On the biological role of a proteolytic-enzyme inhibitor from the ectoparasitic tick *Boophilus microplus*

Peter WILLADSEN and George A. RIDING

CSIRO, Division of Animal Health, Long Pocket Laboratories, Private Bag no. 3, Post Office, Indooroopilly, Queensland, Australia 4068

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The tick *Boophilus microplus* contains a protein that inhibits a range of proteolytic enzymes. Variations in the concentration of this protein throughout the life cycle were followed by measuring simultaneously the inhibition of trypsin and chymotrypsin and reaction with an antiserum to the purified inhibitor. The protein is present in large amounts in eggs and in unfed larvae, but its concentration falls very rapidly after the start of the parasitic stage of the life cycle. This, together with previous evidence, suggests that the inhibitor is important both in eggs and in the initial establishment of the parasite on its host. The activity of the protein towards several enzymes has been measured as an indication of its possible function. Bovine trypsin, chymotrypsin and plasmin and pig pancreatic kallikrein are all inhibited. The protein also affects the blood-coagulation system at several points, since it prolongs both activated-partial-thromboplastin time and prothrombin time. It inhibits the complement-dependent lysis of erythrocytes, but is without significant effect on mitogen-induced lymphocyte stimulation. Thus the inhibitor could have several effects on the host that would be beneficial to the parasite.

The naturally occurring inhibitors of proteolytic enzymes are a large and well-investigated group of proteins. Nevertheless, although there is much information on their physical properties and although their interactions with highly purified enzymes have been studied in detail, the physiological function of many of them is still uncertain. For example, although the bovine trypsin-kallikrein inhibitor was first isolated in 1936 and has been intensively studied since, its cellular distribution in cattle has only just been reported (Fritz *et al.*, 1979), and its function is still a matter of speculation. The question of physiological function is particularly interesting for the few proteinase inhibitors that have been isolated from parasites, since these could potentially not only fulfil any of the internal protective or regulatory functions suggested for comparable inhibitors from non-parasitic organisms, but also be involved in the complex interaction between parasite and host.

The tick *Boophilus microplus* is an economically important parasite of cattle in many tropical and subtropical parts of the world. Its life cycle is simple. Larvae attach to a single host and moult successively to nymphs and adults until, after 3 weeks, the females fall from the host and lay eggs. These hatch after about a further 3 weeks and the larvae continue the cycle.

We have recently reported the purification of a protein from this ectoparasite that inhibits both bovine trypsin and chymotrypsin (Willadsen & Riding, 1979). It was found that cattle that had been exposed to the parasite gave a weak but specific immediate hypersensitivity reaction to the protein, that is, that sufficient material had at some stage been transferred from parasite to host to stimulate an immunological response. This suggests that the inhibitor could be involved in the host-parasite interaction, but one cannot eliminate the possibility that the immunological response was due to unintentional transfer, for example, by regurgitation. Further, nothing was known about the function of this inhibitor. In the present paper we have tried to comment on possible functions for this protein in two ways. We report additional evidence that the inhibitor is involved in the host-parasite interaction. We have also investigated its effect on a number of potentially important host enzymes and enzyme systems.

Experimental

Materials

Most of the materials used in the present work were described previously (Willadsen & Riding, 1979). Pig pancreatic kallikrein (lot KZC 2173; specific activity 1290 kallikrein units/mg) was a gift

from Bayer-Werk, Elberfeld, Germany. The bovine trypsin-kallikrein inhibitor, which will be referred to by the trade name Trasylol, was from Bayer, Leverkusen, Germany. Bovine thrombin was obtained from Calbiochem (San Diego, CA, U.S.A.) and Simplastin and Platelin Plus Activator from General Diagnostics. Eagle's basal medium and foetal-calf serum were from Commonwealth Serum Laboratories, Melbourne, Victoria, Australia, and sodium metrizoate from Nyegaard and Co., Oslo, Norway. CNBr-activated Sepharose, SP(sulphopropyl)-Sephadex and Ficol 400 were from Pharmacia, Uppsala, Sweden.

