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# Production of Angiotensin I-converting Enzyme Inhibitor Derived from Egg White Protein Hydrolysates Using a Membrane Reactor

**WEN-DEE CHIANG[1], MAY-JUNE TSOU[2], CHIEN-HUI WENG[1] AND TSUN-CHUNG TSAI[1]***

*[1] Department of Food Science, Tunghai University, 181, Sec. 3, Taichung Port Rd., Situn District, Taichung City 407, Taiwan (R.O.C.)*
*[2] Department of Nutrition and Health Science, Chungchou Institute of Technology, 6, Lane 2, Sec. 3, Shanjiao Rd., Yuanlin Township, Changhua County 510, Taiwan (R.O.C.)*

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## ABSTRACT

Egg white proteins (EWP) were hydrolyzed with four proteolytic enzymes, including Thermolysin, Alcalase, Esperase and Chymotrysin, to produce hydrolysates with angiotensin I-converting enzyme (ACE) inhibitory activity. The result indicated that EWP hydrolyzed for 0.5-24 hr with Thermolysin produced the highest ACE inhibitory activity among the four enzymes. Therefore, EWP-Thermolysin hydrolysate was produced and further fractionated using several membranes with molecular weight cut-off (MWCO) of 10,000, 3,000 and 1,000 daltons, sequentially. The 1 kDa permeate obtained from the hydrolysate treatment using 1,000 daltons MWCO membrane could further reduce its $IC_{50}$ value from 54.1 to 17.2 μg protein/mL. A lower $IC_{50}$ value represented higher ACE inhibitory activity. The operation stability study showed that the membrane reactor system could maintain a steady production of EWP-Thermolysin hydrolysate over 8 hr. The gastrointestinal protease *in vitro* effect on the ACE inhibitory activity of 1 kDa permeate indicated that gastrointestinal proteases have no significant effect ($p > 0.05$) on the ACE inhibitory activity of 1 kDa permeate.

Key words: egg white proteins, ACE inhibitor, hydrolysate, peptide, membrane reactor

## INTRODUCTION

Hypertension is functionally related to the angiotensin I-converting enzyme (ACE) (EC 3.4.15.1). This enzyme plays a key physiological role in blood pressure control by virtue of the renin-angiotensin system. ACE cleaves to the C-terminal of the histidyl-leucine of the inactive decapeptide angiotensin I to form octapeptide angiotensin II, a potent vasoconstrictor. ACE also inactivates the vasodilating nonapeptide bradykinin[(1)]. Therefore, ACE inhibition with its inhibitor reduces the angiotensin-II activity but increases bradykinin levels, and thus can result in lowered blood pressure[(2)].

Enzymatic hydrolysis of food proteins, such as casein, zein, fish protein and gelatin that release ACE inhibitors as potential nutraceuticals, have been extensively studied[(3-5)]. However, only few egg protein-derived peptides have been described. Miguel *et al.* (2004) produced peptides with ACE inhibitory activity from the enzymatic hydrolysis of egg white proteins[(6)]. These peptides were derived mainly from the ovalbumin proteolysis. They concluded that egg white protein hydrolysis with pepsin to obtain a fraction having a molecular weight lower than 3,000 daltons would produce the highest ACE inhibitory activity *in vitro*. Yoshii *et al.* (2001) obtained oligopeptides of 1,000 daltons or less with ACE inhibitory activity *in vitro* from chicken egg yolk hydrolysate using a crude enzyme and dialysis with a semi-permeable membrane filter[(7)]. They also proved oral administration of the oligopeptides for 12 weeks could suppress hypertension development in spontaneously hypertensive rats.

Enzymatic hydrolysis is very costly in conventional batch-type operations because large quantities of enzymes are required and the enzyme cannot be reused[(8,9)]. Other disadvantages of batch process also have been reported such as time-consuming, labor-intensive, low yield, low productivity and inconsistent quality[(10-13)]. The development and application of ultra-filtration membrane reactor for the hydrolysis of proteins has been applied to overcome those problems[(14)]. Several studies have concluded that protein hydrolysate production in a continuous membrane reactor results in higher productivities[(15-17)] and more uniform products[(14,18)] than batch-type reactors.

