

Introduction of Deuterated Aromatic Amino Acids – [2,3,4,5,6-²H₅]Phenylalanine, [3,5-²H₂]Tyrosine and [2,4,5,6,7-²H₅]Tryptophan Into a molecule of Photochrome Trans-Membrane Protein Bacteriorhodopsin

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Abstract

It was carried out the introduction of functionally important deuterated aromatic amino acids – [2,3,4,5,6-²H₅]phenylalanine, [3,5-²H₂]tyrosine and [2,4,5,6,7-²H₅]tryptophan into a molecule of photochrome trans-membrane protein bacteriorhodopsin, synthesized by a photo-organotrophic halobacterium *Halobacterium halobium ET 1001*. The deuterated protein (output 8–10 mg) 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, gel filtration chromatography on Sephadex G-200, reverse-phase HPLC and EI impact mass-spectrometry of methyl esters of N-Dns- [²H]derivatives of amino acids. Deuterium was detected in all residues of aromatic amino acids. However, the presence in the EI mass spectrum of the BR hydrolysate the peaks [M]⁺ of semi-deuterated analogues of aromatic amino acids – phenylalanine 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 the conservation of the minor pathways of biosynthesis of aromatic amino acids de novo.

Keywords: *Halobacterium halobium*, bacteriorhodopsin, [2,3,4,5,6-²H₅]Phe, [3,5-²H₂]Tyr, [2,4,5,6,7-²H₅]Trp, biosynthesis, EI mass-spectrometry, RP-HPLC.

1. 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. Oesterhelt (Oesterhelt & Stoeckenius, 1971). This photo-transforming integral trans-membrane protein with the molecular weight ~26.5 kDa represents a chromoprotein determining the purple-red colour 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 (Oesterhelt, 1988). 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.

On its structure and location in the cell membrane BR refers to integral trans-membrane proteins, 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. 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 helices 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.

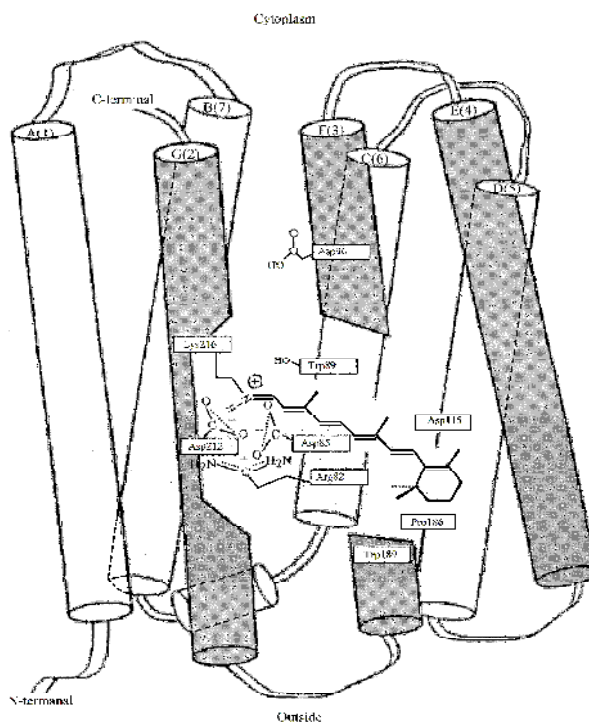


Figure 1: The location of protein and the retinal residue in the BR molecule of halobacterium *H. halobium* according to computer modeling: Latin numerals indicate protein fragments of the BR molecule as 7 α -helical segments (A, B, C, D, E, G, F) with exposed amino acid residues; gray color represents 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 $\sim 0,5 \mu\text{m}$ 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.

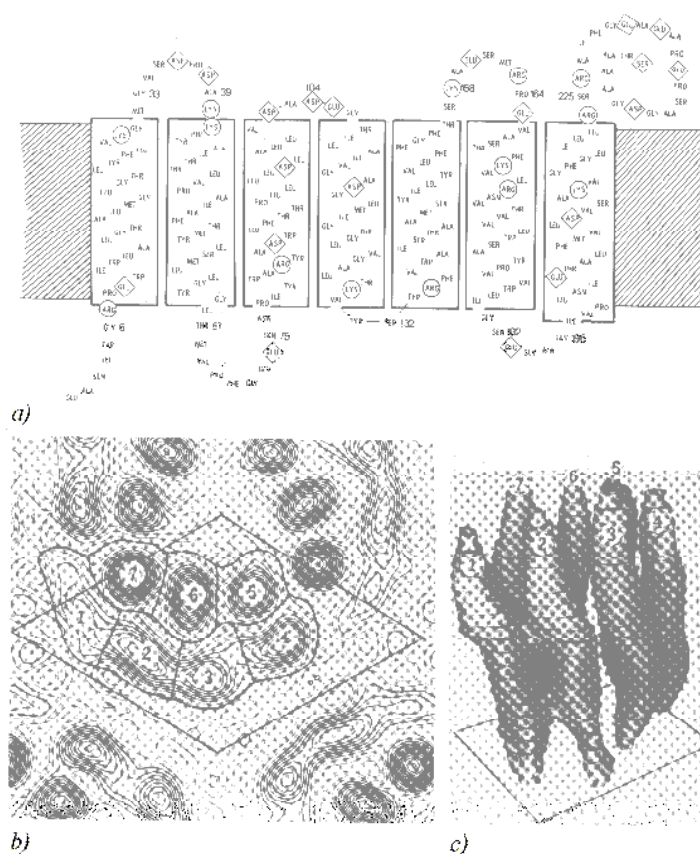


Figure 2: The structure of the BR molecule according to X-ray diffraction analysis:

- 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-*trans*-BR ($\lambda_{max} = 548$ nm) (the quantum yield 0.03 at $t = +20$ °C) into 13-*cis*-BR [$\lambda_{max} = 568$ nm] [3], 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 the BR vary depending on the method of preparation of the PM and the properties of embedded polymer matrix.