Methods

Purification of inhibitor and determination of its concentration. The inhibitor was purified as described previously (Willadsen & Riding, 1979) with the single change that the chromatography on CM (carboxymethyl)-cellulose was replaced by one on SP-Sephadex. In this step, the product of a DEAE-cellulose chromatography was adjusted to pH 2.2, dialysed against 0.1 M-glycine buffer, pH 2.2, then loaded on a column (2.5 cm $\times$ 30 cm) of SP-Sephadex in the same buffer, washed and the inhibitor eluted in 0.1 M-glycine/0.5 M-sodium sulphate, pH 2.2. The effect of this was to increase the final yield of inhibitor from the 11% reported previously to typically 30%.

Inhibitor concentrations were determined either from A_{280} or by the inhibition of trypsin, as described previously (Willadsen & Riding, 1979). To avoid possible anomalous results from the use of partially autolysed commercial preparations of trypsin, the enzyme was purified by affinity chromatography. Soya-bean trypsin inhibitor-Sepharose was prepared from CNBr-activated Sepharose and the inhibitor according to the manufacturer's recommendations, by using 40 mg of inhibitor/g of gel. Trypsin in 0.05 M-Tris buffer/5 mM- CaCl_2 , pH 8.5, was loaded on to the gel at a rate of 20 mg/g of gel, washed with the same buffer and then eluted with a 0.05 M-glycine buffer, pH 2.2. The concentration of eluted trypsin was measured by titration with *p*-nitrophenyl *p'*-guanidinobenzoate (Chase & Shaw, 1970) and from the A_{280} in 1 M-HCl by using a value for $A_{1\text{cm}}^{1\%}$ of 15.0 and a mol.wt. of 24 000 (Walsh, 1970). The two estimates of concentration agreed within 4%. The one from the active-site titration was used for further calculations.

Measurement of changes in inhibitor concentration and enzyme activity throughout the tick life cycle. Eggs were collected from female ticks 4 or 5 days after they had engorged fully. Batches of eggs (50 mg; about 1000 eggs) were weighed into glass vials and maintained at 28°C and 95% relative humidity until larvae hatched and then for about 3 weeks subsequently. Periodically vials were

removed, the contents homogenized in 0.05 M-phosphate buffer, pH 7.5, containing 0.5 mM-EDTA and 1 mM-2-mercaptoethanol by using a glass homogenizer and a tissue concentration of 5–10 mg/ml of buffer, then the suspension centrifuged. The clear supernatant was used for the assays described below.

Developmental changes during the parasite's life cycle were monitored by placing approx. 100 000 larvae, 1–2 weeks after hatching, on a calf that had not previously been exposed to the parasite. Ticks were removed from the host at intervals, counted, weighed, then homogenized in phosphate buffer as described above for the eggs. Samples of parasites were taken from the day of attachment until 2 days before final engorgement of the adults, at which stage the rapid uptake of blood by the tick made interpretation of the results difficult. In addition, pharate nymphs were kept at 28°C and 95% relative humidity after removal from the host until they had moulted and were then extracted as usual.

Several assays were performed on all samples. Inhibitory activity towards both chymotrypsin and trypsin was measured by methods described below. Non-specific esterase was assayed by using fluorescein dibutyrate as substrate (Willadsen & Williams, 1976). Acetylcholinesterase activity was measured in 0.1 M-phosphate buffer, pH 7.5, with 0.48 mM-acetylthiocholine iodide and 3.2 mM-5,5'-dithiobis-(2-nitrobenzoic acid). The increase in A_{412} was monitored and the rates converted into units of enzyme activity (μmol of substrate hydrolysed/min) by using an ϵ value of $14150\text{ M}^{-1}\cdot\text{cm}^{-1}$ (Riddles *et al.*, 1979). Enzyme contents of ticks are given as units per g of material extracted. Protein concentrations were measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Calculation of dissociation constants for enzyme-inhibitor complexes. Dissociation constants for enzyme-inhibitor complexes were measured as described previously (Willadsen & Riding, 1979), by using the method of Green & Work (1953). A range of inhibitor concentrations was incubated with a known amount of enzyme at 25°C, then a portion diluted into an assay solution containing substrate. Formation of inactive enzyme-inhibitor complexes was at least partially reversible, since with all enzymes except trypsin the rate of substrate hydrolysis in an assay increased with time. Initial rates were measured within the first minute after dilution of the enzyme-inhibitor mixture into an assay solution. With each enzyme, theoretical curves, calculated by assuming the reversible formation of an enzymically inactive complex by reaction of enzyme and inhibitor in a 1:1 molar ratio, were fitted to the experimental data to give a value for the dissociation constant of the complex. Although these

assumptions have not been proved experimentally, the theoretical curves calculated in this way fitted the experimental data well. The derived dissociation constants are thus at least an accurate description of the experimental results in the concentration ranges specified for each enzyme.

