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ISOLATION OF PURE C-PHYCOCYANIN FROM *ARTHROSPIRA MAXIMA* AND *ARTHROSPIRA FUSIFORMIS* BY A MODIFIED NON-CHROMATOGRAPHIC RIVANOL – SULFATE PROCEDURE

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(*Submitted by Academician E. Golovinsky on April 20, 2007*)

Abstract

Recently, a modification of the non-chromatographic, rivanol-sulfate method was developed for purification of *Arthronema africanum* phycobiliproteins. Following this procedure C-phycocyanin from *Arthrospira maxima* and *Arthrospira fusiformis* was purified. The molecular homogeneity of the isolated C-phycocyanin was verified by SDS-PAGE that showed the presence of α and β subunits of the pigment only, by its absorption and fluorescence characteristics (maxima at 620 and 650 nm), respectively, as well as by A_{620}/A_{280} ratio of about 4.50. The overall recovery of *Arthrospira maxima* and *Arthrospira fusiformis* C-phycocyanin of 54% and 55% (w/w) from its content in the crude extract was the same as reported before for *Arthronema africanum*. The data obtained proved the wide applicability of this simple, rapid and inexpensive procedure for C-phycocyanin purification.

Key words: *Arthrospira maxima, Arthrospira fusiformis*, C-phycocyanin, method, purification

Introduction. The commercial significance of natural colouring compounds determines the great interest in microalgal phycobiliproteins. C-phycocyanin (C-PC), the major phycobiliprotein of photosynthetic systems of many cyanobacteria is blue coloured. When isolated and purified it is used as a natural dye in food and cosmetics, and as a fluorescent marker in biomedical research [1, 2]. Due to its antioxidant, anti-inflammatory, fibrinolytic, anticancer and free radical scavenging properties, C-PC is also a potential therapeutic agent [3–7]. The species of *Arthrospira* genus are a rich and inexpensive source of C-PC. Moreover, these prokaryotic cyanobacteria can be easily cultivated and harvested [8].

A number of procedures exist for purification of C-phycocyanin. Most of them involve multi-step unit operations including different chromatographic methods [5, 6, 9–11], which makes the scale-up of these methods difficult and expensive. A non-chromatographic, rivanol-sulfate method for C-PC purification was developed as an alternative [12].

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RESEARCH PAPER

Differential effects of methyl jasmonate on growth and division of etiolated zucchini cotyledons

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Keywords
Compensatory growth; cotyledon plate meristem; *Cucurbita pepo* (zucchini); cytokinins; hormonal interactions; senescence.

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ABSTRACT

The jasmonates are well studied in the context of plant defence but increasingly are also recognised as playing roles in development. In many systems, jasmonates antagonise the effects of cytokinins. The aim of the present work was to elucidate interactions between methyl jasmonate and cytokinin (benzyladenine) in regulating growth of zucchini (*Cucurbita pepo* L., cv. Cocozelle, var. Tripolis) cotyledons, taking advantage of the ability to simultaneously quantify cell enlargement and division from paradermal sections of the first palisade layer. Growth regulators were applied to cotyledons, excised from dry seeds and grown in darkness. Cytokinin stimulated expansion and division whereas, surprisingly, jasmonate stimulated expansion but inhibited division. Jasmonate antagonised the stimulating effect of cytokinin on division but worked cooperatively with cytokinin in increasing expansion. However, expansion with jasmonate was more isotropic than with cytokinin. Jasmonate also stimulated the loss of cellular inclusions and soluble protein. Soluble proteins revealed a partial antagonism between jasmonate and cytokinin. These results illustrate the complex interplay between jasmonates and cytokinin in the regulatory network of cotyledon development following germination.

INTRODUCTION

Following germination, cotyledons supply the seedling with nutrients that were stored during embryonic development. Cotyledons of the epigeal type, once exposed to light, expand and photosynthesise, providing an additional source of fixed carbon for seedling establishment. Because cotyledon expansion initially requires stored reserves that might otherwise be used for other seedling organs, it is possible that this expansion is subject to a complicated net of hormonal interactions, tuning growth to the environment. While cytokinin has long been known as the major, hormonal regulator of cotyledon development (reviewed in Mok & Mok 2001), there is evidence that jasmonic acid and its methyl ester may also be involved, possibly as antagonists to cytokinin (Ueda & Kato 1982; Ananieva & Ananiev 1999; Mukherjee *et al.* 2002). Jasmonates are primarily known for their roles in response to herbivory, which have been well studied; however, it is clear that they also play a role in development (Wasternack & Hause 2002). In terms of growth and division, exogenously applied jasmonate can be either inhibitory (Ueda & Kato 1982; Swiatek *et al.* 2002, 2003) or stimulatory (Kondo *et al.* 2002; Cenzano *et al.* 2003; Capitani *et al.* 2005). Jasmonates are present in relative abundance at the shoot apex (Sembdner & Klose 1985), young leaves and developing fruits (Fan *et al.* 1997; Kondo *et al.* 2002), which led to the proposal that they participate in development of the meristem. However, effects of exogenous jasmonate on cell division parameters during shoot meristem development could not always be detected (*e.g.* Cenzano *et al.* 2003). The nature of the control that jasmonates exert on growth and morphogenesis remains to be elucidated.

The etiolated epigeal cotyledon is a useful system for studying the control of expansion and division because vigorous growth occurs in the excised condition and in the dark, removing complications from interactions with the rest of the seedling, photosynthesis or photomorphogenesis. Etiolated cotyledons are also useful because light

CANCER PROTECTIVE ACTION OF POLYSACCHARIDE, DERIVED FROM RED MICROALGA *PORPHYRIDIUM CRUENTUM* – A BIOLOGICAL BACKGROUND

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ABSTRACT

The cell wall sulfated polysaccharide of the red microalga Porphyridium cruentum (Rhodophyta) (PcrPSH) exhibited strong antitumor activity against Graffi myeloid tumor in hamsters both in vitro and in vivo. When tested in vivo, depending on the concentration and the way of application, this polysaccharide decreased transplantability in all experimental groups till 20 days of observation and mortality rate. The tumor growth was retarded and the mean survival time was prolonged with 10 - 16 days. Applied in in vitro experiments the PcrPSH increased both - spreading and phagocytic ability of peritoneal macrophages from healthy and Graffi tumor bearing hamsters in a dose - dependent manner. Primary Graffi tumor cell culture, cultivated in the presence of PcrPSH showed significant decrease of cell viability, determined by MTT test, while in cells derived from bone marrow it was increased at the same conditions of cultivation and concentration of polysaccharide. Primary Graffi tumor cell culture, treated with PcrPSH showed the appearance of a characteristic DNA ladder on agarose gel electrophoresis, which is a biochemical hallmark of apoptosis. The manifested anticancer activity of PcrPSH could be associated with its immunostimulating action as well as with direct cytotoxic properties. Based on these results, we could suggest that the tested algal PcrPSH is a promising candidate as an antitumor agent. Further studies will be done to clarify the mechanisms of a biological action of PcrPSH.

Keywords: apoptosis, *Graffi* myeloid tumor, macrophages, *Porphyridium cruentum*, red microalga

Introduction

Marine organisms represent a valuable source of new compounds. The marine algae traditionally have been used as a health food and as a source of therapeutic agents with immunomodulating and anticancer effects.

The polysaccharides and peptides isolated from seaweeds have become a matter of great interest for cancer therapy. The mechanisms of their anticancer activity are related to their ability to suppress the growth of cancer cells (cytotoxic or cytostatic effects), to enhance the immune responses and to inhibit tumor angiogenesis (1-3). Several marine algal polysaccharides have been found to induce apoptosis in cancer cells (4-8).

Some interesting studies focus on the investigation of sulfated polysaccharides extracted from red microalgae which inhibit viral infection and/or replication and showed potent antiviral activity against a variety of animal viruses (9-15). Fabregas et al (16) established that *Porphyridium cruentum* polysaccharide displays *in vitro* inhibition of the replication of haemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV). Recent studies demonstrated that the polysaccharide from marine Porphyridium sp. showed a moderate mitogen activity, similar to dextran sulfate but lower than concanavalin A and lipopolysaccharide and have antitumor activity against Sarcoma 180 inoculated in the peritoneal cavity of mice (17).

The aim of the present study was to provide information about the stimulating and antitumor properties of a polysaccharide isolated from *Porphyridium cruentum* (PcrPSH) against *Graffi* myeloid tumor in hamsters.

Materials and methods

Algal strain and polysaccharide isolation. The red alga *Porphyridium cruentum* (AG.) NAG strain Vischer 1935/107, obtained from CCALA (Trebon Collection of Autotrophic

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The biliprotein C-phycocyanin modulates the early radiation response: A pilot study

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ABSTRACT

This study analyzed the effects of biliprotein C-phycocyanin (C-PC) on the enzymatic antioxidant defence system in lymphocytes of nuclear power-plant workers and non-exposed controls. Changes in the protein levels of manganese super oxide dismutase (MnSOD), catalase and glutathione-*S*-transferase (GST) were used as markers for early biological effects of a single *in vitro* exposure of cells to: (i) 2 Gy gamma rays; (ii) 5 μM C-PC; and (iii) a combination of C-PC plus irradiation with 2 Gy. The results showed that C-PC selectively stimulated the lymphocyte antioxidant defence system of occupationally exposed subjects. The activation of the antioxidant protective mechanisms as part of the early radiation response was probably related to the chronic low-dose occupational exposure. The modulating capacity of C-PC at the molecular level may be of interest for the protection of occupationally exposed persons.

