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A family of textilinin genes, two of which encode proteins with antihaemorrhagic properties

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Summary. Two peptides, textilinins 1 and 2, isolated from the venom of the Australian common brown snake, *Pseudonaja textilis textilis,* are effective in preventing blood loss. To further investigate the potential of textilinins as antihaemorrhagic agents, we cloned cDNAs encoding these proteins. The isolated full-length cDNA (430 bp in size) was shown to code for a 59 amino acid protein, corresponding in size to the native peptide, plus an additional 24 amino acid propeptide. Six such cDNAs were identified, differing in nucleotide sequence in the coding region but with an identical propeptide. All six sequences predicted peptides containing six conserved cysteines common to Kunitz-type serine protease inhibitors. When expressed as glutathione S-transferase (GST) fusion proteins and released by cleavage with thrombin, only those peptides corresponding to textilinin 1 and 2 were active in inhibiting plasmin with K_i values similar to those of their native counterparts and in binding to plasmin less tightly than aprotinin by two orders of magnitude. Similarly, in the mouse tail vein blood loss model only recombinant textilinin 1 and 2 were effective in reducing blood loss. These recombinant textilinins have potential as therapeutic agents for reducing blood loss in humans, obviating the need for reliance on aprotinin, a bovine product with possible risk of transmissible disease, and compromising the fibrinolytic system in a less irreversible manner.

Keywords: recombinant textilinin, gene cloning, antihaemorrhagic properties.

The accumulation of thrombin and plasmin in the circulation is characteristic of the clinical course of a number of disease states (De Haan & van Oeveren, 1998; Despotis & Joist, 1999). One such condition, disseminated intravascular coagulation (DIC), is accompanied by active secondary fibrinolysis, leading to diffuse haemorrhage associated with microthrombosis (Rocha *et al*, 1998). Envenomation by a number of Australian snakes leads to DIC as a result of the coagulant component in these venoms, which has prothrombinase activity capable of converting prothrombin to α-thrombin (Masci *et al*, 1990; Williams & White, 1992; Pearson *et al*, 1993; Marsh, 1994; Stocker *et al*, 1994). Thrombin, in turn, converts fibrinogen to fibrin, initiating DIC as observed in a variety of disease states with a subsequent propensity to bleeding (Rocha *et al*, 1998). Whereas the haemorrhage associated with overactive production of thrombin is usually treated with antithrombin agents such as heparin and hirudin, there are other forms of bleeding which are more readily associated with overactive destruction of haemostatic plugs by plasmin at wound sites, especially following surgery. (Royston, 1990; Orchard *et al*, 1993; Ray & Marsh, 1997). The plasmin inhibitor aprotinin (Trasylol®) has been effectively employed to curb fibrinolytic activity via the plasminogen–plasmin pathway and control such haemorrhage during surgery (Royston, 1990; Orchard *et al*, 1993). Aprotinin or bovine pancreatic trypsin inhibitor binds to and inhibits plasmin activity without altering platelet activity (Orchard *et al*, 1993; Ray & Marsh, 1997). In addition to inhibiting plasmin, aprotinin also inhibits

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plasma kallikrein (Cosgrove *et al*, 1992; Samama *et al*, 1994; Willmott *et al*, 1995; Van der Meer *et al*, 1996). It has a slow but tight binding affinity for plasmin (Willmott *et al*, 1995), and an increased incidence of graft occlusion and perioperative myocardial infarction has been reported in patients (Cosgrove *et al*, 1992; Van der Meer *et al*, 1996). Evidence for increased arterial thrombosis in pigs after administration of aprotinin has also been recorded (Samama *et al*, 1994). Thus, while aprotinin is used widely in the prevention of blood loss during cardiopulmonary bypass and other forms of surgery, its relatively broad specificity, its tight binding kinetics for plasmin ($K_d = 10^{-11}$ mol/l) and its current sourcing in bovine lungs suggest that it is a less than ideal material for use in humans.

Other fibrinolytic inhibitors have also been used to reduce haemorrhage, presumably by neutralizing plasmin (Royston, 1990; Menichetti *et al*, 1996; Denda *et al*, 1997; Eaton & Deeb, 1998; Hardy *et al*, 1998; Munoz *et al*, 1999; Torianos *et al*, 1999). We have reported on a partially purified protein, textilinin (Txln), isolated from the venom gland of the Australian brown snake, *Pseudonaja textilis textilis*, which inhibited only plasmin and trypsin, and bound less avidly to plasmin than did aprotinin (Willmott *et al*, 1995). Further purification of Txln revealed the presence of two distinct molecular weight forms, Txln 1 and 2, with $K_I \sim 10^{-9}$ mol/l for plasmin (Masci *et al*, 2000). Txln 1 and 2 differ in sequence by six amino acid residues and show, respectively, 45% and 43% homology with aprotinin and 58% and 55% homology with its most closely related naturally occurring plasmin inhibitor from the venom of the Taipan, *Oxyuranus scutellalus* (Possani *et al*, 1992). Although Txln inhibition of plasmin activity was achieved with significantly less tight binding than with aprotinin, intravenous delivery of native Txln1 and 2 to the tail vein of a mouse reduced blood loss from the excised tail vein as efficiently as aprotinin when used at an equivalent weight basis (Masci *et al*, 2000). This would seem to indicate that a less tight binding inhibitor of plasmin has a haemostatic effect as efficacious as a more tight binding inhibitor of plasmin. The fact that the specificity of Txln is far narrower than that of aprotinin (e.g. reacts only with plasmin and trypsin) would seem to confirm that plasmin inhibition is the major pathway by which these inhibitors maintain clot stability and prevent blood loss.