Purification of bovine plasmin and the effect of inhibitor on this enzyme. Plasminogen was isolated from plasma by affinity chromatography on lysine-Sepharose as described by Sodetz *et al.* (1972). After denaturation, reduction and alkylation, electrophoresis of 50 μ g of protein in 7.5% polyacrylamide gels containing sodium dodecyl sulphate (Weber *et al.*, 1972) showed one major band and a series of faint minor bands. A similar result has been reported for bovine plasminogen by Summari *et al.* (1973). Plasminogen in a 0.05 M-Tris/0.05 M-lysine buffer, pH 8.0, was activated with urokinase bound to Sepharose 4B, as described by Castellino & Sodetz (1976). Plasmin activity was measured with 1 mM- α -N-toluene-*p*-sulphonyl-L-arginine methyl ester in the Tris/lysine buffer, the hydrolysis being monitored at 247 nm. The concentration of active plasmin was measured by titration with *p*-nitrophenyl *p*'-guanidinobenzoate in 0.05 M-phosphate buffer, pH 7.8 (Chase & Shaw, 1970).

The effect of inhibitor on plasmin was measured by incubating 1.05 μ M-enzyme in Tris/lysine buffer, pH 8.0, with inhibitor at concentrations of 0.294–1.2 μ M. After 10 min at 25°C, the degree of inhibition was not time-dependent and the residual enzyme activity was assayed.

Effect of inhibitor on bovine thrombin. Bovine thrombin was dissolved in 0.1 M-Tris buffer, pH 8.05, and the concentration of enzyme determined by titration with *p*-nitrophenyl *p*'-guanidinobenzoate at a final concentration of 45 μ M in 50 mM-phosphate buffer, pH 7.8. Enzyme activity was determined with 0.5 mM- α -N-benzoyl-L-arginine methyl ester in 0.1 M-Tris buffer, pH 8.05, by measuring the ΔA_{255} on hydrolysis. To test the effect of inhibitor, thrombin and inhibitor were incubated together in the Tris buffer at 25.0°C and at final concentrations of 1.3 and 12.8 μ M respectively, and portions assayed periodically.

Effect of inhibitor on pig pancreatic kallikrein. Kallikrein was dissolved and assayed in 0.1 M-Tris buffer, pH 8.05. Activity was measured with 0.5 mM- α -N-benzoyl-L-arginine methyl ester as described for thrombin. Since the amount of enzyme available was small, an indirect procedure was used to estimate the enzyme concentration. Initially, the concentration of a solution of Trasylol was measured in two ways: firstly from the A_{280} in Tris buffer, assuming $A_{1\text{cm}}^{1\%} = 8.3$ and a mol.wt. of 6500 (Kassell, 1970) and secondly by incubation of increasing concentrations of Trasylol with 0.235 μ M-trypsin and measurement of the residual enzyme activity. Extra-

polation of the results to complete inhibition allowed calculation of the Trasylol concentration. Both methods of estimation gave a concentration of 0.23 mM. Then kallikrein at a concentration of 5 μ g/ml was incubated with a range of Trasylol concentrations, the residual kallikrein activity measured and the results extrapolated to 100% inhibition. By assuming equimolar concentrations of Trasylol and kallikrein at this point, the concentration of kallikrein was calculated.

The effect of tick inhibitor on kallikrein was measured by mixing 0.17 μ M-enzyme with inhibitor concentrations of 0.26–3.1 μ M at 25°C and assaying the residual enzymic activity after a minimum of 10 min incubation.

Effect of inhibitor on bovine chymotrypsin. The concentration of active enzyme was estimated by titration with *p*-nitrophenyl acetate (Kézdy & Kaiser, 1970). To measure the dissociation constant, enzyme at a final concentration of 0.263 μ M was incubated at 25°C in 0.1 M-phosphate buffer, pH 7.53, with inhibitor concentrations from 64 to 447 nM. Residual enzyme activity was assayed against 0.8 mM-*N*-acetyl-L-tryptophan ethyl ester in the same buffer, measuring the hydrolysis by the increase in A_{300} . The dissociation constant was calculated as described for the other enzymes. In this experiment, inhibitor concentrations were based on the inhibition of trypsin activity.

