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Procedures of partial purification for phycobiliproteins from cyanobacteria isolated from soils of Republic of Moldova

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SUMMARY. Investigation of the new cyanobacterial strains, for use as potential sources of bioactive substances, including phycobiliproteins, encounters some difficulties due to presence of toxins (microcystins) produced by some cyanobacterial strains. Cyanobacteria phycobiliproteins are natural pigments with high potential for application as colorants in food, cosmetics and pharmaceuticals. The objective of the study was the elaboration of a procedure for *Anabaena propinqua* Setchell. et Gardn. phycobiliproteins separation from microcystins and a procedure of partial purification of phycobiliproteins from cyanobacteria *Anabenopsis* sp. The antioxidant capacity of partial purified phycoerythrin from *Anabenopsis* sp. was established.

Keywords: antioxidant capacity, cyanobacteria, microcystins, phycobiliproteins.

Introduction

Cyanobacteria possess a wide spectrum of actual and potential biotechnological applications in diverse fields, such as agriculture, aquaculture, bioremediation, bioenergy and biofuels, nutraceuticals and pharmaceuticals, food industry, cosmetics and biomedical research (Abed *et al*., 2009; Chu, 2012; Lau *et al*., 2015; Manirafasha *et al.,* 2016).

Investigation of the new cyanobacterial strains for use as potential sources of bioactive substances encounters some difficulties with the presence of toxins, including microcystins, produced by some cyanobacteria. Most microcystins are hepatotoxins (liver toxins). Hepatotoxins are produced by species of the genera *Microcystis, Anabaena, Nodularia, Oscillatoria, Cylindrospermum* (Bulimaga *et al*.,

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2014). Moreover, toxins can be eliminated in the nutritive media or can be extracted together with bioactive substances. The methods used for removing of microcystins from drinking water are mainly based on application of activated carbon (Pyo and Moon, 2005; Yan *et al*., 2006; Drogui *et al*., 2012).

At the same time, publications regarding the removal of microcystins from phycobiliproteins extracts are in very small number (Ehmann and Guthrie, 2011, 2015).

The goal of the present research was to elaborate the procedures for removal of microcystins from *Anabaena propinqua* phycobiliprotein extracts and partial purification of phycoerythrin from cyanobacteria *Anabaenopsis* sp.

Materials and methods

The strains of investigated cyanobacteria *Anabaena propinqua* Setchell. et Gardn. and *Anabaenopsis* sp. (isolated by A. Trofim) were cultivated and offered by SRL "Algology", State University of Moldova, under the leadership of the professor V. M. Şalaru. The strains were isolated from the soils of the Cogalnic River Valley meadow, Cimişlia, Republic of Moldova.

Separation of phycobiliproteins extracted from biomass of cyanobacteria *Anabaena propinqua* from toxins was performed by the chromatographic method. The Amberlite XAD-2 (Sigma-Aldrich) column (20 x 0.5 cm) was washed with 10 volumes–of bidistilled water. The aqueous suspension of *Anabaena propinqua* biomass (20 mg/ml) was supposed to freeze-thawed repeated procedure and subsequent maceration of the frozen mixture, using pestle in a mortar for 1 min. The macerate was centrifuged at 6000 rpm, 10 minutes. Extract (42 ml) was placed in the Amberlite column. The unabsorbed fraction was eluted with bidistilled H_2O and the toxin-free phycobilliproteins solution was obtained. Toxins (microcystins) were adsorbed on the Amberlite and could be eluted with alcoholic solutions. To elute the toxins adsorbed on Amberlite, the column was washed with H_2O, 20% C_2H_5OH, then with 96% C_2H_5OH. The identification of the toxin fractions was performed at 240 nm. The peptidic nature of microcystins was established by reaction with 0.35% ninhydrin.

Partial purification of phycobiliproteins extracts from cyanobacteria *Anabaenopsis* sp. by $(NH_4)_2SO_4$ precipitation. Fractionation of phycobiliproteins was carried out by $(NH_4)_2SO_4$ precipitation (Pandey *et al*., 2011; Chakdar and Pabbi, 2012) with our modification. To 70 ml 9.92 g $(NH_4)_2SO_4$ were added to 25% saturation and after storage 1 hour at 4°C the suspension was centrifuged at 10000 rpm,10 minutes. A pink precipitate containing phycoerythrin was obtained. The precipitate was dissolved in 15 ml of water and after centrifugation the supernatant - (phycoerythrin 1) was collected and the insoluble residue (pink violet precipitate) poorly soluble in water has been removed. Then $(NH_4)_2SO_4$ was added to the supernatant to

60% saturation and, after 1 hour of storage at 4°C, the sample was centrifuged at 10000 rpm, 10 min. The precipitate was dissolved in H_2O and the insoluble residue was removed by centrifugation. The pink violet supernatant was collected (phycoerithrin 3), and the pink residue was dissolved in 1.5 ml H_2O and centrifuged. In the obtained supernatant phycoerythrin 2 was isolated. The preparations of phycoerythrin were further subjected to dialysis for 24 hours against 100 times volume of MilliQ water containing 3 mM sodium azide.

