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BIOCHEMICAL STUDIES ON THE EGGS OF *AMBLYOMMA HEBRAEUM*

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ABSTRACT

VERMEULEN, N. M. J. & NEITZ, A. W. H., 1987. Biochemical studies on the eggs of *Amblyomma hebraeum*. *Onderstepoort Journal of Veterinary Research*, 54, 451–459 (1987).

The isolation, characterization and kinetic properties of a toxic and non-toxic anti-protease from the eggs of *A. hebraeum* are compared with similar data obtained for eggs of other tick species. The biochemistry of various physiological processes in tick eggs is reviewed.

INTRODUCTION

Naturally occurring inhibitors of proteolytic enzymes have been isolated from a wide variety of plant and animal sources and the literature on their properties is extensive (Tschesche, 1974; Ryan, 1978; Laskowski & Kato, 1980; Travis & Salvesen, 1983). In spite of this, very little is known on their prevalence, characteristics and functions in ticks and especially tick eggs.

An investigation into the biochemical basis of invertebrate-symbiont associations (Starr, 1975) is important for the study of ticks as vectors of disease agents on a molecular level. Interest in the toxins present in the eggs of ticks stems largely from their possible bearing on toxins associated with tick toxicoses (Regendanz & Reichenow, 1931). The characterization of tick toxins is a prerequisite for investigations into the possible symbiotic or commensal, prokaryotic origin of the toxins and into tick-microbial symbiotic associations in general (Koch, 1960: Houk & Griffiths, 1980). In addition, a chemical analysis of the composition of tick eggs and the associated biological activity of the individual components may lead to a better understanding of the biochemistry of oogenesis, embryogenesis, tick metabolism, selective anti-microbial defence mechanisms of ticks and tick eggs and transovarial transmission of rickettsiae (Garibaldi, 1960; Board & Fuller, 1974; Schwemmler, 1974; Lackie, 1980; Bezuidenhout & Jacobsz, 1986).

A protein, able to inhibit bovine trypsin, chymotrypsin and plasmin and pig pancreatic kallikrein has been isolated from the eggs and unfed larvae of *Boophilus microplus* (Willadsen & Riding, 1979; 1980). This anti-protease causes an immediate hypersensitivity reaction when injected intradermally into cattle. A non-toxic and a toxic anti-protease have also been isolated from the eggs of *Amblyomma hebraeum* (Neitz, Prozesky, Bezuidenhout, Putterill & Potgieter, 1981; Neitz, Bezuidenhout, Vermeulen, Potgieter & Howell, 1983; Vermeulen, Neitz, Potgieter & Bezuidenhout, 1984). The presence of a trypsin inhibitor has been demonstrated in the saliva of the sand tampan *Ornithodoros savignyi* (Neitz, 1976). Four toxic proteins with inhibitory properties against trypsin and/or chymotrypsin have been isolated from *Rhipicephalus evertsi evertsi, Hyalomma truncatum, B. microplus* and *Boophilus decoloratus* (Viljoen, Neitz, Prozesky, Bezuidenhout & Vermeulen, 1985). Anti-proteases isolated by affinity chromatography on trypsin-Sepharose 4B columns from *B. decoloratus* eggs were monitored by chromatofocusing for the formation of any modified protein (Viljoen, Mills, Neitz, Potgieter & Vermeulen, 1984). The enzyme activities of succinate-cytochrome C reductase, of the NADH-cytochrome C reductase system, the NADH oxidase system and cytochrome C oxidase have been determined spectrophotometrically in eggs and larvae of *B. microplus* (Shanahan & O'Hagan, 1973). These activities remain relatively constant during hatching, but alter during the larval stage. An investigation into the function and chemical composition of the secretion of the porose areas of *R. evertsi evertsi* during oviposition has been conducted by Vermeulen, Gothe, Senekal & Neitz (1986).

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MATERIALS AND METHODS

Two types of anti-proteases have been isolated from *A. hebraeum* eggs, namely a toxic (Neitz *et al.*, 1981), and a non-toxic protein (Vermeulen *et al.*, 1984). The collection of the tick eggs, the preparation of crude extracts, the isolation procedures, determination of toxicity, molecular mass and kinetic constants are described in detail in these papers.

To obtain the toxic anti-protease in an analytically pure state from the eggs of *A. hebraeum* is essentially a 2

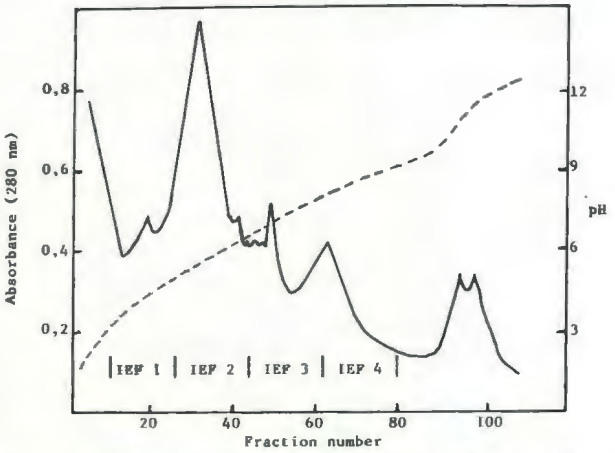

FIG. 1 Iso-electric focusing of the supernatant obtained by dialysis and centrifugation of the crude egg extract. The dotted line represents the pH (Neitz *et al.*, 1981)

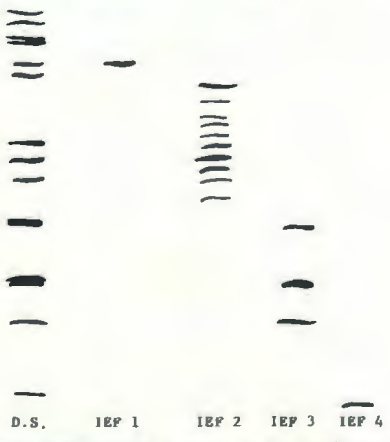

FIG. 2 Separation of proteins present in the crude egg extract and Fractions IEF 1, IEF 2, IEF 3 and IEF 4 by analytical iso-electric focusing. D.S.-supernatant of dialyzed crude egg extract (Sample before preparative IEF) (Neitz *et al.*, 1981)

