UDC 577.37 + 537.86

The Improved Method for Isolation of Photochrome Trans-membrane Protein Bacteriorhodopsin from Purple Membranes of Halobacterium *Halobacterium Halobium ET 1001*

[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 (SRCMB), Bulgaria
Professor, D. Sc., director of SRCMB
1111, Sofia, N. Kopernik street, 32
E-mail: mbioph@dir.bg

Abstract

It was developed the improved method for isolation of photochrome trans-membraine protein bacteriorhodopsin (output − 5 mg from 100 g of wet biomass) capable to transform light energy to electrochemical energy of generated protons H^+ and ATP. The protein was isolated from purple membranes of photo-organotrophic halobacterium *Halobacterium halobium ET 1001* 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, solubilization with 0.5% (w/v) SDS-Na, fractionation by MeOH and column gel permeation chromatography (GPC) of the final protein on Sephadex G-200 with 0.1% (w/v) SDS-Na and 2.5 mM ETDA. The homogeneity of the isolated bacteriorhodopsin was proved by combination of preparative and analytical methods, including elecrtophoresis in 12.5% (w/v) PAAG with 0.1% (w/v) SDS-Na and regeneration of apomembranes with 13-*trans*-retinal.

Keywords: *Halobacterium halobium ET 1001*, purple membranes, bacteriorhodopsin, biosynthesis, biomolecular electronics

Introduction

Bacteriorhodopsin (BR), denoted by analogy to the visual apparatus of mammalian protein rhodopsin, was isolated from the cell membrane of an extreme aerobic photo-organotrophic halobacterium *Halobacterium halobium* in 1971 by D. Osterhelt and W. Stohenius [1]. This photo-transforming integral trans-membrane protein with the molecular weight ~26.5 kDa represents a 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 the Schiff base (as in the visual animal pigments) with Lys-216 residue of the protein.

In halobacteria the BR functions as a light-driven transmembrane proton pump pumping a proton across the membrane. Along with the BR the cell membrane of halobacteria contains a small amount of other related carotenoid pigments, the main of which bakterioruberin determining the stability of halobacteria to solar radiation [2].

The 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 [3, 4]. In addition, the BR is very attractive as a model for studies of functional activity and structural properties of photo-transforming membrane proteins in the composition of native and photo-converting membranes [5, 6].

The BR-containing nano-films produced using the BR-containing purple membranes (PM) of halobacteria were first obtained and studied in this country in the framework of the project "Photochrome", when it was demonstrated the effectiveness and prospects for the use of the BR as a photochromic material for holographic recording. These nanomaterials can reversibly change their structure in response to the physical impact and generate two discrete states, calculable by measurement with using spectral methods. This fact determines their usage as logical computing systems in biomolecular electronics [7–9]. Thus, based on the BR was constructed a photoreceptor with a microelectrode of SnO_2, consisting of 64 cells (pixels) having the size of 2.5×2.5 mm and electric voltage of 0.3–0.7 V [10]. For the signal conversion by this photoreceptor, low electric current (3–10 nA) was enhanced to voltage from 1 to 10 V and then was supplied to the light emitting diodes. This construction suggests the possibility of effective integration of the BR into the modern microelectronic systems.

The main task for the manufacture of BR-containing nanofilms is the orientation of purple membranes between the hydrophobic and hydrophilic media, such as between water and air, as is common in nature. Typically, to improve the characteristics of the BR-containing films the multiple layers were applied to the surface of the polymeric carrier and dried up, preserving their natural structure. The best results are achieved in the manufacture of films based on gelatin matrix [11, 12]. This allows for a high concentration of the BR (up to 50%) in nanofilms to avoid an aggregation of magenta membrane fragments and destruction of the BR in the manufacturing process [13]. Being embedded onto a gelatin matrix the PM fragments are durable ($\sim10^4$ h) and are resistant to many external factors as temperature variations, the intense light exposure, laser radiation, etc. [14]. The dried PM are stacked on the top of each other, focusing in the plane of the matrix. A layer of dried PM with thickness of ~1 μm contains approx. 200 monolayers. When illuminated by light in such nanofilms is recorded the electric potential approximately 100–200 mV, which coincides with the membrane potential of living cells [15].

The structure and mechanism of function of BR have been widely studied in recent years to develop many various approaches of isolation of the protein in native biologically active state that requires the solubilization of BR in a stable, highly purified state free from native lipids [16, 17]. The large scientific and practical interest for obtaining the BR samples for the reconstruction of nanofilms, defined the aim of the present research, related to the development of simple effective method for isolation of the BR from PM in semi-scale quantities.

Material and methods

Bacterial strain

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 to individual colonies on solid 2% (w/v) agarose media with peptone and 4.3 M NaCl.

Chemicals

For preparation of growth media were used *D,L*-amino acids ("Reanal", Hungary), AMP, UMP, biotin, folic acid and vitamin B_{12} − $2 \cdot 10^{-5}$ purchased from "Sigma Corp." (Sigma Aldrich, USA). Organic salts were obtained from Reanal ("Reanal", Hungary). The buffer components were from Reachim-Pharm Ltd. (Russia). All solvents were of HPLC grade. Other chemical reagents

were of analytical reagent grade. Filtrated water was provided by the Milli-Q-Plus water filtration system (Millipore, Bedford, MA, USA).

