Protein Expression and Purification 116 (2015) 50–58

Contents lists available at ScienceDirect

Protein Expression and Purification

journal homepage: www.elsevier.com/locate/yprep

Generation of recombinant destabilase-lysozyme from medicinal leeches in three different expression systems

Valentin A. Manuvera [a,b,*], Alexey S. Kurdyumov [a], Kseniya A. Filonova [a], Vassili N. Lazarev [a,b]

[a] Scientific Research Institute of Physical-Chemical Medicine of the Federal Medical and Biological Agency, Malaya Pirogovskaya Str. 1a, Moscow 119435, Russia
[b] Moscow Institute of Physics and Technology, Institutskiy per. 9, Dolgoprudny, Moscovskaya obl 141700, Russia

ARTICLE INFO

Article history:
Received 24 June 2015
and in revised form 5 August 2015
Accepted 12 August 2015
Available online 12 August 2015

Keywords:
Destabilase-lysozyme
Isopeptidase
E. coli
P. pastoris
Expi293F
Medicinal leeches

ABSTRACT

Destabilase-lysozyme (mlDL) is an enzyme secreted by the salivary gland cells of medicinal leeches. Destabilase-lysozyme possesses lysozyme and isopeptidase activities. We generated recombinant destabilase-lysozyme isoform 2 in three expression systems, i.e., in the bacteria *Escherichia coli*, in the yeast *Pichia pastoris*, and in the human cell line Expi293F. In *E. coli*, we generated both polypeptide in inclusion bodies that was later undergone to the refolding and soluble protein that had been fused with the chaperone SlyD. The chaperone was later cleaved by a specific TEV-protease. In cultures of the yeast *P. pastoris* and the human cell line Expi293F, the soluble form of destabilase-lysozyme was accumulated in the culture media. For the generated enzymes, we determined the lysozyme, isopeptidase and fibrinolytic activities and tested their general antimicrobial effects. The comparisons of the enzymes generated in the different expression systems revealed that all of the destabilase-lysozymes obtained in the soluble forms possessed equal levels of lysozyme, isopeptidase and fibrinolytic activities that exceeded several to ten times the levels of the same activities of the destabilase-lysozyme renaturated from the inclusion bodies. A similar pattern of the differences in the levels of the general antimicrobial effects was observed for the destabilase-lysozymes generated in the soluble form and as inclusion bodies.

1. Introduction

Destabilase from medicinal leeches (mlDL – medicinal leeches destabilase-lysozyme) was the first described member of the i-type lysozymes, which are polyfunctional lysozymes of invertebrates [1]. This enzyme combines lysozyme (muramidase EC 3.2.1.17) and endo-ε-(γ-Glu)-Lys-isopeptidase (EC 3.5.1.44) activities. Later, other i-type lysozymes from invertebrates that possessed similar properties were described [2]. To date, three isoforms of medicinal leech destabilase (i.e., Ds1, Ds2, and Ds3) have been identified [3]. The isoform Ds2 consists of 115 amino-acid residues, and its homologies with the isoforms Ds1 and Ds3 are 75.7% and 66.1%, respectively. All three isoforms are cysteine-rich and presumably have seven disulphide bonds [4].

Lysozymes are a component of the innate immunity system and act as non-specific agents against bacterial pathogens. Muramidase activity involves the cleavage of o-glycoside bonds between N-acetylglucosamine and N-acetylmuramic acid in peptidoglycans and N-acetylglucosamine residues in chitin. The natural substrate of lysozymes is the bacterial cell wall. The muramidase activity of destabilase was determined when the cell wall disruption of *Micrococcus lysodeikticus* [5] was investigated. The isopeptidase activity of destabilase involves the hydrolysis of the stabilized fibrin and its proteolytic degradation product D-dimer [1,6]. Hydrolysis occurs not by common proteolytic cleavage but by disruption of the ε-(γ-Glu)-Lys isopeptide bonds between polypeptide chains [7]. In an experiment using animal models, it was found that this mechanism of action results in the slow disruption of preformed thrombi, i.e., destabilase is a thrombolytic agent. Destabilase exhibits slow thrombolytic action after intravenous injection in rats [6]. The low rates of the induction of thrombolysis induced by this enzyme correlate well with the low rates of the blood vessel wall repair. Imbalances of these rates in urgent thrombolytic therapy result in rethrombosis in more than 30% of cases. However, rethrombosis is nearly absent when hirudotherapy is applied [8].

