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In Vitro Studies and Characterization of Tissue Protein from Green Mussel, *Perna viridis* (Linnaeus, 1758) for Antioxidant and Antibacterial Potential

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Abstract
The antioxidant and antibacterial potential of various solvent extracts (water, 100% and 80% methanol) of *Perna viridis* was evaluated. The tissue analyzed for the macronutrient composition was estimated as the moisture (84%), proteins (63.5%), carbohydrates (24.26%) and lipids (9.99%). The crude extracts subjected to 10% SDS-PAGE analysis showed an array of proteins with a molecular size between 15 and 99 kDa. In the initial antibacterial and antioxidant assays, the 100% methanolic extracts showed maximum activity which was purified using Sephacryl S-100 HR column chromatography. The active fractions CS4-CS7 showed maximum activity in different antioxidant systems such as Total antioxidant activity, Ferric-reducing antioxidant power assay, hydrogen peroxide (H_2O_2) and 2,2-diphenyl-1-picrylhydrazyl radical scavenging assays. In the antibacterial activity, CS47 fraction showed maximum inhibition against *Proteus vulgaris* (15.93 ± 0.115 mm) and minimum against *Klebsiella pneumonia* (13.13 ± 0.152 mm). The FT-IR spectral analysis revealed the presence of bioactive compounds signals at different wave ranges which confirmed the presence of amino groups. Reverse phase high performance liquid chromatography analysis showed the presence of peptides at the RT of 5.86 and 12.189 min. The percentage composition of amino acids was determined through the amino acid analyzer. From this present study, it was confirmed that the active molecule was a peptide, showing strong antioxidant and antibacterial activity.

Keywords *P. viridis* · SDS-PAGE · Antioxidant · Antibacterial · RP-HPLC · Amino acid profiling

Introduction

Marine environment provides shelter for diverse forms of organism. It bestows target specific abilities to the organisms that thrive in the vast condition by producing biologically active secondary metabolites. There are enormous natural resources harbouring potential bioactive peptides, among which marine environment is the top spot for discovering new drug molecules (Ana et al. 2014). Nowadays, researchers are targeting bioactive peptides associated with the food protein, to discover new drug candidates for treating various human diseases and to minimize the side effects (Joseph et al. 2011).

Peptides from marine organisms occupy the largest portion of bioactive compounds (Hu et al. 2015). Most of these bioactive peptides are about 2–20 amino acids in length and usually resides within the parent protein in the inactive form (Erdmann et al. 2008). They become activated on detachment from the parent structure by enzymatic hydrolysis using enzymes (trypsin, chymotrypsin, pepsin, alcalase and proteinases) (Najafian and Babji 2012; Senevirathne and Kim 2012). The structure, sequence and amino acid composition determines the biological activities of the target peptide (Pihlanto-Leppala 2000).

Approximately 60% of the marine animals are invertebrates, which are enormous sources for the discovery of many novel natural products since 1990 (Blunt et al. 2013, 2014). Among the marine invertebrates, 23% of the organisms with pharmacologically active molecules constitute the largest phylum, Mollusca. The bioactive peptide

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isolated from several classes of mollusc exhibit antitumor, antileukemic, antibacterial and antiviral properties (Kiran et al. 2014). In the phylum Mollusca, the Bivalve is the second largest class with potent bioactivities.

As marine bivalves thrive on diversified marine environment, they are subjected to certain amount of stress leading to Reactive Oxygen Species (ROS) generation. The ROS formation is unavoidable and essential in almost all the life forms as it is important machinery for producing energy, mediating intercellular signals and regulating defense mechanism against invading pathogens (Sun et al. 2010). The oxidative stress developed through free radicals may cause damages to DNA, proteins and membrane lipids (Weidinger and Kozlov 2015; Luca et al. 2015) which further results in various human diseases such as arteriosclerosis and cancer (Sindhu et al. 2010; Wang et al. 2012).

Few studies have reported the isolation and identification of antioxidant peptides from edible marine invertebrates like mussels, clams and oysters (Wang et al. 2013; Park et al. 2016) and suggested that the antioxidant activity relies on the amino acid composition of the protein (Ranathunga et al. 2006). Antioxidant peptides are rich in histidine and hydrophobic residues (Chen et al. 1995). High hydrophobicity enhances the radical-scavenging activity at the lipid phase by favoring their dispersion at the water–lipid interface (Ranathunga et al. 2006).

Marine organisms rely on their innate immunity (cellular and humoral defense factors) to defend themselves against coexisting pathogenic microorganisms, which is represented by producing antimicrobial substances, primarily peptides or polypeptides, that are synthesized or induced at the time of infection (Mayer et al. 2011). AMPs (Anti-Microbial Peptides) are usually very short peptides (< 60 amino acids) (Brown and Hancock 2006) that are grouped based on their length, structure and presence of disulfide bridges (Ravichandran et al. 2009).

The antibiotic activity of AMPs is represented by its strong hydrophobic, cationic and amphipathic nature. This enables them to interact with the negatively charged cell membrane of the invading pathogens, leading to cell membrane disruption and apoptosis (Hancock and Rozek 2002). To date, several AMPs have been isolated from marine bivalves, among which the mussel peptides are the firstly characterized (Hubert et al. 1996; Charlet et al. 1996; Mitta et al. 1999).