Antioxidant activity assessment of phycoerythrin preparations by ABTS radical cation scavenging assay (Re *et al.,* 1999). Antioxidant activity of phycoerythrin 1 isolated by 25% $(NH_4)_2SO_4$ fractionation, as well as the fractions obtained by 25-60% $(NH_4)_2SO_4$ precipitation - phycoerythrin 2 and 3 was determined by the reaction with the cation $ABTS^+$ (2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid). $ABTS^+$ was generated by oxidation of ABTS with potassium persulfate. 7 mM ABTS solution and potassium persulfate (2.45 mM) were dissolved in deionized water. The reaction mixture was preserved at the room temperature for 12-16 hours in the dark before using. $ABTS^+$ from the stock solution was diluted with ethanol to absorbance at 734 nm of 0.700 ± 0.020. Then 1 ml of diluted $ABTS^+$ solution was mixed with 0.1 or 0.3 ml of the test sample (1.0 mg/ml) and after 6 min the absorbance was measured at 734 nm.

The % inhibition was calculated according to the equation:

$$\% \text{ Inhibition} = [\frac{Abs\ t0 - Abs\ t6}{Abs\ t0}] \text{ x } 100\ \%$$

where $Abs_{t0\ min}$ is the extinction value of the $ABTS^+$ and Abs_{t6min} solution is the extinction value of the $ABTS^+$ solution after 6 min of incubation with the samples. All determinations were performed in 3 replicates.

Results and discussion

Removing of microcystins from Anabaena propinqua phycobiliproteins extract. In the present study the *Anabaena propinqua* phycobiliproteins extract has been analyzed. Freezing and thawing method and subsequent maceration of the frozen mixture, using pestle in a mortar, were selected as efficient way to obtain aqueous extract of phycobiliproteins from *Anabaena propinqua.* The extraction of phycobiliproteins was accompanied by the presence of toxins (microcystins).

Separation of toxins (microcystins) from phycobiliproteins was performed by the chromatographic method on Amberlite XAD-2 (Fig.1). Toxins (microcystins) were adsorbed on the Amberlite and could be eluted with alcoholic solutions. To elute the toxins adsorbed on Amberlite, the column was washed with H_2O, 20% C_2H_5OH, then with 96% C_2H_5OH. The identification of the toxin fractions was performed at 240 nm. The peptide nature of microcystins was established by reaction with 0.35% ninhydrin.

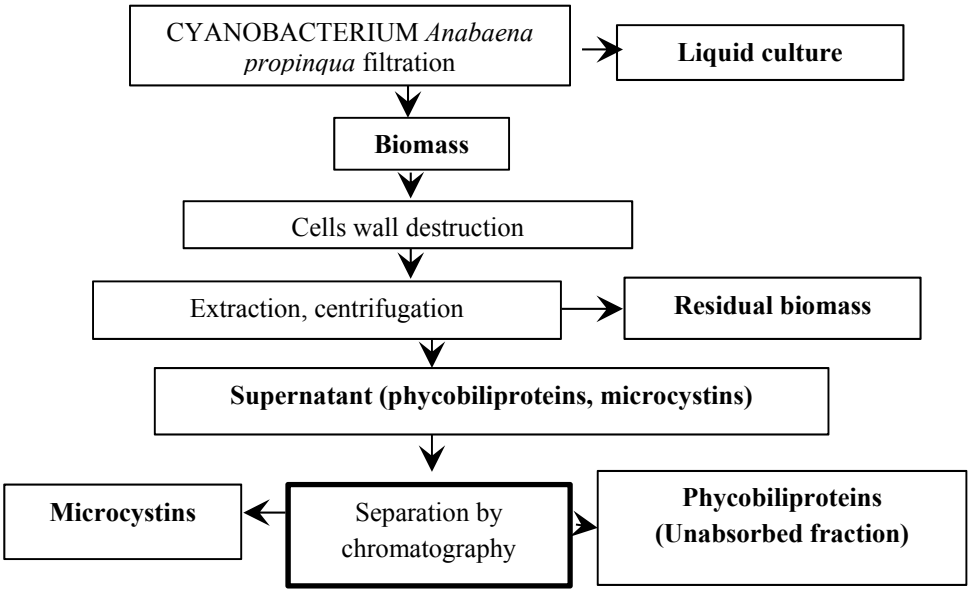


Figure 1. Scheme of microcystins removing from *Anabaena propinqua* phycobiliproteins extract.

