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Peptide ABMOK1 designed from extracellular polymer substance (EPS) of *Nitzschia* sp. (Bacillariophyceae): Disruption of the membrane completeness of *Escherichia coli*

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Abstract

Peptide ABMOK1 is composed of 12 amino acid and can interact with the membrane of Escherichia coli. We have carried out isolation, sequencing, prediction, and synthesis of ABMOK1, which is a peptide of membrane activity. LC-MS/MS mapped the ABMOK1 primary structure, CGYCGACVGVCK; SOPMA predicted secondary structure and random coil, while CAMP was used to predict the antimicrobial probability. LC-MS/MS, SOPMA, and CAMP from this study provide the foundation for comprehensive functional and structural characterization of the peptide ABMOK1. That is first time public peptide from *Nitzschia sp.* ABMOK1 was interacted with membrane of *Escherichia coli* with the features of an unstable membrane. The results may help point to new directions for future peptide research with benthic diatoms.

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Introduction

Pathogenic bacteria are resisted by a type of peptides called antimicrobial peptides (AMPs). AMPs generally exist in organisms, including the space between prokaryotic and eukaryotic cells. AMPs have a molecular weight lower than 10 kDa and amphipathic peptides of variable length (Smith et al. 2010). These amphipathic peptides contain cationic and hydrophobic residues that interact with microbial membranes (Brandenburg et al. 2012) through non-specific interactions with the PG/PE lipids (Arouri et al. 2009). Structural classification of AMPs include a-helical (such as cecropins), cysteine-rich and β-sheet (such as defensins), and rich in His, Arg, Pro, and Trp (tritripticin) (Reddy et al. 2004). Cysteine residue is frequently found in the hydrophobic interior of soluble proteins or as a constituent of catalytically active sites (Creighton 1992). Cysteine cross-linking provides information on proximity relationships in protein and protein complex. Estimation of intramolecular proximity relationships requires the introduction of pairs of cysteine residues at positions of interest since intermolecular distances can be assessed by cross-linking cysteine residues individually introduced at the surface of interacting proteins (Krämer and Jung 2010).

Biofilms are generally found in environments where water exists, and diatoms are general components of a slippery coating that develops on artificial surfaces in marine environments. The micro-biofilm development process consists of two phases in marine environments: initially there is a bacterial phase, and after a few days this changes to to a diatomaceous phase (Molino et al. 2009). Benthic diatoms have developed a ripely secreted system, as raphe; one that is composed of extracellular polymer substances (EPS) that are peptides with molecular weight lower than 10 kDa, secreted by Achnanthes longipes (Wustman et al. 1998). The functions of this group of peptide are still unknown but are presumably related to adhesion and motility, communication, adaptation to

the environment, and protection against toxic influences from the environment.

In this paper, we analyze one of the EPS peptides, ABMOK1, from *Nitzschia sp.* The analysis uses Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS), which mapped the sequences of all the peptides in the same fraction and also produced predictions for AMPs' characteristics to function as peptides. There is only one chance to analyze sequences of all the peptides which molecular weight was very closely. A commercial method makes accurate sequences and high purity and quantity by peptide synthesis.

Materials and Methods

Morphology by SEM

The samples were further dried and prepared according to standard procedures. The fixative used was 2.5% glutaraldehyde/phosphate buffer solution; samples were steam-dried steam with Critical Point Drying, CPD (Emitech K850, Quorum, United Kingdom) and were gold coated by an Ion Sputter Coater (Hitachi E1010, Japan). Then specimens were examined by a scanning electron microscope (SEM, Hitachi S-3000N, Japan) at magnifications ranging from X 8000 to X 35000 to assess ultrastructural features of diatoms and bacteria.

Total proteins and EPS proteins extraction

Proteins were extracted by cell homogeneous buffer (10 mM Tris-HCl, pH 9.0, 1 mM EDTA, 6 mM MgCl2). Upon cell lysis, sonication was performed using an XL2020 sonicator (setting 5, 10 × 15-s bursts, 1-min rests on ice) (Misonix Inc., Farmingdale, NY). The cell debris was separated by centrifugation at 21,500 rpm for 30 minutes at 4^{o}C. The supernatant was total proteins. The cell debris that had become a pellet after centrifugation was removed from the pigment by 95% ethanol and diluted ethanol by deionized water. EPS proteins were extracted from the cell debris by 0.5M NaHCO3 that was modified as in a previous report (Wustman et al. 1997).