Thus, the current study focused to isolate the protein from the tissues of *P. viridis* and to investigate its antioxidant and antibacterial activity through *in vitro* studies. Further, FT-IR spectral analysis, Reverse phase high performance liquid chromatography (RP-HPLC) and amino acid profiling were performed to characterize the active protein.

Materials and Methods

Sample Collection

The fresh samples of bivalve, *P. viridis* were collected from the Vellar estuary in Parangipettai, Cuddalore district of Tamilnadu, along the South-East coast of India at a latitude of 11°29′N and longitude of 79°46′E. The samples were brought to the laboratory in plastic containers with estuary water and washed twice with running tap water to remove the adhered materials.

Morphometric Characteristics

The study animal of three different sizes (minimum, medium and maximum) was morphometrically characterized. The total length was taken by measuring the distance between the anterior and the posterior region. Then the total width was measured from the dorsal side to the ventral side of the animal. The soft tissues withdrawn from the shell were weighed separately to determine the total weight of the animal.

Proximate Composition

The proximate composition of *P. viridis* tissue was performed using standard methods which included moisture (AOAC 1994), protein (Lowry et al. 1951), carbohydrate (Dubois et al. 1956) and lipid (Folch et al. 1957).

Preparation of Crude Extract

Animal shells were opened and the withdrawn soft tissues were rinsed thoroughly with distilled water to remove extraneous material. For crude extraction, 30 g of wet tissue was macerated with 60 ml (1:2) of following solvent systems: Water, 100% Methanol and 80% Methanol respectively. The homogenized mixture was incubated for 48 h at 4 °C and centrifuged at 10,000 rpm for 10 min. The supernatant from each extracts were collected. The pellets were subjected to re-extraction with 30 ml of each solvent (1:1) and centrifuged. The supernatant were pooled and concentrated by rotary evaporator with reduced pressure, freeze-dried and stored at -20 °C until use. The protein content of the different solvent extracts was evaluated (Lowry et al. 1951).

Free Amino Acid Analysis

The protein samples were allowed to precipitate by adding equal volume of ethanol and centrifuged at 10,000 rpm for 1 min at 4 °C. The 1 mg/ml of 20 standard amino acids were prepared. Around, 5 µl of sample and standard

mixture was loaded into TLC aluminium plate silica gel F_{254} (Merck) using Hamilton syringe. The plate was developed in the twin chamber which was saturated with developing solvent system (n-butanol: glacial acetic acid: ethyl acetate: water) at a ratio of 1:1:1:1. The plate was allowed to dry before spraying with 0.3% ninhydrin in n-butanol and heated until the bands appeared.

Bioactivity

Antioxidant Assays

The antioxidant potential of *P. viridis* tissue extracts was evaluated through 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, Total Antioxidant Activity (TAA), Reducing power Assay (RPA) and Hydrogen peroxide (H_2O_2) Assay by the method of Duan et al. (2006), Prieto et al. (1999), Oyaizu (1986), and Ruch et al. (1989).

Antibacterial Activity

Antibacterial activity of the samples was determined by agar disc diffusion method (Bauer et al. 1966). The *P. viridis* extracts were tested against 10 human bacterial pathogens such as *Staphylococcus aureus, Klebsiella pneumonia, Proteus mirabilis, Proteus vulgaris, Shigella sp., Salmonella paratyphi* A, *Salmonella paratyphi* B, *Escherichia coli, Bacillus subtilis* and *Pseudomonas fluorescens*. About 20 ml of Muller Hinton agar (Himedia) was poured into sterile Petri plates and allowed to solidify. The plates were evenly streaked with a sterile cotton swab dipped in the bacterial suspension (10^5 CFU/ml). Sterile disc (5 mm diameter) were immersed in 10 µl of respective crude extracts (20 µg/10 µl) and placed over the agar plates. Amoxycillin (10 µg/disc) was used as positive control. The plates were incubated at 37 °C for 24 h and the zone of inhibition around the disc was measured. Activity was carried out in triplicates and the values were expressed in ± standard deviation.

Gel Filtration Chromatography

The crude 100% methanol extract was purified using Sephacryl S-100 HR resin (Sigma). About 10 ml of concentrated crude extract was loaded on the activated column which was previously equilibrated with 0.15M NaCl and 30 ml of 0.15M NaCl was used as eluent. A final volume of 1 ml was collected for each fraction. The collected fractions were lyophilized and screened for antioxidant and antibacterial activity.

Identification and Characterization of Bioactive Protein

SDS-PAGE Analysis

The protein pattern of the crude and purified samples was determined by SDS-PAGE according to the method of Laemmli (1970). The samples was mixed with 10 µl of sample buffer [0.5M Tris-HCl (pH 6.8) containing 10% (w/v) SDS and 100% (v/v) Glycerol, 1% Bromophenol blue with 10% Beta-mercaptoethanol] at a ratio of 2:1(sample/sample buffer). Samples were loaded into the polyacrylamide gel made of 10% separating gel, 4% stacking gel and subjected to electrophoresis at 50 mA. After electrophoresis, gel was stained with 0.1% Coomassie brilliant blue: glacial acetic acid: methanol and destained with methanol: glacial acetic acid (30:5). The molecular weight of proteins was estimated by comparing with protein marker, Bovine Serum Albumin (BSA) with a molecular weight of 66.5 kDa.

