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Ascaris trypsin inhibitors 1, 2, and 3 have arginine at their reactive P site. This corrects an earlier report that lysine is the reactive P site residue in Ascaris trypsin inhibitor 1 (Peanasky et al., 1974, Bayer Symposium V: Proteinase Inhibitors, pp. 649 - 666). The present work illustrates that the residue modification method of Fritz et al. (1969, Z. Physiol. Chem., 350, 933 - 944) may not be reliably interpreted when trypsin inhibitors have an unusually high lysine content (>12% of the molecular weight of the inhibitor). Thus the following procedure is recommended: treat the inhibitor with maleic anhydride first and second with butanedione reagent; then remove the maleyl groups in an acid environment and determine the activity of the inhibitor.

Immunoperoxidase staining shows that antibody to Ascaris trypsin inhibitor 1 binds to body wall muscle, intestine, eggs and sperm in cross-sections of Ascaris. Antibody to TLCK-porcine trypsin binds to the same tissues and at the same sites as the antibody to Ascaris trypsin inhibitor 1. This is the first demonstration that a protein that originated in the host has been found in the parasite, Ascaris. Analyses of homogenates and of extracts of separated tissues always show an excess of free trypsin inhibitor and no evidence of active trypsin. The host protein is present inside the parasite, probably as the trypsin-inhibitor complex.

The capacity of extracts of Ascaris for inhibiting trypsin has been recognized since the beginning of this century (Weinland, 1903). Our laboratory described the preparation of three different trypsin inhibitors from one extract of Ascaris using affinity chromatography (Goodman & Peanasky, 1982). Each of the species was related to trypsin inhibitors described earlier (Pudles et al., 1967; Portman & Fraefel, 1967; Kucich & Peanasky, 1970). Only two of the inhibitors have ever

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** This research was supported in part by the National Institutes of Health (AI-10992) and the General Research Support Fund of The University of South Dakata School of Medicine.

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been classified: one having lysine and the other arginine at their reactive site (Peanasky et al., 1974). These assignments were made after the trypsin inhibitors reacted with reagents that modified specific amino acid residues, causing the loss of ability to inactivate trypsin. This approach was first applied by Feeney and co-workers (Haynes et al., 1967; Liu et al., 1968). Ascaris trypsin inhibitor 3 retained its activity after the amino groups in it were modified, but was inactive after treatment with 2,3-butanedione reagent which modifies the guanido group of arginine. Consequently, Ascaris trypsin inhibitor 3 was assigned to the class of trypsin inhibitors with arginine at the reactive site. The trypsin inhibiting activity of Ascaris trypsin inhibitor 1 was abolished after treatment of the inhibitor with maleic anhydride which forms a covalent derivative with the \(\alpha\)-amino and the \(\varepsilon\)-amino residues of lysine. Treatment with perchloric acid, which destroyed the covalent derivatives, restored the amino groups and the biological activity. The interpretation was that lysine is certainly at the reactive site (Peanasky et al., 1974).

A more precise determination of the residues at the reactive site of an inhibitor was prescribed by Finkenstadt & Laskowski, Jr. (1965) who observed that under selected conditions of time, pH and appropriate concentrations of enzyme and inhibitor, the interaction of a protein protease inhibitor with its cognate enzyme resulted in the hydrolysis of the reactive site peptide bond in the inhibitor. Such conditions were identified for Ascaris trypsin inhibitor 1 (Peanasky et al., unpublished). Arginine and not lysine was now the C-terminal amino acid released from the \(P_1\) site when Ascaris trypsin inhibitor 1 with the hydrolyzed peptide bond was treated with carboxypeptidase B. Therefore, the assignment of Ascaris trypsin inhibitor 1 has been re-examined and Ascaris trypsin inhibitors 1, 2 and 3 have arginine at their reactive site. A precaution that must be exercised in interpreting the results obtained from the modification method of Fritz et al. (1969) is described.

