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High-Level Expression and Novel Antifungal Activity of Mouse Beta Defensin-1 Mature Peptide in *Escherichia coli*

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Abstract Mouse beta defensin-1 (mBD-1) is a cationic 37-amino acid antimicrobial peptide with three conserved cysterine disulfied bonds. It exhibits a broad antimicrobial spectrum, but mBD-1 against *Candida albicans* (*C. albicans*) and *Cryptococcus neoformans* (*C. neoformans*) is poorly understood. This study describes the *mBD-1* gene, the heterologous fusion expression of the peptide in *Escherichia coli*, and the bioactive assay of released mature mBD-1. By constructing the expression plasmid (pET32a-mBD1), high yields of soluble mBD-1 fusion protein (0.67 g/L) could be obtained in *E. coli* and cleaved by enterokinase. The digested product was further purified and desalted with the final amount of pure mature mBD-1 being 0.14 g/L. Classical fungi growth inhibition assay showed clear antifungal activity against *C. albicans* and *C. neoformans* with IC_{50} of 5 and 2 μM, respectively. The results show that the mBD-1 control fungal colonization through hyphal induction, direct fungicidal activity, and the activity is suppressed by increasing NaCl concentration. Successful expression of the mBD-1 peptide in *E. coli* offers a basis for further studying its antifungal mechanisms and may provide significance in developing this peptide to an antifungal drug.

Keywords Mouse beta defensin-1 · Soluble expression · Fusion protein · Purification · Antifungal activity · *Candida albicans* · *Cryptococcus neoformans*

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Introduction

To maintain sterility, the mucosal surface of the airways must have an efficient innate defense system to combat the constant threat of infection from inhaled microorganisms. Beta-defensins are cationic, cysteine-rich antimicrobial peptides isolated from both myeloid and epithelial tissues [1]. mouse beta defensin-1 (*mBD-1*), whose protein sequence had 57% similarity to human ß-defensin 1 (hBD-1), was expressed in the airway epithelia and whose synthetic peptide was shown to have salt-sensitive antimicrobial activity [2]. Loss of mBD-1 resulted in delayed clearance of *Haemophilus influenzae* from lung; these data show directly that antimicrobial peptides of vertebrates provide an initial block to bacteria at epithelial surfaces [3]. However, the effects of mBD-1 on pathogenic fungi of humans, such as *C. albicans* and *C. neoformans*, have been poorly studied. In the present study, we aimed to establish an efficient method for the production of recombinant mature mBD-1 using His-fusion system in *E. coli.* The recombinant mature mBD-1 showed strong antifungal activity against *C. albicans* and *C. neoformans*. The further study is to investigate the mechanisms of toxic effects of mature mBD-1 peptide with antifungal activity.

Materials and Methods

Strains and Media

E. coli DH5α (*rec*A$^-$ *end*A$^-$) was used as host strain for cloning. *E. coli* BL21 (DE3) was used as host strain for expression. *C. albicans* (ATCC 10231) and *C. neoformans* (clinical isolate) were used for antifungal assay. 2×YT medium (*w*/*v*) containing 1.6% tryptone, 1.0% yeast extract, and 0.5% NaCl was used to achieve high expression level. YPG medium (*w*/*v*) containing 1% yeast extract, 1% peptone, and 2% glucose was used for antifungal assay.

Enzymes, Vector, and Antibodies

All the restriction enzymes, T4 DNA ligase, and Taq DNA polymerase were purchased from Takara Biotech Co. Ltd (Dalian, China). The recombinant enterokinase/His (rEK/His) was purchased from Guangdong Zhongda Marine Biotech (Guangdong, China). pET-32a (+) Vector (Novagen, Madison, WI, USA) was used to construct expression vector. The rabbit anti-mBD1 antibody and horseradish peroxidase conjugated goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Amplification of *mBD-1* DNA Fragment

