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INVESTIGATION OF THE STRUCTURE AND ELEMENT COMPOSITION OF C-PHYCOCYANIN EXTRACTED FROM THE MICROALGAE *SPIRULINA PLATENSIS*

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INTRODUCTION

C-phycocyanin (C-PC) – a special pigment of phycobilin kind – is an element of the blue-green microalga *Spirulina platensis* and it takes an active part in photosynthesis. C-PC absorbs light with a wavelength of 620 nm with a maximum efficiency of the order of 70% and it emits waves with a length of 647 nm.

A great applied interest in the blue-green microalga *Spirulina platensis* is mainly due to that it contains such biologically active substances as gamma-linolenic acid, beta-carotine, C-PC, etc. [1, 2]. In spirulina biomass consisting to 55-70% of proteins playing a vitally important role, the portion of C-PC is 14% on average. It is a globular protein with a complex structure and a molecular weight of 264 000 daltons. The image of the C-PC structure obtained for one of cyanin bacteria by X-ray diffraction with a resolution of 1.66 Å shows what an interesting and complicated organization it has. (http://www.rcsb.org/pdb/cgi/explore.cgi).

Spectral characteristics of C-PC enable its use as fluorescent labels in immunology tests, gel electrophoresis, gel chromatography and isoelectric focusing. C-PC stimulates the immune system is thus a promising means in the treatment of the different kinds of cancer or AIDS.

Having a high radiation protection activity and affinity with such elements as ^{137}Cs and ^{90}Sr, C-PC provides a 66% efficiency of removal of these isotopes when *Spirulina platensis* is used for the rehabilitation of patients, e.g., exposed to radiation during Chernobyl accident in the Ukraine.

In Japan C-PC was used in experiments to treat liver cancer in mice. In [3] the authors discovered that C-PC exhibits hepatoprotective properties when used to treat chemical intoxication in rats. In the last years phycobiliproteins, particularly C-PC, have been tested in photodynamic therapy of tumors in humans [4].

In connection with a great scientific and applied interest in C-phycocyanin-based products there are being carried out intense investigations of the different levels of its organization and physicochemical properties of importance for the use of C-phycocyanin for the purposes of medical treatment and prophylaxis of diseases.

PROBLEM

The processes of photosynthesis in the cells of blue-green algae go with participation of special organelles called phycobilisomes where three types of pigments, phycoerythryn (PE), phycocyanin (PC) and allophycocyanin (APC), are located.

In phycobilisomes light transfer has the so-called cascade type: PE $\rightarrow$ PC$\rightarrow$ APC $\rightarrow$ chlorophyll *a*.

The proportion of phycobilin pigments in different alga species varies greatly and it depends on the conditions they were grown in, including nutrient medium composition, pH, temperature, lighting, etc. [5-8].

Phycobiliproteins are acid water-soluble globular proteins composed of α and β polypeptide chains with one or two covalently conjugated chromophore groups that form four linear pyrrol rings with two carboxyl groups. The spectral characteristics of phycobiliproteins (absorption and fluorescence) are both due to the presence of chromophore groups and the existence of tertiary and quaternary structures of protein globula.

C-PC extracted from *Spirulina platensis* consists of α and β polypeptide chains with one chromophore group being attached to an α chain and two chromophore groups attached to a β chain. The primary structure of α and β polypeptide chains in C-PC from the different biological objects was studied in [9, 10]. C-PC in solution is a mixture of monomer $(\alpha\beta)$, trimer $(\alpha\beta)_3$ and hexamer $(\alpha\beta)_6$ structures. Depending on the temperature, pH and other conditions, the quantitative proportion of these structures changes greatly.

The goal of the reported work was to study the structural organization and element composition of C-PC extracted from the blue-green microalga *Spirulina platensis*. The investigation of the behavior of metals in the process of C-PC purification is of special interest for the understanding of their possible role in the formation of biocomplexes of the protein-metal-chromophore type and estimation of C-PC-based product safety for medical purposes [11].

