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Assessment on multilayer ultrafiltration membrane for fractionation of tilapia by-product protein hydrolysate with angiotensin I-converting enzyme (ACE) inhibitory activity

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ABSTRACT

Ultrafiltration membrane is a convenient system to fractionate fish protein hydrolysate and recovery of peptide fraction with high ACE inhibitory activity. However, the limitation of applying membrane system in protein fractionation is its poor selectivity. This study aiming to assess the application of multilayer ultrafiltration in order to improve the selectivity of the tilapia by-product protein hydrolysate (TBH) separation by achieving higher amount of small peptides. Flat sheet regenerated cellulose (RC) membrane with molecular weight cut-off (MWCO) of 10 and 5 kDa were used for the separation of peptide mixtures. The membrane were arranged in the orientation of 10/5 kDa and 5/5 kDa, in which these two membrane were stacked together in one device. The performance of multilayer membrane were evaluated based on the permeate flux and peptide transmission, and compare with the single membrane system (5 and 10 kDa). The performance ultrafiltration membrane for fractionating TBH was studied under two different conditions (rotation speed and pH). The highest permeate flux and peptide transmission were obtained at membrane with pore size of 10 kDa (single), followed by 10/5 (multilayer), 5 (single) and 5/5 kDa (multilayer). Based on selectivity analysis, most permeate produced were composed of peptides lower than 1500 Da. When the smaller membrane's pore size is used (5 kDa membrane and 5/5 multilayer membranes), the amount of small-sized peptide (<500 Da) increased, indicating that selectivity (specifically on small-sized peptide) can be improved. The permeate from multilayer 5/5 and 10/5 kDa membranes have higher value of ACE inhibitory activity (84.04% and 75.59%, respectively) compared with the single membrane (5 kDa – 71.83% and 10 kDa – 64.32%). This might be due to the permeate enriched with small-sized peptide. Thus, application of multilayer membrane shows the potential to recover high ACE inhibitory activity from TBH.

1. Introduction

Considering the potential uses of fish by-products as a food ingredients, there is a need for a better management of by-products in fishing industries. These by-products are believed to contain a great amount of proteins that can be transformed into a valuable food products through enzymatic hydrolysis. Fish protein hydrolysate (FPH) obtained can be used as food ingredients in order to improve the nutritional properties of food products or bioactive peptides [1]. FPH generated through enzymatic hydrolysis consists of peptide mixtures with various sizes. Peptide sizes are closely related with functional properties; appropriate molecular size can improve the functional properties of end products. Researchers have reported that small peptides (1–5 kDa) from fish protein hydrolysates have strong influence on Angiotensin I-converting enzyme (ACE) inhibition activity [2–6]. ACE plays an important role in the regulation of blood pressure and hypertension [5] and the inhibition of ACE activity (>50%) is a good target for antihypertension treatment. Thus, the separation of this fraction from the protein hydrolysate mixtures becomes a priority.

Membrane technology particularly ultrafiltration (UF) is an effective technology for concentration, extraction, fractionation of molecules and offers a good alternative to traditional separation techniques to achieve environmentally friendly and cost effective process [7]. UF membrane is an established technique in the fractionation of protein hydrolysate because it offers several advantages; simple, selective and energy efficient [8]. Ultrafiltration

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membrane has been used for a couple of decades to fractionate protein hydrolysates from dairy [9–12], animal [13] or vegetable products [14–16], and fish [17–21] for obtaining a specific peptide size.

The application of UF in the separation of protein hydrolysate is a complex process. It may not have a simple and straightforward relationship with the membrane pore size. There are several limitations that can affect the membrane separation process such as the concentration polarization which is the accumulation of retained molecules near the membrane surface, protein adsorption within the porous structure of the membrane, protein deposition on the membrane surface and protein-protein interactions in the feed solution. Such limitations can lead to membrane fouling, thus resulting in membrane performance deterioration [22]. In addition, poor selectivity is one of the common problems for the fractionation of protein using the ultrafiltration membrane. It is usually related to the imperfection of the pore size distribution of the available commercial membranes [23]. A new technique of ultrafiltration membrane called the multilayer membrane has been introduced by Feins and Sirkar [24,25], with ultimate aim to overcome the broad membrane pore size distribution by stacking two or more membranes together in one device, where essentially a completely pure product could be achieved. Later, this technique has been extensively studied by Field and co-workers [23,26,27] for the fractionation of binary protein mixture.

In the present study, ultrafiltration membrane was used to separate TBH produced from enzymatic hydrolysis using alcalase through multilayer membrane in order to enrich the bioactive peptide with small-sized peptide (<500 kDa). The separation of TB protein hydrolysate using UF membrane is conducted not only for obtaining a specific peptide size, but also to enrich peptides with ACE inhibitory activity. Until recently, no study has been reported on the application of ultrafiltration for fractionating TBPH. So far, only one work has been reported on the application of UF in fractionating tilapia muscle protein hydrolysate [5], but it did not discuss in detail with respect to the factors that could affect the UF membrane separation process in order to obtain the desired peptide size. Other studies have reported on the fractionation of FPH from different types of fish [5,18,19], but only used UF as a convenient and simple tool for analytical purpose. Therefore, the objective of this study was to evaluate the performance of the multilayer UF membranes based on permeate flux and peptide transmission, and to understand whether fractionation could improve the recovery of small sized peptide (<500 kDa) with ACE inhibitory activity. The performance of the multilayer UF membrane will also be compared with single UF membrane system.

