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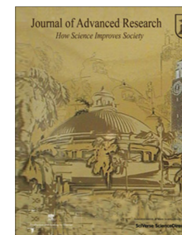

ORIGINAL ARTICLE

Functional analysis of recombinant codon-optimized bovine neutrophil β-defensin

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ABSTRACT

Defensins are cationic antimicrobial peptides with a broad range of activities against bacteria and fungi. In the present study, the entire coding sequence of codon-optimized Bovine Neutrophil β-Defensin 2 (BNBD2) was designed and placed upstream of *Trx* coding sequence into the pET-48b (+) vector. Furthermore, the codon-optimized pelB signal sequences were also added to the upstream of BNBD2 for periplasmic localization. The periplasmic sorting of recombinant β-Defensin 2 was evaluated by osmotic shock and SDS–PAGE on the released proteins. Moreover, the expression of BNBD2-Trx fusion protein was confirmed by the Western blotting technique. Next, the purification of recombinant protein was achieved by Ni^{++} affinity chromatography. BNBD2 was also separated from Trx by chemical cleavage with formic acid. Finally, both of the antibacterial and antifungal activities of the purified protein were examined. Overall, the results indicated successful periplasmic production of BNBD2 protein, which showed antifungal activity against some of *Aspergillus* species as well as the antibacterial activity, expressed as successfully suppressed growth of *Escherichia coli* and *Staphylococcus aureus*.
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Introduction

Antimicrobial peptides, (AMPs), display antimicrobial activity against gram-negative and -positive bacteria, yeasts, and fungi [1,2]. One of the largest families of AMPs is the defensins which are found in vertebrates, invertebrates, and plants. Defensins are cationic peptides with 18–45 amino acid residues. They have a molecular weight of 2–6 kDa with a framework of 6 Cysteine residues (3 disulfide bonds) [3]. Based on the relative positions of disulfide bonds, defensins are classified as α, β or θ-defensins [4]. They exhibit various antimicrobial functions. β-Defensins are able to interact with the charge-negative cellular membranes of bacteria with high affinity. Due to the changes in the membrane structure and electric potential, these peptides can be inserted into the phospholipid layers of the membranes. Hence, they cause membrane depolarization and cell lysis. Antimicrobial effects of β-defensins depend on the hydrophobicity and distribution of positive charge amino acids [5]. In domestic cattle, 13 types of

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β-defensins with conserved sequences were found in blood neutrophil granulocytes [6]. Bovine neutrophil β-defensins (BNBD1–13) are arginine-rich peptides and include 38–42 amino acids with antibacterial activity [7,8]. Therefore, studies on BNBDs could develop a new type of antibiotics in the treatment of mastitis in dairy cows. The N-terminal post-translational modification has been detected in some of BNBDs; however, the main modification of BNBD2 is the formation of disulfide linkages [6]. To the best of our knowledge, no study has been done on the production of recombinant BNBD2 so far. Therefore, BNBD2 can also be a good candidate for the production of recombinant antimicrobial peptides in *Escherichia coli*.

Cytoplasmic production of recombinant proteins in *E. coli* is accompanied by cellular disruption and contamination of the target proteins with other cytoplasmic components [9]. However, targeting recombinant protein in the bacterial periplasmic space has the advantage of having less onerous purification and proteolysis, authentic N-terminus and proper folding, and suitable biological activity [10,11]. In addition, the periplasm has a more oxidizing environment than the cytoplasm, and favors the formation of the disulfide bonds, which is important for the activity of respective proteins [12]. Typically, a signal peptide such as pelB, derived from pectate lyase B of *Erwinia carotovora*, is introduced at the N-terminus of recombinant proteins to help drive them into the periplasmic space [13,14]. Moreover, codon optimization of recombinant proteins or removal of rare codons can increase the efficiency of protein production in *E. coli* [15,16]. In this study, the production of a codon-optimized pelB-BNBD2 with an appropriate direction into the periplasmic space was found. Notably, this protein exhibited antibacterial as well as antifungal activities.

