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# Hybrid Proteins with Organophosphorous Hydrolase Activity and Fluorescence of deGFP4 Protein

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**Abstract**—New genetic constructs encoding synthesis of hybrid proteins possessing organophosphorus hydrolase activity and properties of the pH sensitive analog of the green fluorescent protein were developed. It was established that 0.1 mM of the biosynthesis inducer and cultivation for 10 h after the induction were necessary for the maximum yield of the hybrid proteins in the soluble and active form in *E. coli* cells. The demonstrated synthesis level for one of the new proteins was 2- to 25-fold higher than the yield of the soluble hybrid protein analogs known from the literature. It was found that the organophosporus hydrolase within hybrid proteins demonstrated characteristics (pH optimum, thermal stability and catalytic efficiency) different from the respective characteristics known for the native enzyme. It was shown that the fluorescence of the green fluorescent protein within the hybrid proteins depended on the pH of the medium in a manner similar to the individual protein. Interrelation of the fluorescent characteristics and OPH activity that manifested itself in the hydrolysis of organophosphorus compounds was shown by example of one of the hybrid proteins.

*Keywords*: organophosphorus hydrolase, green fluorescent protein, hybrid proteins, organophosphorus compounds

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Organophosphosphrus hydrolase (OPH, EC 3.1.8.1) is one of the key enzymes in the process of biodestruction of organophosphorus compounds (OPCs), which include pesticides widely employed in agriculture and chemical warfare agents (sarin, soman, VX) [1]. It is know that OPCs possess neurotoxicity with strong cumulative and mutagenic effects [2]. Currently, methods of biological detection and destruction of OPCs, including with the use of OPH, are under development around the world [3].

Generation of the highly effective expression system for the genetic construct encoding recombinant OPH synthesis provided production of the enzyme in high quantities (12 mg from 1 g of cells) in *E. coli* DH5α cells at 30°C and in 0.2 mM IPTG [4]. Genetic constructs encoding synthesis of the enzyme containing hexahistidine ($His_6$-) sequences at the N-terminus ($His_6$-OPH) [5] and C-terminus (OPH-$His_6$) [6] of the protein molecule were created for the isolation and purification of the enzyme using chromatographic media. Enzymes were successfully isolated with the help of macroporous support media on the basis of the
1 polyacrylamide (PAA) cryogel modified by iminodiacetic acid (IDA) and charged by $Co^{2+}$ ions [7]. It was shown therewith that the catalytic parameters of the modified enzyme changed compared to the OPH [6, 8–9].

Investigation of $His_6$-OPH synthesis in *E. coli* cells showed that up to 50% of the protein was formed in an insoluble inactive form, resulting in a yield more than a factor of two lower in comparison with that of the OPH enzyme [10]. However, the presence of such a sequence significantly simplifies the procedure of protein isolation and purification.

It is known that a protein fusion partner—protein connected to the target product through a spacer of a few amino acids—can be used to increase the yield of a soluble protein [11, 12]. The protein fusion partner is synthesized prior to the target protein, goes through the folding process, and keeps the target product in the soluble state. Furthermore, the concept of inhibiting the process of joint protein aggregation is applicable in this case.

Currently the green fluorescent protein (GFP) is often used as a fusion partner for different target proteins, which allows imparting additional properties to the protein on top of the yield increase for the soluble form [13]. In a number of studies, development of the GFP-OPH hybrid protein was described, where GFP and OPH molecules were connected by a sequence of five amino acid residues (-$(Asp)_4$-Lys) [13, 14] as well as a similar hybrid protein containing the $His_6$-sequence on the N-terminus of the molecule [15]. Despite the optimization attempts of the biosynthesis

condition for the proteins, the authors were unable to significantly increase the yield of the soluble form [16].

At the same time, it was established that OPH retains its catalytic activity when bound to GFP. The GFP fluorescence in hybrid proteins was proportional to the OPH catalytic activity, which provided the developers of the hybrid protein with the ability to detect very small quantities of recombinant enzymes in the analyzed media by fluorescence.

