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Characterization of a Proteolytic-Enzyme Inhibitor with Allergenic Activity

MULTIPLE FUNCTIONS OF A PARASITE-DERIVED PROTEIN

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1. A trypsin inhibitor from the tick *Boophilus microplus* was purified by ion-exchange chromatography and gel filtration. 2. It is pure by the criteria of constant specific activity on gel filtration and by electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate. 3. The protein undergoes reversible polymerization, dissociating at low pH. 4. The apparent molecular weight measured by electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate is 18500. 5. Inhibition of trypsin occurs by formation of a 1:1 molar complex. 6. Chymotrypsin is also inhibited, though the dissociation constant of the complex formed is larger than with trypsin. The protein possesses independent sites for the inhibition of chymotrypsin and trypsin. 7. The inhibitor preparation gives an immediate hypersensitivity reaction on intradermal injection into cattle that have been exposed to the tick. The allergenic activity is due to the inhibitor protein itself and not to contaminating material, since the two activities were not separated during purification or in two subsequent affinity-chromatography procedures. 8. The hypersensitivity reaction is a true immunological response, since it is found in almost all cattle that have been exposed to the tick, but not in unexposed animals. In addition, passive cutaneous anaphylaxis can be demonstrated with serum from exposed, but not from unexposed, animals.

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. In spite of this, information on their incidence, characteristics and function in parasites remains fragmentary. The only parasite in which such inhibitors have been studied intensively is *Ascaris lumbricoides* (Kassell, 1970). Apart from this, inhibitors of chymotrypsin or trypsin have been reported in several other nematodes and cestodes, though they have not been well characterized and their function is uncertain. Since the parasites that have been studied are parasites of the digestive system, the obvious function for the inhibitors is as a defence against the host's digestive enzymes. This may be the case whether the inhibitors occur in the body wall of the parasite, as in *A. lumbricoides* (Kassell, 1970), or whether they are actively excreted, as in *Stephanurus dentatus* (Rhoads & Romanowski, 1974). Nevertheless, this function is not universally accepted (von Brand, 1973).

Since proteolytic enzymes are important, not only in digestion, but also in many of an animal's cellular and immunological processes, it is possible that inhibitors could have other roles in the interaction between host and parasite, if they were introduced into the host's tissues. An essential piece of evidence for such roles must be the demonstration that introduction of the inhibitor into the host's tissues does occur. This has not been done, to date, for any host–parasite system.

We have found that unfed larvae of the ectoparasite, *Boophilus microplus*, the cattle tick, contain a trypsin inhibitor. This has been purified and partially characterized. In addition to its inhibitory activity, this protein gives an immediate hypersensitivity reaction in cattle that have been exposed to the tick. This, in turn, is evidence that the protein must be transferred from the parasite into the host's tissues in sufficient quantities to cause an antibody response.

Materials and Methods

Materials

Enzymes and biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., except for *N*-acetyl-L-tryptophan ethyl ester, which was from Mann Research Laboratories (New York, NY, U.S.A.), and *p*-nitrophenyl-*p'*-guanidino-benzoate hydrochloride, from ICN Life Sciences Group (Cleveland, OH, U.S.A.). Sephadex G-50, Sephacryl S-200 and CNBr-activated Sepharose 4B were from Pharmacia (North Ryde, N.S.W., Australia) and ion-exchange celluloses from Whatman (Maidstone, Kent, U.K.). Acrylamide, *NN'*-methylenebisacrylamide and Cyanogum 41 were from BDH Chemicals,

Boronia, Vic., Australia. Other chemicals were of analytical-reagent grade.

Cattle used were *Bos taurus* of various breeds, aged from 1 to 12 years. Cattle exposed to the tick were raised under normal field conditions in south-eastern Queensland, whereas unexposed controls were raised tick-free in pens. Unfed larvae of *B. microplus* were obtained as described by Roberts (1971).

Assay of trypsin, chymotrypsin and inhibitor

Bovine trypsin was assayed with α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester as described by Walsh (1970), the increase in A_{247} accompanying hydrolysis being measured at 25°C on the 0–0.1 absorbance scale of a Varian 635 M spectrophotometer. The concentration of active trypsin was determined by titration with *p*-nitrophenyl-*p'*-guanidinobenzoate as described by Chase & Shaw (1970), with the single change that the buffer used was 0.04M-Tris/HCl/10mM-$CaCl_2$, pH8.1.