The function of protease inhibitors is conceded to be the prevention of unwanted proteolysis. Their participation has been shown, however, in only a few physiological events, i.e. in the control of the activation of zymogens in pancreatic granules and in the pancreatic duct (Fritz et al., 1966; Greene et al., 1966); of reproduction (Zaneveld et al., 1975; Fritz et al., 1975); of infestation of plants by insects (Ryan, 1980), and in some cases of emphysema and cirrhosis of the liver (Laurell & Eriksson, 1963; Jeppsson & Laurell, 1975; Janoff et al., 1975). Ascaris are parasites that live in the intestines of their hosts and are bathed in an environment of digestive enzymes. Most of these digestive enzymes are synthesized in and secreted by the pancreas of the worm's host. The inhibitors of these digestive enzymes are not secreted by the Ascaris worm into the environment. The inhibitors of trypsin, chymotrypsin/elastase, and carboxypeptidases A and B remain inside

\[P_1 \rightarrow P_2 \rightarrow P_1 \rightarrow P_1' \rightarrow P_2' \rightarrow P_1'\] (Schechter & Berger, 1967). The peptide bond cleaved as a result of encounter with trypsin under selected conditions is between residues \(P_1\) and \(P_1'\). Residue \(P_1\) is called the reactive site of the inhibitor.
the worm. How then do these inhibitors function, when they do not seem to come in contact with their cognate enzyme?

Rhodes, Marsh & Kelley (1963) dissected different tissues from the worm, made an extract of each and reported that the inhibitors of trypsin and chymotrypsin seem to be associated with most organs in the worm. We have re-examined this question using antibodies to Ascaris trypsin inhibitor 1 and to porcine trypsin, and a double antibody overlay technique to localize the antigens. Since the antigens for both host trypsin and worm trypsin inhibitor seem to be located at the same sites and since free host enzymes cannot be demonstrated by activity measurements in extracts of the parasite it is suggested that host enzymes are present as their inactive complexes inside the parasite.

**MATERIALS AND METHODS**

*Materials.* Trypsin inhibitor (Trasylol), chicken ovomucoid, trypsin (EC 3.4.21.4) from porcine pancreas, chymotrypsin (EC 3.4.21.1), chicken egg albumin (ovalbumin), Pronase E, penicillin G, streptomycin, thimerosal, N-α-p-tosyl-L-lysine chloromethyl ketone, maleic anhydride, butane-2,3-dione, phenylhydrazine, Triton X-100 and antirabbit IgG raised in goats and coupled to horseradish peroxidase were purchased from Sigma Chemical Company. Fungizone was obtained from E. R. Squibb and Sons, Pharmaceuticals. Osmium tetroxide, 3,3’-diaminobenzidine and the JB-4 Embedding Kit were obtained from Polysciences, Warrington, PA. Diisopropylfluorophosphate was obtained from Aldrich Chemical Company. Permount was obtained from Fisher Scientific. Substrate, hemoglobin was prepared from outdated blood (Kucich & Peanasky, 1970). Ascaris trypsin inhibitors (Goodman & Peanasky, 1982) and Ascaris chymotrypsin inhibitor 5 were prepared by affinity chromatography.

*Trypsin inhibitor* activity is determined as described by Goodman & Peanasky (1982). One unit of trypsin inhibitor activity is the amount of protein needed to inhibit one microgram of trypsin. Specific activity is reported in units/E, where one E is the amount of protein which has an absorbance of 1.00 at 280 nm through a 1 cm light path. A solution of inhibitor (1 mg/ml) has an absorbance at 280 nm of 1.05.

*Butanedione reagent* was prepared according to the method of Grossberg & Pressman (1968).

The determination of reactive sites was done using the method of Fritz et al. (1969), with the precaution that treatment with 2,3-butanedione reagent is always performed on the polymaleylated inhibitor before acid treatment (Scheme 1). An ice cold solution (0.5 ml) of trypsin inhibitor in water, 2.5 mg/ml, was added to a test tube containing 21 mg of NaHCO₃ and 10 mg of maleic anhydride. The reaction was allowed to proceed at room temperature for 30 min, after which aliquots were withdrawn for assay. The reaction mixture was then divided into two parts:

a) One portion of the polymaleylated inhibitor solution (0.1 ml) was added to 0.9 ml of 3% perchloric acid, and the mixture was incubated at 55°C. Aliquots for assay were withdrawn after 60, 120 and 240 min of incubation.
b) The other portion of the polymaleylated inhibitor solution (0.2 ml) was added to 0.4 ml of 0.5 m-sodium bicarbonate, pH 8.6, containing 0.2 ml of butanedione reagent. Aliquots for assay were withdrawn after 30 and 90 min of incubation at room temperature. After 90 min, 0.1 ml of the butanedione treated, polymaleylated trypsin inhibitor was added to 0.9 ml of 3% perchloric acid, and the mixture was incubated at 55°C. Aliquots of the acid treated, butanedione treated, polymaleylated trypsin inhibitor were withdrawn for assay after 60, 120 and 240 min.

