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# Renaturation, Activation, and Practical Use of Recombinant Duplex-Specific Nuclease from Kamchatka Crab

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**Abstract**—We overexpressed duplex-specific nuclease (DSN) from Kamchatka crab in *Escherichia coli* cells and developed procedures for purification, renaturation, and activation of this protein. We demonstrated identity of the properties of the native and recombinant DSN. We also successfully applied the recombinant DSN for full-length cDNA library normalization.

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*Key words*: duplex-specific nuclease (DSN), sugar nonspecific nuclease, renaturation, activation, specificity

Nucleases are widespread in various organisms and involved in processes of replication, reparation, recombination (including transposition), regulation of gene expression, protection from viruses, apoptosis, and digestion [1]. Common methods of molecular cloning, study of DNA–protein interactions, determination of nucleic acid structure, etc. have been developed using various nucleases (primarily restriction endonucleases). Many types of nucleases are known at present, from highly specific restriction endonucleases attacking strictly distinct DNA sequences to sugar nonspecific exo- and endonucleases hydrolyzing not only nucleic acids, but also nucleotides. Therefore, the search for and study of new nucleases is of interest from scientific and practical points of view.

A new unique nuclease (duplex-specific nuclease, or DSN) has been earlier isolated and characterized in our laboratory from hepatopancreas of king crab *Paralithodes camtschaticus*, and its gene has been cloned as well [2]. The amino acid sequence of this nuclease allowed its assignment to the sugar nonspecific endonuclease family. Enzymes belonging to this group hydrolyze bonds between sugar and phosphate residues in DNA, RNA, and nucleotides [1]. However, DSN—in its interaction with nucleic acids—is highly specific hydrolyzing only double-stranded DNA and DNA chain in DNA–RNA hybrids and is inactive toward RNA and single-stranded DNA. Moreover, for effective catalysis DSN requires a minimum ten pairs of ideally complementary nucleotides in double-stranded DNA [2]. DSN is stable and active over a wide temperature range. The specificity and thermostability of this enzyme have allowed development of two methods for molecular biology: normalization of full-length cDNA libraries [3] and SNP detection [2]. However, the absence of active recombinant DSN restricted possibilities of its application and development of new methods with this enzyme and made impossible the study of molecular mechanisms of such unusual specificity of the enzyme.

The aim of this work was the expression of DSN in *Escherichia coli* cells and subsequent renaturation of the enzyme from inclusion bodies.

## MATERIALS AND METHODS

Inorganic salts, antibiotics, and media, as well as protein disulfide isomerase were obtained from Sigma–Aldrich (EU). Restriction endonucleases, T4

*Abbreviations*: BCIP) 5-bromo-4-chloro-3-indolyl phosphate; Cys) cysteine; Cys-Cys) cystine; DSN) duplex-specific nuclease; GSH) reduced glutathione; GSSG) oxidized glutathione; IPTG) isopropyl β-D-thiogalactopyranoside; LB) Luria–Bertani medium; NBT) nitro blue tetrazolium; PBS) phosphate buffered saline; PDI) protein disulfide isomerase; PEG) polyethylene glycol; SDS) sodium dodecyl sulfate.

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DNA-ligase, and buffers for them were purchased from New England Biolabs (UK). Reverse transcriptase (PowerScript), DNA-polymerase (Advantage 2), and buffers for them, human placenta poly(A)-RNA, nucleotides, and metal affinity resin (TALON) were obtained from Clontech (USA). The pET22b(+) vector and *E. coli* strains XLBlue and BL21(DE3) were purchased from Novagen (EU), and proteinase K was purchased from Roche (EU).