RP-HPLC

The active fraction was dissolved in HPLC grade water and subjected to reverse phase—HPLC, C_{18} column. Initially, the column was washed with methanol containing 0.1% TFA for 30 min. Then 20 µl of the active fraction was loaded into the column and eluted using an isocratic gradient of methanol containing 0.1% TFA at a flow rate of 1 ml/min for 1 h at 280 nm.

FTIR Spectral Analysis

The 1 mg of lyophilized sample was dissolved in 1 ml of HPLC grade water and subjected to FTIR spectral evaluation. All spectra were recorded at 24 ± 1 °C along with infrared range from 400 to 4000 cm^{-1} at a data acquisition rate of 2 cm^{-1} per point using FTIR spectrometer (Shimadzu, Japan). Typically, 18 signals were averaged for a single spectrum. Each spectrum was displayed in terms of absorbance as calculated from the reflectance - absorption spectrum using the Hyper-IR spectrum.

Amino Acid Profiling

The active fractions were hydrolyzed under reduced pressure in 6M HCl at 110 °C for 24 h and the hydrolysates were analyzed on a Merck-Hitachi La Chrome amino acid analyzer (D-7000 RP-HPLC System, Darmstadt, Germany).

Results

Morphometric Characteristics

The measured animal size and its weight were as illustrated in Table 1. It was observed that the tissue weight was proportional to the body size.

Proximate Composition

The proximate composition of *P. viridis* was estimated. The mean moisture, protein, carbohydrate and the lipid content was found to be 84%, 63.5%, 24.26% and 9.99% respectively. The protein content was high when compared to other biochemical components.

Table 1 Animal size and weight measurement

Animal size	Total length (in cm)	Total width (in cm)	Tissue weight (in g)
Minimum	4.7	2.8	1.6294
Medium	5.9	3.5	4.1552
Maximum	10	5	10.0264

Protein Estimation of Different Solvent Extract

The amount of protein content present in 1 mg of the sample was found to be dependent on the solvent used for the extraction. The protein concentration was found highest in the water sample (705.9 µg/ml), whereas the 100% methanol sample showed 672.7 µg/ml and 80% methanol as 548.1 µg/ml.

SDS-PAGE Analysis

The crude extracts of *P. viridis* were subjected to 10% SDS-PAGE analysis and the banding pattern was observed after electrophoresis (Fig. 1a). The banding pattern observed was nearly similar between water and 100% methanol extract when compared with standard, BSA.

