for 10 h of operating time. The protein conversions (Figure 6a) showed that after 60 min, the protein conversion was almost unchanged throughout 10 h of operation. Cheryan and Deeslie [23] have suggested that the capacity might not be taken into account the effect of long term operation because the only measured variable (conversion, *X*) in Equation 2 was measure at its steady state values. Therefore, the productivity of EMR was calculated and present in Figure 6b. It appeared that the productivity of the EMR linearly increased as the operating time increased. This tendency could be due to the stable conversion through the EMR operation as mentioned above in Figure 6a. Moreover, this result agreed with previous studies on the productivity of EMRs [18, 23-25].

In addition, the TMP of EMR was monitored against times and is also shown in Figure 6a. It was observed that the TMP was unchanged during the first 7 h and then remarkably increased and finally reached to 1.2 bar at the end of operation (10 h). This trend may be due to membrane fouling causing by an increasing of substances when the EMR progressed with operating time [24-25]. This result also suggested that pre-hydolysis of collagen with Alcalase for 60 min prior introducing to membrane separation unit could reduce some degree of membrane fouling [16].



(a)



(b)

Figure 6 Changes in protein conversion and TMP during EMR operation (a) and productivity (b) (substrate concentrations, 5 g.L^{-1}; enzyme-substrate ratio, 0.08; permeate flow rate of 0.4 ml min^{-1}; hydrolysis at pH 8 and 50 °C)

Regarding to this result, it is crucial for further study required to enhance the performance of the system using operational strategy of EMR. An increase in TMP due to fouling during permeate flux constant mode possibly should be reduced by mitigating fouling of the membrane. The critical flux possibly is one of operation strategy that can be used to control the deposition and removal rate of foulant during filtration to avoid severe fouling. Gas-bubble two-phase flow can be possibly used to enhance the removal rate of foulants from the membrane surface. Self-cleaning strategy using protease enzyme presented in the EMR system is also worthy to study.

Characteristic of Peptide Produced by EMR

Permeate sample produced by EMR operating using E/S ratio of 0.08 and permeate flow rate of 0.4 ml min^{-1} was freeze-dried and then analyzed to characterize the peptides obtained.

The freeze-dried powder of collagen hydrolysate contained 87% (w/w) peptides. The molecular weights of peptides were evaluated by size exclusion chromatography (Sephadex G-15 gel filtration resin). The elution chromatogram at 220 nm shown in Figure 7 indicating that the hydrolysate contained peptides with a majority molecular weight around 500 to 800 Da. Knowing that ACE-inhibitory activity of peptide strongly relates to both peptide size and structure. Moreover, Wu *et al*. [29] have reported that ACE-inhibitory peptides in general contain between 2 to 12 amino acids [11]. In this study, 1 kDa MWCO membrane thus was chosen to separate low molecular weight peptides expecting to exhibit high ACE-inhibitory activity. As expected, the ACE-inhibitory activity of the collagen hydrolysate powder was quite high as indicated by IC_{50} value (0.27 mg.ml^{-1}). The ACE-inhibitory activity of peptides obtained was obviously higher than those of low molecular weight peptides (90.79% of which lower than 1 kDa) derived from salmon skin collagen hydrolysed with Alcalase and Papain using batch reactor (1.165±0.087 mg.ml^{-1}) [5]. Meanwhile, the ACE-inhibitory activity of this collagen peptide slightly higher than those of squid skin gelatin hydrolysed with pepsin (molecular weight < 2 kDa) (0.33 mgml^{-1}) [30]. However, the use of different enzymes and different methods to test the activity may affect the ACE-inhibitory activity and make it difficult to directly compare the IC_{50} values from different studies [31]. Considering that the sequence of amino

acid residues in peptides play an important role in their ACE-inhibitory activity [32], the amino acid sequence of active ACE-inhibitory peptides derived from collagen hydrolysate produced in EMR will be further identified

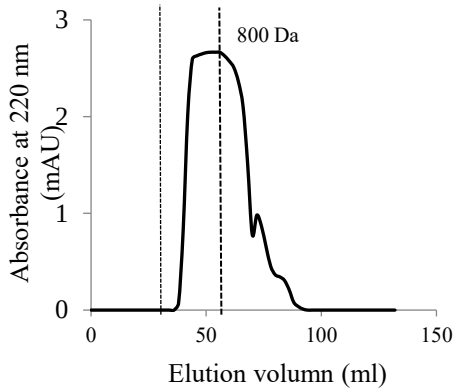


Figure 7 Molecular weight distribution of the collagen peptides produced from EMR