Bovine chymotrypsin was assayed with the substrate 1mM-*N*-acetyl-L-tryptophan ethyl ester in 0.1M-sodium phosphate buffer, pH7.3, containing 1% (v/v) ethanol. The increase in A_{300} on hydrolysis was measured on an absorbance range of 0–0.1. The concentration of active enzyme was measured by active-site titration with *p*-nitrophenyl acetate as described by Kézdy & Kaiser (1970).

Inhibitor was assayed by measuring the decrease in activity of a known amount of trypsin after pre-incubation with the inhibitor. A 200μl portion of the test solution was mixed with 200μl of trypsin in 0.04M-Tris/HCl buffer, pH8.1, containing 10mM-$CaCl_2$. The final enzyme concentration was approx. 0.1μM. After 2–30min at room temperature, a 200μl sample was used to measure the residual trypsin activity. The inhibition was independent of the time of preincubation, within the interval given above, and the degree of inhibition was proportional to the amount of inhibitor added (Fig. 1). Plots of this sort with a range of amounts of inhibitor were always used for the accurate measurement of inhibitor concentration. Concentrations of inhibitor were calculated by assuming the formation of a 1:1 molar complex between inhibitor and active trypsin, and are given as μM. The specific activity is calculated as nmol/mg.

Protein determination

Protein concentrations during purification of the inhibitor were estimated from the A_{280} and A_{260} (Layne, 1957). To calculate the specific activity of the final product of the purification and for all subsequent experiments, the specific absorption coefficient was used.

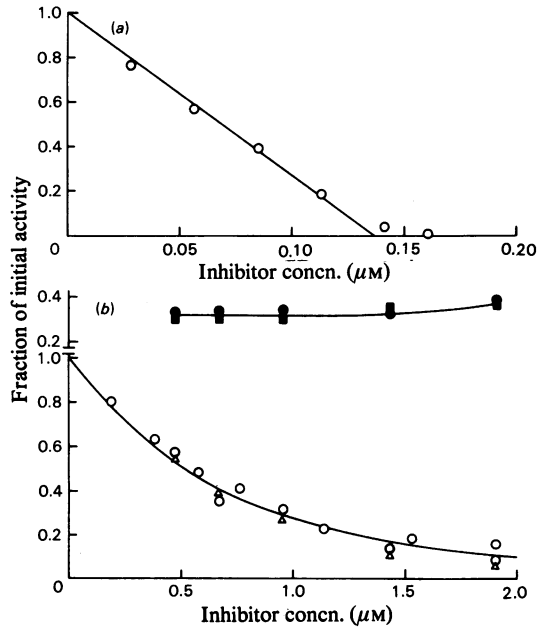

Fig. 1. *Inhibition of trypsin and chymotrypsin*
(*a*) Inhibition of 0.106μM-trypsin by increasing concentrations of inhibitor. (*b*) Inhibition of 0.625μM-chymotrypsin by increasing concentrations of inhibitor in the absence (○) and presence (△) of added trypsin. The upper line in (*b*) shows the residual activity of trypsin mixed in a constant ratio with inhibitor, in the absence (■) and presence (●) of chymotrypsin. Inhibitor concentrations were calculated from the protein concentration and molecular weight of the inhibitor.

Preparation of chymotrypsin–Sepharose

Chymotrypsin–Sepharose was prepared by incubating 3g of pre-swollen CNBr-activated Sepharose with 60mg of chymotrypsin in 30ml of 0.1M-$NaHCO_3$/0.5M-NaCl, pH8.3, for 2h at 25°C. The gel was then filtered and suspended in 20ml of 0.1M-Tris/HCl buffer, pH8.5, for 1h at room temperature, then overnight at 4°C. It was then washed alternately in 0.5M-sodium acetate buffer, pH5.0, and 0.1M-Tris/HCl, pH8.5, to remove excess protein, then stored in 0.05M-sodium phosphate buffer containing 0.1M-NaCl, pH6.6.

Polyacrylamide-gel electrophoresis

Electrophoresis on 7.5 and 10% (w/w) polyacrylamide gels containing sodium dodecyl sulphate was carried out as described by Weber *et al.* (1972). Electrophoresis under non-denaturing conditions used 7.5% gels prepared with Cyanogum 41 in 0.02M-Tris/0.04M-glycine buffer, pH8.8. The gels were pre-

electrophoresed at 300V for about 3h at 4°C before samples were applied and electrophoresed under the same conditions for 2h. Protein bands were stained with Amido Black 10B.