As repeated use of aprotinin is contraindicated in humans for immunogeneic reasons (Wüthrich *et al*, 1992; Diefenbach *et al*, 1995), active Txln may be of value as an alternative in reducing blood loss. To prove the efficacy and safety of recombinant Txln, a large-animal model would need to be established, requiring milligram quantities of Txln. Therefore, a venom source would not provide the quantities necessary for a therapeutic. Accordingly, we performed the cloning of the gene(s) for Txln as an approach to producing sufficient quantities for further experimentation and possible therapeutic use. We describe here the cloning of a family of six genes coding for Txln. Two of the six cDNAs corresponding to Txln 1 and 2 were equally as effective as the native forms in inhibiting plasmin activity and reducing blood loss from the excised tail vein in a mouse model.

MATERIALS AND METHODS

Isolation of RNA. Total RNA was isolated using Trizol reagent (Life Technologies). Frozen venom glands, collected using a permit (WO\002503\00\SAA) supplied by National Parks and Wildlife, Queensland, were homogenized in a Polytron in Trizol, extracted with phenol chloroform (1:1), centrifuged at 12 000 ***g*** for 15 min, and RNA precipitated from the upper aqueous phase with isopropanol and ethanol in succession prior to suspension in H_2O, and stored at −70°C. mRNA was isolated using Dynal magnetic beads as recommended by the supplier. After elution from the beads, mRNA was precipitated with one-tenth of a volume of 3 mol/l sodium acetate and two volumes of ethanol, and stored. Total RNA was used for reverse transcription polymerase chain reaction (RT-PCR).

Cloning of Txln cDNA. RT-PCR was carried out using Promega RT Moloney murine leukaemia virus (MMLV) reverse transcriptase with 1 μg of isolated total RNA or mRNA as template at 42°C for 1·5 h. T4 DNA polymerase was used for second-strand synthesis at 14°C for 3 h. As the primary sequence of Txln had been established (Masci *et al*, 2000), it was possible to design degenerate primers for PCR. The forward primer (FI) was designed based on the N-terminal sequence KDRPD, Txln1-F1 5′-ATGAA(A/G)-GA(C/T)AG(A/G)CC(A/C/T)GA(A/G)(C/T)T(A/G/C/T)GA-(A/G)-3′ while the reverse primer (RI) was based on the C-terminal sequence ESTCGS, Txln1-RI 5′-GT(A/G)CT(T/C)-TC(A/G)TG(T/C)TC(T/C)TC(C/T)-3′. After optimization, PCR conditions were established as 94°C for 1 min for denaturation, 46°C for 1 min for reannealing and 1 min at 72°C for elongation. PCR products were analysed on 2% agarose gels. Amplified product was isolated from the gel using QIAquick PCR purification kit from (Qiagen, Clifton Hill, Vic., Australia), 1–2 μg of Txln-amplified cDNA was blunt ended using T4 DNA polymerase and ligated into pGEX-2T, and nucleotide sequence determined, using a primer based on the vector flanking sequence and the dye terminator method (Perkin-Elmer Corporation, Boston, MA, USA). In order to confirm these results for Txln 1, specific primers based on these sequences were prepared: Txln1-F2 5′-ATA TAT **GGA TCC** AAG GAC CGG CCT GAC TTC-3′, incorporating a *Bam*HI site (bold) to facilitate cloning; Txln1-R2 5′-AAC **GGG AAT TC TCA** GAG CCA CAG GTG CTT TC-3′, including an *Eco*RI site and a stop codon (bold). PCR was carried out, the resulting fragment cloned into pGEX-2T after digestion with *Bam*HI and *Eco*RI, and sequencing carried out for verification.

