Bacteriorhodopsin and its Mutants allude a breakthrough impending to artificial retina construction and strategies for curing blindness

Pardis Saeedi[1], Jafar Mohammadian Moosaabadi[1]*, Mehrdad Behmanesh[2], Akram Eidi[1], Jalil Fallah Mehrabadi[3]

[1] Department of Biology, Science & Research Branch, Islamic Azad University, Tehran, Iran.
[2] Faculty of Sciences, Tarbiat Modares University, Tehran, Iran
[3]MARS Bioinformatics Institute, Tehran, Iran

*Corresponding author: e-mail address: *mohama_j@yahoo.com* (J. Mohammadian Moosaabadi)

ABSTRACT

Bacteriorhodopsin, a model system in nanobiotechnology, is a light-sensitive protein found in the *archaean Halobacterium salinarum* and a very identical protein to visual Rhodopsin. The modification of biological function of BR and its versatile properties is valuable for technical applications including the artificial retina. These photoactive elements of native and particular mutants of bacteriorhodopsin make protein films, used in artificial retinal implants, to treat some retinal diseases and disorders. The two major reasons of retinal photoreceptor cell deterioration are Age-related Macular Degeneration (AMD) and Retinitis Pigmentosa (RP). As in vitro culture of *Halobacterium* is very difficult, and isolation procedure is much time consuming and usually inefficient, so genetic construction of protein is essential. Here, we have produced two types of bacteriorhodopsin, a native and a mutant BR (D85E) and studied their opto-electric responses with respect to wavelength and absorption properties. They are prerequisite for designing artificial retina (sensors) based on biomolecules. Therefore, the new promising technology soon will conceivably eradicate the blindness.

Keywords: Bacteriorhodopsin; Rhodopsin; Photoreceptor; BR Films; Artificial Retina

INTRODUCTION

Microelectronics structures are common in artificial vision systems. Biomimetic technologies are successful systems inspired by nature. So different architectures have been evolved for processing visual information. As a result, some microelectronics can be substituted by functional biomolecules such as bacteriorhodopsin [1]. The potential clinical application of BR is for treatment of blindness caused by the death of rod and cone photoreceptors.

Nowadays genetic engineering is a popular tool for producing and modifying biological macromolecules to function in non-native environments and turning it into an evolutionary optimized biomaterial [2, 3]. In vitro culture of these bacteria is very difficult, and isolation procedure is much time consuming and usually inefficient, genetic production of protein can be helpful. Therefore, the aim of this research is to study the photochemical properties and to produce bacteriorhodopsin and its mutant variant D85E, by site-directed mutagenesis, that can be used in construction of bioelectronics devices, especially in artificial retina. Modification of bacteriorhodopsin comprises the replacement of key amino acids in the protein sequence either by site-directed, semi-random, or site-specific saturation mutagenesis [2].

Eye photoreceptor cells are classified to rod cells and cone cells. Cone cells include photo pigments that are essential for color vision, and need tens to hundreds of photons to become active. Rod cells enclose a photo pigment, Rhodopsin, which is greatly sensitive to light, so it allows vision under dim light circumstances. A rod cell (Figure 1) is sensitive enough to become active by a single photon of light [5].

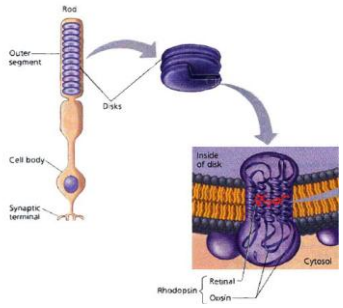

Figure 1. Organization of Rhodopsin inside a Rod Cell. [18]

Upon illumination, Rhodopsin, the photoreceptive pigment of rod cells, will be activated and undergoes a conformational change. Rhodopsin apoprotein is a seven alpha-helical transmembrane protein called opsin, covalently bound to a chromophore (retinal a prosthetic group and a derivative of vitamin A) as shown in Figure 2. Non-active retinal (before exposing to light) is in the 11-cis form, upon light absorption, retinal undergoes a conformational change to the all-trans forms. Retinal conformational change results in a consequent conformational change in the covalently bound opsin polypeptide, so leads to activating a second messenger cascade inside the photoreceptor cell that causes the conduction of signals to the proper retinal neurons. These signals are conveyed along the optic nerve to the visual centers of the brain, which allow the brain to process the visual input and perceive a visual image [5].

