CHEMICAL ENGINEERING TRANSACTIONS
VOL. 106, 2023
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Jeng Shiun Lim, Nor Alafiza Yunus, Peck Loo Kiew, Hon Huin Chin

ISBN 979-12-81206-05-2; **ISSN** 2283-9216
DOI: 10.3303/CET23106148

Investigation into Antimicrobial Activity and Its Stability of Protein Hydrolysate Derived from Earthworms

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This study utilized the earthworm (*Perionyx excavatus*) to generate antimicrobial protein hydrolysate. Alcalase hydrolysis was carried out under the condition including the earthworm:phosphate buffer ratio of 1:6 (w/v), 50 ºC, pH 7.5, enzyme:substrate (E:S) ratio of 500 U/g protein, and 3 h of hydrolysis so that the earthworm protein hydrolysate exhibited the highest antimicrobial capacity against *Escherichia coli* and *Bacillus subtilis*. The inhibition zone diameter (IZD) against *E. coli* and *B. subtilis* were 11.33 ± 0.58 mm and 10.67 ± 0.58 mm, in order; and their minimum inhibitory concentration (MIC) was 0.5 mg/mL for both bacteria. The hydrolysate was further fractionated using ultrafiltration to recover 5 fractions of > 30 kDa, 10-30 kDa, 3-10 kDa, 1-3 kDa and < 1 kDa for testing their antimicrobial activity. The greatest antimicrobial activity was found at the 1-3 kDa fraction, with its IZD against the *E. coli* and *B. subtilis* 2.25 and 1.46 folds lower than that of Ciprofloxacin, in that order. The hydrolysate's antimicrobial activity remained over 55 % after pH treatment in the range from 1 to 11, or after being heated at 100 °C for 180 min. The findings indicate that the earthworm-derived hydrolysate and/or its peptide fractions could be employed as natural versatile antimicrobial agents for different purposes.

1. Introduction

Pathogenic microorganisms cause not only food spoilage but also foodborne diseases that threaten social health and the market economy (Madrazo et al., 2022). Although chemical antimicrobial agents are employed extensively, their side effects such as carcinogenic, teratogenic, and antibiotic-resistant capacity are major challenges for researchers (Mahdavi-Yekta et al., 2023). As a natural antimicrobial agent, antimicrobial peptides (AMPs) express a remarkable inhibitory power against a broad spectrum, preventing the generation of microbial resistance and have fewer adverse effects (Moscoso-Mujica et al., 2021).
AMPs could be generated from the native proteins through a proteolytic process, of which enzymatic hydrolysis is a rapid method, reliable, and easy to control (Sanchez-Reinoso et al., 2021). It has been employed to release AMPs from various protein-rich sources such as cowpea seeds (Osman et al., 2021), monodora myristica seeds (Osukoya et al., 2021) and Indian mackerel by-products (Hasani et al., 2022). In the light of this, in Vietnam, a protein-rich creature (55-70 % protein on a dry weight basis), earthworm, is underutilized and has low economic value (Ding et al., 2019). Accordingly, this study aims to valorize the earthworm to produce antimicrobial protein hydrolysate against *E. coli* and *B. subtilis*, the most regularly tested strains for screening antimicrobial agents. Ultrafiltration was also applied to separate AMPs from the hydrolysate based on the difference of molecular weight. pH and thermal stability of antimicrobial activity of the protein hydrolysate and its peptide fractions were also examined.

2. Materials and Methods

2.1 Materials

Earthworm (*Perionyx excavatus*), purchased at the Biotechnology Center, Ho Chi Minh City, was crushed and stored in polyethylene bags at –20 °C until required. The proximate composition of the earthworm which included 80.99 ± 0.53 % moisture, 69.92 ± 0.23 % crude protein, 6.97 ± 0.10 % crude lipid, 12.78 ± 0.18 % carbohydrate,