1. Introduction

Free radicals and non-radical oxidants, collectively named reactive oxygen species (ROS) can be generated in a biological system by a wide range of endogenous and exogenous processes such as irradiation. Oxidant generation is determined by the balance between the rates of production and clearance of ROS, and is under the control of several radical-producing and radical-quenching pathways. Antioxidants are defined as substances that are able, at relatively low concentrations, to compete with other oxidizable substrates and thus to significantly delay or inhibit the oxidation of these substrates [1]. This definition mainly includes the enzymes super oxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, as well as the non-enzymatic compounds α-tocopherol, β-carotene, ascorbate and glutathione.

ROS generated immediately after irradiation mediate the radiation effects on biological molecules. Therefore, it is reasonable to expect the involvement of the antioxidant defence systems of the cells in the early response to irradiation. Indeed, there are reports on a large increase in manganese SOD (MnSOD) activity in mouse heart [2] and in SOD and catalase activities in the liver of radiation-resistant but not in radiation-sensitive mice, minutes after X-ray exposure [3]. Experimental results suggested that SOD and catalase were involved in the early radiation response and in radioresistance. Association of the inherited radiosensitivity with altered antioxidant activities resulting in more prolonged oxidative stress after radiation exposure has also been demonstrated [4]. In contrast to the increased enzyme activity, no significant changes of the transcriptional levels of these enzymes in the early phase after irradiation have been found. Therefore, it was suggested that the increased antioxidant activity reflected the radiation-induced posttranscriptional or conformational alterations of the enzyme molecules after irradiation [5].

Radio-adaptive response (RAR) has been defined as an enhanced resistance of certain cells exposed to low-dose ionizing radiation to the toxic effects of subsequent irradiation [6]. Data suggested the involvement of the main antioxidant enzymes in RAR. Their contribution has been analyzed by examination of the changes of the antioxidant activities of human lymphoblastoid AHH-1 cells [7]. Soon after irradiation, higher activities of MnSOD, GST, GPx and catalase were registered in the adapted versus non-adapted cells, leading to the conclusion that the increased enzymatic activ-

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The Biliprotein C-Phycocyanin Modulates the DNA Damage Response in Lymphocytes from Nuclear Power Plant Workers

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1. Introduction

The biliprotein C-phycocyanin (C-PC) is a light-harvesting photoreceptor in cyanobacteria and in red algae (Rhodophyta and Cryptophyta) with applications as a natural colorant in nutritional industry and cosmetics (Prasanna et al., 2007) and as a fluorescent marker in medical and biological studies (Glazer, 1994; Sun et al., 2003). The protein is composed of two homologous subunits - α and β (Stec et al., 1999; Contreras-Martel et al., 2007), respectively with one and two phycocyanobilin chromophores, covalently attached to cysteine residues. The subunits form αβ complexes which aggregate into α3β3 trimers and α6β6 hexamers, the latter being the functional unit of the protein. C-PC has been shown to display a variety of pharmacological activities, related to the antioxidant, anti-inflammatory, neuro- and hepato-protective, anti-tumour and wound-healing mechanisms (Romay et al., 2003; Ge et al., 2006; Li et al., 2005; Madhyastha et al., 2008). These properties have attracted attention to the compound as a possible radio-protective agent. It has been demonstrated that rats exposed to 5 Gy of X-rays and fed phycocyanin normalized their antioxidant system within 4 weeks after exposure (Karpov et al., 2000).

Recently, we studied the effects of C-PC in combination with ionizing radiation on lymphocytes, isolated from nuclear power plant workers, exposed to low doses of ionizing radiation (IR), and compared them with the effects on lymphocytes from nonexposed controls (Ivanova et al., 2010). We found that the biliprotein stimulated the expression of the antioxidant enzymes manganese superoxide dismutase (MnSOD), catalase and glutathione-S-transferase (GST) during the early radiation response of lymphocytes from workers, but not from controls. Since the biliprotein positively affects the antioxidant defense pathways, it might be of interest for the radioprotection of occupationally exposed people.

In this study we have further characterized the effects of C-PC on the early radiation response of lymphocytes from unexposed controls and from workers, exposed to low doses of radiation. We quantified the level of persisting radiation-induced DNA double-strand breaks (DSBs) in the presence and absence of C-PC. DSBs are the most dangerous type of DNA lesions, induced by several genotoxic agents, including gamma IR (γ-IR). The ability of cells to readily process DSBs is of vital importance for genomic integrity, as failure to repair these lesions results in

chromosomal breakage, fragmentation and translocation. Moreover, impaired or defective rejoining of radiation-induced DNA strand breaks usually correlates strongly with the individual susceptibility to cancer (Alapetite et al., 1999; Berwick & Vineis, 2000).
The amount of persisting DSBs in cells was determined by the comet assay (CA), a quick, simple and reliable method for analyzing DNA damage and repair that requires a small number of cells and can be performed on both freshly isolated and cryopreserved cells (Decordier et al., 2010). Due to its sensitivity, the method is preferred in human epidemiological studies related to biomonitoring (Möller et al., 2000; Touil et al., 2002). Additionally, the CA is able to provide information on different types of DNA damage/repair and detect cellular damage in a wide dose range of exposures from 0.05 to 10 Gy (Kalthur et al., 2008; Mohseni-Meybodi et al., 2009; Palyvoda et al., 2003). The experiments were performed on human lymphocytes, which, due to their radiosensitivity and circulation throughout the body, reflect the overall state of the organism and are the cellular type most frequently used for assessment of the systematic radiation response (Collins et al., 2008; Decordier et. al., 2010). A major problem with CA is that its sensitivity often leads to detection of a high variation within a single individual. A reliable methodology should be able to detect differences between individuals, but should show a minimal intra-individual variation. Therefore, prior to the epidemiological experiment, in an attempt to achieve minimal intra-individual variation and a linear dose-response curve, we carefully tested a number of conditions. We attained a stable linear dose-response dependence of DNA lesions, persisting 2h after exposure in the dose range from 0.5 to 8 Gy gamma rays.
Our data indicated that C-PC might stimulate the repair of radiation-induced DNA lesions in lymphocytes from both occupationally exposed subjects and non-exposed controls. Moreover, the biliprotein seems to limit the manifestations of high radiosensitivity. Interestingly, we registered a pronounced lower genotoxicity of C-PC in lymphocytes from workers with cumulative doses higher than 20 mSv. Additionally, the effects of C-PC were age-dependent.

2. Experimental procedures

2.1 Subjects and sampling

The exposed group consisted of 44 workers aged between 26 and 62 years, employed at the "Kozloduy" Nuclear Power Plant (NPP), Bulgaria. Cumulative exposure to ionizing radiation (IR), estimated from personal dosimeter records, ranged from 0.32 to 330.77 mSv and represented the sum of the doses collected for the whole period of occupation in the "strictly controlled area". The control group included 12 non-exposed subjects from the NPP administrative staff, aged between 42 and 58 years. In order to exclude external effect on the results of this study, we recorded information on the smoking habits, alcohol consumption, use of medications and previous diagnostic exposure to X-rays. The studied groups were homogenous on the aforementioned criteria and the statistical analysis found no significant effects due to any factor. The study was performed under the National Program "Genomics" of the Ministry of Health and Ministry of Education, Youth and Science of Bulgaria. Informed consent was obtained from all participants.
Blood (2 ml) drawn by venipuncture and collected in EDTA-coated tubes (Vakutainer, Benton Dickinson, Oxford, UK) was delivered to the laboratory and stored at 4^{0}C for up to 24h before processing. The samples from the control and exposed subjects were handled concurrently and the assays were run on coded samples.

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RESEARCH PAPER

Differences in cytokinin control on cellular dynamics of zucchini cotyledons cultivated in two experimental systems

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Keywords
Cell division; *Cucurbita*; cotyledon age; cytokinins; expansion; growth; skotomorphogenesis.

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ABSTRACT

The effect of endogenous cytokinins on the pattern of palisade cell division post-germination does not depend on the conditions of cotyledon development – *in planta* (attached to seedlings) or *in vitro* (isolated from dry zucchini seeds and cultured on water). In cotyledons originating from 4-day-old seedlings (experimental system 1), exogenous cytokinin temporarily (in the first 2 day of cultivation) enhanced post-mitotic cell enlargement of palisade cells, mainly due to enhanced water uptake and use of cell storage compounds, all of which lead to cotyledon senescence. Cytokinin is not able to resume the completed palisade cell division on day 5. As a result, the number of cells and the final areas of treated and control cotyledons are quite similar. By contrast, the effects of cytokinin on cotyledons isolated from dry seeds (experimental system 2) are better expressed, promoting an increase in number of palisade cells accompanied by additional cotyledon area enlargement. However, the prolonged post-mitotic cell expansion in control cotyledons compensates for the reduced speed of cell growth and division activity and decreases differences in final cotyledon area between treatments. The results define cell division as the primary target of cytokinin stimulation in cotyledon tissues competent for division, and determine the temporal patterns of palisade cell cycling related to cotyledon age. This knowledge permits a better choice of experimental system to study effects on cell proliferation and cell growth, as well as cell enlargement and senescence-related events using physiologically homogeneous material.

