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Research Article

INVESTIGATION AND EFFICACY ON PROTEIN HYDROLYSATE OF *PILA GLOBOSA* (A FRESH WATER MOLLUSCS) IN VARIOUS BIOMEDICAL ASPECTS

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ABSTRACT

Objective: The present study is to investigate the antibacterial, antioxidant, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of protein hydrolysate of fresh water Molluscs *Pila globosa* (Swainson, 1822).

Methods: Protein hydrolysate was prepared from a tissue of *P. globosa* by enzymatic hydrolysis. Enzyme digestion was carried out with the enzyme trypsin. The protein concentration was estimated by Bradford's method, and the protein quantification was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. Antibacterial assay was carried against four bacterial pathogens by well diffusion method. Minimum inhibitory concentration (MIC) was assayed by the microdilution method against bacteria. The percentage of free radical scavenging activity was done by DPPH method. The total antioxidant activity also carried out by phosphomolybdenum method.

Results: Digested protein hydrolysate contains 2.2 mg/ml protein concentration, obtained by Bradford's method. The molecular weight of protein hydrolysate was obtained ranging from 13.5 to 96.9 kDa. The maximum zone was observed against *Salmonella typhi* 16±1 mm at 1000 µg/ml. In MIC, activity was found to be maximum at 100 µl concentration of protein hydrolysate. The percentage of total antioxidant activity was increased by increasing the concentration of protein hydrolysate. Maximum of 37% radical scavenging activity was found. The Fourier transform infrared spectroscopy analysis contains peaks ranging from of 579 to 3396/cm.

Conclusion: This finding suggests the possibility that the protein hydrolysate of *P. globosa* may have a potential use for a therapeutic agent like antibiotics.

Keywords: *Pila globosa*, Protein hydrolysate, Antibacterial activity, Total antioxidant activity, Free radical scavenging activity.

INTRODUCTION

The snails belong to the phylum mollusk and class gastropods. The gastropods are the largest class of the phylum mollusk [1]. For centuries snails, and to a lesser extent slugs, have been used both as food and as treatment for a variety of medical conditions [2]. The highly portentous and delicious molluscan meat is on an increasing demand throughout the world. The apple snail, *Pila globosa* (Swainson, 1822) occurs in all types of temporary and permanent water bodies such as ponds, canals, and ditches [3]. In Bangladesh and some part of India, *P. globosa* is used as a protein supplement for humans as well as in aquaculture such as shrimp and catfish farming [4,5]. The *P. globosa* are edible to aboriginal people, and particularly, they have been used as medicines for the cure of a number of ailments such as rheumatism, cardiac diseases, controlling blood pressure, asthma, rickets, calcium metabolism, nervousness, giddiness, and also providing missing vitamins and minerals [6]. In addition, soup prepared from the *P. globosa* flesh is used as medicine that is believed to cure asthma, arthritis, joint swelling, rheumatism, and in quick healing of wounds. Production of protein hydrolysates by enzymatic degradation is a significant research area of recent past. Free radicals are highly reactive oxygen species and among them, superoxide, hydroxyl, peroxyl, peroxynitrite, and nitric oxide radicals are the important ones [7]. These radicals are very unstable and react rapidly with other substances in the body, leading to cell or tissue injury [8]. Antioxidants could be used to overcome the deleterious effect of these free radical induced damages. Antioxidants could be any substance that when present at low concentrations compared to those of an oxidizable substrate [9]. The antioxidants derived from mollusks are easy to obtain, cheap, and safe without any adverse effects in comparison to their synthetic counterparts. Based on these information, the goal of the present study was designed to synthesize the protein hydrolysate of *P. globosa* and evaluate the antibacterial and antioxidant activity.

METHODS

Collection and identification

P. globosa (Fresh water Snails) were collected from Porur Lake, Chennai, Tamil Nadu, India. They were identified by Dr. R. Venkitesan, Scientist - C, Zoological Survey of India, 130, Santhome High Road, Chennai - 600 028. The Registration Number FWM – 552 *P. globosa* (Swainson, 1822).

Sample preparation

The collected snails were brought to the laboratory, and the shells were broken, and the soft body were separated and stored at −20°C until used.

