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ORIGINAL RESEARCH

Is production of protease inhibitors from cyanobacteria nutrient dependent? Comparison of protease inhibitory activities in three species of *Oscillatoria* isolated from Central India

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Abstract Protease inhibitors are produced by a large number of cyanobacterial genera and their motive and mode of production are largely unknown. The present study showcases a comparative study of three isolates: DL02, DL08 and DL018 of *Oscillatoria* species collected from Central India, which possessed the same degree of protease inhibitory activities during isolation. These strains were grown under the same nutritional and environmental conditions in the laboratory for many generations, cultured biomasses were extracted, and screened for their inhibitory activity against three major proteases, i.e., trypsin, chymotrypsin and papain using an endpoint assay with casein and kinetic assay using synthetic chromogenic substrates. When cultured under the same conditions, three *Oscillatoria* strains showed different degrees of protease inhibition against all tested proteases in both the assay systems. *Oscillatoria* sp. DL08 showed highest inhibition (least IC_{50} value) against all the enzymes in both endpoint as well as kinetic assays while *Oscillatoria* sp. DL18 almost lost its protease inhibitory function against tested proteases. Further, papain inhibitory function of *Oscillatoria* sp. is shown for the first time. The study suggests that production of protease inhibitor(s) may not be entirely dependent on abiotic factors such as nutrient availability, light and temperature, but rather a more complex process having several biotic and abiotic factors working in tandem.

Keywords Cyanobacteria · Protease inhibitor · Trypsin · Chymotrypsin · Papain · *Oscillatoria* sp.

Introduction

The diverse physiologies of cyanobacteria serve as an excellent base for production of secondary metabolites, including toxins and enzyme inhibitors (Namikoshi and Rinehart 1996; Harada 2004; Gademann and Portmann 2008). The most pronounce enzyme inhibitors are the inhibitors of proteases. Most of the peptide and

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depsipeptides produced by cyanobacteria have the ability to act as protease inhibitors (Radau 2000; Welker and von Döhren 2006). Though looks simple, the consequences of protease inhibition may lead to various metabolic problems as proteases have a wide presence in the living world. Dysregulation and/or dysfunction of proteases may lead to conditions such as cardiovascular and inflammatory diseases, cancer, osteoporosis and neurological disorders (Turk 2006) apart from the regular digestion impairment in lower animals, i.e., crustaceans (Agrawal et al. 2005a).

Protease inhibitors have been isolated from a variety of freshwater cyanobacterial blooms and mats, i.e., *Microcystis* (Agrawal et al. 2001), *Lyngbya* (Matthew et al. 2007), *Nostoc* sp. (Bajpai et al. 2009) and *Oscillatoria* (Itou et al. 1999). Further, a wide range of proteases has been shown to be inhibited by crude extract or purified compounds from cyanobacteria including *Oscillatoria.* Alike other cyanobacteria, compounds from *Oscillatoria* have also shown to inhibit major serine protease family member enzymes, i.e., trypsin (Itou et al. 1999), chymotrypsin (Fujii et al. 2000), elastase (Shin et al. 1995) and plasmin (Shin et al. 1996).

Most of the earlier research has been oriented toward the presence of newer protease inhibitors in natural as well as cultured cyanobacteria. Scarce information is available related to those conditions that trigger the production of such a variety of compounds from cyanobacteria. A handful of studies show the role of nutrients, light, temperature (Rohrlack and Utkilen 2007; Halstvedt et al. 2008) and bacteria (Sivonen 1990) on production of bioactive compounds from cyanobacteria. Recently, Schwarzenberger et al. (2013)showed that the production of protease inhibitor from *Microcystis aeruginosa* BM 25 is dependent on nitrogen and phosphorus in the culture medium. However, no study compares the production of protease inhibitors from different cyanobacteria under similar culture conditions. The present study is an attempt to investigate whether under the same environmental conditions, different strains of *Oscillatoria* sp. produce the same degree of inhibition toward proteases. For the study, three species of freshwater *Oscillatoria* were collected from the Central Indian region with the ability to produce protease inhibitors. Under axenic conditions, these strains were maintained under similar environmental and nutritional conditions for many generations and the protease inhibitory properties of all three strains were compared against major proteases.

