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Biosynthesis of ^{2}H-labeled Photochrome Trans-membrane Protein Bacteriorhodopsin by Halobacterium *Halobacterium Halobium*

[1] Oleg Mosin
[2] Ignat Ignatov

[1] Moscow State University of Applied Biotechnology, Russian Federation
Senior research Fellow of Biotechnology Department, Ph. D. (Chemistry)
103316, Moscow, Talalihina ulitza, 33
E-mail: mosin-oleg@yandex.ru
[2] The Scientific Research Center of Medical Biophysics (SRC MB), Bulgaria
Professor, D. Sc., director of SRC MB.
1111, Sofia, N. Kopernik street, 32
E-mail: mbioph@dir.bg

Abstract

The semi-preparative biosynthesis of photochrome trans-membrane protein bacteriorhodopsin (output 8–10 mg), labeled with deuterium on functionally important amino acid residues – [2,3,4,5,6-$^{2}H_5$]phenylalanine, [3,5-$^{2}H_2$]tyrosine, and [2,4,5,6,7-$^{2}H_5$]tryptophan was carried out with using a photo-organotrophic halobacterium *Halobacterium halobium*. The protein was isolated from purple membranes by cellular autolysis by distilled water, processing of bacterial biomass by ultrasound at 22 KHz, alcohol extraction of low and high-weight molecular impurities, cellular RNA, carotenoids and lipids, with the subsequent solubilization of final product with 0,5 % (w/v) SDS-Na and fractionation by methanol. The homogenity of the synthesized bacteriorhodopsin and the selectivity of deuterium incorporation into the molecule was proved by combination of preparative and analytical protein methods including elecrtophoresis in 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na, gel filtration chromatography on Sephadex G-200, reverse-phase HPLC and electron impact mass-spectrometry of methyl esters of N-Dns-[^{2}H]derivatives of amino acids.

Keywords: *Halobacterium halobium*; bacteriorhodopsin; [2,3,4,5,6-$^{2}H_5$]Phe; [3,5-$^{2}H_2$]Tyr; [2,4,5,6,7-$^{2}H_5$]Trp; biosynthesis; electron impact mass-spectrometryб RP HPLC.

Introduction

Bacteriorhodopsin (BR), named by analogy to the visual apparatus of mammalian protein rhodopsin, was isolated from the cell membrane of extreme photo-organotrophic halobacteria *Halobacterium halobium* in 1971 by W. Stohenius and D. Osterhelt [1]. This photo-transforming trans-membrane protein with the molecular weight ~26,5 kDa represents chromoprotein determining the purple-red culour of halophilic bacteria, which contains as chromophore group an equimolar mixture of 13-*cis*-and 13-*trans*-retinol C20 carotenoid, bound by Schiff base (as in the visual animal pigments) with Lys-216 residue of the protein [2]. Along with the BR the cell

membrane of halobacteria contains a small amount of other related carotenoid pigments, the primary of which bakterioruberin determining the stability of halobacteria to solar radiation.

In its structure and location in the cell membrane BR refers to integral transmembrane proteins, penetrating the cell membrane, which is divided into three fractions: yellow, red and purple. Purple membrane (PM) fraction comprising on 75 % (w/w) of cell membrane consists from carotenoids, phospholipids (mostly phosphoglycerol diesters with a small amount of nonpolar lipids and isoprenoids) forms a natural two-dimensional crystals which can be investigated using X-ray scattering. BR molecule has seven α-helical protein segments with with symmetrically located retinal residue in the middle. These α-helical segments, arranged in PM in an orderly manner forming trimers with an average diameter ~0,5 μm and a thickness 5–6 nm; each trimmer is surrounded by six others so that to form a regular hexagonal lattice. The BR molecule is arranged in a direction perpendicular to the plane of the membrane, hydrophobic domains represent transmembrane segments and hydrophilic domains protruding from the membrane, connect the individual α-helical intramembranous segments of the BR molecules.

Owing to its structure, BR acts as a light-dependent proton pump, pumping protons across the cell membrane and generates an electrochemical gradient of H^+ on the surface of the cell membrane, which energy is used by the cell for the synthesis of ATP in the anaerobic photosynthetic phosphorylation. This mechanism is called “non-chlorophyll photosynthesis”, in contrast to the plant photosynthesis implemented with the participation of chlorophyll. In this mechanism, at absorbtion of a light photon BR molecule became decolorized by entering into the cycle of photochemical reactions, resulting in the release of a proton to the outside of the membrane, and the absorption of proton from intracellular space [3]. By the absorption of a light photon is occured reversible isomerization of 13-*tras*-BR (λ_{max} = 548 nm) (the quantum yield 0,03 at t = 22 ^oC) into the 13-*cis*-BR ((λ_{max} = 568 nm)), initiating a cascade of photochemical reactions lasting from 3 ms to 1 ps with the formation of transitional intermediates J, K, L, M, N, and O, followed by separation of H^+ from the retinal residue of BR and its connection from the side of cytoplasm (Fig. 1). As a result, between the internal and external surface of the membrane forms a concentration gradient of H^+, which leads that illuminated halobacteria cells begin to synthesize ATP, i.e. convert light energy into energy of chemical bonds. This process is reversible and in the dark flows in the opposite direction, allowing halobacteria develop in the dark by means of switching the heterotrophic photosynthetic metabolism. Thus, the BR molecule behaves as a photochromic carrier with a short relaxation time – the transition from the excited state to the ground state. Optical characteristics for BR are dynamic and vary depending on the method for preparation of purple membranes (PM), the polymer matrix and its composition.