* Author for correspondence. Tel: +886-4-23506747; Fax: +886-4-23506747; E-mail: tct1@thu.edu.tw

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The objectives of this research were to screen both proper enzyme and ultra-filtration membrane to apply in a continuous membrane reactor for EWP hydrolysate production with ACE inhibitory activity and evaluate the membrane reactor performance for hydrolysate production. The product quality was determined by measuring its $IC_{50}$ of the ACE inhibitory activity during processing followed by the effects of hydrolysis of the gastric proteases.

## MATERIALS AND METHODS

I. *Enzymes and C*

Crude dried egg white proteins (from chicken egg white), chymotrypsin (40-60 units/mg protein), pepsin (800-2500 units/mg protein), pancreatin, Thermolysin (84 units/mg protein), angiotensin-I converting enzyme (ACE) (10 units/mg protein), and hippuryl-L -histidyl-L-leucine (HHL) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pancreatin contains many enzymes including amylase, trypsin, lipase, ribonuclease and protease. HPLC standards including cytochrome c, aprotinin, substance P, $(glycine)_6$, $(glycine)_3$ and glycine with molecular weight 125,000, 6,500, 1,348, 360, 189 and 75 daltons, respectively, were purchased from Merck (Damdstadt, Germany) or Sigma Chemical Co. (St. Louis, MO, USA). Esperase type F (7.5 units/g) and Alcalase\ 2.4 L (2.4 units/g) were purchased from NOVO Industry A/S (Copenhagen, Denmark). Polyethersulfone spiral-wound membranes with molecular weight cut-off 1-10 kDa and with membrane area 0.2 $m^2$ were purchased from Osmonics Inc. (California, USA).

II. *Preparation of Egg Wwhite Protein (EWP) Hydrolysates*

The 1.0% (w/v) EWP and 1.0% (w/w of EWP) protease were employed to produce and compare of each hydrolysate with ACE inhibitory activity. The proteases employed in this study included Thermolysin, Alcalase, Esperase and Chymotrypsin. The optimum temperature and pH, reaction conditions for each enzyme were suggested by the supplier. The temperature and pH were adjusted to 55°C and 7.5 for Alcalase, 60°C and 8.0 for Thermolysin and Esperase, and 37°C and 7.5 for Chymotrypsin, respectively. Samples withdrawn at 0.5, 1, 2, 4, 6, 8, 24 hr from each proteolytic mixture were immediately heated in a boiling water bath for 10 min. The degree of hydrolysis (DH) and ACE inhibitory activity of each sample were determined. The DH at designated time was measured using the orthophthaldialdehyde (OPA) method[19].

III. *Assay for ACE Inhibitory Activity*

ACE Inhibitory activity was analyzed spectrophotometricly using hippury-L-histidyl-L-leucine (HHL) as substrate[20]. Five millimolar HHL was prepared with 0.1 M sodium borate buffer (pH 8.3) containing 0.4 M NaCl. ACE from rabbit lungs was dissolved in the same buffer at a concentration of 60 mU/mL. A mixture containing 45 μL of HHL solution and 5 μL of protein hydrolysate or the buffer (control) was incubated at 37°C for 5 min. The 15 μL of ACE solution was then added and incubated for 30 min. The reaction was stopped with 65 μL of 0.1% trifluoroacetic acid (TFA). Hippuric acid (HA) liberated using ACE was determined by RP-HPLC on a LiChrospher C18 column (4 × 250 mm, Merck, Germany). The mobile phase was 0.1% TFA in 50% methanol with a flow rate of 0.8 mL/min. The effluent was monitored with an ultraviolet detector (Shimadzu Co., Kyoto, Japan) at 228 nm. The $IC_{50}$ value was defined as the ACE inhibitor or protein hydrolysate concentration needed to reduce 50% of the height for the HA peak, and determined by regression analysis of ACE inhibitory activity (%) versus protein concentration. The IC50 value was expressed in terms of mg protein/mL. ACE inhibitory activity (%) was expressed as

$$\text{ACE inhibitory activity (\%)} = \frac{\text{Ho - Hp}}{\text{Ho}} \times 100 \qquad (1)$$

where Ho = height for HA peak without protein hydrolysate; Hp = height for HA peak with protein hydrolysate.