Paper Received: 16 July 2023; Revised: 07 August 2023; Accepted: 20 August 2023
Please cite this article as: Bui P.T., Ha D.Q., Pham K.T., Vo T.D.L., 2023, Investigation into Antimicrobial Activity and Its Stability of Protein Hydrolysate Derived from Earthworms, Chemical Engineering Transactions, 106, 883-888 DOI:10.3303/CET23106148

and 10.25 ± 0.10 % ash (on a dry basis) was determined by the methods of Nwachukwu and Aluko (2019). Chemicals with analytical grade (Sigma-Aldrich (USA)) were used for investigations. Proteases including Alcalase® 2.5 L (1226.37 U/mL, 55 ºC, pH 8), Neutrase® 0.8 L (2232.14 U/mL, 50 ºC, pH 7), Protamex® (1315.80 U/g, 55 ºC, pH 6.5), and Flavourzyme® 500 MG (1587.30 U/g, 50 ºC, pH 7) were purchased from Novozymes (Bagsvaerd, Denmark) and stored at 4 ºC. *E. coli* (ATCC 25922) and *B. subtilis* (ATCC 6633) were employed in this research.

2.2 Methods

2.2.1 Experiments

The preparation of earthworm hydrolysates was conducted according to the procedure of Vo et al. (2020). 10 g of the ground earthworms were mixed with 0.02 M phosphate buffer (fixed pH depending on each investigation) with the appropriate ratio, and the mixture was treated at 90 ºC for 10 min to inactivate endogenous enzymes. Then, its pH was adjusted to required pH using 1 M HCl or 1 M NaOH solution and its temperature was brought to hydrolysis temperature before adding enzyme preparation with an appropriate E:S ratio. After a fixed time, the enzyme was inactivated by treating the hydrolysate for 10 min at 90 ºC. The supernatant was then collected by centrifuging at 5000 rpm for 15 min and the gained supernatant was freeze-dried before being stored at -20 ^{o}C.

The effect of hydrolysis parameters including enzyme type, earthworm:water ratio (w/v), temperature (ºC), pH, E:S ratio (U/g protein), and hydrolysis time (h) on the antimicrobial activity of the earthworm protein hydrolysates was investigated using a single factor test method, in which one factor was altered at various levels while the other factors remained constant.

The earthworm protein hydrolysate was further fractionated by using ultrafiltration centrifugal devices of 30 kDa, 10 kDa, 3 kDa, and 1 kDa (Macrosep, Pall Laboratory, USA). Five fractions (<1 kDa, 1-3 kDa, 3-10 kDa, 10-30 kDa, and >30 kDa) were acquired and evaluated for their antimicrobial activity.

2.2.2 Analytical methods

The soluble protein content of the hydrolysate and peptide fractions was analyzed by the protocol of Lowry et al. (1951) while their antimicrobial activity against *E.coli* and *B. subtilis* was evaluated using the procedure of Onyegbule et al. (2014). The practice of Sripokar et al. (2019) was employed to examine the pH and thermal stability of the antimicrobial activity via relative activity which was the percentage of IZD of the treated sample compared to that of the untreated sample.

2.2.3 Data analysis

All experiments were performed in triplicate. Data shown as average ± standard deviation were processed using Excel software. The statistically significant differences were determined using the SPSS software (IBM SPSS Statistics 20).

3. Results and Discussion

3.1 Effect of hydrolysis conditions on antimicrobial activity of the earthworm protein hydrolysates

As seen in Figure 1(a), Alcalase hydrolysate expressed the highest antimicrobial effect on both *E. coli* and *B. subtilis*. In contrast, the others have no antimicrobial activity against B. subtilis. Alcalase, an endopeptidase, prefers hydrophobic and aromatic amino acids at its cleavage sites (Moscoso-Mujica et al., 2021), and usually releases the peptides with N-terminals of Gly, Lys, Arg, Asp, Ala, Trp, Ile (Fu et al., 2018). The positive charge amino acids (Arg, Lys) could interact with negative charges on the bacterial cell surface, while the hydrophobic residues (Gly, Asp, Ala, Trp, Ile) help the containing peptides penetrate into the microbial cell membrane (Rocha et al., 2018). Consequently, the permeability of the bacterial membrane increased, resulting in the release of internal matters and cell death (Osukoya et al., 2021). Alcalase was also used as a proteolytic agent in the study of Osman et al. (2021).

The earthworm:phosphate buffer ratio impacts the probability of collisions between the substrate and enzyme molecules, either fostering or obstructing the production of antimicrobial peptides from the intact protein (Shu et al., 2017). A sufficient amount of solvent could fastly spread out the products of hydrolysis, preventing protein aggregation and feedback effect (Shu et al., 2017). In this study, the antimicrobial activity against both tested strains of the hydrolysate reached the peak at the earthworm:phosphate buffer ratio of 1:6 (w/v) (Figure 1(b)).

