

Antimicrobial and antioxidant activities of protein hydrolysate from terrestrial snail *Cryptozona bistrialis*

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ARTICLE INFO

Received on: 12/09/2018
Accepted on: 25/10/2018
Available online: 30/12/2018

Key words:

Cryptozona bistrialis,
antimicrobial activity,
antioxidant activity, enzyme
digestion, protein hydrolysate.

ABSTRACT

Previously, we have shown that seven different snail proteins for antimicrobial activity. Among them, a protein extract from terrestrial snail *Cryptozona bistrialis* inhibited the growth of pathogenic bacteria and fungi. In the present study, we hydrolyzed *C. bistrialis* protein extract to identify peptides exhibiting the antimicrobial and antioxidant activities. The tissues of the land snail *C. bistrialis* were homogenized and digested with three different enzymes: papain, trypsin, and pepsin. During enzyme hydrolysis, the degree of hydrolysis (DH) and percentage of peptide bond breakage was calculated. Among the protein hydrolysates by the three enzymes, the papain-digested hydrolysate of *C. bistrialis* showed the highest DH ($27\% \pm 0.5\%$) at 37°C temperature, pH 6.0, and enzyme/substrate ratio of 4:100 for 6-hour incubation. Only the papain-digested protein hydrolysate showed antimicrobial activity against *Pseudomonas aeruginosa* and *Staphylococcus aureus* with the maximum zone of inhibition of about 27.3 ± 0.5 mm and 28 ± 0.5 mm, respectively. Based on these results, papain digestion was selected for further antioxidant study. The papain-digested protein hydrolysate showed maximum 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity about 73%, 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid radical scavenging activity is 70%, and ferric reducing antioxidant power was increased when increasing the concentration of the protein hydrolysate of *C. bistrialis*. This hydrolysate is an alternative as natural antimicrobial and antioxidant compounds.

INTRODUCTION

Preparation of protein hydrolysates by using enzyme digestion is an important research area of current times. Hydrolysis of proteins leads to the cleavage of peptide bonds which makes proteins to peptides and amino acids. These mixtures of peptides and amino acids are called protein hydrolysates (Nnanna and Wu, 2006). Many protein hydrolysates possess antioxidant activity (Chen *et al.*, 1998). Enzymatic degradation makes the protein size decrease to peptide, which can modify functional characteristics and improve their quality (Petersen, 1981). Bioactive peptides obtained from various invertebrates by using different commercial enzymes have been reported as effective and safe (Rajapakse *et al.*, 2005).

Number of enzymes has been used for hydrolysis of animal proteins such as papain, trypsin, pepsin, alcalase, etc. (Aspmo *et al.*, 2005; Goldberg, 1993; Venugopal and Shahidi, 1995). Compared to the chemical hydrolysis, the enzymatic hydrolysis has more advantages, such as mild reaction conditions, low undesirable products, and high product quality and yield.

Protein hydrolysates are important ingredients for food and industrial applications because it contains more bioactive compounds (Sarmadi and Ismail 2010). Peptides are not showing any activity when its present inside the sequence of the proteins but its showing more activities when its released by enzymatic hydrolysis such as antimicrobial, antioxidant, antidiabetic, anti-inflammatory, and anti-hypertensive (Raghavan and Kristinsson 2009; del Carmen Millán-Linares *et al.*, 2014; Sila *et al.*, 2015, Maturana *et al.*, 2017; Nongonierma *et al.*, 2017).

In recent years, oxidative stress is the main reason for various degenerative processes and diseases such as acute and chronic inflammatory conditions such as wound (Petersen, 1981).

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Antioxidants can improve the healing of infected and non-infected wounds by reducing the damage caused by oxygen radicals (Venugopal and Shahidi, 1995). The wound healing process may be delayed by the presence of free radicals or microbial infection (Boman, 1995).

Reactive oxygen species and free radicals are the major cause of many diseases such as cancer, diabetes hypertension, neurodegenerative disorders, and aging problems. Based on the possibility of health problems, several commercial antioxidants use is regulated (Ktari *et al.*, 2014, Zeng *et al.*, 2015). In the past few years, there has been an increasing interest to identify antioxidants from different natural sources. Recently, we have shown that a protein extract from terrestrial snail *Cryptozonia bistrialis* among seven different snails collected from India inhibited the growth of pathogenic bacteria and fungi (Ulagesan and Kim, 2018). In this current study, we further analyzed antibacterial and antioxidant activities of protein hydrolysates from the terrestrial snail, *C. bistrialis* prepared by enzymes.