INTRODUCTION

The impact of different effectors on metabolic reactions leading to growth is often studied using cultivated epigeal cotyledons. Generally, there are two main and differing experimental systems used for physiological studies based on epigeal cotyledon growth. The systems differ mainly in the source and age of cultured cotyledons. Usually, cotyledons are removed from 1- to 7-day-old seedlings (*e.g.* Naito *et al.* 1980; Haru *et al.* 1982; Chen & Leisner 1985; Parthier *et al.* 1985; Stoynova-Bakalova *et al.* 2001). To study the effects of exogenous treatments, release of endogenous hormones is usually practiced by floating detached cotyledons in water before treatment. The other experimental system is based on cotyledons directly isolated from the embryonic axes of seeds, where earlier growth and treatment can be studied (Stoynova-Bakalova *et al.* 2002, 2008; Stoynova-Bakalova & Petrov 2006, 2009; Stoynova-Bakalova 2007).

Some authors have noted that cotyledon age and the age of seedlings from which the cotyledons were isolated could influence the magnitude of biochemical results obtained (*e.g.* Naito *et al.* 1980; Haru *et al.* 1982). The basis of these influences has usually been left without discussion, perhaps because data on cellular organisation of epigeal cotyledon growth are very controversial. In particular, participation of cell division in cotyledon development has long been neglected and practically denied. According to Lovell & Moore (1970), the post-embryonic growth of epigeal cotyledons in 11 plant species (including *Cucurbita*) is based only on cell expansion, with the exception of cucumber cotyledons, whose growth has been found to include cell division. Even when cell division is recognised, its temporal and spatial pattern and relationship with cell growth have remained controversial. For *Cucurbita* cotyledons, Nelson (1932) found cell division took place in all tissues (cotyledons grown *in planta*), with cell enlargement beginning after the end of proliferation; Fofanova & Khokhlova (1983) considered cell division as the only process in cellular dynamics responsible for early post-embryonic growth of palisade tissue and the upper epidermis; and Stoynova-Bakalova (2007) demonstrated simultaneous cell division and growth in palisade tissue and both epidermises of cultured *Cucurbita* and *Cucumis* cotyledons isolated from dry seeds. This latter meristem activity and its cytokinin stimulation are restricted to the first 2–5 post-embryonic days of cotyledon development (Stoynova-Bakalova 2007). However, some biochemical studies have shown cytokinin-provoked cell division in relatively older cotyledons isolated from 5-day-old seedlings and treated during subsequent cultivation (Tsui *et al.* 1980; Li & Ma 2007).

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Antitumor activity of B-phycoerythrin from *Porphyridium cruentum*

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ABSTRACT

The *in vitro* effect of highly purified B-phycoerythrin from *Porphyridium cruentum* on the proliferation of Graffi myeloid tumor cells was studied. The MTT assay showed a dose-dependent inhibition of tumor cell proliferation. About 50 and 63% cell growth suppression was recorded using 50 and 100 µg/ml B-phycoerythrin, respectively. In contrast, the pigment stimulated the proliferative response of normal hamster bone marrow cells (113 ± 4.34 and 154 ± 8.6% for 50 and 100 µg/ml, respectively). Fluorescence microscopy of acridine orange and propidium iodide - double stained tumor cells revealed characteristic apoptotic features like nuclear shrinkage, condensed chromatin and membrane blebbing, as well as formation of apoptotic bodies and DNA fragmentation in B-phycoerythrin-treated cells. A concomitant dose-dependent increase in the activities of glutathione reductase and especially of superoxide dismutase (both Mn SOD and Cu/Zn SOD) in Graffi cells in response to the treatment was observed. These results suggest that apoptosis induced by B-phycoerythrin in tumor cells might be at least partly the reason for its antitumor activity.

Key words: Bioactivity; B-phycoerythrin; Graffi tumor; *Porphyridium cruentum*

INTRODUCTION

Phycobiliproteins (phycoerythrin, phycocyanin and allophycocyanin) are a family of colored photosynthetic accessory pigments of red algae and cyanobacteria. Because of their excellent spectral properties, stability, high absorption coefficient, and high quantum yield they are extensively commercialized for fluorescent applications in clinical and immunological analysis and their therapeutic value has been demonstrated, too [1, 2].

B-phycoerythrin (B-PE, a red colored protein) is one of the three main types (B-, R- and C-) of phycoeryhtrin that is widely used as fluorescent labelling reagent in biomedical research and highly sensitive fluorescent techniques [2, 3, 4], in the development of biosensors [5, 6], and as natural colorant in foods and cosmetics [7]. Phycoerythrin isolated from *Nostoc* species was used as a protein marker for electrophoretic techniques [8]. It has been reported that R-phycoerythrin (R-PE) could scavenge free radicals and exhibits antitumor activity [9]. R-PE is a new kind of photosensitizer and could potentiate apoptosis in cancer cell lines subjected to photodynamic therapy [10, 11]. C-phycoerythrin was shown to protect rats from CCl4-induced toxicity [12] and from diabetic complications [13]. It also provides protection against permanganate-mediated DNA damage [14], against $HgCl_2$-caused oxidative stress and cellular damage in kidney [15]. However, no data has been reported on the biological activity of B-PE whose main rich source is the red microalga *Porphyridium cruentum.*

The aim of the present study was to examine the *in vitro* effects of B-PE from *P. cruentum* on the proliferation of Graffi myeloid tumor cells. To gain insights into the mechanisms of B-PE bioactivity, changes in cell morphology, DNA integrity, izoenzyme profile and activity of Mn SOD, Cu/Zn SOD, and glutathione reductase (GR) of Graffi tumor cells were studied, too.

MATERIALS AND METHODS

Algal strain and B-PE purification

The red alga *Porphyridium cruentum* (AG.) NAG. strain VISCHER 1935/107 was obtained from CCALA (Treboón Collection of Autotrophic Organisms, Czech Republic). The procedure for B-PE extraction and purification was previously described [16]. B-PE concentration was determined using the equations of Bennet and Bogorad [17].

Cell lines and culture

Graffi myeloid tumor cells, obtained from Graffi tumor in hamsters were used. The isolation of tumor cells was conducted as described by Toshkova *et al*. [18]. Bone marrow cells (BMC), prepared in a conventional way from femurs of healthy hamsters were used as fast-dividing normal cells. The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (100 units/ml penicillin, and 100 units/ml streptomycin) in a humidified incubator at 37°C and 5% CO_2 atmosphere.

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Cytotoxicity evaluation

Cultured tumor cells or BMC were collected during their exponential growth phase. Cell viability was greater than 98% as determined by trypan blue exclusion. One hundred microliters of cell suspensions (tumor cells or BMC in complete medium) containing 5 × 10^4 cells were seeded in each well of a 96-well microtiter plates and were treated with B-PE at a final concentration of 50 and 100 µg/ml. Untreated cells were used as a negative control. The plates were incubated for 24 h at 37°C in a humidified incubator with 5% CO_2. Cell proliferation was evaluated *in vitro* by the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT) assay [19]. Proliferation activity of B-PE-treated cells was expressed as a percentage of control cells. The experiments were carried out in triplicate, and data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was performed to compare the data from the control group and the treated one. Values of *p < 0.05 and ***p < 0.001 were considered significant.

Acridine orange (AO)/propidium iodide (PI) dual staining and fluorescent microscopy

Graffi tumor cells from exponentially growing cultures were seeded on 13 mm diameter glass slides within 24-well microplate and incubated with B-PE (50 and 100 µg/ml) or Doxorubicin hydrochloride (DOX) as a positive control (10 µg/ml) for 24 h in CO_2 incubator. After treatment, the cells were washed twice with PBS to remove culture media. Then they were stained with equal volumes of acridine orange (10 µg/ml) and propidium iodide (10 µg/ml) according to Abdel-Wahab *et al.* [20], with minor modification. Freshly stained Graffi cells were examined under fluorescence microscope (Leika DM 500B, Wetzlar, Germany) within 30 min before the fluorescence color started to fade.

DNA fragmentation analysis

Graffi tumor cells (5 × 10^4/well) were treated with 50 and 100 µg/ml B-PE for 24 h and DNA was isolated from each control and treated group. The isolation of total DNA and analysis of DNA fragmentation were carried out according to the protocol of Morimoto laboratory (http://groups.molbiosci.northwestern.edu/morimoto/research/Protocols/IV. DNA/B. Prep of DNA). DNA samples were electrophoretically separated on 1.2% agarose gels at 80 V for 90 min in TBE buffer, the gels were stained with ethidium bromide (1 µg/ml), and DNA was visualized under UV light and photographed.

In-gel enzyme activity staining

Whole-cell extracts from Graffi cells were prepared after Bravard *et al*. [21]. Equal amounts (20 mg) of protein from B-PE-treated and untreated tumor cells were subjected to PAGE as described by Okajima *et al*. [22], except that SDS was omitted. The in-gel activity staining of SOD and GR was performed after Beauchamp and Fridovich [23] and Anderson *et al*. [24], respectively. Gel patterns were recorded immediately after staining using the UVItec gel documentation system (Cambridge, UK). Image analysis of the gels was performed on a PC using Gel-Pro32 Analyzer software (Media Cybernetics Inc., Bethesda, MD USA). The antioxidant enzyme activities were recorded as total integrated optical density (IOD) in arbitrary units for each isoenzyme resolved.