**DSN cloning and expression.** Total RNA was isolated from king crab hepatopancreas according to the established protocol [4]. The first chain of cDNA was synthesized with this RNA using a SMART cDNA Amplification Kit (Clontech) according to the manufacturer's protocol. The DSN-encoding cDNA site was amplified from the produced first chain using the following gene-specific oligonucleotides: 5′-TTGGATCCTGTCAATGGCCAGGACTGTGTGTGG-3′ and 5′-AAAAGCTTAGTGAGGAGTCCGACATTGCCCAG-3′. The PCR-product was purified with a QIA-Qwick PCR purification kit (Qiagen, EU) and then treated with restriction endonucleases *Bam*HI and *Hind*III and cloned into the vector pET22b(+). The resulting construct was checked for the absence of mutations by sequencing. The strain BL21(DE3) was transfected with the DSN–pET22b(+) construct. Transformed cells were grown on agarized Luria–Bertani (LB) medium containing 0.2 mg/ml of ampicillin for 14-16 h. Individual colonies were inoculated into 4 ml of liquid LB medium containing ampicillin and grown under continuous stirring (300 rpm) at 37°C for 14-16 h. Thereafter, 1 ml of the final cell suspension was diluted in 100 ml of liquid LB medium with ampicillin and grown at 37°C to the optical density of cell suspension $A_{600}$ = 0.8-1.0. Expression of the recombinant protein was initiated by addition of isopropyl β-D-thiogalactoside (IPTG) to the cell suspension to the final concentration of 1 mM. Thereafter, the cells were grown at 37°C for 5 h.

The following methods were employed for optimization of growth conditions to obtain soluble DSN: cultivation of the producer strain at either 30°C or room temperature (carried out similarly to the above-described technique, but at temperature decreased to ambient or 30°C); decrease in time after the induction (following the general technique, sampling aliquots of the producer strain 0.5, 1, and 2 h after the addition of IPTG); activation of self-generated chaperones of the producer strain (single colonies were inoculated into 4 ml of liquid LB medium containing ampicillin and grown under continuous stirring (300 rpm) at 37°C for 14-16 h; thereafter, 1 ml of the resulting cell suspension was diluted in 100 ml of liquid LB medium containing ampicillin and 3% ethanol and cultivated at 37°C to optical density of the suspension $A_{600}$ = 0.8-1.0; thereafter, IPTG was added to the final concentration of 1 mM, and the culture was grown at 37°C for 5 h).

**Purification of the recombinant enzyme.** After the induction, the cells were centrifuged at 4000*g* for 15 min under cooling. The pellet was resuspended in 50 mM Tris-HCl, pH 8.0, and homogenized by sonication. The resulting lysate was centrifuged at 15,000*g* for 10 min. The supernatant and pellet were analyzed for the presence of the target protein by SDS-PAGE. The inclusion bodies were washed following the scheme:

– the pellet after the preceding stage was resuspended in 50 mM Tris-HCl, pH 8.0, followed by centrifugation at 15,000*g* for 15 min; the procedure was repeated twice;

– the pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 1% Triton-X-100 and 0.5 M NaCl and centrifuged at 15,000*g* for 15 min; the procedure was repeated thrice;

– the pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 2 M urea and centrifuged under the same conditions.

The washed inclusion bodies were dissolved in 50 mM Tris-HCl, pH 8.0, containing 8 M urea. Insoluble particles were removed by centrifugation, and the resulting solution was mixed with 50% TALON suspension (Clontech) previously washed in 0.05 M Tris-HCl, pH 8.0, containing 8 M urea. The amount of the sorbent was calculated from the following ratio: 1 ml of resin to the protein amount obtained from 50 ml of expressing culture. The resulting suspension was incubated for 30 min under stirring followed by centrifugation at 1000*g* for 2 min; the supernatant was removed and the settled sorbent was washed five times with the application buffer. Elution was carried out by addition of 100 mM EDTA in the application buffer. DSN from soluble fraction was isolated in the same way, but 0.05 M Tris-HCl, pH 8.0, without urea was used as the working buffer.

