Partial Purification and Characterization of an Elastase-inhibitory Lipid Derivative from a Cyanobacterium, *Microcystis* Ku2

Suvendra Nath Bagchi · Shubhro Kamal Ghosh

Received: 23 January 2009 / Accepted: 22 May 2009 / Published online: 26 January 2011
© Association of Microbiologists of India 2011

Abstract A unicyanobacterial isolate of cyanobacterium, identified as *Microcystis* Ku2, produced a mammalian elastase-inhibitory lipid derivative. Protease inhibitors in cyanobacteria are unequivocally peptides. Since this metabolite appeared in lipid phase, we worked on a hypothesis that whether metabolite other than peptides could be responsible for the characteristic inhibition. It was purified by saponification and reverse phase column chromatography. The resulting compound was tentatively characterized as a glycolipid with structure of sulfated dipentose derivative of ceramide (MW = 956 Da). The apparent IC₅₀ for elastase was 1.3 μM.

Keywords Elastase inhibitor · Mass spectrometry · *Microcystis* · Purification · Sulfated glycolipid

Introduction

Microcystis sp. produces a variety of linear- and depsipeptides (cyanopeptolins, aeruginosins, microviridins etc.) inhibiting mammalian serine proteases viz. trypsin, thrombin, chymotrypsin and elastase [1]. Usually, abundance of such metabolites in natural population is recorded by MS techniques, mainly the MALDI-TOF, and subsequently clonal cultures of *Microcystis* sp. are raised for further characterization of the specific protease inhibitor. Since isolated *Microcystis* sp. in lab cultures do not maintained any of the colonial morphological features that

they show in nature, PCR amplification of *Microcystis* genus-specific 16S rRNA gene becomes necessary to confirm their identify unequivocally [2].

Earlier, we have isolated a unicyanobacterial strain of *Microcystis* Ku1 from a local water body, and characterized a trypsin-inhibitory metabolite, microviridin [3]. Another strain, termed *Microcystis* Ku2, was found to strongly inhibit porcine pancreas elastase activity. In this communication, we have purified and characterized a novel elastase-inhibitory lipid derivative from cyanobacterium.

Materials and Methods

Several clonal strains of *Microcystis* were raised to unicyanobacterial state from a *M. aeruginosa*-dominant phytoplankton material collected in 1999 from a nearby Kundam lake [4], and were routinely grown in Parker's medium. The purity of the cultures was verified by using phase-contrast and bright field microscopy and by plating aliquots of the cultures on Luria–Bertani medium. One such strain, *Microcystis* Ku1, was characterized earlier [3]. Another strain, subsequently identified as *Microcystis* Ku2, was used in present investigation. Cells from ca. 5 l cultures were harvested by centrifugation (3,000×g) and were immediately lyophilized. *M. aeruginosa* PCC7806 was provided in freeze-dried form by Prof. J. Weckesser, Albert-Ludwigs Universität, Freiburg i. Br, Germany. *Synechococcus elongatus* PCC7942 was a lab culture grown in BG11 medium.

Isolation of genomic DNA (method II), PCR amplification using MIC primers—F: 5'-GCCGCRAGGTGAAA MCTAA-3' and R: 5'-AATCCAAARACCTTCCTCCC-3' [2], and MALDI-TOF MS of whole cells were carried out as given earlier [5].

S. N. Bagchi (✉) · S. K. Ghosh
Department of Biological Sciences, Rani Durgavati University,
Jabalpur 482001, India
e-mail: snbagchi_in@yahoo.com

The freeze-dried cell material (0.5 g dry weight) was extracted with three batches of 40 ml of methanol. The supernatant was then evaporated under vacuum, and the residue dissolved in 0.5 ml of 20% methanol. For inhibition tests, methanolic extracts corresponding to 0.2 mg dry wt of original cell mass or equivalent methanol were added to 1 ml of elastase (Type IV porcine pancreas; Sigma E0258) assay mixture [6].

