Purification and partial characterization of kininogenase activity from *Schistosoma mansoni* adult worms

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SUMMARY

An enzyme presenting kallikrein-like activity (designated sK1) was purified from the supernatant of *Schistosoma mansoni* adult worm homogenate. The enzyme cleaves bradykinin from purified rat plasma kininogen. Activity was optimal at pH 9·0 and the enzyme showed amidolytic activity, since it hydrolysed the kallikrein synthetic substrate D-Pro-Phe-Arg-*p*-nitroanilide. The activity of sK1 upon rat plasma kininogen was strongly inhibited by the serine proteinase inhibitors phenylmethanesulfonyl fluoride, aprotinin or soybean trypsin inhibitor, but not by ethylenediaminetetraacetic acid or sodium tetrathionate. The molecular mass of sK1, as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, was 66 kDa and the pI value, estimated by analytical chromatofocusing, was 4·2. Physical and chemical properties suggest that sK1 is a serine proteinase of the kallikrein family. Evidence is presented which suggests that sK1 is a component of the tegumental surface of the parasite and the levels of its activity in the male adult worm are approximately 21 times higher than those in the female adult worm. The intravenous injection of 3 μg of sK1 into an anaesthetized rat induced a drastic reduction in the arterial blood pressure of the animal. This effect lasted for about 1 min, and was followed by a progressive recovery of the arterial pressure. Neither bradycardia nor cardiac arrhythmias were noticed, suggesting a peripheral vasodilation effect. The presence of sK1 on the surface of adult male worms could play an important role in the wandering capacity of coupled worms into the visceral vasculature of the host.

Key words: kallikrein (EC 3.4.21.35), kinin, kininogenase, *Schistosoma mansoni*, serine proteinase, vasodilation, wandering capacity.

INTRODUCTION

Schistosoma mansoni is a parasite that lives in the visceral blood vessels of animal and man. Proteolytic enzymes play important roles in development of different stages of the parasite's complex life-cycle and have been ascribed a variety of functions including penetration of cercariae through the epidermis (Lewert & Lee, 1956; Stirewalt & Fregeau, 1966; Stirewalt & Walters, 1973), nutrient acquisition (Zerda, Dresden & Chappell, 1988), and evasion of host anti-parasite immune responses developed against the parasite (Verwaerde *et al*. 1988). Serine proteinases present in the cercariae, in the schistosomula, and in the adult worm are involved in these processes (Landsperger, Stirewalt & Dresden, 1982; Verwaerde *et al*. 1986, 1988; Marikovsky, Arnon & Fishelson, 1990). Among them are 2 serine proteinases presenting a molecular mass of 25 kDa (Landsperger *et al*. 1982) and 28 kDa (McKerrow, Newport & Fishelson, 1991), which were purified from *S. mansoni* cercariae and may be involved in the penetration of cercariae into the vertebrate hosts. The 28 kDa proteinase is present in the surface of transformed schistosomula and adult worms (Ghendler *et al*. 1996) and it is associated with the surface of the parasite as it moves through the tunnel created during skin penetration (Fishelson *et al*. 1992).

Of interest, the adult worm augments the diameter of the surrounding vessel approximately 10-fold (Pessoa & Martins, 1982) and the mechanism or substance(s) involved in this vasodilation process is not known.

We believe this augmentation in the diameter of the vessel may be explained by local liberation of bradykinin (BK)-like substance(s) known to play an important role in processes related to the cardiovascular system such as capillary permeability, regulation of fluid and electrolyte balance (Cuthbert & Margolius, 1982), and inflammation (Sharma & Mohsin, 1990). They are generated by the action of kallikrein (EC 3.4.21.35) upon its natural substrate kininogen. Kallikreins are a subfamily of the serine proteinases widespread in the mammalian

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tissues, which selectively cleave 2 peptide bonds in kininogen to liberate the peptide bradykinin or lysylbradykinin (Fiedler, 1979). They have reported molecular masses ranging from 25 to 65 kDa and isoelectric points close to 4·0 (Nustad, Gautvik & Orstavik, 1978; Fiedler, 1979). The presence of kinin-generating enzyme(s) in *S. mansoni* might be important for the migration of the parasite, which presents a surprising migratory capacity behaviour into the portal system of the host (Pellegrino & Coelho, 1978). However, little is known about the presence of kinin-liberating enzymes in the adult worm of *S. mansoni*. The main proteolytic fraction of cercarial extracts was not able either to destroy the biological activity of bradykinin or to release bradykinin from purified dog plasma kininogen (Gazzinelli, Mares-Guia & Pellegrino, 1972). However, the injection of a cercarial extract into guinea-pig skin leads to an inflammatory response that is reduced by simultaneous injection with kallikrein inhibitors (Teixeira *et al*. 1993). As the kinin peptides are known as inflammatory mediators, a mechanism of bradykinin generation may be responsible for all the oedema-inducing activity of cercarial extract.