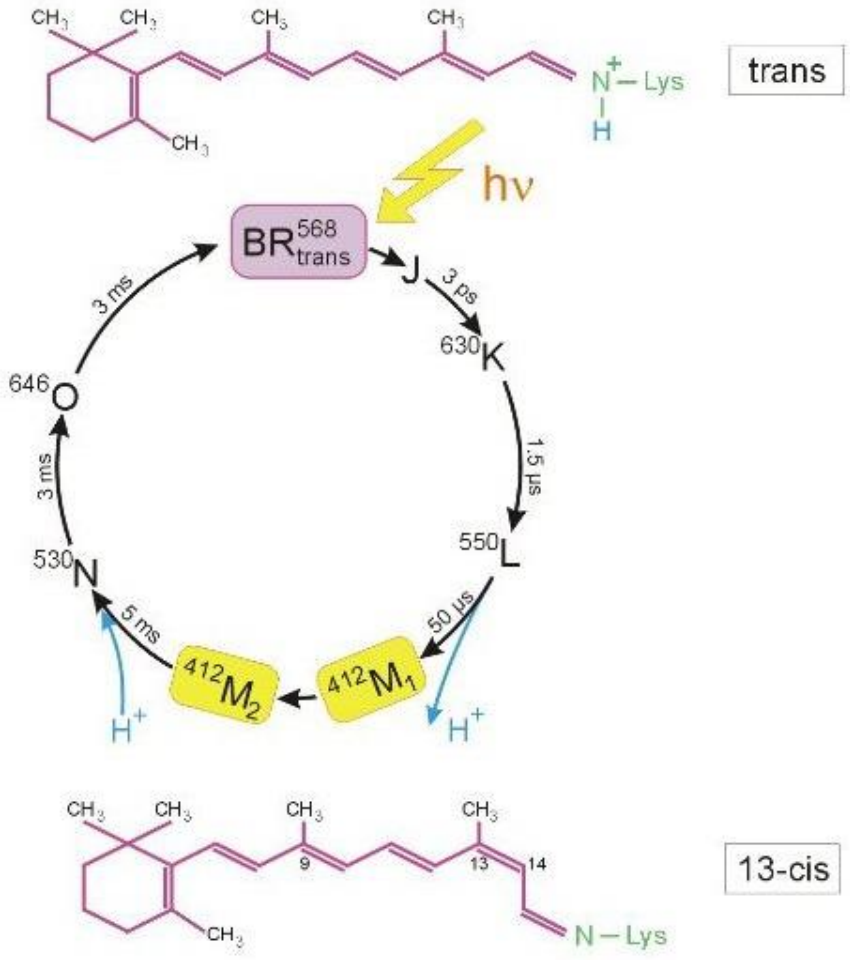


Figure 1. Photocycle scheme of BR (aqueous solution, pH = 7,2, t = 22 °C). Latin numbers J, K, L, M, N, O denote the spectral intermediants of BR. M_1 and M_2 represent spectral intermediants of *meta*- bacteriorhodopsin with the protonated and deprotonated aldimine bond. The superscripts correspond to the position of the absorption maximum intermediatorov photocycle (nm).

BR is the focus of bio-and nanotechnology mainly because of its high sensitivity and resolution, and is used in molecular bioelectronics as natural photochromic material for light-controlled electrical regulated computer modules and optical systems [4–6]. Additionally, BR is very attractive as a model for studies of functional activity and structural properties of photo-transforming membrane proteins in the native and photo-converting membranes. For these studies it is useful to enter into the protein molecule a deuterium label (^{2}H), which allows to apply for evaluating of the structure by NMR-method [7]. In this aspect great scientific and practical interst has BR labeled with deuterium on the residues of functionally important aromatic amino acids – phenylalanine, tyrosine and tryptophan involved in the hydrophobic interaction of the polypeptide chain of the protein with the lipid bilayer of the cell membrane. ^{2}H-labeled aromatic amino acids can be synthesized in gram scale quntities by isotopic exchange (H-^{2}H) in molecules of the protonated amino acids; for analyzing deuterium enrichment levels the EI mass-spectromerty of N-5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl) amino acid derivatives may be used [8–10].

The purpose of this research was to study the micro preparative biosynthesis of BR labeled with deuterium on the residues of functionally important aromatic amino acids – [2,3,4,5,6-$^2H_5$]phenylalanine, [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan, for the reconstruction of

artificial membranes, as well as to study of the levels of deuterium enrichment of the BR molecule by EI mass spectrometry in combination with RP HPLC.

Material and methods

As a BR producer was used a carotenoid strain of extreme photo-organotrophic halobacterium *Halobacterium halobium* ET 1001, obtained from Moscow State University (Russia). The strain was modified by selection of individual colonies on solid agarose media with 2 % (w/v) peptone and 4,3 M NaCl.

For the synthesis of aromatic [^{2}H]amino acids were used the protonated *D*,*L*-amino acids ("Reanal", Hungary), ^2H_2O (99,9 atom.% ^{2}H), ^{2}HCl (95,5 atom.% ^{2}H), and $^2H_2SO_4$ (97,5 atom.% ^{2}H), purchased from the Russian Research Center "Isotope" (St. Petersburg, Russia). For the synthesis of methyl esters of DNS-amino acids used 5-(dimethylamino) naphthalene-1-sulfonyl chloride (Dns-chloride) ("Sigma", USA) and diazomethane, prepared from N-nitrosourea ("Merck", Germany). The inorganic salts were crystallized in 99,9 atom.% ^2H_2O, ^2H_2O was distilled over $KMnO_4$ with subsequent control of the isotope purity by ^{1}H-NMR spectroscopy on a Brucker WM-250 ("Brucker Daltonics" Germany) with a working frequency 70 MHz (internal standard Me_4Si).

Chemical synthesis of *L*-[2,3,4,5,6-$^2H_5$]phenylalanine (95 atom.% ^{2}H), *L*-[3,5-$^2H_2$]tyrosine (96 atom.% ^{2}H) and *L*-[2,4,5,6,7-$^2H_5$]tryptophan (98 atom.% ^{2}H) was performed according to the method of isotopic exchange (^{1}H-^{2}H) in molecules of protonated aromatic amino acids, reported yealier [11].

Synthesis of methyl esters of N-Dns-[^{2}H]amino acids was performed as discribed in the article [12].

