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Research Article

Test for the Cholinesterase Activity in Diatoms *Ulnaria ulna* Cells

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Abstract

Testing of cholinesterase, the enzyme that hydrolyzed acetylcholine, in diatoms *Ulnaria ulna* has been carried out by the histochemical methods with Ellman reagent, Fast Red TR, and azo analogue of Ellman reagent dithio-bis-(p-phenyleneazo)-bis-(1-oxy-8-chloro-3,6) sodium disulfate. Last dye test was better observed visually and under microscope. Inhibitors of cholinesterase neostigmine, and physostigmine decreased the hydrolysis of acetylthiocholine by the cells.

Keywords: *Acetylthiocholine; Ellman Reagent; Fast Red TR Salt; Red Analogue of Ellman Reagent; Neostigmine; Physostigmine*

Introduction

The presence in cells of the enzyme of the hydrolysis of acetylcholine - cholinesterase at the molecular level has now been established not only in animals, but also in plants [1,2]. The gene encoding this protein has been found in some plants [3,4]. It is also known that unlike membrane-bound forms, the free form of this enzyme can be released into the environment by various organisms. Cholinesterase is used in cellular studies and as a marker on acetylcholine. The enzyme was found in some algae [5] except diatoms.

For unicellular water microorganisms like diatoms with rigid shells named frustules, the determination of cholinesterase activity is a complex problem because unlike many algae they can move on the bottom surface in natural conditions.

Purpose of the Study

The purpose of this work is to consider the possibilities of detecting this enzyme in river-living diatoms of *Ulnaria ulna* and its excretions by histochemical method.

Materials and Methods

Objects of research and their cultivation: The objects of research on whole cells objects of the study were samples of diatom algae *Ulnaria ulna* (Nitzsch) Compare such as the living cells and shells primary received from laboratory of Karadag Biostation (Feodosia, Black Sea) [6,7]. Then the cells lines 2.0-419 and 2.0-903 were cultivated in Pushchino laboratory of microspectral analysis of cell and cellular systems on in the Petri plates - 3 cm in a diameter on nutrient medium, which included K phosphate 6.63, $CaCl_2$ 6.51, NaCl 3.47, $MgCl_2$ 5 **µg**/l and silica gel (*Merk*, Austria) 2 **µg**/ml as the source of silicium for the frustules formation (shells/valves). The observation of diatoms

was on cover glasses (slides), which are put on the Petri plates or on watch glasses.

Determination of cholinesterase activity: The cholinesterase activity of cells and isolated organelles was determined by histochemical staining with reagents for cholinesterase Ellman's reagent [8], Fast Red TR salt [9] and an analogue of Ellman's reagent such as 2-dithio-bis-(p-phenylenazo)-bis-(1-oxy-8-chloro-3,6)-sodium disulfate [10] before and after treatment with cholinesterase inhibitors physostigmine and neostigmine. As a substrate, **β-**naphthyl acetate or acetylthiocholine was used. The exposure with the substrate was 40 - 60 minutes. All experiments were carried out at room temperature of 20 - 22°C. The sequence of procedures was as follows. Samples on slide were poured in the optimal substrate concentration for the hydrolytic reaction of 10^{-3} M β-naphthyl acetate or acetylthiocholine (0.05 ml), dissolved in 0.05 M-potassium-phosphate buffer pH 7.25 - 7.5. Pretreatment (before the addition of the substrate) in variants with cholinesterase inhibitors with neostigmine or physostigmine (10^{-6} - 10^{-4} M) lasted 20 - 30 minutes. The most complete inhibition of coloration was noted at a concentration of 10^{-4} M, as described in the work using the Karnovsky-Ruth cholinesterase detection method for electron microscopy [11].

The absorbance spectra of histochemical products were recorded by microspectrofluorimeter MSF-15 (LOMO, Sankt-Petersburg). The autofluorescence used as the test-reactions of the diatom cells on the object glasses (slides) as described earlier for unicellular probes]. All experiments were performed at room temperature 20 - 22°C. The images of living cells and separated volves were recorded and photographed by luminescent microscopes Leica DM 6000 B (USA-Austria), microspectrofluorimeter MSF-15 (LOMO, Sankt-Petersburg) with photocamera Levenhuk M300 Base (USA).

Reagents: The work used dyes for cholinesterase Ellman's reagent, Fast Red TR salt (Sigma, USA) and a red analogue of the Ellman reagent 2-dithio-bis - (p-phenylenazo) - bis-(1-oxy-8-chloro-3,6)-sodium disulfate (Chimanalyt, Sankt-Peterburg). Cholinesterase substrates (β-naphthyl acetate and acetylthiocholine iodide, as well as cholinesterase inhibitors neostigmine and physostigmine were taken from Sigma (USA).