MATERIALS AND METHODS

Chemicals and snail

Trypsin, papain, pepsin, DPPH (2, 2-diphenyl-1-picrylhydrazyl), ABTS [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)], butylated hydroxytoluene, potassium ferricyanide, trichloroacetic acid, ferric chloride, and potassium persulfate were procured from Sigma-Aldrich. Analytical grade other chemicals and reagents were used in this experiment. The reagent was prepared freshly with deionized water. Land snails *C. bistrialis* of live species were collected from Thirumazhisai agricultural field, Chennai, Tamil Nadu, India and they were brought to the laboratory. The snail's shells were broken, and the soft body was stored at -20°C prior to use.

Protein hydrolysate preparation

Three different enzymes were used to produce peptides from the protein of *C. bistrialis*. The enzymes were used at their optimal conditions: pepsin (0.1 M Glycine-HCl buffer, pH 2.0, 37°C temperature, and enzyme/substrate ratio 1:100 w/w), papain (0.1 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer, pH 6.0, 37°C temperature, and enzyme/substrate ratio 1:100 w/w), and trypsin (0.1 M $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ buffer, pH 8.0, 37°C temperature, and enzyme/substrate ratio 1:250 w/w). The enzymatic digestion was carried out based on the method described elsewhere (Je *et al.*, 2007). Enzymatic degradation was performed with three different enzymes such as pepsin, papain, and trypsin separately. The *C. bistrialis* protein was mixed with different enzymes and incubated for 6 hours in constant stirring, after incubation the mixture was heated in water bath at 100°C for 10 minutes, this inactivates the enzyme activity. Then, the mixture was centrifuged at 10,000 rpm in 4°C for 15 minutes, the collected supernatant was the protein hydrolysate.

Degree of hydrolysis

Degree of hydrolysis (DH) was determined based on the procedure described elsewhere (Tang *et al.*, 2009). The *C. bistrialis* protein was mixed with trypsin, pepsin, and papain enzymes with different enzyme/substrate ratios separately 1:100, 2:100, 4:200 w/w and the reaction was conducted at optimal conditions of the

respective enzymes, from the first to sixth hour the hydrolytic yield was measured continuously. The reaction mixture pH was maintained stable during the hydrolysis by using 2 M NaOH, after hydrolysis, the pH was neutralized and the enzymes were inactivated by heating the reaction mixture at 100°C for 10 minutes. The protein hydrolysate was centrifuged for 15 minutes at 10,000 g, after centrifugation the supernatant was collected and used in this study. Percentage of the DH was calculated by using the pH-stat method by Adler-Nissen (1986). DH is described as the ratio between the number of broken peptide bonds (h) and the total number of peptide bonds per mass unit (h_{tot}) as shown below:

$$\text{Percentage of DH} = \frac{h}{h_{\text{tot}}} \times 100$$

The DH of hydrolyzed protein was determined by measuring the amount of free α -amino groups based on the reaction between Sanger's reagent of 1-fluoro-2,4-dinitrobenzene and the amino groups in the amino acids which resulted in a yellow complex of amino acids (Goodwin, 1968). The absorbance was measured at 410 nm.

Estimation of protein hydrolysate concentration

The protein hydrolysate concentration of *C. bistrialis* by papain digestion was determined by Bradford's method and bovine serum albumin used as a standard (Bradford, 1976).

Antibacterial activity

Antibacterial assay was performed for three different enzyme digested protein hydrolysates by Agar well diffusion method against *Staphylococcus aureus* (MTCC 3160) and *Pseudomonas aeruginosa* (MTCC 424) (Bauer *et al.*, 1966). The bacterial cultures were regularly subcultured and maintained in respective medium at 4°C as stock cultures, and for antibacterial assay, a loop full of culture from the stock cultures were transferred to Luria Bertani broth and were incubated for 24 hours at 37°C , after incubation 24 hours broth bacterial cultures were swabbed in nutrient agar plates and kept for 15 minutes in a laminar chamber for absorption of cultures and 5mm wells were made by using a sterile cork borer, 20 μl of the three different enzyme digested protein hydrolysates at different concentrations, such as 12.5, 25, and 50 $\mu\text{g/ml}$, were prepared and added to each well. Antibiotic chloramphenicol (100 $\mu\text{g/ml}$) used as positive control and distilled water was used as the negative control. The plates were incubated for 24 hours in 37°C . The zone of inhibition was measured in millimeters by using the antibiotic zone measuring scale.

