Fish & Shellfish Immunology 41 (2014) 362–370

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Characterization of a novel antimicrobial peptide with chiting-biding domain from *Mytilus coruscus*

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ARTICLE INFO

Article history:
Received 27 June 2014
Received in revised form
1 September 2014
Accepted 14 September 2014
Available online 22 September 2014

Keywords:
Mytilus coruscus
Antimicrobial peptide
Chitin-biding domain
Chitinase

ABSTRACT

Using reverse phase high performance liquid chromatography (RP-HPLC), a novel antimicrobial peptide with 55 amino acid residues was isolated from the hemolymph of *Mytilus coruscus*. This new antimicrobial peptide displays predominant antimicrobial activity against fungi and Gram-positive bacteria. The molecular mass and the N-terminal sequence of this peptide were analyzed by Mass Spectrometry and Edman degradation, respectively. This antimicrobial peptide, with molecular mass of 6621.55 Da, is characterized by a chitin-biding domain and by 6 Cysteine residues engaged in three intra-molecular disulfide bridges. The full-length of cDNA sequence of this new peptide was obtained by rapid amplification of cDNA ends (RACE) and the encoded precursor was turn out to be a chitotriosidase-like protein. Therefore, we named the precursor with mytichitin-1 and the new antimicrobial peptide (designated as mytichitin-CB) is the carboxyl-terminal part of mytichitin-1. The mRNA transcripts of mytichitin-1 are mainly detected in gonad and the expression level of mytichitin-1 in gonad was up-regulated and reached the highest level at 12 h after bacterial challenge, which was 9-fold increase compared to that of the control group. These results indicated that mytichitin-1 was involved in the host immune response against bacterial infection and might contribute to the clearance of invading bacteria.

1. Introduction

Antimicrobial peptides (AMPs), which constitute a first line of host defense against pathogens, are widely expressed in organisms and have been linked to innate immunities in invertebrates. Marine molluscs are widespread, extremely diverse, and constantly under an enormous microbial challenge from the ocean environment. In order to defend themselves against such conditions, marine molluscs have developed very effective mechanisms that are part of their innate immunity [1]. AMPs are the major component of the innate immune system in molluscs [2–4] and antibacterial activity was first reported in mollusks in the '80s [5]. Most of the AMP researches of marine mussels has focused on species *Mytilus*, including *Mytilus edulis* and *Mytilus galloprovincialis*, whereas the isolation and characterization of true AMPs from marine mussels date back to 1996 [6,7].

Among the various natural AMPs, those containing pairs of cysteine residues forming intra-molecular disulfide bridges are particularly common [8–11]. In the last decades, AMPs have been isolated and characterized from marine mussels and classified into seven groups: defensins [6], mytilins [12], myticins [13,14], mytimycin [15], mytimacin [16], big defencin [16], and myticusin [17]. These AMPs have also commonly been identified and characterized in other marine bivalve mollusk. For example, defensins have been described in oysters *Crassostrea virginica* and *Crassostrea gigas* [18,19] and abalone *Haliotis discus* [4]; mytilins and myticins were also described in clams *Ruditapes decussates* [20]. These peptides belong to the cysteine-rich family of AMPs with strong activity against bacteria, fungus and virus [21–23], making them not only a promising template molecule for investigating the relationship between protein structure and function, but also a source of new leads for the treatment of bacterial disease [23].

The molecular diversity of AMPs increases the antimicrobial capabilities of mussels against a high diversity of pathogens. However, AMPs have been explored only in a few species. Therefore, there is still a great potential to unveil new molecules in this phylum. *Mytilus coruscus* is one of the most important economic

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http://dx.doi.org/10.1016/j.fsi.2014.09.019
1050-4648/

shellfish and widely distributed in eastern coast in China. Several antimicrobial peptides have been characterized from *M. coruscus* in our previous work, including mytilins (FJ973154 ~ FJ973155 for mytilin-A and -B; GU324718~GU324723 for mytilin3~8), myticins (GU324724~GU324732 for myticin1~9), and a big antimicrobial peptide named myticusin-1 [17]. Here we present the isolation and biochemical characterization a novel antimicrobial peptide, mytichitin, from hemolymph of *M. coruscus*.

2. Material and methods

2.1. Animals and hemolymph collection

Adult mussels (*M. coruscus*) were collected locally from Zhoushan (Zhejiang province, P. R. China) and were maintained in tanks with fresh sea water at 25 °C. Hemolymph was prepared using the methods described by Mitta [12], the plasma was clarified by centrifugation (12,000 × g, 20 min, 4 °C) and then kept at −80 °C until further analysis.

2.2. Antimicrobial assays

Bacteria and fungus (about 1–2 * 10^8 cfu/mL) used in present study were obtained from China General Microbiological Culture Collection Center (CGMCC, Beijing, P. R. China). The bacterial strains were *Escherichia coli* (CGMCC1.1583), *Vibrio Parahaemolyticus* (CGMCC1.1616), *Pseudomonas aeruginosa* (CGMCC1.0102), *Proteus vulgaris* (CGMCC1.1527), *Vibrio.harveyi* (CGMCC1.1601), *Bacillus subtilis* (CGMCC1.1630), *Staphylococcus aureus* (CGMCC1.128), *Sarcina luteus* (CGMCC28001), and *Bacillus megaterium* (CGMCC1.1487). The fungus used here were *Candida albicans* (CGMCC2.2086) and *Monilia albican* (CMCC(F)98001). Antimicrobial activity was monitored by a liquid growth inhibition assay according to the method described previously [17]. During the various steps of peptide purification, antibacterial activity was monitored on the Gram-positive strain *S. luteus* and the Gram-negative strain *E. coli*.