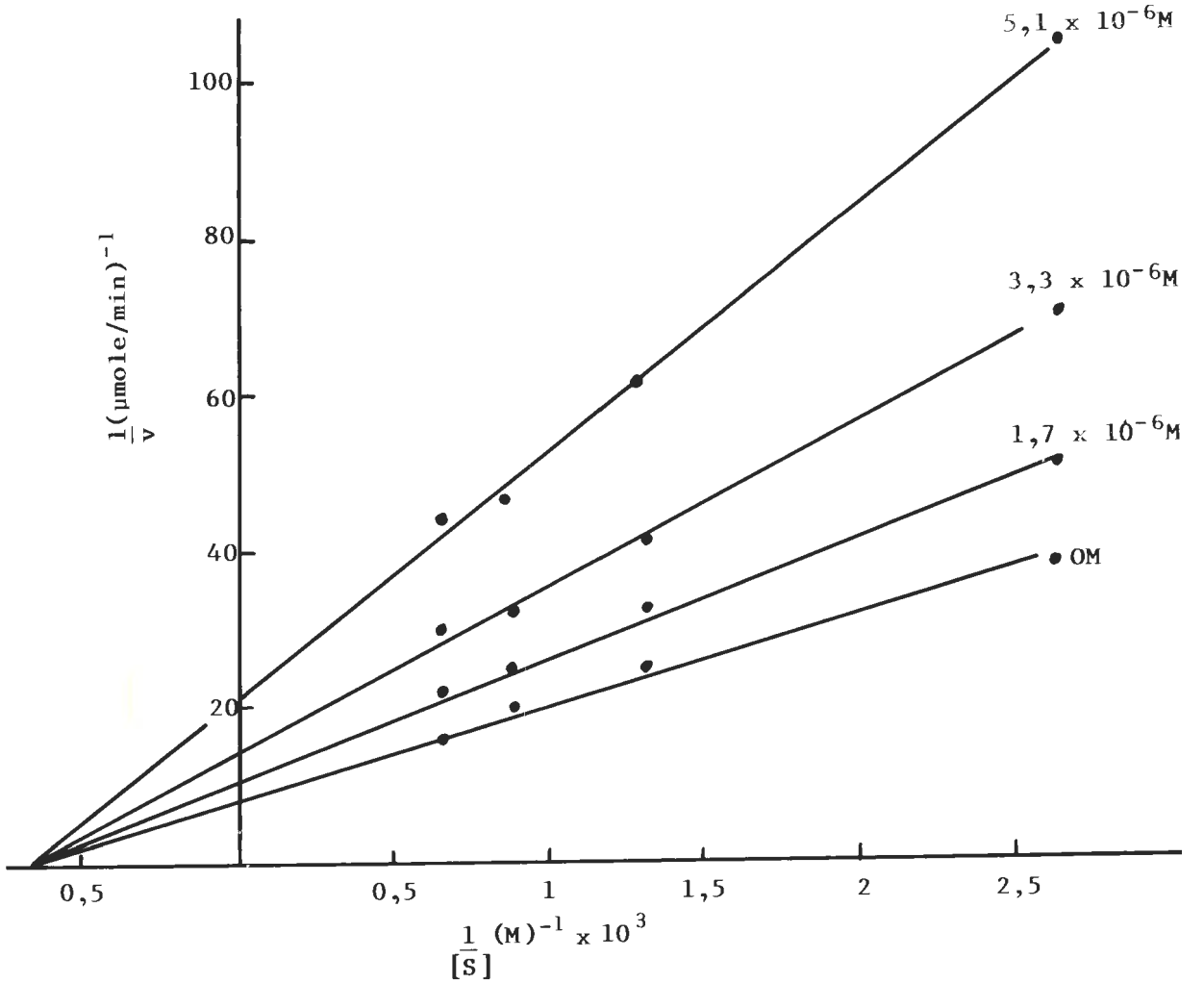

FIG. 3 Reciprocal plots (1/v *versus* 1/s) in the presence of different concentrations of Fraction IEF 4. The trypsin concentration was 7×10^{-7} M (Neitz *et al.*, 1981)