When it was verified that the cDNAs amplified with the Txln primers (F2 and R2) were related to that of the native proteins Txln 1 and 2, the full-length cDNA was cloned using RACE (rapid amplification of cDNA ends). The BD Biosciences Clontech (Palo Alto, CA, USA) SMART RACE kit was used according to the manufacturer's instructions to obtain the adjacent 5′ and 3′ sequences. The primer set used for RACE was the universal primer mix, consisting of long and short universal primer, and nested universal primer. For 3′-RACE double-stranded cDNA, we used Tx1n1 R2 primer and, for 5′-RACE cDNA, Tx1n1 F2 primer. The

cDNA for 3′-RACE was synthesized using 1 μg of total RNA primed with a lock-docking primer, incorporating a SMART sequence at the 5′-end. The cDNA for 5′-RACE was synthesized using a modifying lock-docking oligo (dT) primer and the SMART II oligo. PCR was carried out using cDNA, Advantage 2 Taq Polymerase (Clontech), 92°C for 1min, 50°C for 1 min, 1 min at 72°C for 30 cycles. The amplified fragments were cloned into a T-tailed vector and several clones sequenced. It became apparent from forward and reverse sequencing that six different cDNAs were represented in the amplified products. To subclone these six full-length cDNAs in pGEM-2T, two primers were used: Tx1n1-F 5′-ATC AGC **GGA TCC** ATG TCT GGA GGT-3′ and Tx1n1-R 5′-TCT CCT **GAA TTC** TCA GGC AGC ACA GGT-3′. These full-length cDNAs were used to create six cDNAs corresponding to Txln peptides (59 amino acids). The forward primer sets (TX1F-TX6F) appear below:

- Forward primer for Txln 1 (TXIF) 5′-ATT ATA GGA TCC AAG GAC CGT CCG GAT-3′ (27)
- Forward primer for Txln 2 (TX2F) 5′-ATT ATA GGA TCC AAG GAC CGT CCA GAG-3′
- Forward primer for Txln 3 (TX3F) 5′-AAC GTC GGA TCC AAG GAC CGT CCA AAT-3′
- Forward primer for Txln 4 (TX4F) 5′-AAC GTC GGA TCC AAG GAC CAT CCA AAA-3′
- Forward primer for Txln 5 (TX5F) 5′-AAC GTC GGA TCC AAG GAC CGT CCA AAA-3′
- Forward primer for Txln 6 (TX6F) 5′-ATT GTC GGA TCC AAG GAC CGT CCA AAG-3′.

We used Txln1-R as a reverse primer. The PCR conditions were as described above, and the cDNAs were cloned into pGEX-2T and sequenced.

Expression of glutathione S-transferase (GST) fusion proteins. Colonies representing each of the cDNAs were selected and grown in 2YT medium with 100 μg/ml ampicillin and 0·2 mmol/l isopropyl-β–D-thiogalactoside (IPTG) to induce expression. Direct detection of fusion proteins was performed with 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Laemmli, 1970). Txln–GST fusion proteins produced were purified using affinity chromatography glutathione–Sepharose 4B (Amersham-Pharmacia Biotech). Glutathione–Sepharose 4B gel was washed in phosphate-buffered saline (PBS) four times to ensure that all thrombin inhibitors were removed before incubating with Txln–GST fusion proteins. The bound Txln–GST fusion protein was washed six times with PBS, followed by incubation with human thrombin [5 international units (IU) per mg of fusion protein; this thrombin was kindly donated by Dr John Fenton, Albany, NY, USA]. For a typical reaction, 1 ml of packed gel containing washed Txln–GST fusion protein from 1 l of culture was used with 50 IU of thrombin and incubated at room temperature. Aliquots of the supernatant were taken at 2, 7 and 21 h to investigate the optimal reaction time for cutting Txln from the GST fusion protein. Aliquots were examined by SDS-PAGE. Prior to experimentation with this recombinant Txln in the mouse tail vein bleeding model, it was necessary to remove thrombin according to the method of Schmer (1972). To remove thrombin the digest was applied to a 2 × 20 cm benzamidine–Sepharose (Amersham Pharmacia Biotech, Castle Hill, NSW, Australia) column. The flow-through was collected and concentrated using an Amicon concentrator with a YM3 membrane. Residual thrombin activity was assayed using a chromogenic thrombin substrate, S-2238 (Chromogenex, Molndal, Sweden). Failure to clot citrated plasma was used as an additional detection method.

Recombinant (M) Txln activity. To maximize the efficiency of refolding of the recombinant Txln, two procedures were combined (Shafqat *et al*, 1990a). Briefly, recombinant Txln in 20 mmol/l NH_4HCO_3, pH 8·3, was reduced with 45 mmol/l dithiothreitol in the presence of 2 mol/l guanidine hydrochloride for 15 min at 50°C. The reduced and unfolded Txln was then quickly diluted by 100-fold by adding to 20 mmol/l NH_4HCO_3 buffer, pH 8·3, and left to stand for 18 h. Concentration and purification of active recombinant Txln was carried out by applying the diluted Txln solution to DEAE–Sepharose (1·0 × 10 cm) ion-exchange column as described for native Txln (Masci *et al,* 2000). Active recombinant Txln was assayed by inhibition of plasmin (0·1 IU/ml), using an S-2251 (0·3 mmol/l) chromogenic assay (Friberger *et al*, 1978).

