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High-Level Expression of Soluble Human β-Defensin-2 Fused With Green Fluorescent Protein in *Escherichia coli* Cell-Free System

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Abstract

Human β-defensin-2 (hBD2), a small cationic peptide, exhibits a broad range of antimicrobial activity and does not acquire any microbial resistance. To produce this uneasily detectable, degradable, and toxic polypeptide efficiently, an alternative approach based on the *Escherichia coli* cell-free biosynthesis system was proposed. The approach implies that a polypeptide of interest is synthesized as a fusion protein linked to a green fluorescent protein (GFP) through a cleavable spacer. With batch-mode operation, a significant amount of hBD2 fused with GFP (0.25 mg/mL) can be expressed in this cell-free system. The productivity of the fusion protein can be improved up to 1.2 mg/mL by employing a continuous-exchange cell-free system. Furthermore, the GFP moiety provides directly visible and quantitative monitoring of the polypeptide synthesis, and the product is soluble and stable. This work will be helpful in allowing the rapid and visible expression of other similar defensins using an in vitro cell-free system.

Index Entries: Antimicrobial peptide; human β-defensin-2; cell-free system; green fluorescent protein; fusion protein.

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Introduction

Human β-defensin-2 (hBD2), first discovered in human skin, is a cysteine-rich cationic peptide with 41 amino acids *(1)*. The broad epithelial distribution of hBD2 throughout many organs suggests that it plays an important systemic role in innate immunity, and its synergistic effects on antimicrobial activity have been observed and compared with those of other antimicrobial molecules, such as lysozyme and lactoferrin *(2)*. Unlike α-defensins and hBD1, hBD2 is the first human defensin that is synthesized on inflammatory stimulation, and it appears to be mainly localized in the respiratory tract epithelia and skin *(3)*. Moreover, it seems difficult for microorganisms to acquire resistance to it, which makes hBD2 very attractive for therapeutic use as an antibiotic *(1)*.

During the past decade, biosynthetic production of polypeptides has attracted much attention. Compared with chemical synthesis, it has several advantages, such as low cost, high specific activities of the products, and the possibilities of performing manipulations and alterations at the gene level. At the same time, many difficulties are encountered in the in vivo expression of genes encoding for antimicrobial polypeptides, especially their cytotoxicity and the sensitivity to proteolytic degradation *(4)*. The fusion strategy has been utilized in the production of small antimicrobial cationic peptides in *Escherichia coli* to alleviate these problems, but the fusion proteins always tend to form inclusion bodies, which lead to the inactivation of the expressed proteins *(5)*. The alternative approach based on a cell-free biosynthesis system for the synthesis of toxic and vulnerable polypeptides is devoid of the aforementioned difficulties. Without cell wall and plasma membrane, the cell-free protein synthesis system is of full ability in gene transcription and protein translation and, therefore, is favorable for the synthesis of proteins with antibiotic properties in soluble and functionally active form *(6)*. The *E. coli* combined transcription/translation cell-free system is popularly used because of its capability in the direct synthesis of protein from an exogenous gene. The system can be operated in batch or continuous mode. Batch-mode operation is relatively simple and convenient; however, the efficiency of protein synthesis is quite low *(6)*. Recently, one continuous-exchange cell-free (CECF) system was developed that adopted a "semicontinuous operation" using a simple dialysis membrane to supply substrates and to remove low molecular weight products continuously *(7)*. Various functionally active proteins, such as dihydrofolate reductase, chloramphenicol acetyltransferase, luciferase, green fluorescent protein (GFP), Ras, and Nef, were reported to be successfully produced in this CECF system *(8,9)*.

Although the expression of hBD2 in *E. coli* as a fusion protein has been reported by our laboratory, the expression level was relatively low *(10,11)*. In our previous study, one new strategy of the *E. coli* cell-free system was developed as an alternative method to express hBD2 as a fusion protein with thioredoxin A (TrxA) *(12)*. To visualize and study the kinetics of the

biosynthesis of hBD2 more effectively, GFP was tried here to replace TrxA as a fusion partner for efficient expression of hBD2 in the *E. coli* cell-free system. In addition, further efforts were made to improve the productivity and solubility of the hBD2 fusion protein by using the CECF system.