The demonstration of the presence of kallikrein-like activity in the homogenate of cercariae and the possibility that this activity may, in part, facilitate parasite's migration led us to conduct the present investigation on the presence of the kallikrein–kinin system in the homogenate of *S. mansoni* adult worms.

MATERIALS AND METHODS

Peptides and chromatographic systems

Peptides used in this study were synthesized by the solid-phase method (Merrifield, 1963) using butoxycarbonyl protected amino acids and a Biosearch (Foster City, CA, USA) model 9600 peptide synthesizer. The procedures used for purification and characterization of the peptides were previously described (Chagas *et al*. 1992). The pure peptides were characterized by analytical high performance liquid chromatography (HPLC), amino acid analysis and, when applicable, by biological activity. The B2 kinin receptor antagonist D-Arg,(Hyp^3,Thi^{5,8},D-Phe^7)-BK (B2KRa) was purchased from Sigma Chemical Co. Rat submandibular gland kallikrein was purified as previously described (Araujo *et al*. 1991). HPLC and fast protein liquid chromatography (FPLC) systems, Superdex 75 HR 10/30, Mono P HR 5/20, MinoRPC S 5/20, Polybuffer 74 were acquired from Pharmacia (Uppsala, Sweden) and diethylaminoethyl (DEAE)-cellulose was a product from Whatman.

Parasite

The L.E. strain (Pellegrino & Katz, 1968) of *S. mansoni* was used and cercariae were obtained 48 days post-infection according to Pellegrino & Macedo (1955) by perfusion of the portal and mesenteric veins with saline solution containing 2·5 U/ml heparin (Pellegrino & Siqueira, 1956). In order to induce the worms to regurgitate their gut content, they were rinsed in deionized water 6-fold for 5 min, at room temperature (Chappell & Dresden, 1986).

Purification procedures

All procedures were carried out at 4 °C.

Enzyme. Approximately 2000 worms (330 mg of adult worms of both sexes) or approximately 1000 worms of each sex (300 mg of male or 30 mg of female worms) were used for each experiment. After regurgitation, they were immediately homogenized in 20 mM Tris–HCl buffer, pH 8·4, containing 5 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM sodium tetrathionate (50 mg of worm/ml of buffer). The homogenate was centrifuged at 10000 ***g*** for 30 min and the supernatant dialysed for 15 h against 3 l (1 l/5 h) of 20 mM Tris–HCl buffer, pH 8·4, containing the inhibitors mentioned above. After dialysis, the supernatant was loaded on a DEAE-cellulose column (2·5 × 8·5 cm). The column was eluted with 20 mM Tris–HCl buffer, pH 8·4, containing NaCl at the following concentrations (mM): 25, 50, 100, 200, 300 and 1000. The fractions which hydrolysed the synthetic substrate D-Pro-Phe-Arg-*p*-nitroanilide (amidolytic activity) were pooled and loaded on a Superdex HR 75 column in a FPLC system. The column was eluted with 50 mM sodium phosphate buffer, pH 7·0, containing 150 mM NaCl. Fractions with enzyme activity were pooled and stored at −20 °C.

Rat kininogen. Kininogen was purified from male Wistar rat plasma by single-step DEAE chromatography. Plasma was heated to 61 °C for 60 min, centrifuged at 10000 ***g*** for 15 min and the supernatant supplemented with EDTA to a final concentration of 1 mM and ammonium sulfate to 50 % (w/v) saturation. The resultant precipitate was separated by centrifugation, resuspended in 20 mM Tris–HCl buffer, pH 8·4, dialysed against the Tris-buffer and loaded on a DEAE-cellulose column. The presence of kininogen in the fractions was determined by incubating them with rat submandibular gland kallikrein. The kinin released was determined by bioassay, using isolated rat uterus (Feitosa *et al*. 1989) and the protein concentration was measured by the method of Bradford (1976).