IV. *Fractionation of Hydrolysates with Ultra-filtration Membranes*

Hydrolysates generated from the enzymatic hydrolysis of chicken egg EWP were subjected to ultra-filtration using a spiral-wound membrane with 10 kDa, 3 kDa and 1 kDa molecular weight cut-off, sequentially. Each permeate was collected to determine its protein concentration, profile of molecular weight distribution, and $IC_{50}$ for ACE inhibitory activity.

V. *Batch Reactor*

The batch reactor consisted of a reaction vessel (1 L) with hot water jacket and stirring arm to provide uniform heating and mixing. About 0.5 to 1 L of 1% EWP solution was introduced into the reaction vessel and heated. Until the solution reached the desired temperature, 1% of enzyme / substrate (w/w) was added into the reaction vessel.

VI. *Membrane Reactor System*

One percent of EWP solution was prepared by vigorously stirring and heating while passing through a 100-mesh sieve to remove large particles. The reaction vessel with jacket (Figure 1) was filled with the desired volume of 1% EWP filtrate and the tank temperature was maintained at 50°C using Neslab circulator unit (Thermo, USA). One percent of enzyme (w/w of substrate) was added into the EWP filtrate. The inlet pressure and flow

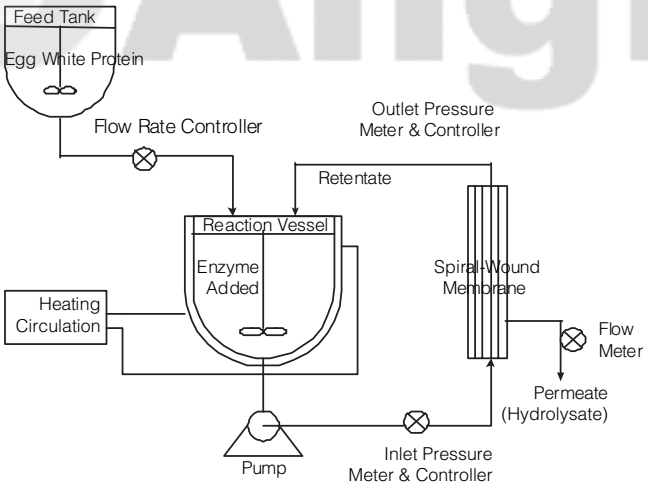


**Figure 1.** Schematic diagram of continuous spiral-wound membrane reactor system.

rate were adjusted properly. The reaction mixture was pumped to the spiral-wound membrane module with an inlet pressure of 4 kg/cm$^2$, where large particles such as intact proteins or enzymes could not penetrate the ultra-filtration membrane pores. These large particles would be recycled into the reaction vessel. The permeate containing particles small enough to penetrate the membrane was collected and lyophilized. The level of reaction mixture in the vessel was controlled by adjusting the flow rates between the EWP solution from the feed tank and the permeate from the membrane.

VII. *Total Nitrogen*

Total nitrogen was measured using the micro-Kjeldahl method[21] using a KjelTec system 1026 with a rapid distillation unit (Tecator, Sweden). All data were corrected for nonprotein nitrogen (NPNi), which was determined as the initial EWP soluble nitrogen in 10% trichloroacetic acid (TCA). The TCA-soluble nitrogen in the product (NPNp) was also determined. The % TCA soluble nitrogen or % conversion X was expressed as

$$X\,(\%) = \frac{NPNp - NPNi}{TN - NPNi} \times 100 \qquad (2)$$

where TN = total nitrogen in the product.

