Production of Antioxidant Peptides from Snakehead Fish Using Batch and Continuous Enzymatic Hydrolysis

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Abstract

Bioactive peptides are promising functional ingredients. Due to its high protein content, snakehead fish (*Channa striata*) extract (SHFE) is one of the suitable parent proteins for bioactive peptides. This study aimed to investigate the production of SHFE-based antioxidative peptides in a conventional batch and continuous system facilitated by an enzymatic membrane reactor (EMR). The effects of different proteases (Alcalase, Neutrase), substrate concentrations, and enzyme-to-substrate ratios were investigated in the batch process. Continuous hydrolysis was then performed under the optimum conditions obtained from the batch process. The optimum conditions based on the antioxidant capacity measured by DPPH and FRAP assays were employing Alcalase with a substrate concentration [S] of 3% (w/v) and an enzyme-to-substrate ratio [E]/[S] of 10% (w/w). Continuous operation was shown to have been performed over a prolonged period, based on the calculated fouling rate. Furthermore, filtration of the resulting permeate with a smaller membrane pore size (2-kDa) increased the antioxidant capacity. This study is expected to increase the production of functional ingredients in snakehead fish.

Keywords: antioxidant, bioactive peptides, enzymatic membrane reactor, snakehead fish

Introduction

Consumers continue to prioritize health and nutrition and depend more on functional foods. Notably, there is an increased demand for functional foods among individuals experiencing post-COVID-19 symptoms (FAO, 2022). Functional foods provide desirable health benefits besides essential nutrition as they contain significant biologically active ingredients. One of these biologically active ingredients is bioactive peptide. Bioactive peptides are protein fragments composed of 2–20 amino acids. These peptides are categorized according to their bioactivities, such as antioxidative, antihypertensive, antidiabetic, antimicrobial, antiproliferative, anti-inflammatory, and anticancer (Akbarian et al., 2022; Berlian et al., 2023; Chakrabarti et al., 2018; Sitanggang et al., 2020). Snakehead fish (*Channa striata*) are a suitable protein source for generating bioactive peptides owing to their high protein content, reaching up to 70% (db, dry basis) (Karnila et al., 2020). Protein hydrolysates from the by-products of snakehead fish (head) obtained using papain have been reported to exhibit antioxidant capacity (Agustin et al., 2023).

Bioactive peptides can be produced by enzymatic hydrolysis, microbial fermentation, or a combination of both (Akbarian et al., 2022). Although enzymatic hydrolysis is costly, it is a commonly preferred method because it uses enzymes with known specificity, thereby generating predictable results and is more accessible in terms of scalability (Peredo-Lovillo et al., 2022; Daliri et al., 2017). Enzymatic hydrolysis can be performed in a batch or a continuous manner. Although the design of a batch system is more straightforward than that of a continuous system, the latter exhibits better efficiency in the production process. Continuous hydrolysis is facilitated using an enzymatic membrane reactor (EMR), which combines product separation and enzyme-catalyzed reactions into a single operation. Parameters such as enzyme-to-substrate ratio and substrate concentration are crucial for utilizing EMR (Sitanggang et al., 2021).

In previous studies, Kurniadi et al. (2023) and Sitanggang et al. (2020, 2021, 2023) demonstrated continuously using EMR to produce plant-based peptides from soybean and velvet beans. Animal proteins are expected to be a better choice for producing antioxidant peptides than plant proteins. These proteins possess notable quantities of leucine, tryptophan, methionine, and cysteine, vital amino acids found in antioxidant peptides (Day et al., 2022; Nwachukwu & Aluko, 2019). In snakehead fish, albumin is a potential antioxidant compound (Hidayati et al., 2018). Albumin contains sulfhydryl (-SH) groups, which function as free radical scavengers (Chubarov et al., 2020; Wen et al., 2020). In this study, we investigated the production of SHFE-based antioxidative peptides in a conventional batch and continuous system facilitated by an enzymatic membrane reactor (EMR).