Immediate hypersensitivity and passive cutaneous anaphylaxis reactions

Immediate hypersensitivity reactions were measured both 20 and 40min after intradermal injection of the test material, buffer being used for control injections: the procedure has been described previously (Willadsen & Williams, 1976). Control injections normally gave no discernible reaction, but occasionally there was a small injection mark. To be scored as positive, a test reaction had to be at least twice the area of controls in the same animal. Concentrations of allergenic material are shown on a logarithmic scale in the Figures, the baseline, however, representing a fraction that gave no detectable reaction. Normally the smallest difference in concentrations of allergen that can be reliably detected is 2-fold.

Passive cutaneous anaphylaxis was carried out after intradermal injections of 0.1ml dilutions of test sera into an animal that had been raised free of ticks. After 3 days, the injection sites were challenged locally and the oedematous reactions measured after 20min. The procedure has been described (Willadsen *et al.*, 1978).

Purification of inhibitor

All operations were carried out at 4°C.

Extraction and pH fractionation. Extracts were prepared by grinding 100g of tick larvae in a mortar, then stirring with 3.5ml of 0.1M-citric acid/g of larvae for 1h. Insoluble material was removed by centrifugation for 30min at 4000*g* ($r_{av.}$ 14cm), the conditions used for subsequent centrifugations also. The pH of the extract was 3.1. This was adjusted with 2.5M-NaOH to pH5.0 and, after 2h, the precipitate centrifuged off. Tris was added to a final concentration of 0.05M, the pH adjusted to 8.8 and the solution dialysed overnight against 2×7vol. of 0.05M-Tris/HCl buffer, pH8.8. Again, insoluble material was removed by centrifugation as above.

Ion-exchange chromatography. The dialysed solution of inhibitor was applied to a column (2.5cm×25cm) of DEAE-cellulose equilibrated with the same buffer as used for dialysis. As soon as the sample was loaded, the column was washed with 0.05M-Tris/HCl buffer, pH8.8, containing 0.05M-NaCl. All fractions containing more than 7nmol of inhibitor/ml were pooled, adjusted to pH4.5 by addition of acetic acid and dialysed for 3h against 10vol. of 0.05M-acetate buffer, pH4.5. The solution was then loaded on a column (2.5cm×40cm) of CM-cellulose, washed with starting buffer, and then a 700ml linear gradient of 0–0.8M-NaCl in the same Tris buffer was applied. The inhibitor was eluted at 0.3M-NaCl, and fractions with a specific activity greater than 15 were pooled. Solid $(NH_4)_2SO_4$ was added to 70% saturation, the precipitate dissolved in a minimum of water and dialysed against 2×40vol. of 0.1M-citric acid/0.5M-NaCl, pH1.7. At 2 days after dialysis was started, the inhibitor solution was centrifuged to remove precipitated material, then loaded on a column (2.5cm×92cm) of Sephadex G-50 (fine grade) and eluted with 0.1M-citric acid/0.5M-NaCl.

Experimental and Results

Preparation of inhibitor

The results of a typical inhibitor preparation are shown in Table 1, and the final chromatographic step in the purification is shown in Fig. 2. The results in Fig. 2 suggest that, in fact, two inhibitors may be present and that, as well as the major active component, there may be a minor one of lower molecular weight. Only those fractions containing the major component at maximum specific activity were pooled to give the final product. Two pieces of evidence suggest that the inhibitor is homogeneous. The fact that constant specific activity is observed across the peak of inhibitory activity on Sephadex G-50 chromatography argues for this. Secondly, polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate showed only a single band of protein (Fig. 3).

Both the purification of the inhibitor and its electrophoresis under non-denaturing conditions as a test for homogeneity were made difficult by two characteristics of the protein, its tendency to polymerize and its tendency to bind to chromatographic and electrophoretic supports at low pH. Thus the inhibitor could not be chromatographed on Sephacryl S-200 in the solvent used for the Sephadex G-50 chromatography, since the protein was tightly bound. Even on Sephadex G-50, it appeared that some abnormal retardation could be occurring, since the ratio of elution volume to void volume for the inhibitor was 1.59, whereas for bovine trypsinogen and horse heart cytochrome *c* under the same conditions the ratios were 1.34 and 1.46 respectively. Electrophoresis of the inhibitor in sodium dodecyl sulphate, as described below, shows its molecular weight to be between those of these two proteins.