Use of the pH-sensitive GFP analog—deGFP4 [17] protein—instead of GFP could allow one to control the synthesis of the hybrid protein and assess the efficiency of the OPH action in the reactions of POC hydrolysis, which proceed with pH changes owing to the release of acidic products. DeGFP4 protein reversibly changes its fluorescence properties depending on the pH changes in the solution. Hence, apart from the yield increase for the soluble form of the enzyme, introduction of the deGFP4 molecule in the hybrid protein could allow one not only to perform OPC hydrolysis but also to have a highly sensitive "witness" of the process directly in the reaction mixture. Production and investigation of the properties of such hybrid proteins was the objective of this study.

## EXPERIMENTAL

Paraoxon (diethyl *n*-nitrophenyl phosphate), diisopropyl fluorophosphate (DFP), imidazole, CHES (2-[N-cyclohexylamino]ethanesulfonic acid, HEPES (N-[2-hydroxyethylpiperazine]-N'-[2-ethanesulfonic acid]), cobalt chloride hexahydrate, nickel chloride, glycerol, bromophenol blue, coomassy brilliant blue R-250, ampicillin sodium salt, kanamycin disulfate, egg albumin, and sodium dodecyl sulfate from Sigma (USA); tryptone and yeast extract from Difco (USA); acrylamide, N,N'-methylenebisacrylamide, and ethylenediaminetetraacetic acid (EDTA) from Merck (Germany); isopropyl β-D-thiogalctopyranoside (IPTG) and molecular weight markers for the protein electrophoresis (kit with the protein molecular masses of 26.0, 34.0, 43.0, 55.0, 72.0, 95.0, 130.0 kDa) from Fermentas (Lithuania); and N,N,N',N'-tetramethylethylenediamine (TEMED) and ammonium persulfate from Bio-Rad (USA) were used in the study. $Co^{2+}$ IDA-
1 PAA cryogel (polyacrylamide gel polymerized at temperatures below 0°C, modified by iminodiacetic acid residues and charged by cobalt ions) from Protiste (Sweden) was used as a support for affinity chromatography. All other chemicals used were of reagent grade quality and were purchased from Labtekhnika and Khimmed (Russia).

Vector pTES-$His_6$-OPH [5] encoding $His_6$-OPH synthesis and vector pGEX-4T2-deGFP4 as the source of the *degfp4* gene [17] encoding the drGFP4 protein provided by V.V. Verkhusha (professor, Albert Einstein College of Medicine, New York, USA) were used for creating genetic constructs. Amplification of the *degfp4* gene using primers pH-F (AGGAGAGGATCCAGTAAAGGAGAAGAACTTTT) and AS-R (AGGAGCGAGCTCGCGCTCGGGCTCTTTTTTGTATAGTTCATCCATGC) or RA-R (AGGAGTGCTAGCGCTTGCATCGGCTTTGTATAGTTCATCCATGC) was used for obtaining genetic constructs encoding hybrid protein synthesis with the intermolecular spacers -$(AS)_5$ and -$(RA)_5$, respectively; pH-F primer was used for insertion of the *Bam*H1 restriction site at the beginning of the coding region of the *degfp4* gene. AS-R or RA-R primers were used for the insertion of the sequence encoding intermolecular spacers -$(AS)_5$ or -$(RA)_5$ at the 3'-end of the gene, respectively, followed by the restriction site *Sac*I or *Nhe*I.

pTES-$His_6$-OPH plasmid was amplified using AS- 2
F of RA-F primers to insert the second part of the gene encoding intermolecular spacers -$(AS)_5$ or -$(RA)_5$ as well as *Nhe*I restriction site at the 5'-end of the *oph* gene encoding OPH. OPH-R primer was complementary to the inner region of the *oph* gene.