Partial purification of Anabaenopsis* sp. *phycobiliproteins by two steps $(NH_4)_2SO_4$ precipitation. As a result of the partial purification of phycobiliproteins aqueous extract by two steps fractionation with $(NH_4)_2SO_4$ (Table 1, Fig. 2) three preparations of phycoerythrin were obtained: phycoerythrin 1 from precipitate isolated after 25% $(NH_4)_2SO_4$ fractionation, phycoerythrin 3 from precipitate obtained by 25-60% $(NH_4)_2SO_4$ fractionation and phycoerythrin 2 from residue resulted after solubilization of the precipitate obtained by 25-60% $(NH_4)_2SO_4$ fractionation.

Table 1.

Partial purification of phycobiliproteins extracts of cyanobacteria *Anabaenopsis* sp by $(NH_4)_2SO_4$ precipitation

Phycoerythrin fractions	**A565**	**A620**	**A650**	**A280**	**Phycoerythrin purity (A565/A280)**
Phycobiliproteins extract	0.400	0.212	0.120	0.400	1.0
Phycoerythrin 1	0.881	0.184	0.122	0.569	1.54
Phycoerythrin 2	1.045	0.220	0.142	0.528	1.98
Phycoerythrin 3	1.210	0.826	0.097	0.481	2.5

Scheme of partial purification of *Anabaenopsis* sp. phycobiliproteins by two steps $(NH_4)_2SO_4$ precipitation is presented in Fig. 2.

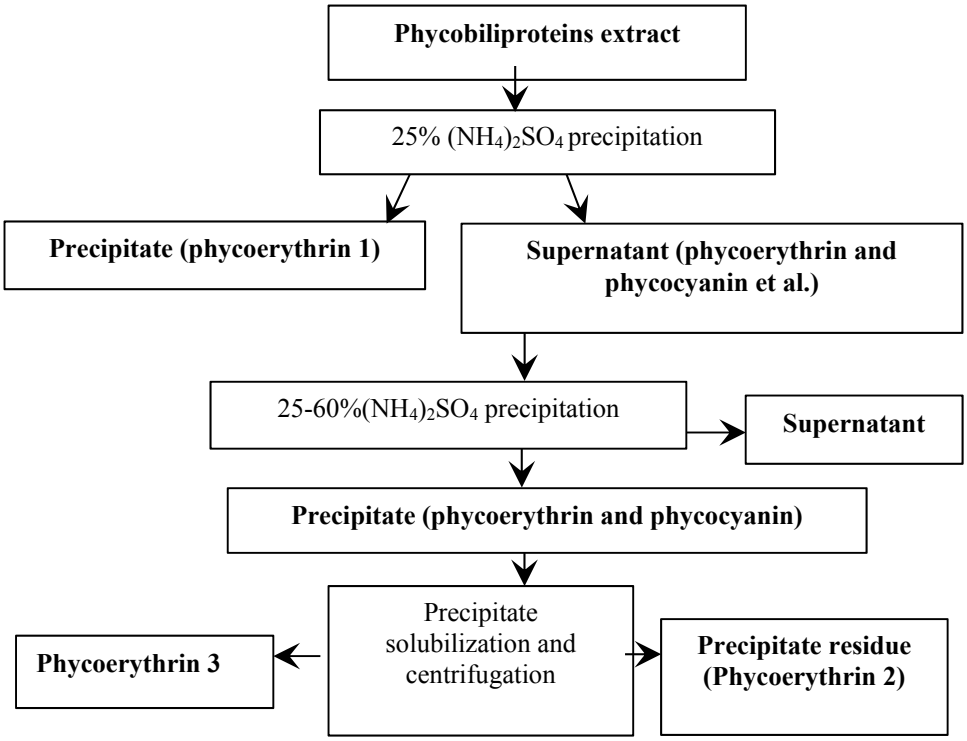
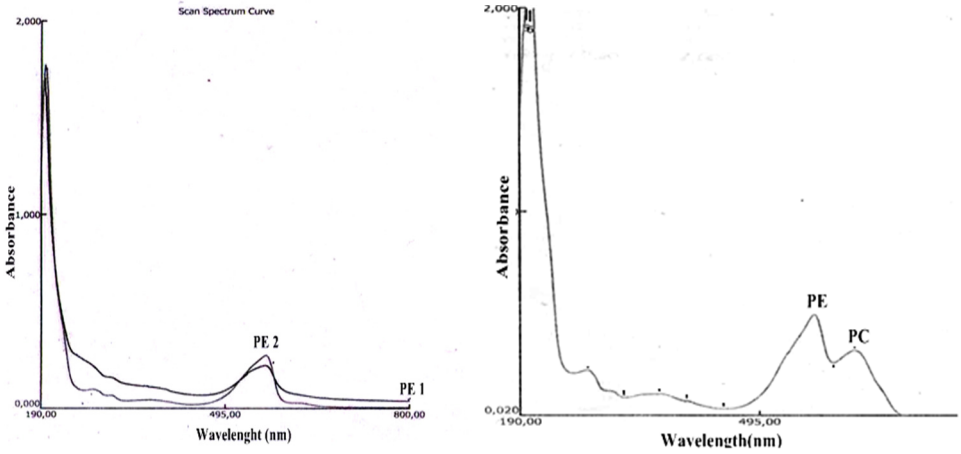