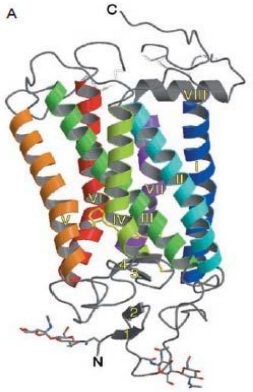

Figure 2. The structure of mammalian Rhodopsin as seen parallel to the plane of the membrane. [19]

Partial or full vision defect can be due to different conditions and diseases that destroy photo- receptor cells of the retina. Age-related Macular Degeneration (AMD) and Retinitis Pigmentosa (RP) are the two major diseases of the retina, which eventually lead to vision loss and blindness. In older adults AMD causes deterioration of both rod and cone photoreceptor cells of the retina, and has also effects on central vision. RP is an inherited disease with degeneration of the rod photoreceptor cells of the retina. Deterioration of the rod cells impairs the capability of vision in dim light and progressively diminishes peripheral vision, then tunnel vision and, finally, ends up in blindness [5 & 6].

Both AMD and RP affect a considerable portion of the population and primarily implicate the degeneration of retinal photoreceptor cells, whereas other neurons of the retina, including retinal ganglion cells whose long axons form the optic nerve, are substantially conserved [7].

More improved and less surgically invasive retinal implants are considered necessary to restore at least partially the vision of patients suffering from retinal perturbation or disease of Retinitis Pigmentosa and Age-Related Macular Degeneration [5].

Employing a visual input that is competent to activate retinal ganglion cells is a promising method for treatment of patients afflicted with a photoreceptor disorder. Visual input activation rebuilds different degrees of vision. A "retina chip" or "silicon retina" is an electronic stimulator that activates the retinal ganglion cells electrically via a visual input. Due to the toxicity of a foreign object in the body, electronic stimulators are not suitable for clinical application. Consequently, there yet remains a great need for proper cure of these patients [7]. Thus, it is better to use a biomaterial to avoid of these problems.

BR, an identical protein to Rhodopsin of the retina, is a light-harvesting membrane protein of a halophilic arcean, *Halobacterium salinarium* [4 and 8]. BR, Like Rhodopsins, is a seven α helical protein bound to the functional chromophore derivative of vitamin A (retinal) via a protonated Schiff base to a lysine residue. Again, like Rhodopsin, the natural function of BR is to operate as a light-driven proton pump, converting sunlight into chemical or electric energy [4 and 9]. During a photo cycle of BR, the proton pumping occurs with photochemical conversions like Figure 3 [4 and 10]. Therefore, BR characteristics make it greatly appropriate for optical and photoelectric applications.

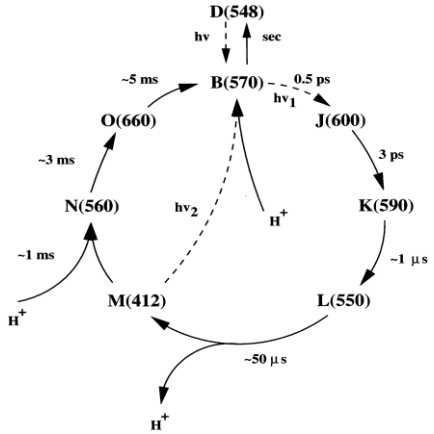

Figure 3. BR photo cycle [4]

Under anaerobic conditions, upon light absorption, BR undergoes a photo cycle, by

translocation of protons from the inside to the outside of the cell [11]. The electrochemical gradient thus generated applies for ATP synthesis by the cell. Based on spectral properties of mutants, they show absorption spectrum similar or different from native BR [12]. BR is capable to maintain the photo cycle even when isolated from the purple membrane and integrated into an artificial membrane, or a thin polymer-based film [1].