VIII. *Productivity Study*[22]

For the batch reactor, productivity was defined as

$$P_{batch} = \frac{XTN_i}{E} \qquad (3)$$

where E = enzyme concentration (mg/mL); $TN_i$ = total nitrogen in unhydrolyzed substrate corrected for NPNi.

For continuous HF membrane reactor, productivity was expressed as

$$P_I = \frac{P\ Jt}{EV} \qquad (4)$$

or

$$P_c = \Sigma P_I \qquad (5)$$

where $P_I$ = instantaneous productivity; P = average product output (mg N/mL) in a time period t (min); J = flow rate (mL/min); E = enyzme concentration (mg/mL); V = substrate volume (L); $P_c$ = cumulative productivity. $P_c$ was calculated over all periods studied. The unit of productivity was mass hydrolysate/ mass enzyme (mg N/ mg enzyme).

IX. *Molecular Weight Distribution*

The molecular weight distribution of the hydrolysate was analyzed using high-performance size-exclusion chromatography (HPSEC). The HPSEC was equipped with Superdex HR 10/30 column (Amersham Biosciences Ltd., Pittsburgh, PA., USA) connected to an UV detector (Shimadzu Co., Kyoto, Japan) set at 214 nm. The mobile phase was 0.02 M phosphate buffer (pH 7.2) containing 0.25 M NaCl and flow rate was set at 0.5 mL/min.

X. *Stability Study*

The stability against *in vitro* gastric proteases was assessed as followed[23]. One percent (w/v) of EWP hydrolysate was treated in 0.1 M KCl-HCl (pH 2.0) buffer with pepsin for 4 hr in a water bath at 37°C. The reaction was stopped by boiling in a water bath for 15 min and neutralized to pH 7.0 with the addition of 2N NaOH solution. One milliliter of neutralized suspension was centrifuged (10,000 ×g, 40min) and the supernatant was used for ACE inhibitory activity determination. The remaining neutralized suspension was digested further by 2% (w/w) pancreatin at 37°C for 4 hr. The enzyme was inactivated by boiling for 15 min followed by centrifugation. The supernatant was used for ACE inhibitory activity determination.

## RESULTS AND DISCUSSION

I. *Effect of Different Protease Hydrolysis on ACE Inhibitory Activity*

Egg white proteins (EWP) were hydrolyzed using Thermolysin, Alcalase, Esperase and Chymotrypsin for 0.5-24 hr, respectively. Figure 2 shows the ACE inhibitory activity and the degree of hydrolysis (DH) in comparison of the four enzyme hydrolysates as a function of the hydrolysis time. Both DH and ACE inhibitory activity increased with the increase in hydrolysis time for each enzyme. The graphs clearly showed that enzymatic hydrolysis was required to release ACE inhibitory peptides from

