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Production of the recombinant antimicrobial peptide UBI_{18-35} in *Escherichia coli*

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ABSTRACT

Radiolabeled peptides derived from ubiquicidine (UBI) are of great interest for early and highly accurate scintigraphic detection and differentiation of infection and sterile inflammation. In the present work the recombinant antimicrobial peptide UBI_{18-35} - a fragment of the human natural cationic peptide ubiquicidine - was produced in *Escherichia coli* for the first time. The insoluble expression of the peptide in fusion with ketosteroid isomerase provided high yield, about 6 mg of UBI_{18-35} per liter. We developed an approach to produce the antimicrobial peptide UBI_{18-35}, that encompasses inclusion body isolation and size exclusion chromatography. This method could be the basis for industrial biotechnological production of diagnostic system components that are in high demand.

1. Introduction

Inflammatory diseases, such as osteomyelitis, arthritis, and vasculitis as well as surgery complications, especially the ones following endoprosthetics of joints and blood vessels in the clinical setting, are a serious problem worldwide. The most serious complication of not timely detected infection is septic shock - a condition with very high mortality. Therefore, diagnostic tools that allow clinicians to differentiate infection and sterile inflammation in humans are in great demand. Timely and accurate distinguishing of infection and sterile inflammation is essential for the choice of further treatment strategy and allows to avoid severe complications. Nuclear medicine imaging procedures play an important role in assessment of inflammatory diseases [1]. Currently, several radionuclide tracer compounds have been developed for scintigraphic detection of inflammation in humans. Unfortunately, many of them are non-specific and do not offer a possibility of distinguishing between sterile and septic inflammation [1]. To solve the problem of early and highly accurate infection diagnosis, scintigraphy with radiolabeled antibiotics and antimicrobial peptides is proposed [2] [3]. Emerging antibiotic resistance and false uptake of antibiotic (e.g., ciprofloxacin) in the sterile inflammation site bring into focus the use of radiolabeled antimicrobial peptides for differential detection of infection [4]. The key advantage of antimicrobial peptides is high specificity and ability to bind only to viable bacteria and penetrate the lipid bilayer [5]. These peptides are derived from antimicrobial domains of human natural peptides, and are able to penetrate in the microbial cell membrane; thus, their accumulation in the area of sterile inflammation does not take place.

Among existing antimicrobial peptides the highest specificity to the site of infection is shown for the radiolabeled fragments of the cationic antimicrobial peptide ubiquicidine (UBI) [6]. The first antimicrobial peptide UBI was isolated from mouse macrophages; later the identical peptide was detected in epithelial cells of the human respiratory tract. The key advantages of UBI peptides are human origin, non-immunogenic, linear structure. Peptides derived from ubiquicidine (UBI) are of great interest not only for differential diagnosis of microbial inflammation, but also for monitoring of the effectiveness of antimicrobial therapy. The efficiency of radiolabeled UBI peptides is shown for patients with osteomyelitis, septic arthritis, bacillemia, mediastinitis after heart surgery, and prosthetic joint infection caused by various species of pathogenic microorganisms [7]. The most promising UBI peptides are considered to be UBI_{29-41} and UBI_{18-35}, which have demonstrated effective imaging of infection and absence of non-specific accumulation in experimental inflammation on animal models [2]. The UBI_{18-35} peptide is less studied than the UBI_{29-41} peptide, despite the fact that the efficiency of its use is not inferior, but even exceeds that of the former, for example, in the selectivity of binding to *Staphylococcus aureus* and *Candida albicans* microbial cells at the sites of inflammation

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[8]. The preferential usage of UBI_{29-41}, which has shorter amino acid chain length, is determined by simplicity of its obtaining.

Currently, peptide derivatives of UBI are produced by solid-phase synthesis. However, obtaining the necessary amount of peptides, especially hydrophobic peptides longer than 15 amino acid residues (a.a.r.), by solid-phase synthesis faces a number of challenges [9,10]. Additional negative factors are the use of toxic solvents and reagents during chemical synthesis as well as high cost of original reactants.