The Japanese researchers have made a multilayer film coated by bacteriorhodopsin. These films enclose bacterial membrane onto a transparent tin oxide electrode hold up on a glass substrate. Then an aqueous electrolyte gel squeezes in between the membrane and a gold-coated glass [8].

Nowadays, some prototypes of artificial retina apply for curing of such retinal diseases and conditions, but each has distinct disadvantages. One prototype, utilizes a diode of silicon to create electrical stimulation upon light activation. The silicon diode photoreceptor generates a sufficient current just in presence of intense light. It stimulates and provides only dim vision. The Artificial Silicon Retina (ASR) is a silicon chip enclosing approximately 3,500 micro photodiodes, each having its own stimulating electrode. The ASR can be surgically implanted under the retina and powered exclusively by incident light and does not require the external wires or batteries [6]. Another prototype occupies an external camera, mounted on a pair of eyeglasses, connected to a microelectrode array by a connecting cable. The electrode array provides electrical stimulation directly to the ganglion cells. A subject is able to perceive light on all electrodes of the array, detect motion, and recognize simple shapes. Yet, distinct disadvantage of this design is that it requires surgically implanted external hardware, like glasses [5]. Finally, BR photosensitive proteins can provide an efficient prototype of artificial retina for treating the cases.

The sensor made from only wild type BR, lacks all wavelengths and has just different levels of light, so black and white images can be perceived, but not colored. By modifying the BR biotechnically, the various wavelength responses can be produced [4].

Bacteriorhodopsin films include a multilayer of BR proteins, in which each layer of the protein alternates with a layer of a cationic polymer. The bacteriorhodopsin protein is chosen from the first main layer of the first bacteriorhodopsin mutant and a second main layer of a native bacteriorhodopsin protein, a second bacteriorhodopsin mutant, or a combination thereof [5].

The steps of producing a bacteriorhodopsin film include; first, a layer of gold is fixed upon a substrate layer. Then, an individual layer of the first BR mutant is deposited upon the gold. After that, a layer of a cationic polymer lay down upon the individual layer of the gold-binding BR mutant, and additional alternating layers of the last and the cationic polymer mounted up (2-5 times) to form individual layers of BR mutant. Next, a layer of the cationic polymer lies down upon the last layer of the BR mutant. Now, an individual layer of a native BR protein, second bacteriorhodopsin mutant, or combination thereof are added upon the layer of the cationic polymer. Again, this last step is repeated until about 200 to about 400 individual layers of the native bacteriorhodopsin protein, second bacteriorhodopsin mutant, or combination thereof. Thereby, BR films will be used in subretinal and epiretinal implants. As a result, providing BR mutants containing one or more of the amino acid substitutions is essential [5].

Such films respond to illumination with discrepancy responsively familiar in motion detection, and when spatially located in the vertebrate eye, they can achieve edge enhancement [1].

Oriented purple membrane (PM) films consist of the BR-based photosensitive protein immobilized in an electrochemical photocell by means of bio affinity bonding with bispecific (BS) antibodies that recognize BR. There are methods based on this technology for designing intelligent optoelectronic devices, which contain “image sensors” for motion sensing, “photoreceptor tips” for artificial vision of human eyes, and “environmental sensors” for using in direction and speed sensing [13].

MATERIALS AND METHODS

Solutions

PBS Buffer: 1.9 mM NaH2PO4.2H2O, 8 mM Na2HPO4, 0.15 M NaCl. Lysis Buffer: Nacl 0.5 M,Tris-Hcl 20 mM pH=8.0, Imidazole 10 mM,0.005% PMSF. Buffer A: 0.01 M Tris-Hcl, 0.15 M NaCl, 0.25% (w/v) deoxycholic acid, and 0.025% (w/v) NaN3

titrated to pH 8.0 with NaOH. Buffer B: 0.01 M Na2HPO4, 1% (w/v) SDS, and 0.025% (w/v) NaN3, pH 8.0

Mutagenesis of the Synthetic bacterio-opsin (sbO) gene

Site-Directed-Mutagenesis supported with SOE PCR method by using the cloned vector(pET21a including sbO gene, considering *E.coli* codon usages, fused with parts of β-gal (13aa) at N-terminus, introducing an NdeI cloning site as the translational start and EcoRI site as the translational stop) as template and specific primers.