In experiments on developmental changes during the parasite's life cycle, an estimate of inhibitor activity towards chymotrypsin was required. For this, 2.63 μ M-chymotrypsin was incubated with various amounts of test material in 0.1 M-phosphate buffer, pH 7.53, and residual enzyme activity assayed in the same way. At these higher concentrations the degree of inhibition was proportional to the amount of inhibitor added to at least 80% inhibition and so the chymotrypsin-inhibitory activity could be calculated exactly as described for trypsin-inhibitory activity previously (Willadsen & Riding, 1979).

Effect of inhibitors on blood-coagulation parameters. Activated-partial-thromboplastin times were measured with Platelin Plus Activator and prothrombin times with Simplastin, both as recommended by the manufacturer. Each measurement was made in quadruplicate. To investigate the effects on these parameters of tick inhibitor and Trasylol, they were first dialysed against phosphate-buffered saline (9.2 mM-phosphate/0.15 M- Na^+ /30 mM- K^+ /0.14 M- Cl^- , pH 7.3) and their concentrations measured against trypsin. Then 50 μ l of inhibitor or phosphate-buffered saline was added to 100 μ l of fresh citrated plasma and, after 5 min incubation at 37°C, the coagulation parameter was measured.

Effect of inhibitor on bovine haemolytic complement. The haemolytic assay of bovine serum

complement was performed as described by Barta & Barta (1970), with guinea-pig erythrocytes sensitized with an optimal concentration of natural bovine antibody. To determine the effect of tick inhibitor on complement titre, it was first dialysed exhaustively against the veronal-buffered sucrose used in the complement assays (Barta & Barta, 1970). Serum, buffer and inhibitor were mixed at 0°C, placed in a water bath at 37°C for 5 min, then sensitized cells added. Complement titres were calculated from the von Krogh equation (Williams & Chase, 1977) and are expressed as CH₅₀/ml, the number of 50% lytic doses of complement/ml of serum.

Isolation and culture of bovine lymphocytes. Fresh defibrinated blood (4 ml) was layered on to 6 ml of Ficoll/metrizoate, prepared by dissolving 10.1 g of Ficoll 400 in 72 ml of water and adding 30 ml of 32% (w/v) sodium metrizoate. The cells were centrifuged at 2500 *g* and 30°C for 45–60 min, the lymphocytes collected, washed three times in cold Eagle's medium and then finally suspended in culture medium at a cell concentration of 2×10^9 /litre. At least 98% of the cells were lymphocytes. The culture medium was Eagle's basal medium to which had been added 4.5% sodium bicarbonate, 2.92 mg of L-glutamine/100 ml and penicillin and streptomycin to final concentrations of 225 units/ml and 125 µg/ml respectively. This was then diluted with 10% (v/v) foetal-calf serum to give the final medium. Cell suspension (100 µl) was mixed with 100 µl of test material, whether mitogen, inhibitor or a mixture of the two, in culture medium, then incubated for 72 h at 37°C in an atmosphere of CO₂/air (1:19). The cells were then pulsed for 5 h with 1.25 µCi of [³H]thymidine per sample, the cells harvested, washed and the incorporated radioactivity measured.

All incubations were in quadruplicate and in most cases one of the samples was withdrawn just before pulsing with [³H]thymidine and the cell viability measured by Trypan Blue exclusion.

Antiserum production and immunodiffusion. An antiserum to purified inhibitor was raised in a rabbit by intramuscular injection of 1.7 mg of protein in Freund's complete adjuvant, followed after 1 month by 0.9 mg intravenously. A series of bleedings starting 1 week after this gave a serum that formed a single strong precipitin line on immunodiffusion against a 10 µg/ml solution of inhibitor. Double immunodiffusion was performed with 1% agar and 2% poly(ethylene glycol) 6000 in phosphate-buffered saline, pH 7.2.

