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Identification of an antimicrobial peptide from human methionine sulfoxide reductase B3

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Human methionine sulfoxide reductase B3A (hMsrB3A) is an endoplasmic reticulum (ER) reductase that catalyzes the stereo-specific reduction of methionine-*R*-sulfoxide to methionine in proteins. In this work, we identified an antimicrobial peptide from hMsrB3A protein. The N-terminal ER-targeting signal peptide (amino acids 1-31) conferred an antimicrobial effect in *Escherichia coli* cells. Sequence and structural analyses showed that the overall positively charged ER signal peptide had an Arg- and Pro-rich region and a potential hydrophobic α-helical segment that contains 4 cysteine residues. The potential α-helical region was essential for the antimicrobial activity within *E. coli* cells. A synthetic peptide, comprised of 2-26 amino acids of the signal peptide, was effective at killing Gram-negative *E. coli, Klebsiella pneumoniae*, and *Salmonella paratyphi*, but had no bactericidal activity against Gram-positive *Staphylococcus aureus*. [BMB reports 2011; 44(10): 669-673]

INTRODUCTION

Antimicrobial peptide is defined as a peptide that can kill microorganisms or inhibit their growth. Antimicrobial peptides are produced in a variety of plants and animals for host defense against microbial infection. A number of different antimicrobial peptides (>880) has been identified or predicted based on nucleic acid sequences (1-3). Antimicrobial peptides can be classified by composition and structure of amino acids into subgroups (1, 4). There are generally four subgroups: anionic peptides, cationic α-helical peptides, cationic peptides enriched for specific amino acids, and anionic and cationic peptides that contain cysteines and form disulfide bonds (1, 5).

Methionine sulfoxide reductases (Msrs) catalyze the reduction of free and protein-based methionine sulfoxide to methionine. Msrs are important enzymes that repair oxidatively damaged proteins, function as antioxidants, and regulate the protein function (6, 7). There are two different types of Msrs, MsrA and MsrB, for the reduction of methionine sulfoxide residues in proteins. MsrA stereospecifically reduces the *S*-epimer of methionine sulfoxide, whereas MsrB only acts on the *R*-form. Three MsrBs occur in mammals where each MsrB is targeted to different cellular locations (8, 9). MsrB1 is a cytosolic and nuclear selenoenzyme, MsrB2 is a mitochondrial protein, and MsrB3 is targeted to the endoplasmic reticulum (ER). Human MsrB3 gives rise to two alternatively spliced forms, which are targeted to different compartments (8). MsrB3A is targeted to the ER, whereas MsrB3B is localized to the mitochondria.

Previously, we observed that human MsrB3A (hMsrB3A) could suppress the growth of *Escherichia coli* cells when overexpressed (8). This observation suggests the possibility that hMsrB3A contains an antimicrobial peptide. In this work, we found that the N-terminal ER targeting signal peptide of hMsrB3A carries the antibiotic effect in *E. coli* cells. We also defined a potential α-helix of the ER signal peptide as an essential region for the antimicrobial activity. We further analyzed that a synthetic peptide (2-26 amino acids of the ER signal peptide) exhibited bactericidal activity against Gram-negative *E. coli, Klebsiella pneumoniae*, and *Salmonella paratyphi*, but not against Gram-positive *Staphylococcus aureus*.

RESULTS AND DISCUSSION

hMsrB3A consists of 192 amino acids and has an ER-targeting signal peptide in the N-terminus (MSPRRSLPRPLSLCLSLCLCLCLAAALGSAQ). We previously observed that the growth of *E. coli* cells was inhibited by overexpression of a recombinant full-length hMsrB3A form (8). However, the *E. coli* growth seemed to be normal when a protein without the signal peptide was overexpressed (8).