Methods

Chemicals

All the enzymes, i.e., trypsin (204844), chymotrypsin (034049) and papain (1648126) along with specialty chemicals, i.e., casein according to Hammerstein (034023) and DL-Dithiothreitol (044929) were purchased from SRL, Mumbai, India. Specific chromogenic substrates, i.e., *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BA*p*NA, B4875) and *N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide [S(Ala)$_2$ProPhe*p*NA, S7388] were purchased from Sigma-Aldrich, USA.

Isolation and culture of *Oscillatoria* sp.

For the present study, three benthic mats comprised mainly of strains of *Oscillatoria* were collected. *Oscillatoria* DL02 was isolated from the mat collected from a local pond near the laboratory. *Oscillatory* DL08 was isolated from mat from the Bhedaghat region while *Oscillatoria* DL18 was isolated from another local lake, Adhartal. The cyanobacteria were identified using identification keys (Desikachary 1959; Whitton and Potts 2000).

The isolations were done using light and dark plate method as described by (Hong et al. 2010). The isolated cultures were maintained in BG11 under white fluorescent lights (1,500 lux) with a light–dark cycle of 16:8 h at 28 ± 2 °C. To make axenic (bacteria-free) culture of cyanobacteria, cultures were constantly given treatment with antibiotics. The cultures were grown in BG11 media supplemented with cycloheximide (20 μg ml^{-1}) (Choi et al. 2008), ampicillin and penicillin G (100 μg ml^{-1}) (Ferris and Hirsch 1991). The cultures were allowed to grow for several generations and the sterility of the cultures was verified by inoculating 100 μl culture broth to 2 ml nutrient broth.

Before starting the experiments, the cultures were allowed to grow exponentially for 30 days and the filaments were harvested by centrifugation and freeze dried. Dried powder was stored at −20 °C until use.

Preparation of cyanobacterial extracts

For extraction, 100 mg dried powder of each *Oscillatoria* sp. was extracted thrice with 100 ml of 75 % methanol. All the three extracts were pooled, dried under vacuum and redissolved in 1 ml of 50 % methanol. This extract was used as a source of protease inhibitors from cyanobacteria (Agrawal et al. 2005a).

Protease inhibitors assays

Endpoint assays

The protease endpoint assays were performed according to (Agrawal 2004) using Hammerstein's casein as a substrate. Casein was dissolved in 100 mM Tris/HCl buffer, pH 8.5, at a concentration of 1 % (w/v). The enzymes (trypsin, papain and chymotrypsin) were prepared in 1 mg ml^{-1} concentration in 100 mM Tris/HCl buffer, pH 8.5, 100 mM Tris/HCl buffer, pH 8.8 and 100 mM phosphate buffer, pH 6.5 with 2 mM DTT for trypsin, chymotrypsin and papain, respectively.

For assay, 50 µl of each enzyme was pre-incubated with 50 µl of each cyanobacterial extract for 30 min at 37 °C. Controls for each enzyme were run that received 50 % methanol in lieu of any cyanobacterial extract. Appropriate blank was also run with all sets that received no enzyme. The reaction was started by the addition of 100 µl substrates (1 % casein) and terminated with 1.2 ml of 10 % tricholoro acetic acid exactly after 20 min. The reaction mixture was centrifuged for 10 min and the absorbance of the supernatant was measured at 280 nm. Release of tyrosine was measured using standard curve of tyrosine in 0.1 N HCl ($R^2 = 0.992$) at 280 nm. The enzyme activity is presented as nM of tyrosine liberated min^{-1} (mg protein)$^{-1}$. All the assays were carried out in triplicate and the data were analyzed by one-way ANOVA and post hoc comparisons (Tukey).

Kinetic assays

Kinetic enzyme assays were performed with specific chromogenic substrates (Agrawal 2004). Trypsin activity was assayed using the substrate BA*p*NA at a final concentration of 1.4 mM in 7.5 % (v/v) dimethylsulfoxide (DMSO). The reaction was carried out in 0.1 M Tris/HCl buffer, pH 8.5. The enzyme inhibitor solution was pre-incubated for 30 min at 37 °C before the addition of substrate to start the reaction. The hydrolysis of substrate was monitored continuously at 390 nm for 10 min. Release of *p*-nitroanilide (*p*NA) was measured using standard curve of *p*NA in DMSO ($R^2 = 1$) at 390 nm.

Papain assay was performed in the same way as trypsin but with 100 mM phosphate buffer, pH 6.5 supplemented with 2 mM DTT.