The assembly of vectors was performed by the ligation of the obtained DNA fragments with the help of T4-DNA ligase for 3 h at 10°C. Competent *E. coli* XL1 Blue cells were transformed by the obtained ligation products. The cells were plated onto LB medium containing bacto agar (1.7%) and 100 μg/ml ampicillin. To assess the correctness of the assembly, six clones were selected, from which plasmid DNA was isolated 2
(after 16–18 h growth at 37°C in 50 ml of LB broth with ampicillin). The performed electrophoretic analysis of the isolated plasmid DNA allowed identifica- 2
tion of the two DNA molecules in each with the 738 bp insert corresponding to the part of the gene encoding the deGFP4 fluorescent protein.

*E. coli* DH5α cells were used for the hybrid protein biosynthesis. *E. coli* DH5α cells for the inoculum were cultivated at 37°C in the LB medium supplemented with ampicillin (100 μg/ml) at constant shaking (180 rpm) in an incubator shaker from Adolf Kuhner AG (Switzerland). Sixteen-hour inoculum (1%) was introduced in the nutrient medium of the following composition to determine IPTG concentration required for obtaining the maximum quantity of the soluble hybrid proteins: tripton—12.0 g/l, yeast extract—24 g/l, glycerol—4.0 g/l, $KH_2PO_4$—6.95 g/l, $K_2HPO_4 \cdot 3H_2O$—12.54 g/l, $CoCl_2$—$10^{-5}$ M, pH 7.0. Induction of the hybrid protein synthesis was initiated following the attainment of the optical density of 0.7 ($\lambda = 540$ nm) by introduction of IPTG in the medium to the final concentration from 0.10 to 0.75 mM. The culture cultivated without IPTG in the medium served as a control. Cells were cultivated at 28°C for 21–24 h.

Cell biomass grown under optimal conditions was separated from the medium by centrifugation (5000*g*, 20 min) using a Beckman J2-21 centrifuge (USA) for further isolation and purification of the hybrid proteins.

Isolation and purification of the $His_6$-deGFP4-$(AS)_5$-OPH hybrid protein was performed according to the well known procedure [18].

$Co^{2+}$-IDA-PAA cryogel was used for isolation and purification of the $His_6$-deGFP4-$(RA)_5$-OPH. The wet biomass was weighed and resuspended in 0.1 M phosphate buffer (pH 7.5) containing 0.3 M NaCl, 0.05 M $NaHCO_3$, and $10^{-5}$ M $CoCl_2$ to the concentration 0.2 g/ml. This was followed by the ultrasound disintegration of cells in suspension (four times for 2 min, 44 kHz). Cell debris was separated by centrifugation (15000 *g*, 30 min). The obtained supernatant was applied to the $Co^{2+}$-IDA-PAA cryogel (5 ml) equilibrated with the same buffer. The support with the immobilized protein was washed with 0.1 M phosphate buffer (pH 7.5) containing 0.3 M NaCl, 0.05 M $NaHCO_3$, $10^{-5}$ M $CoCl_2$, and 0.01 M imidazole at the rate of 2 ml/min until the optical density reached 0.01 ($\lambda = 280$ nm). This was followed by the elution of the hybrid protein by the linear gradient of imidazole (0.01–0.5 M). Imidazole was removed from the collected fractions by dialysis against 0.05 M phosphate buffer (pH 7.5).

The fractions of the hybrid proteins in the entire amount of synthesized cellular proteins as well as the homogeneity of the enzyme preparations were assessed by denaturing and nondenaturing electrophoresis in 12% polyacryamide gel using Mini-Protean II cell (Bio-Rad, USA) followed by coomassie R-250 staining.

Protein concentration was determined by the Bradford method using reagents from Bio-Rad (USA). Arithmetic means and standard deviations were calculated for all processed experimental data. All experiments were performed in triplicate.