Scheme 1. Flow-chart according to Fritz et al. (1969) modified by including treatment of the polymaleylated trypsin inhibitor with 2,3-butanedione reagent before removal of the maleyl groups by acid treatment.

Preparation of the antibodies. The antibodies were developed in New Zealand white rabbits. The antigens were Ascaris trypsin inhibitor 1 and TLCK-porcine trypsin. The initial intradermal injection was with 100 μg of antigen in Freund's complete adjuvant. Three subsequent injections were made at weekly intervals in Freund's incomplete adjuvant using 50 μg of antigen for each intradermal injection. The rabbits were bled after four weeks through the marginal ear vein or by cardiac puncture. Titers were monitored using Ouchterlony plates. The antigen used against the antiserum to TLCK-porcine trypsin was DIP-trypsin.

Purification of the antibodies. Pooled sera were decomplemented by heating at 55°C for 30 min. An equal volume of cold saturated ammonium sulfate was added to the pooled sera at 4°C. The precipitated immunoglobulins were pelleted by centrifuging at 37 000 g, redissolved in phosphate buffered saline, pH 7.4 (PBS)

Abbreviations used are: TLCK-porcine trypsin, porcine trypsin treated with N-α-tosyl-L-lysine chloromethyl ketone at neutral pH; DIP-trypsin and DIP-chymotrypsin, trypsin or chymotrypsin treated with diisopropylfluorophosphatate at neutral pH; PBS, phosphate buffered saline prepared from NaCl, 7.1 g; KCl, 0.2 g; Na₂HPO₄·7H₂O, 2.17 g; KH₂PO₄, 0.2 g, made to 1 liter and adjusted to pH 7.4; PBS-TS, PBS to which has been added sucrose, 68.4 g, and thimerosal, 0.1 g per liter; IgG, immunoglobulin G; EG, ethylene glycol; Hanks’ BSS, Hanks balanced salts solution (Hanks & Wallace, 1949).
and dialyzed against PBS at 4°C overnight. A column of Protein A/Sepharose CL-4B (1.1 × 4 cm) was prepared and equilibrated with PBS. The immunoglobulin fraction from 10 ml of serum was passed through the column and the column was washed until the baseline was reached. The IgG fraction was released with 1 M-acetic acid and the tubes containing the immunoglobulins were immediately neutralized with ammonium hydroxide, dialyzed against PBS and concentrated in an Amicon Cell to 24–40 mg/ml. The antibodies were checked for titers and cross-reactivity on Ouchterlony plates. The antibody against *Ascaris* trypsin inhibitor 1 was used without further purification. The IgG fraction containing antibody against TLCK-porcine trypsin was purified further. Immobilized porcine trypsin (trypsin/Sepharose CL-4B) was treated with diisopropylfluorophosphate at neutral pH to make DIP-trypsin/Sepharose CL-4B. After the DIP-trypsin/Sepharose CL-4B was washed exhaustively, it was mixed with the IgG fraction containing antibody to TLCK-porcine trypsin for 1.5 h and then transferred to a column (1.1 × 5 cm). When all of the protein that was not bound was washed from the column, the protease specific IgG was released from the gel with 100 mm-citrate/3 m-KSCN at pH 6.0 and 4°C. The IgG fraction against porcine trypsin was dialyzed and concentrated by ultrafiltration.

**Preparation of tissues.** *Ascaris* worms were collected at the abattoir and kept at 35°C in a salt medium (Baldwin & Moyle, 1947) to which penicillin G (1 × 10⁶ units/liter), streptomycin (1 g/liter) and Fungizone (10 mg/liter) were added. At the laboratory they were washed exhaustively in saline, separated by sex and incubated for one additional hour in saline to which penicillin G, streptomycin and Fungizone (in the same amounts as above) were added. The worms were then gently stretched to their maximum length, wrapped in aluminum foil and immersed in liquid nitrogen for 30 min. The frozen nematodes were placed on a bed of crushed dry ice and cryofractured into five equal segments with a cold, single-edged razor blade. The second and third segments were then subdivided into 2 mm high cylinders. The cylinders were dehydrated in a graded series of EG and Hanks BSS (Hanks & Wallace, 1949).³

The dehydrating solutions are removed by a graded series of EG and the plastic monomer, JB-4A, containing catalyst.³ The infiltrated tissue was then imbedded in Beem capsules in complete JB-4 medium and was allowed to harden for 48 h. The plastic molds were removed and the blocks were dehydrated over silica gel in an atmosphere of N₂ for 24 h at room temperature. The fully polymerized blocks were rehydrated for 24 h in a 20% constant humidity chamber. The blocks of imbedded tissue were cut with glass knives into cross sections approximately 2 μ thick on an ultramicrotome (Pyramitome-LKB Instruments).