Growth conditions

The bacterial growth was carried out on a synthetic medium (SM) containing (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; $ZnSO_4 \cdot 7H_2O$ – $4 \cdot 10^{-5}$; $FeSO_4 \cdot 7H_2O$ – $5 \cdot 10^{-4}$; $CuSO_4 \cdot 5H_2O$ – $5 \cdot 10^{-5}$; Na-citrate – 0.5; 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 t = +35 $^{\circ}$C on a shaker ("Birad Labs", Hungary) under intense aeration and monochromatic illumination by light fluorescent lamps LDS-40-2 (40 W) ("Alfa-Electro", Russia) (3 lamps × 1.5 lx). Bacterial growth was studied by optical density of the bacterial suspension measured at a wavelength λ = 620 nm on a spectrophotometer Beckman DU-6 ("Beckman Coulter", USA). All further manipulations with BR were carried out with the use of a photomask lamp equipped with an orange light filter PCM -1X (75×50 cm) ("Marbel", Germany).

Isolation of purple membranes (PM)

The row biomass (1 g) was washed with distilled water and pelleted by centrifugation on a 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 $^{\circ}$C. The reaction mixture was centrifuged (1500 g, 15 min), the pellet was re-suspended in 20 ml dist. H_2O and disintegrated by infrasound sonication (22 kHz, 1 min) in an ice bath (0 $^{\circ}$C). The cell homogenate after washing with dist. H_2O was re-suspended in 10 ml of a 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 in the dark for ~2 h at t = +37 $^{\circ}$C. Then 10 ml of the same buffer was added and kept for 10–12 h at 4 $^{\circ}$C. The aqueous fraction was separated by centrifugation on T-24 centrifuge ("Carl Zeiss", Germany) (1500 g, 20 min), the PM precipitate was treated with 50% (v/v) EtOH (5 times × 5 ml) at 4 $^{\circ}$C followed by separation of the solvent. This procedure was repeated 5 times to give colorless washings. The protein content in the samples was determined spectrophotometrically on a Beckman DU-6 spectrophotometer ("Beckman Coulter", USA) by the ratio D_{280}/D_{568} (ε_{280} = $1.1 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$; ε_{568} = $6.3 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [18]. Regeneration of PM was performed as described in [19]. The output of PM fraction makes up 120 mg (80–85%).

Isolation of BR

Fraction of the PM (in H_2O) (1 mg/ml) was dissolved in 10 ml of 0.5% (w/v) SDS-Na, and incubated for ~5–7 h at t = +37 $^{\circ}$C followed by centrifugation (1200 g, 15 min). The precipitate was separated, after that MeOH was added to the supernatant in divided portions (3 times × 2 ml) at 0 $^{\circ}$C. The reaction mixture was kept for ~14–15 h in ice bath at t = +4 $^{\circ}$C and then centrifuged (1200 g, 15 min). The fractionation procedure was performed three times, reducing the concentration of 0.5% SDS-Na to 0.2 and 0.1% (w/v). The crystal protein (output, 8–10 mg) was washed with cold distilled ^2H_2O (2 times × 1 ml) and centrifuged (1200 g, 15 min).

Purification of BR

A protein sample (5 mg) was dissolved in 10 ml of buffer solution and placed on a calibrated chromatography column (150×10 mm) with stationary phase – Sephadex G-200 ("Pharmasia", USA) (specific volume packed beads – 30–40 units per 1 g dry Sephadex), and equilibrated with buffer containing 0.1% (w/v) SDS-Na and 2.5 mM ETDA. The device was e**quiped with Waters** 2487 dual absorbance (**UV/VIS**) d**etector with the w**avelength range at λ = 190-700 nm. Elution proceeded by 1 mM Tris-HCl buffer, pH = 7.4 at a flow rate 10 ml/cm^2·h. The combined

protein fraction was subjected to freeze-drying, sealed in glass ampoules (10×50 mm) and stored in frost camera at t = -10 °C.

Electrophoresis of BR

The procedure 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). The 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.

Proton translocation

The proton translocation was recorded in visicles with a gel-filled pH electrode (Sensorex No. SG900C) combined with PHM85 Radiometer pH meter. The data were collected (0.8 s per time interval) and analyzed using Lotus Measure (Lotus Development). Initial proton pumping rates were determined over 10 s interval. Changes in pH were calibrated using 1μm of 10 mM HCl.

Preparation of apomembranes (AP)

50 mg of PM was suspended in 50 ml of 1 M NH_2OH (pH = 6.0). The reaxion mixure was kept for 10 h with stirring in ice bath (4 °C) under illumination with a xenon lamp. The precipitate was separated by centrifugation (1000 g, 10 min), washed twice with distilled water and centrifuged. AP fraction was resuspended in 2 ml of 5 mM 2-(N-morpholino)-ethane sulfonyl amide in 100 mM NaCl.

Regeneration of AP with 13-trans-retinal

To 2 ml suspension of AP ($2 \cdot 10^{-5}$ mol) in a quartz cuvette was added with stirring 0.1 ml of 2 mM solution of 13-*trans*-retinal in methanol and kept for 6–8 h in the dark at t = +40 °C. The degree of regeneration of PM was determined by spectrophotometry on CDS-200 laser densitometer ("Beckman", USA) by the ratio: $D_{nat.280} \cdot D_{nat..568}/D_{reg..280} \cdot D_{reg.568}$, where D_{280} and D_{568} – the absorbance of a suspension of native and regenerated PM at $\lambda = 280$ and $\lambda = 568$ nm.