Only the papain-digested protein hydrolysate of *C. bistrialis* has activity against *S. aureus* and *P. aeruginosa*. Based on these results, papain-digested protein hydrolysate of *C. bistrialis* was selected for further studies.

DPPH radical scavenging assay

Papain enzyme digested protein hydrolysate of *C. bistrialis* was analyzed for its DPPH radical scavenging activity by using the method by Burits and Bucar (2000) with slight modification. Aliquots of the sample at different concentrations such as 0.5 mg/ml to 3.0 mg/ml were dissolved in 1 ml distilled water separately and 4 ml DPPH (0.004%) in methanol added to

the sample, the reaction mixture was incubated in dark at room temperature for 30 minutes. The optical density of the reaction mixture was read at 517 nm. Control was prepared by using the same protocol but methanol was used to replace the sample. Percentage of DPPH radical scavenging activity was measured by using the formula,

$$\text{Percentage of radical scavenging activity} = \frac{(B - A)}{B} \times 100$$

where *A*—absorbance of sample and *B*—absorbance of the blank.

ABTS radical scavenging assay

ABTS radical cation decolorization assay was performed to determine the free radical scavenging activity of the papain enzyme digested protein hydrolysate of *C. bistrialis* (Re *et al.*, 1999). ABTS assay was performed by using the reaction mixture of (1:1) 7 mM ABTS in water and 2.45 mM potassium persulfate and incubated for 12–16 hours in room temperature at dark. After incubation, the ABTS reaction mixture was diluted by using methanol to get an absorbance about 0.700 ± 0.02 at 734 nm. Samples were prepared in various concentrations such as 0.5–3.0 mg/ml, they were dissolved in 1 ml distilled water separately. After the addition of 20 μ l of protein hydrolysate to 980 μ l of diluted ABTS solution, the mixture was incubated at dark in 30°C for 10 minutes, the absorbance measured at 734 nm. An appropriate solvent used as a blank. All measurements were performed in triplicates, absorbance read at 734 nm, and the percentage of inhibition was calculated by using the formula,

$$\text{ABTS scavenging effect (\%)} = \frac{(AB - AA)}{(AB)} \times 100$$

where AB is absorbance of ABTS radical with methanol and AA is absorbance of ABTS radical with sample extract/standard.

Ferric reducing antioxidant power (FRAP) assay

The ferric ion (Fe^{3+}) reduce the ability of the protein hydrolysate was analyzed by the protocol previously described

by Pulido *et al.* (2000) with modifications. The ferric reducing antioxidant power (FRAP) reagent consists of acetate buffer 25 ml (pH 3.6), 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) 2.5 ml in 40 Mm HCl, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ 2.5 ml. Protein hydrolysate dissolved in distilled water as an aliquot of 30 μ l and FRAP reagent of 900 μ l, incubated for 30 minutes at 37°C, after incubation the absorbance of the mixture read at 595 nm. Ferric reducing antioxidant power indicates the increased absorbance of the reaction mixture.

Statistical analysis

All experiments were performed in triplicate, and results are presented as the mean \pm standard deviation (SD). Analysis of significant differences done by one-way analysis of variance. Differences at $p < 0.05$ were considered as significant.