2. Materials and methods

2.1. Preparation of tilapia by-product mince

Fresh red tilapia (*Oreochromis niloticus*) was obtained live from a local fish farm in Rawang, Selangor, and then immediately brought to the laboratory. Upon arrival at laboratory, tilapia was washed, eviscerated and hand filleted, with the muscle and by-product (head, frames, and tail) separated. Tilapia by-product (TB) were then minced using a blender, packed in small polyethylene plastic bags, frozen, and stored at −20 °C until further use. Prior to the hydrolysis process, a portion of the TB mince was thawed overnight in a refrigerator at 4 ± 1 °C. Alcalase (in solution form with declared activity of 2.4 activity units [AU]/kg and a density of 1.18 g/mL), a bacterial endoproteinase from a strain of *Bacillus licheniformis*, was provided by Novo Nordisk (Denmark) and stored at 5 °C until it was used for hydrolysis experiments. All the chemical reagents used in this study were analytical grade standard reagents with >95% purity.

2.2. Preparation of tilapia protein hydrolysate using alcalase

Minced TB was thawed overnight in a cold room (4 °C), then 15% w/v of minced TB was mixed with 50 mL of 50 mM phosphate buffer solution (PBS, pH 7.5) and the mixture preincubated at 60 °C for 20 min prior to adding the 2.5% w/w alcalase enzyme to initiate the enzymatic hydrolysis reaction. After 60 min of hydrolysis, the reaction was terminated by heating the mixture in a water bath at 90 °C for 15 min with occasional agitation. The mixture was immediately cooled on ice, sedimented at 10,000 rpm for 20 min in a refrigerated high speed centrifuge and the supernatant was collected [28].

2.3. Separation of tilapia protein hydrolysate using ultrafiltration membrane

Fractionation of TB protein hydrolysate was performed using a dead-end ultrafiltration membrane with two different configurations; single and multilayered membrane. 200 ml of TB protein hydrolysate (feed solution) was used for each fractionation process and prior to its use in the experiment, the peptide content of the feed solution was measured according to the methods described by Church et al. [29] and Nielsen et al. [30] with only a slight modification.

Fractionation of TB protein hydrolysates was performed using ultrafiltration membranes (Amicon Model 8200 stirred ultrafiltration cell, Amicon Corp., Danvers, MA) in a dead-end mode system. The experimental setup for UF membrane is shown in Fig. 1. Stirred ultrafiltration cell is equipped with a suspended bar impeller of 4.9 cm inside the test cell and was magnetically driven by stirring hot plate (Favorit). The gap between the impeller and the membrane was about 1.5 mm. The stirring speed was monitored using a digital phototachometer. Two different MWCO of flat sheet regenerated cellulose (RC) membrane of 5 kDa (Millipore, PLCC 06210) and 10 kDa (Millipore, PLGC 06210) were used 28.7 cm^2 surface area and diameter of 63.5 mm. The conditions of single (10 kDa and 5 kDa membranes) and multilayer (two membranes were combined together with the skin side on the top for both membranes – 10/5 kDa and 5/5 kDa membrane) ultrafiltration membrane in fractionating TB protein hydrolysate was conducted at pressure of 2.5 bar, stirring speed of 600 rpm, and pH 8 for 70 min.

2.4. Permeate flux

The volumetric permeate flux, J_v (filtration rate per unit membrane surface area) represents the productivity of a membrane separation process. It depends on the properties of the membrane, the transmembrane pressure, the system hydrodynamics, the protein concentration in the feed and the properties of the solvent and the protein. The following equation was used to measure permeate flux:

$$\text{Flux} = \frac{\text{Total quantity passed through membrane}}{\text{Membran earea} \times \text{time}} \text{L}/(\text{m}^2.\text{h}) \qquad (1)$$

2.5. Peptide transmission

Transmission of peptide was used to calculate peptide concentration in the feed and permeate as the observed transmission is defined as the ratio of the solute concentration in the permeate