Preparation of protein hydrolysate

The proteolytic digestion of *P. globosa* was performed according to the method described by Je *et al*., (2007) [10]. To produce peptides from the tissue of *P. globosa*, enzymatic hydrolysis was carried out with the enzyme trypsin. The enzyme trypsin in 0.1 M phosphate buffer under optimal conditions (pH-8, temperature, 37°C) at the enzyme/substrate ratio of 1:250 (w/w). The tissue of *P. globosa* was homogenized with blender and then thoroughly mixed with the enzyme. The enzyme substrate mixture was incubated for 6 hrs with constant stirring at the end of the incubation period the content was heated in a boiling water bath for 10 minutes at 100°C. This heating inactivates and stops the enzyme activity. Then, the mixture was centrifuged for 15 minutes at the speed of 10,000 rpm. The supernatant obtained was the protein hydrolysate. The hydrolysates were lyophilized to get a powdered sample and were stored at −20°C.

Determination of protein concentration

The concentrations of protein hydrolysate were estimated by the Bradford's method using bovine serum albumin as a standard [11].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis

The molecular weight of the protein hydrolysate was confirmed by the SDS-PAGE analysis [12] with the molecular weight marker ranging from 97.4 to 14.4 kDa.

Antibacterial assay

Test bacterial cultures were obtained from King Institute of Preventive Medicine, Guindy, Chennai. They are *Escherichia coli, Salmonella typi, Vibrio cholerae,* and *Staphylococcus aureus*. The organisms were periodically subcultured and maintained in nutrient agar slant at 4°C. Antibacterial activity was determined by agar well diffusion method [13]. The Muller Hinton agar plates were swabbed with the respective 24 hrs broth culture of organisms and kept for 15 minutes in the laminar chamber for absorption of cultures. Wells were made on agar plates using a sterile cork bore of 5 mm. The protein hydrolysate of various concentrations such as (500 µg/ml, 750 µg/ml, and 1000 µg/ml) was prepared and 20 µl of respective concentrations were added to each well. Distilled water used as negative control and ampicillin (1000 µg/ml) used as positive control. The plates were incubated at 37°C for 24 hrs, and the diameters of the zone of inhibition were measured in millimeter.

Minimum inhibitory concentration (MIC)

The MIC was determined by the microdilution method using liquid nutrient media with different concentrations of the protein hydrolysate. 4.5 ml of sterilized nutrient media was poured into sterilized test tube. Different concentrations of protein hydrolysate such as (12.5 µl, 25 µl, 50 µl, 75 µl, and 100 µl) were added to nutrient media to all the test tubes. Moreover, 0.5 ml of bacterial suspension was added, and the tubes were incubated at 37°C for overnight. In the negative control, the nutrient media contains without the presence of protein hydrolysate sample (4.5 ml nutrient media added with 0.5 ml bacterial culture) and the positive control contains antibiotics by replacing the protein hydrolysate (4.5 ml nutrient media+0.5 ml bacterial culture+chloramphenicol 50 µl). The growth was observed OD at 595 nm spectrophotometrically for the development of turbidity and inhibition by the lowest concentration of sample that inhibits the development of turbidity [14].

Determination of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH radical scavenging activity of the hydrolysate was determined according to the method of Burits and Bucar (2000) [15]. Aliquots of the sample at different concentrations 0.5-3.0 mg were taken in different test tubes and were dissolved in 1 ml of ethanol followed by 4 ml of 0.004% of DPPH solution in methanol solution in each test tube. The test tubes were incubated at room temperature for 30 minutes, and the absorbance was read at 517 nm. Butylatedhydroxytoluene (BHT) was used as standard.

$$\text{Radical scavenging activity (\%)} = [(B-A)/B] \times 100$$

Where A is absorbance at 517 nm of sample and B is absorbance at 517 nm of the blank.

Total antioxidant activity

The total antioxidant capacity of protein hydrolysate was measured by the spectrophotometric method of Prieto *et al*., (1999) [16]. At different concentration, ranges such as (100-500 µl) were prepared and mixed with 1 ml of reagent solution (0.6M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate mixture). The tubes were incubated for 90 minutes at 95°C. The mixture was cooled to room temperature and the absorbance was read at 695 nm against blank. The experiment was conducted in triplicates and values are expressed as equivalents of ascorbic acid in µg per mg of the protein hydrolysate.

Fourier transform infrared spectroscopy (FTIR) spectral analysis

The lyophilized samples of *P. globosa* (10 mg) were mixed with 100 mg of dried potassium bromide and compressed to prepare as a salt disc. The disc was then read spectrophotometrically.

Statistical analysis

All analyzes were carried out in triplicate, and results are reported as the mean±standard deviation (SD). Significant differences were analyzed by one-way ANOVA. Differences at $p<0.05$ were considered significant.

RESULTS

Determination of protein concentration

About 2.2 mg/ml amount of protein was determined at 595 nm, and molecular weight of protein hydrolysate was obtained in SDS-PAGE analysis, ranging from 13.5 to 96.9 kDa shown in Fig. 1.

