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Isolation, identification and functional profile of excretory–secretory peptides from *Onchocerca ochengi*

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ABSTRACT

Parasitic helminths excrete or secrete a variety of functional molecules into the internal milieu of their mammalian hosts and arthropod vectors which reveal distinct immunomodulatory and other biological activities. We identified and initially characterized the low molecular weight peptide composition of the secretome from the filarial parasite *Onchocerca ochengi*. A total of 85 peptides were purified by liquid chromatography and further characterized by mass spectrometry. 72 of these peptides were derived from already described *Onchocerca* proteins and 13 peptide sequences are included in the sequence of uncharacterized proteins. Three peptides, similar to host defense peptides, revealed antibacterial activity. The present analysis confirms the putative involvement of low molecular weight compounds in the parasite-host cross-talk.

1. Introduction

Human onchocerciasis or river blindness, endemic in parts of Africa, Latin America and Yemen is caused by the nodule-dwelling filarial nematode *Onchocerca volvulus* (WHO, 1995). The cattle parasite *Onchocerca ochengi* represents today the most feasible *onchocercid* model parasite (Renz et al., 1995). Based on morphological and biological criteria, *O. ochengi* is the phylogenetically most closely related species to *O. volvulus*, and the two species share the same vector (Achukwi et al., 2004). These facts imply a high degree of similarity at the protein and genome levels (Trees, 2012).

In the present report, the investigation of low molecular weight peptides released by adult *O. ochengi* provides important information contributing to the understanding of the *Onchocerca*-host cross-talk. Furthermore, the helminths excretory–secretory products (ESPs) obviously represent a substantial reservoir of biologically active molecules with potential pharmacological applications. The capacity of parasites to modulate the host immune system is a relevant factor supporting their long-term survival in the mammalian host (Behnke et al., 1992; Maizels and Yazdanbakhsh, 2003). Hence, there is a particular interest to analyse, identify and characterize mediators released by parasites and to investigate how these products, in total and as individual components, influence and interact with the host system (Hewitson et al., 2009). These mediators can ligate, degrade or interact with host molecules or immune cells (Lightlowers and Rickard, 1988). In summary, they are composed of a mixture of different proteins, peptides, lipid mediators and polysaccharides and are termed ESPs (Hewitson et al., 2009; Dzik, 2006). However, the definition fails to distinguish between components actively secreted and

Abbreviations: ESPs, excretory/secretory products; HDM, helminth defense molecules; HDP, host defense peptides; LPS, lipopolysaccharides; RPC, reversed phase chromatography.

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compounds produced as a consequence of other physiological processes (Lightlowers and Rickard, 1988).

Parasites ESP and peptides homologous to host defence peptides (HDP) have been reported to modulate the immune response via molecular mimicry of host defence peptides thus providing anti-inflammatory activities commonly observed in helminth infections (Cotton et al., 2012; Robinson et al., 2011). HDP have distinct activities against bacteria, fungi, eukaryotic parasites and viruses (Mookherjee and Hancock, 2007). The potential of HDP to influence an infection is mediated by direct antimicrobial properties as well as by a modulation of the immune response (Bommarius et al., 2010). Thus, these peptides are known to neutralize LPS-mediated responses and prevent lethal endotoxemia (Giuliani et al., 2010). They also participate in inflammatory responses e.g. by acting as chemotaxins for immune cells (Chertov et al., 1996). HDP can promote phagocytosis while inhibiting oxidant responses of neutrophils or monocytes (Miles et al., 2009; Tecle et al., 2010), they stimulate wound healing as well as angiogenesis (Aarbiou et al., 2002; Murphy et al., 1993). Furthermore, HDP exhibit a modulatory effect on pathways regulating cell survival and apoptosis and they can induce the production of chemokines or other immune mediators (Mookherjee and Hancock, 2007). HDP are low molecular weight short peptides, composed of 12–50 amino acids, with an overall positive charge of +2 to +9 at neutral pH 7.0. Due to the predominance of basic amino acids e.g. arginine, lysine and histidine the interaction with negatively charged bacterial cell wall surfaces is supported (Hancock and Chapple, 1999). These peptides can be divided into subgroups on the basis of their amino acid composition and secondary structure (Hancock and Chapple, 1999; Gennaro and Zanetti, 2000) including cationic peptides rich in proline, arginine, lysine, histidine, phenylalanine or tryptophane, anionic compounds and fragments of larger proteins that arise through metabolic events (Otvos, 2002). The secretion of molecules mimicking HDP-type peptides with antibacterial activities was described before for helminths (Robinson et al., 2011). The objective of the present study was to identify peptides in ESPs of the filaria *O. ochengi* and to analyse these peptides for potential biological activity. Because of the limited amount of available experimental material obtained from the *O. ochengi*-endemic area in Cameroon, we could only focus towards analyzing anti-bacterial activities.