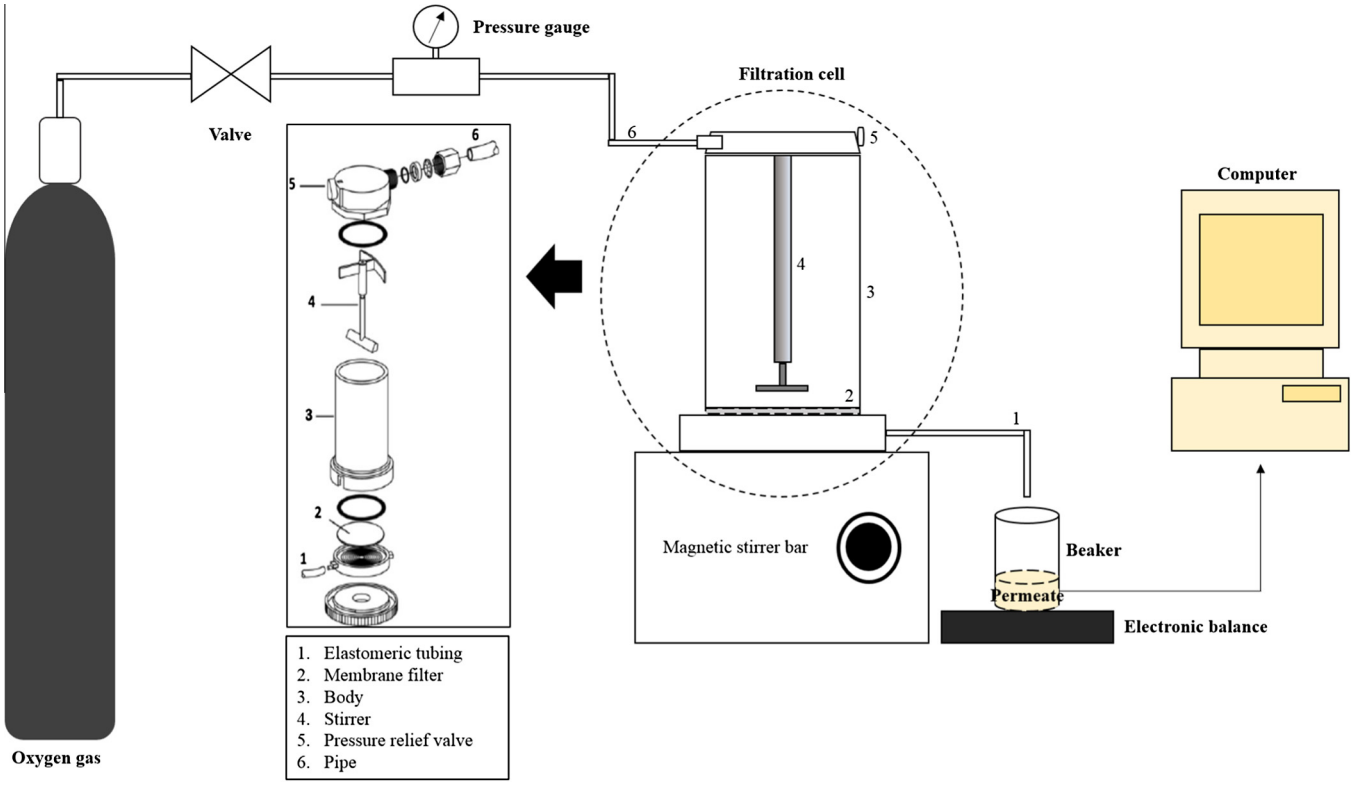

Fig. 1. The schematic diagram for ultrafiltration membrane process.

(C_p) to that in the feed (C_f). Peptide Transmission [23] was calculated using the equation below:

$$Tr(\%) = \frac{C_p}{C_f} \times 100 \quad (2)$$

where

C_P: concentration in the permeate

C_f: concentration in feed solution.

2.6. Measurement of peptides content

The peptides content in the feed solution, permeate and retentate were measured according to the method described by Church et al. [29] and Nielsen et al. [30] with some modifications using the *O*-phthaldialdehyde (OPA) reagent. Fig. 2 shows the standard curve for peptide content measurement.

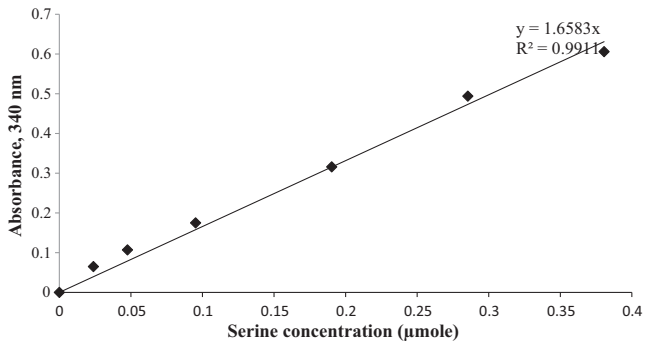

Fig. 2. Standard curve for peptide content measurement.

2.7. Selectivity of the fractionation

Permeates obtained at the best fractionation conditions (600 rpm, and pH 8) for each membrane (10, 5, 10/5, 5/5 kDa) were further analyzed for peptides selectivity based on size distribution. Selectivity of the peptides was analyzed by AKTA Fast Pressure Liquid Chromatography (FPLC; Amersham Pharmacia Biotech). The samples were filtered through a column (Pharmacia Superdex Peptide® GL 10/300) whose matrix was made up of a porous gel (diameter 13–15 lm) consisting of agarose and dextran, of a total volume of 24 mL. The range of fractionation between 100 and 7000 Da. 20 µL of filtered sample were injected and eluted at 25 °C with mobile phase containing 0.05 M of sodium phosphate buffer (pH 7) with 0.1% of NaCl at a constant flow rate of 0.4 mL min $^{-1}$. Peptides of known molar mass (cytochrome C; 12,384 g mol^{-1}, neurotensin; 1678.9 g mol^{-1}, leucine enkaphaline; 686.8 g mol^{-1}, purchased from Sigma) were used as standards to calibrate the column.

