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Reactivation of *Serratia marcescens* Mutant Endonuclease by Hydroxilamine

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Abstract
It is known that histidine plays an important role in the catalytic activity of many nucleases. Performing the function of a common base of these enzymes, it activates the formation of hydroxyl from the water molecule, which, in turn, by attacking the phosphorus atom of the diester bond causes its rupture. It was previously shown that in the endonuclease Serratia marcescens, the replacement of histidine with glycine results in its inactivation. We were able to restore the hydroxylamine activity of the mutant enzyme Serratia marcescens endonuclease, in which histidine in the 89th position is replaced by glycine.
Key words: endonuclease, plasmid, basal level of expression, hydroxylamine, histidine.

INTRODUCTION

Serratia marcescens endonuclease refers to enzymes that can cleave both DNA and RNA [1]. Previously, the endonuclease gene was cloned in the plasmid pHisNuc*Sma* [2,3], and mutant proteins [4] were obtained, and the mechanism of action of this enzyme was also studied [5].

At present, the method of chemical reduction of enzymes is widely used. When low-molecular compounds similar in chemical properties with absent amino acid residues are added to the medium with the inactive mutant enzyme, the activity of this enzyme is restored.

The effect of recovery correlates to the fact that as a result of the mutations, the initial amino acids are replaced by a lower molecular weight. As a result, the active center can form singular "pockets" in which exogenous molecules containing reaction groups lost due to mutagenesis can lead to the restoration of the activity of the mutant enzyme [6]. It has been suggested that such substances can be the precursors to both amino acids and low-molecular amines [7]. It has similarly been shown that an increase in the size of the molecule of the exogenous substance in the reaction mixture reduces the restoration of the enzyme activity [8]. The method of chemical reduction for DNase was first applied by Chen with co-authors to a mutant pancreatic DNase, in which in the active site, histidine in positions 134 and 252 is replaced by glycine and alanine. The addition of imidazole, as well as primary amines to the reaction medium led to an increase in the activity of mutant enzymes. The same effect was observed in the mutant enzyme DNase II (H115A and H297A) when imidazole was added to the medium [8].

Later, Midon and coauthors were able to restore the catalytic activity of a number of endonucleases with the imidazole: Steptococcus pneumonia EndA (H160A), Anabaena sp. NucA (H124A) and Serratia brand Nuc*Sma* (H89G). They suggested that the restoration of the enzyme activity would not take place completely, as by replacing the lost histidine radical with imidazole, its location in the active site will not be optimal [9].

Along with the above substances, some low molecule amines (ethyl-, hydroxylamine, hydrosine, etc.) are also able to restore the activity of enzymes, performing the function of a common base, which in wild-type enzymes is performed by histidine residue.

Based on the fact that histidine, imidazole and amines are nucleophiles, can restore the activity of the mutant endonuclease Serratia marcescens (in which histidine in the 89th position, is replaced by glycine [9]), we assumed that hydroxylamine, as well as histidine, can activate the formation of hydroxyl, which, by attacking the phosphorus atom of the diester bond, causes its rupture.

MATERIALS AND METHODS

1.1. **Research subjects.** Recombinant strains of *E. coli* TGE900 [4] bearing plasmids pHisNuc*Sma* (H89G) or pHisNuc*Sma* (wt). Plasmids contain a mutant or wild variant gene of Nuc*Sma* possessing 0% or 100% nucleic acid activity, respectively. The genes in the plasmid pHisNucSma are under the control of the α-promoter phage λ, the transcriptional activity of which *E. coli* TGE900 is regulated by a temperature-sensitive C1 protein repressor (Fig. 1) [10].

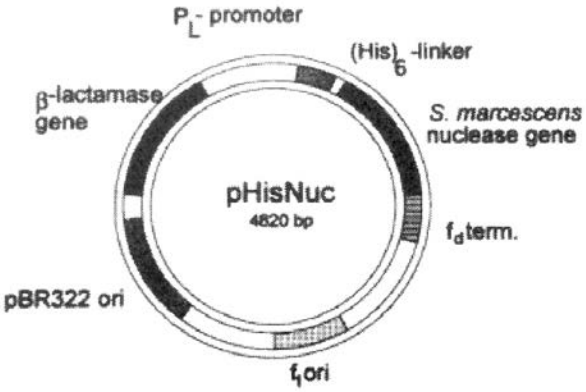

Fig. 1. The structure of the plasmid pHisNuc*Sma* [4].