An advantageous alternative for scaling up the process of peptide production is recombinant DNA technology. Obviously, expression and purification of antimicrobial peptides in microbial cells remains challenging. Realization of this approach involves the need, on the one hand, to overcome a risk of proteolytic degradation of the peptide within microbial cells, and, on the other hand, to mitigate negative effects of the peptide on the producer cell. Thereby, optimization of conditions for peptide production and purification is required.

The approach to expression in the form of insoluble inclusion bodies (IBs), i.e., production of the peptide combined with the insoluble fusion protein, is very advantageous, for it allows to protect both the peptide and the host cell. In this way, an inclusion-body directing peptide - ketosteroid isomerase (KSI) - was chosen as a fusion partner for UBI_{18-35} production.

Therefore, the aim of the current study is to obtain the recombinant *E. coli* strain-producer of UBI_{18-35} and to develop the purification procedure of the peptide.

2. Materials and methods

2.1. Bacterial strains, plasmid and reagents

E. coli XL-Blue and *E. coli* Rosetta(DE3)pLysS were used for cloning and protein expression, respectively. *S. aureus* ATCC 19258 was kindly provided by Dr. Chubik, National Research Tomsk Polytechnic University.

pET-31b(+) expression vector (Novagen, Germany), bacteriological agar (Helicon, Russia), LB Broth, Miller (Amresco, USA), antibodies Anti-His6-Peroxidase (Roche, Switzerland), trypsin (Promega, USA), Ni-NTA agarose (Qiagen, Germany), DDT (Panreac AppliChem, Spain), imidazole (Panreac AppliChem, Spain), Spectra Multicolor Low Range Protein Ladder (Thermo Scientific, USA), ECL Chemiluminescent Substrate Reagent Kit (Thermo Scientific, USA), Encyclo PCR, Plasmid Miniprep Cleanup Standard kits (Evrogen, Russia), Triton X-100 (Acros Organics, USA), cyanogen bromide (CNBr) (Acros Organics, USA), trifluoroacetic acid (TFA) (Fluka, USA), and formic acid (Fluka, USA), SYBER Green I (Thermo Fisher Scientific), Cyanine5-ester-NHS (Lumiprobe, Russia) were used. Ammonium persulfate, NaCl, acrylamide, bis-acrylamide, tetramethylethylenediamine (TEMED), ethylenediaminetetraacetic acid (EDTA), Coomassie brilliant blue R-250, bromophenol blue, acetonitrile, isopropyl β-D-1-thiogalactopyranoside (IPTG), Tris, and urea were purchased from Sigma-Aldrich (USA). Oligonucleotides were synthesized by Biosynthesis (Russia). Restriction enzymes *PstNI* (*Alw*NI prototype), alkaline phosphatase, T4 DNA ligase, and protein molecular weight markers were from SibEnzyme (Russia). Plasmid Miniprep, Cleanup Standard, and Encyclo PCR kits were produced by Evrogen (Russia).

2.2. Construction of the UBI_{18-35} gene and cloning into the pET31b (+) expression vector

The UBI_{18-35} gene containing doubled UBI_{18-35} encoding sequence (*italic*) flanked with methionine codons (**bold**) and *Alw*NI recognition sites (underlined) was synthetized by Encyclo polymerase using designed oligonucleotides with the overlapping region (underlined, *italic*) (forward 5′-TCACCAGATGCTG**ATG***AAAGTGGCGAAACAGGAAAAGA AAAAGAAAAAGACCGGTCG*

*TGCGAAACGTCGT***ATG***AAAGTGGC*-3′ and reverse 5′-GACACAGCA TCTG**CAT***ACGACGT*

TTCGCACGACCGGTCTTTTTCTTTTTCTTTTCCTGTTTCGCCACTTT **CAT***ACGACGTT*-3′) in the thermal cycler 2720 (Applied Biosystem, USA), according to the following program: initial denaturation at 95 °C for 1 min, then 15 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s min, elongation at 72 °C for 3 min, and final elongation at 72 °C for 1 min.