MATERIALS AND METHODS

To carry out experiments, we used the *Spirulina platensis* IPPAS B-265 strain from Timiriazev Institute for Plant Physiology of the Russian Academy of Sciences.

The *Spirulina platensis* was cultivated in a standard Zaroukh nutrient mineral medium for 5 days following the procedure described in [12]. The harvested and rinsed in mineral water biomass of *Spirulina platensis* was centrifuged and the precipitate was freeze-dried in a special absorption-condensation lyophilizer that we designed [13].

C-PC was extracted from the *Spirulina platensis* biomass using a modified version of the Teal and Dale method [14]. The procedure involves a cycle of protein purification operations accompanied with spectrophotometric purity checks of the preparation by the ratio of absorption peaks at λ = 620 and 280 nm (D_{620}/D_{280}). If $D_{620}/D_{280} > 4$, the C-PC preparation is considered to have a high purity.

Spirulina platensis cells were destroyed by lysozyme in a 0.1 M Na-K phosphate buffer, pH being 6.0. The obtained extract containing phycobiliproteins together with other proteins was separated from the residue of destroyed cells by centrifugation. To perform preliminary purification of the preparation, different volumes of water saturated ammonium sulphate $(NH_4)_2SO_4$ were added to the supernatant. The final purification of C-PC was performed by its repeated transmission through a chromatographic column filled with DEAE cellulose equilibrated in a 0.05 M acetate buffer with NaCl at pH 5.2 followed with dialysis against de-ionized water.

The obtained C-PC preparation was freeze-dried and then formed into 50 mg pellets using a titanium press mould to conduct neutron activation analysis.

Earlier we used epithermal neutron activation analysis (ENAA) to determine background concentrations of macro- and microelements in the *Spirulina platensis* biomass [15]. The works by Italian scientists [16, 17] are also devoted to neutron activation analysis of *Spirulina platensis*.

The C-PC samples were irradiated with a neutron flux of 10^{12} n/(cm^2·s) having a high ratio of epithermal to thermal neutrons at the IBR-2 pulsed neutron reactor in FLNP JINR (Dubna). The irradiation channels of the pneumatic transport system and the spectrometer facility of the activation analysis laboratory are detailed in [18]. The ENAA technique of biological sample investigations as applied to *Spirulina platensis* is described in detail in [19].

The investigation of the structural peculiarities of C-PC was carried out by capillary electrophoresis – an effective method of protein from nuclei acid separation getting a firm foothold in ecology, monitoring, biotechnologies, etc. today.

The C-PC experiments were conducted with a capillary electrophoresis facility (CE) of our design described in [20]. The facility employs a flexible silicon capillary with a flare up to 200 μm in the detection area of the type HP – Extended Light Path Capillaries.

To reduce the effect of proteins absorption by the walls of the capillary, electrophoresis was performed in a running buffer at pH 4.8 in the vicinity of the C-PC isoelectric point (I = 4.65). In the regime of cathode detection the mobility of protein subunits and their aggregated is mainly determined by the electroosmotic flux in the direction from anode to cathode.

RESULTS AND DISCUSSION

The results of C-PC capillary electrophoresis experiments are illustrated in Figs. 1 and 2 respectively showing examples of separation of subunits and of their aggregates for PC preparations with different degrees of purity. In the process of CE separate components of the investigated C-PC preparation go through the capillary at different velocities depending on their mass and physicochemical properties. The electrophoregrams show the separation of fragments in time and make it possible to judge about their relative numbers by the intensity of the absorption bands for the light wavelength λ=610 nm.

Figure 1 shows the electrophoregram of a low purity C-PC preparation from where it is seen that α- and β-subunits are not resolved. They form an intense absorption band with a migration time of about 10 min. This band is seen against the background of intense and far from being resolved bands of impurity components. This is observed both in low molecular and high molecular parts of the spectrum.