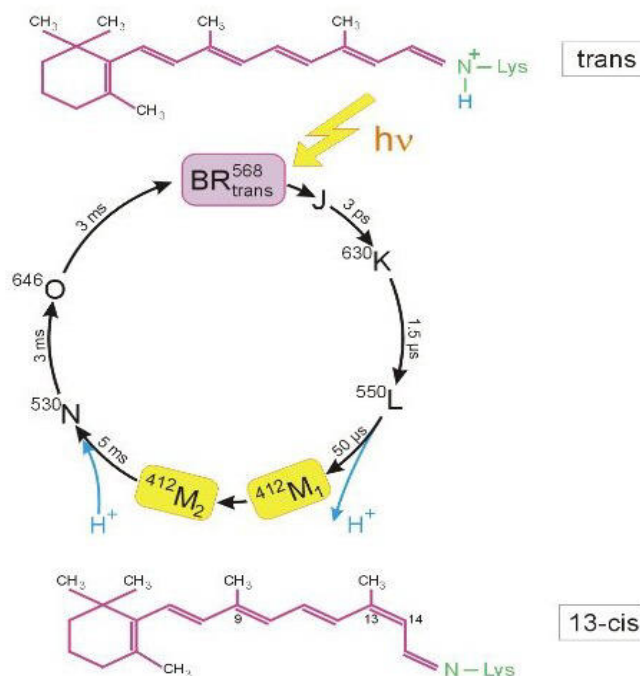


Figure 3: Photocycle scheme of BR (aqueous solution, pH = 7,2, t = +22 °C). Latin numbers J, K, L, M, N, O denote the spectral intermediants of BR. M₁ and M₂ represent spectral intermediants of *meta*- bacteriorhodopsin with the protonated and deprotonated aldimine bond. The superscripts correspond to the position of the absorption maximum intermediates in photocycle (nm)

The main task for the manufacture of BR-containing nanofilms is the orientation of PM between the hydrophobic and hydrophilic media. Typically, to improve the characteristics of the BR-containing films use multiple layers of the PM that are applied to the surface of the polymeric carrier and dried up, preserving their natural structure. The best results are achieved in the manufacture of nanofilms based on gelatin matrix (Fig. 4). This allows achieve high concentration of BR (up to 50 %) in nanofilms and avoid aggregation of membrane fragments and destruction of BR in the manufacturing process. Being embedded in a gelatin matrix PM fragments are durable (~10⁴ h) and resistant to solar light, the effects of oxygen, temperatures greater than +80 °C (in water) and up to +140 °C (in air), pH = 1–12, and action of most proteases. The dried PM is stacked on top of each other, focusing in the plane of the matrix, so that a layer with 1 μm thickness contains about 200 monolayers. When illuminated such nanofilms exert the electric potential 100–200 mV, which coincides with the membrane potential of living cells. These factors are of great practical importance for integration of PM into polymeric nanomatrix with keeping photochemical properties.

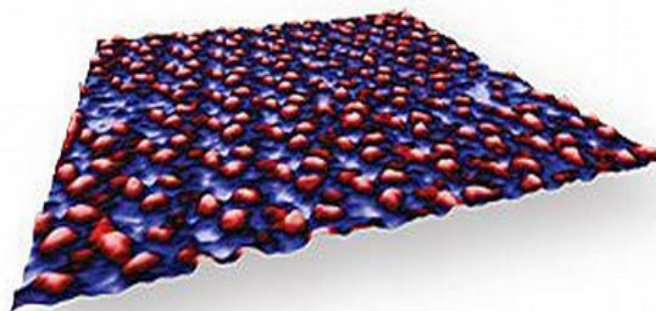


Figure 4: Artificial membrane from the BR-containing PM obtained by the SEM method: scanning area – 100×100 nm, resolution – 50 nm, magnification – 100000 times. PM is shown in purple, BR – in red color

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 (Haupts *et al.*, 1997). Additionally, the 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 (Hampp & Oesterhelt, 2004; Vought & Birge, 1999; Gui-Ying *et al.*, 2012). For these studies it is useful to enter into the protein molecule a deuterium label (^2H), which allows apply for evaluating of the structure by the NMR-method (LeMaster, 1990). In this aspect great scientific and practical interest 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 (Ignatov & Mosin, 2014). ^2H -labeled aromatic amino acids can be synthesized in gram scale quantities by isotopic exchange ($\text{H}-^2\text{H}$) in molecules of the protonated amino acids; for analyzing deuterium enrichment levels the EI mass-spectrometry of N-5-(dimethylamino)naphthalene-1-sulfonyl chloride (dansyl) amino acid derivatives may be used (Mosin *et al.*, 1994; Mosin *et al.*, 1996; Mosin *et al.*, 1999). The purpose of this research was to study the micro preparative biosynthesis of the BR labeled with deuterium on the residues of functionally important aromatic amino acids – [2,3,4,5,6- $^2\text{H}_5$]phenylalanine, [3,5- $^2\text{H}_2$]tyrosine and [2,4,5,6,7- $^2\text{H}_5$]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.

2. Material and methods

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

2.2. Chemicals

For the synthesis of aromatic [^2H]amino acids were used the protonated *D,L*-amino acids (“Reanal”, Hungary), $^2\text{H}_2\text{O}$ (99.9 atom.% ^2H), ^2HCl (95.5 atom.% ^2H), and $^2\text{H}_2\text{SO}_4$ (97.5 atom.% ^2H), 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 $^2\text{H}_2\text{O}$, $^2\text{H}_2\text{O}$ was distilled over KMnO_4 with subsequent control of the isotope purity by ^1H -NMR spectroscopy on a Bruker WM-250 (“Bruker Daltonics” Germany) with a working frequency 70 MHz (internal standard Me_4Si).

2.3. Synthesis of L-[2,3,4,5,6- $^2\text{H}_5$]phenylalanine

40 g of phenylalanine was dissolved in 300 ml of 85% (v/v) $^2\text{H}_2\text{SO}_4$ (in $^2\text{H}_2\text{O}$) and heated with water reflux condenser at 50–60 $^{\circ}\text{C}$ under stirring for 3 days. Upon completion the reaction mixture was cooled, neutralized with 30% (v/v) NH_4OH to pH = 5.5. The product was extracted with ethanol. Yield 24 g (58.7%), $T_m = 271\text{--}273$ $^{\circ}\text{C}$; $[\alpha]_d^{25} = 4.47$ (5 M HCl); $pK_a = 2.20$ (COOH), 9.31 (NH_2); $R_f = 0.6$ (A). UV-spectrum (0.1 M HCl): λ_{max} nm [ϵ $\text{M}^{-1}\text{cm}^{-1}$]: 257.5 [ϵ 195]. ^1H -NMR spectrum ($^2\text{H}_2\text{SO}_4$ in $^2\text{H}_2\text{O}$) (δ , ppm): δ 3.25 (2H, H β), δ 4.4 (1H, H α), δ

7.2–7.4 (0.07H), 95 atom.% ^2H . EI mass spectrum $[\text{M}]^+ m/z$ (I, %): 165 (34), methyl ester of N-Dns-[2,3,4,5,6- $^2\text{H}_5$]Phe: 417 (14), 418 (6).