Restriction, dephosphorylation and ligation were performed according to supplier's instructions. For pET31b(+)-2xUBI_{18-35} vector propagation, *E. coli* XL-Blue competent cells were transformed by the standard $CaCl_2$/heat-shock method. Positive clones were chosen using PCR colony screening. The recombinant pET31b(+)-2xUBI_{18-35} plasmid was sequenced by the genetic analyser ABI 3130XL (Applied Biosystems, USA) using T7 primers (T7 forward 5′-taatacgactcactataggg-3′ and T7 reverse 5′-gctagttattgctcagcgg-3′).

2.3. Expression of the KSI-2xUBI_{18-35} fusion protein

The recombinant plasmid pET31b(+)-2xUBI_{18-35} was cloned into *E. coli* Rosetta(DE3)pLysS. The *E. coli* culture was grown overnight at 37 °C in 5 mL LB-media with ampicillin (50 μg/mL) at constant shaking. The overnight cultures in the amount of 1 mL were used for inoculation of 100 mL fresh LB media supplemented with 1% (v/v) glucose in 1-L flasks at a dilution rate of 1/100, grown at 37 °C for 3 h and shaken at 200 rpm until OD_{600} reached 0.6. Induction of fusion protein expression was tested under various conditions. IPTG was added to its final concentration from 0.1 to 1 mM. The temperature varied from ambient to 37 °C, the cultures were grown for 4 h with shaking at 200 rpm. The cells were harvested by centrifugation at 5000 *g* at 4 °C for 20 min, and the pellet was frozen at – 80 °C.

2.4. Cell lysis

One gram of the thawed cell pellet was resuspended in 5 mL of washing buffer (50 mM Tris HCl; 1% Triton X-100, 100 mM NaCl, 10 mM DTT, pH 8.0 adjusted with HCl) supplemented with lysozyme at its final concentration of 1 mg/mL and sonicated (Bandelin Sonopuls, German) in the ice-water bath. The cell lysate was incubated for 30 min in ice and precipitated by centrifugation at 12,000 *g* at 4 °C for 20 min.

2.5. Purification of KSI-2xUBI_{18-35} fusion by IMAC

The target KSI-2xUBI_{18-35} fusion protein was purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA under denaturing conditions. Briefly, the precipitate after cell lysis was resuspended in 100 mL of binding buffer (40 mM Tris-HCl; 500 mM NaCl, 15 mM imidazole, 8 M urea, pH 8.0) and centrifuged at 10,000 *g* at 4 °C for 20 min. The clarified lysate was applied on the pre-equilibrated Ni-NTA agarose column with the inner diameter of 10 mm and the height of 120 mm. Afterwards, the column was washed with five column volumes (CV) of binding buffer. The fusion protein was finally eluted with 3 CV of elution buffer (40 mM Tris-HCl; 500 mM NaCl, 300 mM imidazole, 8 M urea, pH 8.0). The eluted samples were collected and the buffer was replaced with water by dialysis. Analytical KSI-2xUBI_{18-35} protein expression studies and purification of the recombinant protein were monitored both by 16% SDS–PAGE, according to Laemmli protocol [11], and Western blotting with peroxidase-conjugated anti-His6 monoclonal antibodies. The protein concentration was quantified by Bradford assay using normal rat IgG as a standard.

2.6. Purification of KSI-2xUBI_{18-35} inclusion bodies

The pellet after cell lysis was resuspended in 100 mL of IBs washing buffer (50 mM Tris, 0.5% vol. Triton X-100, 100 mM NaCl, pH 8.0). The IBs were washed with 2 M NaCl (in 50 mM Tris, pH 8.0), collected by centrifugation at 10,000 *g* at 4 °C for 10 min and resuspended in 1.5 M