Figure 2 shows the electrophoregram of a pure C-PC preparation obtained by a repeated purification procedure using a chromatographic column.

Sufficiently well resolved α- and β-subunits with a migration period of 19 or 21 min are clearly seen as well as the lines due to hexamers with a migration period of an order of 28 min.

In addition to them there are observed less intense but quite resolved fragments which actually could be the chains of high molecular C-PC structures.

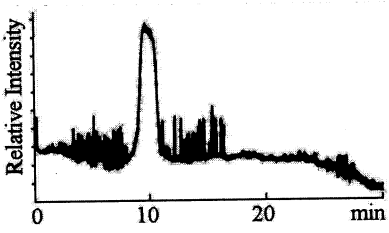

Fig. 1. CE of phycocyanin. The degree of purity (D_{615}/D_{280}) nm in the interval 2-3; conditions of separation: 20 mM phosphate at pH 4.8; silicon capillary: 50/56 cm; 75 μm ID; voltage 10 kV; current 30 μA; $t_{inj.}$ = 30 s (hydrostatically); λ=610 nm.

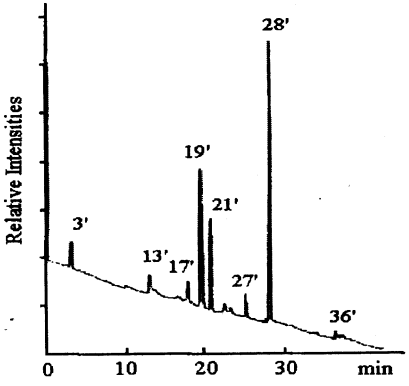

Fig. 2. CE of phycocyanin. The degree of purity (D_{615}/D_{280}) nm in the interval 4.0-4.4; conditions of separation: 20 mM phosphate at pH 4.65; silicon capillary: 37/40 cm; 75 μm ID; voltage 12 kV; current 30 μA; $t_{inj.}$ = 30 s (hydrostatically); λ=610 nm.

The ENAA method was applied to study pellet samples of native *Spirulina platensis* biomass, low purity C-PC (D_{620}/D_{280} = 2.04) and of high purity C-PC ($D_{620}/D_{280} \geq 4$).

The results obtained for long-lived isotopes are summarized in Table 1. The sample irradiation time was 5 days. After irradiation the samples were rewrapped and measured twice in 4 and 20 days. The measuring time for the different elements varied from 1.5 to 10 hours.

Table 1. The ENAA results of *Spirulina platensis* and C-PC extract samples

Element	Content in *Spirulina pl.* biomass, mg/kg	Error, ± %	Content in low purity C-PC, mg/kg	Error, ± %	Content in high purity C-PC, mg/kg	Error ± %
K	20 000	8	183	20	287 800	10
Na	13 050	15	1017	10	42 770	15
Fe	3 800	20	1400	8	2 200	10
Sr	1<	-	3.950	33	77	30
Cr	5.6	10	41.00	16	48	15
Mo	0.39	20	5.510	5	11	4
As	0.57	30	0.19	11	10	11
Ni	1.3	11	14.300	14	9	20
Au	0.068	15	0.00814	5	7	20
Ag	0.63	15	0.72	10	6.7	20
Ba	9.80	10	42	10	5.9	15
Rb	0.12	12	0.146	20	4,0	12
Co	0.10	13	0.018	10	3.1	10
Br	0.53	12	0.530	20	0.7	5
Hg	0.0035	30	-	-	0.61	30
Sb	0.065	10	0.219	10	0.55	16
Se	0.1<		-	-	0.08	27
Zn	10	12	68.3	10	385	14
W	2.5	10	0.408	8	0.5	50
Sm	0.0054	10	0.0023	25	0.1	40

Increased concentrations of K and Na in the samples of pure C-PC are due to that the C-CP solution was freeze-dried in a Na-K phosphate buffer at pH 6.0.