2.4. Synthesis of L-[3,5- $^2\text{H}_2$]tyrosine

100 g of tyrosine was dissolved in 150 ml of 3 M $^2\text{H}_2\text{SO}_4$. The reaction mixture was heated for 2 days at 40–50 $^{\circ}\text{C}$ with water reflux condenser under a slow stream of dry nitrogen. The solution was neutralized with 30% (v/v) NH_4OH to pH = 4.5 and cooled for 24 h at 4 $^{\circ}\text{C}$. The crystalline product was filtered, washed with $^2\text{H}_2\text{O}$ and dried at 10 mm Hg. Yield 90 g (86.5%); $T_m = 316\text{--}317$ $^{\circ}\text{C}$; $[\alpha]_d^{25} = 10.03$ (5 M HCl); $pK_a = 2.20$ (COOH), 9.21 (NH_2); $R_f = 0.45$ (A). UV-spectrum (0.1 M HCl) λ_{max} nm $[(\epsilon \text{ M}^{-1}\text{cm}^{-1})]$: 223 $[(\epsilon 8200)$, 274.5 $(\epsilon 1340)]$. $^1\text{H-NMR}$ spectrum (1M ^2HCl) (δ ppm): $\delta 3.32$ (2H), $\delta 4.35$ (1H), $\delta 6.9$ (1H), $\delta 7.2$ (2H), 96 atom.% ^2H . EI mass spectrum $[\text{M}]^+ m/z$ (I, %): 181 (21), methyl ester of N-Dns-[3,5- $^2\text{H}_2$]Tyr: 429 (15), 430 (5).

2.5. Synthesis of L-[2,4,5,6,7- $^2\text{H}_5$]tryptophan

100 ml of 40% (v/v) $^2\text{H}_2\text{O}$ was placed in a round bottom flask and cooled in an ice bath. 80 ml of trifluoroacetic anhydride (0.5 mol) was added dropwise with stirring. The reaction mixture was kept for 2 h at 4 $^{\circ}\text{C}$ then 25 g of tryptophan was added portionwise. The reaction mixture was kept for 3 days in the dark at 22 $^{\circ}\text{C}$, the solvent was removed at 10 mm Hg., neutralized with 30% (v/v) NH_4OH to pH = 5.9, and cooled for 1 day at 4 $^{\circ}\text{C}$. The crystalline product was filtered, washed with $^2\text{H}_2\text{O}$ and dried at 10 mm Hg. Yield 19 g (60.3%); $T_m = 283\text{--}285$ $^{\circ}\text{C}$; $[\alpha]_d^{25} = 2.8$ (1 M HCl); $pK_a = 2.46$ (COOH), 9.41 (NH_2); $R_f = 0.5$ (A). UV-spectrum (0.1 M HCl) λ_{max} nm $[(\epsilon \text{ M}^{-1}\text{cm}^{-1})]$: 218 $[(\epsilon 3350)$, 278 $(\epsilon 5550)$, 287.5 $(\epsilon 4550)]$. $^1\text{H-NMR}$ spectrum (CF_3COOH in $^2\text{H}_2\text{O}$) (δ ppm): $\delta 3.4$ (2H, H β), $\delta 4.4$ (1H, H α), $\delta 7.3$ (1H, H ϵ), $\delta 7.2\text{--}7.4$ (0.1H, In-H), 98 atom.% ^2H . EI mass spectrum $[\text{M}]^+ m/z$ (I, %): 204(28), methyl ester of N-Dns-[2,4,5,6,7- $^2\text{H}_5$]Trp: 455(9), 456(11).

2.6. Synthesis of N-Dns-[^2H]amino acids

To 4 mg of the dry hydrolyzate BR 1 ml of 2 M NaHCO_3 (pH = 9–10) was added portionwise with stirring 25.6 mg of Dns-chloride in 2 ml of acetone. The reaction mixture was kept for 1 h under stirring at 40 $^{\circ}\text{C}$, acidified with 2 N HCl to pH = 3.0 and extracted with ethyl acetate (3 times \times 5 ml). The combined extract was washed with distilled $^2\text{H}_2\text{O}$ to pH = 7.0 (2 times \times 2 ml), dried over anhydrous Na_2SO_4 . The solvent was removed at 10 mm Hg. Yield 5.3 mg (78%).

2.7. Synthesis of methyl esters of N-Dns-[^2H]amino acids

3 g. of wet N-nitroso-N-methylurea was added to 20 ml of 40% (v/v) KOH in 40 ml of diethyl ether and then stirred on a water bath with ice for 15–20 min for obtaining diazomethane. After the completion of gas release, the ether layer was separated, washed with distilled water to pH = 7.0, dried with anhydrous Na_2SO_4 , and used for the treatment of N-Dns-amino acids. Yield 17.4 mg (69%).

2.8. Biosynthesis of BR

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- $^2\text{H}_5$]phenylalanine, [3,5- $^2\text{H}_2$]tyrosine, and [2,4,5,6,7- ^2H]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-lysine – 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; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ – 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; $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$ – $3 \cdot 10^{-4}$; $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ – 0.065; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ – $4 \cdot 10^{-5}$; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ – $5 \cdot 10^{-4}$; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ – $5 \cdot 10^{-5}$; 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 $^{\circ}\text{C}$ on a shaker (“Birad Labs”, Hungary) under intense aeration and monochromatic illumination (3 lamps \times 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.

2.9. Isolation of purple membranes (PM)

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 $^{\circ}\text{C}$. 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 \times 5 min) in an ice bath (0 $^{\circ}\text{C}$). The cell homogenate after

washing with dist. H₂O was resuspended in 10 ml of buffer containing 125 mM NaCl, 20 mM MgCl₂, 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 PM precipitate 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} ($\epsilon_{280} = 1.1 \cdot 10^5 \text{ M}^{-1} \text{ cm}^{-1}$; $\epsilon_{568} = 6.3 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$) (Neugebauer *et al.*, 1978; Mosin *et al.*, 2012). PM regeneration is performed as described in (Rudiger *et al.*, 1997; Mosin *et al.*, 2013). The yield of PM fraction, 120 mg (80–85%).

2.10. Isolation of BR

Fraction PM (in H₂O) (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, then 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. ²H₂O (2 times × 1 ml) and centrifuged (1200 g, 15 min).

2.11. Purification of BR

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 (“Pharmacia”, 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 EDTA. Elution proceeded by 0.09 M Tris-borate buffer containing 0.5 M NaCl, pH = 8.35 at a flow rate 10 ml/cm²h. Combined protein fraction was subjected to freeze-drying, in sealed glass ampoules (10 × 50 mm) and stored in frost camera at -10 °C.