³ The graded series used for dehydration was: 10% EG/90% Hanks BSS (30 min); 65% EG/35% Hanks BSS (10 min); 100% EG (10 min). The graded series used for infiltration was: 50% EG/50% JB-4A* (10 min); 30% EG/70% JB-4A* (10 min); 10% EG/90% JB-4A* (10 min); 100% JB-4A* (24–48 h). JB-4A* is JB-4A containing the catalyst. Each step in both series was performed at 4°C.
Double antibody overlay procedure. The cross sections of tissue were expanded by floating them on the surface of a 37°C water bath. They were then attached to ultraclean glass microscope slides and placed in a 37°C incubator overnight to increase adhesion between the plastic matrix and the glass surface. The sections were then washed by immersion in PBS-TS for 10 min at 4°C. (Each washing reported from here on was done by immersion for 10 min at 4°C). Excess PBS-TS was allowed to drain off the slides and each cross-section of tissue was covered with 20 μl of Pronase E (0.5 mg/ml) and incubated at 37°C for 1 h. This and all subsequent incubations were performed in a moist chamber. The slides were again washed six times by immersion in PBS-TS and then each cross-section was overlaid with 0.1% phenylhydrazine solution at pH 7.0 for 1.5 h. Following six washes in PBS-TS, 3% hydrogen peroxide was applied to each cross-section for 10 min at 37°C. Excess hydrogen peroxide was washed off and replaced with 10% aqueous solution of ovalbumin for 1 h at 37°C. After the excess ovalbumin was washed away with PBS-TS, the tissue was ready for incubation with the primary antibody. As a control for the primary antibody, the antibody was treated with a small excess of the antigen for 24 h at 4°C. The precipitated antigen/antibody complex was removed by centrifuging at 37 000 g for 20 min. Enough Triton X-100 was added to the solutions of the primary antibody and the antigen precipitated control to bring its concentration to 0.3%. Cross-sections of tissue were incubated for 3 h with the antibody or the antigen precipitated antibody. This was followed by a second application of the antibody and the antibody control and an additional 3 h incubation. Following five washes with PBS-TS containing 0.3% Triton X-100, a sixth wash was performed with the Triton X-100 omitted. The cross-sections were incubated twice, each time for 3 h, with goat antirabbit IgG-horseradish peroxidase conjugate. The cross-sections were then immersed overnight in a wash of PBS-TS containing 0.3% Triton X-100. Following several more washes the next day, the antigens were visualized enzymatically. The substrate solution contained 3,3'-diaminobenzidine·4 HCl (10 mg/50 ml), imidazole (0.1 M), hydrogen peroxide (0.0005%), all in 0.05 M-Tris buffer adjusted to pH 7.6. The cross-sections were overlaid and incubated for 30 min at room temperature with substrate solution. Following six washes in distilled water, each section was exposed to two drops of 2% aqueous osmium tetroxide at room temperature for 10 min. The sections were put through a final wash, mounted with Permount and recorded by photomicroscopy using an Olympus PM-10-A automatic exposure photomicrographic camera system and Kodak TRI-X pan film.

RESULTS AND DISCUSSION

Reactive sites of trypsin inhibitors

The treatment performed on each of the Ascaris trypsin inhibitors is shown in Scheme 1. The trypsin inhibitor activities present after each step of the procedure are shown in Table 1. Two other trypsin inhibitors were utilized as controls: Trasylol,
which has a lysine residue at its reactive site (Kress & Laskowski, Sr., 1968) and chicken ovomucoid, which has an arginine residue at its reactive site (Ozawa & Laskowski, Jr., 1966). After maleic anhydride treatment, Trasylol was inactive and chicken ovomucoid was fully active, as expected. The trypsin inhibiting activity of Trasylol was regenerated by removal of the maleyl groups with perchloric acid. Treatment with butanedione abolished the trypsin inhibiting activity of chicken ovomucoid but did not affect the activity of the already inactive polymaleylated Trasylol. The key point in this procedure is that treatment with perchloric acid to remove maleyl groups restored the trypsin inhibiting activity of the polymaleylated butanedione treated Trasylol but not that of the polymaleylated, butanedione treated, chicken ovomucoid.

