Spectroscopic investigation of vegetable pectin - fluorescent protein EGFP interaction in tumor cells in vitro

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Abstract Using spectroscopic methods, solutions of zosterin and CT26 cell culture were investigated. Fluorescent spectroscopy revealed a negative dynamics of growth of a malignant culture containing a green fluorescent protein in the presence of zosterin. It was observed two-component luminescence kinetics of EGFP protein.

1. Introduction
Pectin substances (pectin polysaccharides, pectins) are the most complex and interesting in terms of structural organization and functional activity in present. Despite significant advances in the field of structural studies of polysaccharides, the complexity of construction and the irregular nature of the carbohydrate chains of pectin macromolecules do not allow us to consider their structure established. Therefore, at the present time, there is no generally accepted model for the structure of pectin macromolecules. Our work is devoted to the study of a drug known under the commercial name "Zosterin-Ultra". The raw material for its production is the perennial aquatic plant Zostera marina, which grows exclusively in the pure water areas of the Russian Federation. Marine pectin (zosterin) has the formula C₆H₈O₆ and is 90-95% represented by a mixture of polygalacturonic and polyglucuronic acids [1, 2]. The properties of this pectin proved to be promising, some of them have already found application in medicine and biology. Currently, Aquamir CJSC, based on its own original technology, produces BADs under the commercial names "Zosterin-Ultra 60%" and "Zosterin-Ultra 30%", which are positioned as natural remedies with qualities of sorbents and immunomodulators.

The most of pectin studies are carried out by IR spectroscopy, chromatography [3]. The main aim to a more detailed study of the properties of zosterin is to obtain information on the driving forces of protein transformation when they interact with pectin. In the literature there is no full information about spectral and optical characteristics of this substance and its solutions. In this paper, we want to pay attention to the possibility of a negative effect of zosterin on the morphology of malignant cell cultures besides.
In the work the commercial preparation "Zosterin-Ultra 60%", which is a brown powder, was investigated. The 1 g powder was dissolved in 50 ml phosphate buffered saline (PBS, 150 mM NaCl, HyClone, USA) at the 60° C temperature using a magnetic stirrer for 10 min. The concentration of stock solution of Zosterin was 20 mg/ml. The fraction of undissolved particles and aggregates were removed by centrifugation of the solutions at 300xg rate regime for 5 minutes. The supernatant was isolated in individual 15 ml tubs and used for further in vitro experiments.

2.2 Cell cultures
A CT26 murine colon carcinoma cell line (ATCC CRL-2638) stably expressing EGFP (CT26-EGFP) was obtained by lentiviral transduction in the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry RAS headed by Dr. Sergey A. Lukyanov. The cells were cultured in RPMI-1640 medium (HyClone, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, USA), 10 units/mL penicillin and 10 μg/mL streptomycin in a humidified incubator with 5% CO₂ at 37° C. For experimental study the CT26-EGFP cells were seeded in 6-well plates at a density 5·10⁵ cells/well and incubated in RPMI medium 10% FBS. 24 hours after seeding 100 ml zosterin solution was added into cellular medium and pipetted. The concentration of zosterin in medium was 2 mg/ml. 24 h after incubation the fluorescence signal of CT26-EGFP in control and experimental groups were analyzed. The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues (26.9 kDa) that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. EGFP variant contains chromophore mutations that make the protein 35 times brighter than wild-type GFP. The fluorescence quantum yield (QY) of EGFP is 0.60. EGFP have a single emission peak of 509 nm by 488 nm excitation.

2.3 Fluorescence imaging
The fluorescence signal from CT26-EGFP cells was obtained using confocal microscope Carl Zeiss Axio Observer Z1 (Zeiss, Germany). The fluorescence of EGFP was measured using Yokogawa CSU-X1 spinning disk, objective 20x/0.5, the 488 nm continuous wave (CW) laser Sapphire LP (Coherent Inc., USA), exposure time is 2000 ms. The fluorescence signals were taken from CT26-EGFP cells as pixel brightness value and analyzed using the software AxioVision Rel. 4.9 (Zeiss, Germany). The results of fluorescence were reported as the mean values of fluorescence intensity with standard deviation.

2.4 Measurement of fluorescence kinetics
The luminescence kinetics was measured with MicroTime 100 (PicoQuant) laser scanning fluorescence microscope. To excite the luminescence, a pulsed diode laser was used (λex = 405 nm, τpulse = 80 ps), to register the fluorescence a photomultiplier (185-800 nm) was used also.

3. Results and discussions

The spectrum of zosterin on Figure 1 is weakly characteristic and smoothly decreases to the IR region. Aggregation plays a key role in gelling, as well as in self-assembly of proteins into supramolecular systems. Therefore, the ability to directly monitor protein aggregation is extremely important when studying the properties of protein-containing systems. Apparently, the transmission of zosterin in the visible range is due to scattering on the micellar structures.

![Figure 1 The absorption spectrum of zosterin aqueous solution in the visible and UV regions](image-url)
The method of fluorescence spectroscopy is one of the most common for studying the physicochemical properties of biological systems and, in particular, the structure of proteins. This method allows one to monitor changes in the microenvironment of the protein's own fluorophores or the introduced fluorescent label.

**Figure 2.** Confocal fluorescence imaging of CT26-EGFP. Top panel is control CT26-EGFP cells and the bottom panel CT26-EGFP cells incubated with zosterin solution 24 h. BF is bright field imaging, EGFP means fluorescence imaging of EGFP, Merge is combined image of BF and EGFP.

In the experimental sample, the cytotoxic effect of Zosterin on tumor cells CT26-EGFP is observed. The survival of cells is reduced in comparison with the control. There is a change in the morphology of the cells and their adhesion to the surface decreases. The presence of Zosterin in the experimental sample changes the viscosity of the culture medium. There is no quenching of the fluorescence signal of EGFP in cells compared to the control. There is no change in the distribution of the fluorescence signal across the cell. There is a slight increase in the average fluorescence signal EGFP from cells in the experimental sample and this may be due to the cytotoxic effect of the Zosterin preparation. Mutations of amino acid residues in the immediate vicinity of the chromophore can significantly alter the fluorescent properties of the protein [5]. On Figure 2 the luminescence kinetics of cell cultures and zosterin is shown.
Figure 3. Luminescence kinetics decay of the cell culture containing the EGFP protein (experimental points) is 1; the approximation by two-exponential dependence (line) is 2; the approximation by exponential dependence with one decay time (dashed line) is 3.

It is known that in a green fluorescent protein, the 203 amino acid residue (Thr203 or tryptophan) is located near the chromophore and is potentially capable to influence on the spectral characteristics [6]. Fluorescent properties of tryptophan are extremely sensitive to changes in its microenvironment, and mainly, polarity. The fluorescence duration of tryptophan in water is 3 ns, and in the protein it varies from 2 to 4.6 ns [7]. Therefore, in the measured luminescence kinetics of the EGFP protein being studied, the presence of two decay life-times 3.7 ns and 0.6 ns may be due to the presence of different forms of the protein. Complexation with low molecular weight ligands and macromolecules, denaturation, aggregation, and other processes cause different connections with the environment and affect fluorescence duration of tryptophan, including. The effect of zosterin on the luminescence kinetics of cell cultures containing the EGFP protein is the subject of further study.

Conclusions

The absorption spectra of "Zosterin-Ultra 60%" aqueous solution were obtained. Fluorescent spectroscopy revealed a negative dynamics of growth of CT26 malignant culture containing a green fluorescent protein in the presence of zosterin. The luminescence decay kinetics of the EGFP protein has two components, what indicates about two forms of the protein in cells in solution.

References

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