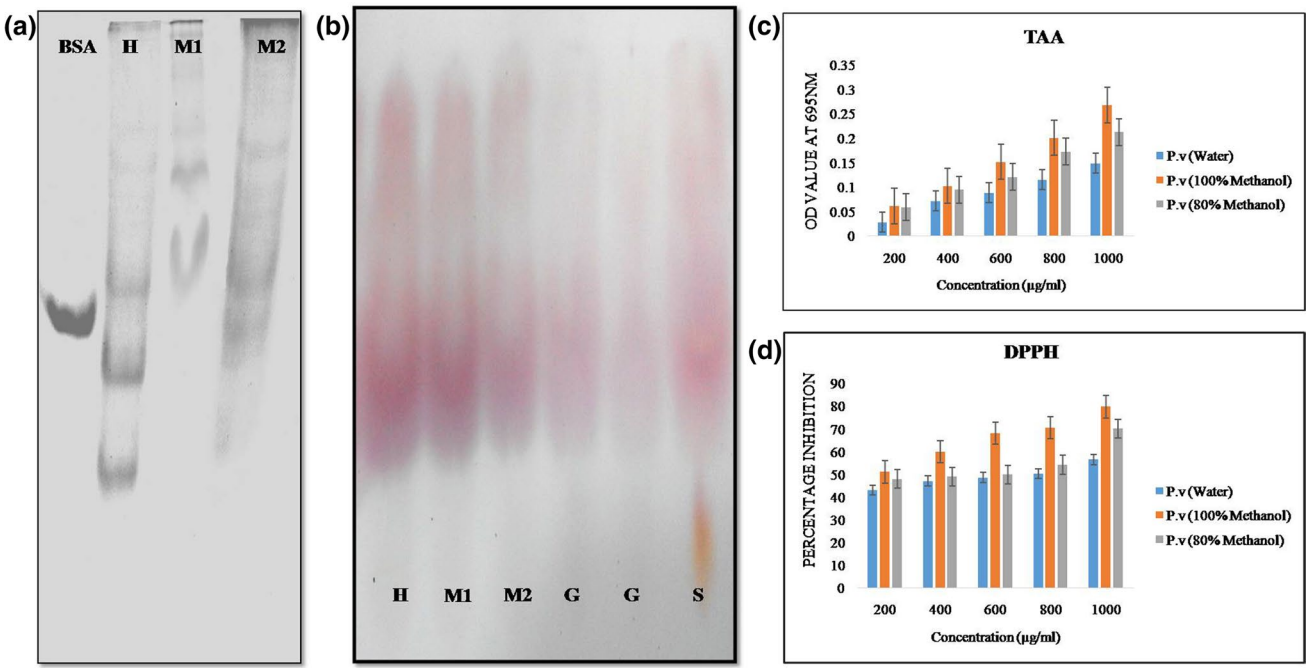

Fig. 1 **a** Represents the SDS-PAGE analysis of *Perna viridis* crude extracts on 10% gel and H denotes as *P. viridis* (Water), M1 as *P. viridis* (80% Methanol) and M2 as *P. viridis* (100% Methanol). Bovin serum albumin (66 kDa) was used as standard for comparison. **b** Shows the presences of free amino acid in *P. viridis* crude extracts. The figure was labeled *P. viridis* Water extract as H, 80% Methanol extract as M1, 100% Methanol as M2, G—Glycine (1 mg/ml) and S − 20 Amino acid standard mix. **c** Antioxidant activity of *P. viridis* crude extracts with different solvents such as water, 100% methanol and 80% methanol at different concentrations evaluated by Total Antioxidant activity. **d** Antioxidant activity of *P. viridis* crude extracts with different solvents such as water, 100% methanol and 80% methanol at different concentrations evaluated by DPPH scavenging activity. Results were expressed as means SD (n = 3) and all the measurements were done in triplicate

Free Amino Acid Analysis

The presence of free amino acids in crude extracts was determined using TLC and compared with glycine and standard amino acid mixture. A pink colored spot was visualized after the plate sprayed with ninhydrin reagent which indicated the presence of free amino acids (Fig. 1b).

Bioactivity of Different Solvent Extracts

Antioxidant Assays

The TAA potential of the samples was as shown in the Fig. 1c. It was found that the antioxidant activity was concentration-dependent. The maximum activity was found in 100% methanol extract and minimum activity in water extract. The DPPH radical scavenging activity of the samples at different concentrations was shown in the Fig. 1d. The results indicated that 100% methanol extract showed higher DPPH radical scavenging activity of 79.86% at 1 mg/ml. While, standard ascorbic acid exhibited 91.76% DPPH scavenging at 100 µg/ml concentration.

Antibacterial Assay

The different extracts of *P. viridis* exhibited significant antibacterial activity (Table 2). Among the three samples tested, 100% methanol extract showed highest activity against *P. vulgaris* (14.83 ± 0.208 mm) and minimum activity against *S. paratyphi B* (6.2 ± 0.26 mm). Subsequently, 80% methanol extract showed maximum activity against *P. vulgaris* (11.06 ± 0.115 mm) and minimum against *P. fluorescens* (5.96 ± 0.251 mm). However, it showed no significant inhibition against *S. paratyphi A, S. paratyphi B, E.coli* and *B. subtilis*, whereas the aqueous extract showed activity against only few pathogens with maximum in *Shigella* sp. (8.06 ± 0.115 mm) whereas minimum against *K. pneumonia* (6 ± 0.3 mm). All the three samples were ineffective on both *B. subtilis* and *E. coli*.

Gel Filtration Chromatography

The protein content of the collected 1 ml fractions were as shown in Fig. 2a. The results showed maximum protein concentration in fractions CF3 to CF14. The 29 fractions (CF1 to CF29) were further pooled into 14 fractions (CS1 to CS14) based on the protein content.

Biological Activity of Fractions

Antioxidant Assays

The purified fractions were evaluated for their antioxidant potential through TAA, DPPH, RPA and H_2O_2 scavenging assays. The fractions (CS1 to CS14) showed varied antioxidant potential. The TAA of CS4 fraction exhibited maximum activity in comparison with the other fractions [Fig. 2b(i)]. According to DPPH radical scavenging assay, the CS5 fraction showed the highest scavenging potential of 70% [Fig. 2b(ii)]. Further, the same CS5 fraction showed highest RPA potential when compared to other fractions [Fig. 2b(iii)] and maximum H_2O_2 scavenging potential as 75% [Fig. 2b(iv)].

Antibacterial Activity for Column Purified Samples

The fractions CS4 to CS7 were pooled as CS 47 based on their antioxidant potential and screened against three human pathogens such as *P. vulgaris, K. pneumonia* and *Shigella* sp. The CS 47 and positive control, Amoxycillin showed the maximum inhibition on *P. vulgaris* (16.66 ± 0.351; 15.93 ± 0.115 mm) followed by *Shigella sp*. (16.93 ± 0.208; 15.23 ± 0.251 mm) and *K. pneumonia* (13.13 ± 0.152; 13.13 ± 0.152 mm).

