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Multimer of the antimicrobial peptide Mytichitin-A expressed in *Chlamydomonas reinhardtii* exerts a broader antibacterial spectrum and increased potency

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In this study, an antimicrobial peptide composed of three tandem repeats of Mytichitin-A (3 × Mytichitin-A) with a designed molecular weight of approximately 25 kDa was expressed in the green alga *Chlamydomonas reinhardtii*. The yield of 3 × Mytichitin-A reached 0.28% of the total soluble protein of the 3 × Mytichitin-A-expressing transgenic cells and the expression level was stable following continuous passaging of the cells for six months. Compared to its natural and yeast-produced recombinant counterparts, which showed a very low level of growth inhibition of gram-negative bacteria, the 3 × Mytichitin-A inhibited the growth of gram-negative bacteria at a minimum inhibition concentration value ranging between 60 and 80 μg/ml. The expressed 3 × Mytichitin-A did not show toxicity to HEK293 cells. Its bioactivity was hardly affected by temperature and pH but was impaired to some extent by the proteinase treatment. Taken together, our study showed that *C. reinhardtii* can be used as a cellular factory to produce bioactive Mytichitin-A in a multimeric format.

[**Key words:** *Chlamydomonas reinhardtii*; Antimicrobial peptide; Mytichitin-A; Antimicrobial activity; Tandem multimer]

Antimicrobial peptides (AMPs), which inhibit or kill a various of microorganisms, are generally short peptides consisting of 5–100 amino acids (1). To date, more than 2000 different AMPs have been identified from organisms including amphibians, mammals, plants, invertebrates, and prokaryotes (2,3). AMPs possess broad-spectrum antimicrobial activity against fungi, viruses, parasites, and bacteria and also can inhibit tumor cell proliferation without causing cellular toxicity (4–7). Compared to conventional antibiotics, AMPs show little effect to induce drug-resistant microorganisms and thus are regarded as promising candidates to replace conventional antibiotics in the near future (8–10). In 2014, Mytichitin-A, consisting of 55 amino acids and having a molecular weight of 7.5 kDa, was isolated from the serum of *Mytilus coruscus* (11). Bioactivity study indicated its strong inhibitory effect on gram-positive bacteria with a minimum inhibition concentration (MIC) value ranging between 8 and 30 μg/ml (11). However, its inhibitory effect on gram-negative bacteria was very weak, with an MIC value ranging between 150 and 310 μg/ml (11). Our previous study showed that recombinant Mytichitin-A, when expressed in *Escherichia coli*, was nonfunctional against bacteria (12). This lack of activity of the recombinant protein was probably because active Mytichitin-A contains 6 cysteines that form three disulfide bonds, which would not form in *E. coli*. In contrast, yeast-derived recombinant Mytichitin-A was found to be potent in inhibiting typical gram-positive bacteria including *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus subtilis* with an MIC value ranging between 30 and 70 μg/ml (12). However, the yeast-derived recombinant Mytichitin-A exhibited little inhibition of the growth of gram-negative bacteria (12). To increase the potency of recombinant AMPs against bacteria, one effective strategy is to design and express AMPs as tandem multimers (13–16). For example, a recombinant multimeric hPAB-β containing eight copies of hPAB-β tandem repeats and a recombinant multimeric *Momordica charantia* 6 (MC6) consisting of ten copies of MC6 tandem repeats have been successfully expressed in *E. coli* with improved antimicrobial activity (13,15).

Increasing attention has been paid to the use of the green alga *Chlamydomonas reinhardtii* as a next generation green cell factory for the synthesis of therapeutic proteins and other industrial enzymes (17–24). Compared to heterotrophic cell factories like *E. coli*, *Pichia pastoris*, *Saccharomyces cerevisiae*, and many cultured mammalian cells, *C. reinhardtii* features a number of advantages including: (i) an eukaryotic expression system similar to that of higher plants, capable of post-translational modifications such as protein glycosylation, processing, and folding, (ii) cost effective phototrophic cultivation with simple and inexpensive medium, and (iii) a safe food additive, so oral delivery of AMP-containing algae is possible. In this study, we aimed to test the use of *C. reinhardtii* as a cell factory to produce recombinant Mytichitin-A containing three copies of Mytichitin-A repeats (3 × Mytichitin-A) and then analyzed the antibacterial spectrum and potency of the purified 3 × Mytichitin-A.

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MATERIALS AND METHODS

Strains, vectors, and reagents *E. coli* XL1-blue competent cells were purchased from Stratagene (La Jolla, CA, USA). *C. reinhardtii* CC-125 was obtained from the *Chlamydomonas* Genetic Center (University of Minnesota, Twin Cities, MN, USA). *E. coli* O157 (ATCC 35150), *S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 21633), and *Salmonella enteriditis* (ATCC 10467) were obtained from the American Type Culture Collection (http://www.atcc.org/). The *C. reinhardtii* nuclear expression vector pHSP-RBCS2-GFP-PARO (25) was used to construct the 3 × Mytichitin-A expression vector used in this study. Regents and chemicals used for DNA manipulation and protein purification were purchased from Fermentas (Waltham, MA, USA) and GE Healthcare (Pittsburgh, PA, USA). All other standard reagents were purchased from Sigma–Aldrich (Saint Louis, MO, USA).