Ethical aspects

The animal test was conducted in accordance with the principles for laboratory animal use and care as found in European Community guidelines, Committee on Care of Laboratory Animal resources, Commission on Life Sciences, National Research Council. Hamsters were housed in the animal care facilities of the EPAM Institute, which are fully accredited for Laboratory Animal Care.

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Growth inhibitory activity of selected microalgae and cyanobacteria towards human cervical carcinoma cells (HeLa)

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ABSTRACT

Although a large number of strains have been shown to possess anticancer activity, the microalgal and cyanobacterial potential is still largely unexplored. The present study was aimed to evaluate and compare the growth inhibitory activity of 33 extracts, 15 fatty acid mixtures and five exopolysaccharides from 19 species on HeLa cells, using MTT assay. All investigated strains showed growth inhibitory activity of at least one tested extract/constituent. More than 80% decrease in Hela cells viability was registered for the crude hot water extract of *Gloeocapsa* sp. and ethanol extracts of the new Bulgarian isolates *Gloeocapsa* sp. and *Chlorella* sp. Most active cell-free culture liquids were these of *Anabaena* sp. and *Synechocystis* sp., while the exopolysaccharides of *Gloeocapsa* sp. exhibited the lowest IC_{50} value (24.4 µg/ml). An interesting and useful finding of our investigation was that the isolated fatty acid mixtures from five out of eight cyanobacterial strains and from four out of seven microalgal strains studied had potent activity against HeLa tumor cells with the IC_{50} values under 30 µg/ml and even under 15 µg/ml. High percentage of the active Bulgarian isolates among the strains tested fulfil the effort of screening cyanobacteria and microalgae of local habitats. In conclusion, the most promising strains for further study are the thermal cyanobacteria *Synechocystis* sp. and especially *Gloeocapsa* sp., which showed the strongest inhibition of tumor cell growth and produced wider range of active components.

Key words: Bioactivity; Cyanobacteria; Exopolysaccharides; Extracts; Fatty acids; Microalgae

INTRODUCTION

Cervical cancer is the second most common and aggressive malignancy in female population [1]. Despite the great progress in developing prevention and screening programs, this disease remains one of the leading causes of cancer-related morbidity and mortality among women in less developed countries [2]. Numerous chemotherapeutic drugs have been successfully used for the treatment of cancers, but severe side effects are predominant. Therefore, during the last few decades renewed interest has emerged towards the finding of safer anticancer agents from natural sources. Focusing on bioproducts, recent trends in drug research have shown that microalgae and cyanobacteria are promising organisms to furnish novel biologically active compounds [3, 4]. Microalgae and cyanobacteria are photosynthetic microorganisms, and a large variety of species with diverse morphological, physiological and biochemical properties are found in almost all earth ecosystems. Their metabolites have been shown to possess antibacterial, antifungal, antiviral, antiplasmodial, protease-inhibitory and other activities, which are targets of biomedical research [5]. One of the fascinating discoveries regarding biological activity is the anticancer property demonstrated among cyanobacterial genera, such as *Nostoc* [6], *Scytonema* [7], *Hapalosiphon* [7], *Lyngbya* [8], *Anabaena* [6, 9], *Symploca* [10], *Synechocystis* and *Synechococcus* [11], as well as among red [12, 13] and green [14, 15] microalgae. Although a large number of strains were screened for biological activity and various cytotoxic compounds were isolated and characterized, the microalgal and cyanobacterial biodiversity is still largely unexplored.

The present study was undertaken to evaluate and compare the growth inhibitory activity of extracts, fatty acid mixures and exopolysaccharides from 19 species towards HeLa cells. It is expected that *in vitro* cytotoxicity screening will permit the selection of extracts and compounds with potentially useful properties to be used for further chemical and pharmacological evaluation. The comparative study may facilitate the selection of strains with a relatively high level of potency and/or with wider range of cytotoxic components.

MATERIALS AND METHODS

Strains and growth conditions

The environmental isolates and the culture collection species/strains used in this study are described in Table 1. Monospecific non-axenic cultures were grown autotrophically in 200 ml flasks, at 28±2 °C under continuous illumination (white fluorescent light, 132 µmol/m^2/s). A carbon source was provided by bubbling sterile 2% (v/v) CO_2 in air through the cultures. The strains were cultured in 200 ml of appropriated liquid medium [16-22] (Table 1). Cultures at the stationary phase of growth were centrifuged (5000 × g, 20 min). Both, cells and cell-free culture liquids were used to prepare samples.

Table 1. List of strains screened for cytotoxic activity

S.No	Phylum/Order	Species	Strain	Locality	Medium
	Cyanophyta				
1	Chroococcales	*Gloeocapsa* sp.	Gacheva 2007/R-06/1	thermal spring, Rupite, Bulgaria	[16]
2	Chroococcales	*Synechocystis* sp.	R10	thermal spring, Rupite, Bulgaria	[16]
3	Nostocales	*Anabaena* sp.	Takacova 1984/1	unknown[a]	BG-11 [17]
4	Nostocales	*Aphanizomenon flos-aquae*		bloom, lake, Czech	Natural sample
5	Nostocales	*Mastigocladus laminosus*	Lukavský 2008/21b	Sample L. Nedbalova	D [18]
6	Nostocales	*Nostoc cf. calcicola*	Desortova 1967/2	Mts.Krkonose, valley Velka Kotelni jama, Czech	Z8 [19]
7	Nostocales	*Nostoc entophytum*	Zehnder/76	stone, gneiss, Cameroon[a]	BG-11 [17]
8	Nostocales	*Nostoc muscorum*	Lukešová 1986/14	soil, Chelcice, meadow, Czech	Z8 [19]
9	Nostocales	*Nostoc sphaericum*		Trebon, Czech	Natural sample
10	Nostocales	*Nostoc* sp.		soil sample, Melnik, Bulgaria	Natural sample
11	Nostocales	*Scytonema ocellatum*	Adhikary 231minus	unknown[a]	Z8 [19]
12	Oscillatoriales	*Arthronema africanum*	Lukavský 1980/1	High saline puddle on sea shore, Kuweit[a]	Z8 [19]
	Ochrophyta				
13	Mischococcales	*Trachydiscus minutus*	Lukavsky & Pribyl 2005/1	technical service water, Temelín nuclear power station, Czech	3-fold Z [20]
	Chlorophyta				
14	Sphaeropleales	*Scenedesmus obliquus*	Lhotsky O. 1966/7	Opatovicky mill, Trebon, Czech	Z [20]
15	Sphaeropleales	*Scenedesmus incrassatulus*	R-83	thermal spring, Rupite, Bulgaria	[21]
16	Chlorellales	*Chlorella* sp.	R-06/2	Thermal spring, Rupite, Bulgaria	[21]
17	ND	Green alga	ND	soil, Levishte, Bulgaria[b]	[21]
18	ND	Green alga	ND	building wall, Sofia, Bulgaria[c]	[21]
	Rhodophyta				
19	Rhodellales	*Rhodella violacea*	Hindak	unknown[a]	[22]

a, from the culture collection of the Institute of Botany ASCR, Trebon, Czech Republic; b, conditionally marked Ga1; c, conditionally marked Ga2.

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RESPONSE OF *TRACHYDISCUS MINUTUS* (XANTHOPHYCEAE) TO TEMPERATURE AND LIGHT[1]

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The effects of different temperatures and light intensities on growth, pigments, sugars, lipids, and proteins, as well as on some antioxidant and proteolytic enzymes of *Trachydiscus minutus* (Bourr.) H. Ettl, were investigated. The optimum growth temperature and light intensity were 25°C and 2 · 132]mol photons Æ m)2 Æ s)1, respectively. Under these conditions, proteins were the main biomass components (33.45% dry weight [dwt]), with high levels of carbohydrates (29% dwt) and lipids (21.77% dwt). *T. minutus* tolerated temperatures between 20°C and 32°C, with only moderate changes in cell growth and biochemical composition. Extremely low (15°C) and high (40°C) temperatures decreased chl and RUBISCO contents and inhibited cell growth. The biochemical response of the alga to both unfavorable conditions was an increase in lipid content (up to 35.19% dwt) and a decrease in carbohydrates (down to 13.64% dwt) with much less of a change in total protein content (in the range of 30.51%–38.13% dwt). At the same time, the defense system of *T. minutus* was regulated differently in response to heat or cold treatments. Generally, at 40°C, the activities of superoxide dismutase (SOD), catalase (CAT), and proteases were drastically elevated, and three polypeptides were overexpressed, whereas the glutathione reductase (GR) and peroxidase (POD) activities were reduced. In contrast, at 15°C, all these enzymes except GR were suppressed. The effect of light was to enhance or decrease the temperature stress responses, depending on intensity. Our studies demonstrate the broad temperature adaptability of *T. minutus* as well as the potential for the production of valuable algal biomass.