2.8. ACE inhibitory activity of tilapia by-product protein hydrolysate

ACE inhibition activity was measured by monitoring the release of hippuric acid (HA) from the hydrolysis of the substrate hippuryl-histidyl-leucine (HHL) using ACE solution according to the method described by Jimsheena and Gowda [31] with slight modification. Firstly, HHL was prepared in 0.05 M of potassium phosphate buffer (pH 8.2) containing of 0.3 M NaCl. ACE from rabbit lungs was dissolved in the same buffer at a concentration 60 mU/mL. About 50 µL of TB hydrolysate and 50 µL of ACE solution was mixed and preincubated at 37 °C for 10 min. Then, 150 µL of HHL solution was added and incubated at 37 °C for 60 min. The reaction was terminated by adding 250 µL of 1.0 M HCl. Then, 400 µL of pyridine was added, followed by 200 µL of BSC. The solution was slowly

mixed using a vortex mixer and then cooled on ice. The yellow color developed was measured at 410 nm using a spectrophotometer. The percentage of ACE inhibition was calculated as follows:

$$\text{ACE} - \text{inhibitory activity } (\%) = \frac{A - B}{A - C} \quad (3)$$

where A = absorbance of solution without sample, B = absorbance of solution with sample, C = Absorbance of blank solution (without ACE solution and sample).

2.9. Statistical analysis

All data were subjected to statistical analysis using the Statistical Analysis System (SAS, 1989) with ANOVA and Duncan's multiple range test were used for multiple comparison. Standard deviation was calculated using the same software.

3. Results and discussion

3.1. Effect of different membrane pore size on permeate flux

The results for the effects of membrane pore size on the permeate flux are presented in Fig. 3. It can be observed that the permeate flux values for single membrane and multilayer membrane have shown a significant difference ($p > 0.05$). The highest flux was achieved at membrane pore size of 10 kDa, followed by 10/5 kDa, 5 kDa and 5/5 kDa with values of 52.28, 40.62, 27.36 and 16.82 L h^{-1} m^{-2}, respectively. The results clearly showed that the larger membrane pore size used, the higher permeate flux was obtained. Vandanjon et al. [21] have studied the fractionation of blue whiting hydrolysates using the tubular UF membrane process by applying four membranes with MWCO of 0.3, 4, 8 and 20 kDa in continuous mode and at different pressures. As expected, the flux increased as membrane pore size increased. Study on the effect of material and molecular weight cut-off on the fractionation of saithe protein hydrolysate was conducted by Chabeaud et al. [32] using five tubular membranes of various material (polyethersulfone-PES, modified-PES, polysulfone) and molecular weight cut-off (MWCO) (4, 6, 8 and 9 kDa) where the main focus is to enrich peptide with low molecular weight. A similar trend was obtained in which permeate flux increased with the increase of membrane MWCO. PES membranes has shown to be less permeable than the modified-PES and PS which probably due to the stronger hydrophobic nature of PES. Highest permeation fluxes were thus obtained with the 8 kDa PS membrane [32].

The impact of a two-step ultrafiltration (4000 Da) and nanofiltration (300 Da) process in fractionating fish protein hydrolysate have also shown a similar behavior where fractionation using UF membrane has higher permeation fluxes compared to NF membrane [17]. Saidi et al. [1] evaluated the performance of six different flat-sheet (different material and pore size) membranes in fractionating prolastin (commercial peptide solution) using dead-end filtration cell under the same conditions of stirring velocities and pressure. Four different membranes (three polyethersulfone membranes from different manufacturer and one regenerated cellulose-RC membrane) with a similar molecular cut-off (10 kg mol^{-1}) were evaluated their performances based on flux and irreversible fouling. A wide range of permeate fluxes (4–34 L h^{-1} m^{-2}) and irreversible fouling (23–71%) were obtained with RC membrane has shown a great performance. When different membrane pore size were evaluated (10 - PES, 8.5 - PA, 5 - PES kg mol^{-1}), at the beginning, the same trend was observed where decreasing permeate flux was obtained when prolastin was filtered with membranes in the following order 10 and 8.5 kg mol^{-1}. However, when 5 kg mol^{-1} membrane was used, it does not show a decline but rise. Therefore, it is very difficult to correlate either the chemical nature or MWCO of the membranes which led to an increase in the flux value.

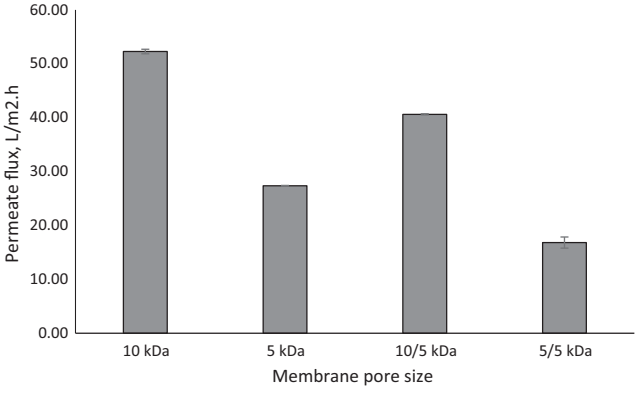

Fig. 3. Effect of membrane pore size on flux of TB protein hydrolysate using single and multilayer UF membranes.