Single amino acid substitutions were carried out by replacement of Asp85, base 294_{th}, with Glu. Primer's sequences were designed applying some soft ware's and on line tools, represented in Table 1. First, forward primer corresponding to the 5` ends was chosen from the initial of the fused gene designed to introduce an NdeI site (CATATG) in primer 1. Mutagenic primers (First reverse primer, ER1, and second forward primer,EF2) around desired replacement and last reverse primers partly from the end of the gene adding XhoI site (CTCGAG) at the end of multiple cloning sites of the vector to obtain a cleavable C-terminally His-tagged construct. Therefore, there is an NdeI restriction site at the N and an XhoI site at the C terminus of recombinant fragments. Primers sequences are presented in Table 1.

Table 1. Primers used in current study

primer	sequences
F1	5´TACATATGACTATGATTACCGACTCTCTGG 3´
R2	5´ GCTCGAGTGCGGAGGTGGCAG 3´
ER1	5´ GAACAGCCATTCTGCATAGCGC 3´
EF2	5´ CGCTATGCAGAATGGCTGTTCACC 3´

The bolded letters signify the alternated nucleotide.

Preparative plasmid (as template) isolation was by the protocols of Roche kit. Every PCR amplification consisted of: a 4µM concentration of each primer, a 100 µM concentration of mixed dNTP (Fermentas), 2µl of template DNA, and 1 unit of *Taq* DNA polymerase (Fermentas) in a final volume of 25µl, by timing shown in Table 2. Calibrated PCR procedure was repeated by *Pfu* DNA polymerase (Fermentas) for the enzyme fidelity. BR produced by just PCRIII and using primers F1 and R2. We extracted the PCR products by High Pure PCR Product Purification Kit of Bioneer (South Korea) as manufacturer's manual.

Table 2. Timetable of PCRs steps

Stages		Temperature			Time
		PCR I	PCR II	PCR III	
Primary Denaturation		94^0	94^0	94^0	4´
	Denaturation	94^0	94^0	94^0	1´
35 Cycle	Annealing	57^0	66^0	65^0	1´
	Elongation	72^0	72^0	72^0	1´
Fine Extension		72^0	72^0	72^0	5´

Bacterial Strains, Cloning and Transformation

E.coli TOP10F` (Stratagene) was employed as cloning host, whereas *E.coli* strain BL21 (DE3) (Novagene) served as expression host. The resulting PCR product was digested using NdeI/XhoI sites and inserted into the Multiple Cloning Site (MCS) of expression vector pET21a, which carries a hexa-histidine (His6) sequence at the C-terminus. Initially, they were transformed into TOP10F' subsequently, extracted plasmids were again transformed into DE3.

Culture and Induction

Transformed BL21 (DE3) cells were grown at 37ºC in 5ml sterile LB Broth (from Scharlau) medium tubes supplemented with 5µl Ampiciline. Upon reaching to an OD 0.6 to 0.7 at 600nm, 10 mM IPTG (Fermentas) was added to induce protein synthesis. After an induction period of 3-4 h, the cells were harvested to identify and determine the level of expression by SDS- PAGE (Coomassie Blue and silver staining) Expression analyses were complemented by Western blots.

Samples of protein underwent electrophoreses in standard SDS polyacrylamide (from Merck) gels (12.5% (w/v)). The gels were electro blotted onto nitrocellulose filters (from Roche) using semidry blotting (Pharmacia Biotech. Nova Blot) and treated with a monoclonal Ab, histidine-tag Ab (from Roche) (dilution 1:5000), as primary antibodies, then utilized Goat anti-mouse IgG conjugated (from Sigma) (dilution 1:4000), as secondary antibodies.