Tegument elution

The components from the tegument of freshly harvested male worms of *S. mansoni* were eluted by incubating the worms (approximately 1000) in 10 ml

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of 0·05 M sodium phosphate buffer, pH 7·4, containing 2·7 mM KCl, 0·9 mM $CaCl_2$, 0·5 mM $MgCl_2$ and 5 mM EDTA (Vasconcelos *et al*. 1993). Incubations were carried out at 37 °C, for 15, 30, 60 or 120 min and then centrifuged at 10000 ***g*** for 30 min and the eluates tested for amidolytic activity.

Kininogenase activity and proteinase inhibitor effects

The kininogenase activity was determined by incubating 1–5 μg of samples from different steps of the purification procedure with 0·1 mg of the purified kininogen, at 37 °C, for 2 h, in a final volume of 200 μl of 0·2 M Tris–HCl buffer, pH 9·0. The products were characterized by comparison with synthetic standards in an HPLC system and by biological assay of the HPLC fractions. The effect of proteinase inhibitors upon kininogenase activity, was determined by incubating the purified enzyme with rat plasma kininogen in the presence of the following inhibitors: 500 U/ml aprotinin, 12 mM phenylmethanesulfonyl fluoride (PMSF), 7·4 μM soybean trypsin inhibitor (SBTI), 10 mM EDTA or 1 mM sodium tetrathionate.

Amidolytic activity

The chromogenic peptide D-Pro-Phe-Arg-*p*-nitroanilide with high specificity for tissue kallikrein (Chagas *et al*. 1992) was used as substrate. Hydrolysis was determined by incubating samples (1–5 μg) from different steps of the purification procedure with 215 mM of substrate in a final volume of 1·5 ml of 0·2 M Tris–HCl buffer, pH 9·0, at 37 °C. The absorbance of the product (*p*-nitroaniline) was monitored at 405 nm ($\epsilon = 8900/\text{M}/\text{cm}$; Juliano & Juliano, 1985).

Optimum pH

Purified enzyme (1 μg) was incubated with 0·1 mg of kininogen or 215 mM of chromogenic substrate, at 37 °C in 0·2 M sodium acetate for pH 5·0 and 5·5, 0·2 M sodium phosphate for pH 6·0, 6·5, 7·0 and 7·5 and 0·2 M Tris–HCl for pH 8·0, 8·5, 9·0 and 9·5.

Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was performed at pH 8·8 in 12·5 % (w/v) acrylamide gels with 0·1 % (w/v) sodium dodecyl sulfate (SDS) (Laemmli, 1970). approximately 1 μg of samples in 0·0625 M HCl buffer, pH 6·8, containing 2·9 % (v/v) 2-mercaptoethanol, 2·0 % (w/v) SDS and 5·8 % (v/v) glycerol were heated in a boiling water bath for 3 min and centrifuged prior to loading. Gels were stained with silver nitrate (Tunon & Johansson, 1984). The molecular mass markers used were bovine serum albumin (BSA) (M_r 67000), ovalbumin (M_r 45000), carbonic anhydrase (M_r 29000) and SBTI (M_r 20 100).

Analytical chromatofocusing

The purified enzyme was loaded on a Mono P HR 5/20 column for pI determination, as described by Gomes, Lima & Pesquero (1994). The column was equilibrated with 25 mM *bis*-Tris–HCl buffer, pH 6·5, and after loading the sample, the column was eluted with a 1:10 diluted solution of polybuffer 74, pH 4·0, for 40 min with a flow rate of 1 ml/min. Fractions of 0·5 ml were collected and tested for amidolytic activity.

High performance liquid chromatography

Kininogen-enzyme incubates were supplemented with 100 μl of 20 % (v/v) trifluoroacetic acid (TFA) and, after filtration (Millipore GSWP01300 filter), samples were loaded on a MinoRPC column in an HPLC system (Pharmacia, Sweden). Peptides were eluted with a linear gradient of 3·7 % (v/v) acetonitrile in 1 % (v/v) TFA at a flow rate of 1 ml/min. The synthetic peptides angiotensin II (Ang II), bradykinin (BK) and lysyl-bradykinin (Lys-BK) were used as standards. The fractions (1 ml) were freeze dried, resuspended in 1 ml of 0·9 % (w/v) NaCl (saline) and submitted to the isolated rat uterus assay.