To first test whether the signal peptide of hMsrB3A indeed had an antimicrobial activity, we separately transformed *E. coli* BL21(DE3) with the pET21-based plasmids pHR3aF and pHR3aΔS, which contain hMsrB3A with and without the signal peptide (amino acids 1-31), respectively, and a C-terminal His-tag. As shown in Fig. 1A, when IPTG was added the growth of cells containing the full-length form construct was severely inhibited compared to that of cells without IPTG.

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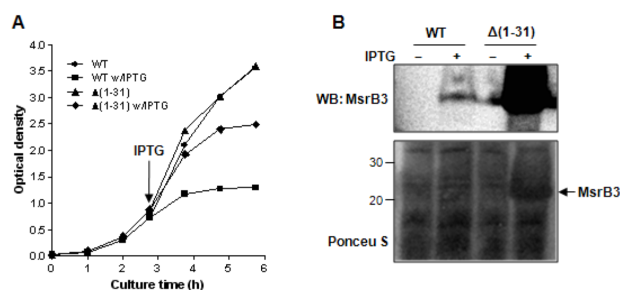

Fig. 1. The N-terminal ER-targeting signal peptide of hMsrB3A shows an antibiotic effect. (A) Growth assays with or without IPTG. *E. coli* BL21(DE3) cells containing the indicated constructs were cultured until the optical density at 600 nm reached 0.6-0.8 and then 500 μM IPTG was added to the cultures. Representative data from two independent experiments are shown. (B) Western blot (upper) and Ponceu S staining (lower). Samples at 2 h IPTG induction were subjected to Western blot analysis. Polyclonal anti-MsrB3 antibodies were used for detection of proteins. WT, full-length hMsrB3A; ⊿(1-31), deletion of 1-31 amino acids of hMsrB3A.

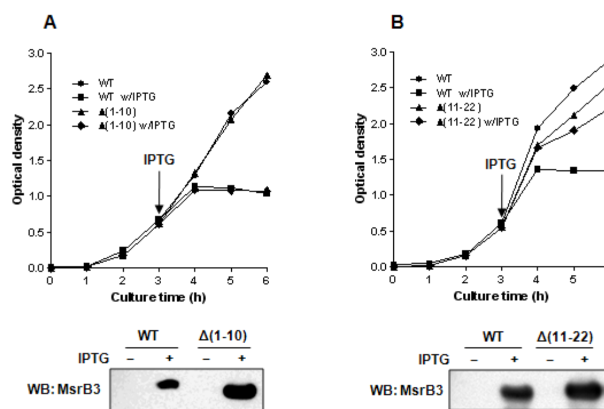

Fig. 2. The potential α-helix motif in the signal peptide is responsible for the antimicrobial effect. *E. coli* BL21(DE3) cells containing the indicated constructs were cultured until the optical density at 600 nm reached 0.5-0.7 and then 250 μM IPTG was added to the cultures. (A) Growth assay of cells containing ⊿(1-10) mutant. (B) Growth assay of cells containing ⊿(11-22) mutant. Representative data from two independent experiments are shown. Samples at 2 h IPTG induction were subjected to Western blot analysis with anti-MsrB3 antibodies (lower panels). WT, full-length hMsrB3A; ⊿(1-10), deletion of 1-10 amino acids; ⊿(11-22), deletion of 11−22 amino acids.

However, the growth of cells containing the signal peptide-deleted construct was only slightly inhibited by the addition of IPTG. This decrease in growth is most likely due to the fact that forced protein expression by IPTG induction drains metabolic energy of cells, resulting in reduced growth. Western blot assays verified that both the full-length and signal peptide-deleted hMsrB3As were only expressed by the IPTG induction (Fig. 1B, upper panel). It was also found that the expression level of the full-length MsrB3A was astonishingly lower than that of the signal peptide-deleted form, which in comparison showed a strong protein band even in the Ponceu S stained membrane (Fig. 1B, lower panel). We also conducted similar growth test but with variable concentrations of IPTG (25-500 μM). The growth of cells transformed with the full-length hMsrB3A was highly inhibited even at 25 μM IPTG (Supplementary Fig. S1). The growth inhibition became fully saturated between 50-100 μM IPTG. In addition, the N-terminally His-tagged hMsrB3A driven from a pET28a-based construct also showed an antibiotic effect in *E. coli* cells (Supplementary Fig. S2). Taken together, the data indicated that the signal peptide of hMsrB3A indeed had an antibacterial activity.