Materials and Methods

Strains and Plasmids

E. coli DH5α was used as the host strain for cloning and for the preparation of template plasmids. The restriction endonucleases, T4 DNA ligase, and *Taq* DNA polymerase were purchased from Takara Biotech (Dalian, China). The plasmids pIVEX2.4c-shBD2 and pIVEX2.4c-GFP were constructed in our laboratory.

Construction of Expression Vector

The GFP coding sequence flanked with *Not*I, *Nco*I, and enterokinase recognition sites was amplified by polymerase chain reaction (PCR) from the plasmid pIVEX2.4c-GFP. The antisense primer for GFP gene was CATGCCATGGC*CTTGTCATCGTCATC*CTTGTACAGCTCGTCCATGCCGAGAG, in which *CTTGTCATCGTCATC*, the encoded enterokinase recognition site, would later be cleaved by enterokinase to release the mature hBD2. The conditions of PCR were as follow: initial denaturing at 94°C for 5 min followed by 30 cycles of 1 min at 94°C, 30 s at 65°C, and 30 s at 72°C. After digestion with *Not*I and *Nco*I, the amplified GFP coding sequence was subcloned into pIVEX2.4c-shBD2 plasmid to construct pIVEX2.4c-GFP-shBD2, in which shBD2 gene was an artificial synthesized gene with favorable codons of *E. coli*, designed by our laboratory. The expression vector was then confirmed by restriction endonuclease digestion and sequencing.

In Vitro Synthesis of GFP-hBD2 in E. coli *Cell-Free Systems*

The rapid translation system (RTS) ProteoMaster instrument, the RTS 100 *E. coli* HY kit, and the RTS 500 ProteoMaster *E. coli* HY kit were from Roche (Grenzacherstrasse, Switzerland). The plasmid midi kit was purchased from Qiagen GmbH (Hilden, Germany).

A batch reactor and a CECF system harboring a semipermeable membrane (Fig. 1) were used for cell-free protein expression. The CECF system contained a 1-mL reaction compartment and a 10-mL feeding chamber. In the CECF system, the supply/waste compartment acts as a reservoir to supply precursors and energy sources continuously to the transcription/translation compartment and to remove low molecular weight byproducts via a dialysis membrane.

The pIVEX2.4c-GFP-shBD2 was extracted from the cell culture using a Qiagen plasmid midi kit, and then it was expressed in batch and CECF format by using an RTS 100 *E. coli* HY kit and an RTS 500 ProteoMaster

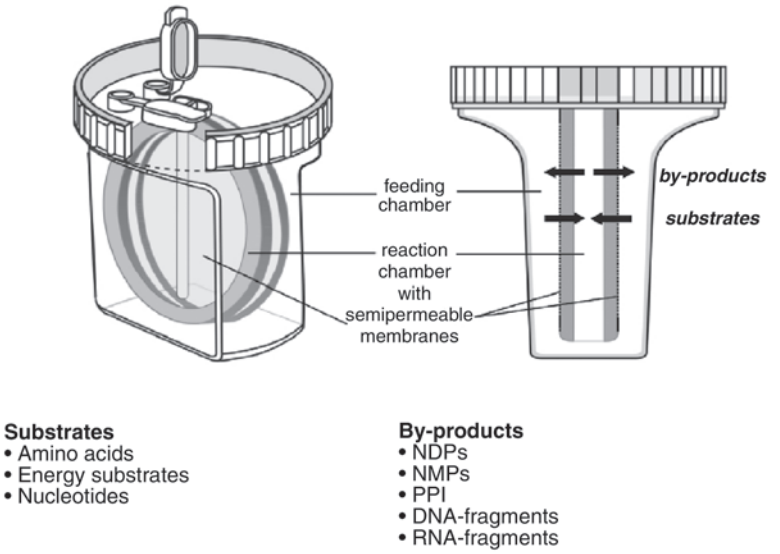

Fig. 1. Schematic diagram of CECF cell-free expression system. NDPs, nucleoside diphosphates; NMPs, nucleoside monophosphates; PPI, pyrophosphate.