Key index words: antioxidant enzymes; biomass composition; growth; light; protein profi temperature; *Trachydiscus minutus*

Abbreviations: AOE, antioxidant enzymes; CAT, catalase; DAB-POD, diaminobenzidine-specific-peroxidase; dwt, dry weight; GR, glutathione reductase; H_2O_2, hydrogen peroxide; KCN, potassium cyanide; L1, light intensity of 132]mol photons Æ m)2 Æ s)1; L2, light intensity of 2 · 132]mol photons Æ m)2 Æ s)1; SOD, superoxide dismutase; T1, 15°C; T2, 20°C; T3, 25°C; T4, 32°C; T5, 40°C

Microalgae are photosynthetic microorganisms that occur in all ecosystems. They represent a large variety of species, living in diverse ecological habitats (Falkowski and Raven 1997). To survive in any particular environment, microalgae have had to develop adaptive strategies to tolerate quite wide fluctuations in their chemical and physical environment and enable the synthesis of a tremendous diversity of compounds from different metabolic pathways.

Due to their extraordinary and diverse biosynthetic potential, and the possibility for controlled cultivation, microalgae are increasingly being used as a source of biomass and the source of a vast range of valuable products of ecological and pharmaceutical interest (reviewed in Cardozo et al. 2007, Raja 2008, Mata et al. 2010). The growing number of applications of algal products requires an increasing production of algal biomass and its useful components. Genetic modification of the existing strains has been used in the past and will continue to be employed in the future (Leon-Banares et al. 2004) as a tool to enhance the synthesis of high-value products. However, the search for new algal strains of promise, and studies on the manipulation of growth conditions for enhanced production of desired products may be more viable options, as they are environmentally sustainable and broaden our fundamental knowledge of algal taxonomy, ecology, physiology, biochemistry, and molecular biology.

The main physical factors controlling microalgal growth and metabolism are light and temperature. Light is the driving force for photosynthesis, as well as being a major factor in cell photoacclimation, in the course of which algal cells undergo dynamic changes in their composition, along with alterations in their ultrastructural, biophysical, and physiological properties, thus increasing photosynthesis and growth (Dubinsky et al. 1995). The effect of temperature is associated mainly with changes in cellular structural components (especially lipids and proteins) and reaction rates. As a consequence of these primary effects, there are also secondary effects on metabolic regulatory mechanisms, specificity of enzyme reactions, cell permeability, and cell composition (Richmond 1986).

The effect of temperature and light on growth, biochemical composition, and enzyme activities of some microalgal species is well documented. However, it is difficult to generalize on the particular

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PHARMACEUTICAL BIOTECHNOLOGY

CYTOTOXIC AND APOPTOGENIC POTENTIAL OF RED MICROALGAL POLYSACCHARIDES

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ABSTRACT

The efforts of scientists are aimed at finding anti-cancer agents of natural origin which have high biological activity, low toxicity and broad spectrum of therapeutic activity. This study was designed to determine the cytotoxic properties of polysaccharides derived from the red microalgae Dixoniella grisea and Porphyridium cruentum and to elucidate the mechanism of their action on two permanent human cell lines MCF-7 (breast adenocarcinoma) and HeLa (cervical carcinoma), as well as on primary culture from Graffi myeloid tumor in hamsters. Our investigations indicated that both algal polysaccharides showed high cytotoxic and apoptogenic activities on cancer cells and may be a promising alternative to synthetic substances.

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Keywords: apoptosis, algal polysaccharides, human tumor cell lines, Graffi myeloid tumor

Introduction

Polysaccharides (PSHs) are essential constituents of all living organisms and play a significant role in a variety of functions in the cells. In the recent years, PSHs have attracted more attention in the biochemical and medical areas because of their anti-cancer effects. The mechanism of the anti-cancer activity is related to the ability of PSHs to inhibit the growth of cancer cells (cytotoxic or cytostatic effect), to stimulate the immune response, to inhibit tumor angiogenesis and to induce apoptosis (3, 21, 24, 40, 42).

The investigations on microalgae offer some advantages such as their ability to produce large amounts of extracellular PSHs at controlled cultivation conditions (42) and the possibility for efficient isolation of these exopolysaccharides (21). Because of the complexity of red microalgal PSHs they are relatively poorly studied. Available data have been devoted mainly to *Porphyridium* sp., *Porphyridium cruentum* and *Dixoniella grisea*. These red microalgae are encapsulated within cell-wall PSHs, the external part of which dissolves in the culture medium (13, 16). Investigation of the soluble cell wall PSHs was focused on their chemistry and rheology (13, 16, 17). These sulfated acidic heteropolymers have also been shown to exert biological activities. The polysaccharide of *Porphyridium* sp. was found to have anti-inflammatory and anti-irritating (25), anti-tumor (34) and antiviral (38) activities. *P. cruentum* polysaccharide exhibited antiviral activity (20, 26) and reduced the blood glucose level in experimental diabetic mice (19). All, *Porphyridium* sp., *P. cruentum* and *D. grisea* PSHs have been reported to be potent antioxidants (11, 37, 39). In a series of experiments, samples of sulfated polysaccharides and samples of natural polysaccharides from red microalgae were found to inhibit the cell growth of FD (early myeloid cell line) and EL-4 and 24-1 T-lymphoma cell lines (18). The anticancer activity of these red microalgal PSHs seems to be promising, but has not been intensively studied yet.

Our previous research has shown the potent *in vivo* antitumor activity of extracellular PSHs produced by *P. cruentum* and *D. grisea* (14, 15). Both PSHs decreased the transplantability of Graffi myeloid tumor in hamsters, retarded the tumor growth and reduced the mortality rate. Additionally, in *in vitro* experiments the PSHs increased the spreading and the phagocytic ability of peritoneal macrophages from healthy and Graffi tumor bearing hamsters in a dose-dependent manner. Direct toxic effect of the PSHs on Graffi tumor cells was suggested, based on the decrease in the viability of cells treated with 100 $\mu g.ml^{-1}$ of *P. cruentum* polysaccharide. In the present investigation we studied *in vitro* the effect of these red microalgal PSHs on two permanent human cell lines MCF-7 (breast adenocarcinoma) and HeLa (cervical carcinoma) and on primary Graffi tumor cells. The mechanism of activity of these PSHs on cancer cells was also examined.

Materials and Methods

Algal strains and cultivation conditions

The red microalga *Porphyridium cruentum* (AG.) NAG strain Vischer 1935/107 obtained from CCALA (Trebon Collection of Autotrophic Organisms, Czech Republic) was grown in batch cultures for 7 days under the following conditions: nutrient medium of Brody and Emerson (9); bubbling with air (10 $l \cdot h^{-1}$) enriched with 2-3% CO_2; 25 °C; continuous lateral illumination with cool-white fluorescent lamps at a photon flux density of 150 $\mu E \cdot m^{-2} \cdot s^{-1}$ measured at the surface of 200 ml flasks. The microalgal strain *Dixoniella grisea* (Geitler) (35), former *Rhodella reticulata* (Rhodophyta) strain

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EFFECTS OF TEMPERATURE ON *SYNECHOCYSTIS* SP. R10 (CYANOPROKARYOTA) AT TWO IRRADIANCE LEVELS. I. EFFECT ON GROWTH, BIOCHEMICAL COMPOSITION AND DEFENSE ENZYME ACTIVITIES

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Summary. The influence of five different temperatures (20, 26, 32, 39 and 44°C) under two irradiance levels (132 µmol photons m^{-2} s^{-1}, unilateral and 2×132 µmol photons m^{-2} s^{-1}, bilateral) on the growth, biochemical composition and enzymatic antioxidant defense of a newly isolated unicellular *Synechocystis* sp. strain R10 was studied. This photoautotrophic blue-green alga grew well in the temperature range 26-39°C under lower light intensity, but the cultures could grow, although slower, at 20°C and 44°C. Temperature, under higher irradiance level, did not significantly affect the growth, except for 44°C, where the growth was greatly reduced. *Synechocystis* sp. R10 responded to the unfavorable conditions by changes in its biochemical composition and enzyme activities. At 20°C and lower light, total carbohydrates content as well as the activities of superoxide dismutase, catalase, glutathione reductase and defined protease isoforms were increased. The response to the combination of high temperature (44°C) and lower light consisted in accumulation of carotenoids and C-phycocyanin, but not in the induction of antioxidant enzymes activity. A significant enhancement in carbohydrate content was observed at 44°C and higher light, simultaneously with the reduction in protein content and activity of all studied enzymes. The observed modulation of antioxidant enzyme and protease activities, and changes in carbohydrate and pigment contents could be a prerequisite for the thermal tolerance and ability of the cyanobacterium to adapt to different environment. Because of its fast growth rate, high phycobiliprotein, carbohydrate and protein contents, and wide adaptability, this strain is of biotechnological interest.