Chymotrypsin activity was assayed with [S$(Ala)_2$ProPhe*p*NA] at a final concentration of 0.95 mM in 7.5 % (v/v) DMSO. Specific activity was determined as nM *p*NA min^{-1} (mg protein)$^{-1}$. All the assays were performed in triplicate and the data were analyzed by one-way ANOVA and post hoc comparisons (Tukey).

For calculation of 50 % inhibitory activity by each cyanobacterial extract, the assays (both endpoint and kinetic) were performed with varying amount of cyanobacterial extracts that corresponded to 0, 1, 10, 100, 1,000 and 10,000 µg dry weight equivalent. The IC_{50} was calculated by fitting a sigmoidal dose–response curve using Sigma Graph Pad Prism® version 6.0.

Results

The aquatic mats from different regions of Central India were collected for the study. All the mats comprised <98 % of *Oscillatoria* species as seen under the microscope. These mats were freeze dried and extracted with 75 % ethanol. The extract was subjected to the in vitro estimation of enzyme activities of trypsin, papain and chymotrypsin. The extracts of all three cyanobacterial mats in a concentration of 5 mg equivalent were able to

decrease the activities of all three enzymes and in both the assay systems (Table 1). The enzyme activity was reduced significantly from control. However, no significant difference was found among the three cyanobacterial extracts, ensuring that the protease inhibitory potential was statistically similar in all three cyanobacteria. Five milligrams equivalent dried mass of all three extracts showed more than 90 % inhibition toward trypsin, papain and chymotrypsin. From each of the cyanobacterial masses, one strain of *Oscillatoria* was successfully isolated, cultured and maintained in an axenic state in the laboratory. The three *oscillators* strains, thus isolated were given the names as *O.* sp. DL02, *O.* sp. DL08 and *O.* sp. DL18 isolated from samples NI02, NI08 and NI18, respectively.

Protease inhibition during endpoint assays

When the cultured biomasses of all three *Oscillatoria* strains were extracted with 75 % aqueous methanol, the degree of inhibition by crude extracts of three different strains of *Oscillatoria* differed a lot against all tested enzymes in both the assays: endpoint and kinetic.

The activity of trypsin in endpoint assays was reduced by all three *Oscillatoria* extracts when used in 5 mg dry weight equivalent concentrations as compared to control which showed trypsin activity as 44.19 ± 2.64 μM tyrosine min^{-1} (mg protein)$^{-1}$. The trypsin activity with *Oscillatoria* sp. DL02 showed 49.1 % inhibition, while *Oscillatoria* sp. DL08 inhibited 79.1 % enzyme activity. *Oscillatoria* sp. DL18 was able to inhibit 41.4 % enzyme activity. All three *Oscillatoria* strains showed significantly different activity from the control (one-way ANOVA: $F_{5,12} = 319.1$, $p < 0.001$). The activity by *Oscillatoria* DL02 was significantly different from that of *Oscillatoria* DL08, but not significantly different from *Oscillatoria* DL18 (Tukey's HSD).

Similarly chymotrypsin activity was also reduced by the three extracts as compared to control that showed chymotrypsin activity as 76.91 ± 0.39 μM tyrosine min^{-1}(mg protein)$^{-1}$. Crude extract of *Oscillatoria* sp. DL02 inhibited chymotrypsin and showed reduced activity as 45.05 ± 1.15 μM tyrosine min^{-1}(mg protein)$^{-1}$ (41.4 % inhibition). The chymotrypsin activity by 5 mg dry weight equivalent of *Oscillatoria* sp. DL08 and *Oscillatoria* sp. DL18 were found to be 35.05 ± 0.39 (54.4 % inhibition) and 65.31 ± 0.61 μM tyrosine min^{-1} (mg protein)$^{-1}$ (15 % inhibition), respectively. The inhibitory activities shown by all three cyanobacterial strains were significantly different from control (one-way ANOVA: $F_{5,12} = 1{,}320$, $p < 0.001$). However, activity with *Oscillatoria* sp. DL08 was not significantly different from activity with *Oscillatoria* sp. DL02, but was significantly different from activity with *Oscillatoria* sp. DL18 (Tukey's HSD).

