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A Tick Protein with a Modified Kunitz Fold Inhibits Human Tryptase

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TdPI, a tick salivary gland product related to Kunitz/BPTI proteins is a potent inhibitor of human β-tryptase. Kinetic assays suggest that three of the four catalytic sites of tryptase are blocked by TdPI, and that the inhibition of one of these involves a peptide flanking the Kunitz head. In the course of the inhibition, tryptase cleaves TdPI at several positions. Crystal structures of the TdPI head, on its own and in complex with trypsin, reveal features that are not found in classical Kunitz/BPTI proteins and suggest the mode of interaction with tryptase. The loop of TdPI connecting the β-sheet with the C-terminal α-helix is shortened, the disulphide-bridge pattern altered and N and C termini separated to produce a highly pointed molecule capable of penetrating the cramped active sites of tryptase. TdPI accumulates in the cytosolic granules of mast cells, presumably suppressing inflammation in the host animal's skin by tryptase inhibition.

Keywords: Kunitz; tick; trypsin; tryptase; structure

Introduction

Human β-tryptases (EC 3.4.21.59) are mast-cell-specific serine proteases with roles in inflammation and tissue remodelling. They contribute to the pathogenesis of asthma and are implicated in other inflammatory, autoimmune and fibroproliferative disorders.[1] Human βII-tryptase is a flat, rectangular complex of four trypsin-like protomers bordering a central, oval hole.[2] Like trypsin, it cleaves substrates at the carboxyl end of lysine and arginine residues. Tryptase seems designed to cleave small peptides, since its catalytic sites can only be reached *via* the central opening, which is too narrow for most proteins to enter. Among the few protein substrates that have been identified is PAR-2, a G-protein coupled receptor that may mediate several of the cellular responses attributed to tryptase.[1] Naturally, the tetrameric structure of tryptase also restricts catalytic-site access to macromolecular inhibitors, LDTI, a leech product that blocks two subunits per tetramer, being the only competitive polypeptide inhibitor hitherto identified.[3]

Like leeches, ticks are slow-feeding, haematophagous ectoparasites that use saliva compounds to counter their hosts' defence reactions at the feeding site.[4] Of the protease inhibitors identified in tick saliva, many are clearly related to Kunitz/BPTI proteins. Others, such as the anticoagulants TAP (Tick Anticoagulant Peptide) and ornithodorin (both from *Ornithodoros moubata*), diverge considerably from traditional members of the family in their sequences, but still assume the typical Kunitz fold.[5] Here we describe TdPI (Tick-derived Protease Inhibitor) a *Rhipicephalus appendiculatus* protein that has primary structure resemblance with both traditional Kunitz/BPTI-type inhibitors and ornithodorin, but assumes a modified tertiary structure. We find that TdPI is cleaved by tryptase to produce a potent tryptase inhibitor and have determined its three-dimensional structure, alone and in complex with trypsin, which allows us to propose a model for the inhibition.

Results

cDNA, native and expressed protein

Clone *Ra*∑ contained the full-length coding sequence for TdPI. Recombinant, insect-cell expressed TdPI (^{eu}TdPI) accumulated in the culture medium, indicating that it is secreted. Protein sequencing consistently revealed the absence of a

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20 amino acid signal sequence. The 97-residue primary structure of mature TdPI (Figure 1) has a mass of 11,102 Da and a p*I* of 9.1. ^{eu}TdPI is a glycoprotein, as indicated by a ~3 kDa reduction in molecular mass following treatment with PNGase F (Figure 2(a)). Native TdPI, which is selectively produced in salivary glands of adult, female ticks during the early feeding stage (Figure 2(b)), carried ~1–2 kDa more carbohydrate than TdPI expressed in insect cells.

TdPI belongs to the family of Kunitz/BPTI domains, which consist of ~50-residue, globular heads flanked by non-conserved *pre-* and *post-head* sequences (Figure 1). In TdPI, the *post-head* region harbours the carbohydrate-attachment site and an imperfect (pseudo) repeat of the peptides PGFKRP and PGFRP, interrupted by a threonine.

Trypsin, plasmin and tryptase inhibition

Urokinase, thrombin, factor Xa, factor XIIa, elastases, kallikreins, cathepsin G, granzyme B, chymase and chymotrypsins were not significantly inhibited by ^{eu}TdPI. The inhibition of trypsin was potent (K_i = 5.6(±2.0) nM), that of human plasmin moderate (K_i = 55(±10) nM).

Tryptase inhibition experiments were carried out using recombinant human βI tryptase (rhβ$_I$T) and β tryptases from human lungs (hLβT), possibly consisting of βII-tryptase and other isoforms.[6] βI and βII tryptases differ in a single residue: N113 in βI is replaced by a lysine in βII, which thereby loses a glycosylation site. The inhibition was assayed at pH 5.7 (close to the pH inside mast cell granules[7]) and at pH 7.6. Figure 3(a) shows the inhibition of rhβ$_I$T by recombinant TdPI, monitored using a peptide substrate. As the concentration of TdPI increases, the tryptase activity drops sharply, before levelling off to ~20–25% of the initial value, consistent with the blocking of three catalytic sites per tetramer. A tenfold increase of the heparin concentration in the assay buffer does not affect the inhibition (results not shown), suggesting that TdPI does not interfere with the stabilisation of tryptase by heparin. Similar inhibition profiles were obtained with native hLβT (Figure 3(b)) and also with mMCP-6, a tetrameric, murine tryptase that is also insensitive to other polypeptide inhibitors[8] (Figure 3(c)).

