A novel coagulation inhibitor from *Schistosoma japonicum*

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SUMMARY

Little is known about the molecular mechanisms whereby the human blood fluke *Schistosoma japonicum* is able to survive in the host venous blood system. Protease inhibitors are likely released by the parasite enabling it to avoid attack by host proteolytic enzymes and coagulation factors. Interrogation of the *S. japonicum* genomic sequence identified a gene, *SjKI-1*, homologous to that encoding a single domain Kunitz protein (Sjp_0020270) which we expressed in recombinant form in *Escherichia coli* and purified. *SjKI-1* is highly transcribed in adult worms and eggs but its expression was very low in cercariae and schistosomula. *In situ* immunolocalization with anti-SjKI-1 rabbit antibodies showed the protein was present in eggs trapped in the infected mouse intestinal wall. In functional assays, SjKI-1 inhibited trypsin in the picomolar range and chymotrypsin, neutrophil elastase, FXa and plasma kallikrein in the nanomolar range. Furthermore, SjKI-1, at a concentration of 7·5 μM, prolonged 2-fold activated partial thromboplastin time of human blood coagulation. We also demonstrate that SjKI-1 has the ability to bind Ca^{++}. We present, therefore, characterization of the first Kunitz protein from *S. japonicum* which we show has an anti-coagulant properties. In addition, its inhibition of neutrophil elastase indicates SjKI-1 have an anti-inflammatory role. Having anti-thrombotic properties, SjKI-1 may point the way towards novel treatment for hemostatic disorders.

Key words: Kunitz protein, trypsin inhibitor, anti-inflammatory agent, anti-coagulant.

INTRODUCTION

When schistosome cercariae penetrate through the mammalian host epidermis, host immune reactions are triggered but a reduced inflammatory action is evident around live parasites suggesting that, as opposed to those dead or dying, the live larvae may be producing substances that down regulate host inflammatory responses (Rao and Ramaswamy, 2000). In addition, schistosomes can live for a considerable time in the mesenteric veins of mammalian hosts but the mechanisms or molecules used by the adult worms which allow them to escape host immune attack remain largely unknown.

According to Virchow's triad, an altered normal blood flow, damaged endothelium or hypercoagulability of plasma initiates blood coagulation (Bagot and Arya, 2008; Wolberg *et al*. 2012). Adult schistosome worm pairs occupy most of the vessel lumen and cause blood turbulence and disturb endothelial cell functions activating platelets and blood coagulation. Furthermore, the schistosome surface has many electronegative charges which lead to the activation of platelets and the intrinsic coagulation cascade (Mebius *et al*. 2013). Extravasations of schistosome eggs also cause endothelial damage (Mebius *et al*. 2013). Unlike adult female worms of *Schistosoma mansoni*, which individually deposit hundreds of eggs, those of *Schistosoma japonicum* deposits thousands of eggs daily into the mesenteric veins (Cheever *et al*. 1994). Even though these eggs are large enough to obstruct blood flow in the smaller veins, clotting does not occur (File, 1995). Consequently, it appears that schistosomes are potent activators of blood coagulation. However, hepatosplenic schistosomiasis patients do not have an increased risk of thrombus formation (Tanabe, 2003). Their sera have a reduced level or activity of clotting factors and exhibit prolonged coagulation times (Omran *et al*. 1995). Also adult schistosome surfaces do not induce platelet adhesion (Wu *et al*. 2007). Therefore it is clear that schistosomes must have evolved several mechanisms to manipulate host hemostasis and to actively inhibit blood coagulation ensuring long-term survival in the host blood stream. It has long been known that whole worm extracts of *S. mansoni* prolonged the activated partial thromboplastin time (APTT) (Tsang and Damian, 1977). Even though several molecules produced by *S. mansoni* have been identified as inhibiting secondary hemostasis (Mebius *et al*. 2013), none have been identified from *S. japonicum*.

Kunitz type proteins, which belong to the I2 family of protease inhibitors, are involved in diverse biological roles such as coagulation, inflammation, fibrinolysis and ion channel blocking (Ranasinghe and McManus, 2013). The amino acid

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at the P_1 reactive site is the major determinant of the energetic and specificity of protease recognition by Kunitz protease inhibitors. Typical trypsin inhibitors contain Arg or Lys while typical chymotrypsin inhibitors contain Leu or Met at the P_1 site (Krowarsch *et al*. 1999). To date, the characterization of Kunitz proteins from parasites suggest they play important roles in modulating host defence mechanisms to favour parasite survival (Chu *et al*. 2004; Corral-Rodríguez *et al*. 2009; Gonzalez *et al*. 2009).

The availability of genomic information and predicted proteomic sequences provide valuable resources to expand knowledge of the parasite's functional biology using recombinant protein approaches (Yan Zhou, 2009; Mulvenna *et al*. 2010; Liao *et al*. 2011*a*). In this study we isolated a gene sequence, *SjKI-1*, homologous to that encoding a single domain Kunitz protein identified after interrogation of the *S. japonicum* genome database (Yan Zhou, 2009). We then performed functional studies using recombinant SjKI-1 produced in *Escherichia coli* and showed it has anti-inflammatory and anti-coagulant properties. This is the first anti-coagulant identified and characterized from *S. japonicum*.