2. Material and methods

2.1. Peptide preparation

In the context of the Cameroonian-German Cooperation Project "Analysis of host-parasite cross-talk based on the bovine model for human onchocerciasis, *O. ochengi*" funded by the German Research Foundation (DFG, via project PAK296) adult female *O. ochengi* filariae were isolated from nodules extirpated from the skin of Gudali Zebu cattle slaughtered in the municipal abattoir in Ngaoundéré, Adamawa region, Cameroon. Pieces of the ventral skin from the inguinal region, where most of the nodules are found (Wahl et al., 1994) were shaved, thoroughly cleaned and antiseptically treated. Then the worm nodules were removed and individually incubated at 35 °C in 0.125% collagenase RPMI solution to digest the nodule capsule. Male and female worms were carefully cleaned from any debris and host tissue with PBS.

Only adult females of *O. ochengi* were used to reduce the gender influence in the peptide composition. Isolated intact and motile female worms were multiple times washed, pre-incubated for 1 h, then the medium changed and subsequently incubated individually at 37 °C for 96 h in RPMI media (Sigma-Aldrich, Taufkirchen, Germany), supplemented with 10 mM Hepes, 200 U/ml penicillin, streptomycin 200 μg/ml, 50 μg/ml gentamycin (pH 7.0–7.2). Every 24 h the supernatant was harvested and the medium was changed. After the incubation period, vitality of the nematode and sterility of the culture supernatants were checked microscopically and the microscope and on blood agar plates (Soblik et al., 2011). Only sterile cultures were used for further processing. Analyzed were supernatants of TCA-precipitated culture fluids from three experiments each performed using five adult female *O. ochengi*.

Proteins in the supernatant were precipitated by trichloroacetic acid (TCA)-treatment (10%) and removed by centrifugation. Only the unprecipitated low molecular fractions were used for further investigations. The peptides were separated from lipids, carbohydrates and salts using an Oasis HLB Plus cartridge (Waters 186000132, Milford, USA). The matrix was rinsed with 2 ml methanol, and equilibrated with 3 ml 0.2% formic acid. A 5 ml sample was loaded, and afterwards washed with 5 ml 0.2% formic acid. The flow through wash step was collected and stored at −20 °C for further investigation. Bound peptides were eluted with 1 ml 30% acetonitrile, 0.2% formic acid followed by 1 ml 60% acetonitrile, 0.2% formic acid. The desalted elution fractions were lyophilized and further separated via reversed phase chromatography (RPC). The lyophilized desalted samples were solved in 0.2% formic acid. Separation was performed using a chromolith® RP-18e 100 × 4.6 mm column (Merck Millipore). Buffer A (sample and washing buffer) consisted of 0.2% formic acid. Buffer B (elution buffer) consisted of 0.2% formic acid and 60% acetonitrile. In total 45 sample fractions were collected, lyophilized and stored at −80 °C until used for mass spectroscopic analysis. The peptide concentration of each fraction was determined by measuring the absorbance of the peptide bond at 205 nm according to the method of Scopes (1974) a standard curve of the peptide with the sequence SAVLQSGFRK (Genescript, USA) to calculate the concentrations of the peptide samples.

2.2. Bactericidal agar plate assay

E. coli BL 21 Star™ (DE3) (Invitrogen, Darmstadt, Germany) were used for the agar plate assay. The cells were cultured in LB Medium until OD_{600} reached a value of 0.4. Aliquots were placed on antibiotic-free LB agar plates. Subsequently the peptides were poured into a 60 mm-diameter wells of the LB plates and incubated for 12 h at 37 °C. Desalted peptide mixtures were used to analyze antibacterial activity and efficiency following a procedure described by Lin et al. (2010). Identified peptides with antibacterial activity were compared with so far known antibacterial peptides deposited within the LAMP (Zhao et al., 2013) and CAMP (Thomas et al., 2010) databases.

2.3. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Three replicates of MALDI-TOF and TOF/-TOF analyses were performed applying fresh purified *O. ochengi* secretome samples without proteinase inhibitors but processing the samples consequently at 4 °C to exclude proteolysis. Further, the samples for the MALDI-TOF and TOF/-TOF analyses were not tryptically digested.

These samples were lyophilized after reversed phase chromatography. Resolved in 30% acetonitrile, 0.1% trifluoroacetic acid (TFA) in H_2O and 0.75 μl and spotted on a MALDI target plate (MTP AnchorChip 384, Bruker Daltonik). After drying 0.75 μl MALDI matrix (0.7 mg/ml cyano-4-hydroxy-cinnamic acid (Bruker Daltonik)), dissolved in 85% acetonitrile, 1 mM $NH_4H_2PO_4$ and 0.1% TFA in H_2O were spotted on the sample dots. Data acquisition was performed in positive ion mode under the control of the FlexControl software 3.3. The instrumental parameters of the UltrafleXtreme MALDI-TOF-TOF-MS (Bruker Daltonik) were set as follows: ion source 1: 25 kV, ion source 2: 23.6 kV, lens: 7.5 kV. MS data were collected automatically using autoXecute. Parameters were set as

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