Table 2 Antibacterial activityof *P. viridis* crude extracts

Strain	Water (negative control)	Amoxycillin (1 mg/ml)	Water	100% methanol	80% methanol
S. aureus	NIL	6.26 ± 0.251	7.5 ± 0.458	8.23 ± 0.208	6.06 ± 0.057
K. pneumoniae	NIL	17.06 ± 1	6 ± 0.3	12 ± 0.3	10.23 ± 0.208
P. mirabilis	NIL	7.93 ± 0.115	0.166 ± 0.152	8.96 ± 0.05	6.4 ± 0.36
P. vulgaris	NIL	17.03 ± 0.251	7.1 ± 0.36	14.83 ± 0.208	11.06 ± 0.115
Shigella spp.	NIL	18.43 ± 0.55	8.06 ± 0.115	12.06 ± 0.305	8 ± 0.1
S. paratyphi A	NIL	9.93 ± 0.208	0.26 ± 0.251	8.26 ± 0.305	0.06 ± 0.115
S. paratyphi B	NIL	9.03 ± 0.351	0.06 ± 0.115	6.2 ± 0.26	0.233 ± 0.208
E. coli	NIL	8.96 ± 0.251	0.366 ± 0.115	0.26 ± 0.251	0.033 ± 0.057
B. subtilis	NIL	0.233 ± 0.208	0.06 ± 0.115	0.06 ± 0.115	0
P. fluorescens	NIL	6.86 ± 0.23	0.333 ± 0.305	8.2 ± 0.264	5.96 ± 0.251

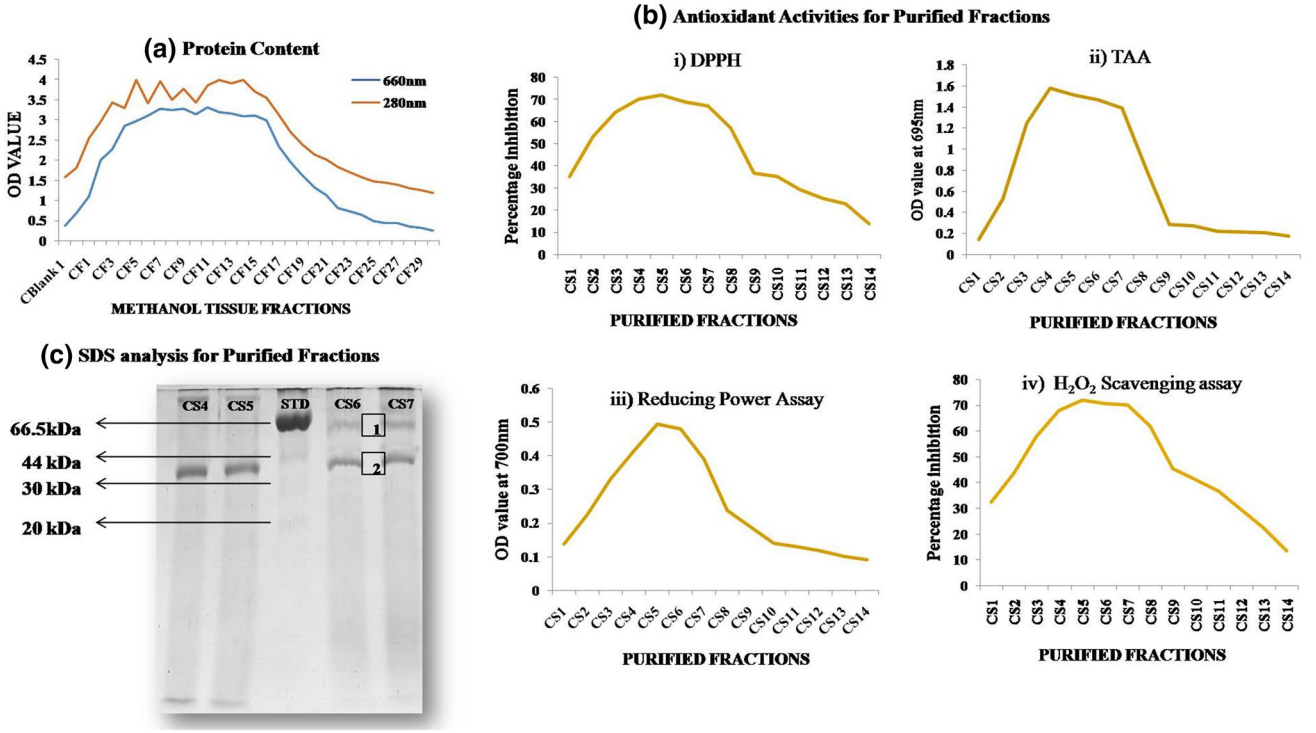

Fig. 2 **a** 100% Methanol extract of *P. viridis* fractions were read at 280 nm and 660 nm which showed the protein content in the collected fractions. **b** Antioxidant activity of methanol extract of *P. viridis* fraction was determined in different assay. (*i*) Antioxidant activity for the *P. viridis* (100% Methanol) pooled purified fractions was evaluated by DPPH assay. (*ii*) Antioxidant activity for the *P. viridis* (100% Methanol) pooled purified fractions was evaluated by TAA assay. (*iii*) Antioxidant activity for the *P. viridis* (100% Methanol) pooled purified fractions was evaluated by reducing power assay. (*iv*) Antioxidant activity for the *P. viridis* (100% Methanol) pooled purified fractions was evaluated by hydrogen peroxide assay. Results were expressed as means SD (n = 3) and all the measurements were done in triplicate. **c** 12% SDS page analysis for the active fractions of *P. viridis*. CS4, CS5, CS6, CS7 denotes the bioactive fractions and STD as Low molecular weight marker

Characterization of Purified Fractions

Molecular Weight Determination

The fractions CS4 and CS5 showed identical bands with a molecular weight less than 66.5 kDa. Similarly, CS6 and CS7 showed two bands among which one band was found to be less than 66.5 kDa as shown in the Fig. 2c.