Material and Methods

Material

Snakehead fish (*Channa striata*) extract (SHFE) was purchased from Toko Raden Saleh, Bogor, Indonesia, in December 2022. The raw materials' protein, fat, and carbohydrate contents were 73.2%, 3.7%, and 6.0% (db), respectively. UF flat-sheet polyethersulfone (PES) membranes with molecular weight cut-off/MWCO of 2-kDa (TRI-SEP® SBNF), 4-kDa (NADIR® UH004), and 5-kDa (NADIR® UP005) were purchased from MANN + HUMMEL (Ludwigsburg, Germany). Alcalase® 2.5 L (EC 3.4.21.62, ~12.500 U/mL), and Neutrase® 0.8 L (EC 3.4.24.28, ~12.000 U/mL) were purchased from Novozymes A/S (Bagsværd, Denmark). Pure water (water one™) was purchased from Jayamas Medica Industri (Indonesia). Bovine Serum Albumin (BSA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,4,6-Tris(2-pyridyl)-s-triazine/TPTZ were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA).

Methods

Experimental Design

Batch hydrolysis of SHFE using Alcalase and Neutrase was initially performed. The effects of substrate concentration *[S]* and enzyme-to-substrate ratio *[E]/[S]* were investigated using a one-factor-at-a-time design. The optimum conditions were determined based on the antioxidant capacity of the permeate, as measured using the DPPH and FRAP methods. Continuous hydrolysis using EMR was then performed under the optimum conditions obtained from the batch process, utilizing a 5-kDa membrane. The resulting permeate from the continuous hydrolysis was subsequently filtered using 4- and 2-kDa membranes to obtain fractions containing lower molecular weight peptides.

SHFE Batch Hydrolysis

Batch hydrolysis was performed using a batch reactor (Fig. 1a). The SHFE was dissolved in phosphate buffer solution (1% w/v, pH 7.5) for 2 h at room temperature under constant agitation. Subsequently, the substrate solution was refrigerated overnight at 4 °C and filtered through Whatman filter paper no. 3 before being utilized. The prepared substrate was then transferred to the reactor, and enzymes were incorporated. The reaction was performed for 7 h at 50 °C, with constant agitation *N* at 300 rpm. Samples were withdrawn throughout the reaction (i.e., at 0, 1, 2, 4, 5, and 7 h) and frozen until further analysis. The performance of the two proteases (Alcalase and Neutrase) and the effects of the enzyme-to-substrate ratio [E]/[S] (*i.e.*, [E]/[S] = 5, 10, and 15% w/w) and substrate concentration [S] (*i.e.*, [S] = 1, 2, and 3% w/v) were investigated.

Continuous SHFE Hydrolysis

The continuous hydrolysis of SHFE was performed using EMR. A 5-kDa membrane (6, Fig. 1b) was placed in the reactor (5, Fig. 1b). The substrate solution was prepared as previously described and placed in a substrate tank (4, Fig. 1b) and a reactor (5, Fig. 1b). Continuous hydrolysis of SHFE was performed under a constant flux (hence, constant residence time τ = 9 h), as described by Sitanggang et al. (2023). The reaction was performed for 9 h at 50 °C with *N* at 300 rpm. The permeates were withdrawn throughout the reaction (*i.e.*, at 0, 1, 3, 5, 7, and 9 h) and frozen until further analysis. The increase in transmembrane pressure (TMP, ΔP) throughout the reaction resulted from fouling on the membrane surface was recorded. The accumulated permeates collected from the container placed on the analytical balance (8, Fig. 1b) were filtered using 4- and 2-kDa membranes to obtain different fractions of SHFE-based peptides. All fractions were measured for their antioxidant half-