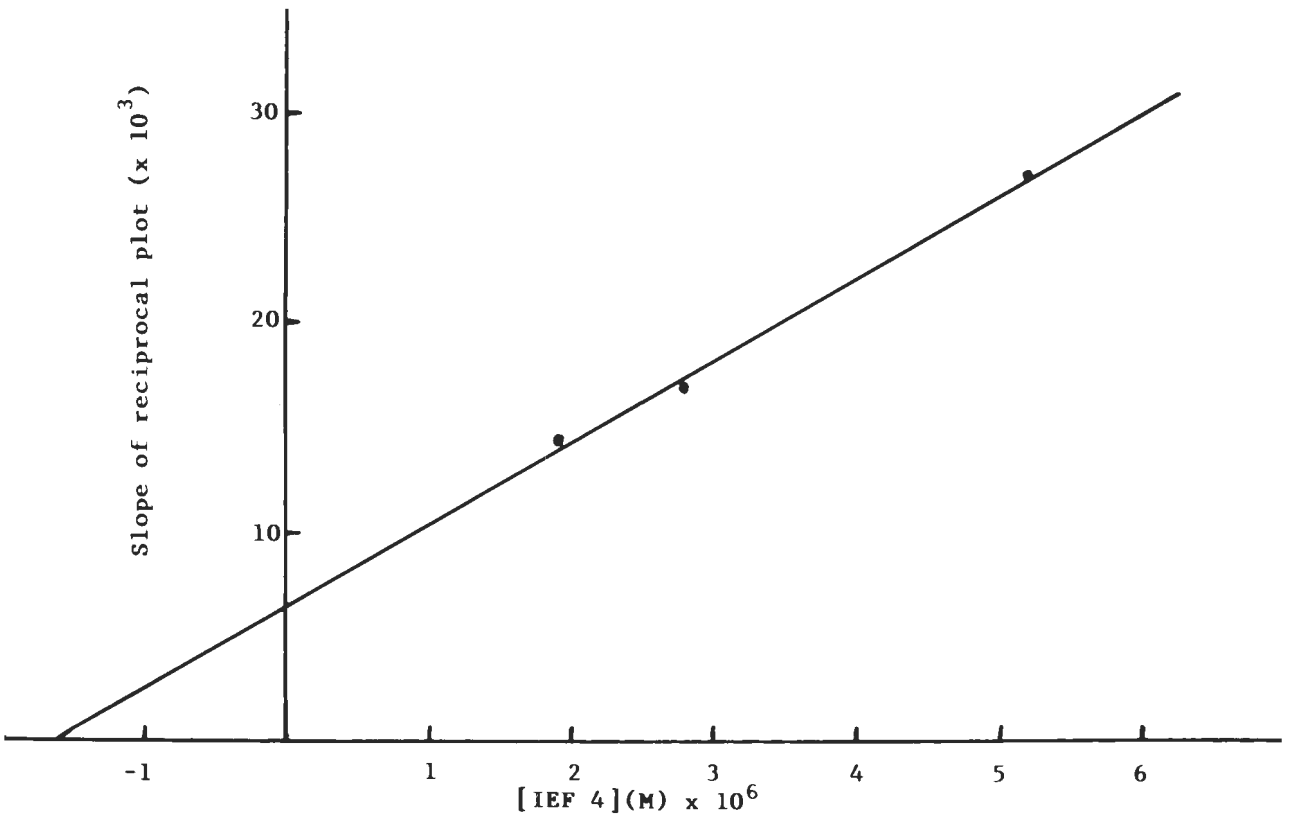

FIG. 4 Replots of the slopes taken from the reciprocal plots (Fig. 3) *versus* Fraction IEF 4 concentration (Neitz *et al.*, 1981)

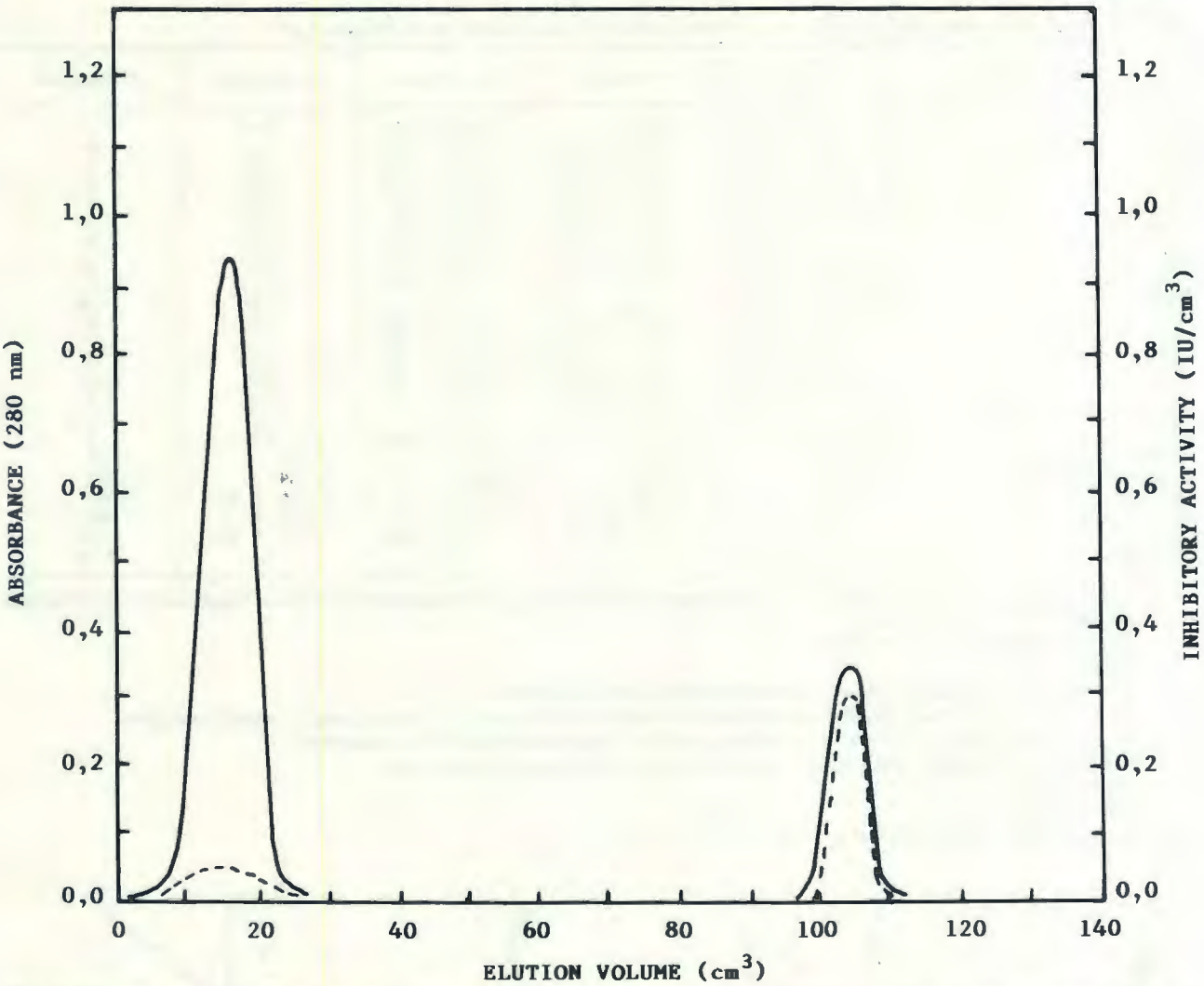

FIG. 5 Affinity chromatography of the freeze dried supernatant of the egg extract in 0,1 M phosphate buffer, pH 7,4. The protease inhibitor was eluted with pH 1,2, 0,01 M HCl solution containing 0,01 M $CaCl_2$. Dotted line represents activity (Vermeulen *et al.*, 1984)

TABLE 1 Purification of a trypsin inhibitor from *Amblyomma hebraeum* eggs[a]

Fraction	Protein (mg)	Specific activity (units/mg)	Inhibitor yield (%)	Purification factor
Homogenate	998	0,33	100	1
Supernatant 9 000 *g*	825	0,37	93,9	1,13
Supernatant 54 000 *g*	91	0,86	24,0	2,63
Affinity chromatography	7,5	32,0	73,8	98,2

[a] Vermeulen *et al.*, 1984

step procedure. In the 1st step extensive dialysis against distilled H_2O precipitates more than half the nitrogen content of the crude extract. The supernatant, after centrifugation of the precipitate, is subjected to preparative iso-electric focusing (IEF) to yield 4 fractions of interest (Fig. 1).