The *mBD-1* cDNA sequence was obtained from the GenBank (accession no. *AA065510*). Total RNA was isolated from a variety of tissues from BALB/c mouse by using Trizol (Gibico BRL) as described by the manufacturer. cDNA synthesis was accomplished using a first strand cDNA synthesis kit (Fermentas Inc.), and the resultant cDNAs were used as a template in a polymerase chain reaction (PCR) with forward primer (mBD1-F) and reverse primer (mBD1-R) and the following conditions: denaturation at 94 °C for 4 min; 30 cycles of 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 40 s. ß-actin was used as a positive control. Double distilled water was used as negative control to prevent contamination. The sequences of primers for mBD-1: mBD1-F, 5′-GCGGGTACCGACGACGACGACAAG GATCAATACAAATGCCTTC-3′ [at the 5′ end containing a restriction site for *Kpn* I

(underlined) and codons of enterokinase (EK) cleavage site (dotted)]; mBD1-R, 5′-GCG CTCGAG**TCA**GCTCTTACAACAGTTGGGCT-3′ (at the 5′ end containing a restriction site for *Xho* I (underlined) and stop codon (boldfaced)), were synthesized by Invitrogen (Shanghai, China).

Construction of Recombinant Expression Vector

The PCR product and vector pET-32a(+) were digested both by *Kpn* I and *Xho* I, respectively. The mature sequences *mBD-1* fragment and the large fragment of pET-32a(+) were recovered. Then, the two fragments were ligated to construct the expression vector pET32-mBD1.

Expression, Purification, and Preparation of Mature mBD-1

A fresh clone of *E. coli*, harboring the pET32-mBD1 vector, grew in Luria–Bertani broth medium containing 100 μg/mL ampicillin at 37 °C overnight with shaking at 200 rpm. Then, the cell suspension was added to 2×YT medium (with 100 μg/mL ampicillin) with the ratio of 1% (*v*/*v*) at 37 °C with shaking at 200 rpm until OD_{600} reached 0.4, expressing was induced with 0.2 mM isopropylthiogalactoside, and cultivation was continued at 32 °C for 8 h. The cells were harvested by centrifugation at 5,000×*g* for 20 min, the pellet was suspended in 5~10 ml of binding buffer (20 mM sodium phosphate, and 500 mM NaCl, 30 mM imidazole, pH 7.4) for each gram of cell paste containing 1 mM phenylmethyl sulfonylfluoride and lyzed by sonication and subsequent centrifugation (12,000×*g* for 25 min at 4 °C). The supernatant (soluble protein fraction) was isolated. Purification was performed on the ÄKTA Purifer system (Amersham Pharmacia Biotech) with HisTrap™ FF crude (GE Healthcare), which was prepacked with the affinity medium Ni Sepharose 6 Fast Flow. The purified fusion protein (TrxA-mBD1) was specifically digested with rEK/His which had a C-terminal His-tagged at 25 °C for 16 h by buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0). The mixture buffer was further purified by HisTrap™ FF crude. The mature mBD-1 was obtained by the effluent of loading sample of digestion mixture and further desalted by Amicon® Ultra-15 3 K Centrifugal Filter Devices. Standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) method was used to check the fusion protein and mature mBD-1. The mature mBD-1 peptide (theoretical molecular weight of 4.07 kD) was analyzed by Western blot with rabbit anti-mBD1 antibody.

Antifungal Assays

Antifungal activity of mature mBD-1 was examined by microdilution plate assay [4] with the following modifications. Briefly, fungal cells were grown in YPG medium and washed twice with 10 mM sodium phosphate buffer (NaPB; Na_2HPO_4/NaH_2PO_4, pH 7.5), and sodium phosphate buffer containing 1% YPG was resuspended at a concentration of 4×10^4 cells/ml. The cell suspensions were then mixed with mBD-1 (1 to 15 μM) at 37 °C for 3 h. Blank control cultures incubated with 10 mM NaPB containing 1% YPG alone. Cell suspensions were diluted in 10 mM NaPB, and aliquots of 500 cells were spread onto YPG agar plates and incubated for 48 h at room temperature. Antifungal assays were performed in triplicate. Cell survival was expressed as a percentage of the blank control, and the loss of viability was calculated as follows [1-(colonies after mBD-1 treatment/colonies after incubation with buffer only)]×100. When required, the incubation medium (containing 15 μM mBD-1) was supplemented with different concentrations NaCl.

