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Molecular and functional characterization of metalloserrulases, new metalloproteases from the *Tityus serrulatus* venom gland

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ABSTRACT

Tityus serrulatus is a Brazilian scorpion species with great medical significance. While the effects of neurotoxins have been extensively studied, little is known about the proteases expressed in the venom gland of this arthropod. In this study, clones from a *T. serrulatus* (Ts) venom gland cDNA library were selected according to homology to proteases. The sequences were aligned in the database and classified by homology. Similarity and identity analyses of the sequences were carried out, and a phylogenetic tree was constructed with the sequences of other proteases. These cDNA sequences correspond to ten different metalloproteases, named metalloserrulases (TsMS). TsMS 1–9 belong to the metzincin family, which has three domains: signal peptide, propeptide, and metalloprotease domain; while TsMS 10 belongs to the gluzincin family. The proteolytic activity of the venom was inferred from the cleavage of fibrinogen, and the residues recognized by the proteases were determined by cleavage of a tripeptide library using a fluorescence resonance energy transfer assay. The Ts venom showed proteolytic activity on fibrinogen and preferential cleavage close to the basic residues K and R. Its activity could be inhibited by EDTA, indicating that the venom from this scorpion predominantly consists of metalloproteases.

1. Introduction

There are several scorpion species in Brazil, but only species from the *Tityus* genus may cause serious injury to humans (Soares et al., 2002). The Brazilian yellow scorpion *Tityus serrulatus* stands out for its large population, parthenogenetic reproduction, high venom toxicity, and great adaptability to urban environments (Lourenço and Cloudsley-Thompson, 1999). The *T. serrulatus* population is more concentrated in cities, since the urban environment is one of its favorite niches, and causes numerous injuries due to its constant contact with humans (Torres et al.,

Abbreviations: MCA, 7-methoxycoumarin-4-acetic acid; TsMS, metalloserrulase; Ts, *Tityus serrulatus*; EDTA, ethylenediaminetetraacetic acid; DPA, N^b-(2,4-dinitrophenyl)-L-2,3-diaminopropionic acid; PMSF, phenylmethanesulfonyl fluoride; E-64, trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane.

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2002; Bortoluzzi et al., 2007; Ministério Da Saúde, 2001, 2009). *T. serrulatus* is broadly distributed in Brazil. In 2012, there were more than 63,000 accidents due to *T. serrulatus* in Brazil, representing 48% of all accidents involving venomous animals (Academia Brasileira de Ciências, 2010; Ministério da Saúde, SINAN, 2013). Depending on the severity of the case, scorpion envenomation may cause complications and lead to death. These animals are responsible for approximately 36% of deaths arising from accidents with venomous animals, demonstrating the importance of understanding scorpion envenomation in Brazil (Academia Brasileira de Ciências, 2010; Ministério da Saúde, SINAN, 2013).

T. serrulatus venom (Ts venom) is comprised of several substances such as mucus, salts, peptides, high molecular mass proteins, hyaluronidase, proteases, hypotensive peptides, nucleotides, lipids, and amino acids. Neurotoxins are considered the most toxic components of scorpion venom and are included in the peptide group. However, other protein components from the venom, such as hyaluronidases and proteases, also take part in the envenomation process (Gwee et al., 1996; Fukuhara et al., 2003; Cologna et al., 2009; Verano-Braga et al., 2008, 2010; Alvarenga et al., 2012; Horta et al., 2014).