Results

Developmental changes during the tick life cycle

We considered that some information about

possible functions of the tick proteinase inhibitor might be obtained by monitoring alterations in its concentration throughout the whole life cycle. Simple measurement of trypsin-inhibitory activity, however, is not sufficient to give a meaningful result, since it is always possible that several different inhibitors, all acting on trypsin, are produced at various stages of the life cycle. The inhibitor we were interested in, the one present in unfed larval ticks, inhibits both trypsin and chymotrypsin simultaneously (Willadsen & Riding, 1979). We therefore assayed activity against both of these, arguing that if parallel changes occurred in inhibitory capacity towards the two enzymes, this would be evidence that we were measuring the activity of the protein of interest. To confirm this we measured immunological cross-reactivity between an antiserum raised to pure larval inhibitor and tick extracts from the different stages of the life cycle. Since nothing has been reported previously on biochemical changes throughout the tick life cycle, the activities of two enzymes were measured as well, to see if changes in inhibitor concentrations were unique to that protein or part of a general trend. The two enzymes chosen were acetylcholinesterase and non-specific esterase, which was assayed against fluorescein dibutylate. In larval ticks, most of the activity towards this substrate is due to one enzyme (Willadsen & Williams, 1976).

The experiment was in two parts. Changes from the day of egg laying to several weeks after hatching of larvae are shown in Fig. 1. The parasitic part of the life cycle from the day of attachment of unfed larvae to the host until about 2 days before final engorgement of female ticks is shown in Fig. 2.

The results shown in Fig. 1 are calculated as the amount of enzyme or inhibitor activity/g of eggs, based on the weight of egg material in each sample on the first day of the experiment. The inhibitor was present in large amounts initially and declined only slowly until the eggs hatched. The larvae and egg-shell residue together contained less inhibitor than did the original eggs, and the amount fell gradually as the larvae aged. Larvae typically have their greatest infectivity 1–2 weeks after hatching. The fall in inhibitor concentration after hatching shown in Fig. 1 is paralleled by a fall in esterase activity, and this may be partly due to loss of viability. Acetylcholinesterase activity in the eggs was too low to measure accurately. The very large amount of inhibitor in eggs and larvae is striking. We have noted this for larvae before (Willadsen & Riding, 1979), although our previous statement that 3% of the extractable protein in unfed larvae was inhibitor is true only under the published conditions of extraction. We have since found that far more inhibitor than this is extracted if the ratio of buffer to tick material is increased from 3.5:1 as used in the

Fig. 1. Changes in eggs and unfed larvae

Day 1 is the day on which eggs were laid and the arrow marks the day of hatching. ■, Amount of inhibitor or enzyme left in the egg shells after hatching; □, amount in the larvae.

purification procedure to 100:1. Extraction of larvae into dilute solution as in this experiment shows that the inhibitor can be up to 10–12% of the extractable protein. In eggs, the proteinase inhibitor was 2–3% of the total egg weight and about 7–8% of the extracted protein. It is noteworthy, too, that although the amount of inhibitor in larvae was less than in the eggs originally, its concentration as a proportion of the extractable protein rose. The specific activity of the extract from eggs was initially 3.7 nmol/mg, whereas in 3-day-old larvae it was 6.3 nmol/mg.

The results in Fig. 2 show that the amount of inhibitor in larvae declined very rapidly in the first 3 days of the life cycle to undetectable amounts, and it did not reappear for the duration of the experiment. It was not found in pharate or freshly hatched nymphs or adults, nor in nymphs that had been removed from the host as pharate ticks and kept in an incubator until they hatched. No comparable changes were seen with either acetylcholinesterase or non-specific esterase.

It is probable that most of the inhibitor activity present throughout the life cycle was due to the protein already characterized from larvae. Alterations in the capacity to inhibit trypsin and chymotrypsin occurred in parallel, suggesting that

one protein with activity towards both enzymes was responsible. Ideally, the ratio of activities towards these two enzymes should have been 1. In practice, up to 20% deviation from this occurred. At least three reasons can be suggested for this. Firstly, there is considerable cumulative experimental error involved in this ratio, since it requires the measurement of inhibition of two enzymes separately by a crude extract. Secondly, it is of course possible that some of the activity at a particular stage of the life cycle is due to other inhibitor proteins specific for either trypsin or chymotrypsin. Thirdly, we have evidence that reaction of the known inhibitor with chymotrypsin may destroy the site specific for that enzyme, but leave the trypsin-inhibitory site intact (P. Willadsen & G. A. Riding, unpublished work). Hence variation in the ratio of trypsin to chymotrypsin inhibition could be due to prior reaction *in vivo* of the inhibitor with proteolytic enzymes.