**Antiserum production.** The DSN isolated under denaturing conditions was precipitated with trichloroacetic acid and washed thrice with acetone. Then the pellet was resuspended in complete Freund's adjuvant. A one-year-old rabbit was immunized four times during three months with 1 mg of DSN for each injection. Then an aliquot of blood was taken, blood elements removed, and the obtained serum was used in further experiments. The titer of the serum was determined by Western-dot-hybridization. One microliter of the solution containing 0.1-0.001 mg/ml of recombinant DSN was applied on a nitrocellulose membrane. Following drying, the membrane was incubated with phosphate-buffered saline (PBS) containing 5% defatted milk (blocking solution for saturation of nonspecific sorption sites) for 1 h. Then the anti-DSN-serum diluted (1 : 200)-(1 : 5000) was added and incubated overnight at 4°C. Thereafter the membrane was washed with PBS and incubated for 1 h with anti-rabbit antibody–alkaline phosphatase conjugate dissolved in blocking solution. Subsequently, the membranes were washed and incubated in nitro blue tetrazolium/5-

bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) solution for visualization of signals. To determine specificity of the antibodies, the native and recombinant DSNs were separated by PAGE and transferred onto nitrocellulose membrane, and the Western-blot-hybridization was carried out using the antibodies to DSN as primary antibodies (dilution 1 : 5000).

**Renaturation of DSN.** *One-step dialysis*. The solution of pure recombinant protein in 50 mM Tris-HCl, pH 8.0, containing 8 M urea was diluted with the same buffer to the concentration of 0.01 mg/ml. Thereafter 50 ml of this solution was dialyzed against 1 liter of 50 mM Na-phosphate buffer, pH 8.0, containing 100 mM $Na_2SO_4$ and 10 mM $MgCl_2$ at 4°C for 24 h.

*Stepwise dialysis*. The solution of pure recombinant protein in 50 mM Tris-HCl, pH 8.0, containing 8 M urea was diluted with the same buffer to the concentration of 0.01 mg/ml. Thereafter 50 ml of this solution was dialyzed against 50 mM Na-phosphate buffer, pH 8.0-9.5, containing 2 M urea, 100 mM $Na_2SO_4$, and 10 mM $MgCl_2$ for 24 h at 4°C. Then the second dialysis was carried out against 50 mM Na-phosphate buffer, pH 8.0-9.5, containing 0.4 M urea, 100 mM $Na_2SO_4$, and 10 mM $MgCl_2$ for 24 h at 4°C. The final dialysis was carried out against the same buffer without urea under the same conditions.

*Renaturation via gradual decrease of denaturing agent*. The solution of pure recombinant protein in 8 M urea in 50 mM Tris-HCl, pH 8.0, was dissolved with the same buffer to the concentration of 0.01 mg/ml. Thereafter 50 ml of this solution was dialyzed against 50 mM Na-phosphate buffer, pH 8.0-9.5, containing 100 mM $Na_2SO_4$ and 10 mM $MgCl_2$ with gradual decrease of urea concentration from 8 to 0 M. The dialysis was carried out for 72 h at 4°C. The following additives to the renaturing buffer were taken at the following concentrations: redox system (3 mM GSH/0.3 mM GSSG or 3 mM Cys/0.3 mM Cys-Cys); 0.5% polyethylene glycol (PEG) 3350; 0.4 M glycerol; 1 μM $CuBr_2$; 0.01% Triton X-100 [5, 6]. All these reagents were added into the buffer against which the sample was dialyzed. In the case of stepwise dialysis, the reagents were added at all stages of the dialysis.

*Use of protein-disulfide-isomerase (PDI)* [7]. In one of the cases, one unit of PDI was added to 1 ml of renatured DSN (bypassing all stages of the stepwise dialysis), in another case to 1 ml of DSN at the last stage of the dialysis (from 0.4 to 0 M urea).

**Activation and purification of renatured DSN.** Proteinase K (50 μl; protein concentration 10 mg/ml) was added to 50 ml of the renatured enzyme solution. The resulting mixture was incubated for 30 min at 37°C and can be stored for 3-4 days at 4°C without any loss of enzymatic activity. Following the treatment with proteinase K, the DSN solution was dialyzed against 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$ for 14-16 h at 4°C. Thereafter the sample was loaded onto a Mono-Q column (Pharmacia, Sweden) equilibrated with 50 mM Tris-HCl, pH 7.5. The DSN was eluted by a 0.25-0.6 M NaCl gradient in 50 mM Tris-HCl, pH 7.5. The purified DSN was concentrated on Biomax-5K ultrafiltration membranes (Millipore, USA). The twenty-fold concentrated enzyme was stored at 4°C.