2.12. 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). 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.

2.13. 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 H₂SO₄ to pH = 7.0. The precipitate of BaSO₄ was removed by centrifugation at 200 g for 10 min, and the supernatant was evaporated in a rotor evaporator at 40 °C.

2.14. RP-HPLC

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), $t = 20 \pm 25$ °C, 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). The results on gradient elution are shown in Table 1. The yield of individual methyl esters of N-Dns-[²H]amino acids – 75–89%; chromatographic purity – 95–98%.

Table 1: The results of one-step gradient separation of a mixture of methyl esters of N-Dns [²H] amino acids from the hydrolyzate of the BR by RP HPLC on a column 250×10 mm, t = 20±25 °C, with octadecylsilane silica gel Separon SGX C 18,7 μm

Number processing	Components of the mobile phase, % vol.		Elution time, min
	A*	B**	
1	90	10	10
2	80	20	10
3	60	40	10
5	50	50	10
6	30	60	5
8	20	80	5
9	10	90	5
10	0	100	5

Notes:

* A – acetonitrile-trifluoroacetic acid 100 : 0.1–0.5% (v/v);

** B – acetonitrile–100% (v/v)

2.15. EI mass spectrometry

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.

3. Results and discussion

3.1. BR biosynthesis

The strategy for biosynthesis of ²H-labeled BR using a strain of extreme photo-organotrophic halobacterium *Halobacterium halobium* was determined by the study of the principal possibility for obtaining ²H-labeled photochrome transmembrane proteins in semi-micropreparative quantities for reconstruction of artificial membranes in ²H₂O and BR-containing nanofilms. [2,3,4,5,6-²H₅]phenylalanine, [3,5-²H₂]tyrosine, and [2,4,5,6,7-²H₅]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–²H) 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. ²H-labeled BR was obtained via growth of halobacterium *H. halobium* on synthetic medium (4.3 M NaCl) with [2,3,4,5,6-²H₅]phenylalanine, [3,5-²H₂]tyrosine and [2,4,5,6,7-²H₅]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 D₂₈₀/D₅₆₈ = 1.5 : 1.0 in which was identical to the natural BR. The growth of the halobacterium on a synthetic medium (Fig. 5b) was practically as the same as in the control (Fig. 5a) on the protonated growth medium with protonated amino acids, that significantly simplifies the optimization of conditions for the biosynthesis of ²H-labeled BR, which consists in the equivalent replacing of protonated aromatic amino by their deuterated analogues – [2,3,4,5,6-²H₅]phenylalanine (0.26 g/l), [3,5-²H₅]tyrosine (0.2 g/l) and [2,4,5,6,7-²H₅]tryptophan (0.5 g/l).

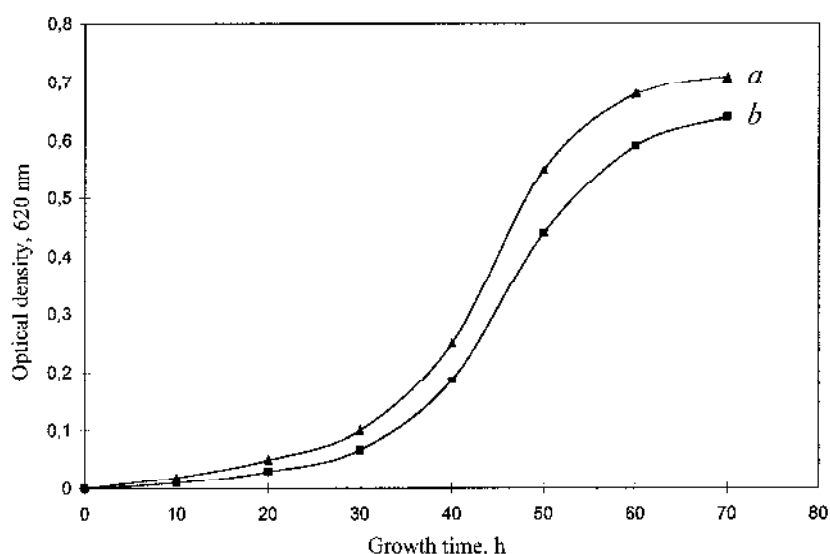


Figure 5: Growth dynamics of *H. halobium* under various experimental conditions: a) – protonated synthetic medium; b) – synthetic medium with [2,3,4,5,6-²H₅]Phe (0.26 g/l), [3,5-²H₂]Tyr (0.2 g/l) and [2,4,5,6,7-²H₅]Trp (0.5 g/l). The incubation period: 4–5 days, temperature: 35 °C, illumination under monochrome light at $\lambda = 560$ nm.

The main stages of the experiment were: growing of a strain-producer *H. halobium* on synthetic medium with [2,3,4,5,6-²H₅] phenylalanine (0.26 g/l), [3,5-²H₂] tyrosine (0.2 g/l) and [2,4,5,6,7-²H₅] tryptophan (0.5 g/l), the separation of cell content, isolation of purple membrane (PM) fraction, removal 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, and final 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 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 to obtain the absorption spectrum of the purified from carotenoids PM suspension (4) and (5) (degree of chromatographic purity of 80–85%), as shown in Figure 6 at various processing stages (b) and (c) relative to the native BR (a). The formation of retinal-protein complex in the BR molecule leads to a bathochromic shift in the absorption spectrum of the PM (Fig. 6c) – the main bandwidth (1) with the absorption maximum at $\lambda = 568$ 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 BR₅₆₈; additional low-intensity bandwidth (2) at $\lambda = 412$ nm characterizes a minor impurity of a spectral form of meta-bacteriorhodopsin M₄₁₂ (formed in the light) with deprotonated aldimine bond between 13-*trans*-retinal residue and protein; the total bandwidth (3) with $\lambda = 280$ nm is determined by the absorption of aromatic amino acids in the polypeptide chain of the protein (for native BR $D_{280}/D_{568} = 1.5 : 1.0$).