Germ Tube Formation Assay

Germ tube formation assay was performed according to Brayman and Wilks [5, 6]. A synchronized 48-h culture of *C. albicans* maintained at 25 °C without shaking. The culture was centrifuged for 10 min to collect the cells (1×10^5) which were resuspended in different concentrations of mBD-1 and cultured in 96-well flat bottom polystyrene plates at 37 °C for 4 h. The medium was discarded by inverting the plates. The cells were washed once by immersion in 70% (*v*/*v*) ethanol, which was discarded, and then 200 μl 0.25% SDS was added to each well. The SDS was discarded, and the plates were washed three times by immersion in distilled water. Germ tubes, attached to the wells, were stained for 15 min with 100 μl 0.02% Crystal Violet dissolved in phosphate-buffered saline. The dye solution was removed by inverting the plates, and the plates were washed three times with water, once with 0.25% SDS and twice with water. After the plates were dried, 200 μl 2-propanol containing 0.04 M HCl and 50 μl 0.25% SDS were added to the wells and mixed briefly. The absorbance at 590 nm was determined by using a microplate spectrophotometer. Optimum culture of *C. albicans* was used as positive control of germ tube formation. All assays were run in triplicates.

Flow Cytometry Assay

Flow cytometry assay was performed according to Vylkova et al. [7], with some modifications. Fungal cells (1×10^5) were mixed with mBD-1 (15 μM) to reach a final volume of 100 μl and were incubated at 37 °C for 3 h. The propidium iodide (PI, final concentration, 50 mg/ml), DNA-binding dye, was added to the cells and incubated for 30 min. To remove excess PI, cells were harvested by centrifugation at 5,000×*g* for 2 min and washed three times with 10 mM NaPB. Then, cell pellets were reconstituted in 1 ml buffer. Control cells were incubated with 10 mM NaPB or PI only. Samples were analyzed on a BD FACSAria flow cytometer (Becton Dickinson).

Data Analysis

Data were obtained from three independent experiments performed each in triplicate and compared by analysis of variance and unpaired two-tailed *t* test. The results were reported as means ± the standard errors (SE). A p value of ≤ 0.05 was considered to be statistically significant.

Results and Discussion

Expression of *mBD-1* in Mouse Different Tissues

A reverse transcription (RT)-PCR using mRNA obtained from mouse tissues was performed to evaluate expression of *mBD-1*. Esophagus, lung, kidney, brain, ovary, tongue, heart, and spleen showed expression of *mBD-1*, but high level expression was seen in kidney, tongue, ovary, lung, and brain (Fig. 1). *mBD-1* is a homolog of *hBD-1*, diffusely expressed through the epithelia of multiple organs. It likely plays an important role in innate immunity at multiple mucosal sites. The putative mature peptide contains six cysteine residues spaced in a typical array and other conserved amino acids that may have important roles in determining the conformation and function of ß-defensins [8].

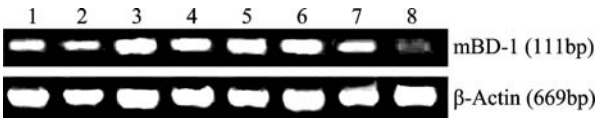

Fig. 1 RT-PCR of mouse mBD-1 from various tissues; total RNA was isolated from mouse tissues and reverse transcribed, and the cDNAs were amplified by using two pairs of mBD-1-specific primers. A single 111-bp band was amplified by using gene-specific primers. As a control, ß-actin cDNA was amplified by using specific primers. Lanes: *1*, esophagus; *2*, lung; *3*, kidney; *4*, brain; *5*, ovary; *6*, tongue; *7*, heart; *8*, spleen

Construction, Expression, and Purification of Mature mBD-1

To construct pET32-mBD1 expression vector (Fig. 2), a cDNA encoding the mature *mBD-1* fragment of 111 bp was successfully amplified by RT-PCR from the mouse lung RNA and cloned into pET32a(+). The recombinant plasmid was transformed into *E. coli* DH5α, and its sequence was identified. The sequence was completely correct by comparison analysis using a BLAST search at the GenBank.