RESULTS AND DISCUSSION

Degree of hydrolysis

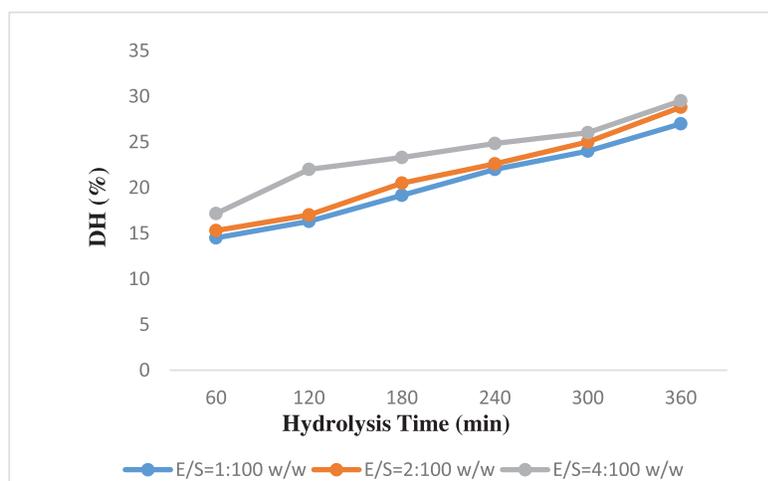
Terrestrial snail *C. bistrialis* (Beck, 1837) was collected and identified with registration number, LM-551. Peptides will be released by enzymatic digestion of proteins. Nnanna and Wu (2006) stated that the enzyme-digested protein leads to the release of peptides and amino acids, they were called as protein hydrolysate. In our present work, the snail *C. bistrialis* protein was digested with three different enzymes such as papain, pepsin, and trypsin to obtain protein hydrolysates. According to Nasri *et al.* (2013), based on factors such as enzyme and hydrolysis time, the protein hydrolysates have variation in their amino acids composition. The percentage of peptide bond breakage was calculated by using the DH, during hydrolysis, the proteolysis monitoring parameter was determined at various time intervals (Adler-Nissen, 1986). Higher percentage of DH indicates that more number of peptide was present in the solution. Sampath Kumar *et al.* (2011) reported that the peptides recovered after cleavage are more bioactive and antioxidant in nature. In our study, the DH of *C. bistrialis* protein was calculated with different enzyme/substrate ratio for three different enzymes and the DH were monitored at various time intervals 1–6 hours presented in Table 1, the percentage of DH was calculated and presented in Figures 1A–C and 2, at the end of 6 hours was,

Table 1. Effect of different enzyme/substrate ratios in the DH of protein hydrolysate derived by papain, pepsin, and trypsin from *C. bistrialis*.

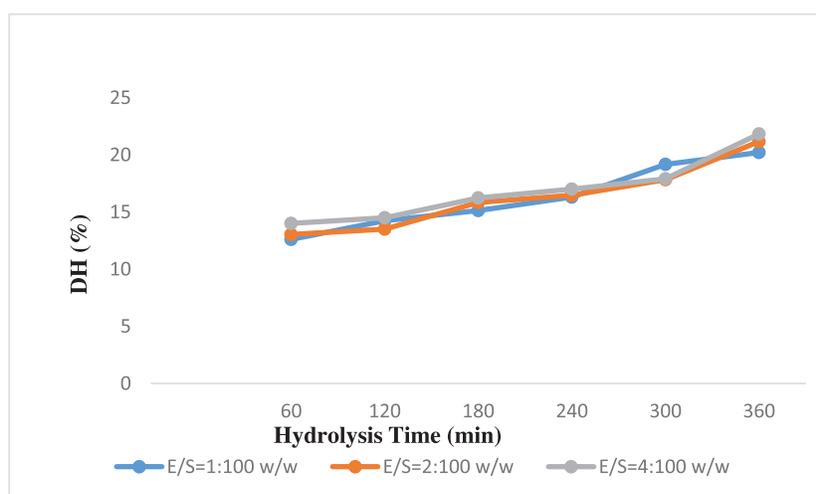
Enzyme	60 minutes	120 minutes	180 minutes	240 minutes	300 minutes	360 minutes
Papain						
E/S = 1:100 w/w	14.5 \pm 0.5	16.3 \pm 0.57	19.16 \pm 0.76	22 \pm 1	24 \pm 1	27 \pm 0.5
E/S = 2:100 w/w	15.3 \pm 0.57	17 \pm 0.86	20.5 \pm 0.5	22.6 \pm 0.76	25 \pm 0.5	28.8 \pm 0.57
E/S = 4:100 w/w	17.16 \pm 0.28	22 \pm 1	23.3 \pm 0.57	24.83 \pm 0.76	26.01 \pm 0.76	29.5 \pm 0.5
Trypsin						
E/S = 1:100 w/w	12.6 \pm 0.14	14.23 \pm 0.4	15.13 \pm 0.70	16.3 \pm 0.30	19.16 \pm 0.40	20.2 \pm 0.5
E/S = 2:100 w/w	13.03 \pm 0.15	13.5 \pm 0.5	15.83 \pm 0.28	16.46 \pm 0.25	17.83 \pm 0.28	21.16 \pm 0.76
E/S = 4:100 w/w	14 \pm 0.5	14.5 \pm 0.5	16.23 \pm 0.25	17 \pm 0.2	17.9 \pm 0.79	21.83 \pm 0.28
Pepsin						
E/S = 1:100 w/w	9.1 \pm 0.36	10.23 \pm 0.25	10.4 \pm 0.52	11 \pm 0.2	11.43 \pm 0.40	12.26 \pm 0.25
E/S = 2:100 w/w	9.5 \pm 0.26	10.3 \pm 0.1	10.6 \pm 0.30	11.56 \pm 0.11	11.6 \pm 0.52	12.4 \pm 0.17
E/S = 4:100 w/w	10.6 \pm 0.52	11.43 \pm 0.40	11.86 \pm 0.11	12.23 \pm 0.25	12.5 \pm 0.3	12.66 \pm 0.28