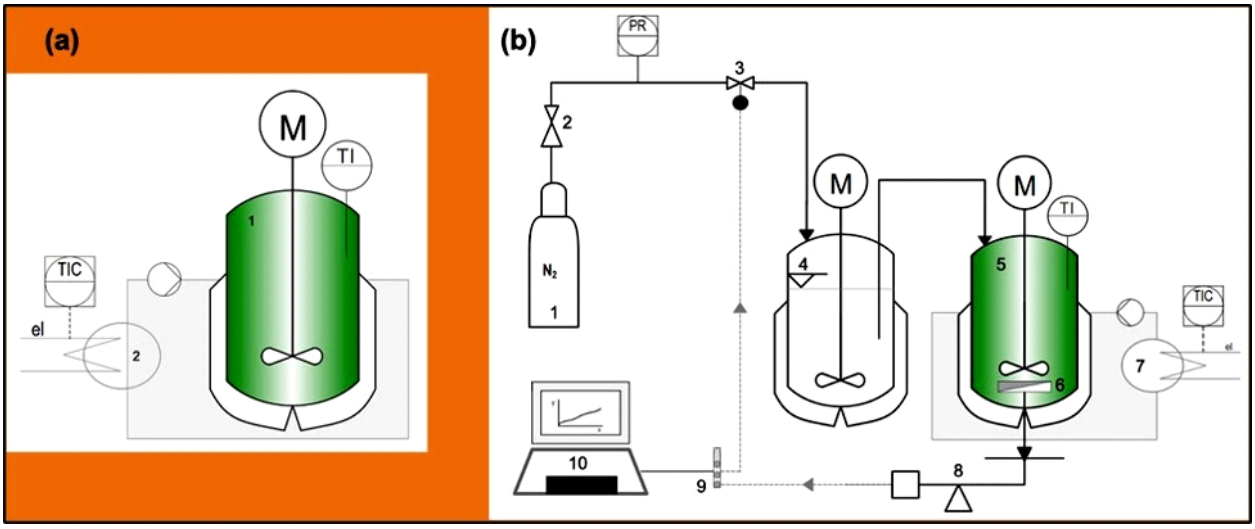

Figure 1 (a) Schematic design of the batch reactor: (1) reactor and (2) water bath system. (b) Schematic design of the enzymatic membrane reactor (EMR): (1) nitrogen tank, (2) pressure reducer, (3) proportional pressure reducer, (4) substrate tank, (5) reactor, (6) UF membrane, (7) water bath system, (8) analytical balance, (9) data acquisition, and (10) PC.

maximal inhibitory concentrations (IC_{50}) using the DPPH assay.

Measurement of Antioxidant Capacity

Antioxidant capacity was measured using two assays: DPPH (2,2-diphenyl-1-picrylhydrazyl) and Ferric Reducing Antioxidant Power (FRAP), as performed by Kurniadi et al. (2023).

Protein Content Analysis

Protein content was monitored using Lowry's method, as described by Sitanggang et al. (2021).

Fouling Rate Calculation (rf)

The fouling rate for continuous production was calculated using the following formula:

$$r_f = \frac{dP}{dt} \times \frac{1}{\eta \times J} \quad \text{.....(1)}$$

where rf is the fouling rate (1/m.h); dP is the transmembrane pressure (TMP, ΔP, Pa); dt is the reaction time (9 h); η is the dynamic viscosity (assumed to be the same as that of water at T = 50°C; 0.5471 mPa · s); and J is the flux (L/m^2.h = LMH).

Statistical Analysis

A completely randomized design was used in this study. One-way analysis of variance (ANOVA) and Duncan's test ($p < 0.05$) were performed.

Results and Discussion

Batch Hydrolysis of SHFE

Effect of Substrate Concentration

The effect of the substrate concentration [S] was evaluated at three different concentrations (1, 2, and 3% w/v). Fig. 2 shows the influence of [S] on the antioxidant capacity throughout the 7-h batch hydrolysis of SHFE. Fluctuations in the antioxidant capacity were observed. This can be explained by the fact that Alcalase and Neutrase are both endoproteases. Endoproteases cleave proteins within the polypeptide chain, which implies that they possess a much wider range of choices for cleaving locations (Tacias-Pascacio et al., 2020). This nature produces various peptide sequences and compositions, influencing the antioxidant capacity (Baehaki et al., 2020). In addition to the sequence and composition of peptides, the antioxidant capacity of protein hydrolysates is influenced by other factors, including the alpha-amino group content, degree of hydrolysate (DH), surface hydrophobicity, and molecular weight (Indriani et al., 2022).

In all [S] treatments, the antioxidant capacity of the permeate was higher than that of the substrate ($p < 0.05$). It can be observed that an increase in [S] led to an increase in antioxidant capacity, indicating an increase in the number of antioxidant peptides produced, as also found previously (Liu et al., 2015). The antioxidant capacity was significantly higher at

higher concentrations (i.e., [S] = 3% w/v). This indicates that the enzymes were yet to be saturated by the substrate and could produce even more peptides. Notably, the FRAP method showed higher antioxidant capacity than the DPPH method. The DPPH assay depicts the radical-scavenging capacity of an antioxidant, whereas the FRAP assay measures its ferric-reducing capacity. Hence, SHFE-based bioactive peptides performed better as metal-reducing antioxidants than radical-scavenging ones. With the highest