MATERIALS AND METHODS

Parasite materials

Naturally infected *Oncomelania hupensis hupensis* snails were collected from an endemic focus in Anhui Province, China, and imported to Australia. All animal work was carried out in the animal facility at QIMR Berghofer Medical Research Institute in strict accordance to the protocols approved by its animal ethics committee (project number P288). Infected snails were induced to shed by exposure to bright light in conditioned water and cercariae were collected from loops into a tube of water containing 10% (v/v) fetal calf serum. Adult *S. japonicum* worms were perfused from ARC female Swiss mice 6–7 weeks after percutaneous challenge with 60 cercariae. Schistosomules were obtained by mechanical transformation of cercariae as described (Brink *et al*. 1977). Soluble parasite antigens were prepared by homogenizing adult worms, cercariae, schistosomules, eggs and miracidia in phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice followed by centrifuging the homogenates at 16 000 ***g*** for 30 min at 4 °C. Excretory/secretory (ES) products from adult worms were obtained following the culture of *S. japonicum* worm pairs in perfusion buffer for 1 h at room temperature and subsequently collecting the supernatants (Liu *et al*. 2009).

Cloning and expression of SjKI-1

Gene sequences homologous to Kunitz type protease inhibitors were identified by BLASTn and BLASTp searches of the *S. japonicum* genome and proteome databases available in GeneDB (http://www.genedb.org/Homepage/Sjaponicum). Among the sequences identified, one mature peptide encoding SjKI-1 – contig Sjp_0020270 – was selected and its domain identification was undertaken using the PROSITE database (http://prosite.expasy.org/) (Sigrist *et al*. 2013). The presence of a signal sequence was checked using signalP 4·1 (http://www.cbs.dtu.dk/services/SignalP/) (Petersen *et al*. 2011) and the subcellular location prediction was done using LocTree3, (https://rostlab.org/services/loctree3/) (Goldberg *et al*. 2014). Searches for similar protein sequences were performed using BLAST (http://blast.ncbi.nlm.nih.gov/Blast) on the National Centre for Biotechnology Information (NCBI) web site and multiple sequence alignments were generated with the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers *et al*. 2011). Phylogenetic analysis was performed using online resources (http://www.phylogeny.fr/) (Dereeper *et al*. 2008). Molecular weight and isoelectric point calculations were performed using the ExPASy-Compute pI/Mw tool (http://web.expasy.org/compute_pi/). The PHYRE2 protein fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre2/) was used to generate the three-dimensional (3D) model of SjKI-1 (Kelley and Sternberg, 2009) and binding site predictions were carried out using the 3DLigandSite (http://www.sbg.bio.ic.ac.uk/3dligandsite/) (Wass *et al*. 2010).

The *SjKI-1* gene sequence was amplified with cDNA from adult worms using 5′-CATGCCATGGCACATCATCATCATCATCACGTTAGAGACTTGCATTACTCATTGAATC 3′ and 5′-GATCCTCGAGCTACACATTGATTCTCAT-TTTACACACTG-3′, forward and reverse primers, respectively, and MyTaq DNA Polymerase (Bioline, Alexandria, Australia). Purified polymerase chain reaction (PCR) products were digested with the restriction enzymes *Nco*I and *Xho*I and ligated into the pET28a expression vector. The plasmid was then transformed into *E. coli* BL21 (DE3) cells and a positive recombinant clone was selected after gene sequencing. A starter culture was grown in 5 mL of Luria-Bertani medium containing 30 mg mL^{-1} Kanamycin and recombinant protein production was induced with 1 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG). Harvested induced cells were lysed with lysozyme (10 mg mL^{-1}) in Tris buffer (100 mM NaH_2PO_4, 10 mM Tris-Cl) and homogenized using a Potter-Elvehjem homogenizer followed by sonication. After washing three times with Tris buffer containing 0·5% (v/v) Triton-X 100, inclusion bodies solubilized in 6 M GuHCl, were allowed to bind to Ni charged resin (Novagen, Madison, USA) at 4 °C. The Ni column was washed sequentially with 4 mL Tris buffer containing 40, 50 and 70 mM imidazole. Then, 50 mL of elution buffer (50 mM NaH_2PO_4, 300 mM

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NaCl), without imidazole, were allowed to pass through the column and finally the refolded protein was eluted with elution buffer containing 300 mM imidazole. The SjKI-1 protein was electrophoresed on 15% (w/v) sodium dodecylsuphide polyacrylamide (SDS-PAGE) gels and its protein concentration was determined using the Bradford assay (Bradford, 1976).

Real time PCR

cDNA was synthesized from total RNA, extracted from adult male and female worms, eggs, cercariae and newly transformed schistosomula, using a Sensiscript® Reverse Transcription kit (Qiagen, Limburg, Netherlands) according to the manufacturer's instructions. The quality and quantity of the cDNA samples were determined using a Nanodrop-1000. Real time PCR was performed with 25 ng cDNA per reaction with SYBR Green PCR Master mix (Applied Biosystems). Forward 5′-ACTGGTAAATGCCGTGCAA-3′ and reverse 5′-AGTTATTCTCGTTCGCACCAC-3′ primers were designed using Primer3 (http://simgene.com/Primer3). NADH ubiquinone reductase (Contig 7836) (forward 5′-CGAGGACCTAACAGCAGA GG-3′ and reverse 5′-TCCGAACGAACTTTG AATCC-3′) was used as housekeeping gene for normalization of data (Gobert *et al.* 2009). The confidence threshold of the second set of results was normalized to the first set before evaluation by importing a standard curve of the first set to the second. Real time PCR of each life cycle stage was carried out twice using four technical replicates each time. The results were analysed using Rotor-Gene 6000 software.

