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Recombinant Destabilase-Lysozyme: Synthesis *de novo* in *E. coli* and Action Mechanism of the Enzyme Expressed in *Spodoptera frugiperda*

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Abstract—Destabilase-lysozyme (DL) from salivary gland secretion of the medicinal leech (*Hirudo medicinalis*) is as a member of the invertebrate lysozyme family, which sharply differs from other lysozyme families. In this study, DL lysozyme function was confirmed during expression of a gene encoding DL in *Escherichia coli*. Several constructs of the expression vectors pKK OmpA and pET-3A with or without bacterial, leech, or yeast signal peptides (SP) were used. The use of a construct without signal peptide genes resulted in normal growth of the transformed cells. Transformation of *E. coli* cells with the constructs containing SP was accompanied by the disruption of the forming cells. The use of the expression vector pET-32 LTC-System for production of DL as a fusion protein with thioredoxin also resulted in normal cell growth. However, specific activity of DL isolated from such cells was significantly lower than that of enzyme purified from extracts of *Spodoptera frugiperda* cells, which were infected with the baculovirus vector carrying DL cDNA. It is shown that the action mechanism of invertebrate lysozyme does not differ from that of other families: recombinant DL from *S. frugiperda* extracts catalyzed cleavage of synthetic substrate, hexamer of N-acetylglucosamine, to di- and tetramers, which is typical for enzymatic function of other lysozyme families.

Key words: destabilase-lysozyme, *E. coli* expression, glycosidase activity, medicinal leech

Destabilase-lysozyme (DL) from salivary gland secretion of the medicinal leech (*Hirudo medicinalis*) is a member of a new family of invertebrate lysozymes (i-lysozymes); their primary structure sharply differs from the other lysozymes [1-3]. This family includes 18 lysozymes with known gene structures [3-6]. Recently we determined a family of genes, denominated *Ds1*, *Ds2*, and *Ds3*, which encode some DL forms [7-9]. Two of these genes (*Ds2* and *Ds3*) were expressed in baculovirus system and their protein products exhibited lysozyme-like activity [1]. Attempts to express these genes in *E. coli* resulted in accumulation of DL protein in the form of inclusion bodies [1]. In this study, we evaluated enzymatic activity of DL *de novo* in *E. coli* and investigated some consequences of DL synthesis and of its transportation into certain intracellular compartments.

It is known that lysozymes of different families destroy proteoglycans of Gram-positive cells by hydrolysis of β-1-4-glycoside bond between 2-acetamido-3-O-(1-carboxyethyl)-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose. Hexamer of N-acetylglucosamine, a classic synthetic substrate of different types of lysozyme, is cleaved by them into di- and tetramers [10]. However, such mechanism of action was not demonstrated for invertebrate lysozymes (i-lysozymes). Using recombinant DL synthesized in *Spodoptera frugiperda*, we have investigated the glycosidase mechanism of action of this invertebrate lysozyme. Hexamer of N-acetylglucosamine was used as a classical synthetic substrate.

Abbreviations: DL) destabilase-lysozyme; *Ds1*, *Ds2*, *Ds3*) genes encoding destabilase-lysozymes; i) invertebrate; h) human; c) chicken (designations for types of lysozyme).

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MATERIALS AND METHODS

Synthesis *de novo* and preparation of recombinant DL expressed in *E. coli*. DL gene *Ds2* was expressed in the baculovirus system as described previously [7]. Several constructions were used for expression of DL gene *Ds3* in *E. coli*. The vector pKK Omp A (*lac* promoter) with gene encoding *E. coli* signal peptide of Omp A (the outer membrane protein A) [11] was kindly presented by Dr. P. M. Rubtsov (Institute of Molecular Biology, Russian Academy of Sciences). The constructions of pET-3A (Novagen, USA) (T7 promotor) with gene encoding the signal peptide of *Ds3* isoform of *Hirudo medicinalis* destabilase-lysozyme [8] or with MFα1, sex pheromone from *Saccharomyces cerevisiae* [12], were used. The last one was kindly presented by S. V. Benevolensky and D. G. Kozlov (Institute of Genetics and Selection of Industrial Microorganisms); pET-3A (T7 promotor) without signal peptide was purchased from Novagen, and pQE-30 (*lac* promoter) was from Qiagen (USA). Expression of the DL *Ds3* gene fused to the thioredoxin gene was carried out in *E. coli* (strain Ad494(DE-3); Novagen) under control of T7 promoter using construct obtained on the basis of the vector pET-32 LTC-System (Novagen).