The values of the DH% are reported as mean of triplicates and \pm SD

(A)



(B)



(C)

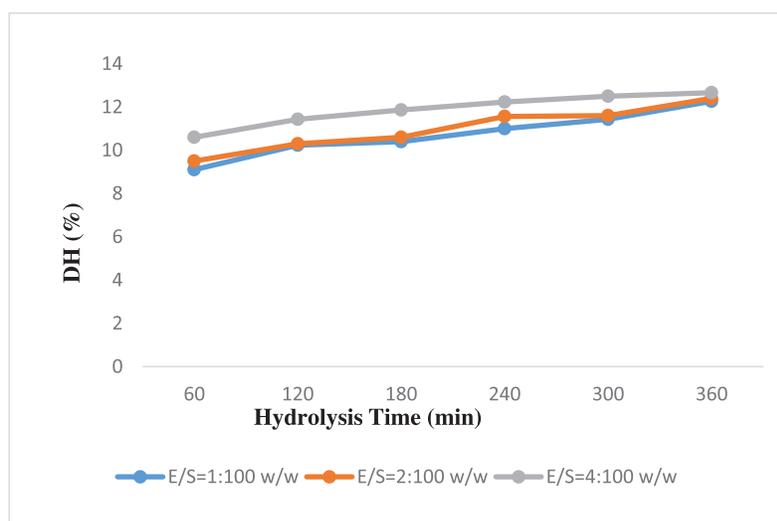


Figure 1. DH of *C. bistrialis* protein by various E/S ratios (A) papain, (B) trypsin, and (C) pepsin. Changes in DH of protein from *C. bistrialis*, hydrolysis by papain at various E/S ratios w/w (1:100, 2:100, and 4:100). The data were expressed as the mean of triplicates \pm SD measurements.

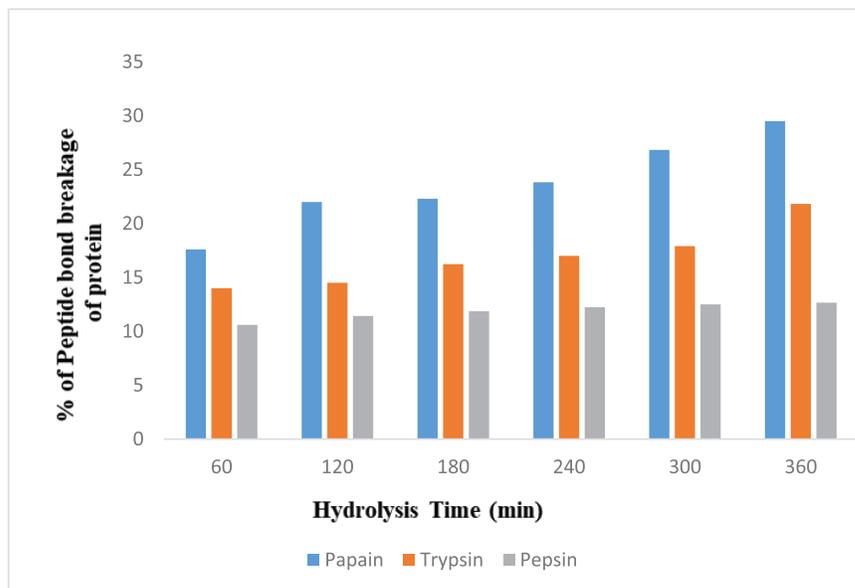


Figure 2. Percentage of peptide bond breakage of protein from *C. bistrialis* by three different enzymes at various time intervals. Percentage of peptide bond breakage of protein from *C. bistrialis* digested by papain, trypsin, and pepsin at various time intervals. The data were expressed as the mean of triplicates \pm SD measurements.