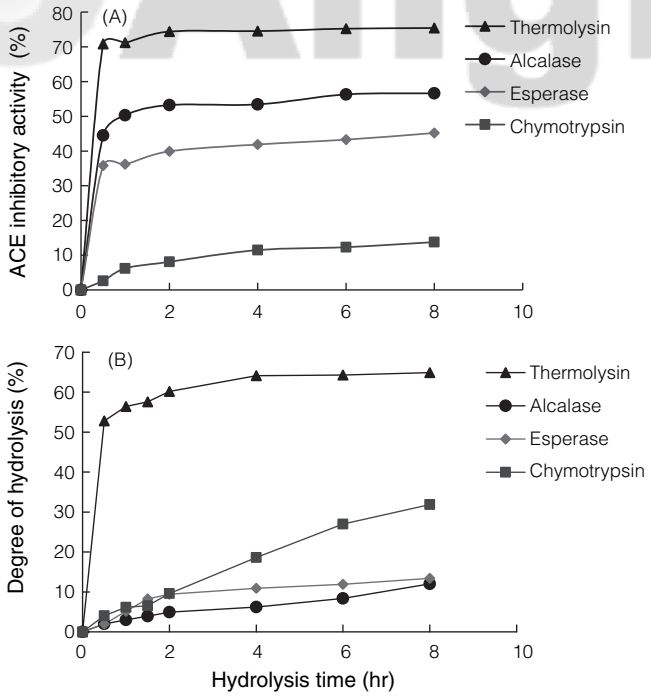


**Figure 2.** Comparison of (A) ACE inhibitory activity; and (B) degree of hydrolysis (DH) within four enzyme hydrolysates as a function of hydrolysis time. Each hydrolysate was diluted 10 times befor measurement of the inhibitory activity. Results are mean values of at least two replicates. Standard deviation did not exceed 3% of the recorded values. The reaction conditions of temperature and pH were adjusted to 55°C and 7.5 for Alcalase, 37°C and 7.5 for chymotrypsin, 60°C and 8.0 for Thermolysin and Esperase, respectively.

**Table 1.** The peptide content, yield and IC50 value of the egg white protein hydrolysate (EWPH) and its permeates, filtered by different molecular weight cut-off membranes in a membrane reactor system

| Sample* | Peptide content (mg protein/mL) | Peptide yield (%) | $IC_{50}$ (μg protein/mL)** |
|---|---|---|---|
| EWPH | 7.95 | 100 | 54.1[a] |
| 10 kDa permeate | 5.98 | 76.2 | 35.1[b] |
| 3 kDa permeate | 3.73 | 48.8 | 22.6[c] |
| 1 kDa permeate | 3.04 | 39.6 | 17.2[d] |

*EWPH filtered by a membrane module with 10 kDa, 3 kDa or 1 kDa MWCO, denoted as 10 kDa, 3 kDa or 1 kDa permeate, respectively.
**It is significantly different ($p < 0.05$) with different letter.

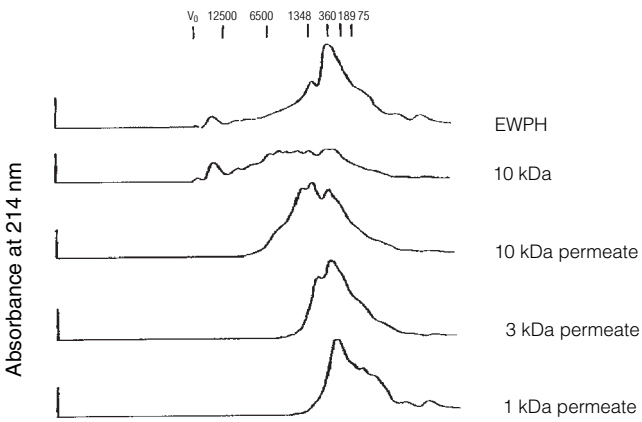


**Figure 3.** The molecular weight distribution of egg white protein hydrolysate (EWPH) and its retentates and permeates obtained from the membrane reactor with different MWCO membranes.

the inactive form of intact EWP. After 2-4 hr of hydrolysis, all enzymes had steady ACE inhibitory activity. Thermolysin showed the highest ACE inhibitory activity and DH among the four enzymes. Thermolysin also had the best ACE inhibitory activity in terms of $IC_{50}$ with the value of 54.1 μg protein/mL at steady state as compared with the $IC_{50}$ value of 78.8, > 750 and >750 μg protein/mL for Chymotrypsin, Alcalase and Esperase, respectively. The lower $IC_{50}$ represented higher ACE inhibitory activity. Therefore, Thermolysin was selected for further study on enzymatic production of ACE inhibitor from EWP. Thermolysin is a bacterial protease that has been studied to hydrolyze porcine skeletal muscle protein, bonito and lactoglobulin to produce ACE inhibitor[24-26].

II. *Effect of Different Ultra-filtration Membranes on ACE Inhibitory Activity*

EWP hydrolysates (EWPH) were prepared by hydrolysis of 1.0% EWP with 1.0% Thermolysin at 60°C and pH 8.0 for 2 hr, heating in a boiling water bath for 10 min, and then centrifugation at 10,000 ×g for 10 min. The EWPH was collected for further study on ACE inhibitory peptides fractionation using spiral-wound membranes.