During endpoint assays with papain, a similar trend was observed when treated with 5 mg equivalent *Oscillatoria* extracts as compared with that of control which showed papain activity as 43.20 ± 3.09 μM tyrosine min^{-1} (mg protein)$^{-1}$. *Oscillatoria* sp. DL02 reduced the papain activity by 58.75 %, *Oscillatoria* sp. DL08 reduced it by 74.2 % and *Oscillatoria* sp. DL18 produced 32.3 % inhibition of papain activity (Table 1). The inhibitory activities shown by all three *Oscillatoria* sp. extracts were significantly different from each other as well as from control (one-way ANOVA: $F_{5,12} = 135.9$, $p < 0.001$).

The 50 % inhibitory concentration (IC_{50}) was evaluated using different concentrations of cyanobacterial extracts equivalent to 1 μg to 10 mg dry weight of respective strains. *Oscillatoria* sp. DL02 showed IC_{50} values of 50.17, 136.2 and 183.5 μg ml^{-1} for trypsin, chymotrypsin and papain during endpoint assays with casein. *Oscillatoria* sp. DL08 showed IC_{50} values of 25.38, 10.84 and 26.88 μg ml^{-1} while *Oscillatoria* sp. DL18 showed IC_{50} values of 238.7, 280.9 and 365.1 μg ml^{-1} for trypsin, chymotrypsin and papain, respectively (Fig. 1).

Protease inhibition during kinetic assays

During kinetic assays, trypsin activity was 242.6 ± 3.51 nM *p*NA min^{-1} (mg protein)$^{-1}$ with BA*p*NA alone. The 5 mg equivalent crude extract of *Oscillatoria* sp. DL02, *Oscillatoria* sp. DL08 and *Oscillatoria* sp. DL18 inhibited trypsin by 88.3, 92.4 and 29.6 %, respectively. The inhibitory activities shown by all three *Oscillatoria* strains extracts were significantly different from control activity (one-way ANOVA: $F_{5,12} = 6{,}829.0$, $p < 0.001$). No significant difference was found between the activities of *Oscillatoria* sp. DL02 and *Oscillatoria* sp. DL08 (Table 2).

Table 1 Enzymatic activity of trypsin, chymotrypsin and papain during endpoint assays as well as kinetic assays with or without to 5 mg dry weight equivalent cyanobacterial extracts in the reaction mixture

	Protease activity					
	Endpoint			Kinetic assay		
	Trypsin [μM tyrosine min^{-1} (mg protein)$^{-1}$]	Chymotrypsin	Papain	Trypsin [nM pNA min^{-1} (mg protein)$^{-1}$]	Chymotrypsin	Papain
Control	42.86 ± 2.8^{A}	79.91 ± 2.91^{A}	47.87 ± 1.91^{A}	249.33 ± 11.21^{A}	824.36 ± 15.61^{A}	330.24 ± 11.01^{A}
Sample NI02	04.21 ± 0.08^{B} (90.2 %)	12.05 ± 1.53^{B} (84.9 %)	05.42 ± 0.93^{B} (88.7 %)	16.18 ± 0.55^{B} (93.5 %)	25.93 ± 32.26^{B} (96.8 %)	26.06 ± 0.86^{B} (92.1 %)
Sample NI08	5.17 ± 0.47^{B} (87.9 %)	14.91 ± 1.78^{B} (81.3 %)	03.78 ± 1.08^{B} (92.1 %)	11.53 ± 1.32^{B} (99.4 %)	29.87 ± 3.25^{B} (96.4 %)	11.73 ± 1.16^{B} (96.4 %)
Sample NI18	6.03 ± 1.24^{B} (85.9 %)	15.48 ± 1.10^{B} (80.62 %)	05.25 ± 0.84^{B} (89.03 %)	8.13 ± 1.98^{B} (96.7 %)	27.73 ± 3.19^{B} (96.6 %)	31.1 ± 2.17^{B} (90.6 %)

The three cyanobacterial mats were with *Oscillatoria* sp. as predominant cyanobacteria. Endpoint assays were performed with casein as a substrate while kinetic assays were performed with BA*p*NA for trypsin and papain and [S(Ala)$_2$ProPhe*p*NA] for chymotrypsin. Enzyme activity was represented as amount of end product released per min per mg protein. Data presented are mean ± SD ($n = 3$). Figures in parentheses show percent reduction of enzyme activity as compared to control. Treatment marked with different letters was significantly different (one-way ANOVA, Tukey's HSD, $p < 0.05$)