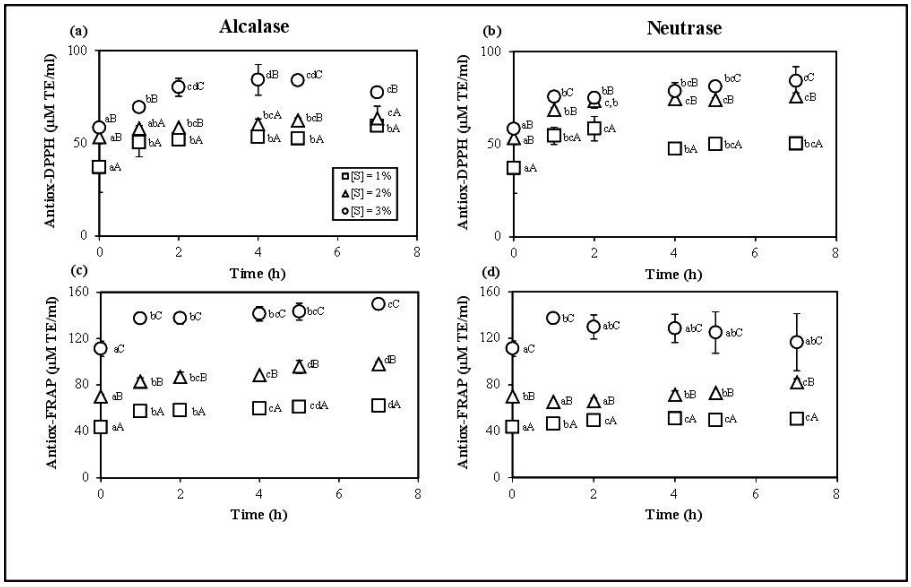

Figure 2. Influence of substrate concentration [S] on antioxidant capacity measured by (a-b) DPPH assay and (c-d) FRAP assay during batch hydrolysis. Different superscript letters indicate significant differences at a confidence level of 95% ($p < 0.05$). The lower case indicates the influence of reaction time for the same concentration, whereas the upper case indicates the influence of concentration for the same sampling time. Reaction conditions: *[*E]/[S] = 10% *w/w*, pH 7.5, *N* = 300 rpm, T = 50 °C.

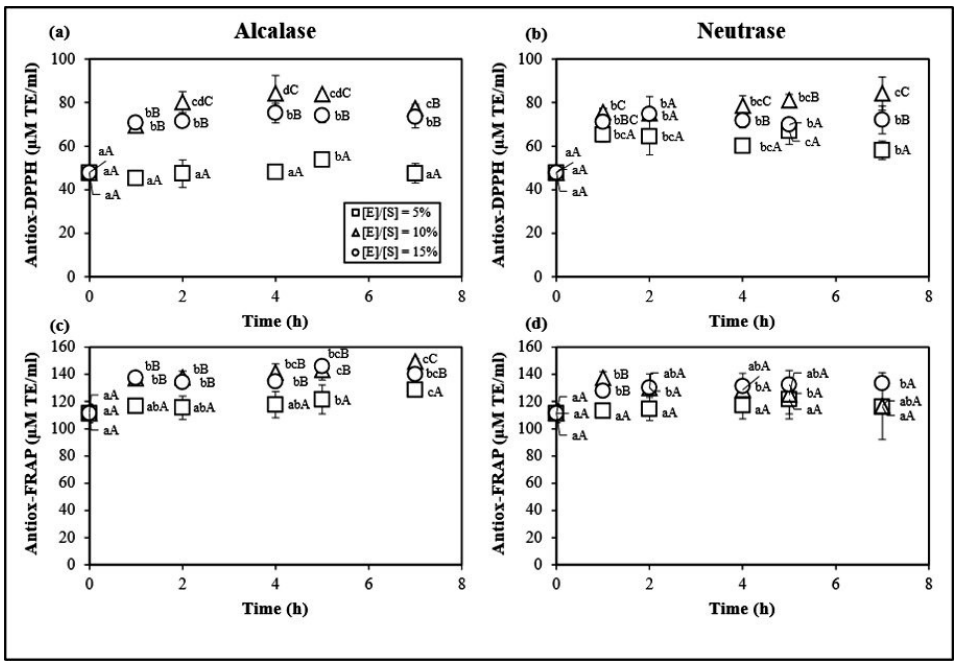

Figure 3. Influence of *[E]/[S]* on antioxidant capacity measured by (a-b) DPPH assay and (c-d) FRAP assay during batch hydrolysis. Different superscript letters indicate significant differences at a confidence level of 95% ($p < 0.05$). The lower case indicates the influence of reaction time for the same concentration, whereas the upper case indicates the influence of concentration for the same sampling time. Reaction conditions: [S] = 3% *w/v*, pH 7.5, *N* = 300 rpm, T = 50 °C.