Bioassays

The isolated rat uterus assay was performed according to the method described by Feitosa *et al*. (1989). The contractions of the preparation were recorded with a frontal writing lever (10 times magnification). Air was bubbled through the bath fluid. The samples tested remained in contact with the muscle for 2 min, and the bath fluid was changed twice between samples. The time cycle was 5 min. The contractions were compared to that of a standard curve constructed using synthetic bradykinin. The kinin response was characterized by specific inhibition with 5·0 μM of the B2 bradykinin receptor antagonist (B2KRa). In order to determine the kinin-induced vasodilation, male albino rats weighing about 200 g were anaesthetized with urethane (1·4 g/kg) and the femoral artery and vein were cannulated with polyethylene tubing (PE 10). The arterial tubing was connected to a Gould P 23 ID pressure transducer (Statham, USA) and the arterial pressure recorded in a polygraph Grass model 7D. In the same polygraph the ECG and the respiratory movements were also recorded in a Grass lead selector model 7LSA and in a Grass volumetric pressure transducer PT 5A, respectively. Saline or a selected dose of sample at the same volume (100 μl) were injected through the femoral vein.

RESULTS

Kallikrein-like activity was present in the supernatant of the homogenate of *S. mansoni* adult worms

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Table 1. Parameters for kininogenase purification from 1 g of adult worms of *Schistosoma mansoni* of both sexes

Procedure	Total protein (mg)	Specific activity*	Total activity†
HS‡	59·30	0·81	48
DEAE-cellulose	1·30	508	660
Superdex HR 75	1·00	649	649

* Expressed as nanomoles of *p*-nitroaniline liberated/min/mg of protein.
† Expressed as nanomoles of *p*-nitroaniline liberated/min.
‡ HS, supernatant from crude homogenate.