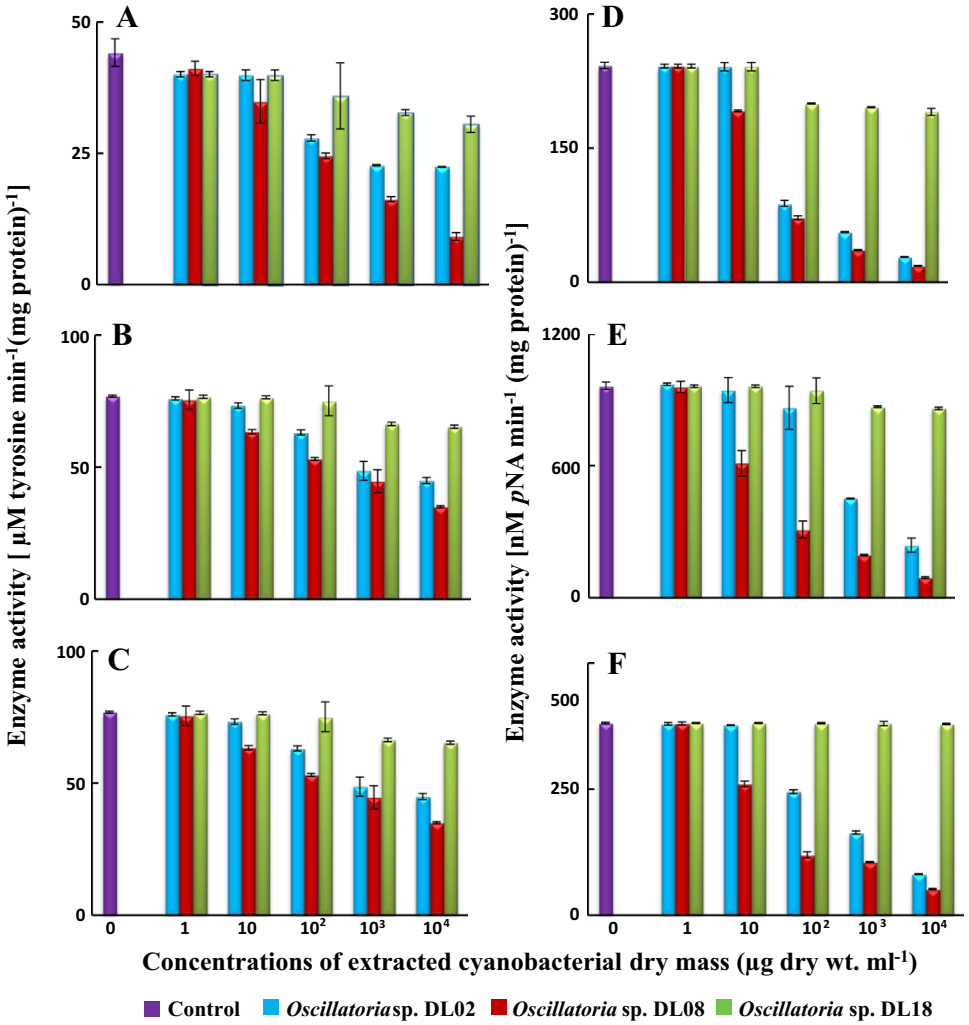

Fig. 1 Effect of extracts of three *Oscillatoria* strains on trypsin, chymotrypsin and papain during endpoint and kinetic assays. Enzyme activity is shown as mean ± SD ($n = 3$). Endpoint assays (**a**–**c**) measured enzyme activity as the amount of tyrosine released as µm tyrosine min^{-1} (mg protein)$^{-1}$ from casein. Kinetic assays (**d**–**f**) measured enzyme activity as nM pNA ml^{-1} (mg protein)$^{-1}$ from respective specific substrates. Amount of extract that caused 50 % enzyme activity inhibition (IC_{50} values) in terms of µg extracted dry mass ml^{-1} was calculated fitting a sigmoidal dose–response curve

The chymotrypsin activity with [$S(Ala)_2ProPhepNA$] alone (control) was 964.36 ± 13.28 nM *p*NA min^{-1} (mg protein)$^{-1}$. The chymotrypsin activity was inhibited by crude extract of three species of *Oscillatoria* though in varying degree. The chymotrypsin activity with 5 mg equivalent dry weight of *Oscillatoria* sp. DL02, *Oscillatoria* sp. DL08 and *Oscillatoria* sp. DL18 was 239.6 ± 32.26 (75.1 % inhibition), 91.87 ± 3.89 (90.4 % inhibition) and 861.98 ± 6.01 nM *p*NA min^{-1} (mg protein)$^{-1}$ (10.6 % inhibition). The inhibitory activities shown by all three *Oscillatoria* strain extracts were significantly different from each other as well as from control (one-way ANOVA: $F_{5,12} = 1{,}401.0$, $p < 0.001$).