E. coli HY kit, respectively. The *E. coli* extract (in RTS 100 and RTS 500) and its feeding solution (in RTS 500) were used as provided by the supplier. Briefly, and as indicated by the manufacturer, the extract contained the following: a buffer that maintains pH between 7.4 and 8.0; crude extract (ribosomes [70s], tRNA, translation initiation, elongation, and termination factors); T7 RNA polymerase; the 20 amino acids between 20 and 100 µ*M*; the four ribonucleotides adenosine triphosphate (ATP), UTP, GTP, and CTP between 0.2 and 2 m*M*; 8–15 m*M* magnesium salt; 100–250 m*M* potassium salt; an ATP-regenerating system; and sulfhydryl compounds (2-mercaptoethanol or dithiothreitol). The feeding solution contained the same components except the crude extract, tRNA, the kinase for the ATP-regenerating system, and the RNA polymerase. In the CECF system, 15 µg/mL of template plasmid was added and the reaction was carried out at 30°C with a stirring speed of 990 rpm for 24 h.

Analytical Methods

The GFP-hBD2 fusion protein was quantified by recording its fluorescence emission spectrum of 492 nm with an excitation wavelength of 535 nm on an HTS 7000 plus Bio Assay Reader (Perkin-Elmer).

Electrophoretic analysis of the synthesized products was performed in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the gel was stained with Coomassie brilliant blue for visualization of the bands.

Western blotting was carried out as follows: Proteins were transferred to a nitrocellulose transfer membrane (Schleicher & Schuell GmbH, Germany) by electroblotting (100 V, 2 h). The membrane was blocked with

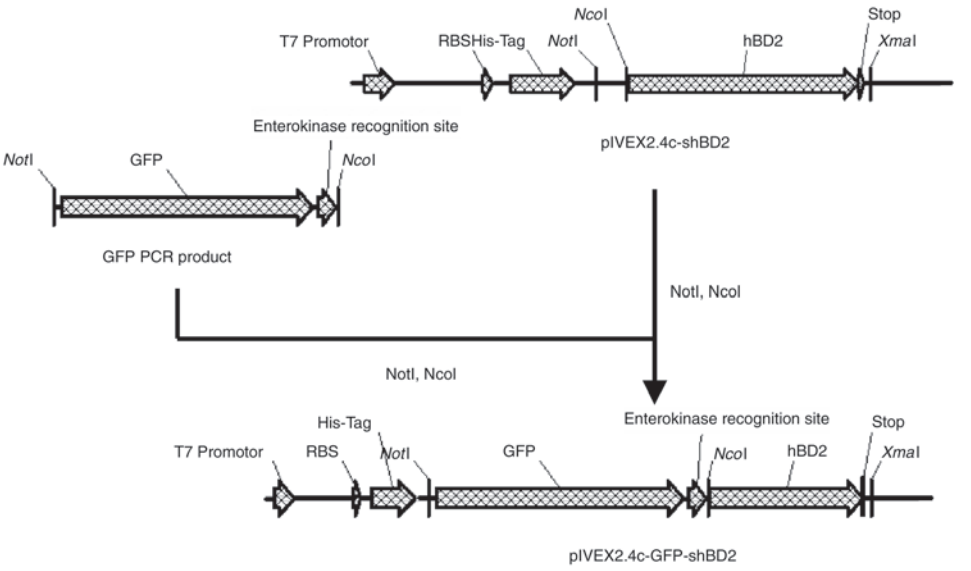

Fig. 2. Construction of plasmid pIVEX2.4c-GFP-shBD2.

3% bovine serum albumin in TBS (10 m*M* Tris-Cl, pH 7.5; 150 m*M* NaCl) and probed with mouse Penta·His Antibody (Qiagen GmbH, Germany) followed by horseradish peroxidase-conjugated goat antimouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The protein bands were visualized with diaminobenzidine.

The total protein concentration was determined with a Bradford protein assay, and the percentage of target protein in total protein was quantified using Quantity One software (Bio-Rad, Hercules, CA).