Results and discussion

Structure and mechanism of action of BR

On its structure and location in the cell membrane BR refers to integral trans-membrane proteins (Fig. 1), penetrating the cell membrane, which is divided into three separate fractions: yellow, red and purple. Purple fraction comprising 75% (w/w) of cell membrane consists from carotenoids, phospholipids (mostly phosphoglycerol diesters of phosphatidyl glycerol phosphate (PGP) with a small amount of non-polar lipids and isoprenoids) forms natural two-dimensional crystals which can be investigated using the electron microscopy, X-ray diffraction methods as X-ray crystallography [20]. These methods have established the existence in the BR molecule of seven α-helical protein segments which span the lipid matrix, while in the middle part is symmetrically located a retinal residue being covalently linked to Lys-216 residue in the G helix so that that each protein molecule has one retinal moiety (vitamin A) (Fig. 1). In the native membrane the polypeptide chain is oriented with the amino-end N-terminal in the extracellular medium and the carboxyl C-terminal – inside the cytoplasmic side. The carboxyl C-terminal is non-helical consists of 24 amino acid residues, four of them are negatively charged and two – are positively charged. Other chains connecting the helixes on the cytoplasmic side contain a total of five additional negatively charged residues, but no more positively charged residues. The amino-end N-terminal of the protein is also nonhelical and consists of six residues.
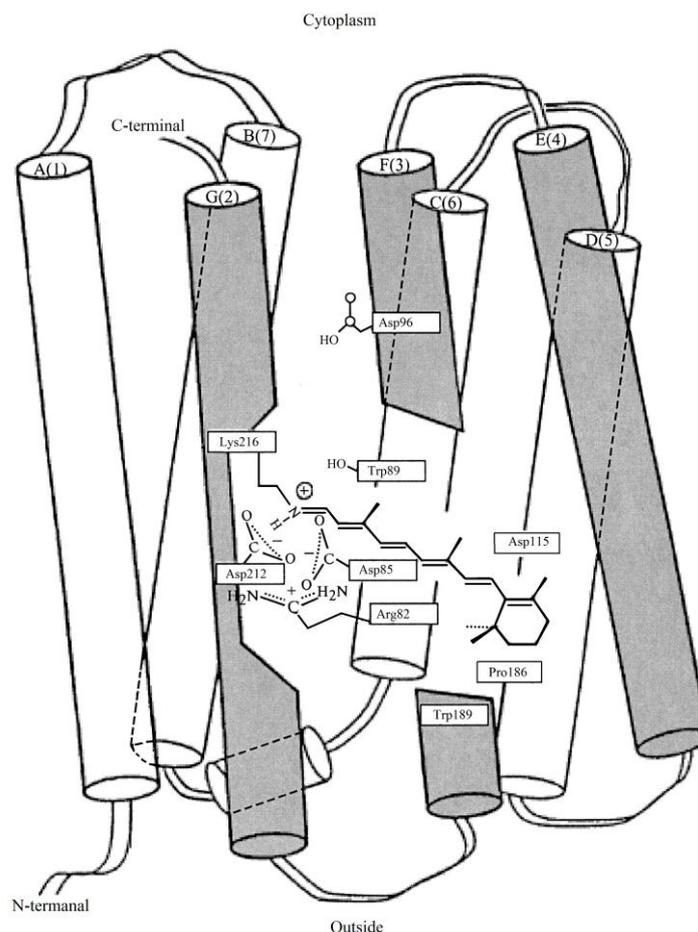
Figure 1: The location of protein and the retinal residue in the BR molecule of halobacterium *H. halobium* according to computer modeling: Latin numerals indicate protein fragments of the BR molecule as 7 α-helical segments (*A, B, C, D, E, G, F*) with exposed amino acid residues; gray color represents the segments responsible for binding of retinal residue to the G-helical segment of BR molecule.