antioxidant capacity, [S] = 3% *w/v* treatment was selected for further investigation.

Effect of Enzyme-to-substrate Ratio

The influence of the enzyme-to-substrate ratio [E]/[S] was investigated at three different concentrations (5, 10, and 15% w/w). Fig. 3 shows the antioxidant capacity during the 7-h batch hydrolysis of SHFE under different [E]/[S]. Similar to the effect of substrate concentration (Fig. 2), there were fluctuations in antioxidant capacity. Regardless, hydrolysis indeed caused an increase in the antioxidant capacity, observing that the antioxidant capacity of the permeate was higher than that of the substrate, generally speaking. Interestingly, the middle point of [E]/[S] 10% *w/w* resulted in a higher antioxidant capacity. On one hand, a lower antioxidant capacity presumably occurred because of excessive hydrolysis at [E]/[S] 15% *w/w*, which resulted in free amino acids that possess antioxidative properties (Xu et al., 2019). Moreover, excessive hydrolysis could induce protein aggregation (including co-extractant), which can also play a role in lowering antioxidant capacity (Indriani et al., 2022). In contrast, at [E]/[S] 5% *w/w*, the lower antioxidant capacity was presumably due to insufficient enzyme present, resulting in enzyme saturation by the substrate. In summary, an increase in [E]/[S] did not equate to higher antioxidant capacity and there was a middle point in which the quantity of both the enzyme and substrate was at optimum. This phenomenon was observed for both proteases. Considering its high antioxidant capacity with fewer enzymes, *a* [E]/[S] of 10% *w/w* was selected for further continuous production.

Effect of Different Proteases

The antioxidant capacity of the hydrolysate produced using Alcalase was higher than that produced using Neutrase under the same conditions (Fig. 2 and Fig. 3). In cleaving the substrate, enzymes have their preferred cleavage site or specificity. This specificity is unique to each enzyme. Protease selection is essential for producing bioactive peptides because the specificity of the chosen protease determines the amino acid composition and sequence of the resulting peptides. Alcalase has specificity mainly for hydrophobic amino acids, whereas Neutrase has specificity mainly for leucine and phenylalanine (Kunst, 2003). Due to its wide range of cleaving sites, Alcalase can produce more bioactive peptides than Neutrase, which explains the higher antioxidant capacity exhibited by Alcalase hydrolysate. Therefore, Alcalase was selected for continuous production. Alcalase has been reported many times to be one of the most efficient enzymes for generating bioactive peptides from different protein sources, such as marine fish processing by-products, mung bean, chicken viscera, *Trachinus Draco*, etc. (Tacias-Pascacio et al., 2020).

Continuous SHFE Hydrolysis in an Automated EMR

Continuous hydrolysis of SHFE to produce bioactive peptides was conducted under optimum conditions previously obtained from batch production (*i.e.*, [S] = 3% *w/v*, [E]/[S] = 10% w/w, using Alcalase). The continuous system was facilitated by maintaining a constant flux (and, hence, a constant residence time) throughout the operation. A residence time of 9 h was selected based on several previous studies, which demonstrated a residence time of 9 h as the optimum condition (Sitanggang et al. 2021, 2023).

As shown in Fig. 4b, an increase in the antioxidant capacity was observed during the continuous process. The antioxidant capacity profile observed in the continuous process showed an increasing trend compared with that in the batch process, which tended to have a more constant trend. This demonstrated that the constant removal of enzymatic products (*i.e.*, peptides) in the continuous process can lead to a higher product yield over time. Furthermore, in an enzymatic operation, such as bioactive peptide production, some factors need to be considered, such as production time and the quantity of the enzyme. In the batch process, there was an unproductive time at the start and end of the reaction (preparation, cleaning, filling, etc.), which had to be repeated for each batch cycle.