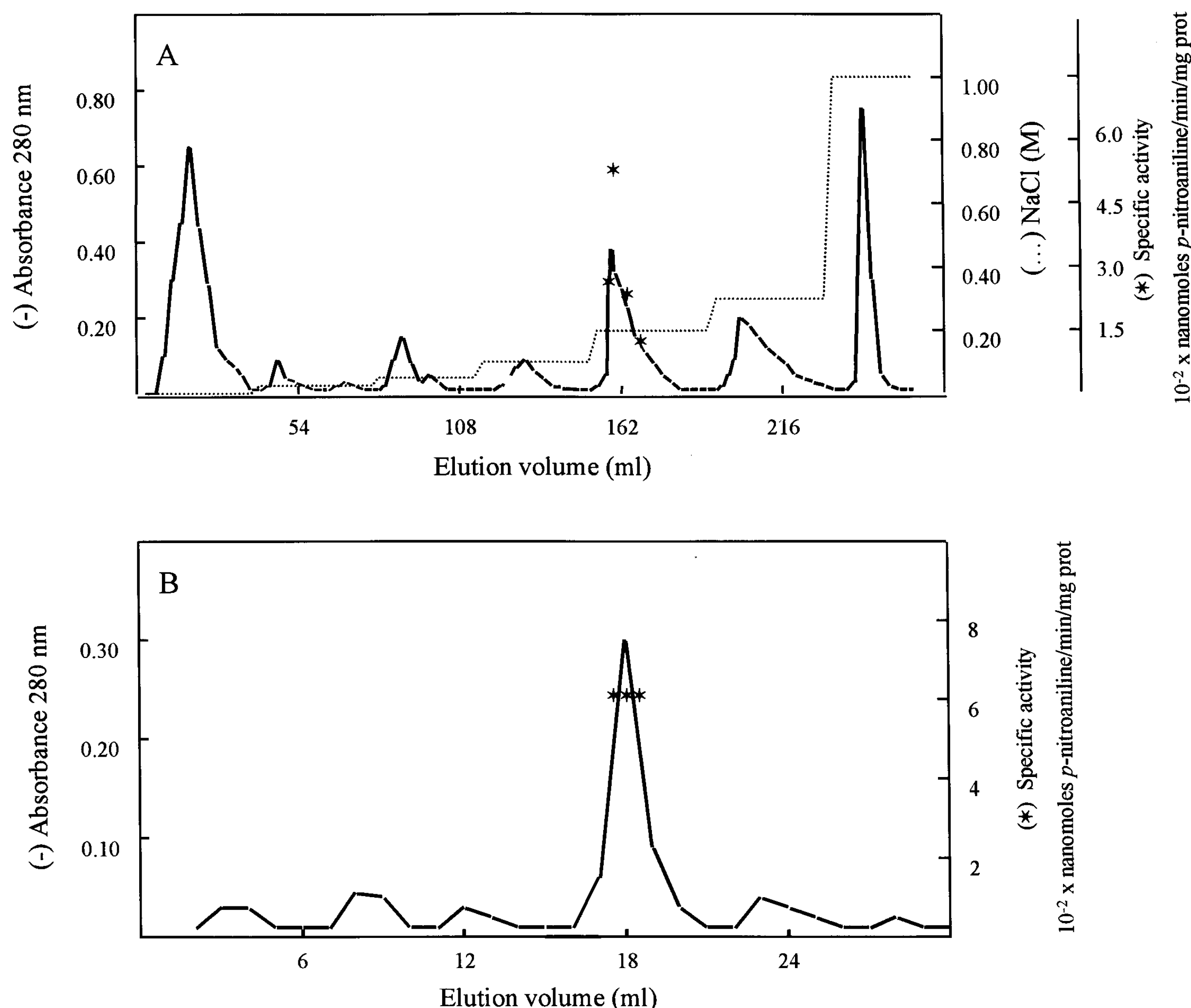

Fig. 1. (A) DEAE-cellulose chromatography of the supernatant from homogenate of adult worms of *Schistosoma mansoni*. The column was equilibrated with 20 mM Tris–HCl buffer, pH 8·4. The adsorbed proteins were eluted with a sodium chloride gradient as shown by dotted line. (B) Superdex HR 75 chromatography of the active material from DEAE-cellulose. The activity of fractions was determined incubating with 215 mM D-Pro-Phe-Arg-*p*-nitroanilide as substrate.

as determined using rat plasma kininogen (kininogenase activity) or the synthetic chromogenic peptide D-Pro-Phe-Arg-*p*-nitroanilide (amidolytic activity) as substrate (Table 1). To purify the kallikrein-like activity the supernatant was applied to a DEAE-cellulose chromatography column and fractions eluted with 20 mM Tris–HCl, pH 8·4, containing 200 mM NaCl possessed the capability to hydrolyse the substrate D-Pro-Phe-Arg-*p*-nitroanilide (Fig. 1A). The contents of these tubes were pooled and loaded on a Superdex HR 75 column (Fig. 1B). The kininogenase activity (not shown in Fig. 1A and 1B)

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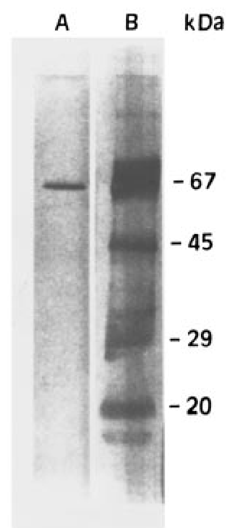

Fig. 2. SDS–PAGE of 1 μg of sK1 (lane A) and molecular mass markers (lane B).