1.2. Nutrient medium. Liquid and solid LB-media were used in the study, to which ampicillin was added at a concentration of 100 μg / ml to create selective conditions.

1.3. The influence of hydroxylamine on the growth of recombinant strains of *E. coli* TGE900 pHisNuc*Sma* (H89G) and E. *coli* TGE900 pHisNuc*Sma* (wt). Grown overnight at room temperature, the culture was diluted in LB-broth containing ampicillin at optical density D = 600 0.05. The resulting culture was poured into three 10 ml flasks, and a hydroxylamine solution was added to the test versions so that the final hydroxylamine concentration in the medium was 0.2 mM. The strains were grown in a shaker at room temperature. During the growth process, samples were taken at one hour intervals to measure the optical density. The optical density was measured on a KFK-2 photoelectric colorimeter at a wavelength of 600 nm.

Based on the figures obtained, a data plot showing the dependence of optical density on the time of cultivation in the presence of hydroxylamine was constructed. Static data processing was conducted using Microsoft Excel.

1.4 Purification of the enzyme. 2 ml of a culture grown overnight at room temperature was added to 100 ml of medium with ampicillin and cultivated at 28 ° C. on a shaker to achieve an optical density of A_{600} = 0.5.

To induce of expression of S. marcescens endonuclease genes, the cultivation temperature was increased to 42 °C and the cultures were cultivated for 4-5 hours. After that, the cells were pelleted in the centrifuge Awell MF20R (France) at 5000 rpm, t = 4 ° C, resuspending the sediment in 20 ml of buffer (10 mM Tris, pH 8.2 + 1 mM EDTA + 20 mM imidazole), and the cells were destroyed in an ultrasound disintegrator 6 times a minute with an interval of one minute. Cell debris was precipitated at 14,000 rpm for 30 minutes, and the supernatant was taken and used to purify the enzyme.

Columns with nickel agarose were washed with 10 ml of buffer (10 mM Tris, pH 8.2 + 1 mM EDTA + 10 mM imidazole). 20 µl of supernatant precipitated at 14,000 rpm were applied to the columns, and washed with 10 µl of buffer (10 mM Tris, pH 8.2 + 1 mM EDTA + 20 mM imidazole). The linked nickel agarose to nuclease was eluted with 1 ml x 3 buffer (10 mM Tris, pH 8.2 + 1 mM EDTA + 200 mM imidazole). 10 µl of the sample was taken from each eluate.

The eluates were placed in dialysis bags and dialyzed against 1000 ml of a buffer containing 10 mM Tris, pH 8.2 and 1 mM EDTA. After dialysis, the concentration of enzymes was determined using the NanoDrop instrument. Glycerol was added to the dialysate to a final concentration of 30%. The enzymes were stored at -20 ° C.

1.5. SDS-electrophoresis in 15% polyacrylamide gel (Laemmli, 1970).

Electrophoresis was conducted for 2-3 hours at a current strength of 35 mA, after which the gel was stained with Coomassie solution and analyzed.

1.6. The restoration of the activity of the mutant nuclease *Serratia marcescens* (H89G) hydroxylamine. Restoration of enzyme activity was determined by the hydrolysis of plasmid DNA. Plasmid DNA of pBSK, 3.19 kb in size, was used as the substrate.

The purified Nuc*Sma* (wt) enzyme was diluted 1000 times (1 µl of enzyme and 999 µl of buffer). The following buffer was used for dilution: 1 µl 2M Tris pH 8.2 + 1 µl 50 mM MgCl 2 + 8 µl water.