Hydrolysis temperature and pH play a crucial role in the enzyme activity, thus influencing the hydrolysate's antimicrobial activity. Temperature could impact the force keeping the shape of the enzyme while the pH governs the charge of the amino acid side chains at the enzyme's catalytic site, altering enzyme's configuration and catalytic activity (Sanchez-Reinoso et al., 2021). The most suitable values for those factors to acquire the

earthworm protein hydrolysate with the greatest antimicrobial activity were 50 ºC and 7.5, in order (Figure 1 (c) and (d)).

Figure 1 (e) and (f) showed that the antimicrobial activity of the earthworm protein hydrolysate increased up to a threshold of E:S ratio (500 U/g protein) and hydrolysis time (3 h of hydrolysis) then declined afterward. It could be due to the fact that with the increase in enzyme amount or hydrolysis time, more antimicrobial peptides were generated from the intact protein, but they could be further hydrolyzed into minor peptides having a lower inhibitory activity (Hasani et al., 2022). Similar patterns could be found in several previous publications (Osukoya et al., 2021).

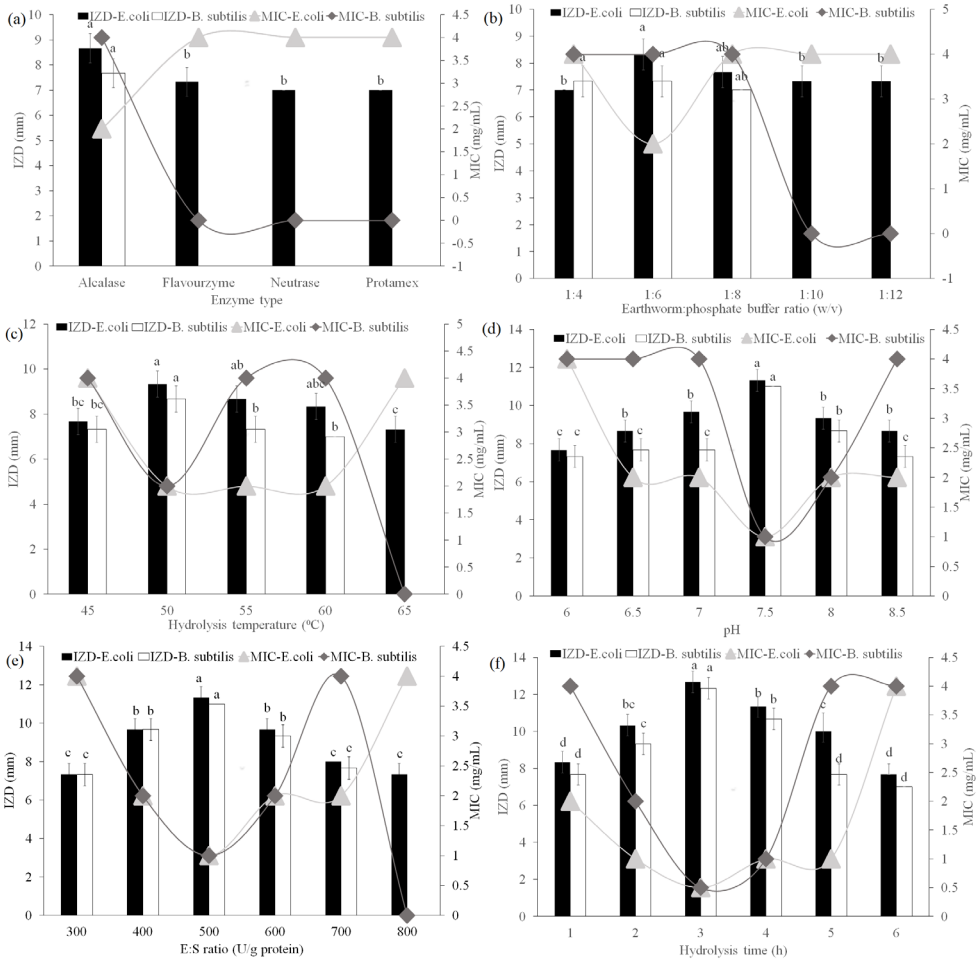

Figure 1: Effect of (a) hydrolysis enzyme type, (b) earthworm:water ratio, (c) temperature, (d) pH, (e) E:S ratio and (f) hydrolysis time on antimicrobial activity of the earthworm protein hydrolysate. The same color bars with various letters show significant differences ($p<0.05$)