Key words: carbohydrates; catalase; cyanobacteria; glutathione reductase; growth; isoenzymes; phycobiliproteins; protease; superoxide dismutase; *Synechocystis*

Abbreviations: AOE – antioxidant enzymes; APC – allophycocyanin; C-PC – C-phycocyanin; CAT – catalase; DW – dry weight; EST – esterase; HL – high light intensity; GR – glutathione reductase; LL – low light intensity; PAGE – polyacrylamide gel electrophoresis; POD – peroxidase; ROS – reactive oxygen species; SOD – superoxide dismutase; *Synechocystis* - *Synechocystis* sp. strain R10

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EFFECTS OF TEMPERATURE ON *SYNECHOCYSTIS* SP. R10 (CYANOPROKARYOTA) AT TWO IRRADIANCE LEVELS. II. EFFECT ON ANTIBACTERIAL, ANTIFUNGAL AND CYTOTOXIC ACTIVITIES

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Summary. The influence of five different temperatures (20, 26, 32, 39 and 44°C) under two irradiance levels (132 µmol photons m^{-2} s^{-1}, unilateral and 2×132 µmol photons m^{-2} s^{-1}, bilateral) on the biological activity of a newly-isolated unicellular cyanobacterium *Synechocystis* sp. strain R10 was studied. Water cellular extracts, fatty acids, culture liquids and extracellular polysaccharides of *Synechocystis* sp. were found to have activities against bacteria, fungus and tumor cells. The exopolysaccharides showed the most potent antibacterial and antifungal activities (MIC=0.25 mg mL^{-1}). The mixture of substances, excreted in the culture medium, inhibited a broad range of target pathogens. The cyanobacterial fatty acids had the strongest growth inhibitory effect on HeLa cells (IC_{50}<15 µg mL^{-1}). The cultivation conditions (temperature, light intensity) and the culture age had considerable and specific effects on the activity of the different groups of metabolites tested. Because of the pronounced potential of *Synechocystis* sp. R10 to produce various biologically active substances and the possibility to increase the activity of its intracellular and extracellular metabolites through manipulation of the cultivation conditions, this strain is of undoubted biotechnological interest.

Key words: antimicrobial activity; blue-green algae; *Candida albicans*; cellular extracts; culture liquids; cytotoxicity; extracellular polysaccharides; fatty acids; Gram-negative bacteria; HeLa cells; *Synechocystis*

Abbreviations: EPS – extracellular polysaccharides; HL – high light intensity; IC_{50} – the concentration required for 50% inhibition; LL – low light intensity; MIC – minimum inhibitory concentration

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Влияние на културационните условия върху производството и биологичната активност на ценни метаболити от цианопрокариота *Synechocystis* sp. R10

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Abstract

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The influence of different temperatures (20, 26, 32, 39 and 44 °C) and illumination (132 μmol photons·m^{-2} s^{-1}, unilateral and 2 × 132 μmol photons·m^{-2} s^{-1}, bilateral) on the growth, pigment con- tent and biological activity of a newly isolated unicellular cyanoprokaryotic strain *Synechocystis* sp. R10 was studied. The best growth was detected at 39 °C, bilateral illumination. Chlorophyll *a* and carotenoid content practically did not change at the whole temperature range except for 44 °C, bilateral illumination, at which it declined significantly. From biotechnological point of view this strain is valuable for its high phycobiliprotein content (C-phycocyanin – 12.0 – 18.7 % of dry weight and allophycocyanin – 3.2 – 7.3 %) and its high potential as a producer of various biologically active substances. Among the cyanobacterial metabolites tested for bioactivity (lyo- philized culture media, cellular water extracts, fatty acids and exopolysaccharides) the extracel- lular polysaccharides of *Synechocystis* sp. strain R10 had the highest antibacterial and antifungal activity (MIC = 0.25 mg/ml), while the mixture of substances, excreted in the culture medium inhibited a broadest range of target pathogens. The cyanobacterial fatty acids showed the strong- est growth inhibition effect on HeLa cells (IC_{50} < 15 μg/ml). Cultivation conditions (temperature and illumination) had substantial but specific effect on the bioactivity of the different groups of metabolites tested.

Key words: biological activity, fatty acids, pigments, polysaccharides, *Synechocystis*

Биологична активност на мастни киселини от *Gloeocapsa* sp. (*Cyanoprocariota*), отглеждан при различни условия

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Abstract

Iliev, I., Gacheva, G., Gigova, L., Petkov, G., Toshkova, R., Gardeva, E., Yossifova, L.S., Naj- denski H. & Kussovski, V. 2012. Biological activity of fatty acids from the cyanobacterium *Gloeocapsa* sp. grown at different conditions. – In: **Petrova, A.** (ed.), Proc. VII Natl. Conf. Bot., 29–30.09.2011, Sofia, pp. 477-483. Bulg. Bot. Soc., Sofia. ISBN 978-954-92808-2-1

Gloeocapsa sp. strain R-06/1 was grown at three different temperatures and two light intensi- ties. The fatty acid composition of this blue-green alga was determined for the first time. The relative content of almost all fatty acids was changed depending on the temperature of cultiva- tion. Biological activity of the tested fatty acids and ethanol extracts isolated from *Gloeocapsa* sp. were also influenced by the culture conditions of the alga. For antibacterial and antifungal assay seven pathogens (*Streptococcus pyogenes*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli, Pseudomonas aeruginosa*, *Salmonella typhimurium* and *Candida albicans*) were used. The cytotoxicity was tested on a human cervix epitheloid carcinoma cell line, HeLa. Fatty acids in- hibited the growth of Gram-positive bacterium *S. pyogenes* and exhibited a cytotoxic effect on HeLa cells with IC_{50} values in the range of 23.2–18.1 μg.ml^{-1}. Ethanol extracts from biomass, ob- tained at all culture conditions decreased the viability of HeLa cells in a dose-dependent manner. Extracts, obtained at 40 °C, 2 × 8000 lx and 15 °C, 8000 lx had the strongest effect (IC_{50} = 71.2 μg. ml^{-1} and IC_{50} = 92.3 μg.ml^{-1}, respectively). In conclusion, the fatty acids and lipophilic extracts from *Gloeocapsa* sp. express significant biological activity which can be improved by changing the culture conditions. Current studies show that this cyanobacterium is a source of substances with medical and pharmaceutical importance.

Key words: antibacterial activity, cyanobacterium, cytotoxicity, ethanol extracts, fatty acids, light intensity, temperature

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Suboptimal growth temperatures enhance the biological activity of cultured cyanobacterium *Gloeocapsa* sp.

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Abstract The cytotoxic, antibacterial, and antifungal activities of cyanobacterium *Gloeocapsa* sp. strain Gacheva 2007/R-06/1 were investigated and the possibility for an enhancement of these activities by changing the culture conditions evaluated. Fatty acids of this cyanobacterium were found to be active against *Streptococcus pyogenes*. Exopolysaccharides inhibited the growth of both Gram-positive and Gram-negative bacteria and the fungus *Candida albicans*. Both exopolysaccharides and fatty acid mixtures also significantly decreased the viability of human cervical carcinoma cells, HeLa. Greater biological activities were exhibited by *Gloeocapsa* sp., cultured at suboptimal temperatures (15–26°C) than at optimal and supraoptimal ones. In comparison with higher light intensity, the low-light cultivation stimulated the cytotoxicity of the fatty acids. In general, low temperatures decreased the growth of *Gloeocapsa* sp., but promoted its biological activity. Prolonged cultivation also had a beneficial impact on the bioactivity. Compared to 4 days, the 17-day cultivation resulted in fourfold higher antibacterial and antifungal activities of exopolysaccharides and more than twice increases in their cytotoxicity. The study revealed that this cyanobacterial isolate is a new source of natural products with potential for pharmacological and medical applications.

Keywords Antibacterial activity · Antifungal activity · Cytotoxicity · *Gloeocapsa* sp. · Physiological factors

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Introduction

Cyanobacteria have been studied for a long time for their particularly interesting morphology, physiology, and diversity. In the last few decades, these prokaryotic oxygenic phototrophs have gained much attention as a rich source of bioactive compounds (Borowitzka 1995). Cyanobacterial metabolites show antibacterial (Chlipala et al. 2009), antifungal (Kajiyama et al. 1998), antiviral (Schaeffer and Krylov 2000), anticancer (Martins et al. 2008), antiplasmodial (Linington et al. 2007), algicidal (Volk and Furkert 2006), immunopotentiating (Pugh et al. 2001), and protease inhibitory activities (Chlipala et al. 2009), which are targets of biomedical research (Wase and Wright 2008). Although a large number of strains were screened for various biological activities and there are numerous reports on isolated active compounds, cyanobacterial biodiversity is still largely unexplored.

Sometimes, the cyanobacteria are collected directly from their natural habitats; however, this biomass is a mixed assemblage, which casts doubt on the true origin of an isolated natural product (Dobretsov et al. 2011). The use of cultured cyanobacteria has several advantages over field collections. Controlled culture conditions allow the investigation of species

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Original article

Antibacterial and antifungal activities of selected microalgae and cyanobacteria

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Summary *In vitro* activity of nine cyanobacterial and ten microalgal newly isolated or culture collection strains against eight significant food-borne pathogens has been evaluated and compared. Water extracts and culture liquids of *Gloeocapsa* sp. and *Synechocystis* sp. demonstrated the widest spectrum of activity with minimal inhibitory concentration (MIC) ranging from 1.56 to 12.5 mg mL^{-1}. Culture liquid of *Anabaena* sp. had the highest activity (MIC = 0.39 mg mL^{-1}) but only to Gram-positive bacteria. Ethanol extracts and fatty acids from all cyanobacteria and microalgae were active against *Streptococcus pyogenes* and/or *Staphylococcus aureus*. The fatty acids of *Synechocystis* sp. inhibited the growth of *Bacillus cereus*, *Escherichia coli* and *Candida albicans* (MIC values of 2.5–1.25 mg mL^{-1}, respectively). Exopolysaccharides (EPS) of *Gloeocapsa* sp. were the sample that exhibited activity against all test pathogens with lowest MIC values (0.125–1 mg mL^{-1}). High activity with a narrower range of susceptible targets demonstrated the exopolysaccharides of *Synechocystis* sp. and *Rhodella reticulata*. Antimicrobial activity was proven for phycobiliproteins isolated from *Synechocystis* sp., *Arthrospira fusiformis*, *Porphyridium aerugineum* and *Porphyridium cruentum*, respectively. In conclusion *Gloeocapsa* sp. and *Synechocystis* sp. and especially their exopolysaccharides showed the most promising potential against the examined food pathogens.