The polypeptide chain of the BR consists of 248 amino acid residues, 67% of which are hydrophobic, formed with the aromatic amino acids, and 33% – hydrophilic residues of aspartic and glutamic acids, arginine and lysine; the protein does not contain histidine or cyctein [21]. These residues play the important structural and functional role in the spatial orientation of the α-helical segments of the BR molecule, arranged in PM in an orderly manner forming 7 trimmers 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 [22]. The BR molecule consists of seven α-helix segments, arranged in a direction perpendicular to the plane of the membrane (Fig. 2). Hydrophobic domains representing trans-membrane segments and hydrophilic domains protruding from the membrane connecting the individual α-helical intra-membranous protein segments of the BR molecules. Along with BR the PM contain lipids, carotenoids and water in their composition.
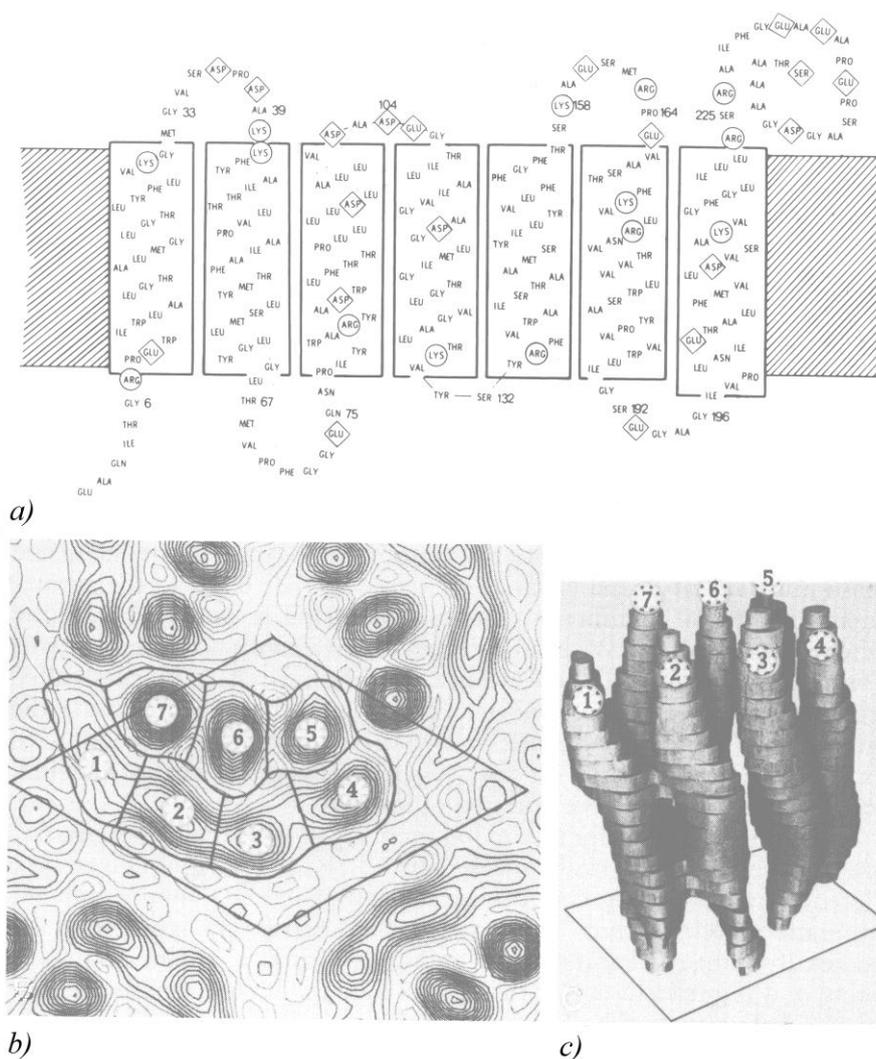
Figure 2: The structure of the BR molecule according to X-ray diffraction analysis:
a) – the primary structure of the BR molecule: amino acids indicated in Latin characters, circles and rhombs show the functionally important amino acids responsible for spatial orientation of α-helical segments of the protein moiety of the BR molecule and the formation of channels for the transfer of protons H+ across the cell membrane;
b) – the electron density map of PM (a single molecule of the protein is encircled in the center). Numbers 1–7 are designated α-helical segments of the BR molecule: – 1– A-segment; 2 –B-segment; 3 – C-segment; 4 – D-segment; 5 – E-segment; 6 – F-segment; 7 – G-segment; *c)* – the spatial structure of the BR molecule: 1 – A-segment; 2 – B-segment; 3 – C-segment; 4 – D-segment; 5 – E-segment; 6 – F-segment; 7 – G-segment.

Owing to the unique structure, the BR molecule 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. The mechanism of ATP synthesis is denoted as "non-chlorophyll photosynthesis", in contrast to the plant photosynthesis implemented with the participation of chlorophyll. In this mechanism, at absorption 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 a proton from intracellular space. By the absorption of a light photon is occurred a reversible isomerization of all 13-*tras*-BR (λ_{max} = 548 nm) (the quantum yield 0.03 at t = +20 °C) into 13-*cis*-BR [(λ_{max} = 568 nm) [23], 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, and finally returns to its 13-*trans*-conformation while remaining bonded to the protein throughout the photo-cycle (Fig. 3). In this process a proton originating at the Schiff base of the retinal residue is passed across by being transferred to the hydrophilic Asp-85 residue lying in sterically favorable positions, to the other side of the cellular membrane; right after that the vacancy is filled up with a proton transferred from Asp-96 residue [24]. As a result, between the internal and external surface of the membrane forms a concentration gradient of H+, resulting that illuminated by light the halobacteria cells begin to synthesize ATP, i.e. convert light energy into the energy of chemical bonds. This process is reversible and in the dark flows back in the opposite direction, allowing to halobacteria developing in the dark by means of switching of photosynthetic metabolism to the heterotrophic 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. The optical characteristics of BR vary depending on the method of preparation of PM and the properties of embedded polymer matrix.