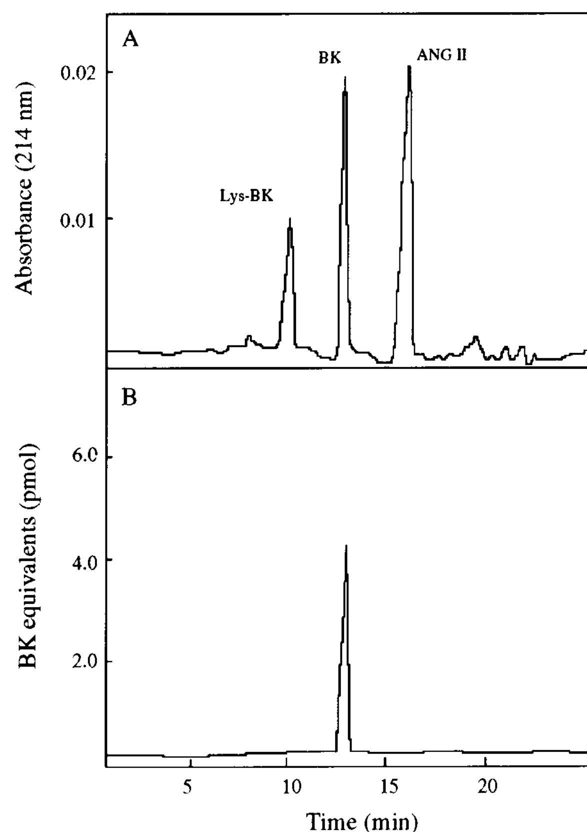

Fig. 3. Elution profiles obtained by HPLC of the components of kininogen-sK1 incubate. (A) Synthetic peptides used as standards: bradykinin (BK), lysyl-bradykinin (Lys-BK) and angiotensin II (ANG II). (B) Bradykinin equivalents in the fractions determined by isolated rat uterus assay.

co-eluted with the amidolytic activity in both chromatographies. The active fractions from Superdex HR 75 chromatography were pooled and submitted to SDS–PAGE (Fig. 2). The enzyme appeared to be homogeneous and to have an apparent molecular mass of 66 kDa. As this fraction contained the kininogenase activity it was designated sK1, as proposed by the common nomenclature for tissue kallikreins (Berg *et al*. 1992), since it is the first kallikrein described from *Schistosoma*. The iso-electric point of sK1, obtained by analytical chromatofocusing using a Mono P column, was 4·2 (not shown). The parameters for sK1 purification are summarized in Table 1. D-Pro-Phe-Arg-*p*-nitro-anilide hydrolysis and kininogenase activity co-eluted throughout the purification procedure (Fig. 1) which resulted in a single protein band as judged by SDS–PAGE (Fig. 2). Taken together these observations strongly suggest that both activities are due to sK1. Therefore, the parameters for sK1 purification are expressed as a function of the amidolytic activity. The enzyme sK1 induced bradykinin release when incubated with rat plasma kininogen as characterized by HPLC and further evidence of this activity was shown by the contractile response of the isolated rat uterus elicited by active fractions from HPLC of kininogen-sK1 incubate (Fig. 3). In addition, the B2 bradykinin receptor antagonist inhibited this contraction (Fig. 4). The optimum pH for bradykinin release and for hydrolysis of the synthetic chromogenic substrate by sK1 was 9·0. At this pH, sK1 had a specific activity of 18 picomoles of bradykinin liberated/min/mg of protein. sK1 was totally inhibited by the serine proteinase inhibitors PMSF, SBTI and aprotinin under experimental conditions while EDTA and sodium tetrathionate showed no effect upon the activity of sK1.

The total sK1 activity, purified/g of adult worm, expressed as nanomoles of *p*-nitroaniline released/min were 715 ± 96 ($n = 3$) and 34 ± 12 ($n = 3$) for male and female worms, respectively. More than 70% of total amidolytic activity present in the male adult worm co-eluted with the components of the tegument after 60 min of incubation of the parasite in phosphate buffer (Table 2).

The effect of sK1 upon the mean arterial pressure of anaesthetized rats is shown in Fig. 5. The control mean arterial pressure was about 100 mmHg and the heart rate varied from 375 to 428 beats/min. Following the injection of sK1, arterial pressure dropped in proportion to the dose. With the higher dose (3·0 μg) the values for arterial blood pressure dropped to around 5 mmHg. This decrease lasted for about 25 sec and after this time a rapid increase to levels higher than 140 mmHg was seen before pressure returned to the control levels 1 min after the sample injection. No changes in the heart rate or abnormal rhythms were noted. The injection of a corresponding volume of saline did not elicit any alteration of the cardiovascular parameters.