Kinetics of plasmin inhibition. Procedures for investigation of plasmin inhibition kinetics by the recombinant Txlns were in accordance with that described previously (Masci *et al,* 2000) and differed from the method used earlier to study the impure Txln preparation (Willmott *et al*, 1995), in that fourfold and 36-fold higher enzyme concentrations were used. This approach allowed reduction of time scale from 1 h to 10 min or less. Enzyme inhibitor assays were performed at 25°C in 0·1 mol/l Tris/HC1, pH 7·4, containing 0·1% (v/v) Tween-80. A concentration of either 2 nmol/l or 18 nmol/l plasmin (supplied through the good offices of Dr F. Toulemende, Choay-Sanofi, Paris, France) was used in these experiments with 75 μmol/l chromogenic substrate (S-2251; Chromogenix). The residual plasmin activity was determined by continuous measurement of the absorbance at 405 nm. Plasmin was supplied in 50% glycerol (8 IU/ml); 1 IU is the rate of digestion of 1 μg of casein/s at 37°C. On the grounds that the pattern of plasmin inhibition was of the form associated with slow, tight binding inhibition, progress curves were analysed for initial rates over the range 0–0·20 ΔA_{405},1–400 nmol/l Txln.

Mouse tail vein bleeding model. A bleeding model was essentially as described elsewhere (Masci *et al,* 2000). These experiments were carried out after obtaining ethical approval from the Princess Alexandra Hospital Ethics Committee (Medical/134/00/PAHRDF). The single dose of the plasmin inhibitors used in these experiments (100 μg/mouse) was selected from a dose range for native Txln (Masci *et al,* 2000) and was approximated to mimic that of aprotinin used during cardiac by-pass (CPB) surgery adjusted to the mouse weight of 20 g. Blood loss was measured by collection into preweighed Eppendorf tubes. Accuracy dictated that blood loss was measured by weight rather than volume. All mice were euthanized by cervical dislocation. The dose of the Txlns used was observed not to induce any adverse effects on the mice over a period of 2–14 d.

RESULTS

Cloning of Txln cDNA

We had previously purified two forms of Txln from the venom of *Pseudonaja textilis textilis* that inhibit plasmin activity and reduce blood loss in an experimental animal model (Masci *et al,* 2000). Based on the protein sequences for Txln 1 and 2, we designed degenerate primers to amplify a fragment of approximately 180 bp using RT-PCR with total RNA isolated from the venom gland of the snake (Fig 1A). Cloning and DNA sequencing enabled us to design additional primers for further amplification of the cDNA. The nucleotide sequence obtained corresponded approximately to the amino acid sequence for Txln. Because of the restrictions imposed by design of the primers for Txln amplification, we were unable to verify the sequence at the N- and C-termini. Accordingly, to complete this sequence and obtain a full-length cDNA, we carried out 3′ and 5′ RACE with primers based on the partial cDNA.

PCR bands of approximately 400 bp (3′ RACE) and 350 bp (5′ RACE) were detected (Fig 1B, lanes 2 and 3), cloned into pGEM-2T and multiple clones sequenced. As

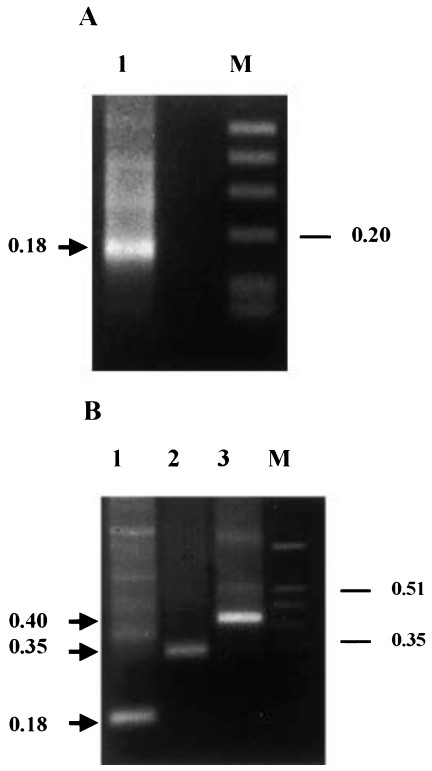

Fig 1. RT-PCR cloning of a fragment coding for Txln1 of *Pseudonaja textilis textilis* and isolation of full-length cDNA. (A) Degenerate primers based on peptide sequence for Txln1 were employed to amplify a fragment of 180 bp, using standard RT-PCR with RNA isolated from the venom gland of the snake. M refers to molecular-weight markers. (B) Oligonucleotide primers based on cDNA sequence were used for 3′ RACE, lane 3 (400 bp) and 5′ RACE, lane 2 (350 bp) to obtain a full-length cDNA sequence. Lane 1 contains initially amplified 180 bp fragment and M refers to markers.

ggagcttcatc ATG TCT TCT GGA GGT CTT CTT CTC CTG CTG GGA CTC CTC ACC CTC TGG GAG GTG CTG ACC CCC GTC TCC AGC AAG GAC CGT CCA GAG TTG TGT GAA CTG CCT CCT GAC ACC GGA CCA TGT AGA GTC AGA TCC CCA TCC TTC TAC TAC AAC CCA GAT GAA CAA AAA TGC CTA GAG TTT ATT TAT GGT GGA TGC GAA GGG AAT GCT AAC AAT TTT ATC ACC AAA GAG GAA TGC GAA AGC ACC TGT GCT GCC TGA atgaggagaccctcctgg attggatcgacagttccaacttgacccaaagaccctgcttctgccctggacc accctggacaccctcccccaaaccccaccctggactaattccttttttctctgc aataaagctttggttccagctgg(A)$_{21}$