Hybrid recombinant protein was purified by metal-affinity chromatography on the sorbent TALLON (Clontech Labs. Inc., USA) according to the protocol of the supplier. For DL excision, the recombinant fusion protein was treated with bovine enterokinase (Institute of Bioorganic Chemistry, Russian Academy of Sciences). Briefly, 20 μg of enterokinase was added to 200 μl of solution (20 mM Tris-HCl buffer, pH 7.4) containing 100 μg of the hybrid protein. The mixture was incubated at 37°C for 4 h and after dialysis against the same buffer the reaction products (thioredoxin and DL) were analyzed by 15% SDS-PAGE [13]. Lysozyme activity of the recombinant products was assayed before and after enterokinase treatment.

Expression of DL *Ds2* in baculovirus system. *S. frugiperda* cell line IPLB-Sf 9 was used to express DL *Ds2* gene encoding DL Ds2 mature enzyme. The methods for preparation of a recombinant virus and cell extracts were described previously [1, 7].

Antibodies to DL. Mice were immunized with native DL purified as described in [7]; 20 μg of DL in complete Freund's adjuvant was injected subcutaneously (s.c.) to the right hind paw of each animal. Two weeks later mice were injected with 10 μg of DL in incomplete Freund's adjuvant into the tail vein. Two weeks later after the evaluation of serum DL-specific IgG titers by ELISA, the mice were injected with Ehrlich ascites to increase the volume of immune sera. Animals were sacrificed two weeks after the injection. Serum and ascites fluid were collected, pooled, aliquoted, and kept at −20°C until use. DL-specific IgG was estimated using reagents from Sigma-Aldrich (Russia) according to the supplier's instructions [14]. Briefly, 2 μg/ml of DL was coated on the ELISA plates (Costar, England) in phosphate buffered saline (PBS). All incubations were performed in 1% bovine serum albumin (BSA) in PBS (BSA-PBS) at room temperature. Serum dilutions made in BSA-PBS were added to the antigen-coated wells and incubated for 2 h. The plates were then washed, and the anti-mouse IgG was conjugated with horseradish peroxidase. In the ascites, the titer of specific antibodies against DL was 1 : 5000. Polyclonal antibodies were obtained and purified by double sedimentation with 15% polyethylene glycol [15]. The yield of antibodies was 9.6 mg per 1.2 ml ascites.

Immunosorbent preparation. Sepharose CL-4B (Pharmacia, Sweden) (1 ml) was activated by CNBr (Fluka, Germany) as described [16]. The concentration of CNBr in acetonitrile was 1 mg/ml. Excess CNBr was removed by sequential wash with 1 mM HCl and 0.1 M phosphate buffer, pH 7.5; after washing, a solution of antibodies in 150 mM NaCl and 10 mM phosphate buffer was added. Antibody immobilization was monitored by protein disappearance from the solution after its contact with the sorbent. After immobilization of 80% of the protein onto Sepharose, the reaction was terminated by addition ethanolamine up to 0.2 M (pH 8.0). The immunosorbent was initially washed on a filter with 1 M sodium acetate buffer (pH 2.5) and then with 0.1 M borate buffer (pH 8.0). Binding capacity of the resultant sorbent determined using partially purified leech extract was 0.15 mg DL per 1 ml of Sepharose. The sorbent was kept in 0.1 M borate buffer (pH 8.0) containing 0.02% sodium azide at 4°C.

Purification of recombinant DL by affinity chromatography. *S. frugiperda* cell extracts (0.8 ml, protein concentration 10 mg/ml) were applied onto the immunosorbent column (0.8 × 2 cm) in the total volume of 2 ml at flow rate 10 ml/h. The column was washed with 15 volumes of 10 mM phosphate buffer, pH 7.4, containing 150 mM NaCl. The antigen was eluted with 7 volumes of buffer containing 0.1 M NaCl and 0.1 M sodium acetate, pH 2.5. The antigen-containing eluate was immediately neutralized using dry sodium tetraborate (up to pH ~ 7.0). This procedure was repeated two or three times using antigen that remained unbound during the first pass through the column. Eluates were pooled and concentrated on a UM-05 membrane filter (Amicon). Protein concentration in the eluate was determined either by absorbance at 280 nm or by a colorimetric method as described in [17].