Western blotting and immunolocalization

Polyclonal rabbit antibodies against SjKI-1 were custom made by GenScript (Piscataway, USA). Protein samples were fractionated on a 15% (w/v) SDS-PAGE gel and transferred to an Immun-Blot® low fluorescence- polyvinylidene difluoride membrane (Bio-Rad Inc, CA, USA). Overnight blocking was performed with Odyssey buffer at 4 °C. Then, the membrane was subjected to incubation with the rabbit anti-SjKI-1 anti-serum (1:2000 dilution in Odyssey buffer and 0·1% Tween-20) for 1 h followed by incubation with IRDye-labeled 800CW goat anti-rabbit antibody (1:15 000 diluted in Odyssey buffer plus 0·1% Tween-20 and 0·01% SDS) for 1 h on a shaker in a dark chamber. The membrane was allowed to dry in the dark and visualized using the Odyssey imaging system.

Paraffin blocks were made from adult worms and portions of the mid intestine of an infected mouse (6 weeks post cercarial challenge) were excised and tissue sections (4 μM) cut and placed on charged adhesive microscope slides. Following de-paraffinization and rehydration, antigen retrieval was done with RevealtA solution (Biocare Medical, Concord, USA). Then the tissue sections were blocked with 1% (v/v) bovine serum albumin in Tris buffered saline (TBS) for 60 min at RT in a humidified chamber and incubated with anti-SjKI-1 antibody (1:200) at 4 °C overnight. After three washes, the sections were incubated with Alexa fluor 488 donkey anti-rabbit IgG (1:500) (Invitrogen, Carlsbad, USA) at 37 °C for 60 min. Nuclei in the tissue sections were counterstained with diamidino-2-phenylindole (DAPI)gold (Invitrogen) and observed under an EVOS fluorescence microscope (Thermo Fisher Scientific, Waltham, MA, USA).

Functional assays with SjKI-1

Protease inhibitor assays – Bovine trypsin, bovine chymotrypsin, porcine pancreatic elastase (PPE), human neutrophil elastase (NE) and human cathepsin G were used to test the inhibitor activities of SjKI-1. Different concentrations of enzymes and SjKI-1 were first incubated together in 96 well plates at 37 °C for 10 min. Subsequently, a chromogenic or fluorogenic substrate was added at concentrations ranging from 5 μM to 100 mM and products released were measured using a plate reader each minute for 30 min. Bovine pancreatic trypsin, bovine pancreatic α-chymotrypsin and the fluorogenic substrates N_{α}-Benzoyl-L-arginine-7-amido-4-methylcoumarin hydrochloride and N-Succinyl-Ala-Ala-Pro-Phe-7-amido-4-methylcoumarin were purchased from Sigma Aldrich (St Louis, USA). The kinetic rate of substrate hydrolysis was measured at excitation/emission wavelengths of 370/460 nM. Human neutrophil elastase and Cathepsin G with their relevant substrates N-Methoxysuccinyl-Ala-Ala-Pro-Val-7-amino-4-methylcoumarin and Suc-Ala-Ala-Pro-Phe-pNA respectively, were purchased from Enzolifesciences (NY, USA). The inhibitory activity of PPE was observed using the *Enzcheck* elastase assay kit (Life Technologies, Carlsbad, USA) following the manufacturer's instructions. Human plasma kallikrein and fluorogenic kallikrein substrates (EMD Millipore, Billerica, MA, USA) were used to test for kallikrein activity in the presence of SjKI-1. An activated Factor Xa (FXa) inhibitor assay kit (Biovision Inc., Milpitas, CA, USA) was used to determine the inhibitory effect of SjKI-1 on FXa.

Results were expressed as a percentage of the relative activity of the SjKI-1 protein using the formula:

Percentage of relative activity = (ΔRFU (relative fluorescence units) of inhibitor/ΔRFU of enzyme control) × 100%

Values were corrected after subtracting background signals and all experiments were performed in triplicate. The concentration of the SjKI-1 protein needed for 50% inhibition of the protease was calculated as the IC_{50}.

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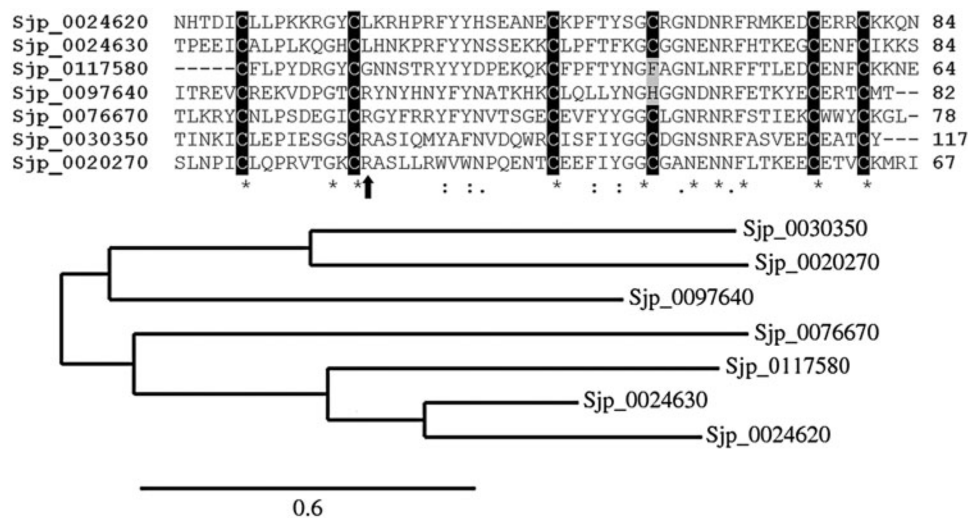