Abbreviations: MIC, minimal inhibitory concentration; HEWL, hen egg white lysozyme; Ds, destabilase; IB, inclusion bodies; TLC, thin-layer chromatography; mlDL, medicinal leeches destabilase-lysozyme; SlyD-Dest2, fused protein SlyD-destabilase isoform 2.

* Corresponding author at: Scientific Research Institute of Physical-Chemical Medicine of the Federal Medical and Biological Agency, Malaya Pirogovskaya Str. 1a, Moscow 119435, Russia.

E-mail address: vmanuvera@yandex.ru (V.A. Manuvera).

http://dx.doi.org/10.1016/j.pep.2015.08.012
1046-5928/

It has also been found that in addition to its enzymatic activities, destabilase possesses heat-inactivated lysozyme activity with general antimicrobial effects against the archaea *Methanosarcina barkeri*, the fungi *Botrytis cinerea*, the yeast *Schizosaccharomyces pombe*, and gram-positive (*Micrococcus luteus*) and gram-negative (*Escherichia coli*) bacteria [9,10]. Antimicrobial effects unrelated to muramidase activity have also been revealed for other lysozymes [1].

To date, only the generation of destabilase by the refolding of *E. coli* inclusion bodies has been described [9]. Destabilase was isolated from the natural salivary gland secretions of medicinal leeches in the form of the destabilase complex and not as a pure enzyme. This complex contains destabilase, prostaglandin, hirudin and kallikrein inhibitors [11]. Thus, it has previously been impossible to compare the enzyme activities of the recombinant and natural proteins. Consequently, we had some doubts concerning the accuracy of the three-dimensional structure of the recombinant protein. These doubts were confirmed in the progression of our present work. Therefore, we obtained recombinant destabilase 2 in soluble form using different expression systems (i.e., bacteria, yeast, and a human cell line) to analyse the properties of the generated enzymes and to determine the optimal generation method. The production of considerable amounts of active destabilase 2 offers opportunities for the investigation of its applications as a thrombolytic agent.

2. Materials and methods

2.1. Strains and plasmids

In this work, the *E. coli* strains Top10 and BL21-Gold (DE3) (Novagen, USA), the plasmids pET (Novagen, ClIIA) and pcDNA3.1 (Life Technologies, USA), and the *P. pastoris* strains and plasmids from the PichiaPink Expression System kit (Life Technologies, USA) were used.

2.2. Construction of plasmids

DNA fragments encoding destabilase 2 were synthesized from oligonucleotides. We generated two variants of the encoding fragment that had different codon compositions that were optimized for *E. coli* and eukaryotic cells (i.e., yeast and human cell lines). Both the generation of the DNA fragments with codon compositions optimized for *E. coli* and the construction of the plasmid pET15/N-Dest2 are described in the Supplementary materials (sections 1–2). The plasmid had a region encoding mature destabilase 2 (without the signal peptide) with an N-terminal 6HisTag. The transcription was driven by the bacteriophage T7 late promoter.

The pET-SlyD plasmid [12] was used for the generation of the fused protein SlyD-Dest2 and was kindly provided by Dr. Mikhail Shneider (Shemyakin-Ovchinnikov, Institute of Bioorganic Chemistry, Moscow, Russia). The synthetic fragment that encoded destabilase with the codon composition optimized for *E. coli* was amplified using the oligonucleotides DsEc-Bam and DsEc-Sal (see Supplementary Table 1). The amplified fragments combined with the plasmids were treated with the BamHI and SalI endonucleases. The reaction products were ligated and subsequently subjected to standard *E. coli* transformation procedures, PCR-selection of the colonies, and extraction of the plasmids and their sequencing. Thus, we generated the pET-SlyD-Dest2 plasmid that contained the gene of the fusion protein SlyD-Dest2 with the N-terminal 6HisTag under the control of the bacteriophage T7 late promoter.