A mixture of 10 µl of buffer, 10 µl of pBSK DNA (15 ng / µl) and 80 µl of water was made or the reaction. 54 µl was then withdrawn from the resulting mixture and mixed with 5 µl of the diluted enzyme (H89G) and 1 µl of NH 2 OH (200 pM) or, in the case of control, 6 µl of water, or as a control for the wild type 6 µl of the enzyme (wt). The reaction was carried out for 2 minutes at room temperature and then stopped by applying 10 µl of the electrophoresis application buffer. Then 10 µl of the sample was taken from each sample and 2 µl of the buffer was applied to the phoresis.

1.7. Electrophoresis in agarose gel

Agarose gel electrophoresis was performed at a constant voltage of 8 V / cm. The gel composition was 1% agarose; 100 mM Tris-borate buffer, and distilled water with a pH of 8.0.

RESULTS

As stated before, there has recently been a wide application of the chemical enzyme reduction method, which allows the recovery of inactive mutant variants of enzymes by introducing inactive enzyme substances similar in properties with a catalytically important amino acid replaced during mutagenesis [7, 9] into the reaction medium. Thus, we investigated the possibility of reduction under the influence of the hydroxylamine inactive mutant variant of H89G nuclease S. marcescens, in which histidine in the 89th position is replaced by glycine.

It is known that hydroxylamine is a weak mutagen that specifically causes cytosine deamination leading to CG → TA transitions. Since in our inactive mutant variant histidine in the 89th position is replaced by glycine, we had previously checked whether glycine encoded triplets would be replaced during the transcription by triplets encoding another amino acid. The results of such a comparison showed that there is only one triplet that can be transitioned, but it does not lead to a substitution of glycine for another amino acid (Table 1).

Codons	Amino acids
GGT	Glycine
GGC→GGT	
GGA	
GGG	

Table. 1. Genetic code of glycine

In addition, hydroxylamine may have a toxic effect. In order to determine the concentration of hydroxylamine, which does not exert any toxic effect and influence the growth of the culture of the recombinant *E. coli* strain *TGE900* pHisNuc*Sma* (H89G), we took only the recombinant *E. coli* strain TGE900 carrying the wild type gene Nuc*Sma* (wt). If, as according to our assumptions, the activation of the mutant enzyme with hydroxylamine occurs, it can affect the growth of the recombinant strain bearing the inactivated mutant Nuc*Sma* gene. As a control, a culture was used without the addition of hydroxylamine.

We used the following concentrations of hydroxylamine: 50 mM, 10 mM, 5 mM, 1 mM, 0.2 mM, 0.1 mM. To this end, the cells of recombinant *E. coli* strain TGE900 pHisNuc*Sma* (wt) were inoculated in 1 ml of media containing different concentrations of hydroxylamine. No hydroxylamine was added to the control test tube. The tubes were incubated on a shaker at room temperature overnight. The next day, the growth of the cultures cultivated in different concentrations were compared with the control. It turned out that only the hydroxylamine concentration of 0.2 mM and 0.1 mM had no effect on cell growth. Therefore, for further experiments, we used the concentration of hydroxylamine 0.2 mM.

3.1 The effect of hydroxylamine on the growth of recombinant *E. coli* strains TGE900 pHisNuc*Sma* (H89G) and *E. coli* TGE900 pHisNuc*Sma* (wt)

It has been established that at a basal level of extracellular expression of Nuc*Sma* in E. *coli* cells, a small amount of the enzyme can be activated inside the cell and cause the hydrolysis of the host DNA / RNA, which will lead to a deceleration of the growth of the culture [9]. Therefore, given that a substance potentially capable of restoring mutant nuclease activity should, upon addition in vivo, cause a decrease in culture growth, we cultivated recombinant strains of *E. coli* TGE900 pHisNuc*Sma* (H89G) and *E. coli* TGE900 pHisNuc*Sma* (wt) at room temperature in the presence and absence of hydroxylamine, and the optical density of these cultures was measured throughout their growth. Based on the data obtained, the growth curves of the cell cultures were constructed, and are shown in Fig. 2.

As shown in Fig. 2, the culture of *E. coli* TGE900 cells pHisNuc*Sma* (H89G) showed a significant deceleration in growth in the presence of hydroxylamine. At the same time, this influence was not found in the recombinant strain *E. coli* TGE900 pHisNuc*Sma* (wt).