The cysteine protease, papain, showed the control value with BA*p*NA alone as 381.23 ± 1.40 nM *p*NA min^{-1} (mg protein)$^{-1}$. *Oscillatoria* sp. DL02 has shown inhibitory activity by reducing papain activity to 81.73 ± 0.86 (78.5 % inhibition). *Oscillatoria* sp. DL08 and *Oscillatoria* sp. DL18 reduced papain activity to 51.73 ± 0.86 (86.4 % inhibition) and 379.4 ± 1.16 nM *p*NA min^{-1} (mg protein)$^{-1}$ (0.4 % inhibition), respectively (Table 1). The inhibitory activities shown by *Oscillatoria* sp. DL02 and *Oscillatoria* sp. DL08 extracts were significantly different from control (one-way ANOVA: $F_{5,12} = 46.37$, $p < 0.001$). Extracts of *Oscillatoria* sp. DL18 failed to inhibit papain during kinetic assays.

When the enzymes were treated with varying concentrations of cyanobacterial extracts, *Oscillatoria* sp. DL02 showed IC_{50} values as 61.74, 105.3 and 186.4 µg ml^{-1} for trypsin, chymotrypsin and papain,

Table 2 Enzymatic activity of trypsin, chymotrypsin and papain during endpoint as well as kinetic assays with or without 5 mg dry weight equivalent extracts from isolated species of *Oscillatoria* in the reaction mixture

	Protease activity					
	Endpoint assays			Kinetic assays		
	Trypsin [μM tyrosine min^{-1} (mg protein)$^{-1}$]	Chymotrypsin	Papain	Trypsin [nM *p*NA min^{-1} (mg protein)$^{-1}$]	Chymotrypsin	Papain
Control	44.19 ± 2.64^{A}	76.91 ± 0.39^{A}	43.20 ± 3.09^{A}	242.6 ± 3.51^{A}	964.36 ± 16.26^{A}	381.23 ± 1.40^{A}
Oscillatoria sp. DL02	22.49 ± 0.05^{B} (49.10 %)	45.05 ± 0.94^{B} (41.4 %)	17.82 ± 1.20^{B} (58.75 %)	28.2 ± 0.36^{B} (88.3 %)	239.6 ± 32.26^{B} (75.1 %)	81.73 ± 0.86^{B} (78.5 %)
Oscillatoria sp. DL08	9.20 ± 0.74^{C} (79.1 %)	35.05 ± 0.39^{B} (54.4 %)	11.11 ± 1.48^{C} (74.2 %)	18.2 ± 0.36^{B} (92.4 %)	91.87 ± 3.89^{C} (90.4 %)	51.73 ± 0.86^{C} (86.4 %)
Oscillatoria sp. DL18	25.89 ± 1.55^{B} (41.41 %)	65.31 ± 0.61^{C} (15 %)	29.22 ± 0.98^{D} (32.3 %)	170.75 ± 3.90^{C} (29.6 %)	861.98 ± 6.01^{D} (10.6 %)	379.4 ± 1.16^{A} (0.47 %)

Endpoint assays were performed with casein as a substrate while kinetic assays were performed with BA*p*NA for trypsin and papain and [S(Ala)$_2$ProPhe*p*NA] for chymotrypsin. Enzyme activity was represented as amount of end product released per min per mg protein. Data presented are mean ± SD ($n = 3$). Figures in parentheses show percent reduction of enzyme activity as compared to control. Treatment marked with different letters was significantly different (one-way ANOVA, Tukey's HSD, $p < 0.05$)

respectively. *Oscillatoria* sp. DL08 showed IC_{50} values as 29.49, 9.35 and 13.54 μg ml^{-1} and *Oscillatoria* sp. DL18 showed IC_{50} values of 133.5, 219.2 and 1,442 μg ml^{-1} for trypsin, chymotrypsin and papain, respectively (Fig. 1).

Discussion

Cyanobacterial protease inhibitors are of great interest (Agrawal et al. 2001; Czarnecki et al. 2006). These protease inhibitors have shown to cause inhibition of digestive proteases belonging to a variety of classes and hence can be related to subacute toxicity (Rohrlack et al. 2003, Agrawal et al. 2005a, b, Agrawal 2010).