4.0 CONCLUSION

The effect of collagen concentration and enzyme to substrate ratio on conversion, capacity and ACE-inhibitory activity of permeate were investigated. The increase in substrate concentration caused a lower conversion and capacity since a higher collagen concentration would cause fouling due to the accumulation of high molecular mass collagen. It was also observed that the increasing of enzyme to substrate ratio could induce protein conversion but decrease the EMR capacity. Therefore, optimization of collagen concentration and enzyme to substrate ratio required precise evaluation. In addition, the EMR could not only provide a stable conversion and TMP throughout the operating but also maintain acceptable level of enzyme activity. Moreover, the collagen peptides from EMR permeation exhibited high ACE-inhibitory activity.

ACKNOWLEDGEMENT

This study was supported by the Higher Education Research Promotion and the National Research University Project of Thailand, Office of the Higher Education Commission, the Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program (PHD/022/2552) and the Graduate School, Prince of Songkla University, Songkhla, Thailand.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

[1] Z. Du, Y. Li. (2022). Review and perspective on bioactive peptides: A roadmap for research, development, and future opportunities. *J. Agri. Food. Res., 9*, 100353.

[2] J. Jia, Y. Zhou, J. Lu, A. Chen, Y. Li, G. Zheng. (2010). Enzymatic hydrolysis of alaska pollack (Theragra chalcogramma) skin and antioxidant activity of the resulting hydrolysate. *J Sci Food Agric., 90*, 635-640.

[3] S. W. Himaya, D. H. Ngo, B. Ryu, S. K. Kim. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. *Food Chem., 132*(4), 1872–1882.

[4] J. K. Seo, M. J. Lee, H. J. Go, Y. J. Kim, N. G. Park. (2014). Antimicrobial function of the GAPDH-related antimicrobial

peptide in the skin of skipjack tuna, Katsuwonus pelamis. *Fish Shellfish Immunol*., *36*(2), 571–581.

[5] R. Z. Gu, C. Y. Li, W. Y. Liu, X. W. Yi, M. Y. Cai. (2011). Angiotensin I-converting enzyme inhibitory activity of low-molecular-weight peptides from Atlantic salmon (Salmo salar L.) skin. *Food Res Int., 44*(5), 1536–1540.

[6] Y. Zhang, X. Duan, Y. Zhuang. (2012). Purification and characterization of novel antioxidant peptides from enzymatic hydrolysates of tilapia (Oreochromis niloticus) skin gelatin. *Peptides*. *38*(1), 13–21.

[7] J. K. Lee, J. Jeon, H. Byun. (2014). Antihypertensive effect of novel angiotensin I converting enzyme inhibitory peptide from chum salmon (Oncorhynchus keta) skin in spontaneously hypertensive rats. *J Funct Foods. 7*, 381–389.

[8] D. H. Ngo, K. H. Kang, B. Ryu. 2015. Angiotensin-I converting enzyme inhibitory peptides from antihypertensive skate (Okamejei kenojei) skin gelatin hydrolysate in spontaneously hypertensive rats. *Food Chem., 174*, 37–43.

[9] T. Ichimura, A. Yamanaka, T. Otsuka, E. Yamashita, S. Maruyama. (2009). Antihypertensive effect of enzymatic hydrolysate of collagen and Gly-Pro in spontaneously hypertensive rats. *Biosci Biotechnol Biochem., 73*(10), 2317–2319.

[10] J. K. Lee, J. K. Jeon, H.G. Byun. (2011). Effect of angiotensin I converting enzyme inhibitory peptide purified from skate skin hydrolysate. *Food Chem., 125*(2), 495–499.

[11] Wu, J., Aluko, R. E., Nakai, S. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship study of Di- and tripeptides. *J Agric Food Chem., 54*, 732–738

[12] B. Sitanggang, A. Drews, M. Kraume. (2022). Enzymatic membrane reactors: Designs, applications, limitations and outlook. *Chem. Eng. Processing Proc. Intens., 80*, 128709

[13] Y. Wang, H. Zhang, R. Fan, Y. Wan, M. Huang, S. Huang, L. Pan, J. Luo. (2024). Stabilizing enzymatic membrane reactor for precise production of oligodextran with tailored molecular weight. *Chem. Eng. Sci., 5*, 120077.

[14] H. Zhang, L. Liu, M. Pinelo, Y. Huang, W. Zhou, Y. Wan, J. Luo. (2022). Integrated microsphere-packed bed enzymatic membrane reactor for enhanced bioconversion efficiency and stability: A proof-of-concept study. *J. Mem. Sci., 658*, 120732.