Besides the membrane pore size, the mass transfer rate was also dependent on the arrangement of the membrane, particularly when two membranes were combined together on one device, called the multilayer membrane [33]. By stacking the membrane with different orientation such as 10/5 and 5/5 kDa arrangement, the mass transport rate was also different. The permeate flux for both membrane orientations showed a significant difference (Fig. 3). The 10/5 multilayer membrane showed higher permeate flux compared to the 5/5 multilayer membrane. Interestingly, the permeate flux values for the 10/5 multilayer membrane is much higher compared to the 5 kDa membrane and slightly lower than the 10 kDa membrane, indicating that permeate yield can also be improved by using multilayer membrane, depending on the appropriate membrane orientation.

Most of the studies conducted using multilayer membrane (using 2 or more membranes) involve of fractionation binary protein mixture [23–27]. According to Feins and Sirkar [26], with the addition of each membrane, the permeation flux is reduced. This is an agreement with the result obtained for multilayer 5/5 kDa membrane which shown a lower permeate flux compared to single membrane (5 kDa membrane). Unlike for the two different membranes pore sizes which was combined together between 10 kDa (top) and 5 kDa (bottom), a higher permeate flux was obtained. This might be due to the extent of concentration polarization encountered by 10 kDa membrane is lesser since it has more open-structure compared to the 5 kDa membrane. Therefore, the flux is significantly higher and can be easily sustained by the next 5 kDa membrane. This finding could be supported from the study on sieving characteristics of multilayer ultrafiltration membranes in separating of dextrans using different membrane orientation and pore size (50 and 30 kDa membranes - 50 kDa membrane on the top followed by 30 kDa (the skin layers on the top for both membranes) [33]. They found that by placing a 30 K membrane under the 50 K membrane resulted in a significant reduction of the sieving coefficients of the larger dextrans compared to 50 K membrane alone. The sieving coefficients for the small molecular weight dextrans through this 50–30 K sandwich are essentially identical to those for the 50 K membrane alone. This phenomena could be explained by the reduction in the contribution of solute diffusion relative to convection in which the membrane at the bottom is relatively flat, thus the sieving behavior of this two layer membrane would be essentially identical to that of a single membrane with properties equal to the top layer membrane. This findings indicate that the ability of solute to pass through the membrane not only depend on membrane pore size but also their orientation.

3.2. Effect of different membrane pore size on peptide transmission

The highest peptide transmission was achieved at membrane pore size of 10 kDa (87.3%), followed by 10/5 kDa (54.4%), 5 kDa (36.1%) and 5/5 kDa (20.0%) (Fig. 4). It shows that the transmission of peptide through membrane is strongly depends on the membrane pore size and their orientation. The results obviously show that the transmissions of peptide through membrane with a small pore size (5 kDa – single and 5/5 – multilayer) are lower compared to those of largermembrane pore size, and this may be due to the tendency of a larger peptide size to be retained and accumulated on the membrane surface of the smaller membrane pore size resulting a severe membrane fouling.

Vandanjon et al. [21] have compared the transmission of peptide using two different membrane pore size with MWCO of 8 and 4 kDa, and found that peptide with a larger size tend to retain more on a membrane with small pore size and the peptide transmission can be increased by increasing the pressure. Chabeaud et al. [32] have studied the impact of material and membrane pore size on the peptide transmission from saithe protein hydrolysate. Fractionation was conducted using five tubular membranes of various material (polyethersulfone-PES, modified-PES, polysulfone) and molecular weight cut-off (MWCO) (4, 6, 8 and 9 kDa). As expected, retention factor decreased with the increased in membrane pore size based on the following order: polysulfone (8 kDa) < modified-PES (6 kDa) < PES (4 kDa) < modified-PES (4 kDa). The higher retention factor indicates a high tendency of peptide to accumulate on the membrane surface resulting in low peptide transmission. This results was in agreement with Saidi et al. [1] who conducted the performances of different membrane material (PES, PA, RC) and pore size in fractionation of tuna dark muscle hydrolysate using dead-end stirred cell ultrafiltration. They found that the retention rate of PES membrane with MWCO of 10 kDa has shown higher yield followed by PES-5 kDa, PA-8.5 kDa and RC-10 kDa. These findings provide evidence that a reduction in the flux not only depends on membrane pore size but also closely related with chemical nature material of the membrane.

Boyd and Zydney [33] have evaluated the sieving characteristics of polydisperse dextran using asymmetric membranes (30 and 50 kDa) in a sandwich arrangement either with their support substructures together (i.e., the skin layers on the two outer surfaces) or with the skin layers together (with the porous substructures at the upstream and downstream surfaces). They found that sieving coefficient for asymmetric membrane increased over an order of magnitude when used in the reverse direction with the skin layers together. Higher selectivity was achieved for small molecular weight dextrans through 50–30 kDa (50/30) sandwich than the conventional membrane due to having a tighter (smaller pore) layer beneath a more open layer. When three regenerated cellulose membrane in the same molecular weight cut-off (30 kDa) are stacked together in a parallel combination (both feed side membrane and permeate side membrane with skin side up) and used to fractionate myoglobin and β-lactoglobulin [26]. They found that complete rejection of β-lactoglobulin was achieved from the feed mixture when membranes are stacked together, resulting in permeate that contains myoglobin only with percentage of 80.25, 98.31 and 100 for 1, 2 and 3 membrane, respectively for 900-min experiment. This indicate that high rejection of protein can be amplified with the additional of membrane, thus allowing a desired protein to pass through the membrane.