Lysozyme activity. This activity was determined as described previously [1] using *Micrococcus luteus* (ICN Biomedicals, USA) cell wall suspension (0.2 mg/ml). The activity was registered by a decrease in absorbance at 450 nm; the linear part of the kinetic curve (observed during the first min of the reaction) was used for calculation of the reaction rate. One unit (U) of the enzymatic activity corresponded to the amount of the enzyme causing a decrease in A_{450} by 0.001 per 1 min at 25°C.

Glycosidase activity. The reaction products of degradation of N-acetylglucosamine hexamer $(GlcNAc)_6$ catalyzed by wild type and recombinant DL were analyzed by mass spectrometry. The substrate, $(GlcNAc)_6$, was prepared by acylation of products of crab crust chitosan proteolysis by chitinase followed by subsequent gel filtration on Sephadex G-25 [18].

Mass-spectrum analysis. Mass spectometric characterization was carried out using a VISON 2000 time of flight (TOF) mass spectrometer (Teremobivalysis, England) with matrix assisted laser desorption/ionization (MALDI). Laser parameters were: nitrogen, 337 nm, pulse 3 nsec, maximum pulse energy 250 μJ.

Chemicals. All chemicals of the highest grades available were purchased from Sigma, Fluka, and Calbiochem (USA) and Reakhim (Russia).

RESULTS

Lysozyme activity of recombinant DL. Structural resemblance between DL and members of a new family of proteins exhibiting lysozyme function [2] suggests the existence of similarities in the antibacterial effect, which results in bacterial cell wall disruption. The appearance of such properties was observed during DL gene expression in *E. coli* cells (Table 1).

The use of expression vector pKK OmpA (*lac* promoter) including the gene encoding signal sequence of OmpA or the other vector pET-3A (T7 promoter) in combination with genes encoding signal peptides Ds3 or MFα1 was accompanied by disruption of growing cells.

Table 1. Effect of various expression vectors used for destabilase-lysozyme gene expression in *E. coli* on cell disruption

Expression vector	Promoter	Signal peptide (SP)	Cell state
PKK OmpA	Lac	OmpA (*E.coli*)	disruption
PET-3A	T7	Ds3 (*Hirudo medicinalis*)	disruption
PET-3A	T7	MFα1 (*Sacharomyces cerevisiae*)	disruption
PET-3A	T7	without SP	no disruption
PQE-30	Lac	without SP	no disruption
pET-32 LTC-System	T7	without SP	no disruption

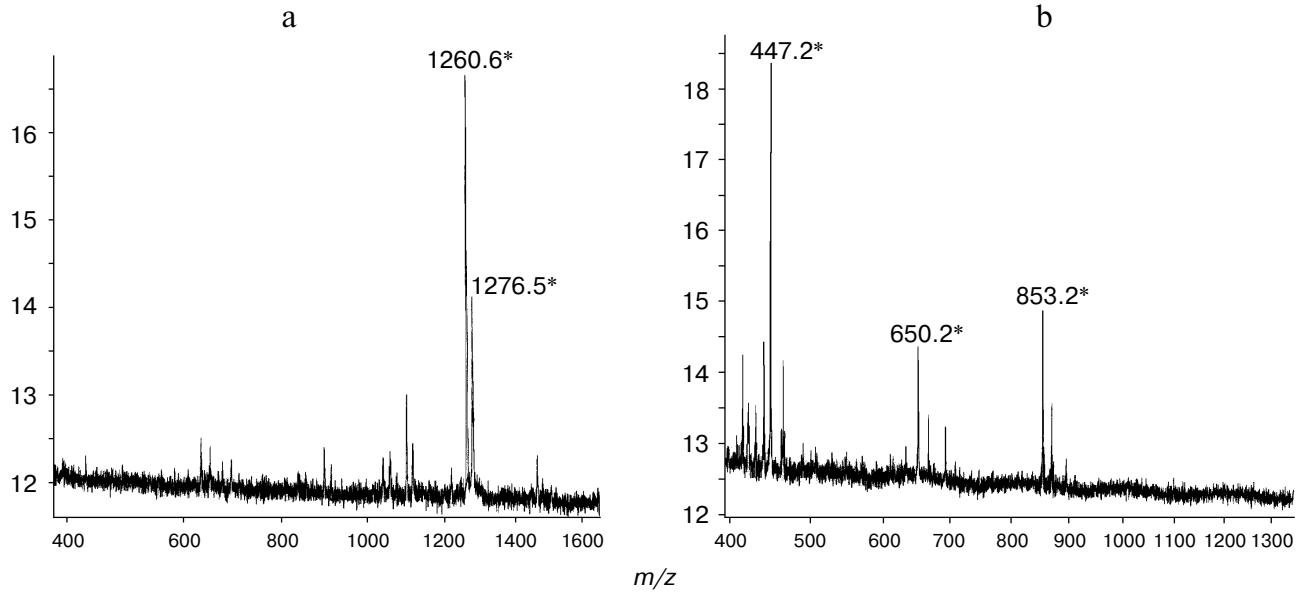
Table 2. Specific lysozyme activities of recombinant DL preparations