To determine the minimal inhibitory concentration (MIC), serial doubling dilutions were carried out following the protocol described by Mitta [12]. The MIC values are expressed an interval (a–b), where (a) represents the highest peptide concentration tested at which bacteria are still growing and (b) the lowest concentration that causes 100% growth inhibition.

2.3. Peptide purification

All HPLC purification steps were carried out on a Waters Delta 600 HPLC system equipped with a Waters 2487 absorbance detector. Fractions were collected and assayed for antimicrobial activity.

The plasma sample was directly subjected to a Sunfire™ prep C8 column (10 × 250 mm Waters) equilibrated with deionized water containing 0.1% trifluoroacetic acid. The sample was eluted with a gradient of 0–5% acetonitrile containing 0.1% trifluoroacetic acid over 5 min followed by 5–60% over 40 min and 60–95% over 5 min at a flow rate of 2.5 mL/min. The eluted fractions were collected manually, dried under vacuum, and reconstituted in MilliQ water (prepared by Millipore synergy system, France). The presence of antibacterial activities was detected by the liquid growth inhibition assays described above. Active fractions were applied to an analytical Vydac C18 RP HPLC column (218TP54, 4.6 × 250 mm) and eluted at the flow rate of 0.75 mL/min using 5–45% acetonitrile/water/0.1% trifluoroacetic acid as a linear gradient. To obtain the peptides with high purity, an additional step was performed on a SunFire™ C18 Column (100 Å, 3.5 μm, 3 × 150 mm, Waters) developed in the same diphasic gradients as above but at a flow rate of 0.25 mL/min. The eluted fractions with antimicrobial activities were vacuum-dried and used for determination of molecular mass and amino acid sequences.

2.4. Peptide sequencing and modeling

The molecular mass of peptide was determined using Matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (Voyager-DE STR biospectometry workstation, Applied Biosystems). Purified peptide was submitted to reduction and alkylation, and Edman degradation was performed with a normal automatic cycle program using an Applied Biosystems Model 491 gas-phase sequencer.

The 3-Dimensional structure of peptide was predicted from amino acid sequences by SWISS-MODEL platform on line (http://swissmodel.expasy.org/interactive).

2.5. The full-length cDNA cloning

The amino acid sequence detected from Edman sequencing was searched against the dbEST database of *Mytilus* using tblastn search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The matched EST sequence with high similarity was used as the template for designing specific primer of PCR amplification. Using Rapid Amplification cDNA Ends (RACE) method; the full length of cDNA was obtained by SUPERSWITCH™ RACE cDNA Synthesis Kit (Shanghai, China) according to the manufacturer's instructions. For 3'-RACE, a sense primer (5'-TAAAATGTGGCATGAATGG-3') and a 3'-Adaptor primer were used; Meanwhile, The antisense (5'-GGGTCTTGTATGGAATCC-3') corresponding to the 3'-end of amplified the cDNA fragment, together with 5'-Adaptor primer was used to obtain the cDNA 5'-end. The full-length cDNA was confirmed by specific primers (sense: 5'-CAGACCTACACTGTTCACGATGAT-3', antisense: 5'- GTTCTCAATCTAATATTCCAAAGTCA-3') designed in the 5'- and 3'-UTR, respectively, from sequences retrieved through RACE assays.

The PCR was performed in 25 μL volume, including of 2.5 μL 10 × SUPERSWITCH™ Hot Start Buffer, 0.1 μL of each primer (10 μM), 2 μL dNTP Mix (2.5 mM), 0.5 μL of the cDNA template, and 0.5 μL SUPERSWITCH™ Hot Start DNA polymerase for 41 cycles on a Thermal Cycler (Bio-Rad, USA). Each cycle consisted of 30 s at 94 °C, 30 s at 60 °C, and 120 s at 72 °C, with a final extension of 5 min. The PCR products were subjected to 1.0% agarose gel electrophoresis, purified, cloned into pMD19-T vector (TaKaRa), and transformed into competent DH5α.

The resulting cDNA sequences were analyzed using the software of DNAstar lasergen 7.0 after nucleic acid sequencing. Signal peptides were predicted using the SignalP 4.0 [24] online tool (http://www.cbs.dtu.dk/services/SignalP/) and conserved domains were predicted using simple modular architecture research tool (SMART) software (http://smart.embl-heidelberg.de/). Following peptide signal removal, the theoretical MW and *pI* were determined on Expasy-ProtParam online tool (www.expasy.org/tools/protparam.html). Multiple sequence alignments were performed with DNAMAN (version 7.0.2.176). Phylogenetic analyses based on the amino acid sequences were conducted by MEGA 6.0 using Maximum Likelihood algorithm with 1000 bootstrap replicates.

2.6. Quantitative analysis of mytichitin-1 expression

The tissue-specific expression and the temporal expression of transcripts in mussels challenged with *S. luteus* were determined by quantitative real-time PCR. Mussels of 5–6 cm shell length were collected from East China Sea. Total RNA was individually purified