Biosynthetic BR (yield 8–10 mg from 1 g biomass) was obtained in synthetic (SM) medium in which the protonated phenylalanine, tyrosine and tryptophan were replaced by their deuterated analogs – [2,3,4,5,6-$^2H_5$]phenylalanine, [3,5-$^2H_2$]tyrosine, and [2,4,5,6,7-^{2}H]tryptophan (g/l): *D*,*L*-alanine – 0,43; L-arginine – 0,4; *D*,*L*-aspartic acid – 0,45; *L*-cysteine – 0,05; *L*-glutamic acid – 1,3; *L*-lycine – 0,06; *D*,*L*-histidine – 0,3; *D*,*L*-isoleucine – 0,44; *L*-leucine – 0,8; *L*-lysine – 0,85; *D*,*L*-methionine – 0,37; *D*,*L*-phenylalanine – 0,26; *L*-proline – 0,05; *D*,*L*-serine – 0,61; *D*,*L*-threonine – 0,5; *L*-tyrosine – 0,2; *D*,*L*-tryptophan – 0,5; *D*,*L*-valine – 1,0; AMP – 0,1; UMP – 0,1; NaCl – 250; $MgSO_4 \cdot 7H_2O$ – 20; KCl – 2; NH_4Cl – 0,5; KNO_3 – 0,1; KH_2PO_4 – 0,05; K_2HPO_4 – 0,05; Na^+-citrate – 0,5; $MnSO_4 \cdot 2H_2O$ – $3 \cdot 10^{-4}$; $CaCl_2 \cdot 6H_2O$ – 0,065; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $FeSO_4 \cdot 7H_2O$ – $5 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$; glycerol – 1,0, biotin – $1 \cdot 10^{-4}$; folic acid – $1{,}5 \cdot 10^{-4}$, vitamin B_{12} – $2 \cdot 10^{-5}$. The growth medium was autoclaved for 30 min at 0,5 atm, the pH value was adjusted to 6,5–6,7 with 0,5 M KOH. Bacterial growth was performed in 500 ml Erlenmeyer flasks (volume of the reaction mixture 100 ml) for 4–5 days at 35 °C on a shaker ("Birad Labs", Hungary) under intense aeration and monochromatic illumination (3 lamps × 1,5 lx). All further manipulations with BR isolation were carried out with the use of a photomask lamp equipped with an orange light filter.

Biomass (1 g) was washed with distilled water and pelleted by centrifugation on T-24 centrifuge ("Carl Zeiss", Germany) (1500 g, 20 min). The precipitate was suspended in 100 ml of dist. H_2O and kept for 3 h at 4 °C. The reaction mixture was centrifuged (1500 g, 15 min), the pellet was resuspended in 20 ml dist. H_2O and disintegrated by infrasound sonication (22 kHz, 3 times × 5 min) in an ice bath (0 °C). The cell homogenate after washing with dist. H_2O was resuspended in 10 ml of buffer containing 125 mM NaCl, 20 mM $MgCl_2$, and 4 mM Tris-HCl (pH = 8,0), then 5 mg of RNA-ase (2–3 units of activity) was added . The mixture was incubated for 2 h at 37 °C. Then 10 ml of the same buffer was added and kept for 10–12 h at 4 °C. The aqueous fraction was separated by centrifugation (1500 g, 20 min), the precipitate of cell debris containing purple membranes (PM) was treated with 50 % (v/v) ethanol (5 times × 7 ml) at 4 °C followed by separation of the solvent. This procedure was repeated 6 times to give a colorless washings. The protein content in the samples was determined spectrophotometrically on DU-6 spectrophotometer ("Beckman Coulter", USA) by the ratio D_{280}/D_{568} ($\varepsilon_{280} = 1{,}1 \cdot 10^5$; $\varepsilon_{568} = 6{,}3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [13]. PM regeneration is performed as described in [14]. Yield of PM fraction 120 mg (80–85 %).

For isolation of BR the fraction of PM (in H_2O) (1 mg/ml) was dissolved in 1 ml of 0,5 % (w/v) SDS-Na, and incubated for 5–7 h at 37 °C followed by centrifugation (1200 g, 15 min). The

precipitate was separated, than methanol was added to the supernatant in divided portions (3 times × 100 ml) at 0 °C. The reaction mixture was kept for 14–15 h in ice bath at 4 °C and then centrifuged (1200 g, 15 min). Fractionation procedure was performed three times, reducing the concentration of 0,5 % SDS-Na to 0,2 and 0,1 % (w/v). Crystal protein (output 8–10 mg) was washed with cold distilled. ^2H_2O (2 times × 1 ml) and centrifuged (1200 g, 15 min). Protein sample (5 mg) was dissolved in 100 ml of buffer solution and placed on a column (150 × 10 mm), stationary phase – Sephadex G-200 ("Pharmasia", USA) (specific volume packed beads – 30–40 units per 1 g dry Sephadex) equilibrated with buffer containing 0,1 % (w/v) SDS-Na and 2,5 mM ETDA. Elution proceeded by 0,09 M Tris-borate buffer containing 0,5 M NaCl, pH = 8,35 at a flow rate 10 ml/cm^{2}h. Combined protein fraction was subjected to freeze-drying, in sealed glass ampoules (10 × 50 mm) and stored in frost camera at -10 °C.

Electrophoresis of BR was performed in 12,5 % (w/v) polyacrylamide gel (PAAG) containing 0,1 % (w/v) SDS. The samples were prepared for electrophoresis by standard procedures (LKB protocol, Sweden). Electrophoretic gel stained with Coomassie blue R-250 was scanned on a CDS-200 laser densitometer ("Beckman", USA) for quantitative analysis of the protein level.

For hydrolysis of BR 4 g of dry protein was placed into glass ampoules (10 × 50 mm), and 5 ml 4 N $Ba(OH)_2$ was added. The mixture was kept at 110 °C for 24 h. The reaction mixture was suspended with 5 ml of hot distilled water and neutralized with 2 N H_2SO_4 to pH = 7,0. The precipitate of $BaSO_4$ was removed by centrifugation at 200 g for 10 min, and the supernatant was evaporated in a rotor evaporator at 40 °C.

RP-HPLC was carried out on a liquid chromatograph Knauer Smartline ("Knauer", Germany) equipped with a pump Gilson ("Gilson Inc.", USA), UF-2563 detector and integrator CR-3A ("Shimadzy", Japan) using a column (250 × 10 mm) with a stationary reverse phase Separon C18 ("Kova", Slovakia), eluent: (A) – acetonitrile-trifluoroacetic acid 100:0,1–0,5 % (v/v) and (B) – acetonitrile – 100 % (v/v); elution rate – 1,5 ml/min; gradient: from 0 to 20 % B – 5 min, 20 to 100 % B – 30 min, 100 % B – 5 minutes, 100 % B to 0 – 2 min 0 % B – 10 min.

EI mass spectra of methyl esters of N-Dns-amino acid derivatives were obtained by electron impact on MB-80A device ("Hitachi", Japan) at energy of ionizing electrons 70 eV, accelerating voltage of 8 kV and the cathode temperature 180–200 °C. Scanning was carried out at a resolution of 7500 relative units using 10 % sharpness.