Elastase-inhibitory metabolite from *Microcystis* Ku2 was purified by modification of Bagchi and Ray [7]. Methanolic extract (250 ml) corresponding to 5 g dry wt was refluxed for 6–8 h at 85°C with 2.5% of KOH and the condensed fraction was hydrolyzed with 15 ml of 0.1 M HCl at 45°C. The GF/C filtered above fraction and the left out methanol were tenfold diluted with de-ionized water and passed through LiChrolut RP-18 (ODS) cartridges (Merck, Germany; 500 mg sorbent). After 20% methanol wash, the bound material was eluted with 20 ml methanol, dried and the residues dissolved in 0.2 ml methanol. Elastase inhibitory metabolite so collected was purified on a HPLC (Shimadzu LC 10AT, Japan) using 250 × 4.6 mm column (Phenomenex Type Luna 5 µm C-18, USA) and methanol: acetonitrile: water (7:2:1 v/v) solvent system at a flow rate of 1 ml min⁻¹. Compounds were detected using a photodiode array detector (SPD-M10AVP, Shimadzu, Japan) at 220 nm. One ml fractions corresponding to the peak positions were collected, dried, weighed and re-dissolved in 1 ml of 50% methanol. Aliquots equivalent to 20 µg dry wt of the residue were assayed for their ability to inhibit elastase. IC₅₀ values were calculated from dose-dependent curve using Microsoft Excel[®]. The assays were carried out at least in triplicate and the values were averaged. TLC of the active fractions was carried out to identify the compound(s). The plates were developed with hexane, diethyl ether and acetic acid (8:2:0.1 v/v), and sugar-containing lipids were detected by 2.2% diphenylamine in acetone, aniline and phosphoric acid (9:0.1:0.1 v/v). ESI MS experiments were performed on a Quattro II TOFSPEC2E Micromass triple quadrupole mass spectrometer. Samples were dissolved in 1:1 (v/v) aqueous methanol. Electrospray samples were typically introduced into the mass analyzer through a syringe pump at a rate of 5 µl min⁻¹. The ESI capillary was set at 3.5 kV and the cone voltage was at 40 kV. ¹H NMR was carried out in CDCl₃ solvent using Bruker DRX-300.

Results and Discussion

A unicyanobacterial culture of putative *Microcystis* Ku2 consisted of single spherical cells (diameter 4.5 µm, with a fine gelatinous sheath) and a few doublets. The cells grew with a generation time of approx 56 h. For an authentication

of identity of the strain, amplification of *Microcystis*-specific 16S rRNA gene was carried out. *M. aeruginosa* PCC7806 and *S. elongatus* PCC7942 were used as positive and negative controls, respectively. The MIC-PCR reactions revealed amplification of ca. 240-bp fragment for both the strains (Fig. 1).

The *Microcystis* Ku2 cells in lab conditions consistently produced an elastase inhibitory metabolite. Absence of apparent bacterial contamination indicated the metabolite to be of cyanobacterial origin. The recognized elastase-inhibitory metabolites in cyanobacteria are invariably the peptides-cyanopeptolins (scyptolins, planktopeptins etc.) and microviridins with Leu and other hydrophobic moieties [8–10]. Since it is possible to recognize these peptides from their diagnostic peaks by MALDI-TOF MS [11, 12], a spectrum of the dried cells of *Microcystis* Ku2 was run. As shown in Fig. 2, signals of neither class of the peptides were detected, albeit prominent signals between *m/z* 655 and 720 did appear, which were identified as unknown lipids (M. Erhard, personal communication). Abundance of high lipid content was rather unique for this strain, and has not been observed in the other lab cultures. It was reasonable to assume that the putative elastase inhibitor is a lipid compound, rather than a peptide. The compound was soluble in methanol and other polar solvents, and during saponification it got resolved into the condensed fraction,

Fig. 1 PCR amplification of MIC 16S rRNA gene with DNA extracted from *M. aeruginosa* PCC7806 (1), *Microcystis* Ku2 (2) and *S. elongatus* PCC7942 (3). MW = 0.1–1 kb DNA ladder