Table 1

| Trypsin inhibitor | Initial activity (units/E) | maleylation | Percent of initial activity after: | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | perchloric acid treatment of polymaleylated inhibitor (min) | butanedione treatment of polymaleylated inhibitor (min) | perchloric acid treatment of polymaleylated, butanedione treated inhibitor (min) | | | | | |
| | | | 60 | 120 | 240 | 30 | 60 | 60 | 120 | 240 |
| Inhibitor 1 | 2320 | 5 | ND | ND | 100 | <1 | <1 | <1 | <1 | <1 |
| 2 | 2220 | 9 | 42 | 70 | 86 | ND | <2 | <2 | <2 | <2 |
| 3 | 2190 | 46 | 59 | 81 | 87 | ND | <2 | <2 | <2 | <2 |
| Trasylol | 2190 | 4 | 60 | 93 | ND | <1 | <1 | 54 | 71 | 95 |
| Ovomucoid | 909 | 100 | 100 | 86 | 84 | ND | 5 | <2 | <2 | <2 |

ND = Not determined

Ascaris trypsin inhibitors 1 and 2 each lost activity after maleylation and regained activity after treatment with acid. Similar results led Peanasky et al. (1974) to conclude that their peak I trypsin inhibitor (Ascaris trypsin inhibitor 1) contained a lysine residue at its reactive site. Peak II trypsin inhibitor (Ascaris trypsin inhibitor 3) remained active after maleylation and when activity disappeared after treatment with butanedione reagent, an arginine residue was reported at its reactive site (Peanasky et al., 1974). However, Table 1 shows that perchloric acid treatment of polymaleylated butanedione treated Ascaris trypsin inhibitors 1 and 2 as well as 3 failed to regenerate their trypsin inhibiting activities. This suggests that an arginine residue is the reactive P, site residue in each of the Ascaris trypsin inhibitors and is inconsistent with the presence of a lysine residue at the reactive site of Ascaris trypsin inhibitor 1. Since two lines of evidence now support an arginine residue at the reactive P, site in Ascaris trypsin inhibitor 1, this laboratory withdraws its earlier conclusion (Peanasky et al., 1974) that a lysine residue is at the reactive P, site of that inhibitor.
If an arginine residue does reside at the P₁ site of *Ascaris* trypsin inhibitor 1, then why did this inhibitor lose activity after maleylation of the lysine residues? We considered that a lysine residue in the inhibitor though not in the P₁ site, is in the contact zone when enzyme and inhibitor form a complex. Modification with maleic anhydride of such a residue leads to spurious results. In numerous experiments, we formed a trypsin and inhibitor complex and treated it with reagents such as acetic anhydride and trinitrobenzene sulfonic acid, which modify the ε-amino group of lysine residues. The strategy was that a lysine residue in the contact zone would be protected from reacting with the modifying reagents and could be located by dissociating the complex and examining tryptic fragments of the inhibitor after peptide fingerprinting. We did not succeed in this strategy, as the complex always dissociated during modification. We then wondered whether there was anything unusual about the lysine content of these *Ascaris* trypsin inhibitors, as compared to other trypsin inhibitors. A review and analysis of 62 different trypsin inhibitors⁴ from various sources revealed that the average content of lysine was 6.3 ± 2.7% (mean ± S.D.). Table 2 lists the lysine content of five of the 62 trypsin inhibitors. The *Ascaris* trypsin inhibitors by comparison have an unusually high lysine content. The percent lysine in *Ascaris* trypsin inhibitors 1 and 2 is more than two standard deviations above the mean; the percent lysine in *Ascaris* trypsin inhibitor 3 is within the two standard deviation limit.

**Table 2**

*Lysine content and active site of selected trypsin inhibitors*

These trypsin inhibitors are representative of the sample of 62 trypsin inhibitors. They contain the lowest, the average ± one standard deviation, and the highest concentrations of lysine residues in trypsin inhibitors. %Lys is expressed as [total residue weight of lysine/molecular weight of the protein]×100.

