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Efficient production of a soluble fusion protein containing human beta-defensin-2 in *E. coli* cell-free system

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Abstract

Human beta-defensin-2 (hBD2), a small cationic peptide, exhibits a broad range of antimicrobial activity and does not cause microbial resistance. In order to produce hBD2 efficiently, an *Escherichia coli* cell-free biosynthesis system has been developed as an alternative method. A specific plasmid pIVEX2.4c-*trx*A-shBD2 was constructed for the cell-free expression of fusion protein (hBD2 linked with His-Tag and Trx-Tag). This allowed enhancement of protein stability and facilitated downstream purification process. Significant amount of target fusion protein was synthesized in the batch-mode bioreactor by optimizing the reaction conditions. About five-fold improvement of productivity (ca. 2.0 mg/ml soluble fusion protein) could be achieved by using a continuous exchange cell-free (CECF) system compared to batch system. One-step affinity chromatographic process was developed to recover high purity fusion protein (95.2%) with overall recovery ratio of about 50%. The fusion protein was cleaved by cyanogens bromide (CNBr), and the mature hBD2 had demonstrated strong inhibition on the growth of *E. coli* D31 at low concentration.

Keywords: Human beta-defensin-2; Cell-free system; Soluble expression; Purification

1. Introduction

Human beta-defensin-2 (hBD2), first discovered in human skin, is a cysteine-rich cationic peptide with 41 amino acids (Harder et al., 1997). The broad epithelial distribution of hBD2 throughout many organs suggests that it plays an important systemic role in innate immunity. Its synergistic effects on antimicrobial activity is observed and compared with other antimicrobial molecules, such as lysozyme and lactoferrin (Bals et al., 1998). Unlike alfa-defensins and human beta-defensin-1, hBD2 is the first human defensin that is synthesized upon inflammatory stimulation, and it appears to be mainly localized in the respiratory

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tract epithelia and skin (Schröder and Harder, 1999). Moreover, it seems difficult for microorganisms to acquire resistance, which makes hBD2 very attractive for therapeutic application as antibiotics (Harder et al., 1997). Thus, it is desirable to produce hBD2 in large amount.

For the production of recombinant proteins, various expression systems, such as prokaryotes cells, yeast cells or mammalian cells have been established. To achieve high-level expression of target protein, *Escherichia coli* as host cell is the most commonly employed expression system because of its distinct genetic background and abundant available plasmids, whereas difficulties are encountered in the expression of genes encoding for antimicrobial polypeptides because of their cytotoxicity and sensitivity to proteolytic degradation. These obstacles could be partly solved when antibacterial polypeptides are synthesized in the form of insoluble fusion proteins forming inclusion bodies in the cell (Haught et al., 1998), while in many cases the production of polypeptides in a soluble form is more appreciated (Buchner et al., 1992). The antibiotic mechanism of human defensins generally involves membranolytic disruption, permeability change, or pore formation against bacteria, fungi, and viruses (Epand and Vogel, 1999). Even without cell wall and plasma membrane, the cell-free protein synthesis system is of full ability in gene transcription and protein translation, therefore, is favorable to synthesize proteins with antibiotic property (Martemyanov et al., 1996). The *E. coli* combined transcription/translation cell-free system is popularly used because of its capability in the direct synthesis of an exogenous gene. The system can be operated in either batch or continuous mode. The batch-mode operation is relatively simple and convenient, however, the efficiency of protein synthesis is quite low (Pratt, 1984). In 1996, Kim and Choi reported a continuous exchange cell-free (CECF) system, in which, with continuous supply of substrates and continuous removal of low-molecular-weight products through a dialysis membrane, proteins can be synthesized continuously. The results indicated that the protein synthesis efficiency in the CECF system is much higher than that in the batch-mode. After then, some commercial *E. coli* cell-free systems were developed, such as rapid translation system (RTS) 100 *E. coli* HY kit for batch operation mode and RTS 500 proteomaster *E. coli* HY kit for continuous mode.

In this laboratory, the hBD2 gene was cloned and the fusion protein was expressed in *E. coli* successfully (Fang et al., 2002), however, the expression level was very low. In this study, the *E. coli* transcription/translation cell-free system was used to evaluate its applicability in hBD2 production. Both batch and continuous operation modes will be compared, and the antibiotic activity of hBD2 will be examined after separation and subsequent cleavage of fusion protein.

2. Materials and methods

2.1. Strains and plasmids

E. coli DH5α was used as host strain for cloning and for the preparation of template plasmids. *E. coli* D31 was applied to test the antibacterial activity of hBD2. The restriction endonucleases, T4 DNA ligase, and Taq DNA polymerase were purchased from Takara Biotech (Dalian) Co. Ltd. (Dalian, China). The plasmid, pGEM-shBD2, was constructed in our laboratory (Peng et al., 2004), pET-32a(+) was from Novagen (Madison, USA), and pIVEX2.4c (carrying T7 promoter and His-Tag sequence) was kindly provided by Professor J. Cao (Cancer Institute of Zhejiang University, Hangzhou, China).

2.2. Construction of expression vector

The original hBD2 gene was modified to obtain shBD2 gene in favor of *E. coli* as host cell. The shBD2 coding sequence flanked with *Nco*I and *Xma*I sites was PCR amplified from the plasmid pGEM-shBD2. The sense primer for shBD2 gene was 5′-CATGCC*ATG*GGCGGCATTGGTGATCCAGTC-3′, where *ATG* encoded methionine residue, would later be cleaved by cyanogens bromide (CNBr) to release the mature hBD2. The *trx*A gene flanked with *Not*I and *Nco*I sites was PCR amplified from the plasmid pET-32a(+). The amplified shBD2 coding sequence was sub-cloned into pIVEX2.4c plasmid after digestion with *Nco*I and *Xma*I to construct pIVEX2.4c-shBD2. The amplified *trx*A fragment was inserted between the same sites of *Not*I and *Nco*I into pIVEX2.4c-shBD2 to obtain the expression vector pIVEX2.4c-*trx*A-shBD2. The detailed procedures were shown in Fig. 1. The expression vectors were