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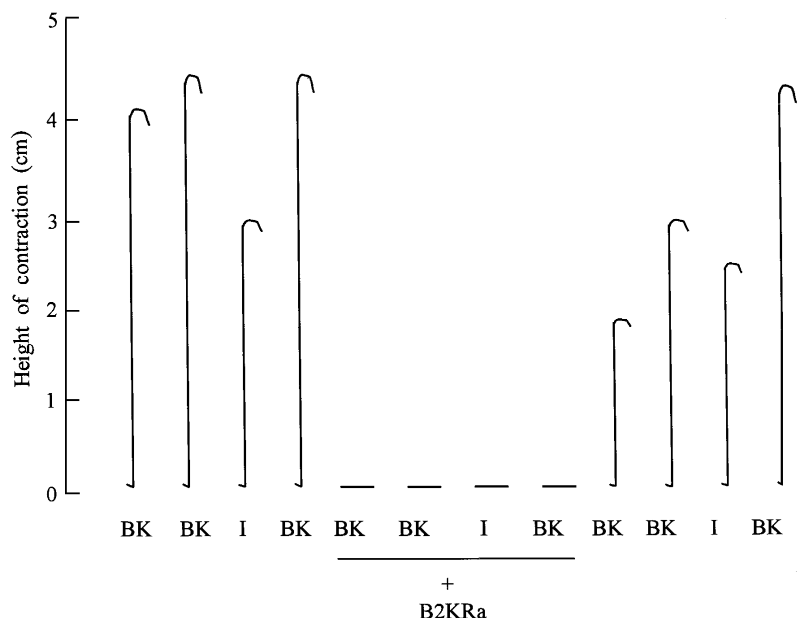

Fig. 4. Effect of the B2 kinin receptor antagonist (B2KRa) on the contractions of the isolated rat uterus induced by bradykinin (BK, 3·0 ng) or by the HPLC active fraction of the kininogen-sK1 incubate (I).

Table 2. Elution time-course of sK1 from tegument of 1 g male adult worms of *Schistosoma mansoni*

Elution time (min)	Eluate activity*
15	350±53 (49)
30	417±55 (58)
60	505±60 (71)
120	445±59 (62)

* The values represent the means±S.D. of 3 experiments and are expressed as nanomoles of *p*-nitroaniline liberated/min.
Values in parenthesis represent the percentage from the total activity observed in the male adult worm (715±96).

DISCUSSION

S. mansoni adult worms are many times bigger than the diameter of the vessel where they live and, in addition to the well-coordinated rhythmical movement observed by Chance & Mansour (1953), may require a mechanism to augment the diameter of the vessel for migration. The bigger vessel diameter seen when the *S. mansoni* is present might be explained by the local release of substances such as kinins, which are potent vasodilators. Our results show the presence of a kinin-forming enzyme in homogenates of adult *S. mansoni*. The purified enzyme was able to generate kinin when incubated with a purified rat plasma kininogen and was designated sK1. The principal evidence for the ability of the enzyme to release kinin comes from the results obtained by incubating sK1 with rat kininogen. At least 1 component of this incubate was able to elicit contraction of isolated rat uterus, this being abolished by the specific B2 kinin receptor antagonist. HPLC data indicated that the kinin generated in this incubate was bradykinin. sK1 belongs to the serine proteinase family, since its activity upon rat kininogen was strongly inhibited by aprotinin, by phenylmethanesulfonyl fluoride or by soybean trypsin inhibitor, but not by ethylenediaminetetraacetic acid or sodium tetrathionate. The physicochemical data obtained for sK1 as apparent molecular mass (66 kDa), isoelectric point (4·2) and optimum pH (9·0) are consistent with those described for enzymes of the kallikrein family (Nustad *et al*. 1978; Fiedler, 1979). Thus, these are the first data providing evidence for the presence of kallikrein-like activity in the adult worm of *S. mansoni*. Our results showing that the levels of sK1 total activity in the worm are sex dependent is evidence that sK1 is not a contaminant from the host blood. However, if the enzyme is acquired from the host blood has yet to be determined. The presence of a gene that codes for kallikrein-like proteinase in the adult *S. mansoni*, shown by Cocude *et al*. (1997), reinforce the possibility that sK1 is synthesized in the parasite. These authors cloned a DNA fragment from *S. mansoni* genomic DNA whose expressed protein is part of a kallikrein-like molecule which shows the greatest similarity to the mouse plasma kallikrein. Teixeira *et al*. (1993) showed the presence of a kallikrein-like activity in cercarial extracts of *S. mansoni* but they did not purify the enzyme. Therefore, we could not define the relationship between this activity and the one described by us in this report.