A non-toxic anti-protease was isolated from the crude extract of eggs of *A. hebraeum* by affinity chromatography of the supernatant on a trypsin-Sepharose 4B column (Fig. 5), after 2 successive centrifugations at 9 000 and 54 000 *g* respectively

RESULTS

A. Toxic anti-protease

Four fractions of interest have been isolated from the eggs of *A. hebraeum* (Fig. 1).

TABLE 2 Amino acid composition of the trypsin inhibitor from *A. hebraeum* eggs[a]

Amino acid	Residues per mole
Lysine	5
Histidine	1
Arginine	8
Cysteine	5
Aspartic acid	8
Methionine	1
Threonine	7
Serine	3
Glutamic acid	14
Proline	8
Glycine	10
Alanine	4
Valine	3
Isoleucine	5
Leucine	3
Tyrosine	7
Phenylalanine	5

[a] Vermeulen *et al.*, 1984

Analytical iso-electric focusing indicates that fraction IEF_4 is pure (Fig. 2). Sedimentation equilibrium centrifugation, SDS gradient electrophoresis and minimum molecular mass calculated from amino-acid composition (Table 3) of the toxin give molecular masses of 10 114, 10 047 and 10 073 respectively.

A kinetic analysis of the toxic protein showed inhibition of trypsin. With the aid of the Lineweaver & Burke (1934) method it has been shown that the toxic protein is

TABLE 3 Amino acid composition[a], molecular mass, minimum lethal dose[b] and isoelectric point of tick egg toxins

Amino acid	*R. evertsi evertsi*	*H. truncatum*	*B. microplus*	*B. decoloratus*	*A. hebraeum*[c]
Asp	2,08 ± 0,03	7,29 ± 0,1	4,4 ± 0,1	8,32	7
Thr	1,86 ± 0,03	7,14 ± 0,2	5,43 ± 0,05	6,17	6
Ser	2,12 ± 0,05	4,33 ± 0,06	3,80 ± 0,00	3,9	5
Glu	3,73 ± 0,09	9,61 ± 0,09	7,00 ± 0,02	9,76	9
Pro	1,58 ± 0,03	5,39 ± 0,06	4,10 ± 0,2	6,85	7
Gly	3,82 ± 0,09	7,43 ± 0,06	9,54 ± 0,5	9,23	9
Ala	2,13 ± 0,06	5,17 ± 0,04	3,68 ± 0,12	4,73	6
Cys	1,18 ± 0,08	5,35 ± 0,05	1,10 ± 0,06	2,85	nd
Val	1,24 ± 0,03	6,13 ± 0,06	5,26 ± 0,06	6,23	5
Met	1,21 ± 0,07	mq	mq	0,85	nd
Ile	0,76 ± 0,06	3,20 ± 0,04	2,63 ± 0,09	3,87	8
Leu	1,53 ± 0,03	5,39 ± 0,02	4,16 ± 0,09	5,78	9
Tyr	1,08 ± 0,04	3,32 ± 0,04	2,62 ± 0,05	4,23	5
Phe	1,18 ± 0,05	3,17 ± 0,04	2,85 ± 0,05	4,01	4
Lys	1,21 ± 0,03	9,26 ± 0,06	5,82 ± 0,12	9,23	4
His	1	1	1	1	1
Arg	1,05 ± 0,04	4,21 ± 0,01	2,96 ± 0,16	3,87	5
Min mol. mass	2 859	9 251	7 157	10 036	9 802
Mol. mass (multiple)[d]	5 718	27 753	28 629	40 144	9 802
Mol. mass (S.E.C.)[e]	5 016 ± 51	26 058 ± 73	30 497 ± 82	40 156 ± 50	10 000
Mol. mass (SDS)[f]	6 518 ± 25	27 806 ± 49	35 908 ± 78	40 020 ± 12	10 000
PI[g]	5,98 ± 0,03	8,32 ± 0,04	9,05 ± 0,04	9,19 ± 0,01	8,0
MLD/mg	0,6	2,5	1,3	1,3	nd

[a] Expressed as mole ratios with respect to histidine. Values include the mean error. Except for *B. decoloratus* (single determination) analyses were performed at least in triplicate

[b] Calculated from amino acid composition

[c] Neitz *et al.* (1981)

[d] Nearest multiple in agreement with mol. mass determined by ultracentrifugation

[e] Determined by sedimentation equilibrium centrifugation. Values include the mean error. At least duplicate samples investigated

[f] Mol. mass according to SDS-PAGE. Values include mean error. Analysis performed at least in quintuple

[g] Isoelectric point from IEF. Values include mean error. Analyses performed at least in sextuple

nd Not determined

mq Minute quantities

MLD Minimum lethal dosage (Viljoen *et al.* 1985)

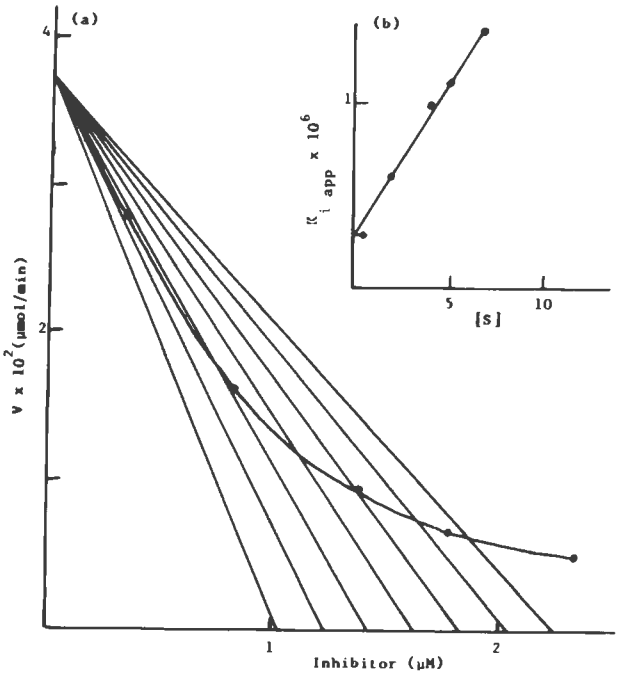

FIG. 6 (a) Dixon plot of enzyme velocity against different inhibitor concentrations in the presence of constant substrate concentration ($9,9 \times 10^{-4}$ M). (b) Replot of $K_{i\ app}$ against different substrate concentrations ranging from 2 to $9,9 \times 10^{-4}$ M (Vermeulen *et al.*, 1984)