Sequence and secondary structure prediction analysis using NetSurfP program showed that the signal peptide of hMsrB3A has two very noticeable motifs. The first 10 amino acids contain three basic amino acid arginines and three prolines. The next 12 amino acids (11-22) were highly predicted to form a coiled α-helix. To test which motif (or both) causes the antibacterial activity against *E. coli* cells, we made two deletion mutants, ⊿(1−10) and ⊿(11-22), by site-directed mutagenesis. Then we performed the same growth experiments as above for ⊿(1-10) and ⊿(11-22) mutants. When IPTG was added, cells containing the ⊿(1-10) mutant form showed very similar growth to cells containing the wild-type, i.e. severe inhibition of growth (Fig. 2A). In contrast, cells containing the ⊿(11-22) mutant form showed no significant inhibition of growth by IPTG induction (Fig. 2B). The data demonstrated that the motif consisting of amino acids 11-22 (potential α-helix) is responsible for the antimicrobial effect. Western blot analyses showed the expression of both mutant proteins by IPTG induction. The ⊿(1-10) mutant band was lower than the wild-type full-length form (Fig. 2A, lower panel). However, unexpectedly, the ⊿(11-22) mutant band appeared to be slightly higher than the wild-type band (Fig. 2B, lower panel), suggesting that the potential α-helix motif may facilitate migration of hMsrB3A on the gel. To test a possibility that the hMsrB3A proteins can be secreted from *E. coli* cells, we assayed extracellular MsrB activity of cells carrying full-length, signal peptide-deleted, ⊿(1-10), or ⊿(11-22) constructs using culture broth after 2 h IPTG induction. As a result, none of samples showed MsrB activity, suggesting that hMsrB3A could not be secreted extracellularly.

The signal peptide sequence (1-31) was analyzed by the Antimicrobial Peptide Data prediction program (10) whether it functions as antimicrobial peptide. This sequence was predicted to have antimicrobial activity with 58% hydrophobic ratio, +3 net charge, and 0.23 kcal/mol protein-binding potential (Boman index). The program also predicted that it may form a disulfide-bond linked structure due to the presence of Cys residues (Cys14, Cys18, Cys20, and Cys22). The alignment program was also conducted to compare it with all sequences stored in the Antimicrobial Peptide Database (10). The three peptides most similar to the signal peptide sequence of hMsrB3A were human LEAP-2 (AP00811) (11), Ci-MAM-A24 (AP01614) (12), and chicken LEAP-2 (AP00745) (13) with

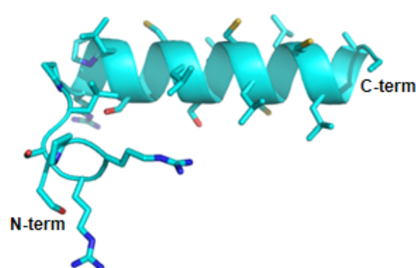

Fig. 3. Estimated structure of peptide B3 using PEP-FOLD program. The lowest energy model among the predicted structures is shown by PYMOL.

39.5%, 38.7%, and 37.8% similarity, respectively.