Moreover, the enzymes used in the batch process could not be recovered (*i.e.*, they must be inactivated after each cycle). In contrast, in the continuous process, the enzymes were retained inside the reactor and exhibited catalytic capacity for a prolonged duration. In summary, a continuous system is expected to reduce both the amount of enzyme used and the unproductive time; hence, it is preferably utilized on an industrial scale (Sitanggang et al., 2022).

The relationship between flux J and transmembrane pressure ΔP throughout the 9-h reaction is shown in Fig. 4a. The average flux fluctuated throughout the reaction but generally remained within the set flux, implying the robustness of the control system of the automated EMR, as previously demonstrated (Kurniadi et al., 2023; Sitanggang et al., 2023), and an increase in TMP concerning the reaction time was observed. An increase in TMP is necessary to compensate for membrane fouling and maintain a constant flux. Based on the increase in TMP, the calculated fouling rate was 3.58×10^{12} 1/(m.h). Membrane fouling occurs because

of the interaction between the membrane surface and macromolecules. The membrane surface can interact with the enzyme, substrate protein, or both. At pH 7.5, Alcalase has a positive charge, while it was reported that the PES membrane had a negative charge. As a result, there may be electrostatic interactions between the enzyme and the membrane surface, causing membrane fouling (Kurniadi et al., 2023). Nevertheless, the fouling rate obtained was within the same magnitude as that Kurniadi et al. (2023) obtained from the continuous hydrolysis of velvet bean protein concentrate. This implies that long-term hydrolysis, as performed by Kurniadi et al. (2023) in the same study, is feasible.

IC_{50} Value for Different Peptide Fractions

Bioactive peptides produced from the continuous process were filtered with smaller membrane MWCOs (4- and 2-kDa) to obtain three different fractions of SHFE-based peptides (permeated as <-5-kDa, <-4-kDa, and <-2-kDa). The IC_{50} value, which is the protein concentration required to inhibit DPPH by 50% (Rivero-Cruz et al., 2020), was determined for each fraction. A smaller IC_{50} value signifies the greater antioxidant capacity of the permeate (Aykul & Martinez-Hackert, 2016). The significant decrease in IC_{50} from the substrate to the batch hydrolysate confirmed the generation of bioactive peptides with antioxidative

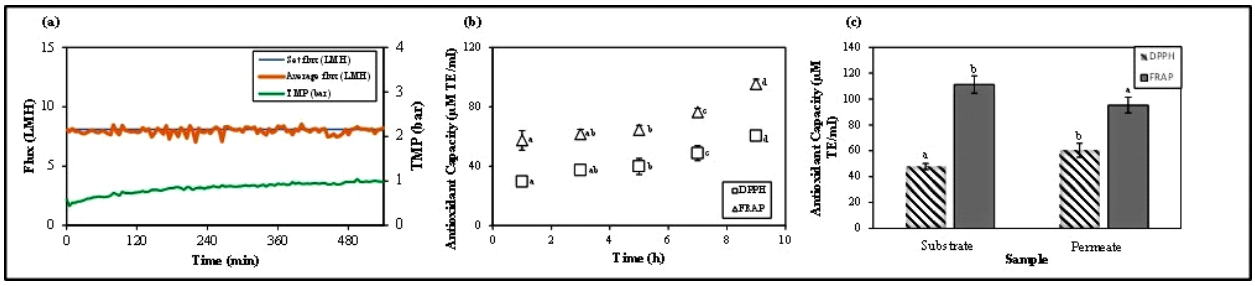

Figure 4. (a) Profiles of transmembrane pressure (TMP), set flux, and average flux during continuous hydrolysis of SHFE in automated EMR; (b) Antioxidant capacity of SHFE-based bioactive peptides during 9-h of continuous hydrolysis. Different superscript letters indicate significant differences with a confidence level of 95% (p< 0.05); (c) Antioxidant capacity of the substrate and permeate of SHFE-based bioactive peptides from continuous hydrolysis. Reaction conditions: [S] = 3% *w/v,* [E]/[S] = 10% *w/w*, pH 7.5, N = 300 rpm, T = 50 °C, employing Alcalase and 5-kDa PES membrane.