### *Determination of OPH Activity of the Hybrid Proteins*

Enzymatic OPH activity of the hybrid proteins was determined spectrophotometrically by monitoring the accumulation of the product of paraoxon hydrolysis (4-nitrophenolate anion) at 405 nm ($\varepsilon = 18000$ $M^{-1}$ $cm^{-1}$, pH 10.5) using an Agilent 8453-UV spectrophotometer (Germany) equipped a thermostatic cell at 25°C. Carbonate buffer (0.1 M, pH 10.5) was used for catalytic activity assay. Aqueous solutions of paraoxon and DFP at concentrations 1 mM were used for investigation of the enzyme substrate specificity. Catalytic reaction was initiated by introduction of the hybrid protein solution in a cuvette containing buffer and substrate so that the concentration of each enzyme in the reaction mixture was $10^{-10}$–$10^{-9}$ M ($E_0$).

A unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol of the substrate per 1 min at 25°C and pH 10.5.

Calculation of the enzyme reaction rates was performed using the initial linear part of the kinetic curves ($v_0 = \tan\alpha$). The maximum rate of the enzyme reaction ($V_{max}$) and the Michaelis constant ($K_m$) were determined from the system of double reciprocal coordinates $1/v_0$–$1/[S]$ (Lineweaver–Burk) and from the entire curve for the dependence of the reaction rate on the substrate concentration.

For investigation of the dependence of enzyme catalytic activity on pH, 50 mM buffers with overlapping pH ranges, HEPES (pH 7.7–8.5), CHES (pH 8.5–10.0), and phosphate-carbonate (pH 9.5–12.0), were used.

A specimen of the enzyme preparation (0.5 ml) in 0.1 M phosphate buffer (pH 7.5) or 0.1 M carbonate buffer (pH 10.5) was placed into the thermostat at different temperatures, 10 μl aliquots were selected at certain time points, and OPH activity was measured for the investigation of the hybrid protein thermal stability. Intervals between the time points were selected in a way to provide a 210-fold decline in the enzyme activity during the experiment.

The reaction rate constant for thermal inactivation was determined from the tangent of the angle for the straight line on the plot of the natural logarithm of the residual enzyme activity versus time.

## RESULTS AND DISCUSSION

Plasmid pTES-$His_6$-OPH that was developed earlier [5] and contained the nucleic acid sequences encoding deGFP4 protein and various interprotein spacers (-$(ArgAla)_5$-[-$(RA)_5$-] or -$(AlaSer)_5$-[$(AS)_5$-]) introduced by genetic engineering techniques was used for the design of genetic constructs encoding the synthesis of the hybrid proteins $His_6$-deGFP4-$(RA)_5$-OPH (**I**) and $His_6$-deGFP4-$(AS)_5$-OPH (**II**). The $His_6$ sequence was introduced at the N-terminus of the hybrid proteins for protein isolation and purification with the help of $Co^{2+}$-IDA-PAA cryogel.

*E. coli* DH5α cells were used for investigation of the expression levels in the obtained pTES-$His_6$-deGFP4-$(RA)_5$-OPH and pTES-$His_6$-deGFP4-$(AS)_5$-OPH genetic constructs. Analysis of the protein electrophoresis data made it possible to establish that the increase in the IPTG inducer concentration in the cultivation medium from 0.1 mM to 0.75 mM resulted in the increased fraction of hybrid proteins among all synthesized intercellular proteins (Fig. 1). However, in parallel with that, the decline in the content of soluble forms of these proteins was observed in the cells. It should be noted that OPH activity was not accumulated in recombinant cells during their growth under the same conditions in the absence of IPTG. Hence, the conditions for the maximum yield of the active form of the hybrid proteins were the following: 0.10 mM IPTG, 10 h growth after protein synthesis induction.