Table 1 shows the peptide content, peptide yield and ACE inhibitory activity $IC_{50}$ of each permeate obtained by ultra-filtering EWPH with 10, 3 and 1 kilo-daltons (kDa) molecular weight cut-off (MWCO) membranes, sequentially. The ACE inhibitory activities and peptide yield of the permeates increased with the decrease in MWCO. The 1 kDa permeate obtained from EWPH treatment using 1,000 daltons MWCO membrane could further reduce its $IC_{50}$ value from 54.1 to 17.2 μg protein/mL. Therefore, 1 kDa MWCO membranes were further applied for continuous ACE inhibitor production.

Figure 3 shows the molecular weight distribution of EWPH and its retentates and permeates obtained from the membrane reactor with different MWCO membranes. By examining the 10 kDa permeate profile, the 10 kDa MWCO membrane could eliminate all peptides with a molecular weight (MW) greater than 6.5 kDa in the EWPH. Furthermore, the 3 kDa and 1 kDa permeate profiles also showed that 3 kDa and 1 kDa MWCO membranes could further remove peptides with MW greater than 1.5 kDa and 0.4 kDa, respectively. These

results indicated that the membrane module was very effective in fractionating EWPH into smaller peptide fractions. The smaller MWCO membrane resulted in the lower $IC_{50}$ value (Table 1). The results also implied that peptides with MW less than 0.4 kDa were responsible for the major ACE inhibitory activity in the EWPH.

Tryptic casein hydrolyaste with an $IC_{50}$ value of 166 μg protein/mL had been reported to have an antihypertensive effect in spontaneously hypertensive rats[27]. The $IC_{50}$ values obtained from ultra-filtered tryptic digests of milk protein ranged from 130-201 μg protein/mL expressed the potential to apply such protein hydrolysates as nutraceuticals in the prevention of hypertension[28]. Furthermore, single oral administration of royal jelly protein hydrolysates with an $IC_{50}$ value of 99 μg protein/mL in 10-week spontaneously hypertensive rats resulted in a significant reduction in systolic blood pressure[29]. Therefore, the $IC_{50}$ values for ultra-filtered EWPH reported herein were even below the concentration range and likely to mediate an anti-hypertensive effect (Table 1).

III. *Continuous Spiral-wound (SW) Membrane Reactor*

Both productivity and operation stability of continuous SW membrane reactors were evaluated using a 1 kDa MWCO membrane. Figure 4 illustrates a productivity comparison between batch and continuous reactors as a function of the volume replacement. The productivity of the batch reactor was independent of the volume replacement, whereas the continuous SW membrane reactor had a direct linear relationship with the volume replacement. This was because that each volume replacement (1 L) required the same amount of enzyme in the batch reactor. However, in the continuous SW membrane reactor the enzyme was introduced only once at the beginning of the run. After three volume replacements, the continuous membrane reactor had a higher productivity than the batch reactor. The result indicated that reusing the enzyme had the advantage of higher productivity in the continuous membrane reactor. Several studies also agreed with these findings[16,18,30].

Figure 5 shows the operation stability of the continuous membrane reactor system in terms of product output and $IC_{50}$. The continuous SW membrane reactor showed steady protein hydrolysate production after a 6 hr operation. There was no detectable enzyme leakage within 8 hr using residue activity detection in the permeate (data not shown). At any operation time the $IC_{50}$ of each permeate from the continuous SW membrane reactor maintained its inhibitory activity below 18 μg protein/mL.

Since some food-protein-derived ACE inhibitors failed to express hypotensive activity after oral administration *in vivo*[4], the mixture of 1 kDa permeates collected from the continuous membrane reactor was investigated for its ACE inhibitory activity *in vitro* by treatment with gastrointestinal proteases including pepsin and the combination of pepsin and pancreatin, respectively. Gastric *in vitro* incubation has been reported to provide a practical and easy process, imitating the fate of these peptides after oral administration[23]. Table 2 shows the effects of gastrointestinal proteases on ACE inhibitory activity of 1 kDa permeate *in vitro*. The result indicated that gastrointestinal protease had very little effect ($p < 0.01$) on the ACE inhibitory activity of the mixture of 1 kDa permeates. Therefore, the result suggested that peptides in 1 kDa permeate be resistant to digestion. Several other reports also have concluded that small peptides had low susceptibility to hydrolysis by gastric proteases[7,23,31].