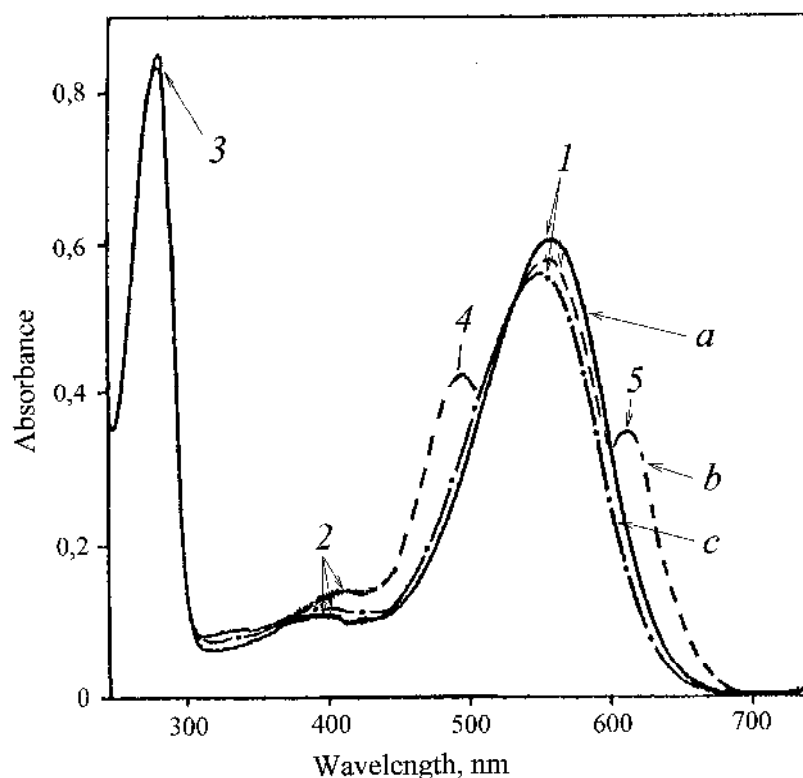


Figure 6: The absorption spectra of the PM (50% (v/v) EtOH) at various stages of processing: (a) – natural BR; (b) – PM after intermediate treatment; (c) – PM purified from carotenoids. The bandwidth (1) is the spectral form of BR₅₆₈, (2) – impurity of spectral form of meta-bacteriorhodopsin M₄₁₂, (3) – the total absorption bandwidth of aromatic amino acids, (4) and (5) – extraneous carotenoids. As a control used the native BR

3.2. Isolation and purification of BR

The fractionation and chromatographic purification of the protein was the next necessary step of the research. As 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 an ionic detergent. Using as the ionic detergent SDS-Na 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 (Nonella *et al.*, 1991). Therefore, there is no need the use organic solvents as acetone, methanol and chloroform for purification of lipids and protein, as the precipitation and delipidization are 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 (GPC). For this purpose the fractions containing BR were passed twice through a chromatography column with dextran Sephadex G-200 balanced with 0.09 M Tris-buffer (pH = 8.35) containing 0,1% (w/v) SDS-Na and 2,5 mM EDTA. The elution was carried out at 20±25 °C with 1 mM Tris-HCl buffer (pH = 7,6) at 10 ml/cm²·h. The data on purification of BR of phospholipids and carotenoids are shown in Table 2. 84% of phospholipids was removed by five washes (65, 70 and 76% was removed by 1st, 2nd and 3rd wash respectively). The total endogenous phospholipid removal on the BR peak was 92% relative to the native PM.

Table 2: Summary results for the isolation and purification of BR by various methods

Sample	PM content, mol PM/mol BR	Phospholipid and carotenoid removal, %	BR yield*, %
PM fraction	20,5	–	–
PM washed with EtOH			
1 wash	16,9	65	93
2 wash	15,1	70	90
3 wash	14,5	76	88
4 wash	13,6	81	84
5 wash	13,2	84	80
BR crystallised from MeOH	12,9	86	75
BR from GPC on Sephadex G-200	10,2	92	86

Notes:

* Percentage yield is indicated in mass.% relative to BR solubilized in 0,5% SDS-Na before concentration.

When the sample was solubilized in 0.1% (w/v) SDS-Na and applied to GPC on Sephadex G-200, a chromatogram shown in Figure 7 was obtained. The total endogenous phospholipid 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.

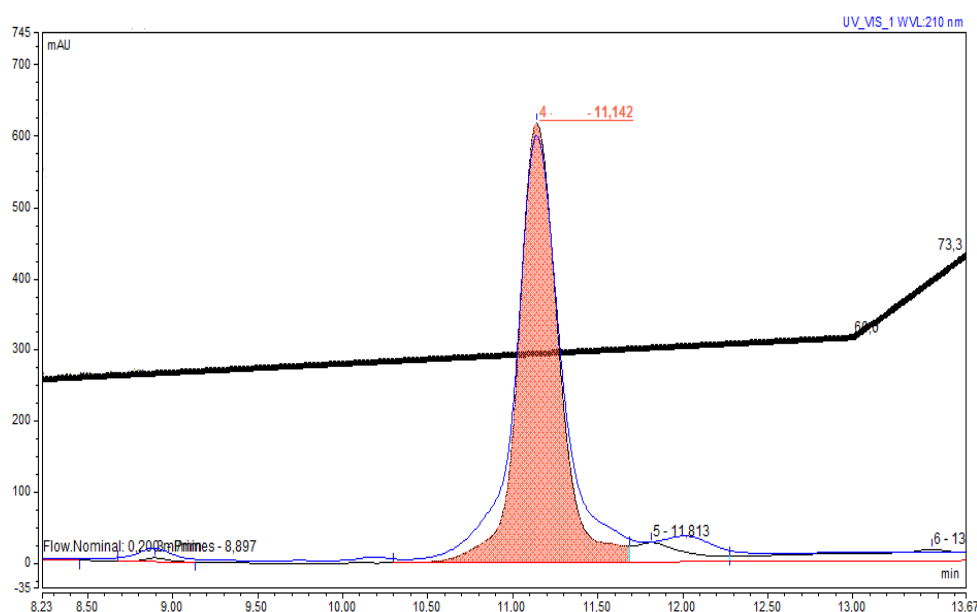


Figure 7: The GPC-chromatogram of the BR sample after purification (shaded as orange shows the separated protein): GPC (150×10 mm) column; stationary phase: Sephadex G-200 (“Pharmasia”, USA) equilibrated with buffer with 0.1% (w/v) SDS-Na and 2.5 mM EDTA; specific volume: 30–40 units per 1 g dry Sephadex G-200; $t = 20 \pm 25$ °C; eluent: 1 mM Tris-HCl buffer (pH = 7.4); flow rate: 10 ml/cm²h; detection UV/VIS at $\lambda = 280$ and $\lambda = 568$ nm

The method for protein fractionation made it possible to obtain 8–10 mg of ²H-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-[²H]amino acids. The micro-preparative 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.

