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Determination of disulfide linkages in antimicrobial peptides of the macin family by combination of top-down and bottom-up proteomics

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ABSTRACT

Macins are a distinct class of antimicrobial peptides (AMPs) produced by leeches and *Hydra*. Their function depends strongly on their three-dimensional structure. In order to support structural elucidation of these AMPs, the knowledge and proper assignment of disulfide bonds formed in these cysteine-rich peptides is a prerequisite. In this report, we outline an analytical strategy, encompassing a combination of top-down MS based analytics and sequence-dependent enzyme cleavage under native conditions followed by high mass accuracy and high resolution MS/MS analysis by LTQ-Orbitrap MS to assign disulfide linkages of three members of the macin family, namely neuromacin, theromacin, and hydramacin-1. The results revealed that the eight cysteine residues conserved in all three macins form the same four disulfide bonds, i.e. [C1:C6], [C2:C5], [C3:C7], and [C4:C8]. Theromacin, which possess two additional cysteine residues, forms a fifth disulfide bond.

Biological significance
Beside the high biological significance which is based on the inherent dependence of biological activity on the structural features of antimicrobial peptides (which holds true for entirely every protein), the presented analytical strategy will be of wide interest, as it widens the available toolbox for the analysis of this important posttranslational modification.

1. Introduction

Antimicrobial peptides (AMPs), a diverse group of molecules consisting of less than 100 amino acid residues are expressed in almost all species, ranging from bacteria and fungi to animals and plants [1]. Up to now, more than 2300 AMPs have been identified or predicted [2]. These "evolutionarily ancient weapons" [3] play an important role in the innate immune system. In addition to their antimicrobial activities, they have been shown to be involved in diverse biological functions, such as inflammatory processes or neuronal regeneration [4]. Hydramacin-1 (HM-1) [5], theromacin (TM), and neuromacin

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(NM) are three AMPs that belong to the family of macins. Despite of the high sequential identity including eight conserved cysteine residues, as shown in Fig. 1, these three polypeptides show significant differences regarding their biological activities [5,6]. As disulfide bond formation acts in stabilizing protein tertiary structures and maintaining biological activity [7], the determination of protein disulfide bonds becomes an essential step to comprehensively characterize cysteine-rich polypeptides.

Assignment of disulfide linkages can be a challenging task, as the number of theoretically possible disulfide bond combinations grow exponentially with the increasing number of cysteine residues. For example, there are a total of 105 or 945 theoretical combinations (i.e., $7\times5\times3\times1$ or $9\times7\times5\times3\times1$) to form four or five internal disulfide bonds from eight or ten cysteine residues, respectively. Proper assignment can become even more challenging when disulfide bonds are formed from closely spaced cysteine residues [8]. Moreover, the presence of free thiol(s) in the molecule and/or exposure of disulfide-linked proteins/peptides in extreme pH can easily induce disulfide scrambling [9].

Methodologies such as NMR and X-ray crystallography have greatly enhanced our knowledge of the three-dimensional structure of proteins including the presence and structural importance of disulfide bonds with minimum disulfide bond interexchange [10]. However, these approaches require a substantial quantity of highly purified protein. Alternatively, modern mass spectrometry coupled with separation techniques has emerged as an informative tool for disulfide bond assignment [11].

In the present study, a simple and straightforward method comprising single or successive enzyme cleavages of non-reduced native protein followed by high resolution and high mass accuracy MS and MS/MS analysis on an LTQ-Orbitrap Velos MS was developed to unambiguously assign the disulfide linkages of three macins. The data obtained by the MS-based assignment of the disulfide bonds delivered important information for the elucidation of the accurate structures of the macins based on three-dimensional NMR [6].

2. Experimental sections

2.1. Chemicals and reagents

Trypsin was purchased from Promega (Madison, MI); endoproteinase Asp-N, formic acid, acetonitrile, and HEPES were from Sigma-Aldrich (St. Louis, MO); water used for all experiments was purified by an arium611VF System (Sartorius, Göttingen, Germany).

2.2. Sample description

Hydramacin-1 from *Hydra magnipapillata*, theromacin, and neuromacin from *Hirudo medicinalis* were recombinantly expressed in the *Escherichia coli* strain BL21 (DE3) as fusion proteins with a thioredoxin-His_6 tag via an enterokinase cleavage site N-terminal to the mature protein; expressed fusion proteins were purified, re-folded and the fusion proteins were cleaved at 37 °C overnight using EnterokinaseMax™ (Invitrogen). Purification of mature hydramacin-1, theromacin, and neuromacin was performed using RP-HPLC on a semi-preparative C18 column (Macherey-Nagel, Düren, Germany) as described before [6].

2.3. Enzymatic cleavage

Depending on the protein sequences, either single enzyme (i.e., trypsin for hydramacin-1 and neuromacin) or successive protease-catalyzed hydrolysis (i.e., trypsin and Asp-N for theromacin) was performed to cleave the purified macins. HPLC-purified macins were diluted with 50 mM HEPES (pH 7.0) to a final concentration of about 2.0 pmol/μL. Trypsin digestion was performed by adding 1 μL (50 ng) endoproteinase trypsin to 10 μg protein solution (enzyme:protein = 1:200 w/w) at 37 °C for overnight incubation. A two step enzymatic hydrolysis of theromacin was performed by adding 1 μL (10 ng) of endoproteinase Asp-N (enzyme:protein = 1:1000 w/w) to the tryptic digests for another 6 h at 37 °C. The digestion was terminated by addition of 5% formic acid and the cleavage products were stored at −20 °C until used for further analysis. A total amount of ca. 1.3 pmol of peptide digests was used for LC-MS/MS analysis.

2.4. Liquid chromatography and mass spectrometry

Peptide separations were carried out with an integrated nano-HPLC system (model UltiMate3000, Thermo Fisher Scientific, Idstein, Germany) equipped with a trap column (Acclaim PepMap 100; 100 Å, 5 μm particle size, 300 μm i.d. × 5 mm; Dionex, Amsterdam, The Netherlands) and an analytical reverse phase C18 column (PepMap; 100 Å, 3 μm particle size, 75 μm i.d. × 15 cm; Dionex). A UV detector with a 45 nL z-shaped capillary detection cell (Model UltiMate, Dionex) was

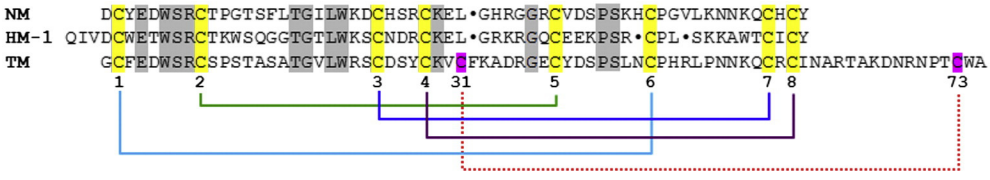

Fig. 1 – Sequence alignment of neuromacin (NM), hydramacin-1 (HM-1), and theromacin (TM). Residues conserved in all macins are highlighted in grey or yellow (cysteines). Four connection lines indicate the four predicted disulfide bonds ([C1:C6], [C2:C5], [C3:C7], and [C4:C8]) conserved in all three macins and the dashed line link two additional cysteine residues (highlighted in pink) in TM stands to a fifth disulfide bond ([C31:C73]).