The present study shows that while grown under similar conditions and nutrients in vitro for many generations, the three isolates of *Oscillatoria* differed in their protease-inhibiting capabilities despite of similar protease inhibitory potential at the time of harvest from their natural environment. Further, the enzyme inhibitory capabilities were different as suggested by differing IC_{50} values in each case of enzyme inhibition with trypsin, chymotrypsin and papain. The papain inhibitory activity by *Oscillatoria* strains is being reported for the first time. The simple reason may be because of less use of cysteine protease while studying the protease inhibitors from cyanobacteria.

Cyanobacteria are known to produce a variety of protease inhibitors with a predominance of peptides and depsipeptides (Weckesser et al. 1996) synthesized via non-ribosomal peptide synthetase and polyketide synthetase pathways (Welker and von Döhren 2006). Although, the conditions that trigger production of so many structural variants at a single point of time in any given cyanobacteria have not been clearly understood, few studies have shown that production of protease inhibitors from laboratory cultures is affected by light, temperature, nitrogen and phosphorus concentrations. The serine protease-inhibiting peptide (anabaenopeptilide) content of *Anabaena* sp. was affected by environmental factors such as phosphate and light (Repka et al. 2004). The highest peptide concentrations were found at light intensities of 23 μmol m^{-2} s^{-1} and phosphate concentrations greater than 100 μg L^{-1}. Rohrlack and Utkilen (2007) showed that the production of anabaenopeptins and microviridins, both protease inhibitors, was linearly correlated with growth rate of their producer organism, *Planktothrix*.

Considering the above studies, in theory, under similar environmental conditions, the same strain should produce a similar degree of enzyme inhibition. However, the case is not the same as shown in the present study. The three strains of *Oscillatoria* sp. showed significant differences in their capability of inhibiting the proteases, despite the fact that they all had shown a similar degree of inhibition at the time of isolation. The studies that investigated biotic factors and nutrient limitation on the production of protease inhibitors have used established laboratory cultures. Because of this, it is impossible to understand the role of nutrients and/or other abiotic factors. Further, no comparative study has been performed using various cyanobacterial strains belonging to one genus.

Earlier it has been shown that changes in nutrition led to dominance of other cyanobacteria in nature. Hambright (2001) showed that low phosphorus triggered the dominance of *Oscillatoria* sp. and *Closterium acutum* over *Aphanizomenon.* Although the role of protease inhibitors has not been investigated in such phenomenon, it is presumable that the production of protease inhibitors under nutrient-limiting conditions is dependent on the intra-generic and intraspecies stress at the given point of time. The data in the present study can be understood in the way, as the load from other biotic factors was reduced during in vitro culturing, the amount of protease inhibitors and their potency also changed accordingly. It is obvious that the three strains of *Oscillatoria* used in the present study may have different gene sets producing different sets of protease inhibitors, but their expression is largely a complex and rather less understood procedure which is dependent upon various factors that may/may not work in tandem.

Conclusion

The results show that abiotic factors, i.e., nutrients, light and temperatures are not the sole factors that trigger and/or regulate the production of protease inhibitor(s) from cyanobacteria. It is clear that other factors do play a major role for occurrence and concentration of cyanobacterial protease inhibitors at a particular time. More

than one factor can act simultaneously for production of protease inhibitors. Rohrlack and Utkilen (2007) discussed the possibility of mutual function of cyanobacterial oligopeptides. Though we agree with this theory, further research in this direction is highly solicited. Complex physiology of cyanobacteria with respect to production of bioactive metabolites, further complicate the understanding of pattern of occurrence of various protease inhibitors. Extensive research has to be done in this field, as there is a large list of biologically active secondary metabolites especially, protease inhibitors, emerging day by day (Welker and von Döhren 2006). It may be tempting to speculate that different protease inhibitors are produced because of different biotic and abiotic pressures a particulate cyanobacterium realizes at a given time and space.

Conflict of interest Authors have no competing interests.

Authors' contributions CP carried out enzyme inhibition studies as well as literature search. NR participated in experiment designs and manuscript writing. MA helped in isolation of cultures and experiments with axenic cultures along with CP. MKA prepared the manuscript, supervised the experiments, analyzed the data statistically and finally approved the manuscript.

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