Results and discussion

The strategy for biosynthesis of ^{2}H-labeled BR using a strain of extreme photo-organotrophic halobacterium *Halobacterium halobium* was determined by the study of the principal possibility for obtaining ^{2}H-labeled photochrome transmembrane proteins in semi-micropreparative quantities for reconstruction of artificial membranes in ^2H_2O and BR-containing nanofilms. [2,3,4,5,6-$^2H_5$]phenyIalanine, [3,5-$^2H_2$]ryrosine, and [2,4,5,6,7-^{2}H5;]tryptophan play important role in hydrophobic interacting of BR molecule with the lipid bilayer of the cell membrane. They are resistant to the isotopic exchange (H-^{2}H) reactions in aqueous solutions under growth conditions and may be easily detected by IE spectrometry after the derivatization to the methyl esters of N-Dns-amino acids. ^{2}H-labeled BR obtained by growth of halobacterium *H. halobium* on synthetic medium (4,3 M NaCl) with [2,3,4,5,6-$^2H_5$]phenylalanine, [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan. Under optimal growing conditions (incubation period 4–5 days, temperature 35 °C, illumination with monochromatic light at λ = 560 nm) in cells were synthesized the purple carotenoid pigment, the spectral ratio of protein and chromophore molecule fragments D_{280}/D_{568} = 1,5 : 1,0 in which was identical to the natural BR. The growth of the halobacterium on a synthetic medium (Fig. 2, *b*) was practically as the same as in the control (Fig. 2, *a*) on the protonated growth medium, that significantly simplifies the optimization of conditions for the biosynthesis of ^{2}H-labeled BR, which consists in the equivalent replacing of protonated aromatic amino by their deuterated analogues – [2,3,4,5,6-$^2H_5$]phenylalanine (0,26 g/l), [3,5-$^2H_5$]tyrosine (0,2 g/l) and [2,4,5,6,7-$^2H_5$]tryptophan (0,5 g/l).

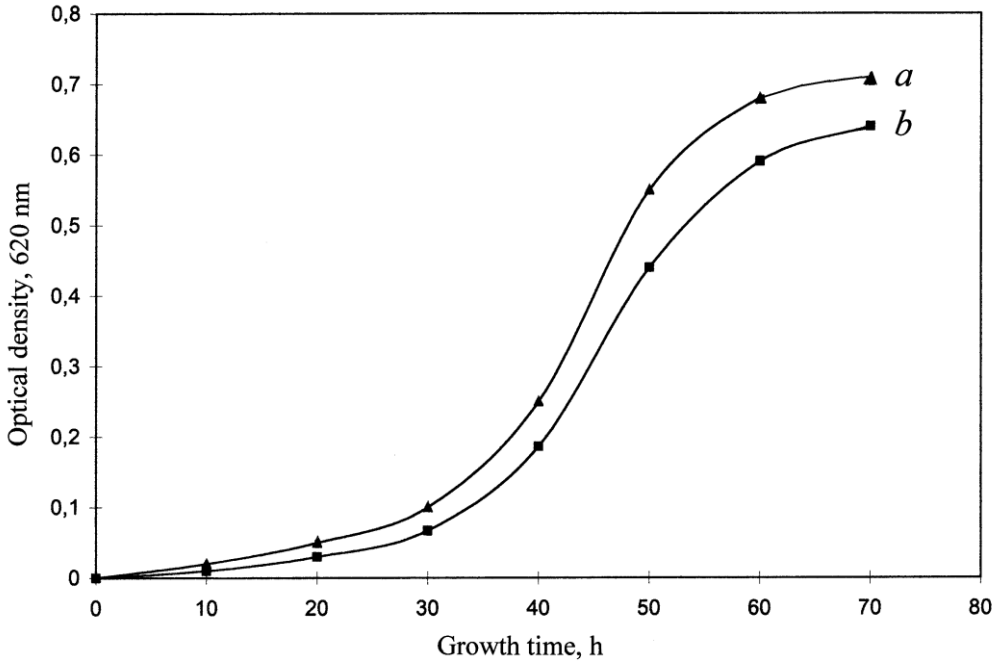


Figure 2. Growth dynamics of *H. halobium* under various experimental conditions: (*a*) – protonated synthetic medium; (*b*) – synthetic medium with [2,3,4,5,6-$^2H_5$]Phe (0,26 g/l), [3,5-$^2H_2$]Tyr (0,2 g/l) and [2,4,5,6,7-$^2H_5$]Trp (0,5 g/l). The incubation period: 4–5 days, temperature: 35 °C, illumination under monochrome light at λ = 560 nm.

The main stages of the experiment were: growing of a strain-producer *H. halobium* on synthetic medium with [2,3,4,5,6-$^2H_5$] phenylalanine (0,26 g/l), [3,5-$^2H_2$] tyrosine (0,2 g/l) and [2,4,5,6,7-$^2H_5$] tryptophan (0,5 g/l), the separation of cell content, isolation of purple membrane fraction (PM), the separation of low-and high-molecular impurities, cellular RNA, pigments (preferably carotenoids) and lipids, fractionation of solubilized in 0,5 % (w/v) SDS-Na protein by methanol, purification on Sephadex G-200, electrophoresis 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na. Because protein is localized in the PM, the release of low molecular weight impurities and intracellular contents was reached by osmotic shock of cells with distilled water in the cold after the removal of 4,3 M NaCl and the subsequent destruction of the cell membrane by ultrasound at 22 kHz. For the destruction of cellular RNA the cellular homogenate was treated with Rnase I. Fraction PM along with the desired protein in a complex with lipids and polysaccharides also contained impurity of related carotenoids and proteins. Therefore, it was necessary to use special methods of fractionation of the protein without damaging its native structure and dissociation. That required applying the special methods of purification of carotenoids and lipids, and the subsequent gel permeation column chromatography on Sephadex G-200. Removing of carotenoids, consisting in repeated treatment of PM with 50 % (v/v) EtOH ethanol at 4 °C, was a routine but necessary step, in spite of the significant loss of chromoprotein. It was used five treatments by 50 % (v/v) EtOH ethanol to obtain the absorption spectrum of purified from carotenoids PM suspension (4) and (5) (degree of chromatographic purity of 80–85 %), as shown in Figure 3 at various processing stages (*b*) and (*c*) relative to the native BR (*a*). Formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption spectrum of PM (Fig. 3, *c*) – the main bandwith (1) with the absorption maximum at λ = 568 nm caused by the light isomerization of the chromophore by the C13=C14 bond is determined by the presence of 13-*trans*-retinal residue in BR_{568}; additional low-intensity bandwith (2) at λ = 412 nm characterizes a minor impurity of a spectral form of *meta*-bacteriorhodopsin M_{412} (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwith (3) with λ = 280 nm is determined by the absorption of aromatic amino acids in the polypeptide chain of the protein (for native BR D_{280}/D_{568} = 1,5 : 1,0).