Figure 2. Scheme of partial purification of *Anabaenopsis* sp. phycobiliproteins by two steps $(NH_4)_2SO_4$ precipitation

It has been established that the two consecutive steps of phycobiliproteins purification by $(NH_4)_2SO_4$ precipitation was efficient for partial purification of phycoerythrin from *Anabaenopsis* sp. aqueous extract (Table 1, Fig. 3a, b).

UV-VIS absorbance of phycoerythrin preparations obtained from *Anabaenopsis* sp. extract at the first and second step of fractionation by $(NH_4)_2SO_4$ revealed that the maximum content of phycoerythrin with the highest purity (A_{565}/A_{280}=2.5) was detected in the phycoerythrin fraction obtained by 25-60% $(NH_4)_2SO_4$ precipitation (Fig. 3b). The preparation contains phycocyanin besides phycoerythrin. The purity of phycoerythrin 1 is the lowest (A565/A280=1.54) in comparison with the other phycoerythrin preparations. A high absorbance at 280 nm was observed, that can be connected with presence of ballast proteins in this solution (Fig. 3a).



a b

Figure 3. UV-VIS absorbance of phycoerythrin preparations obtained from *Anabaenopsis sp* extract at the 1-st and second step of purification by $(NH_4)_2SO_4$: a) phycoerythrin 1 (PE1) and phycoerythrin 2(PE2); b) phycoerythrin 3 (PE3), containing both phycoerythrin and phycocyanin (PC)

Antioxidant capacitaty of phycoerithrin preparations obtained from cyanobacteria Anabenopsis* sp. *assessed by ABTS$^+$ method.

Consumption of natural antioxidants, such as phycobiliproteins, that can possibly scavenge free radicals has often been referred as an effective therapeutic option to alleviate free radicals induced cellular damage. Oxidative stress plays a key role in onset and progression of pathophysiological manifestation of many diseases, including cancer. Intra-cellular oxidative stress takes place under conditions of production of excessive ROS that cannot be mitigated by antioxidant defense system.

Partial purified phycoerithrin preparations obtained from cyanobacteria *Anabenopsis* sp. were tested for determination of its antioxidant capacity (Table 2). The obtained results allow us to conclude that the phycoerythrin 3 fraction constituted from phycoerythrin and phycocyanin, obtained from cyanobacterium *Anabaenopsis* sp. after partial purification by 25-60% $(NH_4)_2SO_4$ precipitation, possesses a maximum antioxidant capacity (100%). In case of phycoerythrin 1 and phycoerythrin 2 lower values of antioxidant capacity (24.9 and 27.48%, respectively) are recorded.

Table 2.