The pET32-mBD1 was transformed into *E. coli* BL21 (DE3). The fusion protein was high percentage (≥95%) in the soluble fraction at this experiment condition, and its volumetric productivity reached 0.67 g/L. The fusion protein was cleaved effectively with rEK/His, and the mature mBD-1 (0.14 g/L) was released by the precise cleavage of TrxA-Tag. SDS-PAGE analysis results are shown in Fig. 3, and about 4.07 kD band could be seen at Western blot (data not shown).

In this work, *mBD-1* gene was fused with thioredoxin (TrxA) under the control of T7 promoter. The major advantages of using TrxA protein as fusion partner are to facilitate the formation of disulfide bonds and accurate of folding of protein [9]. Between TrxA Tag and mBD-1 coding sequence, there is a His Tag, which is beneficial for the fusion protein separation by affinity chromatography and an enterokinase recognition site to facilitate cleaving the fusion protein and release the mature mBD-1 peptide.

Antifungal Activity of Mature mBD-1

The mature mBD-1 exhibited antifungal activities in a dose-dependent manner with *C. albicans* and *C. neoformans*. However, TrxA protein at the same conditions was not

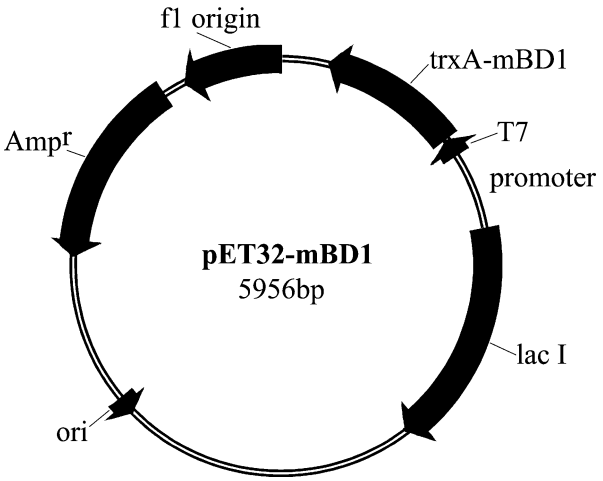

Fig. 2 Schematic representation of expression vector, pET32-mBD1, with T7 promoter, a fusion partner TrxA and an ampicillin-resistance gene

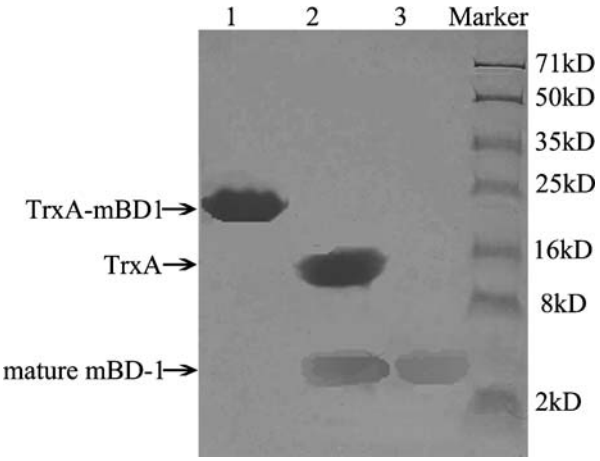

Fig. 3 SDS-PAGE analysis of enterokinase/His digestion and effluent fraction of nickel-affinity; the fusion protein was specifically digested with enterokinase/His (rEK/His), which has a C-terminal His Tag, at 25 °C for 16 h by buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0). The mixture buffer was further purified by HisTrap™ FF crude. The released mature mBD-1 was obtained by the effluent of loading sample of digestion mixture and further desalted by Amicon® Ultra-15 3K Centrifugal Filter Devices. Lanes: *1*, before enterokinase digestion; *2*, after enterokinase digestion; *3*, effluent fraction of nickel-affinity

significantly candidacidal activities (<12.7%). Fungicidal curves are shown in Fig. 4a. Both strains were susceptible to mBD-1 in the 1–15 μM concentration range. The IC_{50} (50% inhibitory concentration) values to *C. albicans* and *C. neoformans* were 5 and 2 μM, respectively, as compared with blank control sample at the same incubation time. This is an indication of an endogenouse defensin of mouse with specific antifungal activity, which is probably central in the defense against infections with fungi.