Study on the effect of sandwich configuration of ultrafiltration membrane on protein fractionation of lysozyme, myoglobin and BSA mixtures with similar MWCO combination (30/30, 50/50 and 100/100 kDa) was investigated by Md. Yunos and Field [27]. They found that the values of transmission of lysozyme with sandwich arrangement are comparable to the transmission for the respective single membrane. Their study shows there is a relationships between membrane pore size and protein molecules of size on the sieving characteristic as well as the effect of system hydrodynamics on the effective pore size of the membrane during protein fractionation.

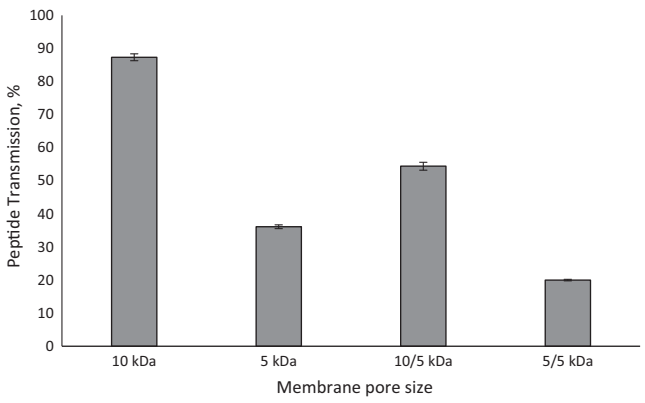

Fig. 4. Effect of membrane pore size on peptide transmission of TB protein hydrolysate using single and multilayer UF membranes.

3.3. Selectivity of fractionates from UF membrane

An analysis by Fast Pressure Liquid Chromatography (FPLC) provided the chromatogram profile of permeate fractions from single and multilayer membranes shown in Fig. 5 and the peptide size distribution was presented in Table 1. There were 6 peaks obtained in permeate for membranes 10, 5 and 10/5 kDa, while permeate from 5/5 kDa membrane showed 7 peaks. From Table 1, it was found that all the permeates were composed of peptides lower than 1500 Da, indicating that the fractionation using the dead-end UF membrane with different membrane configurations (single or multilayer) successfully retained the larger peptide size and allowed only smaller peptide to pass through the membrane. These results showed that there is an effect of concentration polarization phenomena and fouling occurs during fractionation due to the accumulation of the peptides which leads to the formation of a dynamic layer on the membrane surface. This has resulted in no peptide with a large size are able to pass through the membrane and detected from chromatogram profiles. Most of the permeate were enriched with peptide fraction of 895 Da except for the permeate of 5/5 multilayer membrane which was successfully detected a larger peptide fraction with size 1088 Da. This might be due to the permeate compose of peptide with size 1088 Da from 5/5 kDa multilayer membranes has higher concentration if comparing to other membrane configurations, which indicates that multilaying membranes with the same pore size could improve the separation of peptides and thus lead to the increase of peptide yield (Fig. 5). The result of peptide fractionation was clearly dependent on the membrane configuration used.

As expected, fractionation using the 10 kDa membrane produced more peptide with a large size (895 Da) with the value of 46.88%, and peptide size with less than 500 Da in total of 53.08%. For 10 kDa membrane, the percentage of peptide at peak 3–7, decreased as the reduction in size. When a smaller membrane pore size used (5 kDa membrane), the amount of peptide with 893 Da declined as compared to 10 kDa membrane, while the amount of peptide with small-sized peptide (<500 Da) increased. This possibility due to the retention of larger peptide size on the 5 kDa membrane surface and merely allowing peptide with smaller size to pass through the membrane. As for the 10/5 kDa multilayer membrane, a slightly lower peptide with a larger size