RP-HPLC

Two distinct peaks for the collected fraction, CS47 were detected at a RT 5.86 and 12.189 min with an area peak of 2.101 and 97.459% respectively (Fig. 3a).

FTIR Spectral Analysis

The presence of functional group in the active fraction CS47 was analyzed in FTIR spectrometer. The IR spectrum of fractions was as shown in the Fig. 3b.

Amino acid Profiling

The free amino acids present in the CS47 fraction of *P. viridis* were analyzed in a HPLC C_{18} column (4.6 mm × 150 mm) at the flow rate of 1 ml/min. The protein sample and the standard amino acids were determined in terms of RT and peak area (%).The free amino acids were identified in the fraction CS47 in comparison with the standard amino acids (Fig. 3c).

Discussion

Morphometric Studies

The length and weight of the *P. viridis* was recorded in the range of 10–4.7 cm and 10.0264–1.6294 g respectively. Its width ranged from 5 to 2.8 cm. This result was in agreement with the findings of Sundaram et al. (2011), where they evaluated the length-weight relationship of *P. viridis* collected from the Versova creek, Mumbai. Among the 675 specimens collected by them, the length values between 14

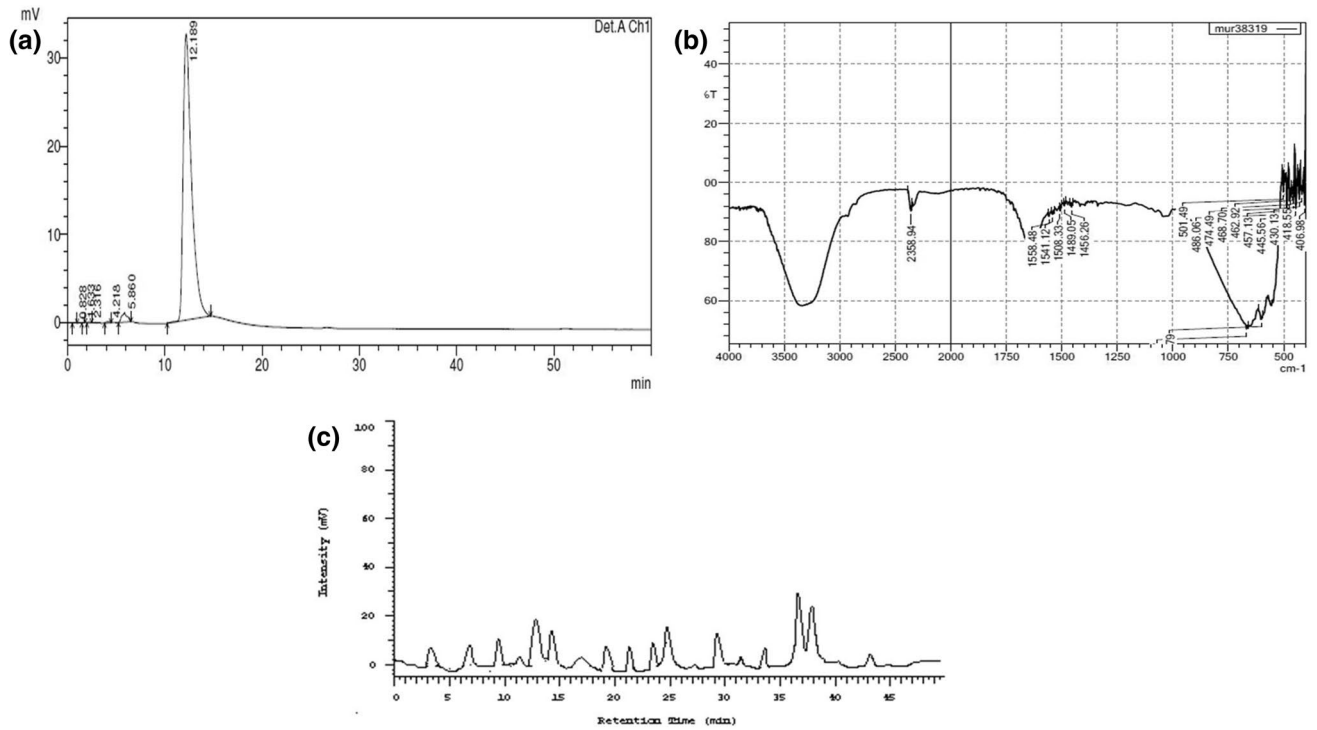

Fig. 3 **a** RP-HPLC analysis of active column fraction (CS47). **b** FTIR spectrum of active column fractions (CS47). **c** Amino acid profile of active column fractions (CS47)

and 89 mm and weight between 0.495 and 37.612 g were recorded during January, 2005–December, 2008.