The ability of the inhibitor to polymerize reversibly could be demonstrated by chromatography on Sephadex. The inhibitor, chromatographed on Sephadex G-50 at pH4.5, was eluted in the void volume. A sample of such a preparation dialysed for 1.5h against 0.1M-citric acid/0.5M-NaCl and chromatographed in the same buffer was still eluted

Table 1. *Purification of trypsin inhibitor from Boophilus microplus*

Fraction	Protein (mg)	Specific activity	Inhibitor yield (%)	Purification factor
Extract	6040	1.51	100	1.0
pH5 supernatant	1740	5.03	96	3.3
pH8.8 supernatant	1070	7.02	83	4.7
DEAE-cellulose eluate	363	14.2	56	9.4
CM-cellulose eluate	107	28.9	34	19.1
pH1.7 supernatant	83	27.6	25	18.3
Sephadex G-50 eluate	20.5	47.9	11	31.7

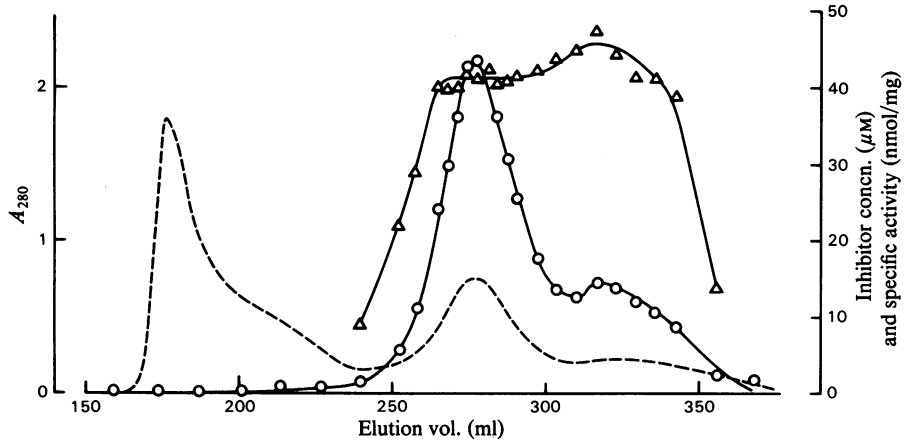

Fig. 2. *Purification of the inhibitor on Sephadex G-50*
The sample was applied to a column (2.5cm×92cm) of Sephadex G-50 and eluted in 0.1M-citric acid/0.5M-NaCl. The Figure shows the activity (○) and specific activity (△) of the inhibitor. ----, A_{280}.

in the void volume. After 15h dialysis, repetition of the chromatography showed two active fractions, one at the void volume, the other retarded. After being left a further 24h at 4°C, the same solution showed only a single active fraction, which was retarded on Sephadex G-50, the behaviour shown in Fig. 2. Conversely electrophoresis of purified inhibitor at alkaline pH gave a pattern suggestive of repolymerization. Thus inhibitor, after Sephadex chromatography at low pH, was dialysed for 15h against 0.02M-Tris/0.04M-glycine buffer, pH8.8, and left for 24h at 4°C. On polyacrylamide-gel electrophoresis in the same buffer this gave the pattern shown in Fig. 3.

Apparent molecular weight of the inhibitor

The molecular weight was estimated by electrophoresis in sodium dodecyl sulphate on polyacrylamide gels, as described by Weber *et al.* (1972). Pig pepsin, bovine trypsinogen, bovine haemoglobin, sperm-whale myoglobin and horse cytochrome *c* were used as standards. Eleven determinations of the inhibitor's molecular weight in three separate experiments on 7.5% gels gave an average of 18500±300. To confirm that electrophoretic behaviour was normal, the experiment was repeated with 10% gels, giving an average value of 18300.

In the gels loaded with large amounts of inhibitor (50 or 100μg), which were used to check the purity of the protein, a faint leading edge was visible after staining. Pre-electrophoresis of the gels in buffer containing 10mM-2-mercaptoethanol, followed by electrophoresis in the same buffer, eliminated this effect, without altering the mobility of the protein.