Plasmid construction A 570 bp nucleotide sequence encoding a codon-optimized C-terminal hemagglutinin-6 × His (HA-6 × His) tagged 3 × Mytichitin-A with an *Eco*RI and a *Xho*I site located at its 5′- and 3′-end, respectively, was synthesized by Genewiz (Suzhou, Jiangsu China). After double digestion with *Eco*RI and *Xho*I, this sequence was inserted into the *C. reinhardtii* nuclear expression vector pBSK-HSP-RBCS2-GFP-PARO (6 kb in size) (25), resulting in the 3 × Mytichitin-A-expressing pBSK-HSP-RBCS2-3 × Mytichitin-A-PARO vector (see Supplementary Fig. S1).

***C. reinhardtii* transformation** Electroporation was performed as described previously (25) to transform foreign DNA into *C. reinhardtii*. Briefly, wild type CC-125 cells were cultured to mid-log phase with a final cell density of about 3–6 × 10^6 cell/ml and then concentrated to a cell density of about 5 × 10^8 cell/ml with TAP/D-sorbitol (60 mM) medium. Two hundred fifty microliters of concentrated cells mixed with 2.5 μg of supercoiled plasmid were added to a 4-mm cuvette (Bio-Rad, Hercules, CA, USA) and then incubated on ice for 10 min. Next, an exponential electric pulse of 2000 V/cm was applied to the cuvette using a Gene Pulser Xcell electroporation apparatus (Bio-Rad). The capacitance was set at 50 μF and no shunt resistor was used. The transformed cells were recovered overnight in the dark at room temperature and then selected on TAP/agar plates containing 20 μg/ml paromomycin.

SDS-PAGE and western blotting analysis Single paromomycin-resistant *C. reinhardtii* colonies were selected and inoculated into each well of a 96-well plate (250 μl TAP medium per well) and then incubated under continuous illumination to reach a cell density of about 4 × 10^6 cell/ml for each well at room temperature. Cells were centrifuged and resuspended in 25 μl of water and then mixed with 25 μl of 2 × SDS-PAGE loading buffer (Solarbio, Beijing, China) per well and boiled for 5 min. The samples were then separated on a 10% (w/v) SDS-PAGE gel by electrophoresis. After the protein samples were electrotransferred onto a nitrocellulose membrane (0.45 μM, PALL, New York, NY, USA), the membrane was then blocked with blocking buffer (5% non-fat dry milk in TBST (10 mM Tris, pH 7.4, 166 mM NaCl) plus 0.05% Tween 20) for 1 h at room temperature. The membrane was then incubated with primary rat anti-HA antibody (1:1000) (Roche, Basel, Switzerland) in blocking buffer for 1 h at room temperature followed by three washes with TBST. The membrane was then incubated with HRP-conjugated goat anti-rat secondary antibody (1:10,000) (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted in the blocking buffer for 1 h at room temperature. After washing three times with TBST, the membrane was then coated with Immobilon western chemiluminescent HRP substrate (Millipore, Darmstadt, Germany). A Mini Chemiluminescent Imaging apparatus (Sage Creation, Beijing, China) was then used to capture the signal.

Inhibition zone assay The effect of temperature, pH and proteinase on 1.5 μg (~0.065 μM) of 3 × Mytichitin-A dissolved in 50 μl solution was determined by measuring the anti-*L. monocytogenes* (ATCC 21633) activity in inhibition zone assays as described previously (12). Before the inhibition zone assays were performed, 3 × Mytichitin-A was incubated at 4 °C, 25 °C, 37 °C, 65 °C, or 90 °C for 1 h to determine the tolerance of 3 × Mytichitin-A to temperature. The lyophilized 3 × Mytichitin-A was first dissolved in Tris/HCl buffer at various pH values of 2, 4, 6, 8, or 10 before incubation with the tested bacteria for inhibition zone assay. To determine the tolerance of 3 × Mytichitin-A to proteinase digestion, 3 × Mytichitin-A was incubated with 0.2 units of papain, pepsin, proteinase K, or trypsin at 37 °C for 2 h before the inhibition zone assays were performed. For all experiments, a protein extract from the wild-type CC-125 strain containing an empty expression vector was used as a negative control and gentamicin (50 μg/ml) was used as a positive control. All experiments were performed in triplicate.