The PM are resistant to solar light, the effects of oxygen, the temperatures greater than +80 °C (in water) and up to +140 °C (in air), pH = 1–12, a high concentration of NaCl (15–20% (w/w)) and action of most proteases [25, 26]. Additionally, the PM are resistant to non-polar solvents such as hexane, but are sensitive to mixtures of polar organic solvents with water. These factors are of great practical importance for integration of the PM into a polymeric nanomatrix with keeping photochemical properties.
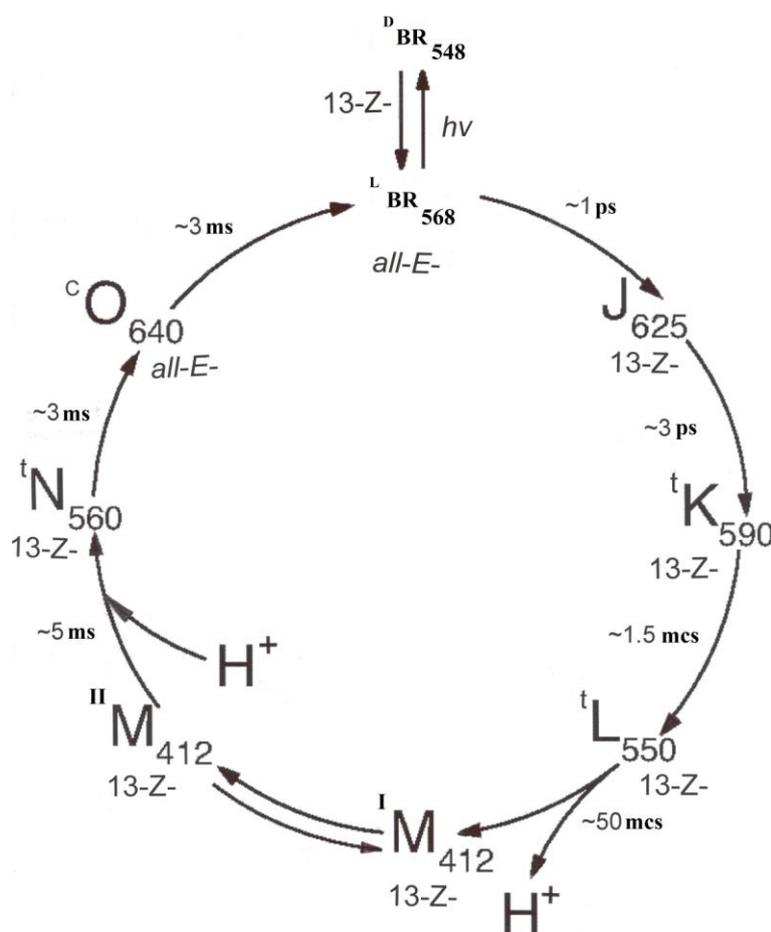

Figure 3: Photocycle scheme of BR (aqueous solution, pH = 7.2; t = +20±25 °C). Latin numbers J, K, L, M, N, O denote spectral intermediates of BR. ^{I}M and ^{II}M represent spectral intermediants of *meta*- bacteriorhodopsin with the protonated (^{I}M) and deprotonated (^{II}M) aldimine bond. L and D denote dark and light forms of pigments. The subscripts correspond to the position of the absorption maximum in the photocycle intermediates (nm)

3.2. Biosynthesis of BR

The technology for preparation of BR consists in growing of halobacteria on liquid synthetic growth media (with 15–20 % (w/w) NaCl) with amino acids, or on natural growth media with peptons – the mixtures of polypeptides and amino acids derived from the partial hydrolysis product or powdered milk, animal meat by proteolytic enzymes (pepsin, trypsin, chymotrypsin), or protein-vitamin concentrate of yeast. The subsequent isolation of BR from purple membranes is carried out by a combination of physical and chemical methods.

For synthesis of the BR used the carotenoid-containing mutant strain of extreme photo-organotrophic halobacterium *H. halobium ET 1001*. The initial strain was modified by selection of BR synthesizing individual colonies with the purplish red color on the solid (2% agarose) growth SM-medium with peptone and 4.3 M NaCl.

For biosynthesis of the BR the resulting cells were grown aerobically under the monochromatic light illumination on a specifically designed SM-medium containing (g/l):

- 18 amino acids: *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;
- Nucleotides: adenosine-5-monophosphate – 0.1; uridine-5-monophosphate – 0.1;
- Inorganic salts: 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;
- Na-citrate – 0.5;
- Growth factors: biotin – $1 \cdot 10^{-4}$; folic acid – $1.5 \cdot 10^{-4}$, vitamin B_{12} – $2 \cdot 10^{-5}$.

In accordance to the daily measurements of optical density of the cell suspension were plotted the curves of bacterial growth of *H. halobium ET 1001* on the SM-medium shown in Fig. 4b relative to the control (Fig. 4*a*) on the peptone medium. In these experimental conditions, the cells synthesizing the purple carotenoid pigment, were identified as a natural BR by the spectral ratio of protein and the chromophore molecule fragments – D_{280}/D_{568} = 1.5 : 1.0. The growth of halobacteria *H. Halobium ET 1001* on the SM-medium (Fig. 4*b*) was insignificantly inhibited compared to the control (Fig. 4*a*) on the peptone medium that significantly simplifies and reduces the cost of biosynthesis of BR. The process consists in growing of halobacteria on SM-medium under illumination by light fluorescent lamps LDS-40-2 (40 W) with monochromatic light with λ = 560 nm for 4–5 days at t = +35 ºC. A significant advantage of this is that unlike the peptone medium, the SM-medium does not contain protein contaminants that could complicate the subsequent isolation and purification of the BR. All further manipulations with the BR were carried out either in the dark or with using of a photomask lamp equipped with an orange light filter.
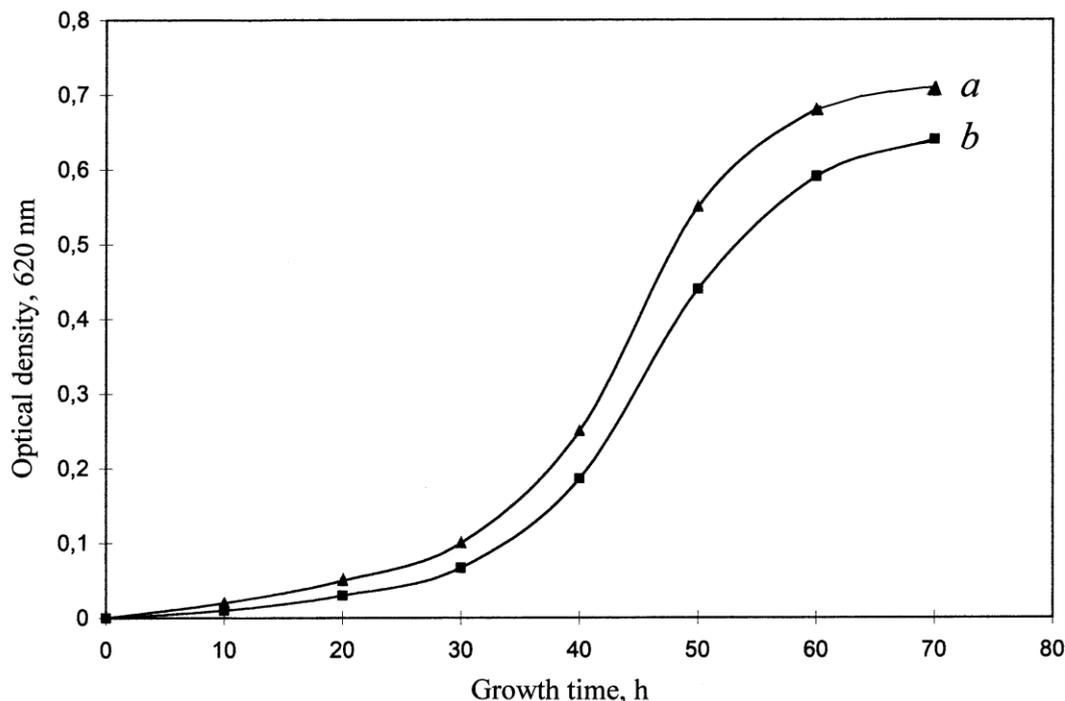
Figure 4: Growth dynamics of *H. halobium ET 1001* under various experimental conditions:
a) – SM-medium; *b*) – peptone medium. Growing conditions: the incubation period: 4–5 days,
temperature: +35 ℃, the illumination under monochrome light at λ = 560 nm