To evaluate a peptidic antibacterial activity, we synthesized a peptide, named B3, which is comprised of 2-26 amino acids of the signal peptide of hMsrB3A (SPRRSLPRPLSLCLSLCLCLCLAAA). First, the peptide B3 structure was predicted using the PEP-FOLD program (14). The lowest energy model structure of peptide B3 shows an α-helical structure starting from Arg9 to Ala25 (Fig. 3). Next, the bactericidal activity of the peptide B3 was examined using Gram-negative *E. coli, K. pneumoniae*, and *S. paratyphi*, and Gram-positive *S. aureus* cells. The peptide B3 exhibited 13% bactericidal activity at 10 μg/ml, 24% at 20 μg/ml, and 34% at 40 μg/ml against *E. coli*, showing a dose-dependent antibacterial activity (Fig. 4A). It also exhibited bactericidal activities against *K. pneumoniae* and *S. paratyphi* (Fig. 4B). However, the peptide showed no bactericidal activity against *S. aureus* (Fig. 4C). The data suggested that the peptide B3 is only effective at killing Gram-negative bacteria.

The peptide B3 has 4 cysteine residues that are predicted to form disulfide bond(s) in the hydrophobic α-helix. Thus, it was of interest to determine whether the potential disulfide bond(s) is involved in the peptide's antimicrobial activity. We assayed the antibacterial activity in the presence of dithiothreitol (DTT) against *E. coli* cells (Fig. 4D). The peptide did not exhibit bactericidal activity in the presence of DTT, suggesting that potential disulfide bond(s) is important for the peptide's antimicrobial activity.

Antimicrobial peptides are divided into four subgroups on the basis of amino acid composition and structure (1, 4). The anionic antimicrobial peptides are small and present in airway epithelial cells, bronchoalveolar lavage fluid, and surfactant extracts. The next subgroup cationic α-helical peptides are short and lack cysteine residues. Generally, the antimicrobial efficiency is proportional to the content of the α-helix. The third subgroup is cationic peptides that are rich certain amino acids, such as proline, arginine or phenylalanine. These peptides lack cysteine residues and are typically linear. The last subgroup anionic and cationic peptides containing cysteines form intramolecular disulfide bonds and stable β-sheet conformation. This subgroup includes protegrin and defensin. The structural analyses using the NetSurP and PEP-FOLD programs highly predicted an α-helical conformation in the ER signal peptide sequence of hMsrB3A. The potential α-helical motif was found to be essential for the antibacterial activity within *E. coli* cells. The antimicrobial peptide driven from hMsrB3A had positive charge and two distinct motifs composed of Arg- and Pro-rich region and hydrophobic α-helix containing multiple cysteine residues. These characteristics seem inconsistent with those of any four subgroups. Presently, the potential disulfide bond(s) formed in the α-helix was involved in the antimicrobial peptide activity. Studies are further needed i) to identify which other residues in the signal peptide of hMsrB3A are important for the antimicrobial peptide activity, ii) to determine a solution structure of the peptide using NMR, iii) to understand a mechanism of the antimicrobial peptide activity, and iv) to increase the peptide's antimicrobial activity for therapeutic use.

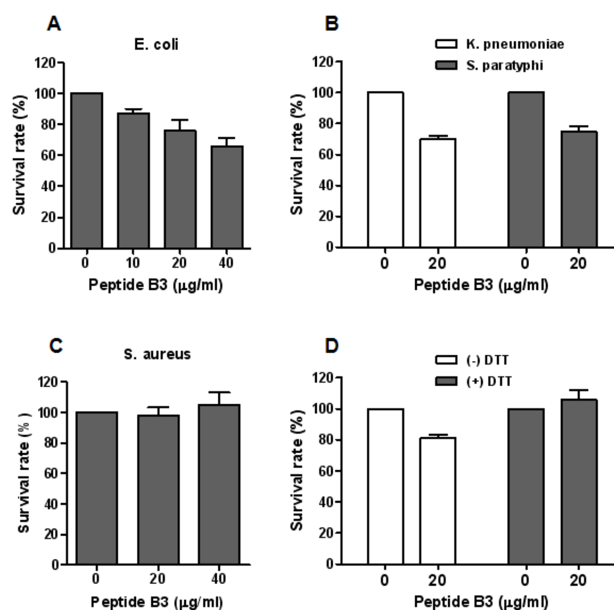

Fig. 4. Bactericidal activity of peptide B3. Gram-negative *E. coli* BL21(DE3) (A) and *K. pneumoniae* and *S. paratyphi* (B), and Gram-positive *S. aureus* (C) were used to evaluate the antibacterial activity of peptide B3. The antibacterial activity against *E. coli* BL21(DE3) was assayed in the presence of 10 mM DTT (D). Data are shown as mean ± SEM from at least two independent experiments.