Fig. 2 MALDI-TOF MS of *Microcystis* Ku2 dried cells, in which signal at m/z 871.5 denotes pheophytin and at 655–720 represent some unknown lipids

Fig. 3 RP-HPLC of 100% methanolic elute of ODS bound saponified material of *Microcystis* Ku2 extract. Arrow indicates the peak corresponding to elastase inhibitory fraction

typical of lipid derivatives. No inhibition was detected in the left out methanol phase. Further purification of the lipophilic extract by HPLC revealed that only the fraction eluted at 23rd min affected elastase at an estimated IC_{50} of $1.25 \mu g\ ml^{-1}$ (Figs. 3, 4). Re-chromatography of this fraction yielded single peak. Upon TLC the residue collected from this elute gave positive reaction for glycolipids as a single spot at apparent $R_f = 0.5$. From 0.5 g of dry cells *ca.* 38 mg of purified elastase inhibitor was recovered.

Upon 1H NMR, the spectrum of the purified sample (Fig. 5) was quite characteristic of fatty acid derivative, i.e. a triplet at δ 0.879, and two singlets at δ 1.28 and 1.64 were detected, which represent protons of $-CH_3$, and $-(CH_2)_n-$. The nature of other minor signals could not be elucidated. The spectrum did not match with any of the known cyanopeptides. Using ESI MS the molecular ion peak also appeared at m/z 956. The fragmentation pattern of this compound is shown in Fig. 6, which can be explained as follows (S. Antonopoulou, personal communication): the

Fig. 4 Effect of increasing concentration of HPLC-resolved fraction (*cf.* Fig. 3) on elastase activity

Fig. 5 Proton NMR of the elastase inhibitory fraction

fragment at m/z 467 corresponds to a di-pentose where three OH-groups have been substituted with two SO_3H - and one CH_3CO - groups. This conclusion is supported by the existence of m/z 255 corresponding to terminal sugar, which upon fragmentation gave rise to m/z 157 and 197 after the removal of SO_3H - and CH_3CO -groups, respectively. Removal of terminal sugar from the molecule gave rise to fragment at m/z 702, which corresponds to the second pentose with an SO_3H -group linked with a ceramide. The structure of the ceramide can not be predicted but assuming

Fig. 6 ESI MS of elastase inhibitory fraction with tentative structure of the metabolite, and suggested fragmentation pattern

the presence of the classical sphingoid chain, the fatty acid chain is expected to be $(\text{CH}_3\text{CH}_2)_5(\text{CH}=\text{CH})_3\text{CO}-$. The most probable structure of the compound is a sulfated di-pentose derivative of a ceramide (MW = 956 Da) (Fig. 6). Further, infrared spectrum of the sample was consistent with the proposed structure, particularly the signals at 1352–1342, 1165–1150 and 910–895 cm^{-1} , characteristic of asymmetric SO_2 stretch, symmetric SO_2 stretch and $\text{S} \rightarrow \text{O}$ stretch, respectively (data not shown).

To best of our knowledge this is first report of an elastase-inhibitory lipid derivative from cyanobacteria. Sulfate-containing glycolipids have been characterized as platelet aggregating factors [13]. Sulfated heparin and its analogues present in blood are strong neutrophil elastase inhibitors [14]. Possibly sulfated di-pentose also has the same function, and the ceramide moiety helps this molecule to bind to the membranes. At the moment, it is difficult to predict upon the function of this metabolite, except that it may serve as a grazing-deterrent substance. Molecules of such large size can not be excreted from the phytoplankton cells, but once ingested, are probably hydrolysed by some esterase action of enzyme cocktail of animal gut, and the released sulfated sugar might inactivate the proteases. Large Cladocera like *Moina* and *Daphnia* graze on planktonic cyanobacteria, including, *Microcystis*. Further, mammalian elastase-like activity has been shown in the body homogenate of *Moina macrocopa* [15]. Further work on anti-inflammatory activity of the metabolite would be desirable, especially because it is produced from a non-toxic strain (absence of microcystins, cf. Fig. 2).