Peptides isolated, sequenced and synthesis

The sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) had three layers composed of one stacking gel of 5% and two resolving gels of 15% and 20%. The gel was prepared to disperse peptides of small molecular weight. Proteins and peptides were run and isolated by SDS-PAGE at 150 V and 50 mA for 100 min. Gels were stained by Coomassie blue. Peptides of small molecular weight were excised from the gel and stain-stripped three times in 50% acetonitrile/25 mM ammonium bicarbonate, pH 8.0, dehydrated with 100% acetonitrile, and dried in an incubator for 5 min at 37oC. Gel pieces were rehydrated with a solution of 50% acetonitrile/1% trifluoroacetic acid. LC-MS/MS was analyzed by Center for Research Resources and Development of KMU. ABMOK1 synthesized by commercial composite (GMbiolab, Taiwan).

Physicochemical characterization

For physicochemical characterization, Expasy's ProtParam server (Gasteiger et al. 2005) was used to calculate the number of amino acids, molecular weight, theoretical isoelectric point (pI), total number of positive and negative residues, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY).

Predict secondary structure and antimicrobial peptides

SOPMA (Self-Optimized Prediction Method with Alignment) (Geourjon and Deleage 1995) was used to calculate the secondary structure feature of ABMOK1. CAMP (collection of antimicrobial peptides) release 2 (Waghu et al. 2014) is provided as a prediction tool for users and it estimates the probability of antimicrobial peptides. The prediction algorithms for antimicrobial peptides are incorporated in the CAMP. These are based on SVM (Support Vector Machines), RF (Random Forests), ANN (Artificial Neural Network) and DA (Discriminant Analysis).

Antimicrobial testing

There were two species of bacteria examined by ABMOK1 peptide. *Escherichia coli* (DH5α) and *Staphylococcus aureus* (ATCC 25923) were examined the cell density at 3 X 107 cells per mL, treated by ABMOK1 peptide of 100 μg/mL in Luria broth (LB) medium for 24 hours at 37 ^{o}C. There were two groups: the control had 4 μL of 25% acetonitrile (ACN) added to 1 mL of LB broth, while the treatment group had ABMOK1 peptide of 100 μg (25 μg/1 mL) added to LB broth.

Results and Discussion

Many different components of EPS are secreted from benthic diatoms in the marine substratum. This specimen was sampled from seawater and it was an example of *Nitzschia sp.* The EPS of *Nitzschia sp.* was shaped like a small ball which had viscosity, while the EPS constructed different shapes that adhered to each other (Figure 1 A). The EPS could help *Nitzschia sp.* attach to the substrate surface and it has been proposed that the forces exerted by continuous secretion against the points of adhesion provide the motive force for locomotion (Drum and Hopkins 1966). We collected the intracellular proteins and EPS proteins, and analyzed them by protein electrophoresis. The sizes of intracellular proteins were quite diverse, and most of the EPS proteins were below 6.5 kDa.

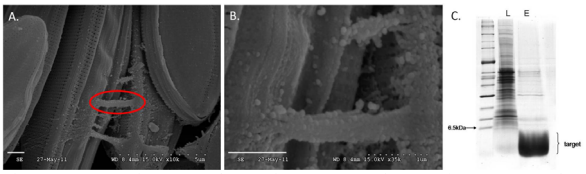

Figure 1: SEM image and protein patterns of *Nitzschia* sp. (A) The bridge-like structure formed by EPS. The region circled in red is expanded in B. (C) The SDS-PAGE resolving the intracellular (L) and EPS (E) proteins. Scale bars = 1 μm.

Identification of the microorganism

Parameters of ABMOK1 peptide were calculated by ExPaSy's ProtParam tool with results as shown in Table 1. ABMOK1 is a peptide of 12 aa, CGYCGACVGVCK;, and its total net charge is +1, which can help attach to negative molecules on the *E. coli* membrane. According to the index definition, ABMOK1 is a stable and hydrophobic peptide. The secondary structure of ABMOK1 peptide was predicted by SOPMA (Self Optimized Prediction Method with Alignment), and this structure shows 100% random coils. The C-terminal, basic lysine contains an ε-amino group (NH3+) that often participates in hydrogen bonding and serves as a general base

in catalytic reactions. Another noteworthy feature of ABMOK1 is that it has four cysteines, almost evenly spaced, which may form disulfide-bonds and which link multiple ABMOK1 peptides into a coiled form that has a stronger structure. In addition, the thiol side chains can also participates in enzymatic reactions, serving as nucleophiles. Those hydrophobic amino acids may interact with fatty acid tails of the membrane.

Table 1: ABMOK1 parameters calculated using ExPaSy's ProtParam tool.