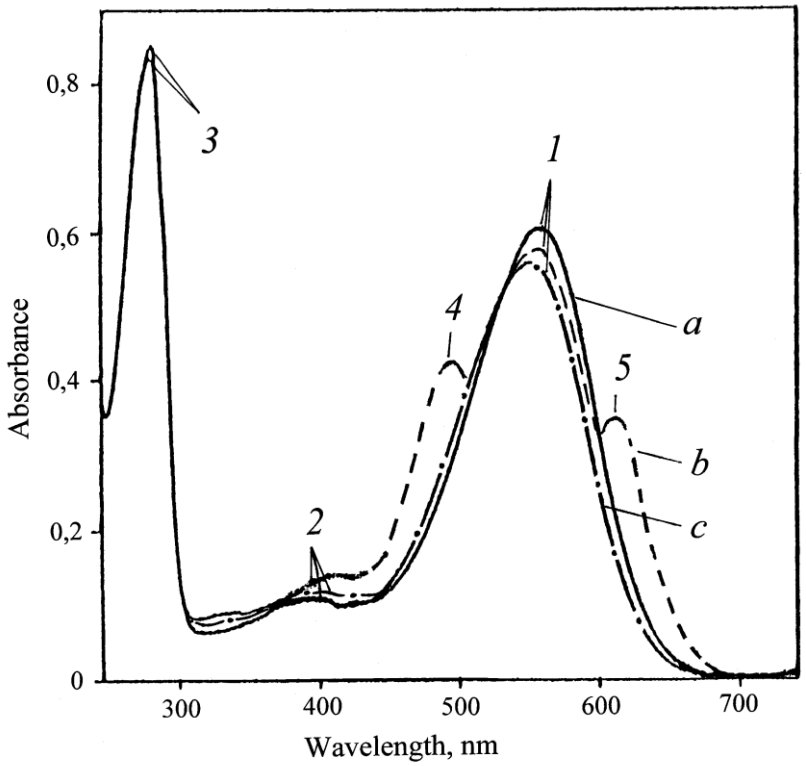


Figure 3. The absorption spectra of the PM (50 % (v/v) ethanol) at various stages of processing: *(a)* – natural BR; (*b*) – PM after intermediate treatment; (*c*) – PM purified from carotenoids. *(b)* The bandwith (1) is the spectral form of BR_{568}, (2) – impurity of spectral form of *meta*-bacteriorhodopsin M_{412}, (3) – the total absorption bandwith of aromatic amino acids, (4) and (5) – extraneous carotenoids. As a control used the native BR

The next necessary step of the research was fractionation and chromatographic purification of the protein. As BR, being a transmembrane protein intricately penetrates bilipid layer in form of seven α-helices, the use of ammonium sulfate and other conventional agents to salting out did not give a positive result for isolation of the protein. The resolving was in the translation of the protein to a soluble form by the colloidal dissolution (solubilization) in an ionic detergent. Using SDS-Na as the ionic detergent was dictated by the need of solubilization of the protein in a native, biologically active form in complex with 13-*trans*-retinal, because BR solubilized in 0,5 % (v/v) SDS-Na retains a native α-helical configuration [15]. Therefore, there is no need the use organic solvents as acetone, methanol and chloroform for purification of lipids and protein, and precipitation and delipidization is combined in a single step, which significantly simplifies the further fractionation. A significant advantage of this method is that the isolated protein in complex with lipids and detergent molecules was distributed in the supernatant, and other high molecular weight impurities – in unreacted precipitate, easily separated by centrifugation. Fractionation of solubilized in a 0,5 % (w/v) SDS-Na protein and its subsequent isolation in crystalline form was achieved at 4 °C in three steps precipitating procedure with methanol, reducing the concentration of detergent from 0,5, 0,25 and 0,1 % (w/v) respectively. The final stage of BR purification involved the separation of the protein from low-molecular-weight impurities by gel-permeation chromatography. The fractions containing BR were passed twice through a column with dextran Sephadex G-200 balanced with 0,09 M Tris-borate buffer (pH = 8,35) containing 0,1 % (w/v) SDS and 2,5 mM EDTA. The method for protein fractionation made it possible to obtain 8–10 mg of ^{2}H-labeled BR from 1 g of bacterial biomass. The homogeneity of BR satisfy the requirements for reconstruction of native membranes, and was confirmed by electrophoresis in 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na, regeneration of apomembranes with 13-*trans*-retinal, and RP-HPLC of methyl esters of N-DNS-[^{2}H]amino aids. Micropreparative output of BR was no barrier to further

mass spectrometric analysis. However, it must be emphasized that to ensure high yields of protein it needs to accumulate more row biomass feedstock.

Conditions of hydrolysis of ^{2}H-labeled protein were determined to prevent the isotopic (H–^{2}H) exchange of hydrogen by deuterium in the molecules of aromatic amino acids, as well as to retain tryptophan in the protein hydrolysate. We considered two alternatives variants - acid and alkaline hydrolysis. Acid hydrolysis of the protein in standard conditions (6 N HCl or 8 N H_2SO_4, 110 ^{0}C, 24 h) is known to induce complete degradation of tryptophan and partial degradation of serine, threonine, and several other amino acids in the protein [16], which do not play a significant role for this study. Modification of this method consists in adding to the reaction mixture of phenol, thioglycolic acid, β-mercaptoethanol, can save up to 80–85 % tryptophan [17]. Using of p-toluenesulfonic acid with 0,2 % (v/v) 3-(2-aminoethyl) indole or 3 M mercapto sulfoacid is also effective to maintain a tryptophan (up to 93 %) [18]. However, these methods are possess an essential disadvantage, because during acid hydrolysis at high speed occurs isotopic (H–^{2}H) excange of aromatic protons(deuterons) in molecules of tryptophan, tyrosine and histidine as well as protons at C3 position of aspartic and C4 glutamic acid [19]. Therefore, even carrying out of hydrolysis in deuterated reagents (6N ^{2}HCl, 4N $^2H_2SO_4$ in ^2H_2O) does not derive the real data about the inclusion of the deuterium into the protein.