Results and Discussion

Construction of Expression Vector

The GFP DNA fragment was successfully PCR amplified from pIVEX2.4c-GFP. Agarose gel electrophoresis (1.5%) of the PCR product showed that the length of the gene was 758 bp, which was in accordance with the calculated length. The GFP gene was confirmed for correct orientation using digestion with restriction endonucleases and sequencing (data not shown). Figure 2 schematically presents the genetic constructs used in this work. With insertion of the GFP coding sequence, the expression product GFP-hBD2 can be easily detected and measured by the GFP fluorescence.

In Vitro Expression of GFP-hBD2 in Batch-Mode Cell-Free System

GFP-hBD2 was produced with the addition of pIVEX2.4c-GFP-shBD2 into batch-mode cell-free systems. Figure 3A shows the time course of accumulation of the fusion protein GFP-hBD2. Figure 3B shows the electro-

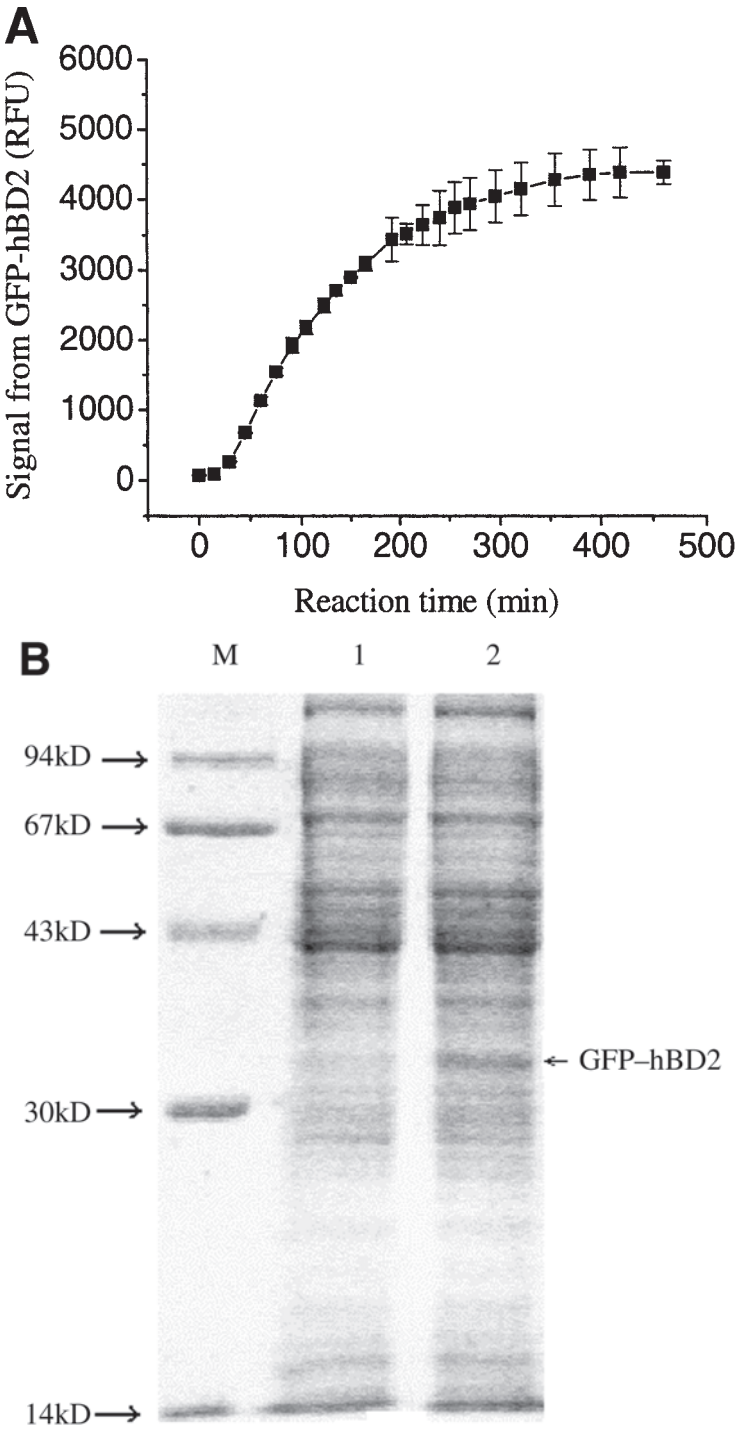

Fig. 3. Cell-free biosynthesis of GFP-hBD2 in batch-mode bioreactor. **(A)** Time course of accumulated fluorescence of GFP-hBD2. Error bars represent the standard deviation (SD) from two separate experiments. **(B)** SDS-PAGE analysis: lane M, protein marker; lane 1, negative control; lane 2, pIVEX2.4c-GFP-shBD2 added.