Purified inhibitor at a concentration of 1 μ M gave a single strong precipitin line on immunodiffusion against a rabbit antibody to this protein. Extracts from the different tick life stages were diluted so the concentration of trypsin inhibitor was also 1 μ M. These similarly gave precipitin lines on immunodiffusion, whereas this did not occur in those fractions lacking measurable inhibitor, although

these were tested as undiluted extracts. This is further evidence that the inhibitor being measured was the same at all stages of the life cycle and that the fall in its concentration in feeding larvae represented a true loss of protein.

Fig. 2. Changes during the parasitic part of the life cycle. The abbreviations for the different developmental stages are: UF, unfed larvae just before attachment to the host; L, larvae; PN, pharate nymphs; N, nymphs; A, adults. Measurements on unfed larvae were made on day 0, the day on which larvae were also placed on the host.

Specificity of inhibitor towards isolated enzymes

The inhibitor was found previously to inhibit bovine trypsin and bovine chymotrypsin (Willadsen & Riding, 1979). It also inhibits the esterase activity of bovine plasmin and pig pancreatic kallikrein. The dissociation constants for the enzyme-inhibitor complexes, calculated as described in the Experimental section, are listed in Table 1. Incubation of $1.3 \mu\text{M}$ -thrombin with $12.8 \mu\text{M}$ -inhibitor decreased the activity of the enzyme by only 4%. If this effect is assumed to be real, and a dissociation constant is calculated from this one value, the value given in Table 1 is obtained. The significance, however, is questionable, and the result simply indicates that thrombin is very poorly inhibited, if at all. The dissociation constant given for trypsin is a maximum estimate calculated on the basis of the absence of curvature in normal inhibitor assays.

Effect of inhibitors on blood coagulation

The effects of tick inhibitor and Trasylol on the partial-thromboplastin times for six cattle are listed in Table 2. The first four cattle were British-breed cattle (*Bos taurus*) that had never been exposed to the parasite, whereas nos. 5 and 6 were zebu (*Bos indicus*) with a history of tick infestation. For all animals, however, $26 \mu\text{M}$ tick inhibitor and $13 \mu\text{M}$ -Trasylol prolonged the activated-partial-thromboplastin times. The differences were significant when analysed by Student's *t* test. The effect of the inhibitors on prothrombin time was tested with three of the same cattle. The mean prothrombin times are listed in Table 2. An analysis of variance for the data gave estimated mean times of 13.55 s for the controls, 14.17 s in the presence of tick inhibitor and 13.56 s in the presence of Trasylol. The increase in prothrombin time in the presence of tick inhibitor was significant ($P < 0.001$).

Effect of inhibitor on haemolytic complement

To investigate possible effects of the inhibitor on complement, titres of the haemolytic activity of bovine sera towards sensitized guinea-pig erythrocytes were measured as described in the Experimental section. Sera were tested after short pre-incubation with the inhibitor and the titres compared

Table 1. Specificity of the inhibitor from the tick *Boophilus microplus*: dissociation constants of enzyme-inhibitor complexes

Enzyme	Buffer	<i>K</i> (μM)
Bovine trypsin	0.04 M-Tris/10 mM- CaCl_2 , pH 8.1	<0.002
Bovine chymotrypsin	0.1 M-Citrate, pH 5.9	0.20*
Bovine chymotrypsin	0.1 M-Phosphate, pH 7.53	0.010
Bovine plasmin	0.05 M-Tris/0.05 M-lysine, pH 8.0	0.10
Pig pancreatic kallikrein	0.1 M-Tris, pH 8.1	0.33
Bovine thrombin	0.1 M-Tris, pH 8.1	~300

* Taken from Willadsen & Riding (1979).

Table 2. Effect of inhibitors on blood-coagulation parameters

Levels of significance: ** $P < 0.001$; * $P < 0.01$.

Coagulation parameter	Species	Animal no.	Inhibitor (concn.)	...	Coagulation times (s)				
					Control	Tick inhibitor (26 μ M)	Trasyolol		
							(26 μ M)	(13 μ M)	(2.6 μ M)
Activated-partial-thromboplastin times	<i>Bos taurus</i>	1			46.80	57.05**	—	84.83**	52.00**
		2			55.25	69.00**	—	100.10**	66.90**
		3			57.65	73.15**	—	103.94**	63.35**
		4			50.75	61.55**	—	—	—
	<i>Bos indicus</i>	5			82.65	88.20*	—	140.53**	88.60*
		6			58.77	66.85**	—	63.13*	62.30*
Prothrombin times	<i>Bos taurus</i>	1			13.63	14.38	13.70	—	—
		2			13.28	13.83	13.35	—	—
		3			13.75	14.30	13.63	—	—

Table 3. Titres of bovine haemolytic complement in the presence and absence of tick inhibitor

Animal no.	[Inhibitor] (μ M)	...	Complement titre (CH ₅₀ /ml)*			
			0	1.0	2.0	4.0
1			600	377	287	189
2			271	n.d.†	193	n.d.
3			315	n.d.	187	n.d.