It has been suggested that a sequence of denaturation in guanidine hydrochloride and 2-mercaptoethanol, alkylation and dialysis against sodium dodecyl sulphate should be used to demonstrate the completeness of denaturation (Weber *et al.*, 1972). This procedure using alkylation with iodoacetamide slightly decreased the mobility of the inhibitor, giving an apparent mol.wt. of 23000 relative to the same standards. There was no evidence of smaller subunits. In summary, the results suggest that the monomeric form of the inhibitor has a mol.wt. of approx. 18500.

The fact that the small, second peak of inhibitory

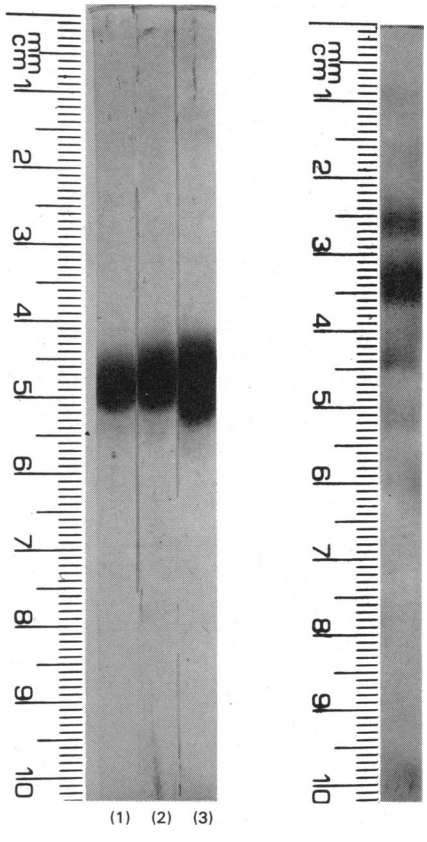

Fig. 3. *Polyacrylamide-gel electrophoresis of the inhibitor*
Gels (1)–(3) show electrophoresis in sodium dodecyl sulphate of 25 (1), 50 (2) and 100 (3) μg of protein. Gel (4) shows the electrophoresis of 40μg of protein at pH8.8.

activity is due to another protein was shown by the fact that on electrophoresis in sodium dodecyl sulphate this peak contained predominantly a protein with mol.wt. 16300. In preparations where both inhibitor peaks were pooled, electrophoresis showed major and minor bands with the expected molecular weights.

Estimation of specific absorption coefficient and stoicheiometry of inhibition

The specific absorption coefficient was measured after dialysis of the inhibitor against 10mM-HCl. The A_{280} was measured both in this solvent and in 0.1M-citric acid/0.5M-NaCl. Samples of the solution were dried at 75°C over P_2O_5, then over P_2O_5 *in vacuo* to constant weight. The value of $A^{1\%}_{1cm}$ in both solvents was 7.2. The inhibitory capacity of the same solution was measured with a freshly prepared solution of trypsin. The stoicheiometry of the inhibition was calculated from the dry weight of the inhibitor, the estimated mol.wt. of 18500 and the trypsin concentration. Since commercial trypsin preparations contain autolysed, inactive enzyme, two estimates of the latter were used: the total protein concentration as calculated from the A_{280} and a specific absorption coefficient of 15.0 (Walsh, 1970) and the concentration of active enzyme obtained by titration with *p*-nitrophenyl guanidinobenzoate. Since the inhibitor might react with some of the autolysis products, the true stoicheiometry may well lie between these two extremes. The molar ratios of inhibitor/trypsin for complete inhibition thus obtained were 1:1.25 (on the basis of total protein) and 1:0.74 (on the basis of active enzyme). These values strongly suggest that inhibitor and enzyme react in a 1:1 molar ratio.

Knowledge of the stoicheiometry of the inhibition reaction and the molecular weight of the inhibitor allows a further calculation. In six consecutive preparations of inhibitor over a period of 9 months, the specific activity of the crude extract ranged from 1.5 to 1.8nmol/mg. Thus the inhibitor is consistently about 3% of the total protein that is soluble under the conditions of extraction.