MATERIALS AND METHODS

Constructs

The pET21-based plasmids pHR3aF that code for full-length hMsrB3A and pHR3a⊿S that lacks 1-31 amino acids of hMsrB3A were described elsewhere (8). These recombinant proteins contained a C-terminal His-tag (LEHHHHHH). The

⊿(1-10) and ⊿(11-22) constructs (deletion of 1-10 and 11-22 amino acids of hMsrB3A, respectively) were generated using pHR3aF as template by site-directed mutagenesis as described (15). To create an N-terminally His-tagged hMsrB3A, a coding region was PCR-amplified and inserted into NdeI/HindIII sites of pET28a. The resulting construct, named pHR3a, encoded the full-length hMsrB3A with an N-terminal His-tag (MGSSH-HHHHHSSGLVPRGSH). All constructs were verified by DNA sequencing.

Growth assay

Transformed *E. coli* BL21(DE3) with each construct were cultured in LB medium containing 100 μg/ml ampicillin at 37°C. When optical density at 600 nm reached about 0.6-0.8 (about 3 h of growth), the culture was separated into two samples in the designated flasks. One of samples was treated with 500 μM or 250 μM IPTG to induce protein expression. Cells were further cultured for 3 h and the growth was monitored by measuring optical density at 600 nm at every hour.

Western blotting

Extracts from *E. coli* cells after 2 h IPTG induction were prepared. The polyclonal anti-hMsrB3 antibodies (9) were used to detect hMsrB3A forms.

Extracellular MsrB activity measurement

To measure extracellular hMsrB3A enzyme activity, 50 ml cultured samples after 2 h IPTG induction were centrifuged and the supernatant fluid was collected. The reaction mixture (100 μl) contained 50 mM sodium phosphate (pH 7.5), 200 μM dabsylated methionine-*R*-sulfoxide, 20 mM DTT, and 50 μl of the supernatant. Reaction was carried out at 37°C for 30 min and the dabsyl-Met was analyzed by HPLC procedure as described (16).

Peptide synthesis

The peptide B3 (SPRRSLPRPLSLCLSLCLCLCLAAA) was chemically synthesized by Peptron (Korea).

Sequence and structure analyses

Secondary structure analysis of hMsrB3A was carried out using NetSurfP program (http://www.cbs.dtu.dk/services/NetSurfP/). The PEP-FOLD (14) and Antimicrobial Peptide Database (10) were used to predict three-dimensional structure and antimicrobial function of peptide, respectively.

Bactericidal assay

Peptide B3 was solubilized in dimethyl sulfoxide (DMSO). Bactericidal assays were performed using Gram-negative *E. coli* BL21(DE3), *K. pneumoniae,* and *S. paratyphi* A and Gram- positive *S. aureus*. All assays were performed in duplicate. *E. coli* cells were exponentially grown at 37°C in LB, while *K. pneumoniae*, *S. paratyphi*, and *S. aureus cells* in Tryptic Soy Broth. The cultures were diluted to 1×10^3 colony forming units (CFU) in a final volume of 100 μl, as determined by the optical density, with the fresh medium. Various concentrations of peptide B3 were added to the cells and the sample was incubated for 1 h at 37°C. Then, CFU were counted by overnight growth on each agar plate at 37°C. The number of CFU of samples treated with peptide was compared to that of controls treated with the same volume of DMSO. The antimicrobial activity of peptide B3 against *E. coli* was also assayed in the presence of 10 mM DTT.