* Unit of haemolytic complement activity described in the Experimental section.

† Abbreviation used = n.d., not determined.

with those from untreated controls. To avoid complications from possible immune reactions towards the inhibitor protein, sera were taken from three cattle that had not been exposed to ticks. The results are given in Table 3. The tick protein at a concentration of 2 μ M clearly inhibited lysis by the three sera and, in the one serum for which a range of concentrations was used, the degree of inhibition was concentration-dependent.

Effect of inhibitor on lymphocyte transformation

Two mitogens were used, phytohaemagglutinin and concanavalin A. Preliminary experiments showed that these caused maximal stimulation of lymphocytes at final concentrations of 25 μ g/ml and 10 μ g/ml respectively and these concentrations were used in all subsequent experiments. For each animal tested, cells were incubated alone, in the presence of mitogen, and in the presence of mitogen and tick inhibitor at concentrations of 10, 100 and 1000 μ g/ml. Ten animals were tested, only two of which had been exposed to the tick. There was no indication that the results from these two animals were different from those of the unexposed ones, so the results from all cattle were pooled.

An analysis of variance of the data for all animals showed that the presence of tick inhibitor had no

effect at any of the three concentrations on the incorporation of ^{3} H into lymphocytes stimulated by either mitogen. The transformation ratio, that is, the ratio of ^{3} H incorporated into stimulated lymphocytes to that incorporated into control cells varied from 19 to 129 for cells incubated with phytohaemagglutinin and from 10 to 110 for cells incubated with concanavalin A. Since most of this variation was attributable to differences in incorporation of label into control cells, the statistical analysis used the data for ^{3} H incorporation into stimulated lymphocytes. For the lymphocytes incubated with concanavalin A, estimates of viability were consistent with a 3% decrease for cells in the presence of 100 μ g/ml inhibitor and a 6% decrease in the presence of 1000 μ g/ml, though only the 6% decrease was statistically significant. The viability of cells was still high. In the eight cattle for which viabilities were measured, the mean value of the controls at the end of the culture period was 60%, for cells in the presence of concanavalin A alone, 78%, and for cells in the presence of concanavalin A and 1000 μ g/ml inhibitor, 72%. Difficulty was experienced in estimating viability of cells incubated with phytohaemagglutinin, since the cells tended to clump. Qualitatively, however, there was no obvious effect of the tick inhibitor on the cells.

Discussion

The proteolytic-enzyme inhibitor studied in the present paper could potentially be involved in one or more of three distinct aspects of the tick's biochemistry. Firstly, it could be involved internally in the control of the tick's own proteolytic enzymes. Secondly, it could be important in the interaction between tick and host. Thirdly, it could be important only for the egg stage of the life cycle, fulfilling the same function as the enzyme inhibitors of avian eggs [which could be, for example, protection against non-specific proteolytic action during hatching (Vogel & Werle, 1970)]. The sequence of changes in inhibitor concentration during the life cycle argues against the first possibility. If the inhibitor was necessary for the internal control of the parasite's own enzymes, it would be expected to persist throughout the growth of the tick and not to show the dramatic fall in concentration within 3 days of the attachment of the larva that is shown in Fig. 2. This fall in concentration would seem to be a genuine loss of protein, since both inhibitory activity and immunological reactivity with a specific anti-serum are lost. The fact that large amounts of inhibitor occur throughout the egg stage suggests the third possibility; that is, that this protein has some function, as yet unknown, in the eggs. Interestingly, unembryonated eggs of the nematode worm *Ascaris lumbricoides* contain large amounts of trypsin and chymotrypsin inhibitors (Peanasky *et al.*, 1974). Our particular interest is the second possibility mentioned: that the inhibitor could be important in the tick-host interaction. The accumulated evidence suggests strongly that it is. The rapid fall in concentration of inhibitor in larvae after attachment, the loss of protein reacting with a specific anti-inhibitor antibody at the same time, and the fact described previously (Willadsen & Riding, 1979) that cattle exposed to the parasite under natural conditions show an immunological response to the inhibitor, all argue that the inhibitor is transferred from parasite to host. More importantly, this transfer occurs at a definite stage of the life cycle. Thus it seems to be a controlled event. To argue that the protein is irrelevant in the tick-host interaction is to say it is a waste product, carried over from the egg stage and eventually excreted into the host just to remove an unwanted protein. This seems unlikely. The amount of material lost is considerable, roughly 10–12% of soluble protein from freshly hatched larvae or 8–9% from larvae at the maximal infectivity. Even if this protein was no longer needed, it should still represent a substantial, metabolizable food reserve.