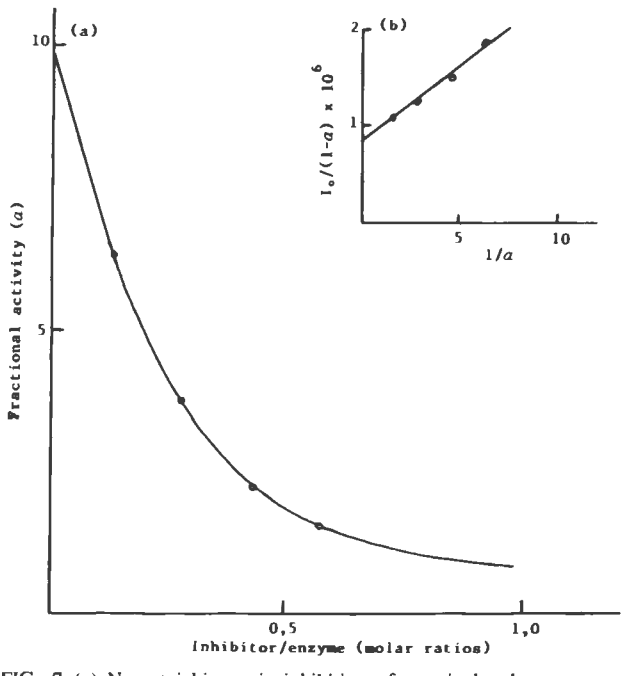

FIG. 7 (a) Non-stoichiometric inhibition of trypsin by the protease inhibitor. (b) Replot of $I_o/(1-a)$ against 1/a from which Ki could be determined. Conditions: 1 µM trypsin and BABNA 0,75 mM final concentration (Vermeulen *et al.*, 1984)

a non-competitive inhibitor of this enzyme with a Ki = 1,6 µM (Fig. 3 & 4).

Clinical symptoms

No difference was observed between the symptoms caused by injection of either the crude egg extract or purified fractions. The symptoms seen in guinea pigs which reacted severely after administration of the fractions were (1) anorexia and severe hyperaesthesia; (2) severe generalized hyperaemia of the skin seen particularly on the hairless areas such as the lips and foot pads; (3) moderate muco-purulent, occulo-nasal discharge; and (4) a severe mucoid diarrhoea, which immediately prior to death of the guinea pig became haemorrhagic. A

TABLE 4 A. Type of inhibition for *R. evertsi evertsi, A. hebraeum, B. microplus* and *B. decoloratus* egg toxins
B. Type of inhibition for the non-toxic anti-protease from *A. hebraeum*

Enzyme	Substrate	Inhibitor	Type of inhibition	Ki
		A. Toxic anti-proteases		nM
Trypsin	BAPNA	*R. evertsi evertsi*[c]	Fast tight-binding[a]	1,6
	Cbz-Arg-AMC	*B. decoloratus*[c]	Slow binding[a]	4,1
	Cbz-Arg-AMC	*B. microplus*[c]	Slow binding[a]	4,6
	Cbz-Arg-AMC	*H. truncatum*[c]	Slow binding[a]	0,34[f]
	BAPNA	*A. hebraeum*[d]	Fast binding[b]	255
Chymotrypsin	SPNA	*R. evertsi evertsi*[c]	No inhibition	
	SPNA	*B. decoloratus*[c]	Fast tight-binding[a]	36,2
	SPNA	*B. microplus*[c]	No inhibition	
	SPNA	*N. truncatum*[c]	Fast tight-binding[a]	23,1
	SPNA	*A. hebraeum*[d]	No inhibition	
		B. Non-toxic anti-protease		
Trypsin	BAPNA	*A. hebraeum*[e]	Fast binding[a]	25,5
Chymotrypsin	SPNA	*A. hebraeum*[e]	No inhibition	

[a] Competitive inhibition
[b] Non-competitive inhibition
[c] Vermeulen, Viljoen, Bezuidenhout, Visser & Neitz, unpublished data, 1987
[d] Neitz *et al.* (1981)
[e] Vermeulen *et al.* (1984)
[f] Overall dissociation constant Mechanism B (Morrison, 1982)

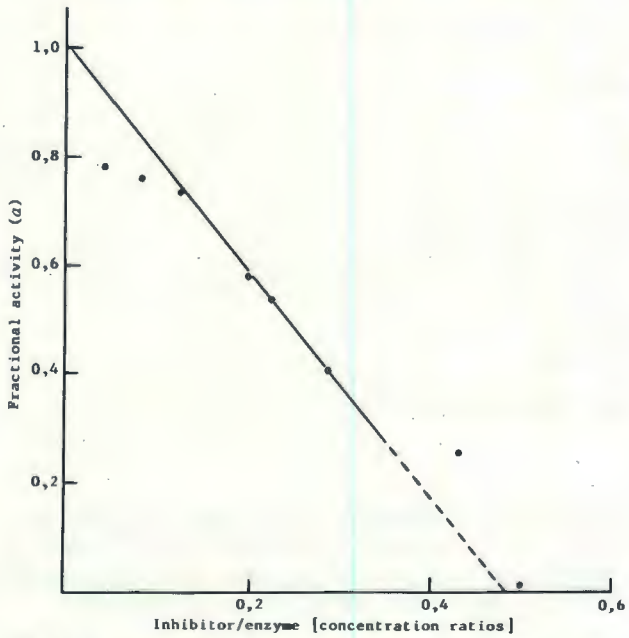

FIG. 8 Stochiometric inhibition of trypsin by the protease inhibitor. E_o/Ki ratio was approx. 50 (Vermeulen *et al.*, 1984)

few of the guinea pigs developed a mild scrotal swelling. Symptoms were noted on the 2nd to 3rd day after administration of the toxins and death of the guinea pigs usually occurred on the 3rd and 4th day.

Histopathology

Kidney lesions were characterized by a peripheral zone of mineralization and necrotic tubular epithelial cells. The liver showed focal areas of coagulative necrosis with mineralization. Degenerative changes in the hepatocytes were observed. Oedema of the urinary bladder was present and infiltration of neutrophils in the mucosa occurred. Oedema of the white matter of the brain was also present.