Results and their Discussion

We analyzed their methods for the determination of cholinesterase activity in *Ulnaria ulna* diatoms by the histochemical staining of the cells and using of the enzyme inhibitors neostigmine and physostigmine. Three possible methods have been applied. Method of Ellman (based on the formation of yellow product with the absorbance maximum 412 nm, when used acetylthiocholine sulfate as a substrate for the cholinesterase) has given weak color of the probes with the diatoms, approximately 80 - 100 cells per ml of the medium. After that we used fast Red TR salt azo dye to stain plant cells using as a substrate β-naphthyl acetate [9]. In this case, the colorless reagent Fast Red TR salt gives a weak red color of the product with the absorbance maximum 546 nm, which is formed during the hydrolysis of its cholinesterase. In both above-mentioned staining, when we put on the cell probe on the subject glass of microscope, we cannot see clearly visible yellow, red or rose color of the cells. Third method in the histochemistry of plant cholinesterases, the red analogue of the Ellman reagent was also used. After hydrolysis of acetylthiocholine the enzyme forms a blue product with thiocholine [12]. Figure 1 shows that after hydrolysis of acetylthiocholine as a substrate of the enzyme in the 1 ml of probes, forms a blue product with thiocholine unlike the red reagent after 1 hour of incubation of samples with the substrate. To identify cholinesterase activity, experiments were conducted on the preliminary treatment of samples with cholinesterase inhibitors. We marked that blue color seen not only in cells, but also in the medium. This shows the excretion of enzyme from the cells. In the absorbance spectra of individual cell on the subject glass of microspectrophotometer one can see maximum 600 nm, differing from maximum 535 - 540 nm, peculiar to red reagent. If preliminary the staining with reagents the inhibitors of cholinesterase neostigmine or physostigmine were added in the probes, we saw a decrease in the height of the maximum 590 - 600 nm. This means that in the probes of the diatoms is a cholinesterase activity.

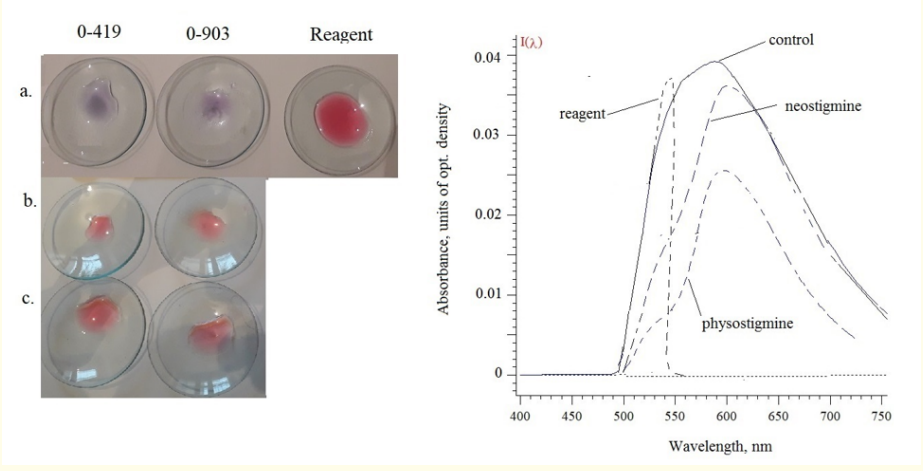

Figure 1: *Histochemical staining of the diatom cells from lines 0-419 and 0-903 with red analogue of Ellman reagent salt on cholinesterase on subject glasses (Left images) before (a) and after the 10-4 M inhibitors neostigmine (b) and physostigmine (c) and example of their absorbance spectra recorded by microspectrophotometer (Right images).*

To see a localization of the enzyme in individual cell is a complex task. Our diatoms are mobile cells, and for photography the cholinesterase reaction in individual transparent cell (Figure 2 left), we use the Whatman 1 chromatographic paper (Figure 2 right). The reaction color was clearly seen if diatoms were put on wet white paper. Control cell became blue in the presence of the substrate acetylthiocholine, and blue prints also were seen on the whole cell surface. Coloration becomes very weak or completely absent if the samples are previously incubated for 20 - 30 minutes with cholinesterase inhibitors neostigmine or physostigmine. If the paper was treated preliminary with the cholinesterase inhibitors, one can see reddish color in the case of neostigmine (only on periphery blue band) and weak blue color of all after the physostigmine variant. Thus, we proposed that the cholinesterase is located mainly on the cell surface and may be excreted to external media. Since the diatoms studies have a motility due to the mucilage from the frustules, it is clear that acetylcholine plays signaling role on the cell surface and the cholinesterase serves as a regulator of the substrate. This mechanism is considered for every living cell-microbial, plant and animal [13].

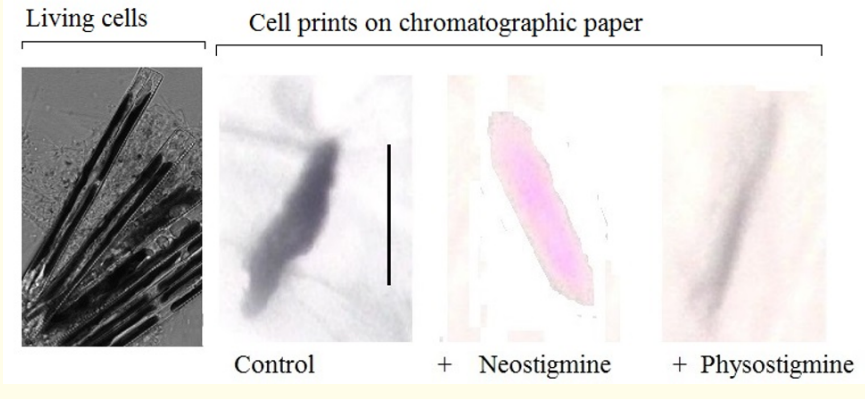

Figure 2: *Common view of the cells of Ulnaria ulna under transmitted light of microscope (Left) and cell-prints on chromatographic paper Whatman 1 after the histochemical staining on cholinesterase (Right). Bar = 75 µm.*

Conclusion

The experiments with test-reaction for cholinesterase in diatoms with various histochemical staining demonstrated most sensitivity for red azo analogue of Ellman reagent. Reaction with acetylthiocholine leads to the formation of blue product with thiocholine that may be inhibited with the cholinesterase inhibitors neostigmine and physostigmine.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Victoria V. Roshchina, the author of main conception of the work, receiver of all experimental data, and she has written the paper.

Data Availability

The datasets generated during and/or analysed during the current study are available in the [NAME] repository [PERSISTENT LINK TO DATASETS].

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