Protein Expression and Purification

Cell cultures of interest were grown in large-scale batches in presence of Ampiciline (100µg/ml) at 37ºC and expression induced by addition of IPTG (10 mM) at an OD 600 of 0.8. Cells were harvested (centrifuged at 6000 g for 6 min at 4º C) 4 h post induction. The yield was ~2 g, wet weight of cells per liter of culture. Pellets were washed and re-suspended in lysis buffer (of Roche plasmid extraction kit). Finally, cells were disrupted by ultrasonication (for 40 cycles of 10 seconds) on ice. Membranes were collected by centrifugation (1 h, 20,000 g, 4 ºC). Cell lysates were filtered using a 0.45 µm (Millipore) filter; then passed through pre packed His-Bind Columns (Novagen) as instruction manual protocol. The purified proteins` concentrations were determined spectrophotometrically by Bioral assay, and then were concentrated in a CF-25 Centricone to 0.5 mg/ml. The eluted protein was dialyzed against PBS (2 × 1 liter), at 4ºC for 1 day, by a dialysis tube (Mw cut-off 3500, spectrum Laboratories, U.S.A.). Then against buffer A (2 × 1 liter) at 25° C for 1 other day (sodium deoxycholate removes any residual cell debris particles), Dialysis was then continued with buffer B (4 × 1 liter) for 2-3 days. The obtained solution (BO/SDS) concentration was the same as before (0.5 mg/ ml). Lyophilized samples were stored at 4ºC.

Spectrometric

bO apoproteins were regenerated with all-trans-retinal (from Sigma). 1mg all trans-retinal was dissolved in 1ml ethanol which should be freshly prepared and kept at -20ºC in the dark for at last two weeks. 0.2ml of each apoproteins was mixed to 2.5µl all-trans-retinal solution (BO/Retinal).

After overnight dark adaptation, reconstituted BR mutants (100mg/ml), irradiated with light for 5min by a 200-W projector lamp to obtained light adapted variants. Spectrum analysis was assayed from 200 to 700nm using instrument UV-Visible (Unicam UV-300) spectroscope.

RESULTS

PCR Products

Three SOE PCRs were done, resulting in desired mutants, as two pairs of primers (F1, xR1and xF2, R2) were employed to produce two separate gene fragments (300bp, 500bp) using the cloned sbo gene as the template. The resulting gene fragments were purified on a 1% agarose gel and served as templates for a third PCR reaction using the first and last primers (F1, R2), PCRIII produces full-length mutant genes (800bp) (Figure 4). All constructs with the desired mutations (D85E) were verified by DNA sequencing (Figure 5) and they aligned by MEGA4 soft ware (Figure6).

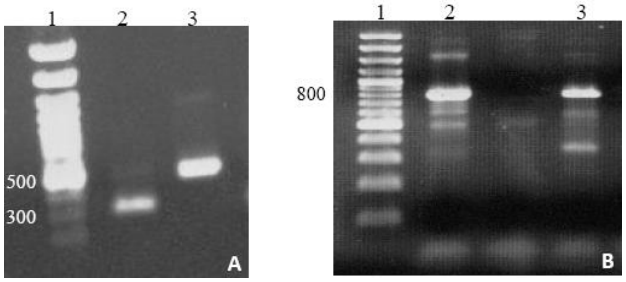

Figure 4. SOE PCR results
A; lane1; DNA ladder 100bp, Lane2; 300bp fragment (E1), Lane3; 500bp fragment (E2)
B; lane1; DNA ladder 100bp, Lane2; 800bp fragment (D85E), Lane3; 800bp fragment (BR)

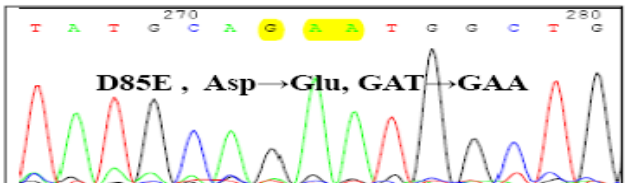

Figure 5. DNA sequencing verified the accuracy of the point mutation

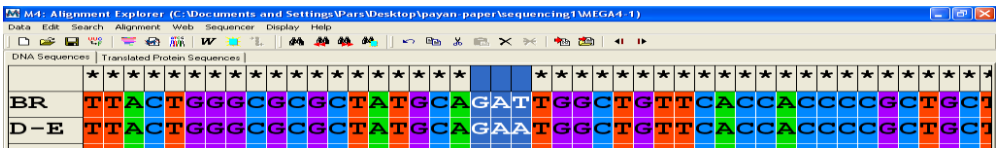

Figure 6. Sequence alignments are showing only point mutation miss matched in mutant construct.