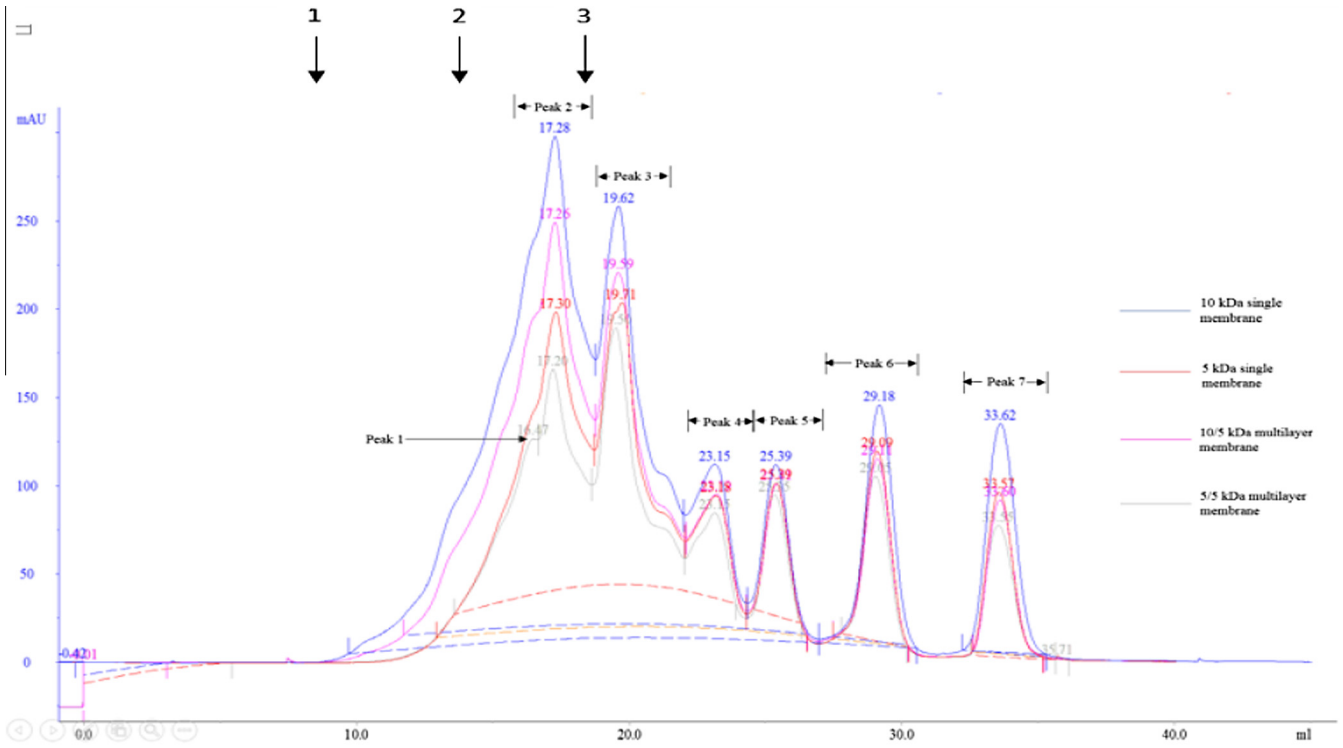

Fig. 5. Chromatogram profiles of tilapia by-product protein hydrolysate which fractionated through ultrafiltration membranes of 10, 5, 10/5 and 5/5 kDa. All permeates (10, 5, 10/5 and 5/5 kDa) were analyzed by AKTA FPLC (Amersham Pharmacia Biotech). The samples were filtered through a column (Pharmacia Superdex Peptide® GL 10/300). Standards: (1) Cytochrome C (MW 12,327 Da) (2) Neurotensin (MW 1673 Da), (3) Leucine enkaphaline (MW 687 Da).

Table 1
Percentage of peptide composition.

Membrane configuration (kDa)	Molecular weight composition (w/w,%)			
	10	5	10/5	5/5
Peptide fraction < 1500 Da				
Peak 1 (1088 Da)	ND	ND	ND	13.97 ± 0.17
Peak 2 (895 Da)	46.88 ± 0.68	36.80 ± 0.33	45.44 ± 0.66	21.35 ± 1.74
Peptide fraction < 500 Da				
Peak 3	24.20 ± 0.69	28.83 ± 0.38	26.64 ± 0.77	27.69 ± 0.11
Peak 4	8.18 ± 0.18	9.51 ± 0.30	8.32 ± 0.59	7.58 ± 0.55
Peak 5	5.68 ± 0.36	7.37 ± 0.31	6.44 ± 0.25	7.19 ± 1.04
Peak 6	7.96 ± 0.52	9.81 ± 0.25	7.51 ± 0.27	12.06 ± 0.38
Peak 7	7.10 ± 0.63	7.68 ± 0.59	5.64 ± 0.54	10.15 ± 0.86
Total (<500 Da)	53.08	63.20	54.55	64.67

ND = not detected.

was obtained as compared to 10 kDa membrane and more in peptide less than 500 Da but significantly lower than 5 kDa membrane. The peptide transmission behavior through 10/5 kDa membrane was similar to 10 kDa membrane which is in agreement with Boyd and Zydney [33] results where the sieving behavior of 50/30 kDa membrane in fractionating of dextran was essentially identical to the top layer of the membrane. Meanwhile for 5/5 multilayer membrane, two peaks of peptides (1088 and 895 Da) were detected with the total percentage of 35.32% which slightly lower compared to the of 5 kDa membrane indicating that the transmission behavior of large peptide size are identical with 5 kDa membrane. Interestingly, more peptides with small size (<500 Da) were obtained for 5/5 multilayer membrane (64.67%), with more small peptide at peak 6 and 7 were produced, indicating that the selectivity of membrane can be improved through multilayer membranes as the basic principles of multilayer membrane essentially could produce a pure protein in permeate [24,25]. These results are consistent with general principles of the UF and NF mass transfer mechanism where the performance and the selectivity of membranes are related to the pore size, shape and charge of the solute [1,34].

Studies on the fractionation of fish protein hydrolysate through two-steps membrane process were conducted by several researchers [1,17]. Bourseau et al. [17] have fractionated fish protein hydrolysate using ultrafiltration (4000 Da) followed by nanofiltration (300 Da). Fractionation using ultrafiltration produced permeate enriched with small peptides-sized of 1000–300 Da (49.66%), and a small amount of peptides with large peptide size (>7000–4000 Da; 14.7%). When permeate obtained was further fractionated using nanofiltration, permeate enriched with small peptides-sized of 300 Da (81.47%) was obtained. A similar behavior of peptide composition was found when fractionation of tuna dark muscle hydrolysate using UF-NF steps in a batch and diafiltration configurations. They successfully produced peptides with size mostly in the range of 1000– <300 Da which is about 80.9–82.8%.