Quantification of 3 × Mytichitin-A by ELISA assay An ELISA assay was performed to quantify the 6 × His-tagged 3 × Mytichitin-A expression as described previously (26). Briefly, purified 6 × His-tagged GFP proteins (>97% purity) at different concentrations of 0, 50, 100, 200, 400, and 800 ng/ml were used to create a standard curve. After 6 × His-tagged GFP protein (100 μl/well) and the total soluble protein of *C. reinhardtii* (100 μl/well) were used to coat the 96-well plate, the primary mouse anti-6 × His monoclonal antibody (1:1000 dilution) were added into each well and incubated for 2 h at 37 °C. After washing with TBST three times, HRP-conjugated goat anti-rat IgG (1:10,000) was then added into each well and incubated for 2 h at 37 °C followed by washing with TBST three times. To perform the enzyme reaction, 100 μl of tetramethylbenzidine substrate solution (CoWin Biosciences, Beijing, China) was added into each well and incubated for 25–30 min at room temperature. The reaction was terminated by the addition of 50 μl of 0.5 M H_2SO_4. The optical density at 450 nm (OD 450 value) was then measured by using a SynergyH1/H1MF ELISA reader (BioTek, Winooski, VT, USA). All experiments were performed in triplicate.

Affinity purification and gel filtration chromatography For the purification of 3 × Mytichitin-A, the 3 × Mytichitin-A-expressing transgenic *C. reinhardtii* cells were cultured in 5 L of TAP medium at 23 °C under continuous illumination until the cell density reached 3–6 × 10^6 cells/ml. After washing with HEPES buffer (pH 7.4) three times, cells were resuspended in 25 ml of G-buffer (20 mM Tris, 50 mM NaCl and 5 mM $MgCl_2$, pH 7.4). After sonicating completely, the insoluble materials were discarded after centrifugation at 12,000 ×*g* for 15 min at 4 °C. The soluble proteins were mixed with 2 ml of Ni-NTA resin equilibrated with G buffer and incubated with shaking at 4 °C overnight. After extensive washing with G-buffer, the elute was collected using elution buffer (G buffer containing 500 mM imidazole). The purified proteins were then concentrated using an Amicon Ultra centrifugal filter with a molecular weight cut-off of 3 kDa (Millipore, Billerica, MA, USA) (25). The eluted material was then separated using a Superdex 200 10/300 GL column (GE Healthcare) according to the manufacturer's instructions.

MIC assay MIC refers to the minimum concentration of the peptide required to inhibit the growth of tested bacteria. In this study, *E. coli* O157 (ATCC 35150), *S. aureus* (ATCC 25923), *L. monocytogenes* (ATCC 21633), and *S. enteriditis* (ATCC 10467) were used to determine the bacteria-specific MIC value of the purified 3 × Mytichitin-A. Briefly, 3 × Mytichitin-A was diluted with PBS to different concentrations (10–120 μg/ml). Twenty micro litter of each dilution was then added into each well of a 96-well plate. After 100 μl of the mid-log bacterial suspension (2–7 × 10^5 CFU/ml) was distributed into each well of the 96-well plate, the plate was shaken at 220 rpm at 37 °C for 12 h. The OD600 value was then measured using a SynergyH1/H1MF ELISA reader (BioTek). All assays were performed in triplicate.

MTT assay The MTT assay was used to detect the cytotoxicity effect of 3 × Mytichitin-A on HEK293 cells. To do this, various concentrations (0, 20, 30, 45, 60 and 80 μg/ml) of 3 × Mytichitin-A were incubated with HEK293 cells in 96-well plates. After 72 h incubation, 20 μl of MTT solution (5 mg/ml in 1 × PBS) was added to the plates and the plates were then incubated for another 3 h. DMSO was then used to dissolve the formazan crystals and the optical density (OD) at 570 nm was read by a SynergyH1/H1MF plate reader (BioTek). The percentage of cell viability was calculated according to the standard formula as follows: Cell viability (%) =OD sample/OD control. All experiments were performed in triplicate.

RESULTS

HA-6 × His double-tagged 3 × Mytichitin is expressed stably in *C. reinhardtii* The vector pHSP-RBCS2-3 × Mytichitin-A-PARO, which directs the expression of a C-terminal HA-6 × His double-tagged tandem multimer of Mytichitin-A, containing three copies of Mytichitin-A was transformed into *C. reinhardtii*. After initial screening by Western blotting assay using an anti-HA antibody that specifically recognized the HA-6 × His tagged 3 × Mytichitin-A, 8 out of 100 paromomycin-resistant *C. reinhardtii* transformants were found to express the 3 × Mytichitin-A peptide with an expected molecular weight of 25 kDa (Fig. 1A). These transformants showed different levels of expression. When the expression of 3 × Mytichitin-A-expressing transformant with the lowest expression of 3 × Mytichitin-A (the 4th transformant) was set to 1, the expression in the other 3 × Mytichitin-A-expressing transformants was between 1 and 4-fold higher expression (Fig. 1B). To determine the yield of 3 × Mytichitin-A in the highest 3 × Mytichitin-A-expressing transformant (the 2nd transformant, named as 3 × Mytichitin-A/CC-125 hereafter), an ELISA assay was performed as described in the materials and methods section. The 3 × Mytichitin-A yield was 0.28% of total soluble proteins of 3 × Mytichitin-A/CC-125 cells, a yield rate similar to that of other recombinant proteins in *C. reinhardtii* as reported previously (25) (Fig. 1C). Together with the observation that the growth of 3 × Mytichitin-A/CC-125 was not altered as compared to the wild-type counterpart (Fig. 1D) and the 3 × Mytichitin-A level did not

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