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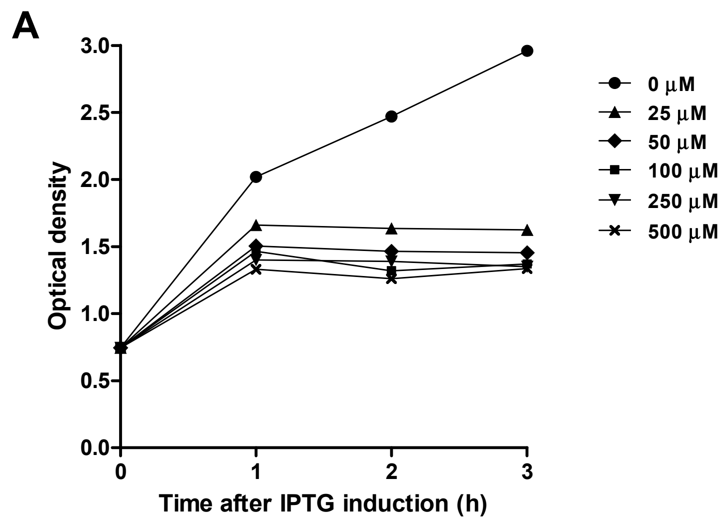
Supplementary Data

Figure S1. Growth assays of cells containing full-length hMsrB3 with variable IPTG concentrations. *E. coli* BL21(DE3) cells containing the C-terminally His-tagged hMsrB3 construct were cultured until the optical density at 600 nm reached 0.6–0.8 and then the indicated concentrations of IPTG (0–500 μM) were added to the cultures. (A) Growth assays. (B) Western blot analysis with anti-MsrB3 antibody.



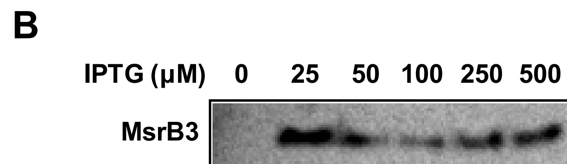
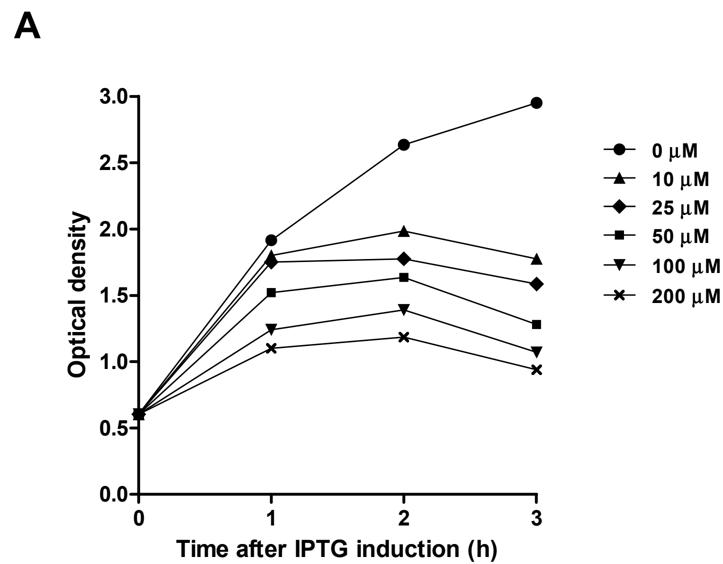


Figure S2. Growth assays of cells containing N-terminally His-tagged hMsrB3 with variable IPTG concentrations. *E. coli* BL21(DE3) cells containing the N-terminally His-tagged hMsrB3 construct were cultured until the optical density at 600 nm reached 0.6–0.8 and then the indicated concentrations of IPTG (0–200 μM) were added to the cultures. (A) Growth assays. (B) Western blot analysis with anti-MsrB3 antibody.

A



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