Toxicon 90 (2014) 308–317

Contents lists available at ScienceDirect

Toxicon

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

Holocyclotoxin-1, a cystine knot toxin from *Ixodes holocyclus*

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ARTICLE INFO

Article history:
Received 5 March 2014
Received in revised form 20 August 2014
Accepted 20 August 2014
Available online 27 August 2014

Keywords:
Ixodes holocyclus
Holocyclotoxin-1
Paralysis tick
ICK motif
SPPS
Native chemical ligation

ABSTRACT

In the past 100 years minimal venom research has focused on ticks despite several species possessing a highly paralytic and lethal venom cocktail of proteinaceous molecules. The saliva of the Australian paralysis tick, *Ixodes holocyclus*, has been responsible for 20 human fatalities from 1900 to 1945, and up to 100,000 domestic animal fatalities annually. In the last 50 years, research on this tick has focused on identifying the neurotoxins present in the saliva and in the last ten years the sequence of a potential neurotoxin, HT-1, has been determined. In this study we chemically synthesised HT-1 using Boc-chemistry in combination with native chemical ligation. Following successful oxidative folding, we determined the three-dimensional structure of HT-1 by NMR spectroscopy and found a novel structural fold with three of the four disulfide bonds comprising the inhibitory cystine knot (ICK) motif. The fourth disulfide bond connects the second loop to the N-terminal, which decreases the flexibility of the structure.

1. Introduction

Ticks are obligate ectoparasites that feed on the blood of a variety of mammals, including humans, livestock and domestic animals (White, 2010). Tick venoms have generally only been sparsely studied (Fry et al., 2009; Steen et al., 2006). Nevertheless, ticks are capable of numerous detrimental effects that are potentially life threatening to their hosts, usually via paralysis (Mans et al., 2004) and allergic reactions (Steen et al., 2006), or indirectly by acting as vectors for pathogen transmission (Katargina et al., 2013; Schulze et al., 2013; Wright et al., 2012). Recent genome and transcriptome analysis has greatly contributed to the understanding of tick saliva (Diaz-Martin et al., 2013; Gibson et al., 2013; Hill and Wikel, 2005; Schwarz et al., 2013).

The Australian paralysis tick, *Ixodes holocyclus*, is found along the eastern coastline of Australia in areas of high humidity and moderate temperatures (Sutherland and Tibballs, 2001). In Australia, there have been 20 fatal human *I. holocyclus* envenomation cases reported between 1900 and 1945, which is more than that of the funnel web spider, red back spider and blue-ringed octopus (Sutherland and Tibballs, 2001). Currently allergic reactions are of a greater concern than paralysis in humans due to effective patient care and the use of antivenom. On the other hand, estimates of the number of domestic animals affected annually range from 10,000 to 100,000 with approximately 10% of these progressing to death despite tick removal and antivenom administration (Atwell et al., 2001; Eppleston et al., 2013; Hall-Mendelin et al., 2011; Stone, 1986). Canines are particularly susceptible, with one in twenty cases presented to veterinarians being fatal regardless of treatment (Atwell et al., 2001; Campbell and Atwell, 2002). This high fatality rate highlights the need for increased research into the neurotoxic and cardiotoxic effects of *I. holocyclus* saliva.

Abbreviations: HT-1, holocyclotoxin-1; SPPS, solid phase peptide synthesis; DDH, disulfide-directed β-hairpin.

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http://dx.doi.org/10.1016/j.toxicon.2014.08.068
0041-0101/

The saliva of ticks has evolved to contain a complex cocktail of components that counter the effects of the host immune system, including anticoagulants, prostaglandins, immunosuppressants, antihistamines, prostacyclin, calreticulins and several others (Fry et al., 2009; Steen et al., 2006). Many components of tick saliva have been characterised recently, including Ir-CPI, a coagulation contact phase inhibitor from *Ixodes ricinus* (Decrem et al., 2009), various defensins, a thrombin inhibitor and peroxiredoxin from *Haemaphysalis longicornis* (Galay et al., 2012; Iwanaga et al., 2003; Tsuji et al., 2001, 2007), and a metalloproteinase and anticoagulant from *Ixodes scapularis* (Francischetti et al., 2002, 2003). The majority of these components have been identified through molecular cloning techniques using excised salivary glands. Very little structural information is available for the identified tick peptides. There are currently only 16 tick protein structures deposited in the PDB, and these are predominantly anticoagulants (Lim-Wilby et al., 1995; Pantoja-Uceda et al., 2008; Sanglas et al., 2008), enzymes (Moraes et al., 2011) and enzyme inhibitors (Chmelar et al., 2011; Salat et al., 2010) (Fig. 1).