Source of cell extract	Purification method	Specific activity, U/mg protein
S. frugiperda cell line infected with the baculovirus vector carrying DL *Ds2* gene	immuno-adsorption	32,000
E. coli cells transformed with the expression vector pET-32 LTC-System carrying DL *Ds3* gene fused to thioredoxin gene	metal-affinity chromatography	8000

However, when *E. coli* cells were transformed with vectors pET-3A or pQE-30, lacking genes encoding signal peptides, normal cell growth was observed. The use of the expression vector pET-32 LTC-System for transformation of *E. coli* cells suggests formation of the fusion protein (including Ds3 isoform of DL and thioredoxin) in the cytoplasm. Under these conditions, normal bacterial growth was observed.

Expression of DL cDNA using the construct based on pQE-30 vector resulted in formation of significant amounts of the insoluble product as inclusion bodies. Using N-terminal sequencing this product was determined as DL. However, the fusion protein of DL with thioredoxin was synthesized in *E. coli* cells as the soluble protein. This fusion protein was partially purified from the cell extracts using metal-affinity chromatography and after the treatment with enterokinase DL was tested for lysozyme activity (Table 2). In this table, lysozyme activity of recombinant DL purified from extracts of *S. frugiperda* by immunoadsorption is introduced. Specific lysozyme activity of recombinant DL isolated from *E. coli* cell extracts was significantly lower than that of recombinant DL isolated from *S. frugiperda*. Taking into consideration these results, the recombinant protein obtained by the expression of gene *Ds2* in the baculovirus system has been chosen for subsequent investigation of DL glycosidase mechanism of action.

Identification of products of N-acetylglucosamine hexamer hydrolysis catalyzed by recombinant DL expressed in *S. frugiperda*. Figure (panel (a)) shows the mass spectrum of $(GlcNAc)_6$ after incubation at 25°C for 24 h. Under these conditions this oligomer is stable and contains corresponding intensive quasi-molecular ion $[M + Na]^+$ of 1260.6 daltons and less intensive ion [M +



Degradation of hexamer of N-acetylglucosamine by preparation of recombinant DL. Mass-spectrogram of starting substrate in 20 mM Tris-HCl buffer, pH 7.4 (a), and of products of N-acetylglucosamine hexamer degradation after incubation for 24 h at 25°C with recombinant DL expressed in *S. frugiperda* cells in 20 mM Tris-HCl buffer, pH 7.4 (b) (enzyme/substrate ratio 1 : 30)

$K]^+$ of 1276.5 daltons. Quasi-ions $[M + Na]^+$ and $[M + K]^+$ of low intensity corresponded to contamination of the oligomer with penta-, tetra-, tri-, and dimers [18]. After the incubation of this substrate with 80 pM recombinant DL in the same buffer at 25°C for 2, 6, 14, and 24 h, full hydrolysis of $(GlcNAc)_6$ was observed only after the incubation for 24 h. The mass spectrogram is characterized by disappearance of the peak corresponding to hexamer and appearance of intensive peaks typical for dimer (quasi-molecular ion $[M + Na]^+$ of 447.2 daltons), tetramer (quasi-molecular ion $[M + Na]^+$ of 853.2 daltons), and trimer of N-acetylglucosamine (quasi-molecular ion $[M + Na]^+$ of 650.2 daltons) (figure, (b)).

DISCUSSION

The enzymatic function of DL was confirmed during expression of the *DL* gene in *E. coli*. Several constructions of expression vectors allowing accumulation of this enzyme in cytoplasm (in the absence of SP) or in periplasm (in the presence of SP) of the bacterial wall were used. Data of Table 1 clearly show that normal cell growth was observed only during transformation of *E. coli* cells with the constructions lacking signal peptide genes. The use of pQE-30 vector resulted in formation of the insoluble enzyme as inclusion bodies. A similar form of lysozyme was also found during expression of canine milk lysozyme gene in *E. coli* cells [19].

