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Toxic potential of five freshwater *Phormidium* species (Cyanoprokaryota)

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Abstract

Among the Cyanoprokaryota (blue-green algae), the genus *Phormidium* has thus far rarely been studied with respect to toxin production and potentially resulting human and environmental health effects. We here show that five previously unexplored freshwater species of this genus (*Ph. bijugatum*, *Ph. molle*, *Ph. papyraceum*, *Ph. uncinatum*, *Ph. autumnale*) are indeed capable of producing bioactive compounds. *Phormidium* extracts caused weight loss as well as neuro/hepatotoxic symptoms in mice, and in the case of *Ph. bijugatum* even death. Very low levels of saxitoxins and microcystins, as confirmed by ELISA, were insufficient to explain this toxicity and the differing toxic potencies of the *Phormidium* species. Qualitative HPLC analyses confirmed different substance patterns and in the future could aid in the separation of fractions for more detailed substance characterisation. The results in vivo were confirmed in vitro using cells of human, mouse and fish. The fish cells responded least sensitive but proved useful in studying the temperature dependence of the toxicity by the *Phormidium* samples. Further, the human cells were more sensitive than the mouse cells thus suggesting that the former may be a more appropriate choice for studying the impact of *Phormidium* to man. Among the human cells, two cancer cell lines were more responsive to one of the samples than a normal cell line, thereby indicating a potential anti-tumour activity. Thus, the five freshwater *Phormidium* species should be considered in environmental risk assessment but as well, as a source of therapeutic agents.

Keywords: Cyanoprokaryota; *Phormidium*; Toxins; Mouse bioassay; Fish and mammalian cell cultures; Cytotoxicity

1. Introduction

Cyanoprokaryota (blue-green algae) have been observed in aquatic environments around the world. Several strains of these microorganisms are known to produce a wide variety of toxins and/or biomedically interesting, bioactive compounds. The chemical structure of these compounds and their mechanisms of action in biological systems in vitro/in vivo have been intensively investigated during recent years. Most investigations in this field examined species belonging to the genera *Microcystis, Cylindrospermopsis, Anabaena, Oscillatoria (Planktothrix)* and *Aphanizomenon* (Lakshmana Rao et al., 2002; Haider et al., 2003).

Cyanotoxins cause direct intoxications of animals and humans through contact with bloom water or indirect

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poisoning due to consumption of contaminated food (Carmichael and Falconer, 1993; Carmichael, 1994, 1997; Jochimsen et al., 1998; Falconer, 1999; Ito et al., 2000). Well-known cyanotoxins can be divided, like algal toxins generally, into hepatotoxins (e.g. microcystins, nodularins, cylindrospermopsins), neurotoxins (e.g. anatoxin-a, homoanatoxin-a, saxitoxins) and dermatotoxins (e.g. lyngbyatoxins, aplysiatoxins). Hepatotoxins are inhibitors of serine/threonine specific protein phosphatases (PP1 and PP2A), neurotoxins block neurotransmission and dermatotoxins are the cause of skin irritations, allergic reactions and gastroenteritis. During the past years, the frequency and global distribution of toxic algal incidents appear to have increased, and human intoxications from novel algal sources have occurred. This has led to the revelation that numerous cyanoprokaryotic species not commonly investigated may be the source of potent toxins.

One genus of Cyanoprokaryota for which information on toxin production exists for only a few species is the genus *Phormidium*. In the marine environment, *Phormidium corallyticum* was identified as causing the black band disease of Atlantic reef corals (Mitsui et al., 1987). *Phormidium persicinum* was reported to produce unidentified compounds toxic to brine shrimps (Lincoln et al., 1991). The stereochemistry and structure of one marine *Phormidium* sp. metabolite highly toxic to brine shrimp was elucidated by Williamson et al. (2002) and termed phormidolide. According to Lilleheil et al. (1997), *Oscillatoria formosa*, or *Phormidium formosum* as classified by Anagnostidis and Komarek (1988), produces homoanatoxin-a. Likewise, Baker et al. (2001) observed lethal toxic effects to mice after intraperitoneal injection (400 mg kg^{-1}) of unfiltered extracts of *Ph. formosum* as well as of *Phormidium amoenum*.