3.3. Isolation and purification of BR

The main stages of isolation of the BR were:
- Growth of halobacteria *H. halobium ET 1001* on the SM-medium;
- Cell disintegration and autolysis;
- Separation of purple membrane (PM) fraction;
- Purification of PM from the low and high-molecular weight impurities, cellular RNA, carotenoids and phospholipids;
- Solubilization of PM in 0.5% (w/v) solution of ionic detergent SDS–Na to form a micro-emulsion;
- Fractionation of the solubilized BR by MeOH;
- Gel permeation chromatography (GPC) on Sephadex G-200;
- Electrophoresis in 12.5% (w/v) PAAG with 0.1% (w/v) SDS-Na.

Since the 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. The resulted fraction of PM along with the desired protein in a complex with lipids and polysaccharides 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 to avoid the dissociation of retinal residue in the protein. That required applying the special methods for purification of carotenoids and lipids, and the subsequent gel permeation column chromatography (GPCC) on Sephadex G-200.
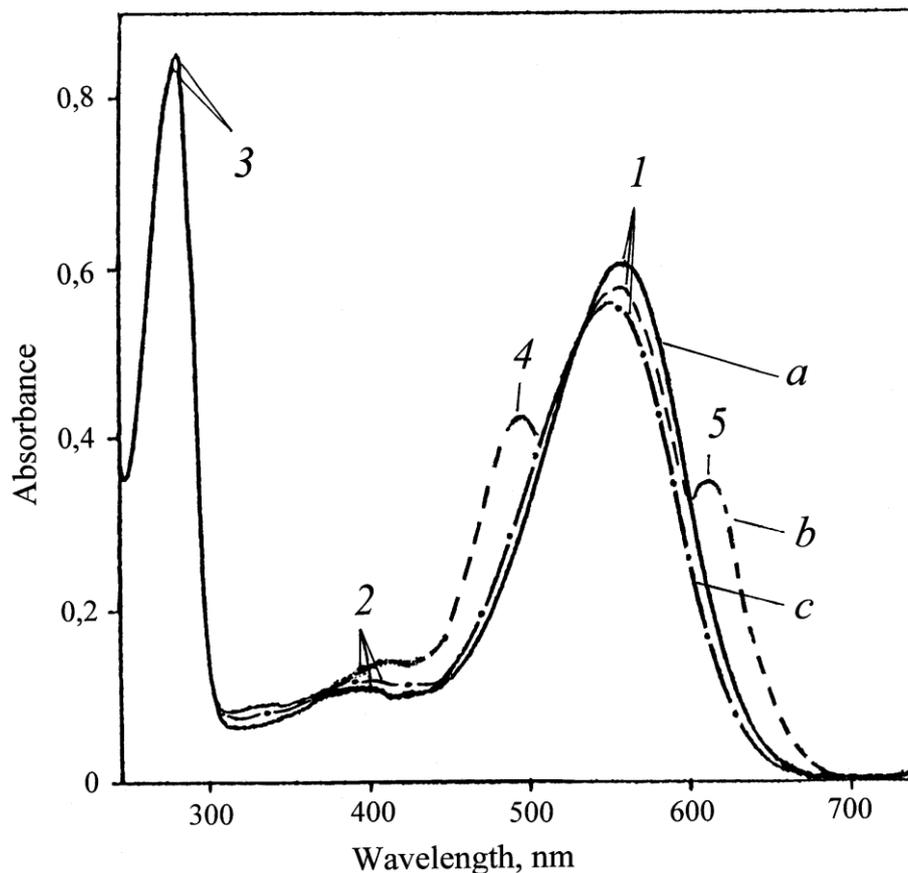
Figure 5: The absorption spectra of PM (50% (v/v) EtOH) at various stages of processing:
(*a*) – natural BR; (*b*) – PM after intermediate treatment; (*c*) – PM purified from carotenoids.
The bandwith (1) is the spectral form of BR_{568}, (2) – impurity of spectral form of meta-bacteriorhodopsin, M_{412}, (3) – the total absorption bandwith of aromatic amino acids,
(4) and (5) – extraneous carotenoids. The native BR was used as a control.