In this work, it should be noted that the value of IC_{50} is also affected by assay conditions such as the used medium, the initial concentration of fungal spore, the incubation time, etc. However, when NaCl was added, the activity of mBD-1 was suppressed by increasing NaCl concentration (Fig. 4b). This phenomenon was also observed in other types of defensins. For insect and mammalian defensins, high cationic strength in solution will hamper the positively charged defensin to interact with the negative microbial membrane surface [10, 11].

Inhibition of Germ Tube Formation

The result of germ tube formation inhibition by mBD-1 to *C. albicans* is shown in Fig. 5a. The germ tube formation of *C. albicans* was strongly suppressed by mature mBD-1, inhibition rates were 40.07%, 57.89%, and 65.06% with 3.4, 6.4, and 51.2 μM, respectively.

C. albicans is a dimorphic fungus, capable of growth in a yeast or filamentous form. The yeast-to-hyphal transition begins with the formation of a germ tube, the initial stage of hyphal formation, making this transition a potentially useful end point for an antifungal drug assay [12]. The end point is particularly relevant for glucan synthesis inhibitors, since the activation glucan synthase is associated with the extensive synthesis of cell wall during the formation of hyphae [13]. Hawser and Islam [14] first demonstrated that inhibition of germ tube formation could be more sensitive to drug intervention than inhibition of growth, permitting the assessment of nonidealized antifungal drugs.

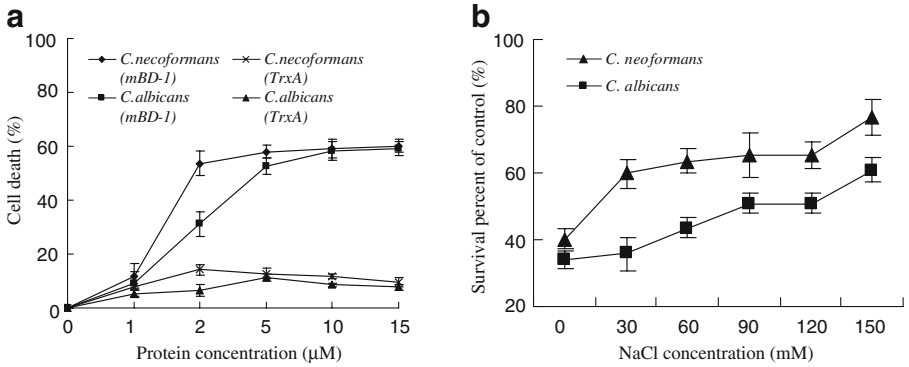

Fig. 4 Functional characterization of mature mBD-1; *C. albicans* and *C. neoformans* were incubated with mature mBD-1 in 10 mM NaPB containing 1% YPG. Triple experiments were carried out, and the average values and variations (*error bars*) are presented. **a** Fungal-killing curve; the cell suspensions were mixed with mature mBD-1 and TrxA protein (as a negative control), respectively (1 to 15 μM), at 37 °C for 3 h. Blank control cultures incubated with 10 mM NaPB containing 1% YPG alone. Cell suspensions were diluted and spread onto YPG agar plates and incubated at room temperature for 48 h. Cell survival was expressed as a percentage of the blank control, and the loss of viability was calculated as follows [1-(colonies after mBD-1 treatment/colonies after incubation with buffer only)]×100. **b** Effect of NaCl concentration on the antifungal activity of mBD-1. The incubation medium (containing 15 μM mBD-1) was supplemented with different concentrations NaCl. Cell suspensions were diluted and spread onto YPG agar plates and incubated at room temperature for 48 h. Cell survival was expressed as a percentage of the control

The assay, measuring the transformation of *C. albicans* from the yeast form to the hyphal form, is especially useful for early drug discovery. Our results showed a strong inhibition of germ tube formation with 57.89% of 6.4 μM. Clearly, the germ tube assay was more sensitive, allowing the determination of a definitive IC_{50} for most of the compounds [5].