Fig 2. Complete nucleotide sequence for Txln2 cDNA (430 bp). The sequence contains 11 nucleotides of 5′ UTR and a 3′ UTR of 146 nucleotides. The open reading frame initiated by AAG is 177 nucleotides in length, and this is preceded by 72 nucleotides coding for a propeptide sequence. A poly-A tail of 21 nucleotides was detected.

predicted from the primer design, overlapping sequences were obtained in the two RACE products, which provided a full-length cDNA sequence of 430 bp (Fig 2). As observed for the naturally occurring Txln 1, 59 amino acids were coded for, followed immediately by a stop codon. It is of interest that the two most C-terminal amino acids, both alanines, differed from the glycine and serine residues that were tentatively identified by N-terminal sequencing. However, an additional 72 nucleotides upstream of lysine (AAG) and inframe provided evidence for a proform of Txln 1. The initial codon in this sequence specified methionine. Untranslated regions were also present, 11 nucleotides in the 5′ UTR and 146 nucleotides in the 3′ UTR. There was also evidence for a poly-A tail at the 3′ end of the mRNA (Fig 2).

After sequencing multiple clones from the RACE products, it became apparent that there were several different cDNAs coding for Txln. This was confirmed by designing primers based on these sequence variants in the N-terminal region of the coding sequence and by combining with long universal primer to produce PCR products for sequencing. In all, six separate Txln cDNA sequences were identified. The relative number of these clones isolated were Txln1 (14%), Txln2 (54%) and Txln3–6 (8% each). Alignment of the predicted amino acid sequences demonstrated that all six proteins coded for were of the same size (59 amino acids), and the 24 amino acids that presumably make up the cleaved-off propeptide were completely conserved in all cases (Fig 3). Four blocks of amino acids (four, six, three and five amino acids in size) in the C-terminal region were conserved, as were six cysteine residues, typical of plasmin inhibitors and presumably responsible for conformational stability (Fig 3). Variation was observed over 39 amino acid positions and was most prominent in the N-terminal half of the proteins.

Expression and activity of recombinant Txln

GST constructs encoding the active peptide (59 amino acids) were produced by PCR, and all six Txln cDNAs were cloned into pGEX-2T which has a thrombin site for cleavage of the

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          1                                                                  83
Txln1  MSSGGLLLLLGLLTLWEVLTPVSSKDRPDFCELPADTGPCRVRFPSFYYNPDEKKCLEFIYGGCEGNANNFITKEECESTCAA
Txln2  -MSSGGLLLLLGLLTLWEVLTPVSSKDRPELCELPPDTGPCRVRFPSFYYNPDEQKCLEFIYGGCEGNANNFITKEECESTCAA
Txln3  -MSSGGLLLLLGLLTLWEVLTPVSSKDRPNFCKLPAETGRCNAKIPRFYYNPRQHQCIEFLYGGCGGNANNFKTIEECESTCAA
Txln4  -MSSGGLLLLLGLLTLWEVLTPVSSKDHPKFCELPADTGSCKGNVPRFYYNADHHQCLKFIYGGCGGNANNFKTIEECKSTCAA
Txln5  -MSSGGLLLLLGLLTLWEVLTPVSSKDRPKFCELLPDTGSCEDFTGAFHYSTRDRECIEFIYGGCGGNANNFITKEECESTCAA
Txln6  -MSSGGLLLLLGLLTLWEVLTPVSSKDRPKFCELPADIGPCDDFTGAFHYSPREHECIEFIYGGCKGNANNFNTQEECESTCAA
consensus-
       MSSGGLLLLLGLLTLWEVLTPVSSKDrPkfCeLpadtGpCrvrfpsFyYnprehkCieFiYGGCgGNANNFiTkEECeSTCAA
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Fig 3. Alignment of the amino acid sequences for Txln 1–6 and derivation of a consensus sequence. Amino acid sequences were aligned using six conserved cysteine residues. The 24 amino acid propeptide sequence is identical in all six Txlns. The consensus sequence was derived manually. The nucleotide sequences have been submitted to the GenBank™ data bank with accession numbers: AF402324–402329 for the textilinin 1–6 nucleotide sequences respectively.

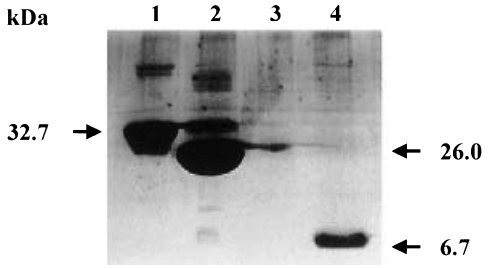

Fig 4. Expression of recombinant Txln1 using a GST fusion construct. All six Txlns were cloned into pGEX-2T and expressed as GST fusion proteins prior to cleavage of the fusion with thrombin. Proteins were separated on a 12% SDS-PAGE gel. Lane 1, GST–Txln1 fusion protein; lane 2, cleaved GST protein retained on beads; lane 3, beads washed after elution of thrombin-cleaved fusion protein; lane 4, elution of Txln1 from the beads after thrombin cleavage.