The assessment of elements concentrations in C-phycocyanin after its purification makes it possible to suggest which of the studied metals could take part in the formation of macromolecular complexes with C-phycocyanin of the protein-metal-chromophore type. It should be noted that a comparative estimation of the concentrations of metals in the investigated preparations was performed accounting for the fact that C-PC is a constituent part of proteins composing 55-70% of the spirulina biomass and its portion is 14% of the total amount of the proteins.

According to estimation taking into account the percentage of C-PC metals in *Spirulina platensis* may follow the sequence: Zn>Cr>Ni>Co>As>Sr>Mo>Ag>Hg.

The content of such toxic metals as Hg, As, Sr, etc. does not exceed the presently accepted off-limit for the human organism (see. http://www.spirulina.com).

Thus, the obtained C-PC preparations can be used for the purposes of pharmacology both in a pure form and after being purposely loaded with some elements. The ENAA method can be successfully applied to perform control of toxic element contents in C-PC, which is extremely important in early stages of such investigations [11].

The obtained results could be of use for the improvement of our understanding of the structural peculiarities of C-PC and further investigations of the specifics of the interaction of heavy and toxic metals with C- phycocyanin.

CONCLUSIONS

1. The behavior of α- and β-subunits in the process of C-PC purification is studied by the method of capillary electrophoresis.
2. A good resolution of α- and β-subunits and of hexamer lines $(\alpha\beta)_6$ is obtained for high purity C-PC.
3. It is shown that capillary electrophoresis can be successfully applied to study the structural peculiarities of C-PC and the role of heavy and toxic metals in the formations of macromolecular complexes. The concentrations of 20 elements are estimated by the ENAA method for C-PC with different degrees of purity.
4. It is established that some of the investigated metals (Zn, Ni, Sr, Cr, Co, Mo, etc.) are still present in C-PC after it is purified, which means that they could be present in the composition of macromolecular complexes with C-PC of the protein-metal-chromophore type.
5. It is shown that the content of toxic metals in C-PC does not exceed the presently accepted off-limit and C-PC can be thus used, both in a pure form and in complex with appropriate elements, to prepare pharmaceuticals.

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Исследование структуры и элементного состава С-фикоцианина, выделенного из клеток сине-зеленой микроводоросли *Spirulina platensis*

Исследованы структурные особенности и элементный состав С-фикоцианина, выделенного из клеток сине-зеленой микроводоросли *Spirulina platensis*. Методом капиллярного электрофореза изучено поведение структурных субъединиц, образующих фикобилисомы в процессе очистки С-фикоцианина. Определено их соотношение для очищенного С-фикоцианина. Методом эпитеплового нейтронного активационного анализа изучен элементный состав С-фикоцианина различной степени чистоты и определена совокупность металлов (Zn, Cr, Ni, Co, As, Sr, Mo, Ag, Hg), способных принимать участие в образовании макромолекулярных биокомплексов с С-фикоцианином. Показано, что содержание таких токсичных металлов, как Hg, As, Sr и др., не превышает уровня, допустимого для человеческого организма.

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Перевод авторов

Mosulishvili L. M. et al. D14-2002-7
Investigation of the Structure and Element Composition of C-Phycocyanin Extracted from the Microalgae *Spirulina platensis*

The structure and element composition of C-phycocyanin (C-PC) extracted from the blue-green alga *Spirulina platensis* were studied. The behavior of structural subunits forming phycobilisomes in the purification process was studied by capillary electrophoresis. Their proportion in high-purity C-PC was determined. The element composition of C-PC of different purity was studied by means of epithermal neutron activation analysis, and metals which may form macromolecular complexes with C-PC were determined (Zn, Cr, Ni, Co, As, Sr, Mo, Ag, Hg). It was shown that contents of toxic metals did not exceed accepted permissible levels for the human organism.

The investigation has been performed at the Frank Laboratory of Neutron Physics, JINR and at the E. L. Andronikashvili Institute of Physics of the Georgian Academy of Sciences.

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