If one assumes that the inhibitor has a function in the attachment of parasite to the host, two questions remain. Why is it present for such a short period in the parasitic stage of the life cycle? What are its

possible functions? The answer to the first question may lie in the large amounts of inhibitor necessary for physiological effectiveness. The concentrations of normal serum inhibitors are high. For example, the normal concentration range of human α_1 -antitrypsin is 1.8–2.5 mg/ml (Küppers, 1974), and if a tick needed to maintain concentrations like this in the vicinity of its attachment site on the host to achieve a beneficial effect, the metabolic cost to the parasite would be large. In fact, release of the inhibitor occurs at the most critical stage of the parasite's life cycle, since the major loss of ticks from the host occurs in the 24 h after first attachment (Roberts, 1968). Perhaps only at this time does potential benefit to the tick outweigh the cost of excreting this protein.

In an attempt to answer the second question we have examined the effect of the inhibitor on separate enzymes. It is effective against not only bovine trypsin and chymotrypsin but also bovine plasmin and pig urinary kallikrein, although it is apparently ineffective against bovine thrombin. This information in itself contributes little to an understanding of possible physiological roles. The ability to inhibit bovine serum kallikrein could be useful to the parasite, but one cannot justifiably extrapolate from inhibition of the pig urinary enzyme to the bovine serum one. The real problem with studies on isolated enzymes, though, is the multiplicity of potential target enzymes and the complexity of the interactions between the clotting, fibrinolysis, kinin and complement systems (Cochrane, 1976; Movat, 1978; Osler, 1976). For this reason, we investigated the effect of inhibitor on blood coagulation, complement lysis and lymphocyte stimulation rather than on further separate enzymes.

Both activated-partial-thromboplastin times and prothrombin times are significantly prolonged in the presence of inhibitor; that is, both extrinsic and intrinsic systems are affected. Trasylol was included as a comparison in this experiment, since it is known to have anticoagulant properties (Vogel & Zickgraf-Rüdel, 1970). It was found to be more effective than the tick inhibitor in prolonging partial-thromboplastin time, but to have no effect on prothrombin times. It is reasonable to conclude that the tick protein would be a useful anticoagulant.

At a concentration 10-fold lower than that necessary for anticoagulant action, the tick protein inhibits complement-dependent cell lysis. Minimization of the many biological activities of complement should clearly be advantageous to the parasite.

Finally, we investigated the effect of tick inhibitor on mitogen-stimulated lymphocyte transformation, since several non-specific proteinase inhibitors inhibit this reaction. The inhibitors shown to have this ability are Trasylol (Thomson *et al.*, 1978),

soya-bean trypsin inhibitor (Moreau *et al.*, 1975) and α_2 -macroglobulin (Goutner *et al.*, 1976). At concentrations of tick inhibitor as high or higher than those used for these proteins, the tick protein had no effect on lymphocyte stimulation. It had only a very slight effect on cell viability, unlike Trasylol, which was found to be cytotoxic to peripheral blood leucocytes (Thomson *et al.*, 1978). The absence of an effect of tick inhibitor on lymphocytes, like its failure to inhibit thrombin, at least demonstrates that there is a degree of specificity in its action and indirectly lends credibility to the suggestion that the demonstrated inhibition of blood coagulation and complement could be physiologically important functions.

The list of possible effects for this protein is obviously far from complete and at least one more deserves serious consideration. The recent demonstration that Trasylol originates from bovine mast cells suggests that it is involved in the regulation of mast-cell proteinases either intra- or extra-cellularly (Fritz *et al.*, 1979). Since the most striking immunological reaction to *Boophilus microplus* in cattle is immediate hypersensitivity (Willadsen *et al.*, 1978), the tick may well find it advantageous to duplicate this function itself.

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