Reaction of the inhibitor with chymotrypsin

Inhibitor was incubated with bovine α-chymotrypsin in 0.1M-citrate buffer, pH5.9, at 25°C for 5–30min, then residual enzyme activity assayed. The degree of inhibition was independent of time in this range. The assays curved upwards, suggesting reversal of the inhibition reaction, so initial rates in the first minute of the assay were used to calculate the results. The concentration of enzyme in the incubation, estimated by active-site titration, was 0.63 μM and the concentrations of inhibitor calculated from the protein concentration and a mol.wt. of 18500 ranged from 0.19 to 1.9μM. The results are shown in Fig. 1. The solid line is the theoretical curve calculated for the formation of a 1:1 molar complex with a dissociation constant of 0.2μM. Although assumption of such a simple equilibrium may be an oversimplification, the curve adequately represents these data.

This experiment was repeated by using the same concentration of chymotrypsin and a range of inhibitor concentrations, with one alteration. Before addition of chymotrypsin to the incubations, the inhibitor was first treated for 1min at 25°C with 1.5 times the amount of trypsin neutralized by the amount of inhibitor added. That is, inhibitor and trypsin were added in a constant ratio such that excess uninhibited trypsin was always present. In

these mixtures of inhibitor, trypsin and chymotrypsin, chymotrypsin was assayed with *N*-acetyl-L-tryptophan ethyl ester and trypsin with α-*N*-toluene-*p*-sulphonyl-L-arginine methyl ester. Chymotrypsin at the concentrations used had no effect on the trypsin substrate, whereas a very small correction was necessary for the effect of trypsin on the chymotrypsin assays. The residual activity was measured for both enzymes and compared with the residual activities after incubation of the enzyme with inhibitor separately. The results are also shown in Fig. 1. Interestingly, the simultaneous presence of two enzymes made no difference, showing that the inhibitor must possess two independent binding sites, one for trypsin, one for chymotrypsin.

Immunochemical properties of the inhibitor

Early experiments suggested that intradermal injection of the inhibitor into cattle that had been exposed to the tick caused an immediate hypersensitivity reaction. It was tempting to assume that the inhibitor itself was allergenic since, on the evidence presented above, the protein was apparently pure. Apparent purity, however, is not sufficient evidence that inhibitor and allergen are the same. Micrograms of inhibitor protein were sometimes necessary to give a dermal reaction, whereas two other proteins from the tick are already known that can give similar reactions in nanogram amounts (Willadsen *et al.*, 1978). Thus a small degree of contamination with another allergenic protein could give misleading results. Consequently, two different affinity-chromatography procedures were used to demonstrate that the inhibitor was in fact the allergenic material.

First, inhibitor was bound to a column of Sephacryl S-200 at low pH and high ionic strength, then eluted by a change in pH. The results are shown in Fig. 4. A small amount (12%) of the inhibitor was rapidly eluted, accompanied by some allergenic activity, the bulk of both inhibitor and allergen being eluted together on the change of pH. Although there is no indication of the specificity of this procedure for the inhibitor, the use of high ionic strength at low pH should decrease non-specific binding of other proteins (Belew *et al.*, 1978). Thus trypsinogen, for example, chromatographed normally in the low-pH buffer used here.

In the second experiment, inhibitor was bound to insolubilized chymotrypsin at pH6.6, then eluted by a decrease in pH intended to dissociate the enzyme–inhibitor complex. Inhibitory activity was measured against trypsin and allergenic activity tested as usual. The results are shown in Fig. 5. Both activities were bound to the chymotrypsin column and were eluted together with the change in pH. Some allergenic activity that was devoid of inhibitory

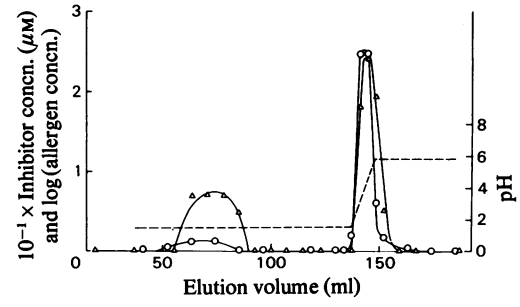

Fig. 4. *Chromatography of purified inhibitor on Sephacryl S-200*

Purified inhibitor (6.3mg) was applied to a column (1.6cm×30cm) of Sephacryl S-200 in 0.1M-citric acid/0.5M-NaCl. The column was washed with this buffer, then eluted with 0.1M-citrate, pH5.8. The Figure shows inhibitor (○) and allergen (△) activities. ----, pH.