In a yeast expression system, the recombinant destabilase was generated using an PichiaPink Expression System kit (Life Technologies, USA). All sixteen possible variants of the plasmids encoding destabilase 2 were constructed according to the manufacturer's recommendations. The construction procedures and plasmid maps are presented in the Supplementary materials (section 3). Thus, we constructed plasmids based on pPink-HC and pPink-LC. These plasmids encoded destabilase isoform 2 fused with one of the eight signal peptides and the C-terminal 6HisTag.

The plasmid for the expression of the Ds2 gene in the human cell line Expi293F was constructed using a pcDNA3.4-TOPO TA Cloning Kit (Invitrogen, USA). The construction procedures and plasmid maps are presented in the Supplementary materials (section 4). Thus, we constructed the pcDNA3.4-Dest2 plasmid that encoded pro-destabilase 2 fused with authentic signal peptide and the C-terminal 6HisTag.

2.3. Generation of the fusion protein SlyD-Dest2 in E. coli

The *E. coli* strain BL21-Gold(DE3) was transformed with the pET-SlyD-Dest2 plasmid. A single colony was inoculated into 100 ml of LB medium containing ampicillin (150 μg/ml) and grown in a shaking incubator for 8 h. Following the incubation, culture was inoculated into bioreactor Brunswick BioFlo 110 Fermentor/Bioreactor (Fisher Scientific) filled with 2.5 l of LB2× containing ampicillin (150 μg/ml) and 10 mM lactose. Fermentation was performed over 18 h in the following conditions: temperature: 28 °C; maximum aeration; and rotational speed: 750 rpm. The culture was centrifuged at 2500*g* for 15 min. The cells were washed with PBS and subsequently centrifuged. Then, the cells were resuspended in 250 ml of distilled water. The ice-cold cells were disrupted using an ultrasonic processor (Branson 250 Sonifier, Branson, USA) at 50% of the maximum power for 10 min. The lysate was centrifuged at 15,000*g* for 15 min. Next, an 8× chromatographic buffer was added to the supernatant (final concentration: 500 mM NaCl, 20 mM NaH_2PO_4, 10 mM imidazole, pH 7.4) followed by centrifugation at 15,000*g* for 15 min. Fifty-millilitre aliquots were transferred into tubes containing 5 ml of Ni Sepharose High Performance (GE Healthcare, USA) equilibrated with chromatographic buffer. The media were resuspended and incubated at room temperature for 15 min with occasional mixing. After centrifugation at 500*g*, the sorbent was washed with the chromatographic buffer and transferred into the column. The column was washed with 200 ml of the washing buffer (500 mM NaCl, 20 mM NaH_2PO_4, 40 mM imidazole, pH 7.4) followed by the addition of the eluting buffer (500 mM NaCl, 20 mM NaH_2PO_4, 500 mM imidazole, pH 7.4). The flow rate was 1.5 ml/min. The inspection of the process and the collection of the fractions were performed via measurements of the eluate absorbance at 280 nm. The solution of the fusion protein was dialyzed against a solution of 50 mM sodium phosphate and 50 mM sodium chloride (pH 7.0) at room temperature overnight. The precipitate was separated by centrifugation at 15,000*g* over 15 min. The protein concentration was measured using the Bradford protein assay. Next, 1% (m/m) TEV-protease (Sigma, USA) and 0.5% (v/v) β-mercaptoethanol were added followed by incubation at room temperature for 16 h. After incubation, the solution was centrifuged at 15,000*g* for 15 min followed by the addition of 8 M urea stock to a final concentration of 1 M. Next, 10% acetic acid was added dropwise with continuous mixing of the solution. The decrease in the pH caused the precipitation of the chaperon SlyD. The addition of the acid was continued until intense precipitation stopped (at a pH of approximately 5.0). The precipitate was separated by centrifugation at 15,000*g* for 15 min. The supernatant was transferred into a Tricorn 10/50 column filled with CM Sepharose Fast Flow (GE Healthcare, USA). The column was preliminarily washed with starting buffer (20 mM NaH_2PO, 1 M urea, pH 5.5, adjusted with NaOH). After loading, the column was washed