Proximate Composition of *P. viridis* Tissue

The application of different solvent extraction method is fundamental in the exploration of potent marine biomolecules. Among the marine organisms, mussel tissues consist of high protein concentration with variable molecular masses (Grienke et al. 2014). Giese (1969) had reported that the protein is the dominant organic constituent in the mollusc. Likewise, the protein content in the *P. viridis* was found to be maximum (63.5%) when compared with the other biochemical parameters. These results connoted with the observations of Saritha et al. 2015, where the protein content ($36.15 \pm 1\%$) was higher than carbohydrates and lipids. Lower protein content ($20.3 \pm 1.56\%$) was reported in the *P. viridis* collected from Kadiyapattinam, Kanyakumari district, South West Coast of India (Immaculate et al. 2018).

Protein Estimation of Different Solvent Extract

In this study, the protein concentration in water extract was found to be higher (705.9 µg/ml) than the other methanolic extracts. Binsi et al. 2006 reported the total protein (g kg^{-1} meat) was 136.5 ($N \times 6.25$) in *P. viridis*. The authors also outlined that the higher moisture content could have led to the decrease in the protein concentration. Hence, as the moisture content was found to be 84% and protein as 63.5% in the present proximate analysis, it correlates with the results of the protein concentration of *P. viridis* solvent extracts.

SDS-PAGE Analysis of Solvent Extracts

The banding pattern of the water extract and the methanol extracts corresponded to the bands of standard BSA, which possessed a molecular weight of 66.5 kDa. Similar report was stated by Praveena and Fathima (2017), who mentioned that the crude protein of *P. viridis* had several bands with a molecular weight ranging between 14.5 kDa to 99 kDa, whereas the larger proteins were eliminated during post dialysis. Madhu et al. (2014) reported five distinct bands from the crude methanolic extract of *P. viridis* at a molecular range of 63 kDa to 29 kDa. The methanolic extract of *Cantharus tranquebaricus* correspond to an intensive band at 47 kDa (Sarumathi et al. 2012). Hence, it is indicative from the results that the methanolic extract of bivalve possesses proteins in the molecular range of 63 kDa.

Bioactivity of Different Solvent Extracts

Antioxidant Activity

Mussel inhabits dynamic marine environments and is exposed to variety of free radicals leading to oxidative stress. Thus, these organisms in their native environment tend to develop defense strategies such as generation of antioxidants and antioxidant enzymes. As these bivalves are great source for human consumption and bioactive compounds, antioxidant activity of the *P. viridis* extracts were evaluated. Among the three extracts, 100% methanol sample showed maximum DPPH radical scavenging activity of 79.86% and TAA value of 25 µg/mg equivalent of ascorbic acid. Likewise, this finding related with the results of Abirami et al. (2014), where the DPPH scavenging effect of *M. meretrix* was 74.52%, followed by *P. viridis* (73.64%) and *T. attenuate* (23.54%). In their TAA studies, 620 µg/mg of *B. spirata* and 23 µg/mg of *M. casta* extract in equivalents of ascorbic acid has been reported.

Antimicrobial Activity

The *P. viridis* tissue extracts exhibited significant activity against the tested human pathogens. Among the three extracts, 100% methanol sample showed maximum activity against *P. vulgaris*. However, 100% methanol and the other two extracts did not exhibit any significance against *E.coli* and *B. subtilis*. The D-amino acids and glycine was an important factor in inhibiting the growth of *E. coli* and *B. subtilis* as suggested in the studies of Hishinuma et al. (1969) and Leiman et al. (2013). Thus, the lower concentration or lack of these D-amino acids in the extract could have resulted in the insensitivity of the 100% methanol sample. Chandran et al. (2009) also reported that the methanolic extract of the *P. viridis* gill exhibited maximum bactericidal activity against *S. aureus* (19 mm) and minimum against *S. paratyphi* (11 mm). However, Madhu et al. (2014) screened the antibacterial activity of methanolic extract of *P. viridis* tissue and observed that the maximum inhibition was against *Vibrio cholera* (15 mm) and minimum against *K. pneumonia* (5 mm).

Purification Through Gel Filtration Chromatography

The 100% methanolic extract was purified using Sephacryl S-100 HR column chromatography, which has a molecular range of 1 kDa to 100 kDa. Since, the molecular range of protein in the *P. viridis* extracts analyzed through SDS-PAGE was around 66.5 kDa, this column was used for purification of the extracted proteins. Tachibana et al. (2005) and Cong et al. (2005) also applied similar purification strategy for the molluscan proteins evaluated in their studies.