Parameters	ABMOK1
Number of amino acid	12
Molecular weight	1162.4
pI	7.88
Negative residues	0
Positive residues	1
Estimated half-life (hours)	>10 hours (*Escherichia coli*, in vivo)
Instability index	18.27
Aliphatic index	56.67
GRAVY	1.15

The prediction algorithms resulted in non-AMP, SVM, and others resulted in AMP (Table 2). In all the algorithms, DA was a chief consideration, while discriminant function analysis is a classification based on the act of distributing a new one into group of AMP function or not.

Table 2: CAMP R2 server prediction of AMP probability

Prediction algorithms	Class	AMP probability
SVM	Non-AMP	0.295
RF	AMP	0.5465
ANN	AMP	--
DA	AMP	0.978

No clear effect of ABMOK1 (100 μg/mL) on the growth of *E. coli* was seen, probably due to the peptide's high affinity to the wall of culture tubes. However, we noticed that dots of aggregated *E. coli* formed on the tube wall in the presence of ABMOK1. SEM images were taken from ABMOK1-treated *E. coli* aggregated on the tube wall (Figure 2 A), ABMOK1-treated *E. coli* suspended in culture medium (Figure 2 B), and untreated *E. coli* as a control (Figure 2 C) to examine their morphologic features. ABMOK1-treated *E. coli* that was isolated from aggregates on the tube wall showed features of an unstable membrane, such as membrane adhesion (Figure 2 a), punctured holes on the membrane (Figure 2 b), and collapsed cellular structure (Figure 2 c). ABMOK-1 may also affect the cell division of non-aggregated *E. coli*, since most of the suspension cells taken from the ABMOK1-treated culture were abnormally elongated (Figure 2 B) in comparison to untreated *E. coli* (Figure 2 C). ABMOK1 would aggregate to tube wall of culture and the LB broth phase had a concentration too low to kill *E.coli*. The growth curve for *E. coli* showed no significant difference (data not shown). ABMOK1 were not affected by *Staphylococcus aureus* in SEM image and growth curve (data not shown).

Conclusions

The statistical results for an antimicrobial peptide from CAMP for the taxonomy of a resource organism show that only 0.02% is contributed from algae (1/5040). Traditional isolation and sorting systems (such as molecular sieve chromatography or C-18) cannot solve problems that are in the same fraction, and there has been little research to discuss how diatoms could produce antimicrobial peptide. We here show that the *Nitzschia sp.* of benthic diatoms could secrete numerous small peptides. Moreover, we found that one of the peptides that could destroy *E. coli* membrane was the

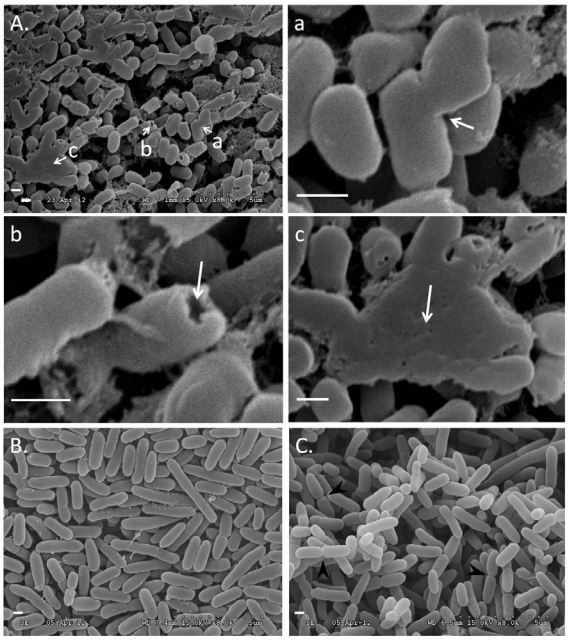

Figure 2: SEM image of *Escherichia coli* treated by ABMOK1. (A) *E.coli* collected on the tube wall of a culture that had been treated by ABMOK1 (100 μg/mL). The areas a, b, and c in A are expanded in the images labeled respectively. Area "a" shows the membrane of two cells with abnormal fusion (arrow), "b" shows punctured holes in the membrane (arrow), and "c" shows collapsed cellular structure (arrow). (B) *E. coli* collected from the same tube as A but with suspension bacteria. (C) *E. coli* collected from LB with 25% ACN of 4 μL, marked with the normal of the cell division. Scale bars = 0.5 μm.

ABMOK1 peptide. This study shows that *Nitzschia sp.* is a good species to consider using for an antimicrobial peptide.

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