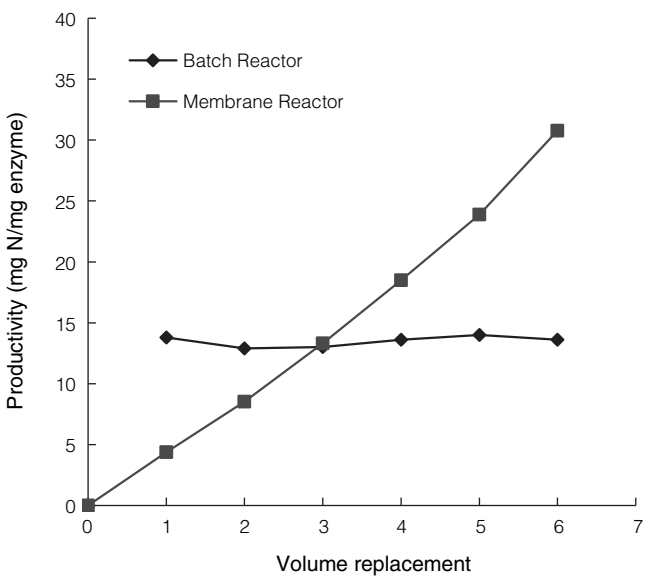


**Figure 4.** Comparison of productivity between batch reactor and membrane reactor. Results are mean values of at least two replicates. Standard deviation did not exceed 4% of the recorded values.

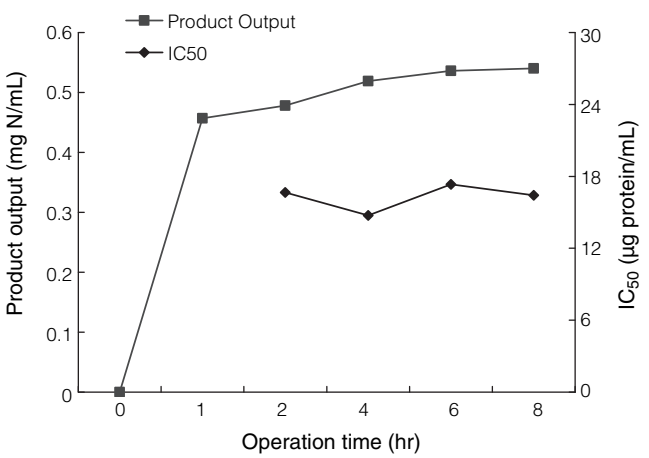


**Figure 5.** Operation stability of the continuous membrane reactor system in terms of product output and $IC_{50}$. Results are mean values of at least two replicates. Standard deviation did not exceed 4% of the recorded values.

**Table 2.** ACE inhibitory activity of 1 kDa permeate followed by digestion using gastrointestinal proteases

| Enzyme | IC50 (μg protein/mL)* |
|---|---|
| Control (1 kDa permeate) | 17.2[a] |
| Pepsin | 17.0[a] |
| Pepsin + pancreatin | 16.5[a] |

*It is not significantly different ($p > 0.05$) with same letter.

## CONCLUSIONS

The results from this study showed that it was possible to continuously produce EWPH with ACE inhibitory activity in a membrane reactor. The major advantages of the membrane reactor over the traditional batch reactor were the large increase in enzyme utilization efficiency and overall reactor productivity. By selecting the optimum MWCO membrane a consistently uniform product with the desired molecular weight characteristics could be achieved. The 1 kDa permeate with $IC_{50}$ less than 18 μg protein/mL obtained from the membrane reactor might represent a potential nutraceutical for the prevention of hypertension. However, further confirmation of the efficacy of this nutraceutical on blood pressure decrease in spontaneously hypertensive rats or in clinically hypertensive human subjects is required.

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