Fig. 1. Clustal alignment of putative Kunitz protein sequences identified in the *Schistosoma japonicum* genome/proteome databases and their phylogenetic analysis showing separation into two clades representing typical trypsin and chymotrypsin inhibitors. The P_1 reactive site is shown by the black arrow head. Proteins which contain Arg (R) at this site are typical trypsin inhibitors, while those having a Leu (L) are typical chymotrypsin inhibitors.

Coagulation assays. To test the effect of SjKI-1 in the coagulation pathway, APTT, prothrombin time (PT) and thrombin clotting time (TCT) were performed at the Pathology Queensland – Central Laboratory, Herston Hospitals Campus, Brisbane, Queensland, Australia. These tests detect the activity of intrinsic and common pathway, extrinsic pathway and thrombin activity, respectively, in the coagulation cascade and are widely used to determine the anti-coagulation properties of human blood (Marder *et al*. 2012). Human blood was collected into sodium citrate vacutainers and plasma was separated. Plasma in 800 μL aliquots was incubated with different concentrations of SjKI-1 for 10 min at 37 °C in a water bath. Aprotinin (5 μM) was used as the positive control for APTT and TCT and FVII negative plasma as positive control for PT. Clot formation was measured by a Sta-R coagulometer (Diagnostica Stago, Asnières, France) and the kits, TriniCLOT APTT HS (Trinity Biotech, Bray, Ireland), Thromborel®S (Siemens, Bavaria, Germany) and STA®-Thrombin (Diagnostica Stago, NJ, USA) were used for the determination of the APTT, PT (Jung *et al*. 2002; Curry and Pierce, 2007; Salmanizadeh *et al*. 2013) and TCT (Ignjatovic, 2013), respectively.

Calcium binding assay. An assay for calcium binding was carried out using a published procedure (Kurien and Bachmann, 2009) with minor modifications. Bovine serum albumin (BSA) as positive control and SjKI-1 were separated on a 15% (w/v) SDS-PAGE gel and transferred to a nitrocellulose membrane. The membrane was washed with wash buffer (10 mM imidazole, 60 mM potassium chloride, 5 mM magnesium chloride, pH 6·8) for 1 h at 37 °C with gentle shaking. After rinsing with distilled water, the membrane was incubated with 1 mM $CaCl_2$ for 1 h. Following washing (3×) with 20% (v/v) ethanol and a final wash with distilled water, the membrane was incubated with 1 mM Quin-2 (AM) (Sigma Aldrich, St Louis, USA) for 1 h prior to visualizing bound calcium using an ultraviolet (UV) transilluminator.

RESULTS

Gene and amino acid sequence analysis

Seven gene sequences, homologous to single domain Kunitz proteins, were identified in the *S. japonicum* genome by Basic Local Alignment Search Tool (BLAST). Phylogenic analysis of these putative Kunitz inhibitors separated them into two clades representing typical trypsin (top clade) and chymotrypsin inhibitors (bottom clade except Sjp_0117580) depending on the precise amino acid residue at the P_1 reactive site (Fig. 1). The Sjp_0117580 sequence contains Gly at the P_1 reactive site and probably does not function as a protease inhibitor. The selected gene of interest – Sjp_0020270 – was first listed as a putative Kunitz inhibitor in a microRNA study using developing schistosomula and adult worms (Huang *et al*. 2009), although no further characterization was undertaken. Further prediction of the biological function of Sjp_0020270 (which we have named SjKI-1) was made using *in silico* predictions of Gene Ontology (GO) with the category of 'response to stimulus' proposed (Swain *et al*. 2011*b*).

The translated amino acid sequence of SjKI-1 contains 69 amino acids, has a predicated molecular mass of 8·023 KDa, a pI of 7·12, contains a single Kunitz domain and is without a signal peptide. Despite no signal peptide sequence being present,

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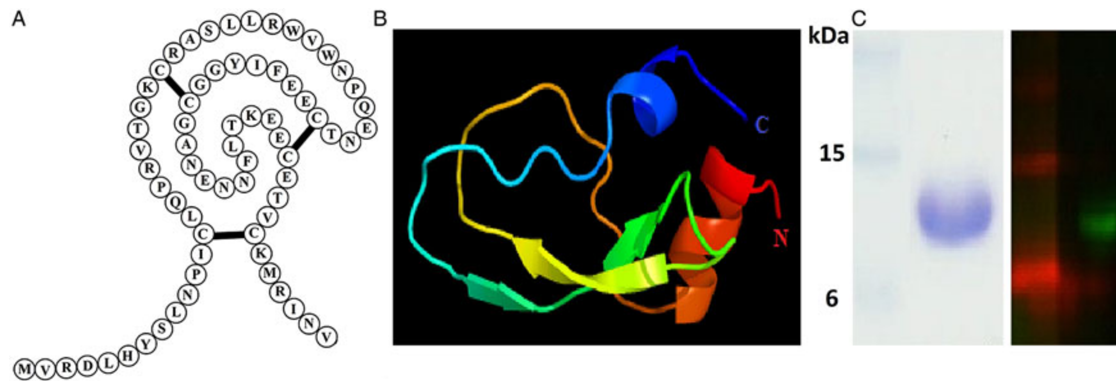