Under these conditions of biomass cultivation, the fraction of the soluble form of the $His_6$-deGFP4-$(RA)_5$-OPH hybrid protein over the total amount of the target protein in the cell was approximately twofold larger than for the known $His_6$-OPH protein [1] (Table 1). In addition, the fraction of the $His_6$-

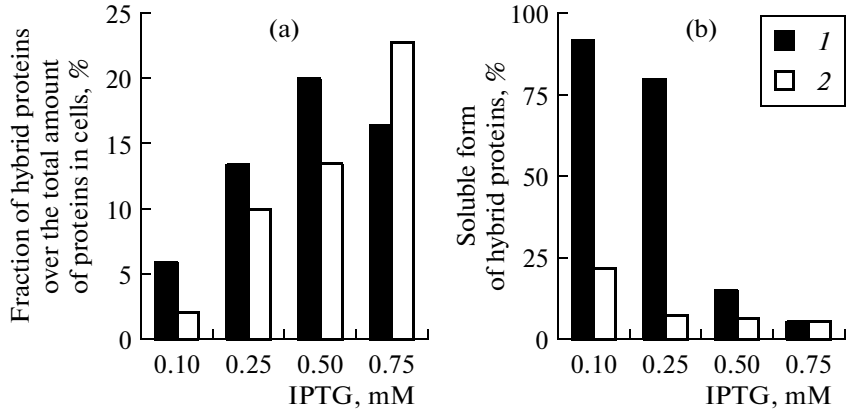


**Fig. 1.** (a) Fraction of $His_6$-deGFP4-$(RA)_5$-OPH (*1*) and $His_6$-deGFP4-$(AS)_5$-OPH (*2*) hybrid proteins in *E. coli* $DH5_\alpha$ cells reached by the tenth hour of growth after protein synthesis induction by different concentrations of IPTG in the cultivation medium (28°C) over the total amount of proteins synthesized by cells; (b) content of the soluble form of proteins in cells.

deGFP4-$(RA)_5$-OPH protein (5.5%) over the total amount of intercellular proteins was found to be 13-fold higher than the similar value typical of the OPH hybrid analog known from the literature (0.4%) [14] (Table 1). On the basis of the fact that the fraction of the $His_6$-deGFP4-$(RA)_5$-OPH protein soluble form (92%) was twice as high as the fraction of the soluble form of the known $His_6$-GFP$Asp_4$-Lys-OPH protein [14], the total obtained amount of the designed hybrid protein was approximately 25-fold higher than in case of its analog.

Macroporous metal chelating support $Co^{2+}$-IDA-PAA cryogel, which was successfully used previously for isolation of $His_6$-OPH and OPH-$His_6$ [6–8, 10], was used for isolation and purification of the $His_6$-deGFP4-$(RA)_5$-OPH. Homogeneity of the obtained protein preparation was $96 \pm 1\%$ as assessed by electrophoresis (data not shown). It was shown that the enzyme can be eluted from the support by the imidazole gradient at a concentration of 125–175 mM. It was established that, from 1 g of *E. coli* DH5α wet biomass (humidity 85%), 3.8 mg of highly purified preparation of $His_6$-deGFP4-$(RA)_5$-OPH could be produced, which is only 7% lower than the yield of $His_6$-OPH from *E. coli* SG3009[pREP4] cells [8].

In the process of the $His_6$-deGFP4-$(AS)_5$-OPH hybrid protein isolation, it turned out to be impossible to reach an acceptable degree of homogeneity using the same support. Probably, this fact is related to the specific features of the spatial structure of the protein, which results in the significant reduction in its attachment to the chromatographic support used in the study. The weak attachment of the protein to the support leads to the early elution of the protein at low imidazole concentrations, which were used to wash out the nonspecifically bound ballast proteins present in the cellular disintegrate supernatant applied to the support.