Cloning and transformation

Digestion of vector and PCR product with NdeI/XhoI and ligation were accomplished as mentioned. Cloned Vectors are standing upper comparing to blank vector, as illustrated in Figure 7. The accuracy of transformation is confirmed through NdeI/XhoI double digestion of fragments simultaneously. Sequencing of full-length plasmids confirmed the precision of the cloned segments in vector.

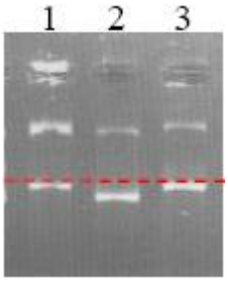

Figure 7. cloned plasmids comparing to blank
Lane2 is blank vector, lane 1 and 3 are plasmids including inserts

Protein expression

Expression of bO and D85E in *E.coli* (DE3), is initiated by culturing and induction of samples. Under various inductions [1–100 mM IPTG], they were over expressed and a 29.6 kDa band (including; short part of β-gal+ *bo* gene+ 6His tag) was detected in SDS–PAGE gel shown in Figure 8, also bands were seen in western blots of using anti His tag conjugated antibodies as discerned in Figure 9.

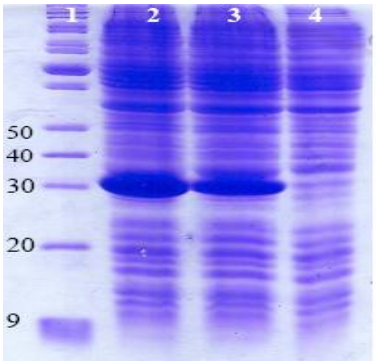

Figure 8. SDS–PAGE gel electrophoresis
Lane 1: Molecular weight marker. Lane 2: 4h after induction D85E
Lane 3: 4h after induction BR. Lane 4: without induction

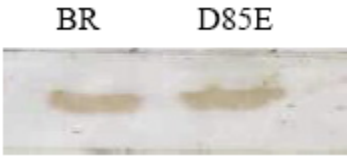

Figure 9. Western blot analysis by monoclonal antibody (anti His tag).

Purification of His tagged bacterio-opsin

His tagged *bo* proteins were Purified by His-Bind Columns due to Histidine affinity to nickel. As shown in Figure 10. 29.6 kD proteins have been eluted but there are some impurities.

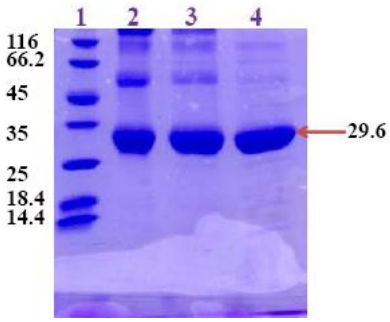

Figure 10. SDS–PAGE gel electrophoresis detecting purified mutant proteins
Lane 1: molecular weight marker, Lane 2: D85E, Lane 4: BR

Absorption spectroscopy

The absorption characteristics of BR mutants were analyzed using just LA (light adapted) forms. Variant D85E and BR exhibited peaks at 560 and 568 nm, respectively. In Figure 11, absorption spectra of the regenerated BRs are shown.

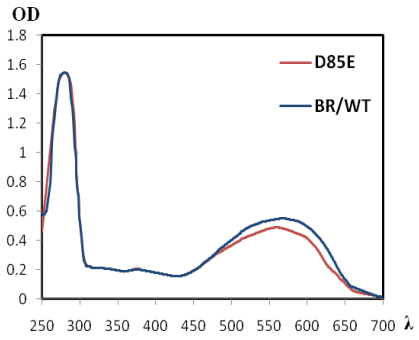

Figure 11. Absorption spectra of the BR and D85E (Asp85Glu)

DISCUSSION

Both structurally and functionally, BR is very similar to Rhodopsin photoreceptors of vertebrate [1]. While in vitro culture of halophilic bacteria and PM isolation are complicated and ineffective, protein production by means of genetic engineering is very imperative. Here, we have produced two types of bacteriorhodopsin, a native and a mutant BR (D85E) and studied their opto-electric responses with respect to wavelength and absorption properties. They are prerequisite for designing artificial retina (sensors) based on biomolecules [1]. Therefore, the new promising technology will soon conceivably eradicate the blindness [6] by using the product of this project.