At pH 7.6 (but not at pH 5.7), the plateau-regions of the profiles obtained with ^{eu}TdPI (24–30% residual activity (*r.a.*)) were consistently higher than the ~20% ones obtained with (non-glycosylated) TdPI expressed in bacteria (^{pro}TdPI). This suggests that the carbohydrate moiety is responsible for the "sub-optimal" activity of ^{eu}TdPI at pH 7.6.

Unlike the hydrolysis of peptide substrates, the proteolysis of fibrinogen (a known tryptase substrate) can be fully blocked (Figure 3(d)). This requires TdPI amounts that bring the *r.a.* (as measured using *n-p-tosyl*-GPK-*pNA*) below 50%, suggesting at least two catalytic sites need to be blocked (in all tetramers in the sample, hence the <50% value).

Its conserved P4-P3′ sequence[9] (Figure 1) suggests that TdPI uses the same mode of inhibition as traditional Kunitz/BPTI domains in which this peptide constitutes the principal protease-recognition (or "reactive") site. Moreover, the P1 residue

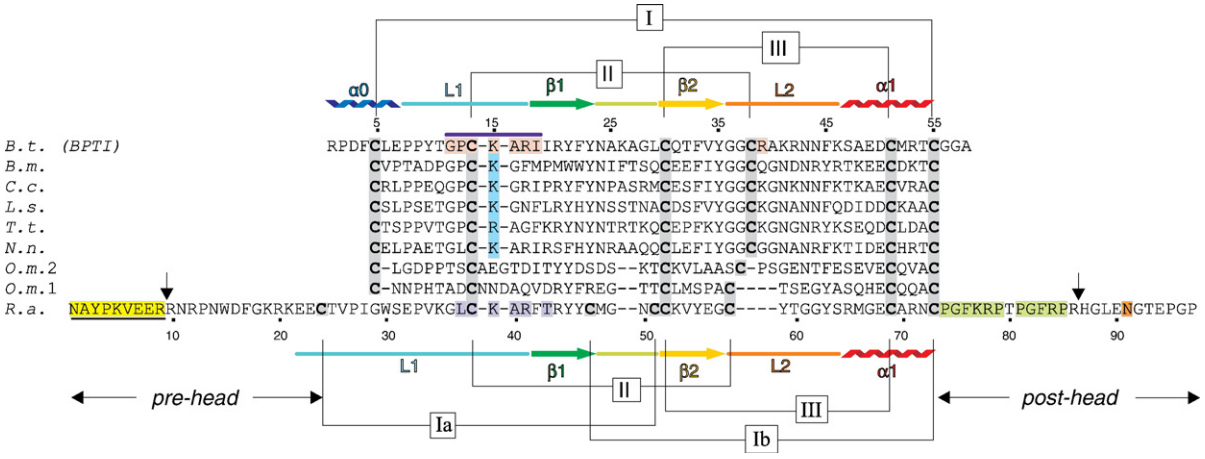

Figure 1. Structural alignment of the full-length TdPI sequence (*R.a.*, shown without the signal sequence MGRTTLIVAIVLVAFVASTLG), and the part of BPTI of which the structure has been determined (PDB code 1BPI, top sequence, *B.t.*, GenBank accession number P00974). In between, the alignment contains the head regions of classical Kunitz domains from the cattle tick *Boophilus microplus* (*B.m.*; P81162), sea turtle *Caretta caretta* (*C.c*; P00993), snail *Lymnaea stagnalis* (*L.s.*; A59204), horseshoe crab *Tachypleus tridentatus* (*T.t.*; P16044), leaf-nosed viper *Eristhocophis macmahoni* (*E.m.*; P24541), parasitoid wasp *Pimpla hydrochondriaca* (*P.h.*; CAD27738), and cobra *Naja nivea* (*N.n.*; P00986), as well as the non-classical heads of ornithodorin (O.m.2 and O.m.1; P56409). In TdPI, the peptide identified by N-terminal sequencing is highlighted in yellow, the glycosylation site in orange, the pseudo-repeat in green. The arrows indicate tryptase cleavage sites. The structural elements (the 3_{10} helix α0, loops L1 and L2, β-strands and α-helices) and disulphide bridges (roman numerals) shown at the top refer to the BPTI structure, those at the bottom to TdPI. The dark blue bar denotes the BPTI reactive-site loop (from P4 to P3′); P1 residues are highlighted in blue. Residues of TdPI (pale blue) and BPTI (pink) that form hydrogen bonds with trypsin are marked.