Antioxidant capacitaty of phycoerithrin preparations obtained from cyanobacteria A*nabenopsis* sp. determined by $ABTS^+$ method

Sample	**A734**	**A734(0) - 734(exp)**	**% inhibition**	
			0.1 ml	**0.3ml**
Phycoerythrin fraction after the 1-st step of fractionation (25% $(NH_4)_2SO_4$)				
1. ABTS 2. Phycoerythrin 1	0.699±0.04 0.641±0.03	0.058	8.30	24.90
Phycoerythrin fractions after the 2second step of fractionation (25-60%$(NH_4)_2SO_4$)				
3. Phycoerythrin 2 4. Phycoerythrin 3	0.635±0.03 0.464±0.02	0.064 0.235	9.16 33.62	27.48 100

From previous research it was established that cyanobacterium *Anabaena propinqua* contains 7.22–8.87% of phycobiliproteins from biomass, at the cultivation on the Drew media supplemented with NH_4NO_3. So, the phycoerythrin content was prevailing (4.29 – 5.10% of biomass) in comparison with phycocyanin and allophycocyanin content. The content of phycocyanin and allophycocyanin varies between 0.53 to 2.3% and 0.67 to 2.09%, respectively (Bulimaga *et al*., 2014). The high content of phycobiliproteins (up to 8.87%) in cyanobacterium *Anabaena propinqua* biomass makes it a source of perspective for obtaining natural colorants.

The extraction of phycobiliproteins can be carried out using phosphate buffers or distilled water. However, the water extraction is more preferable having the advantage of phycobiliproteins obtaining with a higher yield compared to extraction with buffers (Khatoona *et al*., 2018). The method for rapid phycobiliproteins extraction from cyanobacteria *Synechococcus* CCMP 833 is also known (Viskari and Colyer, 2003). The disadvantage of this method is the necessity in dialysis of phycobiliproteins extract for removing of detergent CHAPS, used in high concentration (3%) for culture cells disruption.

The use of *Anabaena propinqua* as a source of phycobiliproteins is limited due to the presence of microcystins that are extracted together with phicobiliproteins. The research carried out in the present paper has shown that AmberliteXAD-2 (hydrophobic copolymer of styrene-divinylbenzene resin) can be used as efficient adsorbent for microcystins.

The toxin-free phycobilliproteins fraction was not adsorbded on Amberlite column and could be eluted by H_2O. For the separation of microcystins from phycobiliproteins, the authors Ehmann and Guthrie have used other resins, such as

Amberlite™ XAD 16HP, Amberlite™ FPX66, Diaion™ PS-DVB or Sepabeads™ SP70 (Ehmann and Guthrie, 2011, 2015).

As a result of purification of *Anabaenopsis* sp. phycobiliproteins by two steps fractionation with $(NH_4)_2SO_4$, three fractions of phycoerythrin: PE-1, PE-2, and PE-3 were obtained. According to the purity values (A620/A280) of phycoerythrin fractions, the purest fraction is PE-3(2.5) followed by PE-2(1.98) and PE-1(1.54) (Table 1).

Although the PE-3 fraction had a higher purity and could be used as a food and cosmetic pigment, it also contains phycocyanin, besides phycoerythrin (Fig. 3b). Further purification of the PE-3 fraction could be performed by chromatographic methods for use in immunodiagnostics or drug preparations. The other two phycoerythrin fractions contained a higher amount of ballast protein (Fig. 3a).

The scheme proposed in this study can be used to fractionate and obtain partially purified phycoerythrin fraction not only at *Anabaenopsis* sp. and *Anabaena propinqua*, but also to other cyanobacteria.

The analysis of the antioxidant activity of the obtained phycoerythrin fractions revealed the maximum antioxidant capacity of phycoerythrin 3, containing some quantity of phycocyanin. This fact is probably due to their synergistic action. The results are in accordance with the research results of various authors who related the high antioxidant capacity of C-phycocyanin from cyanobacteria *Spirulina platensis* (Bulimaga *et al*., 2012) and *Synechococcus* sp. (Sonani *et al.,* 2017), as well as C-phycoerythrin from *Phormidium* sp. and *Halomicronema* sp. (Madamwar *et al*., 2015).

Conclusions

Separation of *Anabaena propinqua* phycobiliproteins from toxins (microcystins) by the chromatographic method on Amberlite was performed**.** The unabsorbed fraction was eluted with distilled H_2O and the toxins-free phycobilliproteins solution was obtained. The procedure of phycobiliproteins isolation from microcystins was proposed. It has been established that the two consecutive steps of purification of phycoerythrin by $(NH_4)_2SO_4$ precipitation were efficient for partial purification of phycobiliproteins from *Anabaenopsis* sp. aqueous extract. The maximum content of phycoerythrin with the highest purity (A_{565}/A_{280}=2.5) was detected in the phycoerythrin fraction obtained by 25-60% $(NH_4)_2SO_4$ precipitation. The antioxidant capacity of phycoerythrin preparations has been established.

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