3.2 Antimicrobial activity of peptide fractions

It has been published that small peptides may accelerate to attack the target sites on the microbial surface, entering the bacterial cell, possibly disrupting various cellular processes and metabolic functions of bacteria (Aguilar-Toalá et al., 2020). The facilitation of the interaction between small peptides and bacterial membranes was brought by the peptide's structure acquisition and the exposure of amino acid side chains and charges (Rocha et al., 2018). The result of this study was in agreement with the above theory that the 1-3 kDa peptide fraction affected both tested strains at the highest level, while the >30 kDa fraction did not show any impact (Table 1). The lower antimicrobial activity of the <1 kDa peptide fraction may be due to its high content of free amino acids which provide a low contribution to bioactivity of the fraction (Sanchez-Reinoso et al., 2021).

The IZD against the *E. coli* and *B. subtilis* of the 1-3 kDa fraction were 2.25 and 1.46 folds, in that order, lower than that of Ciprofloxacin; the MIC value of the 1-3 kDa fraction for the *E. coli* and *B. subtilis* was 1000 and 500 times, in order, higher than that of Ciprofloxacin. It was reported that DNA gyrase and topoisomerase IV which initiated DNA replication and transcription in bacterial cells were the targets of ciprofloxacin (Rehman et al., 2019). This could lead to a high tendency of ciprofloxacin resistance in the bacteria because the mutations in

the targeted enzymes-encoding genes to diminish the affinity of these enzymes for ciprofloxacin may easily happen or the overexpression of efflux pumps could expel the ciprofloxacin from the microbial cell (Rehman et al., 2019). AMPs tend to rapidly disturb bacterial membranes, resulting in a low tendency of resistance due to the requirement of a long time for the membrane redesign in bacteria (Aguilar-Toalá et al., 2020). So, the 1-3 kDa fraction from the earthworm protein hydrolysate may be served as a potent antimicrobial agent.

Table 1: Antimicrobial activity expressed as IZD (mm) and MIC (mg/mL) of peptide fractions and Ciprofloxacin

Peptide fraction	<1 kDa	1-3 kDa	3-10 kDa	10-30 kDa	>30 kDa	Cipofloxacin
IZD for *E. coli*	13.0 ± 0.0^{c}	14.7 ± 0.6^{b}	10.7 ± 0.6^{d}	8.0 ± 0.0^{e}	No effect	33.0 ± 0.6^{a}
IZD for *B. subtilis*	12.3 ± 0.6^{c}	13.7 ± 0.6^{b}	9.7 ± 0.6^{d}	7.3 ± 0.6^{e}	No effect	20.0 ± 1.0^{a}
MIC for *E. coli*	0.5	0.25	1.0	4.0	No effect	$2.5*10^{4}$
MIC for *B subtilis*	0.5	0.25	2.0	4.0	No effect	$5.0*10^{4}$

Different letters in the same row show significant differences ($p<0.05$)

3.3 Thermal and pH stability

Figure 2 indicated that almost antimicrobial activity of the earthworm hydrolysate and its peptide fractions remained unchanged at pH 7-9 and declined as pH moved to acidic or extremely alkaline conditions. It could be explained that strong acidic or alkaline conditions may destroy several amino acids such as Gln, Arg, Cys, and Ser, together with promoting unfavorable reactions, namely deamination and racemization, as a result, changing structure and conformation of AMPs, losing their bioactivity (Zhang et al., 2021). Contrastly, low net electrostatic repulsive energy at around neutral pH would minimize the swell and unfolding of AMPs and thereby remaining their bioactivity (Damodaran and Parkin, 2017). Similar results could be found in the publications of Naimah et al. (2018).