papain 27%, trypsin 20%, and pepsin 12%, respectively. The higher level of breakdown 14.5% for papain, 12.6% for trypsin, and 9% for pepsin were obtained in the first hour of digestion and the percentage of peptide bond breakage increased gradually up to the 6 hours. Among the three enzymes used in our work, papain showed a higher DH than trypsin and pepsin. The present work result showed the result similar to the report of papain digestion of fish protein hydrolysate in which 22% of hydrolysis was obtained during the sixth hour (Abdulazeez *et al.*, 2013). The hydrophobic amino acids are important for antioxidant and antimicrobial properties of protein hydrolysate, they can improve their interaction with lipid targets or entry into target organs through hydrophobic associations. Papain-digested *C. bistrialis* protein was 1.42 mg/ml.

Antibacterial activity of digested protein hydrolysates of *C. bistrialis*

Three different protein hydrolysates were analyzed for their antibacterial activity against *S. aureus* and *P. aeruginosa*. Only the papain-digested protein hydrolysate of *C. bistrialis* had activity against tested pathogens as shown in Figure 3A–C and Table 2. According to Najafian and Babji (2012), usually, the protein hydrolysates which contain cationic amino acids in their composition will show higher antimicrobial activity. Antimicrobial activity was analyzed for protein hydrolysates from fermented fishes such as goby (*Zosterisessor ophiocephalus*), zebra blenny (*Salaria basilisca*), ray (*Dasyatis pastinaca*), and sardinelle (*Sardinella aurita*) (Jemil *et al.* 2014). These hydrolysates inhibited both Gram-positive and Gram-negative microorganisms. The amino acid sequence is closely related to the antimicrobial activity of the protein hydrolysate (Memarpoor-Yazdi *et al.* 2012; Najafian and Babji, 2012). Based on these results, papain-digested protein hydrolysate of *C. bistrialis* was selected for further studies.

Antioxidant activity digested of protein hydrolysate

Antioxidant properties of papain-digested protein hydrolysate were presented in Figure 4A–C. Antioxidant properties were analyzed for different proteins from various natural bio-resources such as bullfrog skin and porcine collagen and the protein hydrolysate of round scad (*Decapterus maruadsi*) analyzed for its antioxidant activity (Thiansilakul *et al.* 2007). Peptides were purified and analyzed for its antioxidant activity from giant squid muscle (Rajapakse *et al.* 2005). In our study, effective results of antioxidant potential protein hydrolysate from *C. bistrialis* were obtained. Two different radicals such as DPPH and ABTS scavenged by protein hydrolysate, it might be governed by a difference in chain length, amino acid side chain, and hydrophobicity. In a protein hydrolysate, the amino acid composition is an important factor for antioxidant activities. Chi *et al.* (2015) stated that the protein hydrolysate of Skipjack tuna showed higher radical scavenging activity because of the presence of hydrophobic and aromatic amino acids.

Normally, the hydrogen-donating potential of protein hydrolysate is determined by DPPH radical scavenging activity (Thiansilakul *et al.*, 2007). DPPH is a free radical, it gave a maximum absorbance at 517 nm at an appropriate environment, the absorbance will be reduced and the color will be changed from purple to yellow, when the DPPH facing a hydrogen-donating substance, such as an antioxidant (Nalinanon *et al.*, 2011; Shimada *et al.*, 1992). This proves the peptide has maximally 73% radical scavenging activity. In this study, when we increased the concentration of protein hydrolysate, the radical scavenging activity also increased. Similar results were reported on *Nemipterus japonicas* and *Exocoetus volitans* (Yousuf Naqash and Nazeer, 2010) in which radical scavenging activity was proportional to peptide concentration. Papain-digested hydrolysate used in the present study reached scavenging activity equal to that of butylated hydroxytoluene, a synthetic antioxidant. Bougatef *et al.* (2009) reported protein hydrolysate from *Mustelus mustelus* contained