These symptoms are the same as those described by Viljoen *et al.* (1985) for toxic anti-proteases isolated from *B. decoloratus, B. microplus, H. truncatum* and *R. evertsi evertsi.*

B. Non-toxic anti-protease

Using affinity chromatography on a trypsin-Sepharose 4B column (Fig. 5), we were able to isolate 7,9 mg of a single protein which was shown to be homogeneous by SDS gel electrophoresis, from 1 g of eggs (Table 1).

The molecular mass, as determined by ultracentrifugation, was taken as 8 400, which is in approximate agreement with that determined by SDS gel electrophoresis and that calculated from the amino acid analysis (Table 2).

The dissociation constant (Ki) for this tightly bound inhibitor was determined by the methods of Dixon (1972) and Bieth (1974). The 2 methods yielded Ki values of 0,3 μM and 0,2 μM respectively as shown in Fig. 6 and 7.

The stoichiometric inhibition of trypsin by the protease inhibitor is shown in Fig. 8

No inhibition of chymotrypsin was observed even after incubation for 30 min at 25 °C with this protein.

The amino acid composition, molecular mass and isoelectric points of 5 tick species are summarized in Table 3. A comparison of the dissociation constants of the toxic and non-toxic anti-proteases with those from 4 other species are shown in Table 4.

The determination of protease activity in tick eggs

Mills (1985), using the linked enzyme assay procedure (LEAP) (Taylor & Andrews, 1983), as shown in Fig, 9, has determined that the eggs of *A. hebraeum* and *B. decoloratus* contain 18 μg and 10 μg protease in 1 mg of wet egg mass respectively when trypsin was used as a standard for the calibration.

DISCUSSION

Mechanism of action of anti-proteases

The importance of the control of intracellular proteolysis has been recognized for some time and has recently received considerable attention (Holzer & Tschesche, 1979; Turk & Vitale, 1980; Katunuma, Umezawa & Holzer, 1983). Proteolytic reactions also play a key role in the control of many other physiological functions such as digestion, translocation of proteins, maturation of hormones, virus assembly, immune responses, inflammation, blood coagulation, fibrinolysis, control of blood pressure, fertilization, sporulation, germination, oncogenic transformation and other morphogenic processes (Holzer & Heinrich, 1980). Since relatively little is

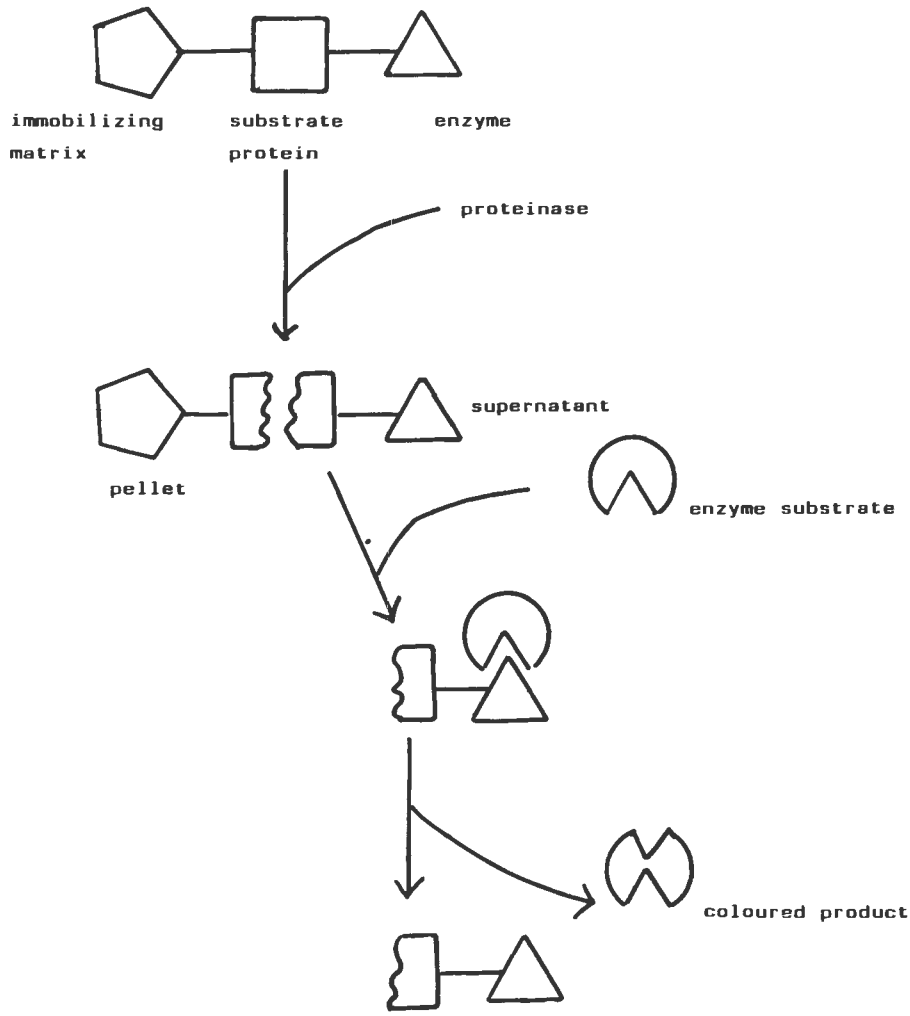

FIG. 9 Detection of proteolytic activity using the linked enzyme assay procedure (Taylor & Andrews, 1983)

known about the role of anti-proteases in tick eggs and in the life cycle of the tick, the available information will be discussed in some detail, while a few functions that have been described for anti-proteases in related and other fields will be mentioned briefly.

Tick eggs

The variation in the concentration of the anti-protease from *B. microplus* was followed throughout the life cycle of the tick by measuring its inhibition of trypsin and chymotrypsin activity (Willadsen & Riding, 1980). This protein was found to be present in large amounts in eggs (7–8 % of extractable protein) and in the unfed larvae (10–12 % of extractable protein). Its concentration stays relatively constant in the tick egg but falls very rapidly after the start of the parasitic stage of the life cycle. It has similarly been reported that the toxicity of *A. hebraeum* eggs for guinea pigs does not change with age (Neitz *et al.*, 1981). It has been suggested that this inhibitor is important both in the eggs and during the initial establishment of the tick on its host. Cattle exposed to the tick under natural conditions exhibit an immunological response to the inhibitor, suggesting that it is transferred from the tick to the host (Willadsen & Riding, 1979). The inhibitor also inhibits complement-dependent lysis of erythrocytes and coagulation of blood (Willadsen & Riding, 1980). The large amounts of protein, roughly 10–12 % of soluble protein from freshly hatched larvae, transferred to the host, are probably required for establishment on the host during the first critical 24 h after larval attachment (Roberts, 1968). It therefore appears that this inhibitor could have several effects on the host that are beneficial to the parasite.