Keywords Antimicrobial activity, cyanobacteria, extracts, food-borne pathogens, microalgae.

Introduction

The escalating levels of antibiotic resistance among pathogenic bacteria forced the efforts of scientists to search for new antimicrobial substances from various natural sources including microalgae and cyanobacteria (Mundt *et al.*, 2001; Safonova & Reisser, 2005; Ghasemi *et al.*, 2007; Prakash *et al.*, 2011). Both groups of photosynthetic microorganisms are represented by a wide variety of species, living in diverse ecological habitats (Falkowski & Raven, 1997). To survive in a highly competitive environment, often exhibiting widely fluctuating chemical and physical parameters, they have developed defensive and adaptive strategies, including the synthesis of a tremendous diversity of compounds from different metabolic pathways. Many of their metabolites have been shown to possess antibacterial, antiviral, antifungal, enzyme inhibiting, immunostimulant, cytotoxic and antiplasmodial activities (Ghasemi *et al.*, 2004; Singh *et al.*, 2005). Although the potential of microalgae and cyanobacteria as biofertilisers is well known, the attention has recently been focused on biotechnological potential for obtaining pharmacologically active secondary metabolites (Carmichael, 1992; Yuzuru, 1993). Recent investigations on biologically active secondary metabolites from microalgae led to the identification of a wide range of compounds.

Antibacterial substance, named 'chlorellin', was firstly isolated from *Chlorella*. This mixture of fatty acids was found to exhibit inhibitory activity against both Gram-positive and Gram-negative bacteria (Pratt *et al.*, 1944). 'Parsiguine', a novel antimicrobial substance from *Fischerella ambigua* has been reported too (Ghasemi *et al.*, 2004). As the pharmaceutical drug discovery has depended heavily on the process of empirically screening of large number of crude extracts from a wide variety of organisms, herewith we present new data about antibacterial and antifungal activities' assessment of various strains of phototrophic microalgae and cyanobacteria.

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Growth, biochemical and enzymatic responses of thermal cyanobacterium *Gloeocapsa* sp. (Cyanophyceae) to temperature and irradiance

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SUMMARY

The present study describes a strain of *Gloeocapsa* sp. designated as Gacheva 2007/R-06/1, originally isolated from a geothermal flow located in Rupite, Bulgaria. To evaluate whether this cyanobacterium is locally adapted to hot environment or has the ability to tolerate lower temperatures, its growth, biochemical composition, enzyme isoforms and activity of the main antioxidant enzymes and proteases were characterized under various temperatures and two irradiance levels. The strain was able to grow over the whole temperature range (15–40°C) under two different photon fluence densities – 132 µmol photons m^{-2} s^{-1} (unilateral, low light, LL) and 2 × 132 µmol photons m^{-2} s^{-1} (bilateral, high light, HL). The best growth occurred at either 34°C and LL or at 36°C and HL, but significant growth inhibition was noted at 15°C and 40°C. Low temperature treatment (15°C) resulted in higher levels of total protein and an increased activity of manganese superoxide dismutase (MnSOD) and glutathione reductase, as compared to optimum growth temperatures. After simultaneous exposure to 15°C and HL, increases in lipid content and activity of iron superoxide dismutase and catalase (CAT) were also observed. Cultivation of cells at 40°C enhanced MnSOD, CAT and peroxidase activities, regardless of irradiance level. Increased total protein content and protease activity at 40°C was only associated with the HL treatment. Overall, these results indicate that *Gloeocapsa* sp. strain Gacheva 2007/R-06/1 used different strategies to enable cells to efficiently acclimate and withstand adverse low or high temperatures. This strain obviously tolerates a wide range of temperatures below its natural habitat temperature, and does not seem to be locally adapted to its original thermal regime. It behaved as a thermotolerant rather than a thermophilic cyanobacterium, which suggests its wider distribution in nature.

Key words: antioxidant enzymes, blue-green algae, esterase, growth, light, pigments, protease, temperature tolerance.

INTRODUCTION

Cyanobacteria are oxygenic, photosynthetic prokaryotes, and a wide variety of species with diverse morphological, physiological and biochemical properties (Vincent 2009) dominate the microbial communities of almost all ecosystems on Earth, including extreme environments (Whitton & Potts 2000). The cosmopolitan distribution of cyanobacteria indicates that they possess the capacity to adapt to specific ecological niches and acclimate to transitory changes in environmental conditions of their respective habitats (Tang & Vincent 1999; Tiwari *et al.* 2005). During the acclimation process, these organisms respond to various external signals, such as nutrient availability, irradiance level and wavelength, temperature, salinity, water availability, mainly through regulation of gene expression and enzyme activities (Los & Murata 1999; Los *et al.* 2008).

Light and temperature are among the most important environmental factors controlling photoautotrophic growth and metabolism of cyanobacteria. Extreme temperatures and light induce formation of reactive oxygen species (ROS; Mishra *et al.* 2005; Suzuki & Mittler 2006), which are highly damaging to proteins, lipids, and nucleic acids (Suzuki & Mittler 2006). To counter the deleterious effects of increased ROS levels, cyanobacteria, like algae and higher plants, have developed enzymatic and non-enzymatic scavenging systems.

There has been a number of studies concerning the effect of temperature and irradiance on growth and biochemical composition, but few reports have addressed antioxidant enzyme activities in cyanobacterial species. Cyanobacteria display considerable differences in their sensitivity, physiological and biochemical responses, and adaptive strategies (Tang & Vincent 1999; Rafiqul islam *et al.* 2003; Tiwari *et al.* 2005;

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RESPONSES OF *SYMPLOCA* SP. (CYANOBACTERIA) TO NITROGEN DEPLETION DURING CULTURING

Liliana Gigova, Natalia Ivanova

(*Submitted by Academician V. Golemansky on September 9, 2013*)

Abstract

The fast-growing cyanobacterium *Symploca* sp. was cultured for three days in liquid medium, containing low nitrogen level, and cell growth, phycobiliproteins (PBP) content and the response of stress-related enzymes were studied. The gradual depletion of nitrogen in the medium during culturing led to a gradual decline in growth rate, and a sharp decrease in the PBP content. In accordance with these phenotypes, the cellular metabolic (esterase) activity was reduced, while protease activity was enhanced and new isoenzymes appeared in the cells of 3-days old culture. The activities of superoxide dismutase (SOD) and peroxidase (POD) did not change considerably during the cultivation. In contrast, an increase in the glutathione reductase (GR) activity was registered, due to the stimulation of certain isoforms of the enzyme. The results demonstrated that besides the bleaching, a process typical for non-diazotrophic cyanobacteria, *Symploca* sp. responded to the nitrogen depletion in the medium by specific enzymatic changes.

Key words: cyanobacterium, enzymes, growth, nitrogen, phycobiliproteins, *Symploca* sp.

Introduction. Cyanobacteria are oxygenic photosynthetic prokaryotes, found in nearly all ecosystems. To survive in any particular habitat, cyanobacteria have evolved various adaptive mechanisms to tolerate quite wide fluctuations in their chemical and physical environment. In many natural habitats, the availability of combined nitrogen is growth-limiting. Non-diazotrophic cyanobacteria respond to nitrogen (N) limitation by a process termed chlorosis or bleaching [1–3]. This process involves proteolytic degradation of phycocyanin and allophycocyanin, accessory light-harvesting biliprotein pigments, and gradual loss

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Antitumor Activity of C-phycocyanin from *Arthronema africanum* (Cyanophyceae)

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ABSTRACT

Pure C-phycocyanin (C-PC) was isolated from Arthronema africanum *to evaluate its potential antitumor effects in vivo and in vitro. Experimental myeloid Graffi tumor in hamsters was used as a model. The cell proliferation assay showed that C-PC treatment, at concentration of 100 μg mL^{-1} for 24 h, significantly inhibited the growth of Graffi tumor cells (51.4% viability). Agarose gel electrophoresis of the genomic DNA of treated cells displayed time- and concentration-dependent fragmentation pattern, typical for apoptosis. Apoptotic process was related to the increase in cellular manganese and copper/zinc superoxide dismutases and glutathione reductase activities, coupled with a low catalase activity. In vivo C-PC administration (5.0 mg kg^{-1} body weight) suppressed the tumor transplantability and growth, while the mean survival time of the tumor-bearing hamsters was increased. The results revealed promising antitumor activities of* A. africanum *C-PC and suggested the potential of this natural biliprotein pigment for future pharmacological and medical applications. The study provided new data on the mechanism of the C-PC-induced apoptosis in which the imbalance of antioxidant enzymes that favoured hydrogen peroxide accumulation might play a leading role.*

Key words: Antitumor activity, *Arthronema africanum*, *In vivo*, *In vitro*, C-pycocyanin, Myeloid Graffi tumor