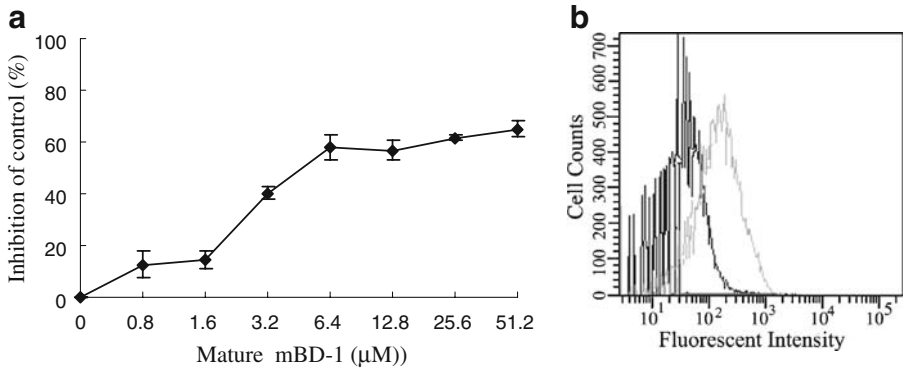

Fig. 5 Antifungal effect of mature mBD-1 on *C. albicans*; **a** germ tube formation of *C. albicans*; a synchronized 48-h cultures of *C. albicans* were suspended in different concentrations of mBD-1 and cultured at 37 °C for 4 h. Germ tubes were stained with Crystal Violet and were treated with 2-propanol containing 0.04 N HCl and 0.25% SDS. Absorbance was determined at 590 nm using microplate spectrophotometer. All assays were run in triplicates. **b** *C. albicans* treated with mature mBD-1 exhibited PI uptake. *C. albicans* strain was incubated at 37 °C for 3 h with 15 μM mBD-1 followed by 30 min incubation with 50 mg/ml PI. Cells were washed three times in 10 mM sodium phosphate buffer (pH 7.4) and analyzed by BD FACSAria. Control cells are indicated with a *black line*. Cells treated with mBD-1 exhibited high level PI uptake, resulting in 40.1% killing at 15 μM mBD-1 (*gray line*, n=3, p<0.001) compared with untreated cells

Flow Cytometry Assay

We examined candidacidal activity of mature mBD-1 through PI uptake. *C. albicans* strain was incubated with mBD-1, and exhibited high level PI uptake, with 40.1% killing at 15 μM (Fig. 5b gray line, $n=3$, $p<0.001$) compared to untreated cells.

Although many antimicrobial peptides have common features, such as net cationic charge, a unifying principle for their mechanism of antimicrobial action has not been demonstrated. While human defensins have been shown to interact with membranes in susceptible bacteria and fungi [15], in this study, we show that mBD-1 induces membrane permeability for PI in the susceptible *C. albicans*. The rationale for the present study was to test the hypothesis that endogenous ß-defensins contribute to the innate defense against *Candida* species by multiple mechanisms which may include antimicrobial action, inhibition of adherence to epithelial cells, and upregulation in response to hyphal growth. Feng et al. have reported that hBDs control fungal colonization through hyphal induction, direct fungicidal activity, and inhibition of candidal adherence [16]; other investigators also have reported that hBD-2 and hBD-3 require Ssa1/2p for antifungal activity [7]. Although the exact mechanism of microbial killing has not yet been fully understood, many studies indicate that an amphipathic conformation is required for membrane perturbation, which is responsible for the subsequent microbial death [1, 17]. Due to the presence of fungal strains with multidrug resistance, there is a need to search for new drugs. Some defensins are inhibitory to *C. albicans* which can cause fatal infections in immunocompromised patients [18]. However, mBD-1 for antifungal activity and mechanism are poorly understood. Our study is the first report that a recombinant mature mBD-1 peptide shows a strong antifungal activity of *C. albicans* and *C. neoformans*. Successful expression of the recombinant mBD-1 peptide in *E. coli* offers a basis for further studying its antifungal mechanisms and may gain further insights into the role of mBD-1.

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