The use of constructions containing SP genes of different origins (leech, *E. coli*, or yeast) resulted in cell disruption due to secretion of the active enzyme into the periplasm. This is consistent with results of Wild et al. [20] where it was demonstrated that chicken egg white lysozyme exhibited antibacterial activity with respect to *E. coli*; this effect was attributed to disruption of outer and inner bacterial envelope. In these experiments, the mechanism of exogenous action of lysozyme on *E. coli* cells depending on their contact duration was investigated. The confirmation of that result in our experiments is important. We observed the action of endogenous lysozyme synthesized *de novo* inside the cell and directed into different cell compartments. Pellegrini et al. [21] demonstrated that chicken egg lysozyme blocked of macromolecular synthesis *E. coli* by inhibition of DNA and RNA syntheses and damaged the outer membrane and inner membrane permeabilization bringing about bacterial cell death.

So, we demonstrated that the use of different constructs resulted in accumulation of DL in cytoplasm or in periplasm of the bacterial cell wall, and this caused different effects on cell growth.

The use of the expression vector pET-32 LTC-System implies formation in *E. coli* cytoplasm of the fusion protein containing Ds3 isoform of DL and thioredoxin. Under these conditions, there was normal bacterial cell growth. From these cells, Ds3 isoform of DL was isolated and purified by using metal-affinity chromatography followed by enterokinase treatment. Its lysozyme activity was 8000 U/mg, which is 4 times less than that of DL purified from *S. frugiperda* cell extracts (Table 2). So, in subsequent experiments we used DL synthesized in the baculovirus system.

It is known that lysozymes cause bacterial wall disruption by hydrolyzing α-1,4-glycoside bonds of bacterial peptidoglycans [10]. The catalytic site of these enzymes contains functionally important Glu and Asp carboxylic residues, which are necessary for catalysis [10] (e.g., human lysozyme contains Glu35 and Asp53 [22], whereas T4 lysozyme has essential Glu11 and Asp20 [23]). Nilsen et al. [2] and Bachali et al. [3] aligned sequences of proteins of c-, h-, and i-lysozyme families including DL. This gave a clear view on the positions of their catalytically active dicarboxylic amino acid residues. The position of Glu residue in all proteins including invertebrate lysozymes corresponded to Glu35 of human lysozyme. However, in contrast to the human [22] and chicken [24] lysozymes, DL lacks Asp53. DL as well as other invertebrate lysozymes has essential Asp48; this is most appropriate position for the catalytically important Asp residue, but 3D structure analysis of i-type lysozyme is needed to test this hypothesis [3].

To evaluate the catalytic mechanism of recombinant DL purified from *S. frugiperda*, we analyzed products of hydrolysis of $(GlcNAc)_6$, a well known lysozyme substrate [10]. Incubation with recombinant DL for 24 h caused complete degradation of $(GlcNAc)_6$ followed by formation of $(GlcNAc)_2$ and $(GlcNAc)_4$ (figure, (b)). Such mechanism of action for a member of the invertebrate lysozyme family is demonstrated for the first time. Appearance of N-acetylglucosamine trimer, $(GlcNAc)_3$, (quasi-molecular ion $[M + Na]^+$ of 650.2; figure, (b)) can be attributed to low DL chitinase activity (Zavalova et al., unpublished data). This activity is typical for invertebrate lysozymes [5]. So, in spite of some differences in the catalytic site structure the mechanism of glycosidase action of DL, the member of invertebrate lysozyme family, is the same as that of the c- and h-lysozyme families.

This glycosidase function of recombinant DL is very important for understanding of DL antibacterial activity as it is directed to the destruction of the peptidoglycan layer of the cell wall. But the thickness of the peptidoglycan layer and the cell wall composition of Gram-positive and Gram-negative bacteria are different. Dissolving of *M. luteus* cell walls by DL is reached by glycosidolysis of smooth thick peptidoglycan layer of this Gram-positive bacterium. In our experiments, dissolving of Gram-negative *E. coli* is provided by destruction of the thin peptidoglycan layer by DL, which is synthesized *de novo* and secreted into the periplasm. However, this mechanism is not unique. It is known that the antibacterial functions of lysozymes are independent of their catalytic function [25-27]. Therefore, the mechanism of DL antibacterial action may be additionally provided by its nonenzymatic properties, such as insertion into the lipid layer of the cytoplasmic cell membrane.

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