**Determination of enzymatic activity.** DNase activity was quantified by a modification of the method of Kunitz [8, 9]. DNase activity was quantitatively evaluated by measurement of degradation of high-molecular-weight nucleic acids by the enzyme samples under study. Two variants of the method were used: with calf thymus genome DNA or λ and M13 viral DNAs. In the first case, the following components were present in the sample: DNA at concentration of 250 ng/μl, reaction buffer (25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$), and the DSN solution under study (1/10 of the sample volume). The reaction mixture was incubated at 60°C for 1 h. Reaction products were analyzed by electrophoresis in agarose gel. In the second case, the reaction mixture contained: λ and M13 viral DNAs (500 ng each), reaction buffer (25 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$), and the DNase solution under study (1/10 of sample volume). The reaction mixture was incubated at 60°C for 1 h and then analyzed by electrophoresis in agarose gel.

**Detection of single nucleotide substitutions and normalization of full-length cDNA libraries.** The samples for detection of single nucleotide substitutions were prepared according to the previously described protocol [2]. Normalization of full-length cDNA libraries was carried out according to the previously described method [3]. A SMART cDNA Synthesis Kit (Clontech) was used for synthesis and amplification of cDNA libraries. Quantitative PCR was used for evaluation of concentration of cDNA-copies of various genes in cDNA library.

## RESULTS

**Expression and purification of recombinant DSN.** DSN cDNA was cloned in the expression vector pET22b(+), which is commonly used for expression of secretory or toxic proteins in *E. coli* cells. This vector endows the recombinant protein with N-terminal signaling sequence specific for *E. coli*, which targets the protein secretion into periplasm, as well as with C-terminal sequence of six histidines, which allows purification of the recombinant protein by affinity chromatography on a $Ni^{2+}$-containing resin. The cloned DSN cDNA was devoid of both non-translated regions and the site presumably encoding its own signaling peptide. However, electrophoretic protein analysis of the recombinant strain demonstrated that the recombinant DSN is present in the water-insoluble fraction (Fig. 1). The *E. coli* strain transformed with the vector without the insertion was used as

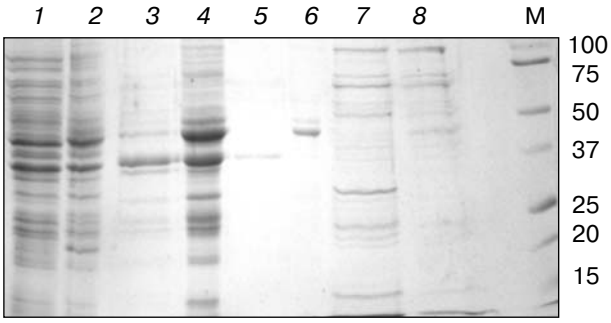


**Fig. 1.** Electrophoregram of proteins for recombinant (pET-DSN) and control (pET) *E. coli* strains. Lanes: *1*, *2*) total protein of pET and pET-DSN strains, respectively; *3*, *4*) fraction of inclusion bodies for pET and pET-DSN strains, respectively; *5*, *6*) protein obtained after purification of inclusion bodies of pET and pET-DSN strains, respectively, on metal affinity resin under denaturing conditions; *7*, *8*) water-soluble fraction of cell lysate of pET and pET-DSN strains, respectively, purified on metal affinity resin; M, molecular mass markers (kD).

a control. Following purification of the recombinant protein on Co-containing sorbent, the distribution of the protein was unchanged: the recombinant protein was only present in insoluble fraction purified under denaturing conditions (Fig. 1). The yield of purified denatured protein was 100 mg per liter of bacterial culture.

We tried to obtain the recombinant DSN in soluble form by varying growth conditions for the strain-producer. However, neither decrease in cultivation time after induction nor temperature decrease resulted in the appearance of DSN in the soluble fraction of the cell lysate (data not shown). We also activated the native chaperons of the producer by its cultivation in medium with low ethanol content. However, the growth of the producer (unlike the control strain) stopped after addition of IPTG. The analysis of proteins in soluble fractions of these cultures also revealed no DSN (data not shown).