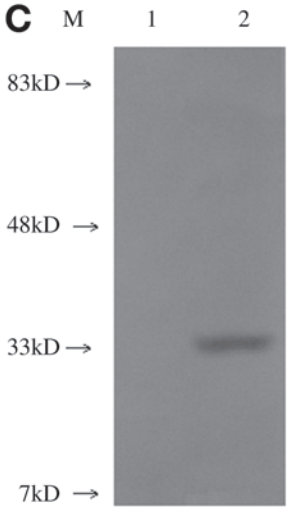

Fig. 3. **(C)** Western blotting analysis: M, protein prestained marker; lane 1, negative control; lane 2, pIVEX2.4c-GFP-shBD2 added.

phoretic analysis of the fluorescent products synthesized after 8 h of incubation. These results showed that the fluorescent fusion protein was accumulated with time, and the target band appeared approximately at a 34.2-kDa position after staining with Coomassie brilliant blue G-250, which was in accordance with the theoretical size. These results were further confirmed by Western blotting assay (Fig. 3C). However, with another plasmid, pIVEX2.4c-shBD2, hBD2 without the fusion part was not detected in this cell-free system under the same reaction conditions (data not shown). This indicated that the expression of this small peptide was stabilized with the GFP-tag in this cell-free system. The solubility of the expressed protein is a major concern because it may obviously influence the bioactivity and downstream purification *(13)*. In this work, in contrast to in vivo expression of hBD2 *(10,11)*, almost all of the hBD2 fusion protein (99.8%) was expressed in soluble form. A quantity of approx 0.25 mg/mL of soluble fusion protein was synthesized. Subsequently, only soluble hBD2 fusion protein was detected and quantified. Apparently, the hBD2 fusion with GFP provided more advantages, such as direct visualization as well as rapid and precise quantitative determination of target protein.

Enhanced Expression of GFP-hBD2 in CECF System

To make the coupled transcription/translation reaction more efficient, a CECF system harboring a semipermeable membrane was used as the cell-free protein synthesis system. Protein expression was carried out according to the procedure recommended by the supplier. Samples were taken at 1.5, 3, 6, 9, 12, 16.5, 21, and 24 h, respectively. The synthesized GFP-hBD2 was detected with fluorescent (Fig. 4A) and electrophoretic analysis (Fig. 4B).