Antibacterial activity

The antibacterial activity was performed by test organisms such as *E. coli*, *Salmonella typhi*, *Vibrio cholerae*, and *S. aureus,* and the zone of inhibition of each organism was recorded and listed in Table 1 and Fig. 2.

MIC of *P. globosa*

In MIC method, the inhibition of microorganisms was found to be maximum at increasing the concentration of protein hydrolysate of each organism was observed in Table 2.

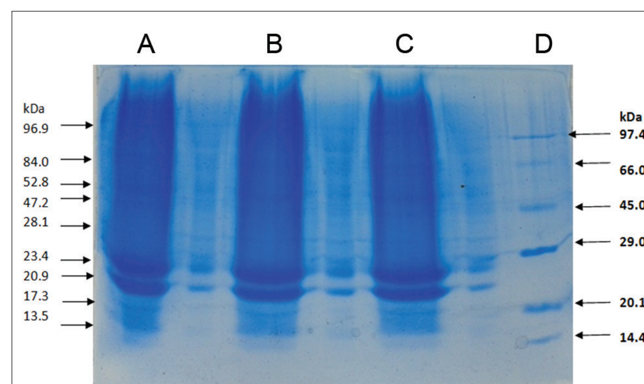

Fig. 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis - *Pila globosa.* (A) Protein hydrolysate of *Pila globosa.* (B) Protein hydrolysate of *P. globosa.* (C) Protein hydrolysate of *P. globosa.* (D) Protein molecular weight marker

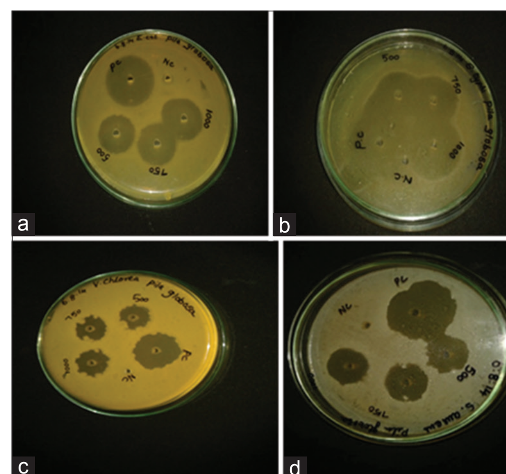

Fig. 2: Antibacterial activity of *Pila globosa* (a) *Escherichia coli.* (b) *Salmonella typhi.* (c) *Vibrio cholera.* (d) *Staphylococcus aureus*

Table 1: Antibacterial activity of *P. globosa*

Microorganisms	Zone of inhibition in mm				
	1000 µg/ml	750 µg/ml	500 µg/ml	Positive control	Negative control
Escherichia coli	13.6±0.57	12.6±0.57	12±115.8	3±0.28	-
Vibrio cholera	11.5±0.5	7.83±2.87.1	6±2.81	1.83±0.76	-
Staphylococcus aureus	10.6±0.57	9.66±0.57	8.5±0.5	12.5±0.5	-
Salmonella typhi	16±113.5	6±0.51	2.9±0.1	18.76±0.25	-

Antibacterial activity of protein hydrolysate of *P. globosa*. The data were expressed as mean of triplicates±SD measurements. *P. globosa: Pila globosa*, SD: Standard deviation

Table 2: Minimum inhibitory concentration of *Pila globosa*

Microorganisms	Microorganisms concentration of protein hydrolysate in µl (OD at 595 nm)						
	Positive control	Negative control	12.5 µl	25 µl	50 µl	75 µl	100 µl
Escherichia coli	0.04	0.92	0.81	0.56	0.43	0.29	0.16
Vibrio cholera	0.02	0.73	0.70	0.49	0.34	0.22	0.08
Staphylococcus aureus	0.06	0.8	0.73	0.61	0.52	0.34	0.18
Salmonella typhi	0.06	1.27	1.09	0.92	0.50	0.31	0.15

Positive control: Nutrient media + bacterial culture + antibiotic. Negative control: Nutrient media + bacterial culture

DPPH activity

In DPPH method, the percentage of free radical scavenging activity was listed in Fig. 3. Maximum of 37% radical scavenging activity was found compared to. BHT was used as a standard.

DPPH radical scavenging activity of protein hydrolysate of *P. globosa*. The data were expressed as the mean of triplicates±SD measurements.