**Raising of antiserum against DSN.** We immunized a rabbit with the recombinant DSN purified on cobalt-containing resin under denaturing conditions. After the fourth immunization, blood was taken and the serum was tested. The titer determined by Western-dot-hybridization was 1 : 5000. The same DSN was used as the antigen and for immunization. We confirmed specificity of these antibodies to recombinant and native DSN by Western-blot-hybridization.

**Renaturation and purification of the recombinant DSN.** We first tried to renature DSN by drastic removal of denaturing agent. With this aim, suspension of metal-affinity resin with immobilized DSN in 8 M urea was diluted by burst addition of 100 volumes of buffer. After overnight incubation, we precipitated the resin and eluted the DSN. However, the resulting samples did not display DNase activity, and the enzyme largely precipitated (data not shown).

Then we tried to renature DSN via dialysis against the buffer without denaturing agents. The recombinant protein purified in the presence of 8 M urea was diluted to the urea concentration of 1 M and dialyzed twice against renaturing buffer (the method proposed by Gusev coauthors [10]). Very weak DNase activity was detected in the recombinant protein sample after the dialysis, with its absence in the control sample (Fig. 2). We supposed that the DNase activity of recombinant protein can be inhibited by heterogeneous N-terminal sequence. According to data obtained previously, proteinase K has no effect on the natural DSN activity. We supposed that the recombinant enzyme possesses analogous resistance. Therefore, for removal of N-terminal leader peptide the treatment of recombinant protein with proteinase K was carried out resulting in drastic increase in its DNase activity, with absence of DNase activity in the control sample (Fig. 2).

Then we tested three different renaturation schemes: one-step dialysis (from 8 to 0 M), three-step dialysis (from 8 to 2, from 2 to 0.4, and from 0.4 to 0 M), and gradual decrease in urea concentration [11]. In the first case, the activity of DSN comprised 410, in the second 2330, and in the third 1100 Kunitz units. Therefore, in subsequent experiments we used the stepwise dialysis for renaturation.

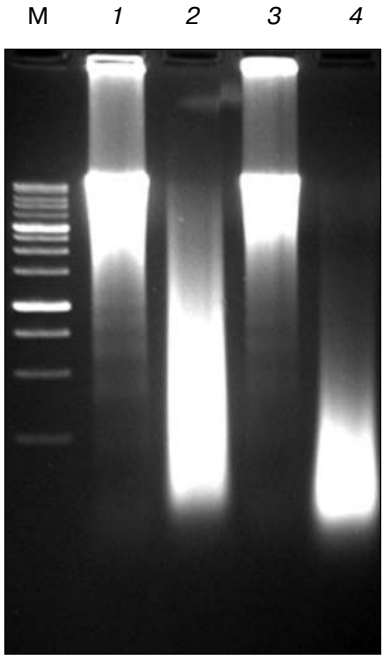


**Fig. 2.** Electrophoresis of genome DNA treated with renatured DSN or specimens obtained under the same conditions as for the control strain. Lanes: *1*) control sample not treated with proteinase K; *2*) renatured DSN not treated with proteinase K; *3*) control sample after the treatment with proteinase K; *4*) renatured DSN after the treatment with proteinase K; M, molecular mass markers.

**Table 1.** Additional reagents influencing the effectiveness of DSN renaturation

| Reagents | Activity, Kunitz units |
|---|---|
| Without additional reagents, pH 8.0 | 560 |
| 3 mM/0.3 mM GSH/GSSG, pH 8.0 | 2330 |
| 3 mM/0.3 mM GSH/GSSG, pH 9.0 | 4450 |
| 3 mM/0.3 mM GSH/GSSG, pH 9.5-10.0 | 6070 |
| 3 mM/0.3 mM Cys/Cys-Cys, pH 9.5-10.0 | 4100 |
| 3 mM/0.3 mM GSH/GSSG + 0.05% PEG 3350, pH 9.5-10.0 | 3950 |
| 3 mM/0.3 mM GSH/GSSG + 0.4 M glycerol, pH 9.5-10.0 | 2640 |
| 3 mM/0.3 mM GSH/GSSG + 1 μM $CuBr_2$, pH 9.5-10.0 | 40 |
| 3 mM/0.3 mM Cys/Cys-Cys + 0.4 M glycerol + 0.05% PEG 3350, pH 9.5-10.0 | 4210 |
| 3 mM/0.3 mM GSH/GSSG + 0.01% Triton X-100, pH 9.5-10.0 | 110 |