We hypothesized that hydroxylamine, as well as histidine, imidazole, and amines, which are nucleophiles [9], can restore the activity of the mutant enzyme. This can be explained by the fact

that, by entering the active site of the inactivated enzyme, hydroxylamine, acting as a common base which is carried out in the wild-type enzyme His89, can activate the formation of hydroxyl (OH^-). Hydroxyl, in turn, attacks the phosphorus atom of the phosphoether bond located in the active center, and thus causes its rupture.

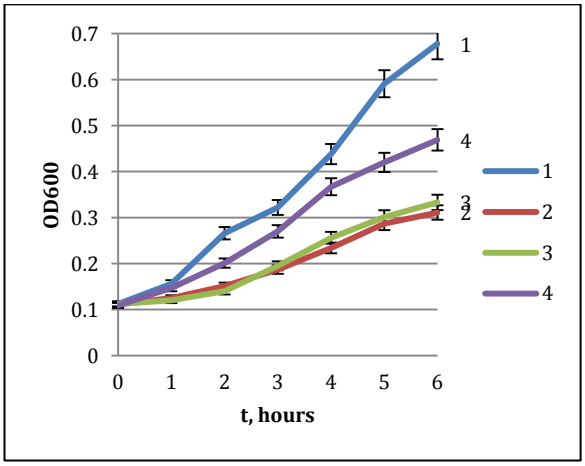

Fig. 2. Graph of the dependence of growth of recombinant *E. coli* strains on hydroxylamine.
1 - *E. coli* TGE900 Nuc*Sma* (H89G) without hydroxylamine;
2 - *E. coli* TGE900 Nuc*Sma* (wt) without hydroxylamine;
3 - *E. coli* TGE900 Nuc*Sma* (wt) with hydroxylamine;
4 - *E. coli* TGE900 Nuc*Sma* (H89G) with hydroxylamine.

3.2 Determination of the effect of hydroxylamine on the restoration of the activity of the mutant variant of Nuc*Sma* endonuclease (H89G)

To study the effect of hydroxylamine on the restoration of nuclease activity of Nuc*Sma* (H89G), we isolated and purified the parent and mutant enzymes with Ni-NTA agarose.

Since the endonuclease gene of the recombinant *E. coli* strain TGE900 pHisNuc*Sma* (H89G) is under the -promotor, which is repressed by the temperature-sensitive repressor protein C1 bacteriophage λ (whose gene is in the chromosome of the host cell), the recombinant strains were first grown on a shaker at a temperature of 28 ° C to an optical density of 0.5 (600 nm), and then the enzyme synthesis was induced by raising the cultivation temperature to 42 ° C, in the span of 5 hours. Induction was analyzed by SDS-electrophoresis (Fig. 3).

As shown in Figure 3, after the induction of recombinant *E. coli* TGE900 pHisNuc*Sma* (H89G) and pHisNuc*Sma* (wt), there is an increase of protein of about ~ 30kDa, indicating an increase in the synthesis of Nuc*Sma* endonuclease under the influence of temperature. In non-induced recombinant strains such an increase is not observed.

The purification of the nuclease was carried out by chromatography on a Ni-NTA column. The eluates of the purified enzymes were checked for purity by SDS-electrophoresis in 15% polyacrylamide gel (Fig. 4).