Material and methods

Construction of the recombinant vector

E. coli, BL21 (DE3) F-ompT hsdsB (r^-Bm^-) gal dcm, and pET48b (+) were used as the host and vector, respectively. Coding sequences (CDS) of the bovine *BNBD2* gene (Accession number, P46160), were obtained via University of California Santa Cruz (UCSC) data bank. Specific sequences, encoding PelB signal peptide and cleavage site of formic acid (Asp-Pro), were placed at the 5′ and 3′-terminal ends of *BNBD2* CDS, respectively (Fig. 1). Two web servers, optimizer (http://www.genoms.uvr.es), and *E. coli* rare codon analyzer 2 (http://www.faculty.ucr.edu), were performed on *BNBD2* CDS. Meanwhile, Codon Adaptation Index (CAI) was used to evaluate the designed sequences to the Gene script web site (http://www.gene script.com) (Table 1). The optimized sequences (250 bp) of *pelB-BNBD2* were ordered from the NedayeFan Company (Tehran, Iran). Synthesized DNA fragments which had been inserted into the pGH vector were digested with *Xba*I/*Nde*I. Subsequently, *Xba*I-*pelB-BNBD2*-*Nde*I was inserted into the same place in pET48b (+). The ligation was achieved using T4 ligase enzyme (TaKaRa, Japan). And finally, the DNA manipulations were carried out according to the standard protocols [17].

Protein expression

A single colony of recombinant bacteria transformed with pET48b (+)-*BNBD2* was cultured overnight in 5 mL of LB broth medium which contained 30 μg Kanamycin/mL at 37 °C. The overnight culture was transferred to 50 mL of LB broth medium. Upon reaching the optical density (OD_{600}) of 0.4–0.6, the protein synthesis was induced with 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). The optical density was measured by the spectrophotometer (Shimadzu, Japan). Afterward, the temperature of the incubator was set to 30 °C. After another 2–6 h incubation period, the cell pellets were harvested and re-suspended in TES buffer (Tris 20 mM-EDTA 1 mM, pH: 8, 20% sucrose). The cells were incubated on ice for 30 min, centrifuged and resuspended in 50 mM of ice-cold $MgSO_4$, and incubated again on ice for another 30 min. The periplasmic proteins were collected via centrifugation at 12,000*g* (4 °C) [18]. Further, the supernatants were concentrated by Trichloroacetic acid (TCA). Later, equal amounts of periplasmic protein and the pellet as cytoplasmic protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). In addition, the total protein was obtained by the sonication method with TE buffer (10 mM Tris, 1 mM EDTA, pH: 7.8) for 30 s and at 50/60*Hz*.

Western Blotting Analysis

A solubilized protein fraction of each sample was subjected to SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF; Bio-Rad, USA) membrane which had been activated by soaking it in methanol for 15 s. After blocking the

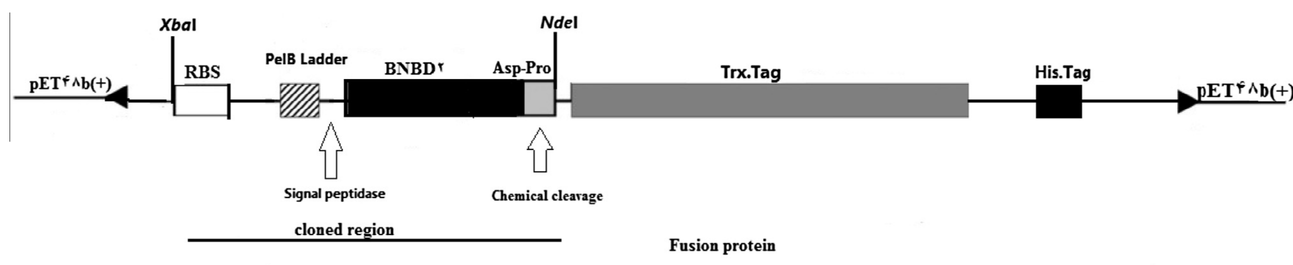

Fig. 1 Schematic representation for a part of the expression vector pET48b (+) - BNBD2. A codon optimized DNA fragment encoding pelB-*BNBD2* was inserted upstream of Thioredoxin (Trx) encoding sequences in pET48b (+) vector. BNBD2 was expressed as a fusion protein with Trx. As depicted, (Asp-Pro) was designed to facilitate chemical cleavage for release of recombinant BNBD2 from the fusion protein.

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