Fig. 2. (A) Secondary structure of the Kunitz domain (B) 3D model of SjKI-1 determined using PHYRE2 (C) SDS-PAGE of 3 μg SjKI-1 (left panel) and a western blot with anti-SjKI-1 antibody and 100 ng SjKI-1, green shows the SjKI-1 band and red shows the protein marker.

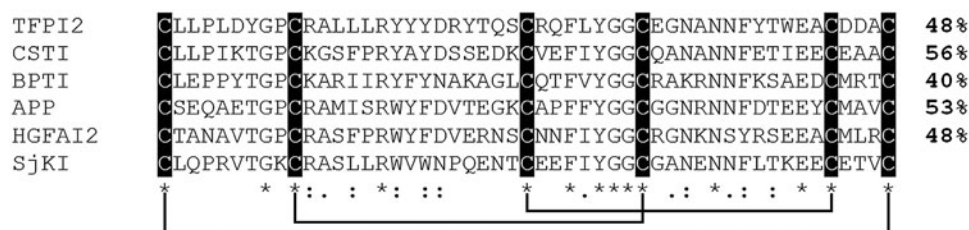

Fig. 3. Clustal alignment with other characterized Kunitz proteins: 1st domain of human tissue factor pathway inhibitor 2 (TFPI2): AAK13254·1, CSTI from *Bombyx mori*: P81902·1, BPTI: P00974·2, APP: 444646 and HGFAI2: BAA25024·1. The three disulphide bonds, C1-C6, C2-C4 and C3-C5, are shown in square brackets. The percentage sequence similarity between SjKI-1 and the other Kunitz inhibitors, obtained by NCBI blast analysis, is indicated to the right. Abbreviations: APP, amyloid precursor protein; BPTI, bovine pancreatic trypsin inhibitor; CSTI, cocoon shell associated trypsin inhibitor; HGFAI2, hepatocyte growth factor activator inhibitor type 2; NCBI, National Centre for Biotechnology Information; TFPI2, tissue factor pathway inhibitor 2.

using LocTree3 we predict that SjKI-1 is a secreted protein bearing the GO term of 'extracellular' (0005576) with 89% accuracy. Therefore SjKI-1 is probably a non-classical secreted protein which is secreted upon stimulus by the mammalian host.

Homology BLAST identified another protein sequence AAW27351·1 which consists of a longer sequence, predominantly at the N-terminal (67 amino acids), when compared with SjKI-1. The amino acid sequence of AAW27351·1 bears the same Kunitz domain as SjKI-1 (100% amino acid homology). A local BLASTn was performed using Bioedit2·2·10 using the SjKI-1 nucleotide sequence and the Sjr2scaffold assembly of the genome resulting in a homology hit of > SJC_S000109, length = 618 314, score = 391 bits (197), expect = e-107, identities = 197/197 (100%). To compare the genomic basis of SjKI-1 and AAW27351·1, and if both of these sequences arise from the same genomic site, we performed a NCBI Splign alignment (Kapustin *et al*. 2008). This alignment compared the same homologous genomic sequence SJC_S000109 (genomic scaffold) and the cDNA sequences of AAW27351·1 and SjKI-1 to demonstrate exon/intron boundaries (Supplementary Table S1). The second exon of both SjKI-1 and AAW27351·1 originated from the same genomic position, thus indicating that SjKI-1 is a post-translational modification of AAW27351·1. Predictions of LocTree3 indicated that AAW27351·1 is also a non-classical secretory protein with 87% accuracy.

Figure 2A is a schematic diagram of the secondary structure of the SjKI-1 protein showing the formation of proposed disulphide bonds between C1-C6, C2-C4 and C3-C5. Figure 2B is a 3D model showing β sheets and α helices from the C terminal to the N terminal. Fig. 2C is a western blot showing the specificity of the rabbit anti-SjKI-1 antibody indicating its suitability for use in the immunolocalization of SjKI-1 in *S. japonicum*.

NCBI blast and clustal alignment analyses of SjKI-1 indicated 40–56% amino acid sequence similarity and high amino acid conservation in the Kunitz domains (Fig. 3). Furthermore, SjKI-1 shares high sequence homology with the human proteins TFPI2, amyloid precursor protein (APP) and HGFAI2.

SjKI-1 gene expression

Real time PCR indicated higher *SjKI-1* gene expression in adult male and female worms and eggs than cercariae and schistosomules (Fig. 4).