[15] C. Martin-Orue, G. Henry, S. Bouhallab. (1999). Tryptic hydrolysis of κ-caseinomacropeptide: Control of the enzymatic reaction in a continuous membrane reactor. *Enzyme Microb Technol., 24*(98), 173–180.

[16] M. Tauntong, N. Sirinupong, W. Youravong. (2014). Effect of pre-hydrolysis by alcalase on enzymatic membrane reactor performance in production of low molecular weight peptide from nile tilapia skin gelatin. *Kasetsart Journal-Natural Science*, *48*(6), 929–941.

[17] M. Tauntong, N. Sirinupong, W. Youravong. (2016). Triple helix structure of acid soluble collagen derived from nile tilapia skin as affected by extraction

Temperature. *J Sci Food Agric., 96*(11), 3795–800.

[18] J. Cui, X. Kong, Y. Hua, H. Zhou, Q. Liu Q. (2011). Continuous hydrolysis of modified wheat gluten in an enzymatic membrane reactor. *J Sci Food Agric., 91*(15), 2799–2805.

[19] S. C. Cheison, Z. Wang, S. Y. Xu. 2007. Preparation of whey protein hydrolysates using a single- and two-stage enzymatic membrane reactor and their immunological and antioxidant properties: Characterization by multivariate data analysis. *J Agric Food Chem., 55*(10), 3896–3904.

[20] L. B. Nielsen, H. H. Nielsen. (2001). Purification and characterization of cathepsin D from herring muscle (Clupea harengus). *Comp Biochem Physiol - B Biochem Mol Biol., 128*(2), 351–363.

[21] C. A. Bailey, P. Bryla, A. W. Malick. (1996). The use of the intestinal epithelial cell culture model, Caco-2, in pharmaceutical development. *Adv Drug Deliv Rev., 22*(1–2), 85–103.

[22] L. E. Shi, G. Q. Ying, Z. X. Tang, J. S. Chen, W. Y. Xiong, H. Wang. (2009). Continuous enzymatic production of 5-nucleotides using free nuclease P1 in ultrafiltration membrane reactor. *J Memb Sci., 345*(1–2), 217–222.

[23] M. Cheryan, W. D. Deeslie. (1983). Soy protein hydrolysis in membrane reactors. *J Am Oil Chem Soc., 60*(6), 1112–1115.

[24] W. Qu, H. Ma, W. Zhao, Z. Pan. (2013). ACE-inhibitory peptides production from defatted wheat germ protein by continuous coupling of enzymatic hydrolysis and membrane separation: Modeling and experimental studies. *Chem Eng J., 226*, 139–145.

[25] A. Perea, U. Ugalde. (1996). Continuous hydrolysis of whey proteins in a membrane recycle reactor. *Enzyme Microb Technol., 18*(1), 29–34.

[26] W. Qu, H. Ma, W. Li, Z. Pan, J. Owusu, C.Venkitasamy. (2015). Performance of coupled enzymatic hydrolysis and membrane separation bioreactor for antihypertensive peptides production from Porphyra yezoensis protein. *Process Biochem., 50*(2), 245–252.

[27] A. Guadix, F. Camacho, E. M. Guadix. (2006). Production of whey protein hydrolysates with reduced allergenicity in a stable membrane reactor. *J Food Eng., 72*(4), 398–405.

[28] D. Belhocine, H.Mokrane, H. Grib, H. Lounici, A. Pauss, N. Mameri. (2000). Optimization of enzymatic hydrolysis of haemoglobin in a continuous membrane bioreactor. *Chem Eng J., 76*(3), 189–196.

[29] S. Wu, X. Feng, X. Lan, Y. Xu, D. Liao. 2015. Purification and identification of angiotensin-I converting enzyme (ACE) inhibitory peptide from lizard fish (Saurida elongata) hydrolysate. *J Funct Foods., 13*, 295–299.

[30] L. Lin, S. Lv, B. Li. (2012). Angiotensin-I-converting enzyme (ACE)-inhibitory and antihypertensive properties of squid skin gelatin hydrolysates. *Food Chem., 131*(1), 225–230.

[31] P. A. Harnedy, M. B. O'Keeffe, R. J. FitzGerald. (2015). Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga Palmaria palmata. *Food Chem., 172*, 400–406.

[32] R. J. Elias, S. S. Kellerby, E. Decker. (2015). Antioxidant activity of proteins and peptides. *Crit Rev Food Sci Nutr.,* 48, 430–441.