In an attempt to remove a large fraction of the carotenoids and phospholipids from the membrane by column GPC, the PM fraction was washed by 50% (v/v) of EtOH before stabilization with SDS-Na. Removing of carotenoids, consisting in the repeated treatment of the PM with 50% (v/v) EtOH at 0 ºC, was a routine but necessary step, in spite of the significant loss of the chromoprotein. It was used five treatments by 50% (v/v) EtOH to obtain the absorption spectrum of PM suspension purified from carotenoids (4) and (5) (degree of chromatographic purity of 80– 85%), as shown in Figure 5 at various processing stages (*b*) and (*c*) relative to the native BR (Fig. 5*a*). Figure 5 shows a dark-adapted absorption maximum at λ = 548 nm. The formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption spectrum of PM (Fig. 5*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 the native BR, D_{280}/D_{568} = 1.5 : 1.0). Upon the light absorption, the maximum absorbance of the PM shifts to λ = 556 nm with 6–8% increase in extinction. The $\lambda_{280}/\lambda_{568}$ = 280/568 nm absorbance ratio for BR is directly related to the ratio of total protein (the native BR) and is a convenient indicator for BR stability and integrity. The identical absorbance ratios are monitored using the conventional optics on a Beckman DU-6

spectrophotometer ("Beckman Coulter", USA) for the detergent-solubilized BR or the purified BR-solubilized in detergent.

The fractionation and chromatographic purification of the protein was a necessary next step of the BR purification. For obtaining protein from a biological material in purified, homogeneous state using various detergents assisted to the cleavage of protein-lipid complexes and the rupture of protein-protein bonds. In particular, for the release of proteins (enzymes) which are firmly connected with biomembranes or other subcellular structures used Triton X-100, sodium dodecyl sulfate (SDS) and sodium deoxycholate as detergents.

As the BR, being an integral membrane 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 appropriate ionic detergent. Using as the ionic detergent SDS-Na was dictated by the need of carrying out the more accomplished solubilization of the protein in a native, biologically active form in complex with 13-*trans*-retinal, because BR solubilized in 0.5% (w/v) SDS-Na retains a native α-helical configuration [27]. However SDS-Na is a strong detergent and its usage for protein fractionation is justified by a limited range of concentration (from 0.1 to 0.5% (w/v)). In addition SDS-Na seems to be more effective for lipid removal that other conventional detergents. Therefore, there was no need the use organic solvents as acetone, methanol and chloroform for purification of phospholipids, while precipitation and delipidization stages being combined in one single step, which significantly simplifies the further fractionation of the protein and reduces its losses during the precipitation procedure. A significant advantage of this method is that the isolated protein in complex with phospholipids and detergent was distributed in the supernatant, while other high molecular weight impurities – in the precipitate, which can be easily separated by centrifugation. Fractionation of the solubilized in 0.5% (w/v) SDS-Na protein and its subsequent isolation in crystalline form was performed at 0 °C in three steps precipitating procedure with MeOH, slowly reducing the concentration of SDS-Na from 0.5, 0.25 and 0.1% (w/v). The final stage of the BR purification involved the separation of the protein from low-molecular-weight impurities by GPC. For this purpose the fractions containing BR were passed twice through a chromatography column with dextran Sephadex G-200 balanced with buffer (pH = 8.35) containing 0.1% (w/v) SDS-Na and 2.5 mM EDTA. The data on purification of BR are shown in Table 1.

Table 1: The data on purification stages of the BR

Sample	mol PM/mol BR	Phospholipid removal, %	BR yield*, %
PM	15.5	–	–
PM washed with EtOH			
1 wash	4.9	65	93
2 wash	4.1	70	90
3 wash	3.5	76	88
4 wash	3.2	81	84
5 wash	2.8	84	80
BR crystallized from MeOH	1.9	86	75
BR from GPC on Sephadex G-200	1.2	92	86

* Note:
Percentage yield relative to the BR solubilized in SDS-Na before the purification procedure

As was shown in Table 1, 88% of phospholipids was removed by five washes (65 and 76% was removed by 1st, 2nd and 3nd wash respectively). When the sample was solubilized in 0.1% (w/v) SDS-Na and applied to the GPC on Sephadex G-200, a chromatogram shown in Figure 6 was obtained. The total endogenous phospolipid removal on the BR peak was 92% relative to the native