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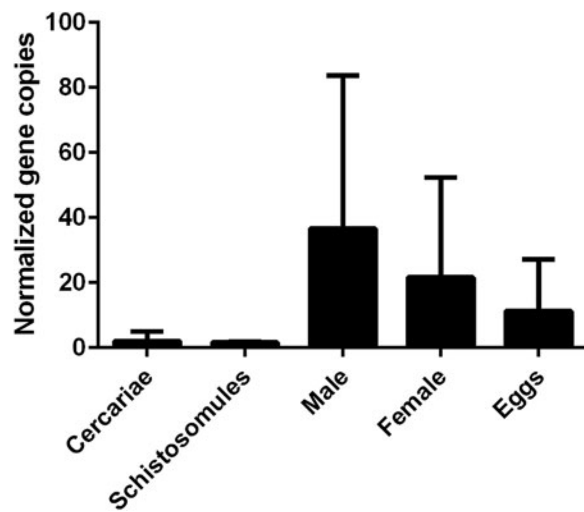

Fig. 4. Real time PCR showing expression of the *SjKI-1* gene in different life stages of *Schistosoma japonicum*. Error bars represent mean gene copy number ± the standard error of the mean (S.E.M.). Abbreviation: PCR, polymerase chain reaction.

Immunolocalization and western blotting

Immunolocalization studies indicated that SjKI-1 was localized between the egg shell and miracidium of eggs trapped in the intestinal wall (Fig. 5). Notably, no positive reactivity was evident in eggs trapped in infected liver sections, nor in sections of adult worms (not shown). None of the soluble parasite antigen fractions or adult ES products reacted positively with anti-SjK-1 antibody in western blot analysis (data not shown).

Protease inhibitor assays

SjKI-1 inhibited trypsin, chymotrypsin, neutrophil elastase, FXa and plasma kallikrein with IC_{50} values of 0·21, 18, 122, 650and 3·2 nM, respectively (Fig. 6). The protein is a potent inhibitor of trypsin as indicated by its inhibition in the picomolar range. Furthermore, SjKI-1 inhibited plasma kallikrein more potently than it did FXa. No inhibition was evident for pancreatic elastase or cathepsin G (data not shown).

Coagulation assays

Clot formation of healthy donor blood in the APTT normally takes between 26 and 41 s (Fig. 7; range shown as a rectangle). SjKI-1 prolonged APTT over the normal time period (Fig. 7) but clotting times for PT and TCT were normal, suggesting no FVII or direct thrombin inhibition. The time taken for clot formation in APTT gradually increased with the concentration of SjKI-1 protein; i.e. there was a 2-fold increase with 7·5 μM SjKI-1.

Calcium binding assay

3D Ligand binding site predictions indicated that the 39th Glu residue of SjKI-1 would bind calcium ions (Ca^{++}). As BSA contains three Ca^{++} binding sites, it was used as positive control in a binding assay (Majorek *et al*. 2012). Calcium-bound Quin-2 (AM) is excited under UV light and is observed as white bands (Fig. 8). As shown in Fig. 8, SjKI-1 is a Ca^{++} binding protein.

DISCUSSION

The presence of Kunitz proteins in many phylogenetically diverse species suggests that these molecules perform important biological roles. Interrogation of the *S. japonicum* genome indicated several gene sequences homologous to Kunitz proteins, none of which had been characterized before. Accordingly, we describe the first Kunitz inhibitor to be functionally expressed from *S. japonicum*.

Despite their intimate contact with the components of the immune system in mammalian blood, schistosomes can survive for many years without being attacked. APTT measures the activity of the intrinsic and common coagulation cascades and can be prolonged by deficiencies in factors XII, XI, IX, VIII or pre-kallikrein. We recorded a prolonged APTT in the presence of SjKI-1 suggesting that this Kunitz protein can inhibit one or several clotting factors involved in the coagulation pathways. The intrinsic pathway, also known as the contact activation system (CAS), is activated by the binding of Factor XII to collagen or a negatively charged surface, such as that presented by the schistosome tegument (Mebius *et al*. 2013). FXIIa activates plasma pre-kallikrein to kallikrein which has different biochemical functions. Kallikrein accelerates the activation of both FXII and pre-kallikrein and cleaves high molecular weight kininogens liberating bradykinin, which is an angiogenic and inflammatory mediator (Lynch and Shariat-Madar, 2012). In addition, kallikrein activates C3 and C5 which are major components of the complement system, and these components subsequently activate both the classical and alternative complement activation pathways (Lynch and Shariat-Madar, 2012). FXa is the convergent factor of all three secondary hemostasis pathways and both FXa and kallikrein have been suggested to be good targets for anti-coagulant drugs (Moreau *et al*. 2005; Ansell, 2007). It was encouraging therefore, that SjKI-1 inhibited both proteases, with higher activity against plasma kallikrein.

The activation of pre-kallikrein in turn activates neutrophils, plasmin, coagulation factors and kinins. The inhibition of plasma kallikrein by SjKI-1 suggests a possible role in inhibiting CAS at the very early stage of coagulation initiation. In addition, inhibition of neutrophil elastase by SjKI-1 may inhibit CAS by down regulating further neutrophil activation (Wachtfogel *et al*. 1993). Local accumulation of FXII leads to its auto-activation and induces a cascade of reactions which activates other clotting