Owing to inefficiency of the $His_6$-deGFP4-$(AS)_5$-OPH hybrid protein isolation and purification with the help of $Co^{2+}$-IDA-PAA cryogel, it was decided to purify this protein by the method used for the isolation

**Table 1.** Accumulation of OPH activity in *E. coli* cells transformed with the plasmids pTES-$His_6$-deGFP4-$(RA)_5$-OPH, pTES-$His_6$-deGFP4-$(AS)_5$-OPH, and pTES-$His_6$-OPH at the optimal concentration of the biosynthesis inducer in the medium

| Parameters | $His_6$-deGFP4-$(RA)_5$-OPH | $His_6$-deGFP4-$(AS)_5$-OPH | $His_6$-OPH [10] | $His_6$-GFP-EK-OPH [14] |
|---|---|---|---|---|
| Final IPTG concentration, mM | 0.10 | 0.10 | 0.20 | 0.75 |
| Maximum specific activity, units/g of cells | 76 | 101 | 62 | 100 |
| Fraction of target protein over the total amount of intercellular protein, % | 5.5 | 2.1 | 12.6 | 0.4 |
| Soluble fraction over the total amount of target protein, % | 92 | 22 | 50 | 46 |

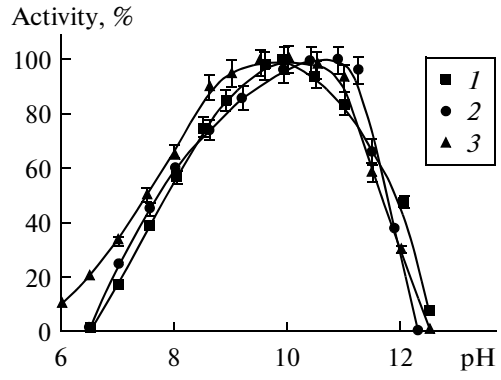


**Fig. 2.** pH dependence of the paraoxon hydrolysis reaction catalyzed by the enzymes $His_6$-deGFP4-$(RA)_5$-OPH (*1*), $His_6$-deGFP4-$(AS)_5$-OPH (*2*), and OPH (*3*).

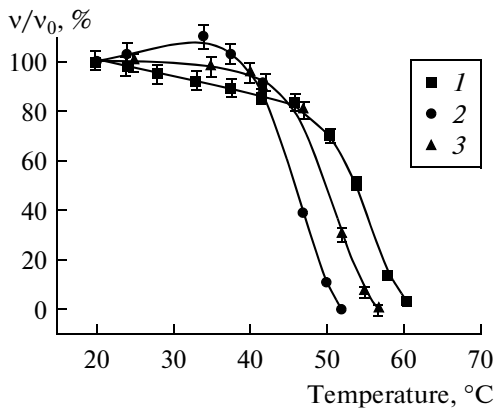


**Fig. 3.** Residual activity of proteins: (*1*) OPH in 0.1 M Tris-HCl buffer (pH 8.5), (*2*) $His_6$-deGFP4-$(RA)_5$-OPH in 0.1 M carbonate buffer (pH 10.5), (*3*) $His_6$-deGFP4-$(RA)_5$-OPH in 0.05 M phosphate buffer (pH 7.5) after 15 min exposure to different temperatures.

of native OPH [18]. The purity of the enzyme preparation obtained by this method was 94 ± 1% as assessed by electrophoresis (data not shown). It was established that 1.3 mg of $His_6$-deGFP4-$(AS)_5$-OPH protein could be obtained from 1 g of wet biomass (humidity 83%) in the process of protein purification.

Investigation of the catalytic parameters of the obtained hybrid proteins revealed that pH optima of their OPH activity were shifted to the alkaline region by 1–1.5 pH units relative to the optimum of the native OPH (Fig. 2).

Catalytic parameters of the enzymatic hydrolysis of paraoxon pesticide catalyzed by the hybrid proteins $His_6$-deGFP4-$(RA)_5$-OPH and $His_6$-deGFP4-$(AS)_5$-OPH at the pH values of the reaction mixture established as optima of their OPH activity were determined (Table 2). An increase in the Michaelis constants ($K_m$) (8- to 12-fold) was observed for the hybrid proteins in comparison with the $K_m$ value characteristic of OPH, and a decrease in the $V_{max}$ values (1.5- to 3-fold), which were determined at a concentration of $10^{-9}$ M for all proteins, was noted.