In conditions of alkaline hydrolysis (4N $Ba(OH)_2$ or 4N NaOH, 110 ^{0}C, 24 h) the reactions of isotopic (H–^{2}H) exchange were almost not occurred (except for a proton (deuteron) at C2 atom of histidine, and tryptophan not destroyed. These factors determined the choice of this method of hydrolysis in our research. Simplification of the procedures for the allocation of amino acid mixture from protein hydrolizate due to the neutralization of H_2SO_4 (in ^2H_2O) was the reason for choosing as hydrolysing agent 4N $Ba(OH)_2$. Possible D,L-amino acid racemization by alkaline hydrolysis did not affect the result of the subsequent mass spectrometric study of the level of deuteration of amino acid molecules.

To study the deuterium labelling of BR molecule EI mass spectrometry was used after modification of amino acid mixture of the hydrolyzate into methyl esters of N-Dns-[^{2}H]amino acids. To obtain reproducible results on the deuteration of ^{2}H-labeled protein first recorded a total scan EI mass spectrum of the mixture of methyl esters of N-Dns-[^{2}H]amino acids derived from the hydrolyzate BR. The level of deuteration was calculated from the peak of the molecular ion $[M]^+$ of amino acid derivatives relative to the control. Then, the separation of methyl esters of N-Dns-[^{2}H] aromatic amino acids was performed by by RP HPLC to record EI mass spectra for each individual amino acid derivate.

El mass spectrum of the mixture of methyl esters of N-DN-amino acids as shown in Figure 4 (scanning at *m/z* 50–640, base peak at *m/z* 527, 100 %), is characterized by continuity: the peaks in the range at *m/z* 50–400 on the scale of the mass numbers are fragments of metastable ions, low molecular weight impurities, as well as products of chemically modified amino acids. Analyzed [^{2}H]-labeled aromatic amino acids occupying scale mass numbers at *m/z* 415–456, are mixtures of molecules containing various numbers of deuterium atoms. Therefore, the molecular ions $[M]^+$ were polymorphously split into individual clusters displaying a statistical set of *m/z* values depending on a number of hydrogen atoms in the molecule. Taking into account the effect of isotopic polymorphism the level of deuterium enrichment in [^{2}H]amino acid molecules was determined using the most commonly encountered peak of the molecular ion $[M]^+$ in each cluster with mathematically averaged value of $[M]^+$ (Fig. 4). As is shown in Figure 4 molecular ion peak of phenylalanine was determined by $[M]^+$ at *m/z* 417, 14 % (instead of the $[M]^+$ at *m/z* 412, 20 % for non-labeled derivative (unlabeled peaks of amino acid derivatives are not shown)), tyrosine – $[M]^+$ at *m/z* 429, 15 % (instead of $[M]^+$ at *m/z* 428, 13 %), tryptophan – $[M]^+$ at *m/z* 456, 11 % (instead of $[M]^+$ at *m/z* 451, 17 %). The level of deuterium enrichment corresponding to the increase of molecular weight was for [^{2}H]tyrosine 1 (90 atom.% ^{2}H), [^{2}H]phenylalanine – 5 (95 atom.%, ^{2}H) and [^{2}H]tryptophan – 5 (98 atom.% ^{2}H) deuterium atoms. This result coincides with the data on the initial level of deuterium enrichment of aromatic amino acids – [3,5-$^2H_2$]Tyr, [2,3,4,5,6-$^2H_5$]Phe and [2,4,5,6,7-$^2H_5$]Trp, added to the growth medium and indicates a high selectivity of inclusion of aromatic [^{2}H]amino acids into the BR molecule. Deuterium was detected in all residues of aromatic amino acids (Table). However, the presence in the EI mass spectrum peaks $[M]^+$ of protonated and semi-deuterated phenylalanine analogues with $[M]^+$ at *m/z* 413–418, tyrosine – with $[M]^+$ at *m/z* 428–430 and tryptophan – with $[M]^+$ at *m/z* 453–457 with different levels of contributions to the deuterium enrichment of molecules testifies about conservation of the minor

pathways of biosynthesis of aromatic amino acids de novo, resulting in the dilution of the deuterium label, that evidently is determined by the conditions of biosynthesis of ^{2}H-labeled BR (Table).

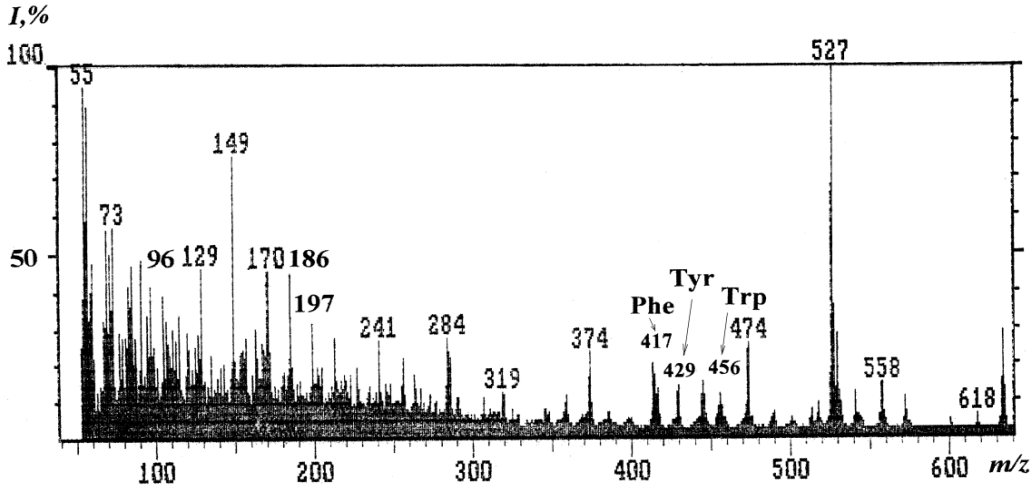


Figure 4. Full scan EI mass spectrum of methyl ester of N-Dns-[^{2}H]derivatives of aromatic amino acids isolated from the BR hydrolyzate, obtained on synthetic medium with [2,3,4,5,6-$^2H_5$]Phe (0,26 g/l), [3,5-$^2H_2$]Tyr (0,2 g/l) and [2,4,5,6,7-$^2H_5$]Trp (0,5 g/l). (energy: 70 eV, accelerating voltage: 8 kV, temperature: 180–200 °C). Hydrolysis conditions: 4 N $Ba(OH)_2$ (in ^2H_2O), 110 °C, 24 h. Molecular ion peaks represented by the symbols of amino acids correspond to their derivatives; *I* – relative intensity (%).