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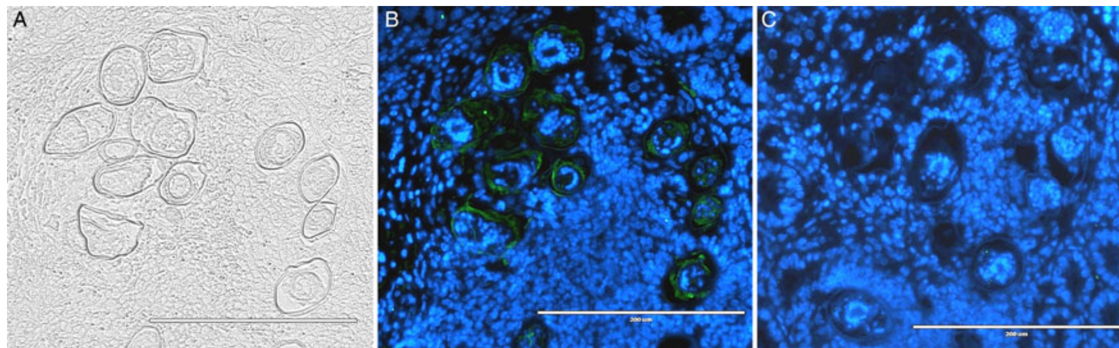

Fig. 5. *Schistosoma japonicum* eggs in mouse intestinal wall; positive fluorescence is shown in green and DAPI stained nuclei are blue. (A) Bright field (B) rabbit anti-SjKI-1 antibody (C) control rabbit serum. Abbreviation: DAPI, diamidino-2-phenylindole.

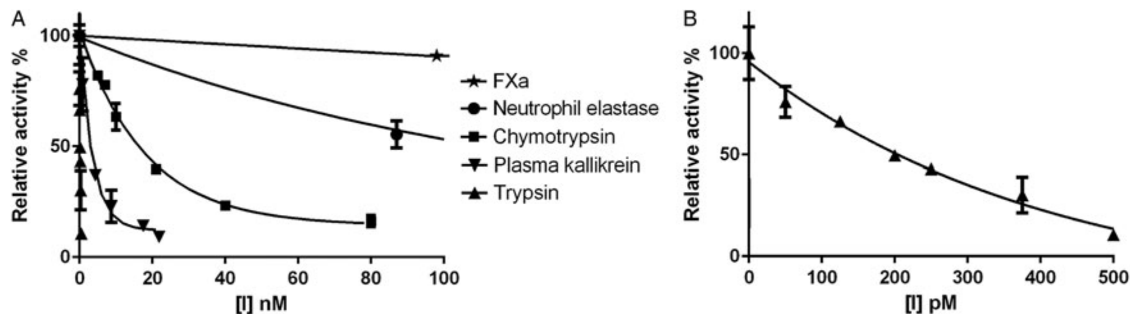

Fig. 6. (A) Relative inhibition of neutrophil elastase, trypsin, chymotrypsin, FXa and plasma kallikrein by SjKI-1 in nanomolar range concentrations and (B) trypsin inhibition in picomolar range. Error bars represent the mean ± S.E.M.

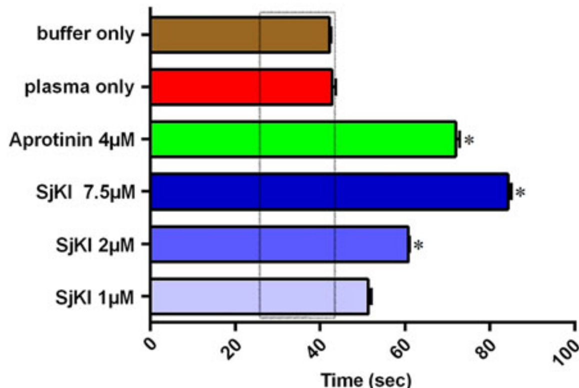

Fig. 7. Action of SjKI-1 on APTT: the normal time period for the clot to form (26–41 sec) is shown by the rectangle. Error bars represent mean ± S.E.M. *P* values of $<0{\cdot}05$, marked by *, were obtained by One-Way analysis of variance with Geisser–Greenhouse correction. Abbreviation: APTT, activated partial thromboplastin time.

factors (Renne *et al*. 2012). Finally, the action of thrombin causes the cleavage of fibrinogen to fibrin leading to formation of a stable fibrin clot. Calcium ions play a pivotal role in blood coagulation as many of the reactions involved are Ca^{++} dependent (Furie and Furie, 1988; Koklic *et al*. 2014). Through its Ca^{++} binding ability, SjKI-1 may contribute also to disruption of the coagulation cascade by reducing the level of free Ca^{++} available for these reactions. It has been shown that whole schistosome worm homogenates were able to block the conversion of FXII to FXIIa (Foster *et al*. 1992) although the mechanism involved was not determined.

Serine proteases comprise over one-third of the known proteolytic enzymes, with the Clan PA (proteases of mixed nucleophile, superfamily A) proteases, which bear the trypsin fold, being the largest family (Di Cera, 2009). Most Clan PA proteins have trypsin-like substrate specificity and are involved in a number of key biological processes, particularly in blood coagulation and the immune response (Di Cera, 2009). As predicted by its P_1 reactive site amino acid, SjKI-1 is a typical trypsin inhibitor with an IC_{50} value of 0·21 nM and is likely involved in various biological roles promoting survival of the schistosome parasite, through its inhibition of serine proteases.