Arachnids are well known for their ability to incapacitate prey using a complex mixture of neurotoxins contained in their venom (Nicholson et al., 2006) that have been relatively well studied. In contrast, tick neurotoxins are relatively unstudied venom components, despite the existence of over 60 paralytic tick species (Mans et al., 2004). Both high and low molecular weight toxins have been isolated from the salivary glands of *Rhipicephalus evertsi evertsi* (Viljoen et al., 1986) and *Argas walkerae* (Maritz et al., 2000). The toxin from *R. evertsi evertsi* has a molecular weight of 68 kDa (Viljoen et al., 1986), whereas the toxin from *A. walkerae* is 11 kDa (Maritz et al., 2000), which is a similar size to snake and scorpion neurotoxins. Interestingly, in *A. walkerae* toxic fractions corresponding to a mass range of 43–115 kDa were identified though at lower pH the toxic fraction had a mass of only 11 kDa (Maritz et al., 2000). The observation that tick neurotoxins form large complexes at physiological pH is in agreement with previous results for *I. holocyclus* (Maritz et al., 2000). Stone and Aylward (1987) reported that the neurotoxin in *I. holocyclus* saliva had a molecular weight of between 40 and 80 kDa though more recently Thurn et al. (1992) identified a 5 kDa peptide,

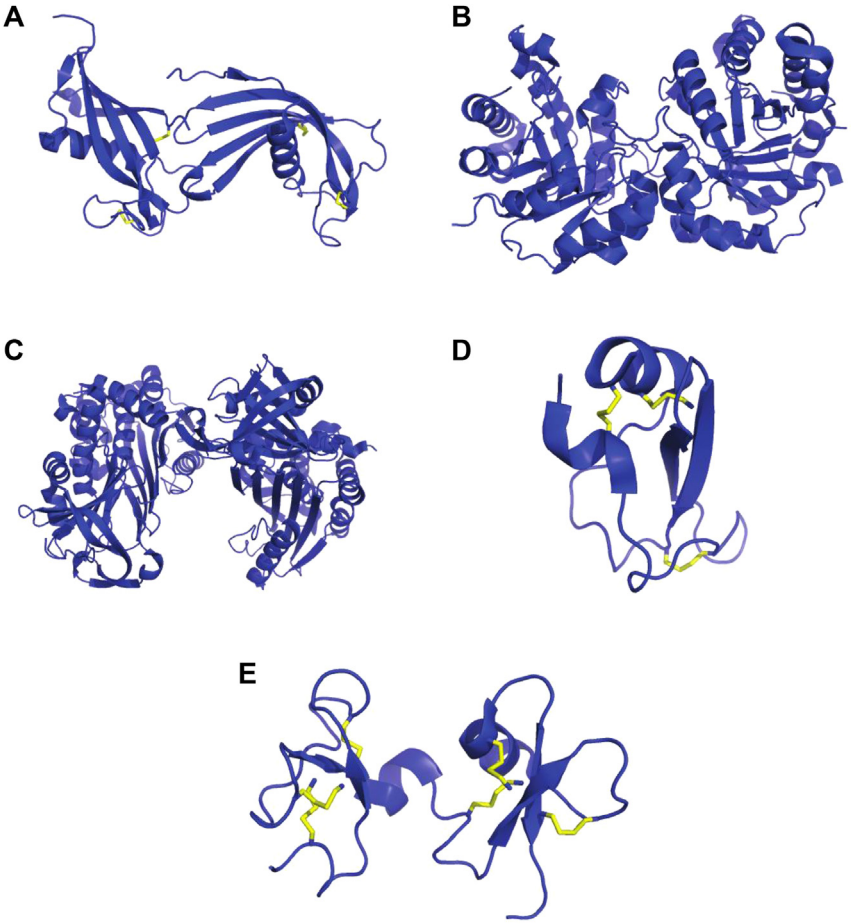

Fig. 1. Examples of structures of proteins identified in ticks: cystatin from *Ornithodoros moubata* (A; PDB code 3L0R), triosephosphate isomerase from *Boophilus microplus* (B; PDB code 3TH6), serpin from *Ixodes ricinus* (C; PDB code 3NDA), tick anticoagulant peptide from *O. moubata* (D; PDB code 1TCP), and tick carboxypeptidase inhibitor from *Rhipicephalus bursa* (E; PDB code 2JTO).