Improving the vision of people suffering from retinal disease or damage requires the development of retinal implants. The artificial retinas replace damaged photoreceptor cells, and thus, are capable of curing any retinal defects, which have not offended the bipolar or ganglion network [5].

Mostly photo sensors, vision chips, smart sensors or artificial retinas, are silicon based [4 and 14], which has its own disadvantages. Light transducing photoelectric protein bacteriorhodopsin in artificial retinas display differential responsivity, motion detection and edge enhancement. Under appropriate circumstances, these artificial receptors mimic the differential responsivity trait of mammalian photoreceptor cells. Applying orientated bacteriorhodopsin to produce the photoelectrical signal allows rapid responsivity, and high quantum effectiveness. The capability of manipulating the properties of the protein through chemical and genetic methods improves devise flexibility [5].

Site-directed mutagenesis of the *bO* gene was the point of our efforts to function studies of BR. Other prior approaches to do so, mutagenesis of the *bO* gene by techniques of mismatched oligo nucleotides or restriction fragment replacement were mostly ineffective. Therefore, in this work we improved a different approach occupying SOE PCR with two pairs of primers and the *bO* synthetic gene as template, introducing mutation in the gene in an expression vector to bring about a specific amino acid replacement. We expressed the bop gene in *E.*coli, using expression vectors that produce BR as fusion proteins with a segment of the protein of *E.*coli, β-gal. The expression is under the control of the translational signals, and there is the additional transcriptional control by the T7 promoter element downstream of the gene on the vector. We used synthetic bop gene with deletion of the 39 nucleotide of the N-terminal leader peptide equal with 13 amino acids. Therefore, problems with inability of *E.coli* signal peptidases for removal of the leader sequence were solved [15]. Sequencing confirmed no extra mutation, except favored single site mutation.

Despite, considering *E.coli* codon usages and changing a number of codons to increase protein expression, it was rather low. After transferring to micro aerobic conditions and simultaneously induction was a suitable condition for heterologous expression of proteins in *E.coli* BL21 (DE3), which made it to be rather effectual for expression being detectable.

In BR production, however, the purification system utilized in this experiment, is the most efficient method, there are some contaminations composed of histidine-rich proteins of *E.coli*, like cytochromeoxidizes or aggregated bOs, which don`t interfere with the spectra assay [16], but, for applying in artificial retina, they should be completely purified.

Films of multilayer bacteriorhodopsin-based photocell have been developed that respond to immediate alterations in light intensity. These photocells are employed to construct a 64-pixel photoreceptor that detects moving images. Therefore, these films could supply the foundation for motion detectors or artificial vision apparatus [8].

The reverse polarity of the photoelectric response of these BRs is not for structural or fundamental changes but due to a reverse orientation of recombinant proteins in micelles [17], this problem is solvable by fixing the proteins on a basis in BR films.

Mutant D85E and BR (WT) show up absorption spectra with peaks at 560 and 568 nm, respectively. The first have blue shifts comparing to BR. Maximum absorption of recombinant proteins either the red shift or the blue shift are desirable and consequently have specific properties; they also have their own certain applications. For instant, they can be the basis of Electrochromic materials of different colors.

In spite of extensive applications in technologies, efforts continue towards their use for information processing. These proteins utilize as light sensitive elements in image

detectors, artificial retina, spatial light modulators and holographic memories; these applications make BR and its mutants as a protein of interest. Moreover, producing proteins with different λ_{max} creates a foundation for color sensitive sensors. Constructing artificial retinas based on bacteriorhodopsin molecules depends on color sensitivity developments. A sensor contains several proteins with different wavelength characteristics.

As a conclusion, properly oriented native protein is capable of producing a considerable ion gradient. Thereby, the photo response and ion pumping properties will improve via genetic engineering and will develop performance of the retinal implant [2].

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