**Table 2.** Final scheme of purification and renaturation of recombinant DSN

| Purification step | Total protein, mg | Target protein, mg | Specific activity, Kunitz units |
|---|---|---|---|
| *E. coli* cells | 20 | 1.0 | 0 |
| Inclusion bodies (after dissolving) | 3.0 | 1.0 | 0 |
| Metal affinity chromatography | 0.5 | 0.5 | 0 |
| Renaturation and activation by proteinase K | 0.2 | 0.1 | 620 |
| FPLC on Mono-Q | 0.1 | 0.1 | 607 |

It is known that isomerization of disulfide bonds is stimulated in the presence of mixtures of reduced and oxidized low-molecular-weight dithiols. We tested the pairs of reduced/oxidized glutathione (GSH/GSSG) and cysteine/cystine (Cys/Cys-Cys). The presence in renaturing buffers of the mixture glutathione reduced/glutathione oxidized at the concentration of 5-3 and 0.5-0.3 mM, respectively, substantially increases the yield of active enzyme (Table 1). The cysteine/cystine mixture at the same ratios and concentrations also substantially improves the yield of renatured DSN (Table 1).

It has been shown earlier for some disulfide-enriched proteins that effectiveness of disulfide bond isomerization increases with increase in renaturing buffer pH from 6 to 10 [12]. We tested three different variants of renaturing buffer with pH 8, 9, and 10. The latter proved to be optimal (Table 1). Besides, we tested other reagents (the detergents Tween-80 and Triton X-100, heavy metal ions ($Cu^{2+}$), glycerol, and PEG 3350) for which a positive effect on renaturation of various proteins has been demonstrated earlier. However, according to our observations, only PEG 3350 has a positive effect on DSN renaturation. When renaturation was finished, DSN was treated with proteinase K and purified by chromatography on a Mono-Q column to homogeneity (Table 2). The developed method provides soluble recombinant enzyme with the yield of 20 mg per liter of bacterial culture. Following renaturation, the major portion of DSN molecules remains in the solution and does not precipitate. However, 20% of the initial denatured protein remain intact after the treatment with proteinase K (Fig. 3). A good fraction of the DSN molecules is apparently properly folded and does not precipitate, but does not form proper disulfide bonds. These molecules are inactive and hydrolyzed by proteinase K.

**Functional probes, catalytic properties, stability, and application of renatured DSN.** The specificity of the recombinant enzyme was determined on single-stranded M13 phage DNA and double-stranded λ phage DNA. The renatured DSN only demonstrated nuclease activity

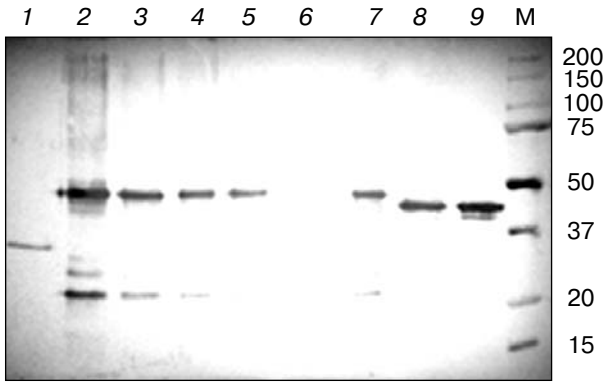


**Fig. 3.** DSN samples before and after renaturation—separated by PAGE, transferred onto membrane, and stained with antibodies against DSN. Lanes: *1*) pET inclusion bodies purified on TALON resin; *2-5*) pET-DSN inclusion bodies purified on TALON resin (respectively 1/5, 2/15, 1/10, and 1/15 of the amount of protein used for renaturation and treatment with proteinase K); *6*) pET after renaturation and treatment with proteinase K; *7*) pET-DSN after renaturation and centrifugation; *8*) pET-DSN after renaturation and treatment with proteinase K; *9*) natural DSN isolated from hepatopancreas of king crab; M, molecular mass markers (kD).