Insects

The presence of protease inhibitors in insect eggs, larvae and adults is well established (Kang & Fuchs, 1980; Kucera & Turner, 1981). It has been suggested by Kucera & Turner (1981) that although the role of protease inhibitors in insect embryogenesis has not been demonstrated, the well documented roles of proteases and their inhibitors in developing and differentiating systems (Holzer & Heinrich, 1980), indicate that these substances may well play a significant role in the regulation of development and differentiation of insects. Kucera & Turner (1981), were the first to isolate protease inhibitors from eggs of insects. They also showed that the trypsin-inhibiting activity of these inhibitors in eggs of *Hemileuca olivial* increases during the latter part of embryogenesis. Willadsen & Riding (1980) found that the anti-protease activity of *B. microplus* eggs stays relatively constant during embryogenesis. The fact that proteolytic activity alters during the embryogenesis of insects (Kucera & Turner, 1973) suggests a possible role for naturally occurring inhibitors.

Other sources

It has been suggested that the anti-proteases in avian

eggs act as natural non-specific anti-microbial defences through the inhibition of bacterial and fungal proteinases (Davis, Zahnely & Donovan, 1969; Board & Fuller, 1974). The fact that egg-white anti-protease stays relatively constant during hatching (Cohen & Balls, 1955) has been interpreted as functioning as a protective measure against unspecific reactors during this time (Vogel & Werle, 1970). The parasitic helminth *Ascaris suum* can remove host trypsin and chymotrypsin from its environment and then sequesters them with its own anti-proteases as inactive complexes (Peanansky & Abu-Erreish, 1971; Martzen, Geize, Hogan & Peanansky, 1985). It has been observed in a number of plant species that severe wounding of a single leaf releases a wound hormone that spreads rapidly through the plant to signal accumulation of proteinase inhibitors as a possible defence mechanism against pests (Ryan, 1978).

Toxic anti-protease

A toxic anti-protease with a molecular mass of about 10 000 has been isolated from the eggs of *A. hebraeum*. This protein is a non-competitive inhibitor of pancreatic bovine trypsin with a Ki of $0{,}16 \times 10^{-7}$ M. No inhibition of chymotrypsin was observed. Four similar toxic anti-proteases have been isolated from *B. microplus, B. decoloratus, H. truncatum* and *R. evertsi evertsi* (Viljoen *et al.*, 1985). The molecular masses of these 4 toxins were about 30 500, 40 000, 26 000 and 5 000 respectively. The 4 anti-proteases were toxic at different levels (Table 3) ranging from 0,6–2,5 MLD/mg when injected subcutaneously in guinea pigs. The tick egg toxins caused only partial paralysis (paresis) which differs from the paralysis caused by adult ticks (Murnaghan & O'Rourke, 1978). The histopathological lesions observed in guinea pigs inoculated with egg toxins isolated from *A. hebraeum* and the 4 other tick species mentioned in Table 4, were similar.

This suggests that the toxins although different in structure and iso-electric point have a similar mode of action. The histopathological findings indicate that the toxins exert their effect on cell membranes (Neitz *et al.*, 1981). Riek (1957) also mentions degeneration and necrosis of hepatocytes as well as degenerative changes in the kidney tubular epithelial cells of guinea pigs inoculated with egg or tick extracts isolated from various Ixodidae. De Meillon (1942) demonstrated the presence of toxins in the eggs of *R. evertsi evertsi, B. decoloratus* and *H. leachi*. He proposed that similar toxins might be present in the eggs of all Ixodidae. Riek (1957) indeed demonstrated toxins to be present in the eggs of 17 species of ixodid ticks and absent in 5 species of argasids. The symptoms observed by the above authors after injection of egg extracts of all the ixodid species investigated, are similar to those described by us.

The toxic proteins isolated from the 5 tick species (Table 4) were assayed as inhibitors of trypsin and chymotrypsin. Four types of inhibition were observed.

The fact that 4 types of trypsin inhibition were observed with the 5 toxins and that only 2 of them also inhibit chymotrypsin, suggests that the toxicity observed in guinea pigs is probably not caused by the toxin activity on these enzymes. This is substantiated to some extent by the fact that soyabean anti-trypsin with a Ki of 1×10^{-11} M for trypsin (Kowalski, Leary, McKee, Sealock, Wang & Laskowski, 1974) was non-toxic to guinea pigs at 5 times the dosage used in the toxicity studies with the tick egg toxins (Vermeulen, Viljoen, Bezuidenhout, Visser & Neitz, unpublished data, 1987).

Antisera against the egg toxins of *R. evertsi evertsi, H. truncatum, B. microplus* and *B. decoloratus* were prepared in mice and an ELISA was developed with the antisera (Vermeulen, Viljoen, Bezuidenhout, Visser & Neitz, unpublished data, 1987). The egg toxins of *B. decoloratus* and *B. microplus* exhibited immunological identity with Ouchterlony micro-immunodiffusion and ELISA. The egg toxins of *H. truncatum* and *R. evertsi* seem to be immunologically unrelated to each other as well as to the above 2 egg toxins. Kinetic data indicate some similarity as far as trypsin is concerned. However, in the case of chymotrypsin only *B. decoloratus* inhibits this enzyme. The kinetic mechanisms observed for trypsin and chymotrypsin inhibition by the egg toxins of *R. evertsi evertsi* and *H. truncatum* support the immunological dissimilarity of these toxins. No immunological data on the toxin of *A. hebraeum* is available yet.