INTRODUCTION

In the last few decades, cyanobacteria have gained much attention as a rich source of bioactive compounds (Singh et al. 2005). One of the fascinating discoveries regarding biological activity is the anticancer property demonstrated among cyanobacterial genera such as *Nostoc*, *Fhormidium*, *Gloeocapsa*, *Anabaena*, *Spirulina*, *Synechocystis* and *Synechococcus* (Surakka et al. 2005; Martins et al. 2008; Gigova et al. 2011; Oh et al. 2011). Cyanobacteria produce a wide range of cytotoxic compounds, many of which are regarded as promising candidates for drug discovery, with applications in pharmacy, because of their natural origin, uniqueness and structural diversity (Singh et al. 2005; Tan 2007; Gademann and Portmann 2008). Recently, the attention of researchers was focused on the phycobiliproteins, and especially on C-phycocyanins (Walter et al. 2011). Phycobiliproteins (phycoerythrins, phycocyanins and allophycocyanins) are light-harvesting protein pigments, characteristic for cyanoprokaryotes and two algal phyla (Rhodophyta and Cryptophyta). C-phycocyanins were established to have various biological activities and pharmacological properties. These water soluble biliproteins have shown

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Biological activity of microalgae can be enhanced by manipulating the cultivation temperature and irradiance

Review Article

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Abstract: The escalating levels of antibiotic resistance among pathogenic bacteria and the side effects of chemotherapeutic drugs in use forced the efforts of scientists to search for natural antimicrobial and anticancer substances with novel structures and unique mechanism of action. Focusing on bioproducts, recent trends in drug research have shown that microalgae (including the cyanobacteria) are promising organisms to furnish novel and safer biologically active compounds. Many microalgal metabolites have been found to possess potent antibacterial, antifungal, antiviral, anticancer and antiinflammatory activities, as well as antioxidant, enzyme inhibiting and immunostimulating properties. In this paper, the studies on the biological activity of microalgae associated with potential medical and pharmaceutical applications are briefly presented. Attention is focused on the impact of cultivation temperature, irradiance and growth stage on the biomass accumulation, activity and pathways of cell metabolism and the possibilities of using these variable factors to increase the diversity and quantity of biologically active substances synthesized by microalgae.

Keywords: *Antibacterial • Anticancer • Antifungal • Biologically Active Metabolites • Growth Stage*

1. Introduction

Microalgae are a large and heterogenous group of photoautotrophic microorganisms, including species from different phyla – Cyanophyta (blue-green algae, cyanoprokaryotes, cyanobacteria), Chlorophyta (green algae), Rhodophyta (red algae), Cryptophyta, Haptophyta, Pyrrophyta, Streptophyta, Heterokontophyta. Microalgae exhibit remarkable ecological plasticity, namely the ability to adapt to changing and frequently extreme environmental conditions such as temperature, light, salinity, pH and moisture, which defines their worldwide distribution [1,2]. To survive in a complex and competitive environment, these organisms have developed adaptive and defense strategies that are related to the synthesis of various, some of which are unique, compounds from different metabolic pathways. Due to their extraordinary and diverse biosynthetic potential, and the possibility for controlled cultivation, microalgae are increasingly being used for biomass production and as a source of a vast range of valuable substances of industrial, ecological and pharmaceutical interest (reviewed by [3-5]).

2. Studies on the biological activity of microalgae associated with potential medical and pharmaceutical applications

A substance with antibacterial activity was first isolated from *Chlorella* in 1944. A fatty acid mixture, named "chlorellin" was shown to inhibit the growth of both

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PROCEEDINGS

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BO14. EXPERIMENTAL TUMOR MODELS DISPLAY DIFFERENT SUSCEPTIBILITY TO C-PHYCOCYANIN

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Abstract

Cells of myeloid *Graffi* tumor in hamsters and ascites tumor of *Guerin* in rats were grown in the presence of pure C-phycocyanin (C-PC) and their susceptibility to the treatment was compared. The changes in tumor cell proliferation, DNA integrity and activities of the main antioxidant enzymes were evaluated as markers for *in vitro* antitumor activity of C-PC. In the myeloid *Graffi* tumor in hamsters' model, C-PC significantly inhibited the growth of tumor cells and induced a DNA fragmentation, coupled with an increase in the cellular manganese superoxide dismutase and the glutathione reductase activities. In contrast to the promising activity against the cells from solid tumor, C-PC had no effect on the cells of ascites tumor of *Guerin*. This study showed a difference in the sensitivity of the cells from both types of tumors to the treatment and highlighted the meaning of the selection of experimental models when looking for new anticancer agents.

Introduction

C-phycocyanin is an accessory light-harvesting pigment in cyanoprokaryotes and two algal phyla (Rhodophyta and Cryptophyta). This water soluble and non-toxic biliprotein has many valuable pharmacological properties, such as anti-inflammatory, fibrinolytic [8], antidiabetic [16], anti-oxidant and free radical scavenging abilities [4], as well as antibacterial [18], antifungal, antiviral

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BIOLOGIE

Immunologie

ANTITUMOUR ACTIVITY OF *STREPTOMYCES* SP. (ACTINOBACTERIA) POLYSACCHARIDE

Liliana Gigova, Reneta Toshkova*, Natalia Ivanova, Liliya Yossifova*, Elena Gardeva*

(*Submitted by Corresponding Member O. Poljakova-Krusteva on April 28, 2014*)

Abstract

The in vivo and in vitro antitumour activities of extracellular polysaccharide derived from the Antarctic *Streptomyces* sp. 1010 (StrEPS) were assessed and compared with those of the red microalga *Dixoniella grisea* polysaccharide (DixEPS). Experimental myeloid Graffi tumour in hamsters was used as a model. In vivo administration of StrEPS suppressed the tumour transplantability and growth, while the mean survival time of the tumour-bearing hamsters was increased. Genomic DNA of polysaccharide-treated cells displayed a concentration-dependent fragmentation pattern, typical for apoptosis. Apoptotic process was related to the increase in cellular superoxide dismutase activity coupled with a decrease in glutathione reductase activity and undetectable catalase activity. StrEPS exhibited antitumour potential and mechanism of action similar to those of DixEPS, despite the differences in the origin, the molecular weight and monosaccharide composition of the two polysaccharides. The results showed that the bacterial polysaccharide, like the red alga polysaccharide, might be a promising alternative to synthetic substances as a natural antitumour compound with potential for future pharmacological and medical applications.

Key words: apoptosis, extracellular polysaccharides, Graffi myeloid tumour, in vitro, in vivo

Introduction. The extracellular polysaccharides (EPSs) of microorganisms offer a wide range of potential applications because of their unique structure, composition, fluid dynamics, and stability, combined with manifestation of vari-

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Microalgae respond differently to nitrogen availability during culturing

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Variations in the exogenous nitrogen level are known to significantly affect the physiological status and metabolism of microalgae. However, responses of red, green and yellow-green algae to nitrogen (N) availability have not been compared yet. *Porphyridium cruentum, Scenedesmus incrassatulus* and *Trachydiscus minutus* were cultured in the absence of N in the medium and subsequent resupply of N to the starved cells. Culture growth and in-gel changes in isoenzyme pattern and activity of glutamate synthase, glutamate dehydrogenase, malate dehydrogenase, aspartate aminotransferase, superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase were studied. The results demonstrated that the algae responded to the fully N-depleted and N-replete culture conditions by species-specific metabolic enzyme changes, suggesting differential regulation of both enzyme activity and cellular metabolism. Substantial differences in the activities of the antioxidant enzymes between N-depleted and N-replete cells of each species as well as between the species were also found. In the present work, besides the more general responses, such as adjustment of growth and pigmentation, we report on the involvement of specific metabolic and antioxidant enzymes and their isoforms in the mechanisms operating during N starvation and recovery in *P. cruentum, T. minutus* and *S. incrassatulus.*

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1. Introduction

Microalgae are oxygenic photosynthetic organisms found in nearly all ecosystems. In many natural habitats, the availability of combined nitrogen is growth-limiting (Sterner and Elser 2002). Nitrogen (N) is an essential major element required for the synthesis of amino acids, proteins, nucleic acids, coenzymes, chlorophyll and other accessory photosynthetic pigments (phycobiliproteins in blue-green, rhodophytes and cryptophytes algae). Therefore, these ecologically successful organisms have evolved a number of mechanisms to overcome stress due to N limitation. The representatives of various phyla show much similarity in their responses to N starvation such as a reduced rate of cell division (Sciandra *et al.* 2000; Sinetova *et al.* 2006; Msanne *et al.* 2012), down-regulation of photosynthesis (Silva *et al.* 2009; Hockin *et al.* 2012; Zhang *et al.* 2013), transition of the electron transport chain to the cyclic regime (Berges *et al.* 1996; Zhang *et al.* 2013), maintenance only of essential protein synthesis in the cell (Hockin *et al.* 2012), accumulation of carbon- and energy-rich storage compounds such as polysaccharides, starch and triacylglycerols (Guschina and Harwood 2006; Hu *et al.* 2008; Silva *et al.* 2009; Msanne *et al.* 2012) and, as a result, cell survival. The reduction of the photosynthetic pigments content leads to yellow appearance of the cells, known as chlorosis or bleaching. Chlorosis

Keywords. Antioxidant enzyme activities; growth; isoenzyme pattern; metabolic enzyme activities; nitrogen recovery; nitrogen starvation