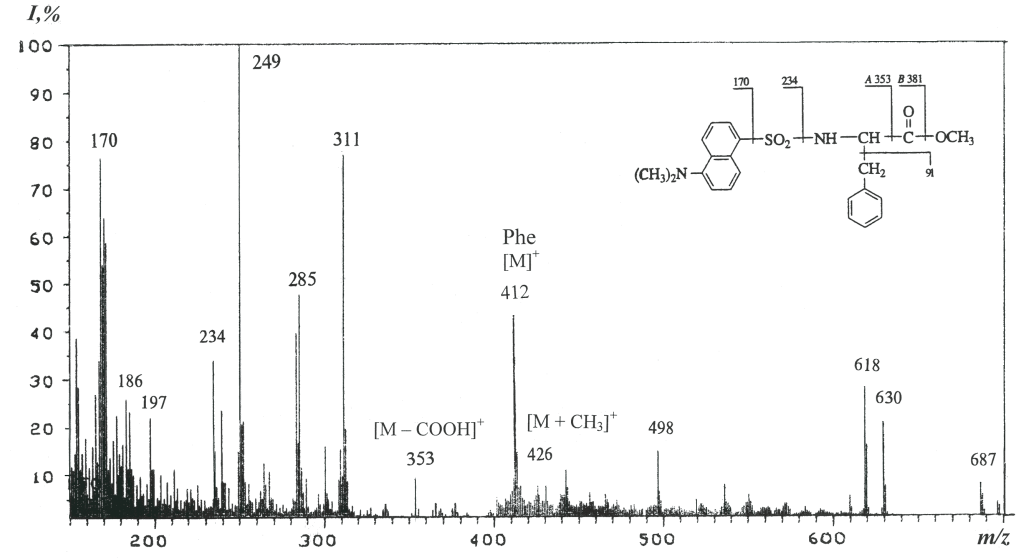
Table

The values of the molecular ion peaks $[M]^+$ in the EI mass spectrum of methyl esters of N-Dns-[2,3,4,5,6-$^2H_5$]Phe, N-Dns-[3,5-$^2H_2$]Tyr and N-Dns-[2,4,5,6,7-$^2H_5$]Trp and levels of their deuterium enrichment

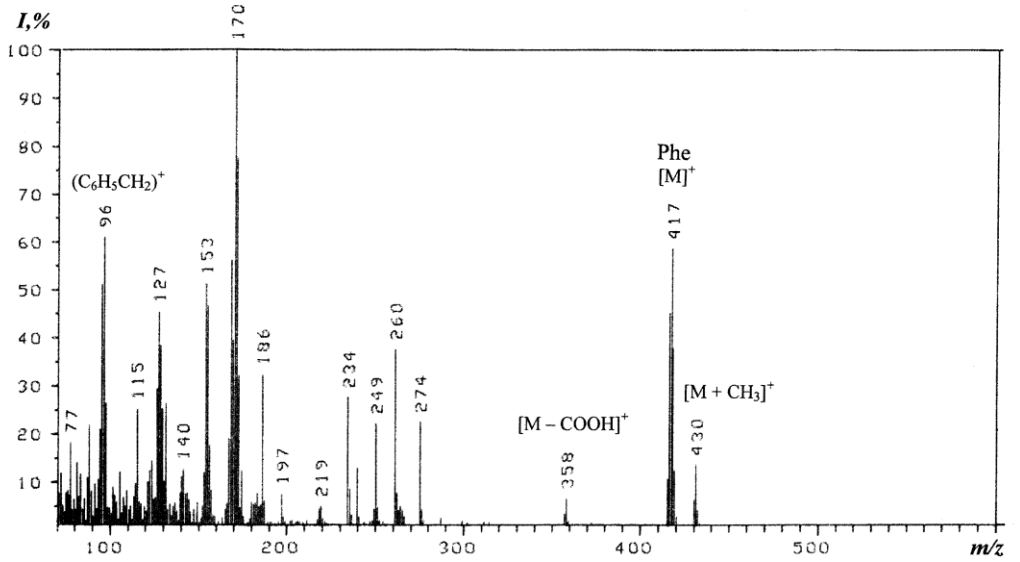
Compound	Value of $[M]^+$	Intensity, %	The total number of hydrogen atoms*	Level of deuterium enrichment, % of the total number of hydrogen atoms **
N-Dns-[2,3,4,5,6-$^2H_5$]Phe-OMe	413 414 415 416 417 418	7 18 15 11 14 6	1 2 3 4 5 6	13 25 38 50 63 75
N-Dns-[3,5-$^2H_2$]Tyr-OMe	428 429 430	12 15 5	– 1 2	– 14 29
N-Dns-[2,4,5,6,7-$^2H_5$]Trp-OMe	453 454 455 456 457	5 6 9 11 5	2 3 4 5 6	26 38 50 64 77

Notes:
* A dash means no incorporation of deuterium.
** In calculating the level of deuterium enrichment protons(deuterons) at carboxyl and NH_2-amino groups of amino acids were not considered due to the easily isotopic (H-^{2}H) exchange.

According to the mass spectrometric analysis the molecular ion peaks $[M]^+$ of methyl esters of N-Dns-[^{2}H] derivatives of aromatic amino acids have a low intensity in EI mass spectra and were polymorphously split, so the areas of of the molecular enrichment were strongly broadened. Moreover, mass spectra of the mixture components are additive, so the mixture can be analyzed only if the spectra of the various components are recorded in the same conditions. These calculations provide for the solution of the system of *n* equations in *n* unknowns for a mixture of *n* components. For components, the concentration of which exceeds 10 mol.%, the accuracy and reproducibility of the analysis makes up ± 0,5 mol.% (at 90% confidence probability). Therefore, to obtain reproducible results it was necessary to chromatographically isolate individual derivatives of [^{2}H] amino acids from the protein hydrolyzate. For this aim was used RP-HPLC on on octadecylsilane silica gel Silasorb C18, the effectiveness of which was confirmed earlier by separation of a mixture of methyl esters of N-Dns-derivatives [^{2}H]amino acids from other microbial objects as methylotrophic bacteria and microalgae [20].



a)



b)

Figure 5. EI mass spectra of methyl N-Dns-[2,3,4,5,6-$^2H_5$]Phe under various experimental conditions: (*a*) – the unlabeled methyl ester of N-Dns-Phe; (*b*) – methyl ester of N-Dns-[2, 3,4,5,6-$^2H_5$]Phe, isolated from the BR hydrolyzate by RP HPLC. Separation conditions: 250 × 10 mm Column C18 ("Kova", Slovakiya), eluents: (A) – CH_3CN–CF_3COOH (100: 0,1–0,5 % (v/v)) and (B) – CH_3CN (100 % (v/v)).