Bioactivity of Purified Fractions

Antioxidant Effect

Among the 29 fractions collected after gel filtration chromatography, 14 fractions were derived based on proteins analysis. Further, these fractions were subjected to TAA, DPPH, RPA and H_2O_2 antioxidant assays. The CS4 fraction possessed higher TAA potential than the other fractions, whereas, CS5 exhibited maximum DPPH, RPA and H_2O_2 activities. Sreejamole and Radhakrishnan (2016) reported that *P. viridis* methanol extract showed 76.5% and 0.75 O.D DPPH radical and RPA potential. Madhu et al. (2014) reported that the methanolic extract of *P. viridis* tissue showed 76.9%, 88.12% and 62.5% inhibition in DPPH radical, Hydrogen peroxide and nitrous oxide scavenging assays, whereas it showed 27.8 and 174 µg equivalent to ascorbic acid standard in TAA and RPA assays respectively. Abirami et al. (2014) reported the methanolic extracts of gastropods and bivalves could be a potential source of natural antioxidant. The scavenging activity was higher in bivalves than the gastropods and echinoderms. Hence, this study was in agreement with the antioxidant potential of the bivalves as reported earlier.

Antibacterial Susceptibility

As the fractions CS 4–7 exhibited similar antioxidant potential, they were pooled as fraction CS47. The fraction CS47 was screened against 3 bacterial pathogens, where the maximum activity was found in *P. vulgaris*. Hence, it is evident from our results that the purified fraction exhibited higher antimicrobial efficacy than the crude samples. It is notable that the purification is an inevitable step in maximizing the specific bioactivities (Berg et al. 2002).

Characterization of *P. viridis* CS47 Fraction

Molecular Weight Determination

After column purification, the active fractions were subjected to 12% SDS-PAGE, where the CS4 to CS7 showed similar band patterning at 66.5 kDa. The purified CAMC- and COMC-Mefp-2 protein from the Blue Mussel *Mytilus edulis* L. also exhibited bands at molecular range of 48-54 kDa and 63-71 kDa, respectively (Rzepecki et al. 1992). Thus, such molecular range of proteins is predominantly found in Bivalves with diverse biological activities. Likewise, a 60 kDa protein, Bursatellanin-P from purple fluid of *Bursatella leachii* (Sea hare) was reported with Anti-HIV activity (Rajaganapathi and Kathiresan 2002).

RP-HPLC Analysis

For the fractions CS47, two distinct peaks were observed at the RT of 5.86 and 12.189 min. Hence, the bands observed in SDS-PAGE and peaks in RP-HPLC were in correlation. Further, the purified protein from *Tegillarca granosa* with a molecular weight of 20 kDa showed strong antitumor activities against various cell lines had a retention time of 10 min in HPLC column (Lv et al. 2015). Hence, the fraction CS47 observed within the similar RT value as *T. granosa* could be developed as a potent lead molecule.

FTIR Spectra

FTIR spectral analysis revealed the presence of functional groups in the *P. viridis* fractions. The band at 1654 $cm^{-1}$and 1647 cm^{-1}, which occurs at a similar wavelength in polyamides and proteins, is commonly assigned to stretching of the C=O group hydrogen bonded to N–H of the neighboring sheet chain. The acetyl amino group was represented by a band at 1022 cm^{-1}, indicates the asymmetric in-phase ring stretching mode and 656 cm^{-1} OH-Out-of plane bending (Focher et al. 1992). Similarly, the FT-IR spectrum of *P. viridis* crude protein revealed the presence of NH stretching at the wavenumber 3411 cm^{-1} and 1500 cm^{-1} indicated the bending of NH coupled with CN stretch. The asymmetrical stretching and skeletal stretch at 1654, 1647, 1022 and 656 – 453 cm^{-1} positions indicated the amide groups (Madhu et al. 2014).

Amino Acid Profiling Through HPLC Analysis

The amino acids are utilized to form various cell structures, which are key components and also serve as a source of energy. The presence of amino acid was analyzed by the amino acid analyzer and compared with the standards. The fraction was found to contain amino acids such as aspartic acid (178.3 µg/g), leucine (293.7 µg/g), isoleucine (274.5 µg/g), histidine (209.3 µg/g) and glutamic acid (205.7 µg/g) in high amount followed by methionine (193.2 µg/g) and asparagine (193.5 µg/g). Thus, concentration of leucine could have influenced the antibacterial activity reported in this study as it has been reported that leucine-rich sequences have a huge impact in the antimicrobial activity of peptides (Torres et al. 2017). However, alanine (23 µg/g) and tryptophan (18.9 µg/g) content were found to be low. Abirami et al. (2014) showed the same range of amino acid level in the *D. cuneateus* extract. Similarly, the other bivalve *Gafrarium tumidum* represented similar ratio of amino acid content. The present results also correlated with the report of Saritha et al. 2015, which showed methionine ($2.145 \pm 0.090\%$) as dominant amino acid and the alanine level was found low ($0.454 \pm 0.104\%$) in the extracts of *P. viridis*.

Conclusion

The present finding clearly depicted that the *P. viridis* purified fraction possessed strong antioxidant and antibacterial activity. Further, purification of the protein was found to be the significant step in distinguishing the bioactivities of crude and purified samples. Hence, it can be concluded that the purified protein from the *P. viridis* could be developed as a potential pharmaceutical agent. This study has further widened new horizons in the exploration of bioactive molecules from the green mussel.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Research Involving Animal and Human Rights This article does not contain any studies with human participants or animals performed by any of the authors.

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