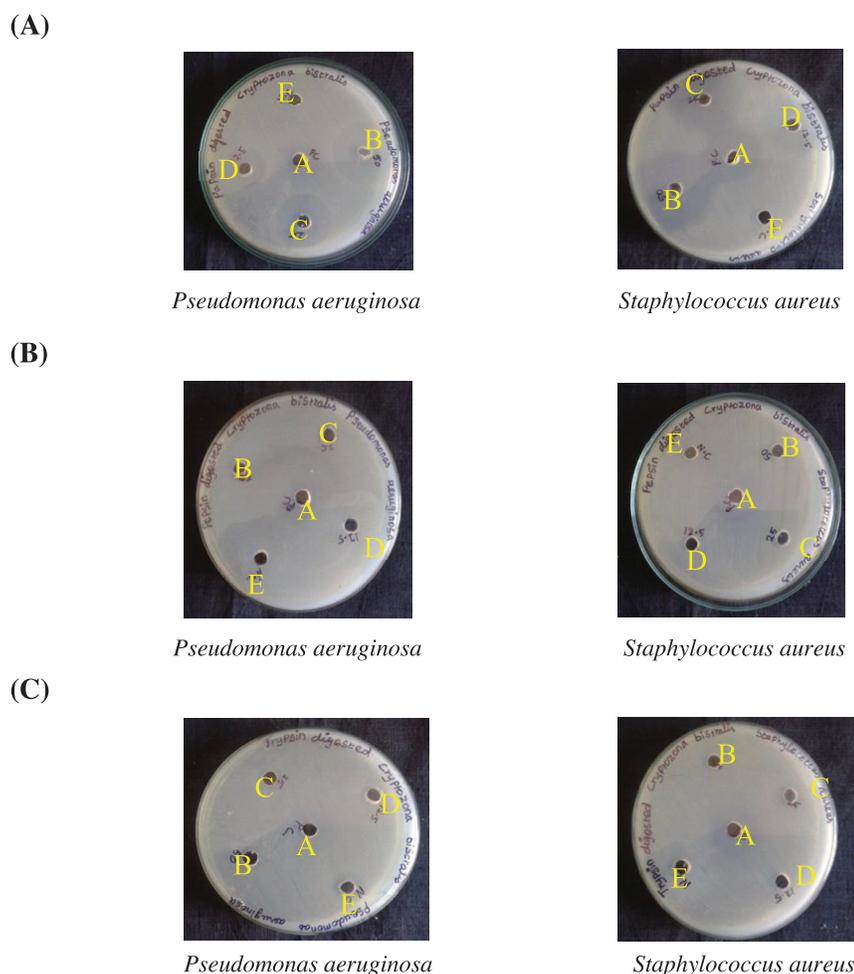


Figure 3. Antibacterial activity of protein hydrolysate of *C. bistrialis* prepared by (A) papain, (B) pepsin, and (C) trypsin. In each plate, positive control (chloramphenicol, 100 µg/ml) and negative control were labeled as A and E, respectively, 50 µg/ml was B, 25 µg/ml was C, and 12.5 µg/ml of protein hydrolysate was D.

Table 2. Antibacterial Activity of three different enzyme digested protein hydrolysates of *C. bistrialis*.

Enzyme digest	Microorganisms	Zone of inhibition in mm				Chloramphenicol (100 µg/ml)
		50 µg/ml	25 µg/ml	12.5 µg/ml	N.C (mm)	
Papain digest	<i>Pseudomonas aeruginosa</i>	27.3 ± 0.5	24 ± 1	18.3 ± 0.5	-	28 ± 0.5
	<i>Staphylococcus aureus</i>	28.3 ± 0.5	25.5 ± 0.5	22.8 ± 1.2	-	30.6 ± 0.6
Pepsin digest	<i>Pseudomonas aeruginosa</i>	-	-	-	-	15.6 ± 0.57
	<i>Staphylococcus aureus</i>	-	-	-	-	17.3 ± 0.5
Trypsin digest	<i>Pseudomonas aeruginosa</i>	-	-	-	-	18.3 ± 0.76
	<i>Staphylococcus aureus</i>	-	-	-	-	30.8 ± 0.28

Antibacterial activity of three different enzyme digested protein hydrolysates of *C. bistrialis*. The data were expressed as the mean of triplicates ± SD measurements.

antioxidant activity which can reduce Fe³⁺ / ferricyanide complex to ferrous form. *Zhu et al.* (2006) showed that peptide hydrolysate could decrease oxidation by binding with iron and chelating preoxidative iron. Commonly, FRAP assay is performed to analyze the ability to reducing TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (*Binsan et al.* 2008). The significant Ferric reducing the antioxidant ability and maximum 70% of ABTS radical scavenging activity obtained in the present study and confirms the antioxidant property of *C. bistrialis* protein hydrolysate.