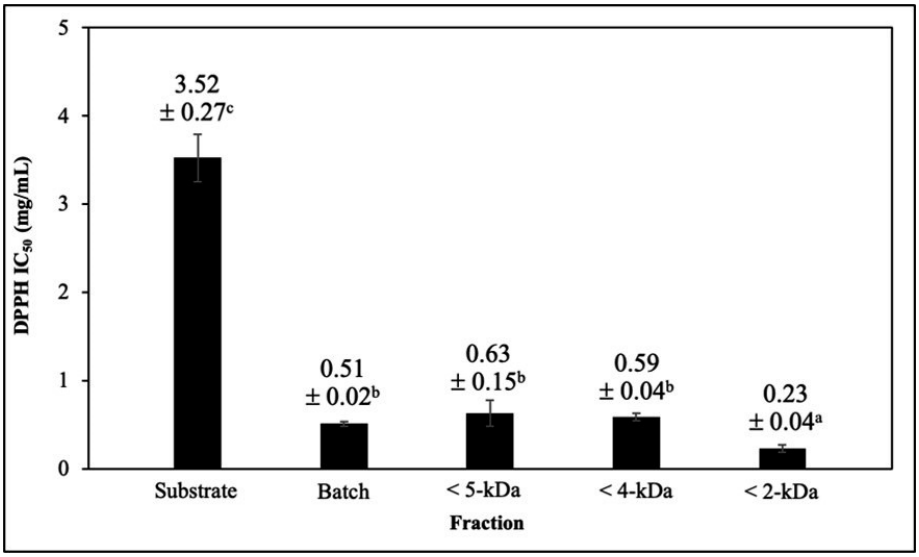

Figure 5. IC_{50} values of antioxidant capacity for different peptide fractions. Different superscript letters indicate significant differences (P < 0.05).

properties. The IC_{50} values for the batch hydrolysate and the hydrolysate filtered by 5- and 4-kDa membrane filtration were not significantly different ($P < 0.05$). The smallest fraction (*i.e.*, the 2-kDa fraction) had the highest bioactivity (Fig. 5). Peptides with lower molecular weights have been known to possess higher bioactivity, including antioxidant capacity (Kurniadi et al., 2023; Sitanggang et al., 2021, 2023). Other studies have also reported increased antioxidant capacity owing to lower molecular weight (Chen et al., 2023; Durand et al., 2021; Saallah et al., 2020).

The IC_{50} value of SHFE-based bioactive peptides filtered with < 2-kDa membrane (0.23 ± 0.04 mg/mL) was comparable to other fish-based peptides in the literature. For instance, isolated peptides from gelatin hydrolysates obtained using Alcalase from scale gelatin of skipjack tuna (*Katsuwonus pelamis*) exhibited DPPH radical scavenging capability with DPPH IC_{50} values ranging from 0.67–1.34 mg/mL after being divided into fractions using 3-kDa ultrafiltration membranes, DEAE-52 cellulose column, Sephadex G-25 column, and purified using the RP-HPLC system (Qiu et al., 2019). Loach protein hydrolysates, which were obtained using Alcalase and separated using gel filtration chromatography (<1-kDa), exhibited a DPPH IC_{50} value of 2.91 ± 0.02 mg/mL (Mao et al., 2023). Both studies produced hydrolysate in a batch system and separated the peptides using various peptides in different unit operations. This study produced SHFE-based bioactive peptides continuously using EMR that combined enzymatic hydrolysis and product separation using ultrafiltration membranes.

Conclusion

Bioactive peptides from SHFE exhibited antioxidant capacity that could be produced through enzymatic hydrolysis using Alcalase and Neutrase, either in a batch or continuous process. Based on batch operation, optimum conditions such as [S] = 3% (w/v), [E]/[S] = 10% (w/w) and using Alcalase were selected to produce bioactive peptides from SHFE in a continuous manner. Furthermore, the continuous production of SHFE-based bioactive peptides using EMR has the potential for long-term use. Permeate filtration using a 2-kDa membrane increased the antioxidant capacity, as indicated by a lower DPPH IC_{50} value (*i.e.*, 0.23 mg protein/ml). Further research is needed to identify the specific peptide sequences responsible for the antioxidant capacity and other bioactivities that SHFE-based bioactive peptides might exhibit.

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Supplementary Materials

Supplementary materials is not available for this article

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