3.3. Hydrolysis of BR

Conditions of hydrolysis of ^2H -labeled protein were determined to prevent the isotopic ($\text{H}-^2\text{H}$) 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 °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 (Zvonkova *et al.*, 1970), 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 (Penke *et al.*, 1974). 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%) (Liu & Chang, 1971). However, these methods are possess an essential disadvantage, because during acid hydrolysis at high speed occurs isotopic ($\text{H}-^2\text{H}$) exchange of aromatic protons(deuterons) in molecules of tryptophan, tyrosine and histidine as well as protons at C3 position of aspartic and C4 glutamic acid (Pshenichnikova *et al.*, 1995). Therefore, even carrying out of hydrolysis in deuterated reagents (6N ^2HCl , 4N $^2\text{H}_2\text{SO}_4$ in $^2\text{H}_2\text{O}$) does not derive the real data about the inclusion of the deuterium into the protein.

Under conditions of alkaline hydrolysis (4N $\text{Ba}(\text{OH})_2$ or 4N NaOH, 110 °C, 24 hours) the reactions of isotopic ($\text{H}-^2\text{H}$) 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 hydrolyzate due to the neutralization of H_2SO_4 (in $^2\text{H}_2\text{O}$) was the reason for choosing as hydrolysing agent 4N $\text{Ba}(\text{OH})_2$. 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.

3.4. Studying of the deuterium labeling of BR

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

EI mass spectrum of the mixture of methyl esters of N-Dns- ^2H amino acids as shown in Figure 8 (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 ^2H aromatic amino acids occupied scale mass numbers at m/z 415–456, are mixtures of molecules containing various numbers of deuterium atoms. Therefore, the molecular ions $[\text{M}]^+$ 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 ^2H amino acid molecules was determined using the most commonly encountered peak of the molecular ion $[\text{M}]^+$ in each cluster with mathematically averaged value of $[\text{M}]^+$ (Fig. 4). Thus, for phenylalanine molecular ion peak was determined by $[\text{M}]^+$ at m/z 417, 14% (instead of the $[\text{M}]^+$ at m/z 412, 20% for non-labeled derivative (unlabeled peaks of amino acid derivatives are not shown)), tyrosine – $[\text{M}]^+$ at m/z 429, 15% (instead of $[\text{M}]^+$ at m/z 428, 13%), tryptophan – $[\text{M}]^+$ at m/z 456, 11% (instead of $[\text{M}]^+$ at m/z 451, 17%). The level of deuterium enrichment corresponding to the increase of molecular weight was for ^2H tyrosine – 1 (90 atom.% ^2H), ^2H phenylalanine – 5 (95 atom.% ^2H) and ^2H tryptophan – 5 (98 atom.% ^2H) deuterium atoms. This result coincides with the data on the initial level of deuterium enrichment of aromatic amino acids – $[3,5-^2\text{H}_2]\text{Tyr}$, $[2,3,4,5,6-^2\text{H}_5]\text{Phe}$ and $[2,4,5,6,7-^2\text{H}_5]\text{Trp}$, added to the growth medium and indicates a high selectivity of inclusion of aromatic ^2H amino acids into the BR molecule. Deuterium was detected in all residues of aromatic amino acids (Table 3). However, the presence in the EI mass spectrum the molecular ion peaks $[\text{M}]^+$ of protonated and semi-deuterated phenylalanine analogues with $[\text{M}]^+$ at m/z 413–418, tyrosine – with $[\text{M}]^+$ at m/z 428–430 and tryptophan – with $[\text{M}]^+$ 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 in molecules, that evidently is determined by methabolic pathways of biosynthesis of ^2H -labeled BR while assimilating by the cell the ^2H -labeled aromatic amino acids, which are synthesized in the cell from the general predecessors – phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) (Fig. 9).

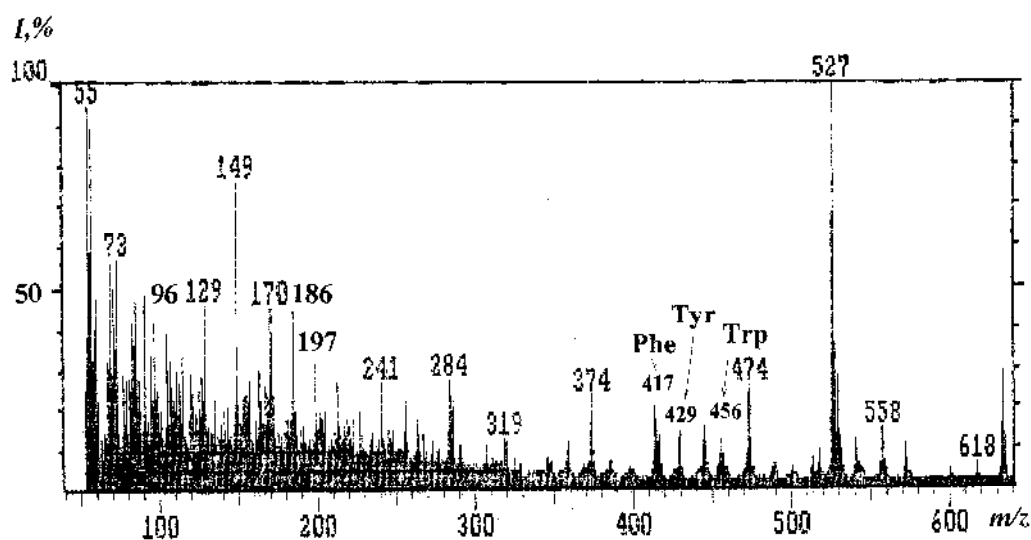


Figure 8: Full scan EI mass spectrum of methyl ester of N-Dns-²H derivatives of aromatic amino acids isolated from the BR hydrolyzate, obtained on synthetic medium with [2,3,4,5,6-²H₅]Phe (0.26 g/l), [3,5-²H₂]Tyr (0.2 g/l) and [2,4,5,6,7-²H₅]Trp (0.5 g/l) (energy: 70 eV, accelerating voltage: 8 kV, temperature: 180–200 °C). Hydrolysis conditions: 4 N Ba(OH)₂ (in ²H₂O), 110 °C, 24 h. Molecular ion peaks represented by the symbols of amino acids correspond to their derivatives; *I* – relative intensity (%).

Table 3: The values of the molecular ion peaks [M]⁺ in the EI mass spectrum of methyl esters of N-Dns-[2,3,4,5,6-²H₅]Phe, N-Dns-[3,5-²H₂]Tyr and N-Dns-[2,4,5,6,7-²H₅]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- ² H ₅]Phe-OMe	413	7	1	13
	414	18	2	25
	415	15	3	38
	416	11	4	50
	417	14	5	63
	418	6	6	75
N-Dns-[3,5- ² H ₂]Tyr-OMe	428	12	–	–
	429	15	1	14
	430	5	2	29
N-Dns-[2,4,5,6,7- ² H ₅]Trp-OMe	453	5	2	26
	454	6	3	38
	455	9	4	50
	456	11	5	64
	457	5	6	77

Notes:

* A dash means no incorporation of deuterium.