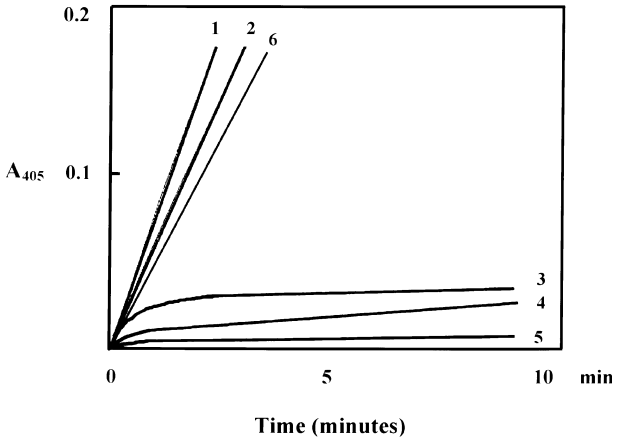

Fig 5. Time course of inhibition of plasmin using Txlns and aprotinin. GST-Txln fusion proteins were cleaved and separated on glutathione beads. Purified Txln was added to incubations containing 18 nmol/l plasmin and assayed for inhibition of plasmin activity using the S-2251 chromogenic assay. Line 1, plasmin activity alone; line 2, effect of recombinant Txln3 on plasmin activity; line 3, effect of Txln 1 on plasmin activity; line 4, effect of Txln2 on plasmin activity; line 5 represents the inhibition of plasmin activity by aprotinin and line 6 is the curve for Txln 1 with Arg-19 replaced by Ala.

fusion protein. A construct with the propeptide form (83 amino acids) was also prepared for Txln 1. Txln–GST fusions were bound to glutathione–Sepharose 4B followed by extensive washing prior to cleavage with thrombin, which resulted in release of recombinant Txln into the supernatant. The results for Txln 1 expression are shown (Fig 4). Efficient expression of all GST fusion proteins was observed after induction of cultures with IPTG (results not shown). Thrombin was subsequently removed by absorbing with benzamidine–Sepharose 4B using a batch procedure as described previously (Schmer, 1972). To ensure that the recombinant Txlns were correctly folded, samples were reduced, diluted in low-salt solution followed by a concentration step on DEAE–Sepharose as described in *Materials and methods*. Activity of Txln was followed by inhibition of plasmin using the S-2251 chromogenic assay.

Kinetics of plasmin inhibition

As observed for native Txln 1 and 2, the recombinant forms of these Txlns also caused a marked inhibition of plasmin activity (Fig 5, curves 3 and 4). The extent of inhibition was similar to that with aprotinin (curve 5). These curves are representative of assays with added plasmin inhibitor concentrations of 400 nmol/l. The dissociation constants (K_i) were calculated using initial rates of inhibition of plasmin by recombinant Txln over a concentration range of 1–400 nmol/l. Curve-fitting analysis was carried out as described previously (Masci *et al*, 2000). The K_i values for recombinant Txlns 1 and 2 at 2 nmol/l plasmin were 7·2 ± 0·2 nmol/l and 5·8 ± 0·4 nmol/l respectively, which were slightly higher than the values for native Txlns (Table I). This may be merely a refolding anomaly. This slight but consistent difference was also observed when

Table I. Summary of data for plasmin inhibition by textilinins.

Inhibitor	K_i (2 nmol/l plasmin) ($n = 6$) nmol/l	K_i (18 nmol/l plasmin) ($n = 6$) nmol/l
S100 pool (Txln 1 and 2)*	7·1 ± 0·2†	13·9 ± 0·3
Txln 1 (native)	3·5 ± 0·3	2·6 ± 0·2
Txln 2 (native)	2·2 ± 0·2	2·8 ± 0·3
Aprotinin	0·053	–
Txln 1 (recombinant)	7·2 ± 0·2	6·8 ± 0·3
Txln 2 (recombinant)	5·8 ± 0·4	4·6 ± 0·2
Txln 3–6 (recombinant)	0	0

*S100 pool refers to venom extract separated on Sephacryl 100 and pooled as described previously (Masci *et al,* 2000).

†± one standard deviation.

18 nmol/l plasmin was employed. Under these conditions, the K_i value for aprotinin was 0·05 ± 0·003 nmol/l, binding to plasmin more tightly by two orders of magnitude than the Txlns. When the propeptide form of Txln 1 was used in this assay, no inhibition of plasmin activity was observed (data not shown). Surprisingly, none of the newly identified Txlns 3–6 had significant inhibitory effect on plasmin activity; only results for Txln 3 are shown (Fig 5, curve 2). When Arg-19 was mutated to Ala, Txln 1 lost its ability to inhibit plasmin. The corresponding residue in aprotinin (Arg-15) is thought to be important in its plasmin inhibitory activity (Markwardt, 1978). The amount of inhibitor added to 18 nmol/l plasmin was the same for each inhibitor.