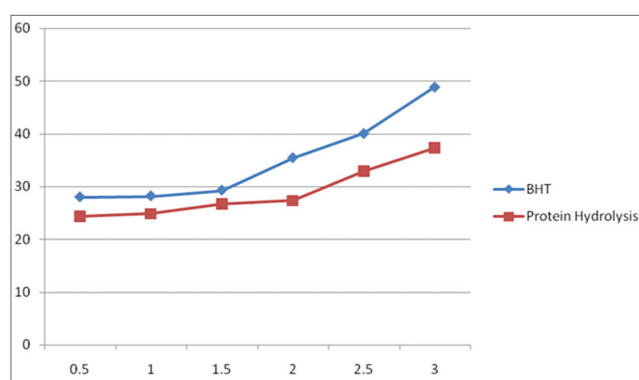

Fig. 3: 2,2-diphenyl-1-picrylhydrazyl activity of protein hydrolysate of *Pila globosa*. DPPH radical scavenging activity of protein hydrolysate of *P. globosa*. The data were expressed as mean of triplicates±standard deviation measurements

Total antioxidant activity

The total antioxidant activity also carried out by phosphomolybdenum method, and it was reported in Fig. 4.

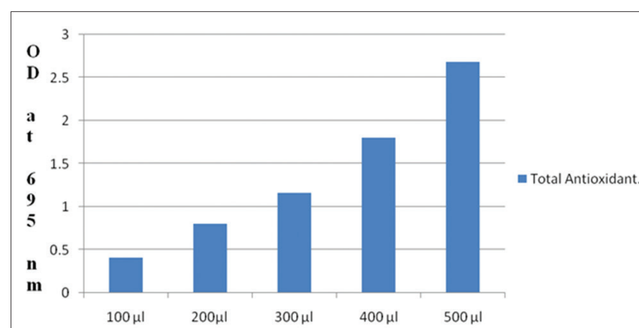

Fig. 4: Total antioxidant activity of protein hydrolysate of *Pila globosa*

FTIR spectral analysis

The FTIR studies of these adsorbents helped for the identification of various forms of the compounds present in the mollusk protein hydrolysate. The vibrations are appreciable due to the availability of various constituents. The observed bands are in the range of 579-3396/cm was observed in Fig. 5.

DISCUSSION

In recent years, great attention has been paid to the bioactivity of natural products because of their potential pharmacological utilization. Molluscs are widely used in world research institution for various studies, but recently they have been recognized as potential sources of antibacterial and antifungal substances. 2.2 mg/ml protein concentrations were obtained in digested protein hydrolysate. Molecular weight ranging from 13.5 to 96.9 kDa was found in the protein hydrolysate of *P. globosa* which acts as bioactive compounds for various biological activities. Antibacterial and antiviral activities have been previously described in the hemolymph of several molluscan species such as sea hares, sea slung, oysters, and mussels [17-23]. The maximum zone was observed against *S. typhi* 16±1 mm at 1000 µg/ml. In MIC, the protein hydrolysate of *P. globosa* showed maximum inhibition of microorganism such as *Vibrio cholera* and least at *S. aureus*. Radical scavenging activity was found to be a maximum of 37%. The scavenging ability increased with an increase in the concentration of hydrolysate. These values are equal for protein hydrolysate to that of synthetic antioxidant BHT in comparison. Similar findings were reported in frog skin [24]. The FTIR studies of these adsorbents helped for the identification of various forms of the compounds present in the mollusk protein hydrolysate; peaks were obtained ranging from of 579 to 3396/cm. In the present study indicated that protein hydrolysate of *P. globosa* may contain potential antibiotics.

CONCLUSION

The present study revealed that the protein hydrolysate of *P. globosa* showed a potent antibacterial and antioxidant activity. This investigation was followed by the screening tactics in the investigation of novel bioactive compounds. It is promising that the tested gastropod synthesis novel antibiotics for bacterial infections. Further investigations intending to purify these active compounds should be considered to clarify their chemical nature.

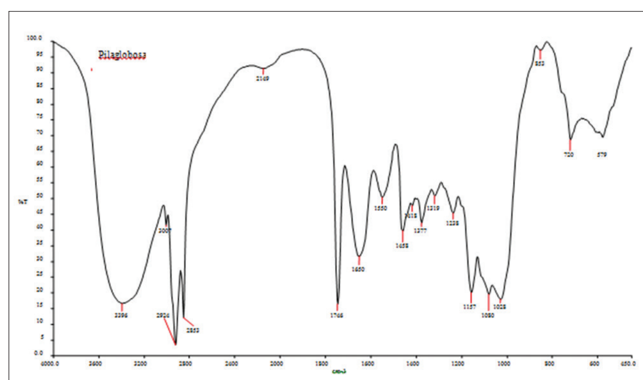

Fig. 5: Fourier transform infrared spectroscopy analysis of protein hydrolysate of *Pila globosa*

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