** In calculating the level of deuterium enrichment protons(deuterons) at carboxyl and NH₂-amino groups of amino acids were not considered due to the easily isotopic (H-²H) exchange.

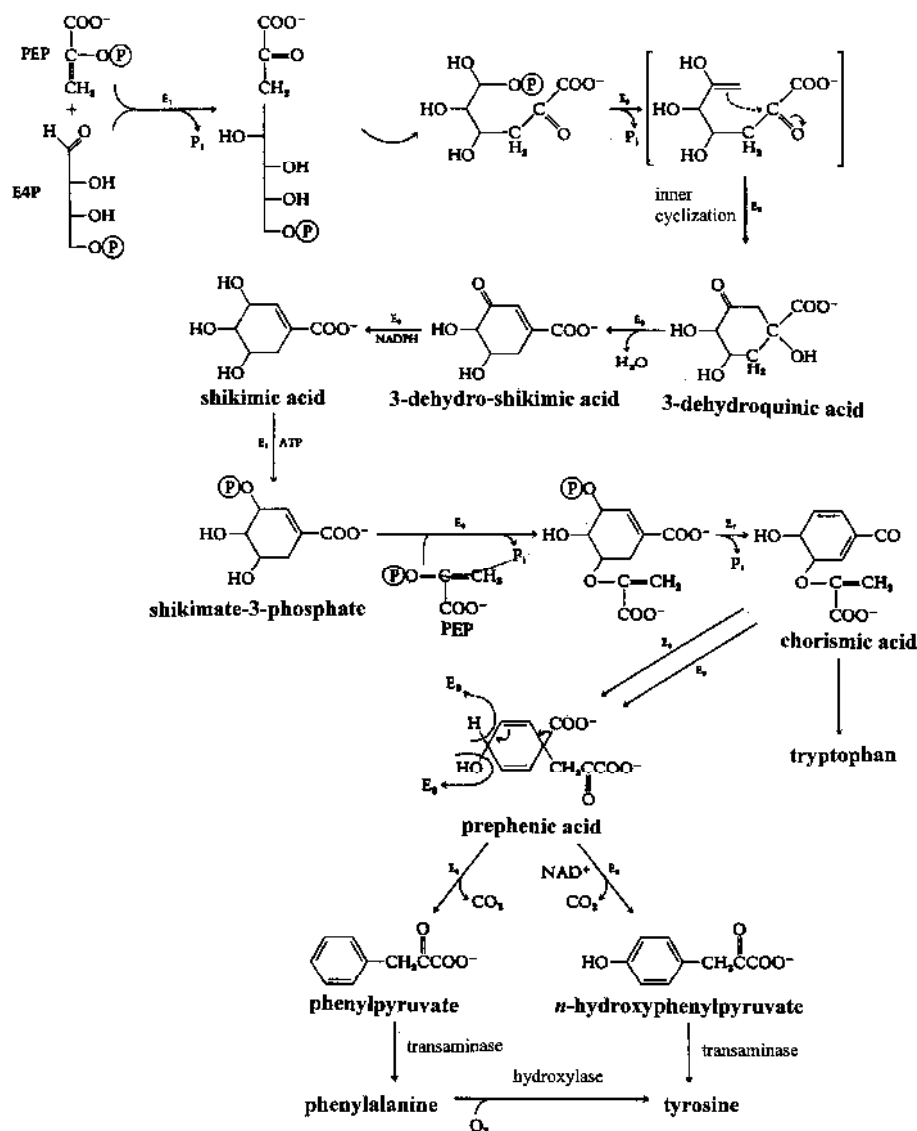
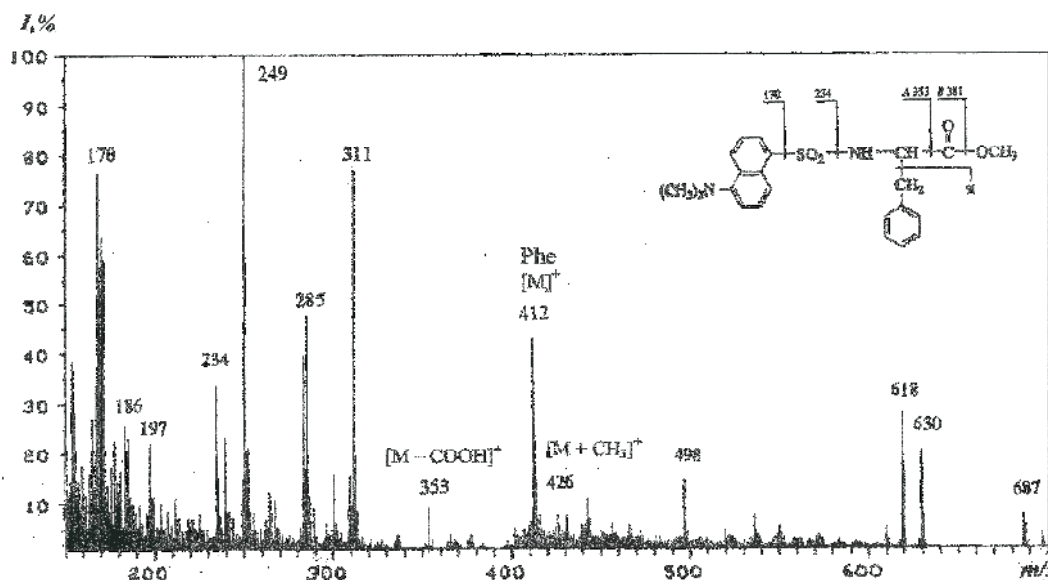


Figure 9. A general scheme of biosynthesis of aromatic amino acids – phenylalanine, tyrosine and tryptophan from the general predecessors – phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) (Mosin & Ignatov, 2012)