Mouse tail vein bleeding model

As recombinant Txln 1 and 2 were effective in inhibiting plasmin activity, we determined whether they would also reduce blood loss from an excised tail vein in the mouse. For these experiments large-scale cultures (10:l) were prepared, yielding 50 mg and 40 mg of Txln 1 and 2, respectively, after purification. The effect of intravenous delivery of Txln 1 and 2 on the blood loss from an excised mouse tail vein is shown in Table II, and for comparison the results for aprotinin are also shown. The amount used was equivalent on a weight basis to the amount of aprotinin used clinically in humans and this was 100 μg of each protein studied per average 20-g mouse. It can be seen from Table II that aprotinin reduced blood loss by approximately 60%, which was similar to the extent of reduction for the recombinant forms of Txln 1 and 2. In keeping with the data on plasmin inhibition, Txln 6 had a negligible effect on blood loss. The native forms of Txln 1 and 2 were equally effective (Table II). It is well to observe that, despite the fact that Txlns bound to plasmin less tightly than aprotinin by two orders of magnitude, they had the same antihaemorrhagic effect on a weight (and molar) basis (see Tables I and II).

DISCUSSION

Snake venoms have proved to be a rich source of neuromuscular toxins and haemostatic agents, including plasmin inhibitors (Takahashi *et al*, 1972; Strydom, 1977; Ritonja *et al*, 1983; Shafqat *et al*, 1990a, b; Possani *et al*, 1992; Joseph *et al*, 1999). Venoms from all species in the *Pseudonaja* genus have plasmin inhibitory activity. We have shown here that two recombinant forms of one of these inhibitors, Txln, were also capable of inhibiting plasmin activity (Fig 5). Two distinct, naturally occurring forms of Txln, Txln 1 (MW 6688) and Txln 2 (MW 6692), isolated from *Pseudonaja textilis* have been described previously (Willmott *et al*, 1995; Masci *et al,* 2000). These two forms differ by six amino acids distributed over the protein (59 amino acids) but have approximately the same inhibitory effect on plasmin ($K_i \approx 10^{-9}$ mol/l) (Masci *et al,* 2000). Based on Txln sequence data, we cloned full-length cDNAs closely related in sequence to Txln 1 and Txln 2. Subsequent expression of the recombinant forms in *E. coli* produced proteins that were as effective as Txln 1 and 2 in inhibiting plasmin activity (Table I). While only two forms

Table II. Blood loss data for a mouse tail-vein bleeding model.

Treatment	Blood loss (g)	Average reduction in blood loss (%)
Saline (control)	0·869 (± 0·145)	–
Aprotinin	0·352 (± 0·152)	59·5
Txln 1 (native)	0·386 (± 0·150)	55·6
Txln 2 (native)	0·329 (± 0·134)	62·2
Txln 1 (recombinant, benzamidine–Sepharose)	0·283 (± 0·156)	67·5
Txln 2 (recombinant, benzamidine–Sepharose)	0·298 (± 0·164)	65·8
Txln 6 (recombinant, benzamidine–Sepharose)	0·856 (± 0·152)	1·4

Native Txln 1 and 2 were adsorbed with conA–Sepharose to remove contaminating prothrombin activator. Recombinant Txln 1, 2 and 6 were adsorbed with benzamidine–Sepharose, to remove thrombin added to cleave the Txln insert from the Txln–GST fusion protein. All groups contained 30 animals with the exception of the group that received recombinant Txln 6, which included four animals.

of the Txln inhibitor were isolated from *Pseudonaja* venom, we identified four additional Txln cDNAs expressed in the venom gland. These were identified by sequencing multiple clones and designing gene-specific primers to confirm separate sequences. None of these additional Txlns were inhibitory towards plasmin. At an initial glance at Fig 3, this may seem somewhat surprising as there are several blocks of conserved sequence common to the Txlns, including the number and spacing of the six cysteine residues. These cysteines form three intra-chain disulphide bonds to stabilize the molecule and maintain it in an active conformation. In the presence of reducing agents, Txln has no inhibitory activity against plasmin. Furthermore, alignment of Txln 1 and 2 with other Kunitz-type serine protease inhibitors, aprotinin and taicotoxin, reveals an exact match and spacing of the cysteine residues (Masci *et al,* 2000). Clearly, other amino acid substitutions in the molecule have an important bearing on activity, and the precise conformation associated with the disulphide bonding relates to stability rather than inhibitory activity, e.g. primary sequences are also relevant to the inhibitory activity. To address this, we looked for amino acid changes in Txln 3–6, which are conserved only in Txln 1 and 2. For these conserved residues across the molecule, there were 13 differences with Txln 3, 15 with Txln 4 and 16 each for Txln 5 and 6. Of these substitutions, seven were common to Txln 3–6 and were largely confined to a single block of conserved residues in Txln 1 and 2 (amino acids 17–22). It was evident that the substitutions vary from five to seven over this eight-residue block (Fig 3). In addition, changes were consistently seen at amino acids 31 and 41. Alignment of taicotoxin, a compound isolated from the venom of the Taipan and active in inhibiting plasmin (Possani *et al*, 1992), reveals 50% conservation of amino acids over the same region. On the other hand, when aprotinin (with 45% identity to Txln) was aligned over this region, five residues were different. However, Arg-19 within this sequence was maintained and was not present in recombinant Txln 3–6. Mutagenesis of this Arg to Ala leads to complete loss of activity for Txln 1 and 2 in the plasmin inhibitory assay. When the two cysteines are aligned, this arginine residue aligns with Arg-15 in aprotinin, which has been suggested to form part of the active plasmin inhibitory region (Markwardt, 1978). Thus, together with other residues, Arg-19 appears to be critical for maintaining an active structure, as has been suggested by others (Powers & Harper, 1986).