Acknowledgments The authors thank the Head, Department of Biological Sciences, Rani Durgavati University, Jabalpur for the use of laboratory facilities, Dr. K.P. Madhusudan, SAIF, Central Drug Research Institute, Lucknow and Dr. M. Erhard, AnagnosTec GmbH,

Luckenwalde, Germany, for helping with ^1H NMR and MS analyses, and Department of Biotechnology, Govt. of India, New Delhi for financial assistance.

References

1. Welker M, von Döhren H (2006) Cyanobacterial peptides—nature's own combinatorial biosynthesis. *FEMS Microbiol Rev* 30:530–563
2. Ouellette AJA, Handy SM, Wilhelm SW (2006) Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molecular characterization of associated cyanobacterial communities. *Microb Ecol* 51:154–165
3. Ghosh SK, Bagchi D, Bagchi SN (2008) Proteolytic activity in *Microcystis aeruginosa* is inhibited by a trypsin-inhibitory cyanobacterial peptide with a partial structure of microviridin. *J Appl Phycol* 20:1045–1052
4. Agrawal MK, Bagchi D, Bagchi SN (2001) Acute inhibition of protease and suppression of growth in zooplankton, *Moina macrocopa*, by *Microcystis* blooms collected in Central India. *Hydrobiologia* 464:37–44
5. Ghosh SK, Das PK, Bagchi SN (2008) PCR-based detection of microcystin-containing cyanobacterial bloom from Central India. *Ind J Exp Biol* 46:66–70
6. Bieth J, Spiess B, Wermuth CG (1974) The synthesis and analytical use of a highly sensitive and convenient substrate of elastase. *Biochem Med* 11:350–357
7. Bagchi SN, Ray S (2001) Extraction and purification of and algicidal metabolite from a cyanobacterium, *Oscillatoria laetevirens*. *Ind J Microbiol* 41:163–167
8. Grach-Pogrebinsky O, Sedmak B, Carmeli S (2003) Protease inhibitors from a Slovenian lake Bled toxic waterbloom of the cyanobacterium *Planktothrix rubescens*. *Tetrahedron* 59:8329–8336
9. Matern U, Oberer L, Falchetto RA, Erhard M, König WA, Herdman M, Weckesser J (2001) Scyptolin A and B, cyclic depsipeptides from axenic cultures of *Scytonema hofmanni* PCC 7110. *Phytochemistry* 58:1087–1095
10. Rohrlack T, Christoffersen K, Hansen PE, Zhang W, Czaenecki O, Henning M, Fastner J, Erhard M, Neilan DA, Kaebnick M (2003) Isolation, characterization and quantitative analysis of

- microviridin J, a new *Microcystis* metabolite toxic to *Daphnia*. J Chem Ecol 29:1757–1770
11. Welker M, Maršálek B, Šejonhova L, von Döhren H (2006) Detection and identification of oligopeptides in *Microcystis* (cyanobacteria) colonies: toward an understanding of metabolic diversity. Peptides 27:2090–2103
 12. Czarnecki O, Henning M, Lippert I, Welker M (2006) Identification of peptide metabolites of *Microcystis* (cyanobacteria) that inhibit-trypsin like activity in planktonic herbivorous *Daphnia* (Cladocera). Environ Microbiol 8:77–87
 13. Antonopoulou S, Oikonomou A, Karantonis HC, Fragopoulou E, Pantazidou A (2002) Isolation and structural elucidation of biologically active phospholipids from *Scytonema julianum* (cyanobacteria). Biochem J 367:287–293
 14. Rode H, Koerbe S, Besch A, Methling K, Loose J, Otto HH (2006) Synthesis of pseudosaccharinamine derivatives as potential elastase inhibitors. Bioorg Med Chem 14:2789–2798
 15. Agrawal MK, Bagchi D, Bagchi SN (2005) Cysteine and serine protease-mediated proteolysis in body homogenate of a zooplankter, *Moina macrocopa*, is inhibited by a toxic cyanobacterium, *Microcystis aeruginosa* PCC7806. Comp Biochem Physiol B 141:33–41