It is clearly seen that the selectivity of the small peptide can be enhanced when it is operated in two-step membrane process. However, two-step membrane process involving relatively high operating costs leading to limited use in peptides separation. In comparison with the multilayer membrane which is adopted in the present study, it can become an alternative to the use of two-stage membrane separation, which involves several unit operations that make it costly. Through the multilayer membrane technique, a single operation system can be achieved with relatively less cost required.

3.4. Effects of different membrane pore's sizes and configuration on ACE-inhibitory activity

This study aimed at producing peptide with a small size through multilayer UF membranes process in order to improve the ability of TB hydrolysate in inhibiting ACE activity. Furthermore, several studies have reported that small peptide size (<10 kDa) obtained from the fractionation process using UF membrane was more effective in inhibiting ACE activity than peptides of large size [5,6,35,19].

Table 2 shows the ACE inhibitory activities of the permeate fractionated from single and multilayer membranes. The ACE inhibitory activity of permeate from 5 kDa membrane was higher compared to 10 kDa membrane with the values of 71.83% and 64.32%, respectively. This indicated that fractionation using small membrane pore size can enhance the biological activity of protein hydrolysate fraction. This is consistent with the findings reported by several researchers [5,6,35], where they also found that ACE inhibitory activities were significantly increased with a decrease in peptide molecular weight.

High ACE inhibitory activity of the permeate obtained from 5 kDa membrane most probably due to the high percentage of small peptide-sized (Table 1). Peptides size have a significant effect on the ACE inhibitory activity in which a high concentration of a large peptides in the permeate, lead to a decrease in ACE inhibitory activity. High ACE-inhibitory activity in the permeate fractionated by membrane with a small pore size could be related to the peptide structure and sequence [36], with the most potency shown by the C-terminal tripeptide sequence. In addition, the effectiveness of the peptides to inhibit ACE activity was also influenced by amino acid groups (hydrophobic amino acids) at the C-terminal or N-terminal [37].

In comparison with the multilayer membrane (Table 2), it was found that permeate from 5/5 kDa membrane yield peptides with the highest ACE inhibitory activity (84.04%). Interestingly, both permeates from the multilayer membrane exhibited a higher ACE inhibitory activity as compared to the single membrane. High ACE inhibition activity for the 5/5 kDa membrane could be due to the high concentration of a small peptide presence in the permeate, which led to a significant reduction in ACE activity. As mentioned previously, the most potent of peptides in inhibiting of ACE activity is strongly influenced by the peptides sequence as well as the presence of certain amino acid at C-terminal of peptides sequence such as Trp, Tyr, Phe, Pro and a hydrophobic amino acid which their tends to interact with the active site of ACE [36,38]. Therefore, with the presence of a small peptides-sized in large quantity could increase the possibility of the presence such group of amino acids in the permeate, thus may contribute to ACE inhibitory activity. The ability of a small peptide-sized to inhibit ACE activity has also reported for peptides derived from a variety of fish species [5,6,35,19]. The result clearly showed that there is a relationship between peptide size and ACE inhibitory activity, in which the highest ACE inhibition can be achieved through the production of a small peptide size in a great amount. Thus, application of multilayer membrane potential increased the production of small peptide size as well as the ACE inhibitory activity.

Table 2
ACE-inhibitory activity of permeate from single and multilayer membranes.

Membrane configuration (kDa)	ACE inhibition activity (%)
10	64.32 ± 2.15
5	71.83 ± 5.07
10/5	75.59 ± 2.15
5/5	84.04 ± 0.81

All data expressed as mean ± standard deviation (n = 3).
Different superscript within each column indicate significant differences ($p < 0.05$).

4. Conclusions

This study had successfully discovered several significant findings on the separation of TB protein hydrolysate using dead-end mode of UF through single and multilayer membrane by manipulating their membrane pore size (10 and 5 kDa) and orientation (10/5 and 5/5 kDa). The separation of TB hydrolysate was greatly influenced by the membrane pore size, in which the highest permeation fluxes and peptide transmission was achieved at 10 kDa membrane, and decreased when 5 kDa membrane was used. Proven also that multilayer membrane does not necessarily provide a low permeate flux and peptide transmission, but also provide better yield as compared to single membrane. All membranes configuration either single or multilayer have exhibit interesting selectivity characteristics, in which all permeates fractions were enriched with peptides lower than 1500 Da. Fractionation using 5/5 kDa multilayer membrane had positive impact on the selectivity of a small peptide size, which more peptides with size < 500 Da was obtained (64.67%). The most potent ACE-inhibitory peptides corresponded to those in permeate fractions (<500 Da). It is worth noting that selectivity of the peptides through ultrafiltration membrane process can be improved by manipulating the membrane pore size as well as membrane orientation. In conclusion, multilayer membrane is suggested to fractionate tilapia by-product protein hydrolysate with great amount of ACE inhibitory activity.

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