PM. The absorbance profile shows the separated protein. For smaller BR loads (0.1 mg), the analytical column (size) worked equally well.
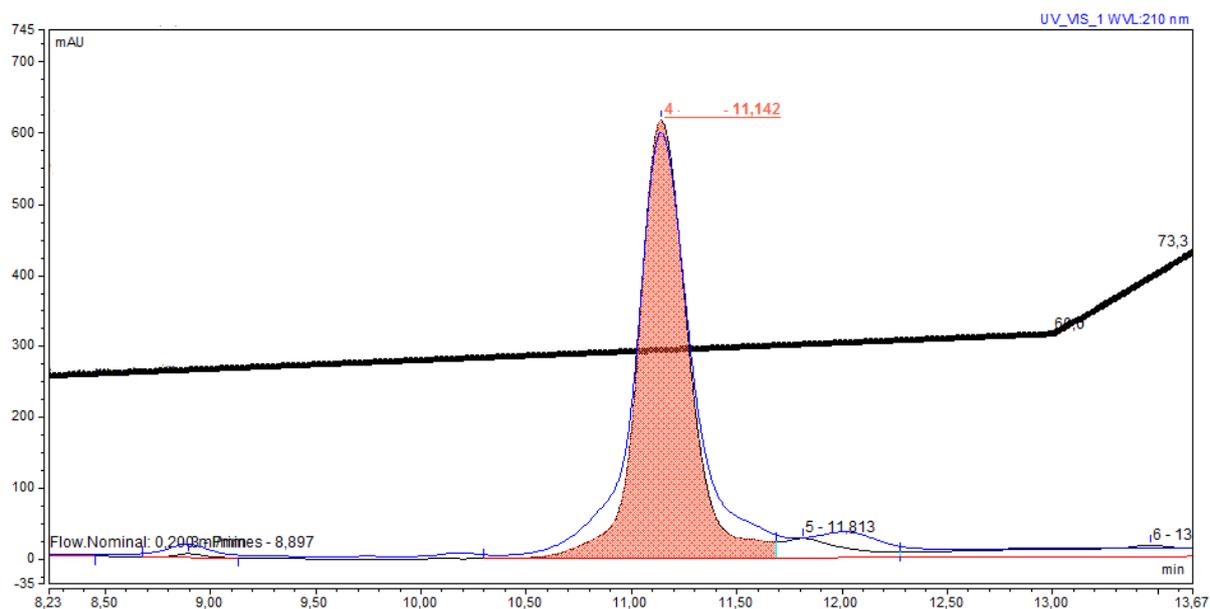

Figure 6: The GPC-chromatogram of the BR sample after purification (shaded as orange shows the separated protein): GPC (150×10 mm) column; stationary phase: Sephadex G-200 ("Pharmasia", USA) equilibrated with buffer with 0.1% (w/v) SDS-Na and 2.5 mM ETDA; specific volume: 30–40 units per 1 g dry Sephadex G-200; t = 20±25 °C; eluent: 1 mM Tris-HCl buffer (pH = 7.4); flow rate: 10 ml/cm²·h; detection UV/VIS at λ = 280 and λ = 568 nm

The homogeneity of the isolated BR satisfies to 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 and *in vitro* regeneration of AP with 13-*trans*-retinal. The degree of regeneration of PM was determined by the ratio: $D_{nat.280} \cdot D_{nat..568}/D_{reg..280} \cdot D_{reg.568}$ (D_{280} and D_{568} – the absorbance of a suspension of native and regenerated PM at λ = 280 and λ = 568 nm) was 65 mol.%. The output of the crystalline protein makes up approximately 5 mg. The isolated protein was washed with cold dist. ^2H_2O, centrifuged (1200 g, 15 min), subjected to freeze-drying, sealed into glass ampoules and stored in frost camera at t = -10 °C.

Conclusion
The technology for isolation of BR indicates the high yields of the protein, as a result of which it was isolated 5 mg of BR from 100 g of wet bacterial biomass. To ensure higher yields of BR it needs to accumulate more row biomass feedstock that can be easily achieved in the mass-scale laboratory conditions. The main advantage of this method is that the isolated BR retains its native configuration in combination with 13-*trans*-retinal, and the ability to photochemical reactions *in vitro* that is important for further use of the BR for the construction of photo-transforming nanofilms and artificial membranes containing BR.

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УДК 577.37 + 537.86

Улучшенный метод выделения фотохромного трансмембранного белка бактериородопсина из пурпурных мембран галобактерии *Halobacterium halobium ET 1001*

[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 (НИЦМБ), Болгария
Профессор, доктор наук Европейской академии естественных наук (ФРГ), директор НИЦМБ
1111, София, ул. Н. Коперника, 32/6
E-mail: mbioph@dir.bg

Аннотация. Разработан улучшенный метод выделения фотохромного трансмембранного белка бактериородопсина (выход 8–10 мг), способного трансформировать световую энергию фотонов в электрохимическую энергию генерированных протонов и АТФ. Протеин был изолирован в чистом кристаллическом виде из пурпурных мембран фотооргнанотрофной галобактерии *Halobacterium halobium ET 1001* за счет клеточного лизиса дист. водой, обработкой бактериальной биомассы ультразвуком при 22 кГц, спиртовой экстракции низко- и высоко-молекулярных примесей, клеточной РНК, каротиноидов и фосфолипидов, солюбилизации белка в 0.5% (масс./об.) ДДС-Na, фракционировании MeOH и колоночной гель-проникающей хроматографии (ГПХ) на сефадексе Sephadex G-200 с 0.1% (масс./об.) ДДС-Na и 2.5 мМ ЭТДА. Гомогенность изолированного бактериородопсина подтверждена комбинацией препаративных и аналитических методов, включая электрофорез в 12.5% (масс./об.) ПААГ с 0.1% (масс./об.) ДДС-Na и регенерацией апо-мембран с 13-*транс*-ретиналем.

Ключевые слова: *Halobacterium halobium ET 1001*, пурпурные мембраны, бактериородопсин, биосинтез, биомолекулярная электроника.