It is of some interest that multiple cDNAs specifying short-chain neurotoxins have also been identified in *Pseudonaja textilis* (Gong *et al*, 1999). In that study, six cDNAs were described based on sequence data from two lethal neurotoxins of sizes rather similar to the Txlns (57–58 amino acids). The products of these cDNAs expressed in *E. coli* displayed similar toxicities and binding constraints for the acetylcholine receptor to those observed with the native proteins (Gong *et al*, 1999). These cDNA sequences showed no significant relationship to Txln cDNAs. The putative prodomain together with the first two amino acids of the protein are absolutely conserved among the Txlns. As it was not possible to detect the prodomain sequence in the native Txlns, it is likely that it is rapidly cleaved on completion of protein synthesis or during the secretion process. Conservation of a serine/lysine sequence at the cleavage site suggests that all six peptides are processed by the same protease, releasing the 24 amino acid propeptide. This sequence is highly hydrophobic, which suggests that it facilitates secretion of the active peptide prior to cleavage, presumably in the venom. Other snake venom proteins also contain a propeptide that is also cleaved for activation (Gong *et al*, 1999). It can be speculated that Txln, being expressed in a propeptide form, may offer the possibility of administering an inactive protein that becomes activated in the body by proteolytic cleavage. One possibility is that this cleavage site could be mutated so that it is cleaved specifically by thrombin, providing controlled release of this inhibitor at the site of a haemostatic plug, which we know has active thrombin available on its surface. This could overcome the potential 'overshoot prothrombotic' effect observed with active aprotinin and, as a bonus, avoid unwelcome clot extension while maintaining clot stability (Cosgrove *et al*, 1992; Van der Meer *et al*, 1996). The highly conserved prodomain and the cleavage site together with other blocks of sequence in the Txlns suggest that those that do not inhibit plasmin (Txln 3–6) may be active against proteases involved in other physiological processes.

Aprotinin is widely used as a therapeutic drug and there are reports of side-effects such as vein graft occlusion, arterial thrombosis and fatal anaphylactic shock during cardiac surgery (Cosgrove *et al*, 1992; Westaby, 1993; Samama *et al*, 1994; Diefenbach *et al*, 1995; Van der Meer *et al*, 1996; Alderman *et al*, 1998). Aprotinin has widespread effects on the components of haemostasis, binding to other proteases such as kallikrein, and it may prevent blood loss by a more complex series of events (Willmott *et al*, 1995). We have shown here that the recombinant Txlns are at least as effective as bovine-lung-sourced aprotinin in reducing blood flow in the excised tail vein of the mouse. As pointed out above, aprotinin has a broad spectrum of activity *in vivo*, which makes it difficult to extrapolate the clinical effect from *in vitro* inhibition of plasmin. In fact, aprotinin is normally calibrated in kallikrein inhibitor units (KIU; 20 KIU corresponds to 1 plasmin-inhibiting unit) while its plasmin and other inhibitory activities are not stated on commercial packs.

Furthermore, Txln is an acidic protein (pI = 4·4) (Masci, 2001) while aprotinin is basic with a pI of 8·9, which could have a major impact on the pharmacological activities of these peptides. In addition, the *in vitro* data are restricted to inhibition of plasmin where the residual enzyme activity is measured by use of a simple chromogenic peptide. *In vivo*, several potential substrates for plasmin exist and the kinetics of inhibition may be markedly different with these large biological substrates from that seen with the simple chromogenic substrate, as has been shown elsewhere (Gaffney *et al*, 1977). Extension of the mouse model studies to larger animals (e.g. pigs or sheep) is necessary to further endorse the efficacy of recombinant Txlns in combating blood loss during surgery and enhance their feasibility for

use in the clinic. Furthermore, the availability of the inactive propeptide form of recombinant Txln, activated preferentially by thrombin attached to fibrin *in vivo*, may add a clinical advantage in targeting such inhibitory activity to potential haemorrhagic sites. Such studies are all the more relevant in the current climate when blood transfusion may involve danger from a variety of virus-based disease states.

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