It can be calculated from reaction rate constants (Bieth, 1980; Fersht, 1984) that the time necessary for 100 % inhibition of a fast binding inhibitor is usually $<<$ 1 s and it is then assumed that when the inhibitor concentration is 10 × that of Ki, it could play a possible physiological role. The concentration of the fast binding, tick egg inhibitors of trypsin and chymotrypsin is at least a 1 000 × higher than their Ki values (Viljoen *et al.*, 1985) and they could therefore possibly play a role in inhibiting trypsin- and chymotrypsin-like enzymes in the egg. A similar calculation can be made from reaction rate constants (Bieth, 1980) for the slow-binding inhibitors (Vermeulen, Bezuidenhout, Visser & Neitz, unpublished data 1987) and it appears that at the concentration at which they are present in the tick egg, they could also possibly play a physiological role in controlling enzymes with trypsin-like activity. Mills (1985), using the LEAP method and trypsin as a standard, has determined that *A. hebraeum* and *B. decoloratus* contain 18 and 10 μg/mg (wet material) of proteolytic activity respectively. This represents concentrations of about 600 and 300 μM respectively, if it is assumed that the average molecular mass of the proteases is 30 000. This suggests that the anti-proteases are probably necessary to prevent unwanted proteolysis that might be caused by the relatively high proteolytic content of the eggs.

The role suggested by Willadsen & Riding (1980) for the protein isolated from *B. microplus* eggs and larvae and which inhibits a number of proteolytic enzymes, as discussed under the section mechanism of action of anti-proteases, is based on the best evidence available. A comparison of some of the characteristics of the 2 proteins isolated from *B. microplus* by Willadsen & Riding (1979; 1980) and Viljoen *et al.*, (1985) respectively is supplied in Table 5.

TABLE 5 Comparison of the characteristics of proteins isolated from *B. microplus*

Characteristics	Willadsen & Riding (1979 & 1980)	Viljoen *et al.* (1985)
Molecular mass (SDS, DTT)	18 500	35 900
pI	ND	9,05
Trypsin inhibition (Ki)	< 0,002 μM[a]	0,0046 μM[c]
Chymotrypsin inhibition (Ki)	0,010 μM[b]	
Type of trypsin ihibition	ND	slow binder
Toxicity to guinea pigs	ND	toxic

[a] α-N-toluene-p-sulphonyl-L-arginine methyl ester was used as substrate.

[b] N-acetyl-L-tryptophan ethyl ester was used as substrate.

[c] 7-Carbobenzoxy-argininamido-4-methylcoumarin •HCl was used as substrate (Vermeulen, Viljoen, Bezuidenhout, Visser & Neitz, unpublished data, 1987).

[d] N-succinyl-L-phenylalanine-p-nitroanalide was used as substrate (Vermeulen, Viljoen, Bezuidenhout, Visser & Neitz, unpublished data, 1987).

Although the 2 proteins differ in molecular mass and their ability to inhibit chymotrypsin and notwithstanding

the fact that some information is not available for a complete comparison, both show trypsin inhibition with similar dissociation constants. Since the toxic anti-proteases isolated from the 5 tick species listed in Table 4 all inhibit trypsin, and in some instances also chymotrypsin, it is tempting to suggest that they will probably act in a way similar to that suggested by Willadsen & Riding (1980) for the protein they isolated from *B. microplus*.

Non-toxic antiprotease

A protein with anti-proteolytic activity against trypsin, but with no activity against chymotrypsin and which was non-toxic to guinea pigs has been isolated by affinity chromatography from *A. hebraeum* tick eggs. This protein was a competitive inhibitor with a dissociation constant of about 0,255 μM. Stoichiometric studies with trypsin (Fig. 8) suggest that this inhibitor has 2 binding sites for trypsin.

It is known that when an inhibitor binds with trypsin on a trypsin-Sepharose 4B affinity column such as we have used, the enzyme could possibly catalyse the cleavage of a single peptide bond in the anti-protease molecule (Tschesche, 1974; Laskowski & Sealock, 1971, Goodman & Peanansky, 1982). The fact that the electrophoretic conditions used by us, which included SDS and dithiothreitol, showed only one component, suggests that no modified inhibitor was formed. Affinity chromatography has also been used successfully to isolate mainly native trypsin inhibitor from *Ascaris lumbricoides* (Goodman & Peanansky, 1982). It has, however, been shown by Viljoen *et al.* (1984), by column chromatofocusing of a *B. decoloratus* anti-protease fraction obtained from a trypsin-Sepharose 4B affinity column, that the virgin anti-protease was in time converted to a modified anti-protease. Mills (1985) demonstrated that this problem could be circumvented by affinity chromatography on a anhydrotrypsin-Sepharose 4B affinity column (Schin-Ishi, Yokosawa, Kumazaki & Nakamura, 1983). Consequently only anhydrotrypsin with no catalytic activity should be used in affinity chromatography of anti-proteases. Viljoen *et al.* (1984) observed, however, that the toxic anti-protease from *B. decoloratus* did not bind to a trypsin-Sepharose 4B affinity column even after 24 h incubation on the column. The fact that the toxin (molecular mass 40 000), was not bound to trypsin on the affinity column could possibly be explained by unfavourable steric interactions with the trypsin-bound Sepharose.

Enzyme kinetics

As shown in Table 4, 4 different mechanisms of trypsin inhibition have been observed with the tick anti-proteases. It is important to identify the type of inhibition and to use the corresponding kinetic velocity equation derived for the particular type of inhibition. The kinetic constants of proteolytic inhibitors are extremely useful in assessing the possible physiological functions of these compounds (Bieth, 1980). It is important in the case of slow or slow tight-binding inhibition to use the methods as described by Baici & Gyger-Marazzi (1982) and Morrison (1982). In the case of fast tight-binding inhibition the method described by Dixon (1972) and Bieth (1980) should be used instead of the classical Lineweaver & Burk (1934) methods.

CONCLUSION AND PERSPECTIVES

At this stage of the investigations there is no evidence to connect the anti-proteolytic activity of the tick egg toxins to the toxic symptoms, immunological properties or the histopathological lesions observed in guinea pigs. By directed mutagenesis of the relevant amino acid in the binding site of the toxins for their particular protease, it should be possible to resolve whether the anti-proteolytic activity plays any role in the toxicity. It is therefore necessary to determine the amino acid sequence of the different toxic and non-toxic anti-proteases.

In the case of the toxic anti-proteases and the non-toxic anti-protease their concentrations during the life cycle of the tick should be studied. The availability of the ELISA for the anti-proteases should facilitate a project of this nature. Their role in the establishment of the larvae on their host should get some attention. It would be useful to determine the amount and types of proteolytic enzymes produced during the tick's life cycle to assess a possible role for the anti-proteases in controlling these enzyme activities. At this stage of the investigation it is difficult to draw any conclusions from the functions described for anti-proteases in other insect eggs, nematodes, avian eggs, plants and mammalian species in regard to possible similar functions for the anti-proteases present in tick eggs.

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