In summary, papain digested the *C. bistrialis* protein with highest DH. The protein hydrolysate showed antibacterial

activity against pathogenic bacteria and antioxidant activity. From these results we concluded that the protein hydrolysate of *C. bistrialis* could be an efficient source for natural antibacterial and antioxidant agents. Further optimization for hydrolysis condition using response surface methodology and characterization may be needed for future biomedical applications.

FUNDING

This work is supported by Pukyong National University (2017).

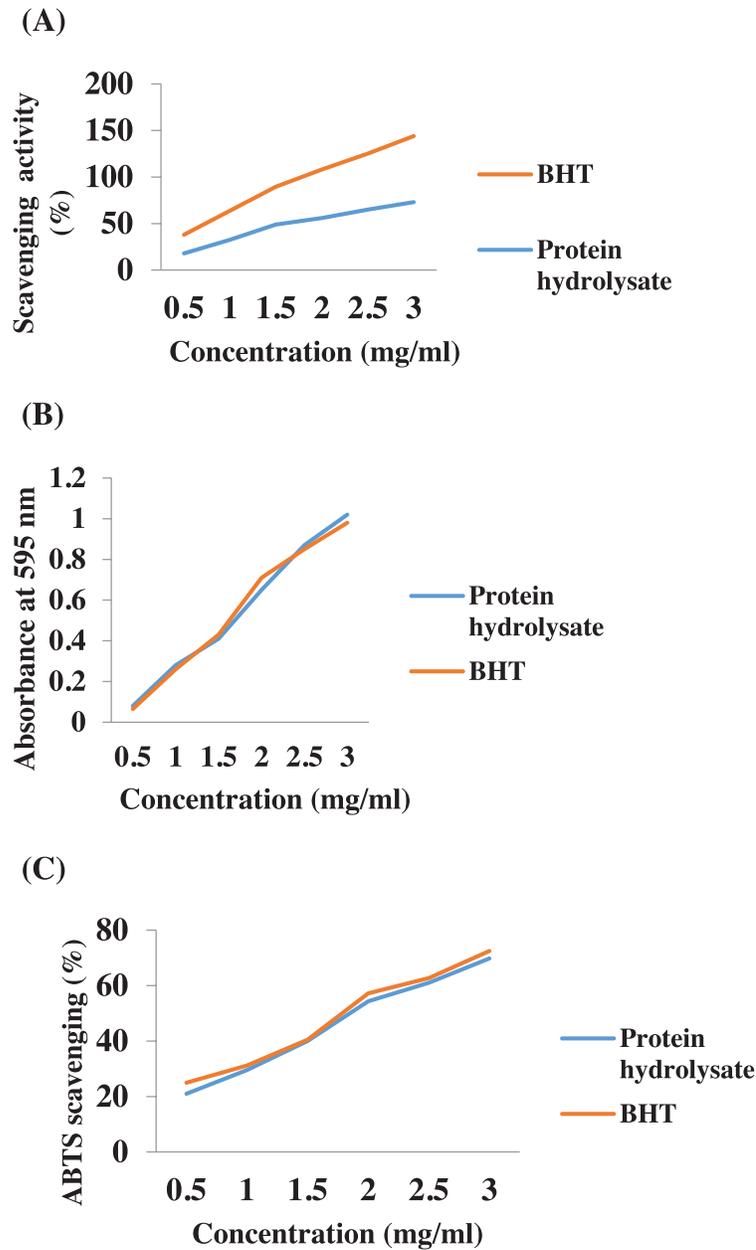


Figure 4. Antioxidant activity of protein hydrolysate from *C. bistrialis*. (A) DPPH radical scavenging activity, (B) FRAP assay, and (C) ABTS radical scavenging assay. The data were expressed as the mean of triplicates \pm SD measurements.

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How to cite this article:

Ulagesan S, Kuppasamy A, Kim HJ. Antimicrobial and antioxidant activities of protein hydrolysate from terrestrial snail *Cryptozona bistrialis*. *J App Pharm Sci*, 2018; 8(12): 012–019.