to double-stranded DNA (Fig. 4). Besides, we studied enzymatic activity to ideal ten-nucleotide duplexes and duplexes with a pair of unpaired nucleotides according to the method proposed by Shagin with coworkers [2]. This method comprises the usage of oligonucleotide substrate, one end of which is fluorescently labeled, and the other is labeled with a quenching group. Such nucleotide is not fluorescent because the energy absorbed by the fluorescent group is transferred to the quencher and is dissipated as heat. If the oligonucleotide is hydrolyzed, the fluorescent label and quencher no longer interact, and the fluorescence arises. To create ideal duplexes or duplexes with a non-complementary base pair, a completely complementary or usual oligonucleotide complementary with an error are added to such oligonucleotide.

According to our results, the enzyme only exerts nuclease activity to ideal duplex and does not interact with duplex containing a substitution, as well as with single-stranded oligonucleotide (Fig. 5). The optimal conditions for catalysis (temperature, pH, and magnesium concentration) for renatured DSN are the same as for native enzyme (data not shown).

We developed earlier a method for normalization of full-length cDNA libraries, in which native DSN played the key role [3]. In living cells the number of RNA copies of various genes can vary 1000-fold and more (in cDNA libraries this ratio is usually the same). However, many experimental tasks require comparable concentrations of cDNA copies of all genes throughout the library. According to our method, the amplified cDNAs from the library were subjected to denaturation and subsequent renaturation and then treated with native DSN and reamplified. As a result, the complexity of the library was decreased insignificantly, average length of cDNA fragments remained unchanged, and concentrations of cDNA copies for various genes were equalized [3]. We used recombinant DSN instead of the native one for normalization of human placenta cDNA library. As in the case of native DSN application, the average length of cDNA remained unchanged, and concentrations of cDNA copies of various genes became closer. So, cDNA concentration of major genes was decreased approximately 1000-fold, cDNA concentration of moderately represented genes remained unchanged, and cDNA of minor genes was several times increased (data not shown). Thus, cDNA libraries can be effectively normalized using the recombinant DSN.

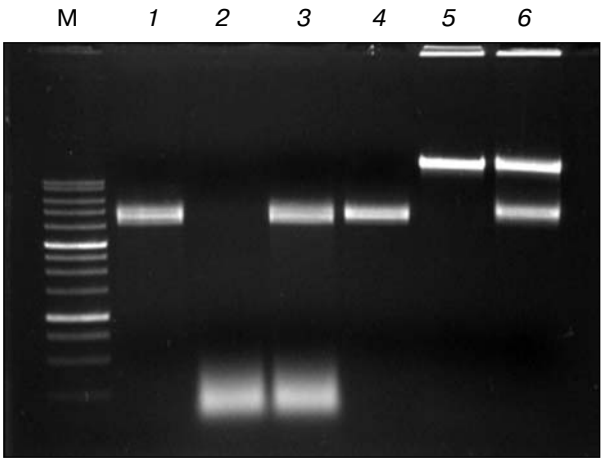


**Fig. 4.** Electrophoresis of single-stranded M13 phage DNA and double-stranded λ phage DNA after treatment with renatured DSN. Lanes: *1*) M13 phage DNA after treatment with renatured DSN for 1 h at 60°C; *2*) λ phage DNA after the treatment with renatured DSN for 1 h at 60°C; *3*) mixture of M13 and λ phage DNA after treatment with renatured DSN for 1 h at 60°C; *4-6*) negative control: M13 phage DNA, λ phage DNA, and their mixture without treatment with DSN, respectively; M, molecular mass markers.