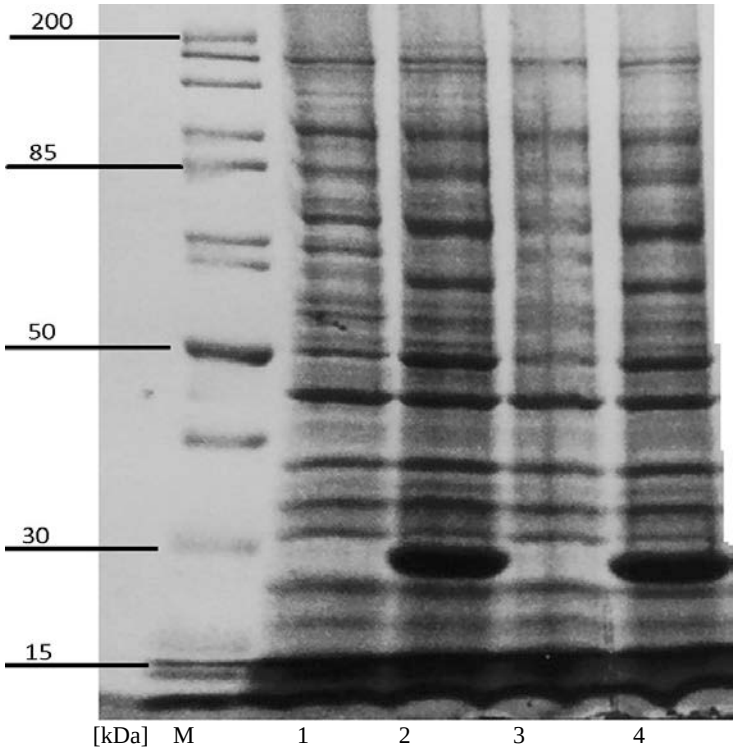

Figure 3 - Electrophoregram of proteins of recombinant *E. coli* strains TGE900 before and after the induction of the *S. marcescens* endonuclease gene.
M - molecular marker RageRuler Unstained Protein Ladder from Fermentas;
1 - Nuc*Sma* (H89G) before the induction of the *S. marcescens* endonuclease gene;
2 - Nuc*Sma* (H89G) after induction of the *S. marcescens* endonuclease gene;
3 - Nuc*Sma* (wt) before the induction of the *S. marcescens* endonuclease gene;
4 - Nuc*Sma* (wt) after induction of the *S. marcescens* endonuclease gene.

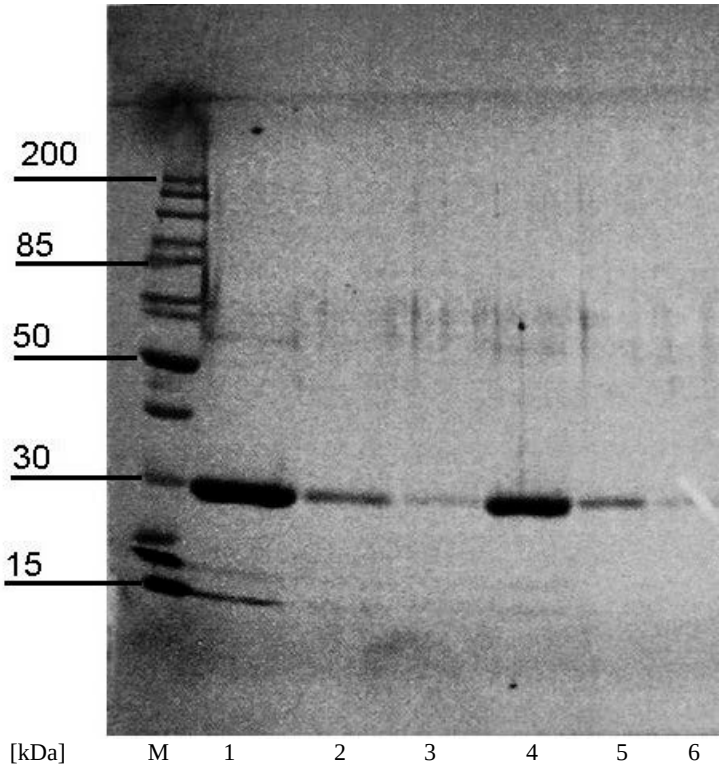

Fig. 4. Electrophoregram of protein fractions, after elution with Ni-NTA agarose. 1-3 eluates - NucSma (H89G); 4-6 eluates - NucSma (wt); M - molecular marker RageRuler Unstained Protein Ladder from Fermentas.