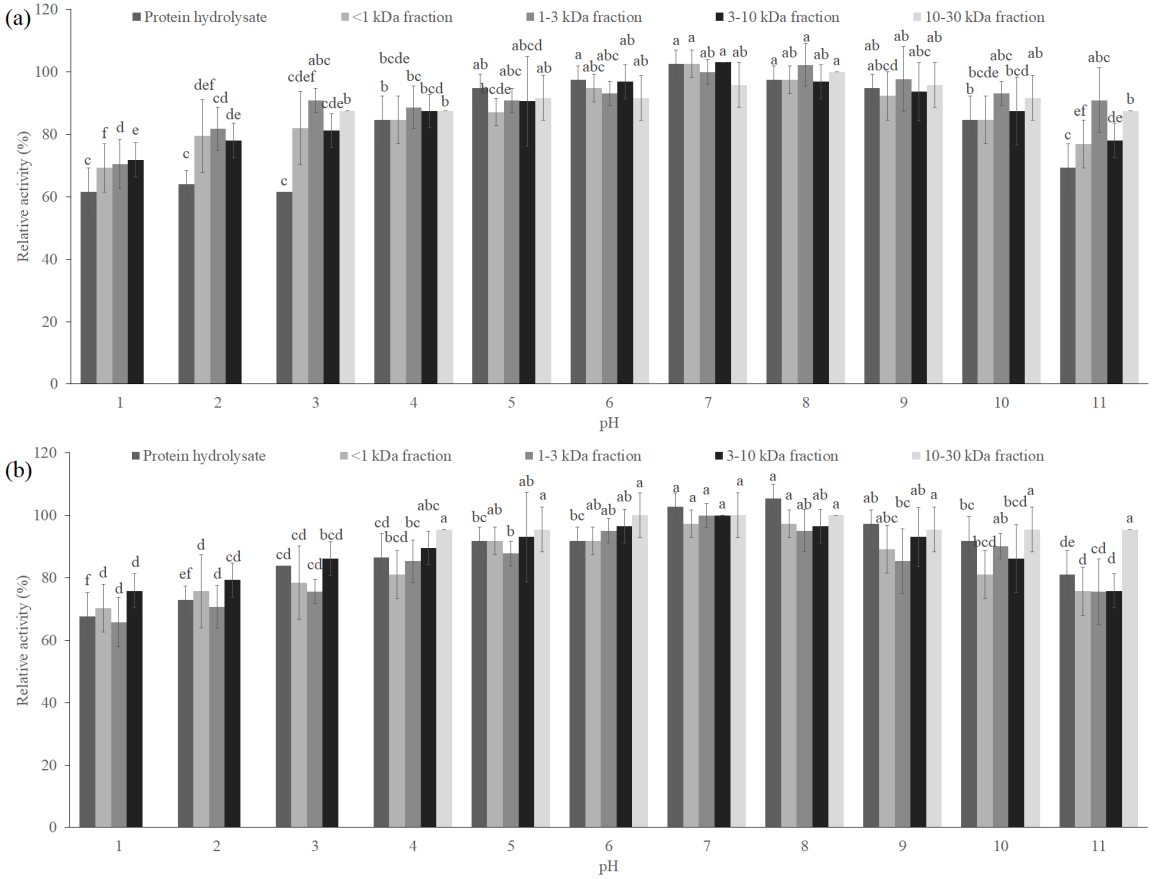

Figure 2: pH stability of antimicrobial activity against (a) E. coli and (b) B. subtilis of the earthworm protein hydrolysate and its peptide fractions. The same color bars with various letters show significant differences ($p<0.05$)

In terms of thermal stability, almost the relative antimicrobial activity against both tested strains of the protein hydrolysate and its peptide fractions remained stable after being treated at 100 ºC for 30 min and decreased afterward (Figure 3). Zhang et al. (2021) reported that most AMPs possess an even-numbered cysteine residue, connected by -S-S- bonds to form rigid structures, thus stabilizing the peptides structure at high temperature. Al-sahlany et al. (2020) concluded that the primary structure and low molecular weight of peptides also contribute to their thermal stability. In contrast, as prolonging heating time, the disintegration of noncovalent interactions (hydrophobic interactions, electrostatic interactions, hydrogen bonds), protein denaturation, protein aggregation and amino acid degradation result in the loss of peptide's bioactivity (Damodaran and Parkin, 2017).