The augmentation of the total sK1 activity found in the DEAE-cellulose fraction, as compared to that of the supernatant agrees with the findings of

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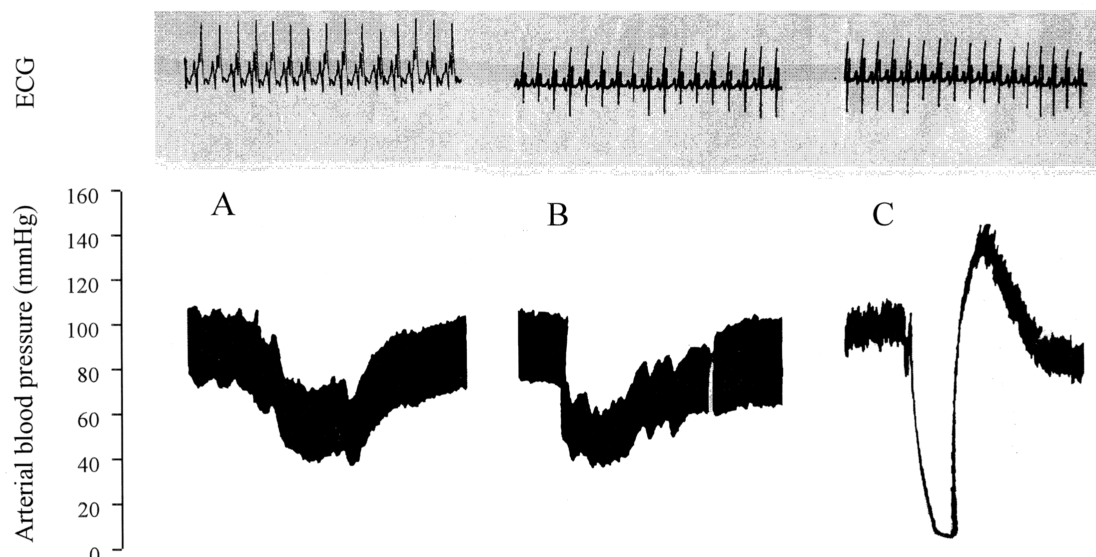

Fig. 5. Effect of sK1 intravenous injection on the arterial blood pressure and electrocardiogram (ECG) in anaesthetized rats. The figure depicts 3 separate experiments. Electrocardiogram in the upper tracing (25 mm/sec) and arterial blood pressure in the lower tracing (25 mm/min). The doses of 1·0 (A), 1·5 (B) and 3·0 μG (C) of sK1 were injected into the femoral vein.

Ghendler, Arnon & Fishelson (1994), who demonstrated that adult worms of *S. mansoni* synthesize a serine proteinase inhibitor designated Smpi56. It seems likely that in the purification procedure sK1 was separated from all the inhibitory activity in the DEAE-cellulose step.

In order to verify the presence of the kallikrein substrate kininogen in the homogenate of *S. mansoni* adult worms, the supernatant of the homogenate and the fractions collected after DEAE-cellulose chromatography of the supernatant were incubated with rat submandibular gland kallikrein. Kinin activity was not detected in these incubates (not shown). This is evidence for the absence of the kallikrein substrate kininogen in the adult worm. Thus, to generate bradykinin *in vivo*, sK1 should be hydrolysing the substrate present in the host plasma and should be localized on the surface of the worm tegument. This proposal is supported by our results, since more than 70% of total sK1 activity eluted from the tegument of the male worm after 60 min incubation in phosphate buffer.

sK1 activity levels in male adult worms, are 21 times greater than in females, providing evidence that the sK1-dependent kinin generation could be important for the host–parasite relationship since the adult female worm normally lives in the male gynecophoral canal and does not need to make any effort to move into the blood vessels. The female adult worm leaves the gynecophoral canal only at the moment of oviposition. The effects of sK1 causing a fall of the arterial blood pressure, allied to other findings presented in this paper, suggest that this hypotension is a kinin-mediated effect. It is well established that kinins can cause vasodilation and hypotension, and can be generated in the blood and tissue fluids by the action of kallikrein that splits small polypeptides away from the kininogen in the alpha-2 globulin fraction (Fiedler, 1979; Cuthbert & Margolius, 1982). Even though we have little knowledge about the function of kinins on the control of circulation, the observed powerful effects of sK1 on the arterial blood pressure, added to the occurrence of local vasodilation of mesenteric vessels housing the adult *S. mansoni*, indicate that the tegument of the adult male worm might play a special role in regulating the diameter of those vessels thus facilitating worm movement inside them.

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