The lower efficiency of the hybrid proteins in relation to such substrate as paraoxon was probably associated with the changes in the spatial conformation of the OPH protein globe due to the interaction of both partners in the hybrid protein.

The inactivation constants at different temperatures (Table 3) and the dependence of the residual catalytic activity after 15 min exposure on temperature (Fig. 3) were determined for the $His_6$-deGFP4-$(RA)_5$-OPH hybrid protein. A sharp drop of the OPH activity of the $His_6$-deGFP4-$(RA)_5$-OPH hybrid protein occurred with increasing temperature in the range 40–52°C (pH 10.5), and at 50°C, its activity was less then 20% of the initial (Fig. 3). Thermal stability of the OPH hybrid analog was less than the thermal stability of the native enzyme at the same pH. However, at the lower pH of 7.5, the hybrid protein retained 70% of the initial activity at 50°C. Hence, it was established that the hybrid protein could be successfully employed for POC hydrolysis at neutral medium pH and elevated temperatures.

A linear character of the $His_6$-deGFP4-$(RA)_5$-OPH protein inactivation kinetics at pH 7.5 and different temperatures was observed in a wide concentration range of the protein (0.1–1 mg/ml).

Thermal inactivation of oligomeric proteins can proceed through dissociative and nondissociative mechanism [19]. According to the theory of the dissociative inactivation of oligomeric proteins, the dependence of $\ln(\nu/\nu_0)$ on time is represented by straight lines connected by an inflection point indicating the protein dissociation into subunits [19]. On the contrary, the dependence of $\ln(\nu/\nu_0)$ on time for mono-

**Table 2.** Catalytic parameters of the hybrid proteins and OPH in the reaction of paraoxon hydrolysis at optimal pH (for hybrid proteins, pH 10.5; for OPH, pH 8.5)

| Catalytic parameters | $His_6$-deGFP4-$(RA)_5$-OPH | $His_6$-deGFP4-$(AS)_5$-OPH | $His_6$-OPH [10] |
|---|---|---|---|
| $K_m$, mM | 0.13 | 0.20 | 0.02 |
| $V_{max}$, $\times 10^{-7}$ $M^{-1}$ $s^{-1}$ | 2.5 | 5.6 | 8.5 |

meric proteins is represented by straight lines without inflection because the activity decline in this case is due to the changes in the tertiary protein structure caused by the increase in temperature [20]. It is logical to presume that thermal inactivation of the oligomeric proteins with high stabilization energy prohibiting dissociation even at elevated temperatures proceeds by the mechanism common to monomeric proteins. The changes in the tertiary structure of the active subunits of these oligomers are caused exclusively by the temperature factor and are not related to the effect of dimerization on their conformation, as in the case of the dissociative mechanism [19]. The dependence of $\ln(v/v_0)$ on time for such protein should be similar to the dependences common to thermal inactivation of monomeric proteins. Hence, thermal inactivation of the $His_6$-deGFP4-$(RA)_5$-OPH hybrid protein proceeds according to a mechanism different from dissociative, namely, in the form of a dimer or monomer.

It is reliably established that OPH forms an exceptionally stable dimer with the stabilization energy of 44 kcal/mol [21, 22]. Electrophoretograms of the homogenized OPH preparations obtained even in denaturing conditions displayed two distinctive bands corresponding to the monomeric and dimeric forms of the protein (data not shown).

The opposite situation was observed for the obtained hybrid proteins. The native electrophoresis of the purified $His_6$-deGFP4-$(RA)_5$-OPH protein showed that 85% of the hybrid protein was in a monomeric form, while only 15% was in a dimeric form (Fig. 4). Low concentration of the hybrid protein dimer in the enzyme preparation indicates that it is functioning predominantly in the monomeric state and could suggest a significant decline in the stabilization energy of the dimer of the enzyme as compared with the native OPH. The ease of the hybrid protein homodimer dissociation into subunits is reflected in the shape of the $\ln(v/v_0)$ dependence on time obtained for its inactivation.