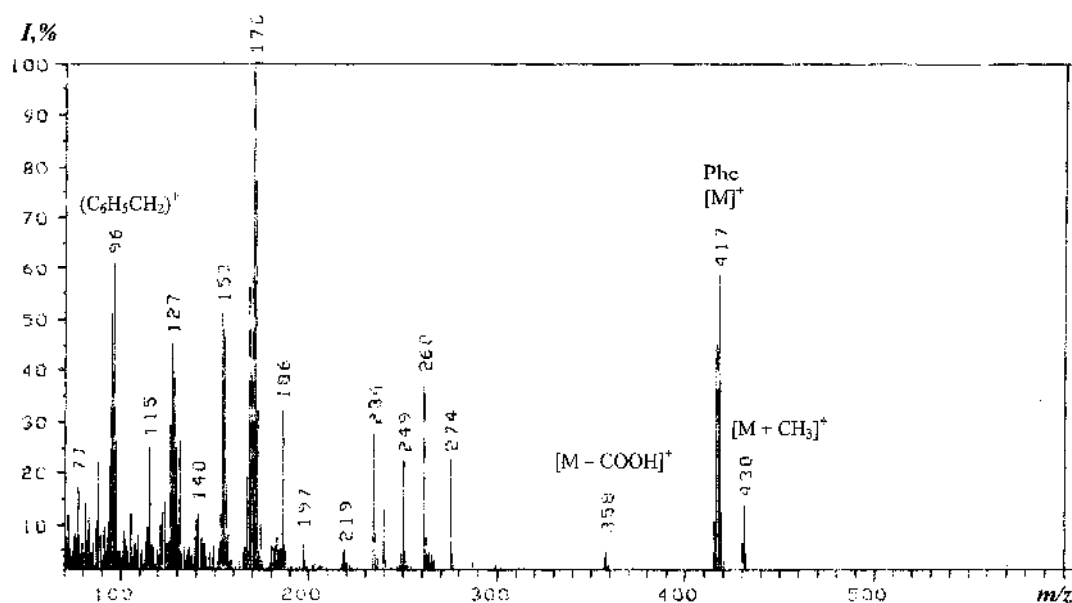
According to the mass spectrometric analysis the molecular ion peaks $[M]^+$ of methyl esters of N-Dns- $[^2H]$ derivatives of aromatic $[^2H]$ amino acids have a low intensity in EI mass spectra and were polymorphously split, so the areas of the molecular enrichment were strongly broadened. Moreover, mass spectra of the mixture components are additive, so the mixture can be analyzed only if the spectra of the various components are recorded under 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 on deuteration levels it was necessary to chromatographically isolate individual derivatives of $[^2H]$ amino acids from the protein hydrolysate. For this aim was used RP-HPLC on octadecylsilane silica gel Silasorb C18, the effectiveness of which was confirmed earlier by separation of a mixture of methyl esters of N-Dns- $[^2H]$ amino acids isolated from other microbial objects as methylotrophic bacteria and microalgae (Mosin & Ignatov, 2012; Mosin & Ignatov, 2013).

The data for chromatographic separation of methyl N-Dns-derivatives of $[^2H]$ amino acids from different natural sources do not differ from those for methyl esters of unlabeled N-Dns-amino acid derivatives, as the isotopic enrichment of molecules virtually has no effect on chromatographic characteristics. The method was adapted to

the conditions of the chromatographic separation of a mixture of methyl esters of N-Dns-[²H]amino acids from the hydrolysate of the BR, consisting in the optimization of the ratio of eluent, form and rates of gradient elution from the column. In addition, while using a gradient the maximum number of peaks in the chromatogram significantly increases – peak capacity, which is very important for the separation of complex multicomponent mixtures, which are the protein hydrolysates. In this case, each component of the mixture is separated under the most optimal composition of the eluent, thereby achieving their full separation quality in much less time than with the isocratic regime. The method was adapted to the conditions of chromatographic separation of a mixture of methyl esters of N-Dns-[²H]amino acids the of BR hydrolysate 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 – CH₃CN–CF₃COOH (100: 0,1–0,5% (v/v)) (A) and (B) – CH₃CN (100% (v/v)) by progressively increasing the concentration of component B in the mixture from 10 to 100%. Samples were dissolved in 10 ml MeOH and applied in an amount of 50-100 μl on a column (250×10 mm) with octadecylsilane silica gel Separon SGX C 18,7 μm. Herewith it was possible to isolate the tryptophan and inseparable pair phenylalanine/tyrosine. The levels of chromatographic purity of methyl esters of N-Dns-[2,3,4,5,6-²H₅]phenylalanine, N-Dns-[3,5-²H₂]tyrosine and N-Dns-[2, 4,5,6,7-²H₅]tryptophan isolated from protein hydrolysate were 89, 91 and 90%, respectively with the output 97–85%.



a)



b)

Figure 10: EI mass spectra of methyl N-Dns-[2,3,4,5,6-²H₅]Phe under various experimental conditions: a) – the unlabeled methyl ester of N-Dns-Phe; b) – methyl ester of N-Dns-[2, 3,4,5,6-²H₅]Phe, isolated from the BR hydrolyzate by RP HPLC. Separation conditions: 250 × 10 mm Column C18 (“Kova”, Slovakia), eluents: (A) – CH₃CN–CF₃COOH (100: 0.1–0.5% (v/v)) and (B) – CH₃CN (100% (v/v)).

The result confirmed Figure 10b which shows the EI mass spectrum of methyl N-Dns-[2,3,4,5,6-²H₅]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 (Figure 6a), scanning at m/z 150–700, base peak at m/z = 250, 100%). The evidence for the inclusion of 5 deuterium atoms into phenylalanine molecule is the presence of “heavy” molecular ion peak of methyl ester of N-Dns-[²H]phenylalanine ($[M]^+$ at m/z 417, 59%, instead of $[M]^+$ at m/z = 412, 44% for non-labeled derivative of phenylalanine), and the additional deuterium-enriched peaks of benzyl C₆H₅CH₂ fragments of [²H]phenylalanine molecule at m/z = 96, 61% (instead of m/z = 91, 55% in the control (not shown)) (Fig. 10b). The peaks of secondary fragments of varying intensity with values at m/z = 249, 234 and 170 are the secondary decomposition products of the dansyl residue to the 5-(dimethylamino)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 (COOCH₃) group of the methyl ester of N-Dns-[²H]phenylalanine, and a peak $[M + CH_3]^+$ at m/z 430, 15% (instead of m/z = 426, 8% in the control) – is the product of further methylation of α -amino group in the [²H]phenylalanine molecule (Fig. 10b). According to the EI mass spectrometry data, the difference between the molecular weight of protonated and deuterated species calculated by the 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 [2,3,4,5,6-²H₅]phenylalanine added to the growth medium. Mass spectrometric data on deuterium enrichment levels of [3,5-²H₂]tyrosine and [2,4,5,6,7-²H₅]tryptophan are also in good correlation and confirmed by ¹H NMR.

4. Conclusion

The experimental data indicate the high efficiency 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 ²H-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 ²H-labeled fatty acids and other biologically active compounds. These studies will provide an answer to the question of how functions the BR in the native membranes under condition of the complete replacement of protons by deuterium.

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