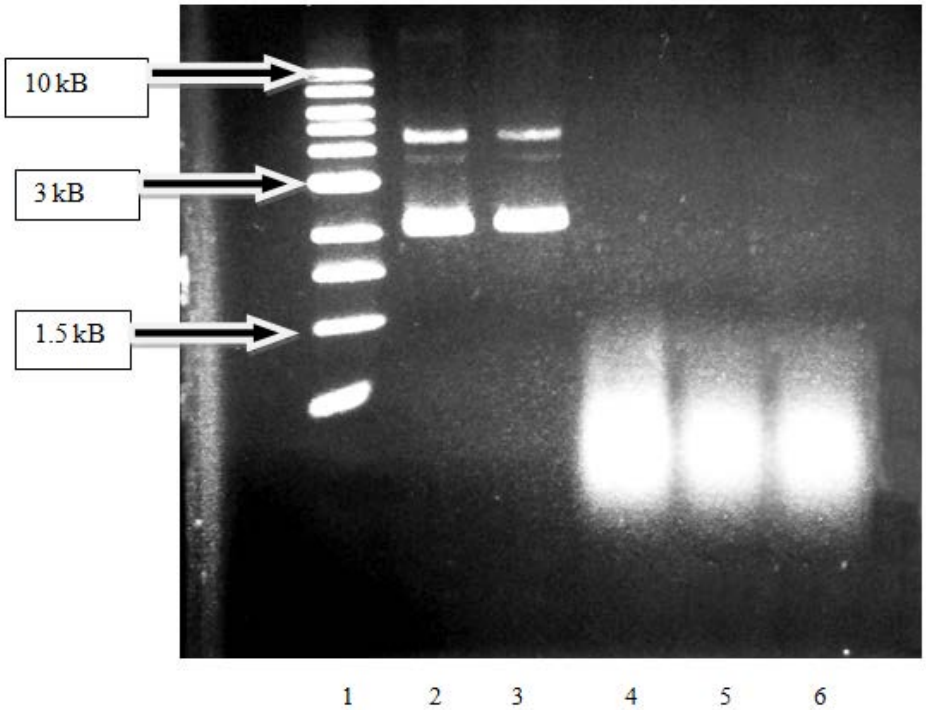

Fig. 5. Electrophoregram of products of plasmid DNA hydrolysis with pBSK endonuclease *S. marcescens* initial and mutant variants in the presence and absence of hydroxylamine.

1. marker from Fermentas;
2. plasmid DNA pBSK (control);
3. Nuc*Sma* (H89G) without addition of hydroxylamine;
4. Nuc*Sma* (H89G) with the addition of hydroxylamine;
5. Nuc*Sma* (wt) without addition of hydroxylamine;
6. Nuc*Sma* (wt) with the addition of hydroxylamine.

As can be seen from Fig. 4, eluates of purified proteins on an electrophoregram are presented in the form of a single band with a molecular mass of ~ 29 kDa, which corresponds to the expected Nuc*Sma* nuclease mass. After dialysis, the enzyme concentration was measured on a Nanodrop (A280) instrument. It was 0.025 μg / μl for Nuc*Sma* (H89G), and 0.022 μg / μl for Nuc*Sma* (wt).

The recovery of the activity of the mutant variant of Nuc*Sma* endonuclease (H89G) was determined by the hydrolysis of plasmid DNA.

The hydrolysis of the plasmid DNA was analyzed by electrophoresis in a 1% agarose gel (Figure 5).

As seen from Fig. 5, the nuclease activity of the mutant variant of the enzyme Nuc*Sma* (H89G) with the addition of hydroxylamine is reduced, which leads to the hydrolysis of plasmid DNA. At the same time, in the absence of hydroxylamine in the reaction medium, hydrolysis was not observed. Unlike the mutant variant, the original Nuc*Sma* (wt) of the enzyme that was in the control hydrolyzed the plasmid DNA both in the presence and absence of hydroxylamine.

Thus, as a result of this study, it was determined that hydroxylamine reduces the growth of the recombinant strain *E. coli* TGE900 pHisNuc*Sma* (H89G) compared to the control without hydroxylamine. Such a decrease in growth may be due to the restoration of the activity of the mutant enzyme, leading to the cleavage of nucleic acids in the cell. This assumption was confirmed by the experiment on the hydrolysis of plasmid DNA by the purified enzyme of the mutant variant of Nuc*Sma* endonuclease (H89G).

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