The method was adapted to the conditions of chromatographic separation of a mixture of methyl esters of N-Dns-[^{2}H]amino derivatives of BR hydrolyzate comprising in optimization of eluent ratios, the gradient type, and the rate of gradient elution from the column. The best separation was achieved by gradient elution with a solvent mixture CH_3CN–CF_3COOH (100: 0,1–0,5 % (v/v)). It was possible to isolate the tryptophan and inseparable pair phenylalanine/tyrosine. The levels of chromatographic purity of methyl esters of N-Dns-[2,3,4,5,6-$^2H_5$]phenylalanine, N-Dns-[3,5-$^2H_2$]tyrosine and N-Dns-[2, 4,5,6,7-$^2H_5$]tryptophan isolated from protein hydrolysate were 89, 91 and 90 %, respectively with the output 97–85 %. The result confirmed Figure 5, *b* which shows the EI mass spectrum of methyl N-Dns-[2,3,4,5,6-$^2H_5$]phenylalanine isolated by RP-HPLC (scanning at *m/z* 70–600, base peak at *m/z* 170, 100 %) (EI mass spectrum is shown relative to the unlabeled methyl ester of N-Dns-phenylalanine (Fig.5, *a*), scanning at *m/z* 150–700, base peak at *m/z* 250, 100 %). Proof for the inclusion of 5 deuterium atoms into the phenylalanine molecule is the presense of "heavy" molecular ion peak of methyl ester of N-Dns-[^{2}H]phenylalanine ($[M]^+$ at *m/z* 417, 59 %, instead of $[M]^+$ at *m/z* 412, 44 % for non-labeled derivative of phenylalanine), and the deuterium-enriched additional peaks of benzyl $C_6H_5CH_2$ fragments of [^{2}H]phenylalanine molecule at *m/z* 96, 61 % (instead of *m/z* 91, 55 % in the control (not shown)) (Fig. 5, *b*). Peaks of secondary fragments of varying intensity values at m/z 249, 234 and 170 are the secondary decomposition products of the dansyl residue to the 5-(dimetilamino)naphthalene, low intensity peak $[M - COOCH_3]^+$ at *m/z* 358, 7 % (instead at *m/z* 353, 10 % in the control) is a product of cleavage of a carboxymethyl ($COOCH_3$) group of the methyl easter of N-Dns-[^{2}H]phenylalanine, and a peak $[M + CH_3]^+$ at *m/z* 430, 15 % (instead of *m/z* 426, 8 % in the control) – the product of further methylation by α-amino group of the [^{2}H]phenylalanine (Fig. 5, *b*). According to the EI mass spectrometry data, the difference between the molecular weight of the protonated and deuterated species of peaks $[M]^+$ of methyl ester of N-Dns-phenylalanine makes up 5 units that corresponds with the primary data on the deuterium enrichment level of [2,3,4,5,6-$^2H_5$]phenylalanine added to the growth medium. Mass spectrometric data on deuterium enrichment levels of [3,5-$^2H_2$]tyrosine and [2,4,5,6,7-$^2H_5$]tryptophan are also in good correlation and confirmed by ^{1}H NMR.

Conclusions

The experimental data indicate the high efficiency of the of incorporation of H-labeled aromatic amino acids into the BR molecule with output of BR 8–10 mg per 1 g of biomass. The main advantage of this method is that the isolated BR keeps its native configuration in combination with 13-*trans*-retinal, and the ability to photochemical reactions in vitro, as demonstrated by electrophoresis in 12,5 % (w/v) PAAG with 0,1 % (w/v) SDS-Na. The method is also applicable for the preparation of other similar to BR transmembrane proteins of halophilic bacteria – sensory rhodopsin and halorhodopsin. The unique properties of natural bacteriorhodopsins provide a wide range of bio- and nano-technological applications in which may find their application ^{2}H-labeled analogues. In the future we plan obtaining fully deuterated analogs of BR for the reconstruction of functionally active systems of transmembrane proteins in heavy water with purified ^{2}H-labeled fatty acids and other biologically active compounds. These studies will provide an answer to the question of how function BR in the native membranes in condition of the complete replacement of protons by deuterium.

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УДК 577.37 + 537.86

Биосинтез ^{2}H-меченного фотохромного трансмембранного белка бактериородопсина галобактерией *Halobacterium halobium*

[1] Олег Викторович Мосин
[2] Игнат Игнатов

[1] Московский государственный университет прикладной биотехнологии, Российская Федерация
Старший научный сотрудник кафедры биотехнологии, канд. хим. наук.
103316, Москва, ул. Талалихина, 33
E-mail: mosin-oleg@yandex.ru
[2] Научно-исследовательский центр медицинской биофизики (РИЦ МБ), Болгария
Профессор, доктор наук Европейской академии естественных наук (ФРГ), директор НИЦ МБ.
1111, София, ул. Н. Коперника, 32/6

Аннотация. Разработан микропрепаративный биосинтез природного фотопреобразующего белка бактериородопсина (выход 8–10 мг), меченного дейтерием по функционально важным остаткам аминокислот – [2,3,4,5,6-$^2H_5$]фенилаланина, [3,5-$^2H_2$]тирозина и [2,4,5,6,7-$^2H_5$]триптофана, с помощью фотоорганотрофной галобактерии *Halobacterium halobium*. Белок был из пурпурных мембран лизисом клеток дистиллированной водой, обработкой клеточной биомассы ультразвуком при 22 кГц, спиртовой экстракцией низко- и высокомолекулярных примесей, клеточной РНК, каротиноидов и липидов, с последующей солюбилизацией конечного продукта в 0,5 % ДДС-Na и низкотемпературным фракционированием метанолом. Гомогенность синтезируемого бактериородопсина И селективность включения дейтерия в молекулу доказаны комбинацией методов выделения и белкового анализа, включая электрофорез в 12,5 % ПААГ с 0,1 % ДДС-Na, гель-проникающей хроматографией на сефадексе G-200, обращённо-фазовой ВЭЖХ и масс-спектрометрией электронного удара метиловых эфиров N-Dns-[^{2}H] производных амино килот.

Keywords: *Halobacterium halobium*, бактериородопсин, [2,3,4,5,6-$^2H_5$]Phe, [3,5-$^2H_2$]Tyr, [2,4,5,6,7-$^2H_5$]Trp, биосинтез; масс-спектрометрия электронного удара, ОФ ВЭЖХ.