Investigation of the catalytic parameters of the hybrid proteins showed that the $His_6$-deGFP4-$(RA)_5$-OPH $K_m$ value for the DFP was approximately twofold lower than the $K_m$ value known for the DFP hydrolysis catalyzed by the native OPH and was 0.5 mM. It was established that the $V_{max}$ value for the DFP hydrolysis by the hybrid protein decreased approximately twofold in comparison with the $V_{max}$ value for the DFP hydrolysis catalyzed by the native OPH and was $10^{-8}$ M $s^{-1}$.

Analysis of the fluorescent properties of the $His_6$-deGFP4-$(RA)_5$-OPH hybrid protein revealed that the deGFP4 protein present in its composition has an absorption maximum at the wavelength of 400 nm and two fluorescence maxima at the wavelengths of 461 and 515 nm, which correspond to the characteristics of the free deGFP4 protein. Investigation of the pH effect of the medium on the fluorescence intensity of the hybrid protein showed that the fluorescence maximum at 515 nm was reached at pH 8.5. This was only 0.5 units lower than that for the individual deGFP4 protein.

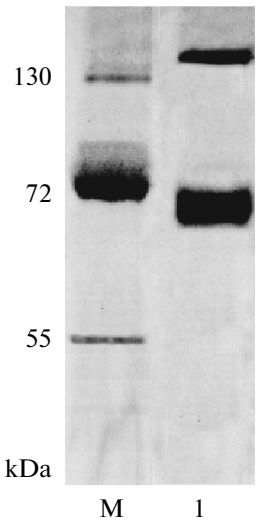


**Fig. 4.** Electrophoretogram depicting the protein composition of the purified $His_6$-deGFP4-$(RA)_5$-OPH protein sample (1) in nondenaturing conditions; M is molecular weight markers.

A 0.2 pH unit decrease was observed in the process of the $4 \times 10^{-3}$ M paraoxon hydrolysis by the $His_6$-deGFP4-$(RA)_5$-OPH hybrid protein in 1 mM phosphate buffer (pH 7.5) supplemented with 100 mM NaCl. In addition, hydrolysis of this concentration of paraoxon resulted in easily registered ~15% changes in the fluorescence intensity at the emission wavelength of 515 nm.

Hence, the deGFP4 protein molecule can serve as an internal "witness" of the POC hydrolysis by the OPH molecule even at low POC concentrations. The soluble form of the $His_6$-deGFP4-$(RA)_5$-OPH obtained in this work can be proposed for application

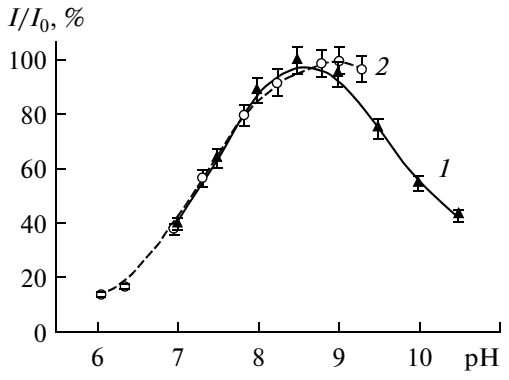


**Fig. 5.** pH dependence of the fluorescence intensity of the $His_6$-deGFP4-$(RA)_5$-OPH (*1*) and deGFP4 (*2*) proteins measured at the wavelengths of excitation of 400 nm and emission of 515 nm.

in studies of POC hydrolysis in vivo in tissue cultures at physiological pH and elevated temperature (37°C) for investigation of the toxicity of POC and the possibility of its reduction by the action of OPH [23].

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SPELL: 1. cryogel, 2. plasmid, 3. nondenaturing, 4. Lyagin, 5. Scholtz