NCBI Blast of SjKI-1 indicated high sequence similarity with the cocoon-associated trypsin inhibitor from *Bombyx mori* and APP. The former has been identified in the middle silk gland at the final stage of larval growth and is thought to play a role in protecting silk proteins from degradation during histolysis (Kurioka *et al*. 1999). APP is a transmembrane protein present in the plasma membranes of all human cell types. Ubiquitously expressed APP isoforms have the characteristic canonical inhibitor Kunitz domain, are secreted and inhibit mitochondrial metabolic

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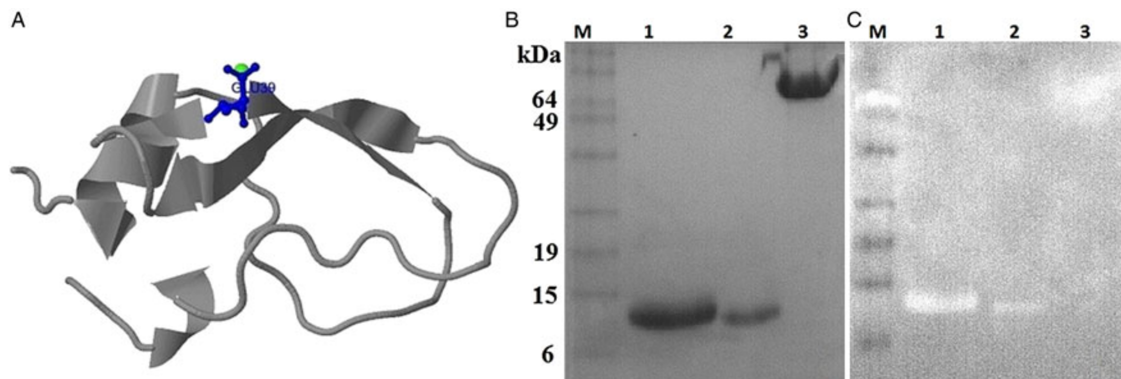

Fig. 8. Demonstration of binding of SjKI-1 with calcium. (A) The predicted binding site of SjKI-1 with Ca^{++} is shown in green and the 39th Glu residue is in blue; (B) SDS-PAGE gel and (C) UV image of the membrane. Lane M, BenchMark Pre-stained protein ladder; Lane 1, 10 μg SjKI-1; Lane 2, 3 μg SjKI-1; Lane 3, 10 μg BSA. Abbreviation: BSA, bovine serum albumin; UV, ultraviolet.

enzymes (Chua *et al*. 2013), FXIa and mesotrypsin (Salameh *et al*. 2010). Mesotrypsin is different from other trypsin isoforms and is upregulated in epithelial cancers including lung, colon, breast, pancreas and prostate (Salameh and Radisky, 2013). APP-containing Kunitz domains are considered as the first endogenous physiological substrate of mesotrypsin and are regarded as promising therapeutic targets (Salameh and Radisky, 2013).

Distinct from the miracidium cell mass, the inner sub-shell envelope and associated Reynold's layer of the mature egg of *S. mansoni* is considered as the primary source of egg secretions which help mediate egg transit through gut tissues and is likely to play a role in initiating hepatic pathology (Ashton *et al*. 2001). The presence of SjKI-1 in the sub-shell envelope of *S. japonicum* eggs suggests a possible role for this Kunitz protein in these two processes. The fact there was no reactivity of the anti-SjKI-1 antibody with soluble parasite antigen fractions or adult ES products in western blots suggests SjKI-1 was either not detectable or is present at low abundance. Alternatively, the SjKI-1 protein may degrade quickly or is only produced *in vivo* in response to the presence of host proteases.

Intragenus *Schistosoma* comparative genomic analysis (Swain *et al*. 2011*a*) and LocTree3 predictions indicate it is likely that SjKI-1 is a secretory protein. As SjKI-1 does not contain a signal sequence this suggests it is not secreted via the conventional process involving cleavage of the signal peptide. Indeed, 48% of the proteins identified in *S. japonicum* ES products were predicted to be non-secretory products suggesting that they are purposely released by the parasite through an unknown excretory mechanism (Liu *et al*. 2009; Liao *et al*. 2011*b*). Further, in addition to the conventional signal sequence-dependent ER-Golgi secretory pathway, it is now known that a number of fundamentally important proteins, involved in cell survival, immune surveillance and tissue organization, secreted by eukaryotic cells follow unconventional modes of secretion depending on the signals received and the particular requirements of the cell (Rabouille *et al*. 2012).

Since schistosomes are natural blood dwellers, *in vitro* studies may not provide the optimal environment to understand their biological activities in the natural environment. Further, proteins that play pivotal roles in the biology of these flukes may degrade rapidly or are synthesized in undetectably low but bioactive levels *in vivo* (Hewitson *et al*. 2009). This could be the reason why proteomic studies have to date failed to identify the presence of Kunitz proteins in *S. japonicum* (Liu *et al*. 2009; Hong *et al*. 2013; Zhang *et al*. 2013).

In summary, the results presented here indicate that SjKI-1 is an important molecule secreted by *S. japonicum* and has anti-inflammatory and anti-coagulant properties. It may play a major role in prolonged survival of the parasite in the mammalian host. Other putative Kunitz proteins we identified may also play key roles but further research is needed to characterize and determine their precise function in the biology of *S. japonicum*. SjKI-1 should now be assessed further as a potential anti-coagulant and offer a novel treatment for hematological disorders.

SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182015001328.

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