Several studies have focussed on a more therapeutic viewpoint of biological activity of *Phormidium* species. Trichloracetic-acid-treated *Phormidium* extracts reduced croton oil-induced oedema in mice by about 60% in a dose-dependent manner (Garbacki et al., 2000). Glycolipids isolated from *Phormidium tenue* were found to inhibit enzymatic activity of HIV-1 reverse transcriptase to different extents (Reshef et al., 1997). Capsular polysaccharides isolated from different *Phormidium* strains showed anti-inflammatory properties. Several investigators presented data for anti-tumour or anti-plasmodial activity of compounds isolated from *Ph. tenue* and *Phormidium ectocarpi*. Two digalactosyl diacylglycerols isolated from the freshwater cyanobacterium *Ph. tenue* effectively inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin papilomas in ICR mice (Tokuda et al., 1986, 1996; Shirahashi et al., 1993). The methylene dichloride extract of *Ph. ectocarpi* showed anti-plasmodial activity towards *Plasmodium falciparum* (Papendorf et al., 1998). Williamson et al. (2002) reported a specific inhibitory activity of phormidolide to Ras-Raf protein–protein interaction, a critical component in the mitogen-activated signal transduction cascade in a number of cancer types.

The purpose of this study was to investigate whether five freshwater species of the genus *Phormidium* (*Ph. bijugatum, Ph. molle, Ph. papyraceum, Ph. uncinatum* and *Ph. autumnale*), unknown so far with regard to toxin production, are a source of intracellular and/or extracellular toxic compounds. Investigations were based both on analytical approaches as well as on in vivo/in vitro bioassays. These experiments revealed that the selected species indeed are toxigenic and produce identified as well as unidentified compounds with significant toxic activity.

2. Materials and methods

2.1. Phormidium cultures and preparation of extracts

Five different freshwater species of the genus *Phormidium* (Cyanoprokaryota) were studied:

- *Phormidium bijugatum* Kongiss. vel foleolarum (Mont.) Gomont—kept in PACC (Plovdiv Algal Culture Collection) as No 8602;
- *Phormidium molle* (Kutzing) Gomont—kept in PACC as No 8140;
- *Phormidium papyraceum* (Agardh) Gomont—kept in PACC as No 8600;
- *Phormidium uncinatum* (Agardh) Gomont—kept in PACC as No 8693;
- *Phormidium autumnale* (Agardh) Gomont—kept in PACC as No 5517.

Phormidium species were grown intensively under sterile conditions as described by Dilov et al. (1972) using a Z-nutrient medium (Staub, 1961). Cultures were synchronised by altering light/dark periods of 16/8 h. The temperature was 33 and 22 °C during the light and dark period, respectively. This culture regime was established in order to closely mimic the conditions for optimal growth of *Phormidium* in natural habitats in the summer months. The intensity of light during the light period was 224 μmol photon s^{-1} m^{-2} (Lux 12,000). The culture medium was aerated with 100 L of air per hour per 1 L of medium, adding 1% CO_2 during the light cycle. The period of cultivation was 14 days.

Extracts of the *Phormidium* species were obtained according to the method of Krishnamurthy et al. (1986) with slight modifications. Briefly, *Phormidium* species were removed from the Z-medium and weighed, then frozen and thawed, and extracted twice (3 h and overnight) with water–methanol–butanol solution (15:4:1, v:v:v, analytical grade) at 22 °C while stirring. The extracts were centrifuged at 10,000 rpm for 30 min. The supernatants of the two extracts were pooled and organic solvents removed via speed-vac centrifugation (SAVANT, Instruments, Inc. Farmingdate,