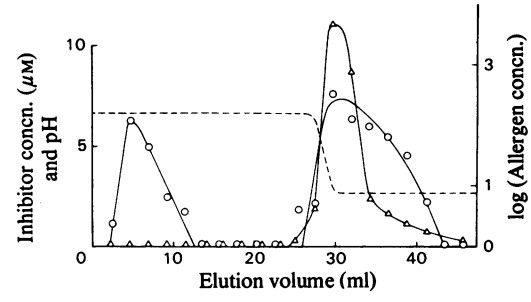

Fig. 5. *Chromatography of purified inhibitor on chymotrypsin–Sepharose*

Inhibitor (0.2mg) was applied to a column (0.5×7cm) of chymotrypsin–Sepharose in 0.05M-sodium phosphate buffer/0.1M-NaCl, pH6.6, at 25°C. The column was eluted first with this buffer, then with 0.05M-sodium phosphate/0.1M-NaCl, pH2.6. The Figure shows inhibitor (△) and allergen (○) activities; ----, pH.

activity was eluted at pH6.6. This may be due to a proportion of inhibitor being either inactive before loading on the column or inactivated by reaction with chymotrypsin.

These two chromatographic experiments provide strong evidence that the major part of the allergenic activity is due to the inhibitor itself and not to an unidentified contaminant.

The fact that the oedematous dermal reactions caused by the inhibitor were immunological in nature was shown in two ways. First, the reactions were dependent on tick exposure. Intradermal injection of 5μg of protein into 21 animals caused positive reactions in 20 of them. With 0.5μg, 14 out of the 21

reacted. However, injection of more than 5μg into six animals that had not been exposed to the parasite gave no reaction. Secondly, passive cutaneous anaphylaxis reactions were carried out with sera from four exposed and four unexposed animals. None of the sera from unexposed animals gave reactions. Three out of the four sera from exposed animals gave reactions with sensitizing doses of 0.1 ml of undiluted serum or serum diluted 1:5. Thus the titres of reaginic antibody, although possibly low, are significant. The test material used in these experiments was purified inhibitor that had been further chromatographed by attachment to Sephacryl S-200 at low pH and then desorbed as described previously. This was done to decrease further the possibility of contamination by other allergens.

Discussion

The study of the complex interactions between a metazoan parasite and its host is usually restricted to just one aspect of the biological, biochemical and immunological interactions that occur. For example, although there have been many studies of the immunology of parasitic infections, the specific antigens involved have rarely been identified and almost never have they been shown to possess functional activity in a biochemical sense. One exception is the production of antibodies to an excreted acetylcholinesterase of several parasitic nematodes (Edwards *et al.*, 1971; Ogilvie *et al.*, 1973; Rothwell & Merritt, 1974). Conversely, if it is simply shown that parasites contain a particular protein, it is not possible to distinguish between a purely internal metabolic role for the protein in the parasite and its involvement in extraparasitic processes.

The protein that we have purified from the cattle tick has features of interest from enzymological, immunological and parasitological points of view. It has been shown that it will inhibit both trypsin and chymotrypsin and that it possesses separate binding sites for these two enzymes. Such double-headed inhibitors are known from a number of sources, for example dog submandibular glands (Fritz & Hochstrasser, 1976), soya beans (Birk, 1968), lima beans (Krahn & Stevens, 1970) and egg-white (Rhodes *et al.*, 1960; Tomimatsu *et al.*, 1966). The protein that we have isolated thus has intrinsic interest as a proteolytic-enzyme inhibitor.

In addition, we have shown that the inhibitor causes an immediate hypersensitivity reaction on intradermal injection into cattle and that this reaction is a specific immunological response. This is of interest in a purely immunological sense. Despite the vast literature on immediate hypersensitivity, very few allergens have been shown to have a biochemical role separate from their allergenicity. Almost the only exceptions are the components of honey-bee venom.

Whether or not the hypersensitivity reaction is important in the host–parasite relationship is unknown, but it does at least show that the inhibitor has been present in the host's tissues. It has been pointed out above that in very few other cases has such contact between a biochemically active molecule of a metazoan parasite and its host been shown to occur. The function of the inhibitor *in vivo* is unknown. It is extremely unlikely that its effect on trypsin or chymotrypsin is physiologically important. There are, however, similar enzymes necessary for many of the processes of the immune system and it is possible that the inhibitor has an effect on one of these that is advantageous to the parasite.

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