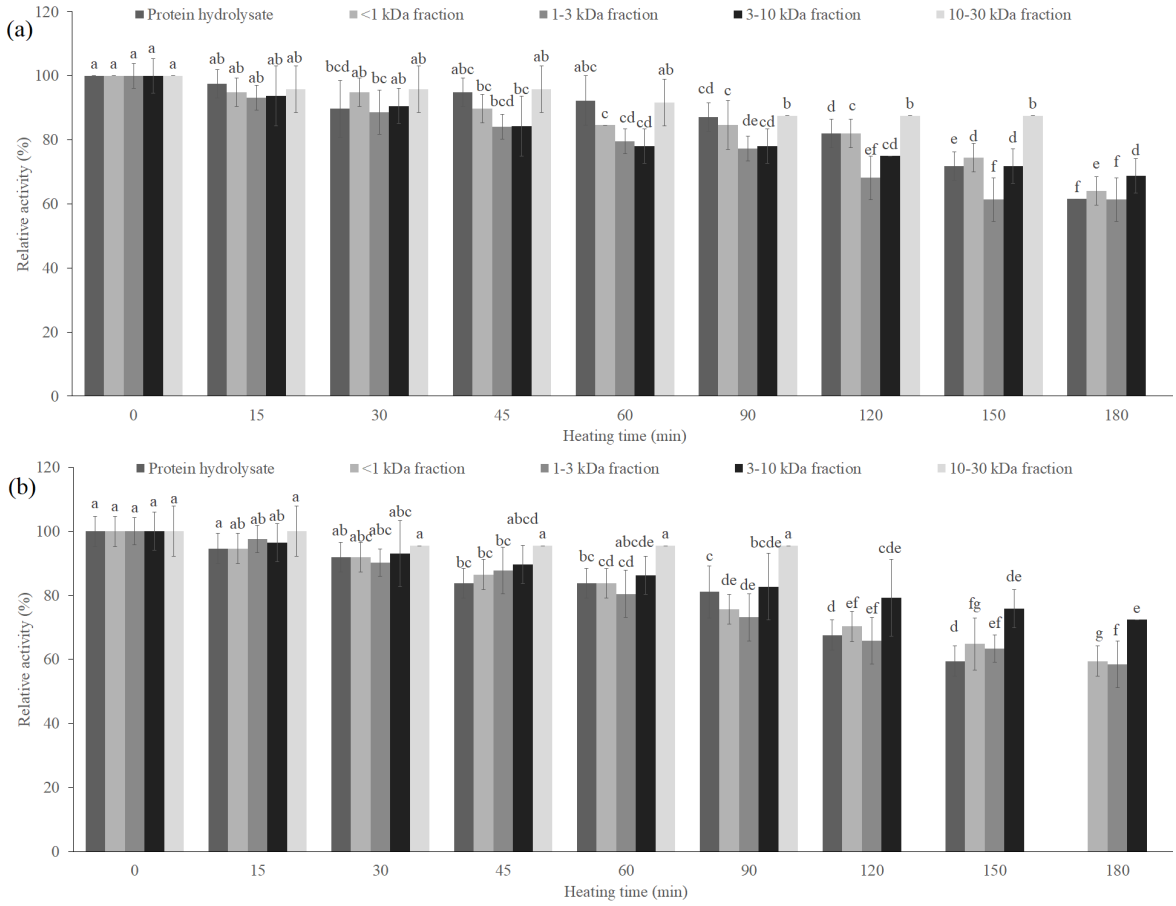

Figure 3: Thermal stability of antimicrobial activity against (a) E. coli and (b) B. subtilis of the earthworm protein hydrolysate and its peptide fractions. The same color bars with various letters show significant differences ($p<0.05$)

4. Conclusion

This study has discovered antimicrobial activity of the protein hydrolysate/peptide fractions derived from the earthworm against the two common foodborne pathogens, *E.coli* and *B.subtilis*. The earthworm hydrolysate exerting the greatest antimicrobial activity was gained at the hydrolysis condition including Alcalase as catalytic agent, the earthworm:phosphate buffer ratio of 1:6 (w/v), 50 ºC, pH 7.5, E:S ratio of 500 U/g protein, and 3 h of hydrolysis. The fraction 1-3 kDa from the hydrolysate exhibited the highest inhibitory effect on both the tested strains and could remain 100 % its initial activity after being treated at 100 ºC for 15 min or in the pH range from 6 to 8. The result suggested that the fraction 1-3 kDa could be employed as antimicrobial agents in a broad range of food products owing to their activity stability to pH and thermal treatment. A deeper research on antimicrobial mechanisms of these samples, their fortification into different food models and peptide identification should be done.

Acknowledgments

We acknowledge Ho Chi Minh City University of Technology (HCMUT), VNU-HCM for supporting this study.

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