Proteases can cleave proteins at specific locations based on the amino acid sequence, next to amino acid residues located at the N- or C-terminus, or randomly. Proteases are classified into four groups, according to the key amino acid (serine, cysteine, or aspartic acid) in the catalytic site or according to the need of a metallic ion to realize its function. These enzymes are important for cell metabolism; for example, they take part in the post-translational process of removing signal peptides and propeptides. Proteases can also act as toxins, which are well characterized in spider and snake venoms (Chaim et al., 2011; Serrano, 2013). Among the proteases described for venomous animals, metalloproteases are the most common. These proteases need a bivalent ion as a cofactor to have proteolytic activity (Markland and Swenson, 2013; Ortiz et al., 2014). The proteolytic function in scorpions is not clear because several species do not present evidence of these enzymes in their venom composition; however, recent studies have characterized proteases in scorpion venom (Costal-Oliveira et al., 2012; Venâncio et al., 2013; Ortiz et al., 2014).

In some accidents involving *T. serrulatus*, symptoms of acute pancreatitis have been observed (Possani et al., 1991; Fletcher et al., 1996; Ortiz et al., 2014). These symptoms are caused by the proteases that cleave proteins from the SNARE complex, which is responsible for the pancreatic vesicular transport from the cytoplasm to the cell membrane. Antarease is one of these proteases that has been described by Fletcher et al. (2010). It specifically cleaves the *vesicle-associated membrane proteins* (VAMPs) from the SNARE family. The cleavage of these proteins disrupts the transport of pancreatic vesicles to outside the organ, causing pancreatitis.

In Ts venom, the only protease that has been described is antarease; however, in this work, a great diversity of proteases revealed from the Ts venom gland cDNA library is presented. All sequences showed similarity and identity to a class of zinc-dependent metalloproteases, thus forming a new family of scorpionic metalloproteases, which are named metalloserrulases (TsMS).

2. Methodology

2.1. Milking of venom

T. serrulatus scorpions were collected in the city of Belo Horizonte, Minas Gerais, Brazil (19° 53.8′ S, 43° 57.8′ W) and kept in captivity for regular venom milking. The crude venom was collected by electrical stimulation and solubilized in 0.01% trifluoroacetic acid (Sigma–Aldrich). Approximately 500 scorpions were used. The venom was centrifuged at 16,000 × *g* and 4 °C for 10 min, and the supernatant was recovered. The soluble fraction was dosed using the Bio-Rad "Protein DC assay" kit (Lowry et al., 1951), and stored in the freezer at −20 °C until use.

2.2. cDNA library construction and protease clone identification

The cDNA library used in this study was described previously by Kalapothakis et al. (2001). The clones used in this work were identified as proteases by homology to the UniProt database using the BLASTx (Consortium, 2010; Alvarenga et al., 2012). DNA sequencing reactions were performed on both strands using M13 universal primers, and the sequences were grouped in contigs by CodonCode Aligner 4.0.

2.3. Bioinformatics analysis

Sequences were aligned using CodonCode Aligner 4.0 for contig formation and searched for similarity using the BLAST platform, including the BLASTx tool against the databank of nonredundant sequences. The cDNA sequences were transcribed *in silico* before the next analysis. The protein sequences were aligned among themselves using the ClustalW program in the MEGA 5 platform (Tamura et al., 2011). After the alignment, the similarity and identity percentages between the sequences were calculated. The primary sequence alignments of the proteases were visualized with the program Jalview 2.8 (Waterhouse et al., 2009). The sequences were analyzed to identify the signal peptide using the online program SignalP 4.1 (Petersen et al., 2011), and the propeptide cleavage site was determined using the online program ProP 1.0 server (Duckert et al., 2004). Additionally, the antarease protein sequence deposited in the databank (P86392.2) was included in the alignment analysis for the identification of possible differences.

The sequence alignments were made using the MEGA 5 platform, and the tree was built with the neighbor-joining algorithm (Zuckerkandl and Pauling, 1965; Tamura et al., 2011). *The search for similar sequences was performed using the BLASTp tool in the UniProt database* (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic tree was constructed using the metalloproteases described in this work and also the ten most similar sequences to the TsMS 1 cDNA sequence in three different taxa: Arachnida (*Mesobuthus eupeus* ABR20110.1, ABR20111.1; *M. martensii* AHA36326.1;