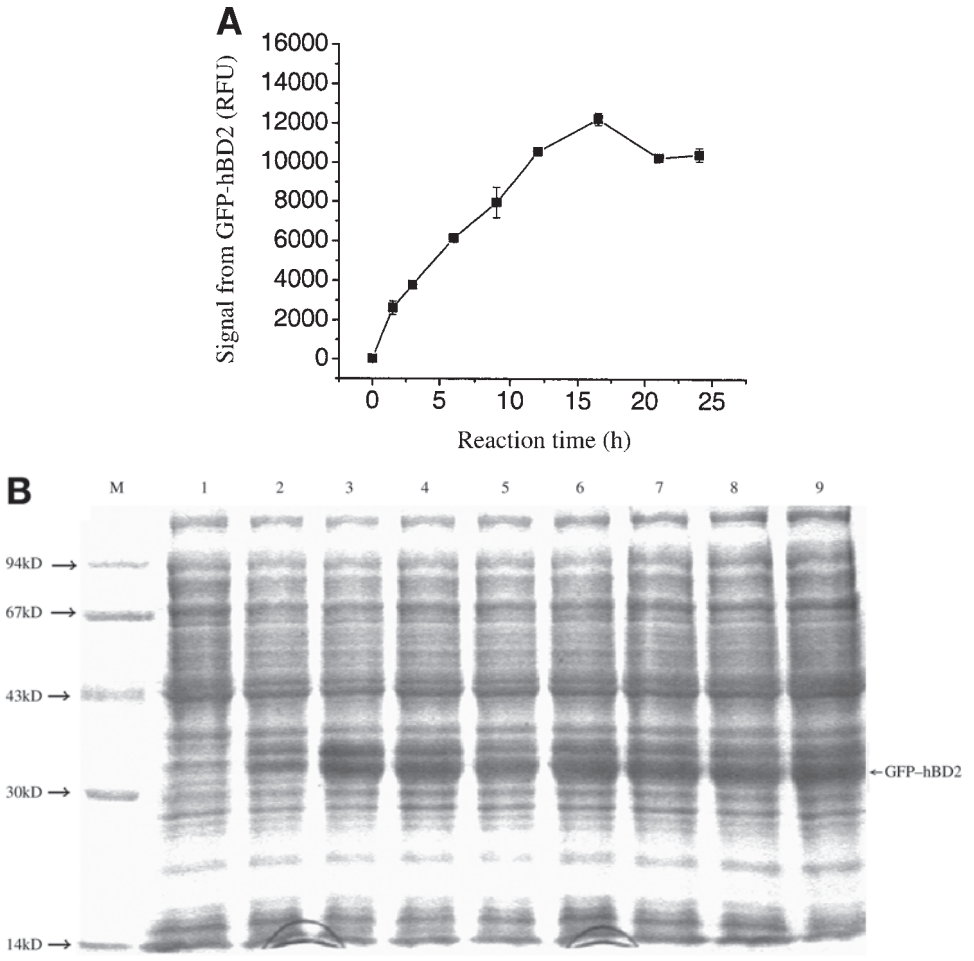

Fig. 4. Cell-free biosynthesis of GFP-hBD2 in CECF system. **(A)** Time course of accumulated fluorescence of GFP-hBD2. Error bars represent the SD from two separate experiments. **(B)** SDS-PAGE analysis: M, protein marker; lanes 1–9, GFP-hBD2 synthesis after 0, 1.5, 3, 6, 9, 12, 16.5, 21, and 24 h of incubation, respectively.

The results indicated that the fluorescent strength increased gradually. The highest expression of GFP-hBD2 fusion protein reached 1.2 mg/mL in the CECF system, which was five times higher than that synthesized in a batch-mode system. The highest fusion protein production was observed after reaction for 17 h and then dropped slightly. In the CECF system, substrates were supplied and the low molecular weight products were removed via a dialysis membrane continuously. The results explain that the continuous supply of precursors as well as energy sources for protein synthesis is extremely important for high expression.

For protein or peptide with antibacterial activity, such as hBD2, if bacteria are used as the expression host, the product may demolish the cell wall and thus cause the death of the host cell; therefore, productivity

will be very low. The advantages of using a cell-free system to express target protein are that no cell wall exists and that the system utilizes only the enzymes related to transcription/translation and energy regeneration. Because the activities of these enzymes are not affected by the accumulation of target protein, the productivity of target protein in the cell-free system will be much higher than that in a living-cell system *(6)*. Although in our laboratory many efforts have been made to express human defensins in *E. coli (11,14,15)*, the expression levels were still limited. However, in our previous work, high soluble expression of hBD2 fused with TrxA (1.0 to 2.0 mg/mL) was achieved in the cell-free system. The present work showed that the fusion of hBD2 with GFP would bring about comparable expression of target fusion protein (approx 1.2 mg/mL). Moreover, all the expressed GFP-hBD2 was almost soluble and could be detected rapidly and precisely. Therefore, this work may be very helpful in allowing the rapid and visible expression of other similar defensins using an in vitro cell free system.

Conclusion

An expression plasmid was constructed and added to an *E. coli* cell-free transcription/translation system for expressing soluble fusion protein of hBD2 to prevent the harmful effect of in vivo-expressed target protein on cell growth. With batch-operation modes, soluble fusion protein was successfully expressed, and the productivity could be further improved up to fivefold using a more efficient CECF system. This in vitro GFP fusion expression strategy provides not only stable and soluble expression of hBD2, but also accurate and visualized detection of the product.

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