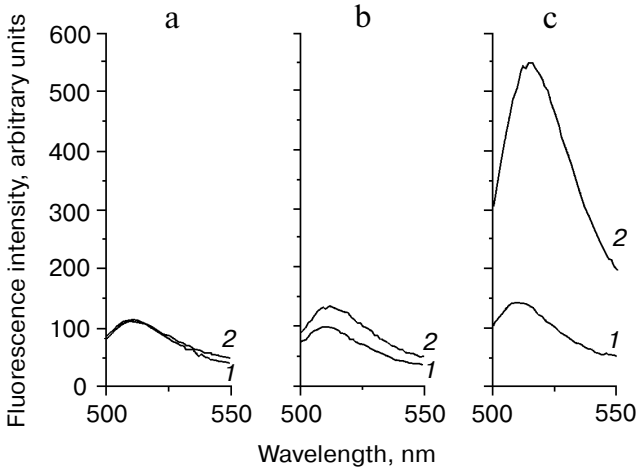


**Fig. 5.** Nuclease activity of renatured DSN to single-stranded oligonucleotide (a), double-stranded oligonucleotide containing a pair of non-complementary nucleotides (b), and ideal oligonucleotide duplex (c). Samples were incubated for 1 h at 37°C. Emission spectra were registered on a fluorimeter at excitation wavelength of 480 nm. Lines: *1*) substrate fluorescence spectrum before the treatment; *2*) fluorescence plot of the same substrates after the treatment with DSN.

## DISCUSSION

Natural DSN is a highly processive and very stable enzyme [2]. Expression of the active enzyme in a prokaryotic cell results in rapid degradation of its genome and cell death. To avoid this, we have cloned a cDNA copy of the DSN gene into pET22b(+) vector providing excretion of the recombinant protein into the periplasm. However, the recombinant DSN molecules aggregated and formed inclusion bodies. Using cobalt-affinity resin, we isolated this enzyme in homogeneous state under denaturing conditions.

Renaturation of a protein containing disulfide bonds is a technically difficult task [13-15]. We have chosen a classic renaturation method—decrease of denaturing agent concentration by stepwise dialysis. In fact, in the preparation obtained by this method a good portion remained in solution. However, more than 98% of the molecules degraded, and nuclease activity remained comparably low after the treatment of this solution with proteinase K. Then we supposed that some of the molecules remaining in the solution after stepwise dialysis are correctly folded but lacked proper disulfide bonds. Probably, this is the cause of their intolerance to proteinase K and catalytic inactivity. An inconsiderable part of the molecules possessing catalytic activity and resistant to proteinase K possesses correct intramolecular bonds. To enhance the yield of properly bonded molecules, we employed the redox pair reduced/oxidized glutathione in the ratio of 10 : 1 [13]. As a result, the yield of active DSN increased fivefold. Elevation of pH value of the renaturing buffer to 10 proved to be effective as well, favoring isomerization of disulfide bonds [16-19]. Eventually, the yield of renatured DSN increased from 1 to 20%.

Recombinant DSN, like the natural one, is resistant to treatment with proteinase K, suggesting the correct folding of the protein. Interestingly, the recombinant enzyme is not only resistant to proteinase K, but becomes significantly more active after the treatment. Two explanations for this fact are possible: the leading peptide encoded by the vector inhibits DSN activity or the enzyme itself contains a peptide temporarily inhibiting its activity, which is cleaved during processing under natural conditions. The identical temperature and pH optima and similar plots of catalytic activity versus divalent metal ion concentrations suggest identity of the native and recombinant enzymes.

On the basis of these data we suppose that the natural DSN is not subjected to any significant posttranslational modification. The natural DSN can effectively hydrolyze only double-stranded DNA and is inactive to single-stranded DNA and RNA [2, 3]. Manifestation of nuclease activity requires a minimum of ten ideally complementary base pairs. The enzyme renatured in our experiments is also active only to double-stranded DNA and is capable of binding only ideal duplex no less than ten base pairs in length. The specificity of the natural DSN has been used to develop a method for full-length cDNA libraries [3]. This is a unique method enabling equalization of the concentrations of cDNA copies for various genes in a library. We have demonstrated that renatured DSN can be highly effectively employed with this aim.

The proposed method for producing active recombinant enzyme can be used for the study of molecular mechanisms of DSN substrate specificity. Using site-directed mutagenesis and renaturation of the resulting mutants, we can determine amino acid residues comprising the substrate-binding and catalytic sites of the enzyme. Besides, a promising technological task is the usage of mutant variants of DSN possessing modified properties, for instance, decreased or increased stability, other substrate specificity, etc.

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