<table>
<thead>
<tr>
<th>Inhibitor source</th>
<th>Molecular weight</th>
<th>%Lys</th>
<th>Reactive site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snail mucus⁴</td>
<td>6463</td>
<td>2.0</td>
<td>Arg</td>
</tr>
<tr>
<td>Leeches⁵</td>
<td>4830</td>
<td>5.3</td>
<td>Lys</td>
</tr>
<tr>
<td>Garden bean⁶</td>
<td>8090</td>
<td>6.3</td>
<td>Lys</td>
</tr>
<tr>
<td>Seminal plasma⁴</td>
<td>7600</td>
<td>8.4</td>
<td>Arg</td>
</tr>
<tr>
<td><em>Ascaris</em> inhibitor 3</td>
<td>7280</td>
<td>10.6</td>
<td>Arg</td>
</tr>
<tr>
<td>2</td>
<td>6330</td>
<td>12.1</td>
<td>Arg</td>
</tr>
<tr>
<td>1</td>
<td>6800</td>
<td>13.2</td>
<td>Arg</td>
</tr>
<tr>
<td>Cuttle fish⁶</td>
<td>6820</td>
<td>15.0</td>
<td>Lys</td>
</tr>
</tbody>
</table>

⁴ Dietl & Tschesche (1974).
⁵ Fritz & Krejci (1976).
⁷ Schiessler et al. (1974).

* The trypsin inhibitors selected are those for which the amino acid composition was known and so far as possible the reactive site P₁ residue has been determined.
Fig. 1. Cross reactivity of the antibodies developed against *Ascaris* trypsin inhibitor I and TLCK-porcine trypsin: In series I, antibody to *Ascaris* trypsin inhibitor I is in the center wells. The satellite wells contain antigens: *Ascaris* trypsin inhibitor I in wells A and C, DIP-trypsin in B and D, DIP-chymotrypsin in E and F, and *Ascaris* chymotrypsin inhibitor 5 in G and H. In series II, antibody to TLCK-porcine trypsin is in the center wells. The satellite wells contain antigens: DIP-trypsin in wells A and C, *Ascaris* trypsin inhibitor I in B and D, DIP-chymotrypsin in E and F and *Ascaris* chymotrypsin inhibitor 5 in wells G and H. The concentrations of the antigens are 100 μg/ml and 25 μg/ml. The antibody reacts only with the primary antigen.

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Only one other trypsin inhibitor of the 62 examined has such a remarkably high lysine content; a cuttle fish trypsin inhibitor has 15% lysine by weight (Tschesche & Rucker, 1973). Interestingly, a lysine residue was reported at its reactive site, using the method of Fritz et al. (1969).

The net charge of the *Ascaris* inhibitors is undoubtedly important in the maintenance of an active conformation. The neutralization of many positively charged amino groups, as with acetylation, or changing of a positive to a negative charge, as with maleylation or succinylation, significantly affects the structure of these proteins. As a result, the trypsin inhibitors modified by these procedures no longer interact successfully with trypsin and cannot inhibit it. Modification of the *Ascaris* trypsin inhibitors with an agent that preserves the positive charges, as with guanidination, does not alter the inhibitor's conformation significantly and does not affect their activity as trypsin inhibitors. From this we conclude that when most of the positive charge on a trypsin inhibitor molecule is contributed by its lysine residues, then the loss of trypsin inhibiting activity which is seen following maleylation may be due only to changes in the conformation of the molecules and not to the presence of a lysine residue at the P₁ site.

The accuracy of the Fritz et al. (1969) method for reactive site determination in trypsin inhibitors has never been questioned. However, it is now clear that this procedure, under some conditions, can yield spurious results. This report describes a precaution that will guard against this problem, so that the method remains a reliable means for determination of the reactive P₁ site of trypsin inhibitors with a high lysine content.

*Localization of trypsin inhibitors and host trypsin in cross-sections of Ascaris*

Antibodies were developed in rabbits to *Ascaris* trypsin inhibitor 1 and to TLCK-porcine trypsin. The antibody to *Ascaris* trypsin inhibitor 1 did not cross react with DIP-trypsin, DIP-chymotrypsin or *Ascaris* chymotrypsin inhibitor 5; the antibody to TLCK-porcine trypsin did not cross react with *Ascaris* trypsin inhibitor 1, DIP-chymotrypsin or *Ascaris* chymotrypsin inhibitor 5 (Fig. 1). The antibodies reacted only with their respective antigens: *Ascaris* trypsin inhibitor 1 and DIP-porcine trypsin.

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**Fig. 2.** Localization of *Ascaris* trypsin inhibitor 1 and porcine trypsin in *Ascaris*. Cross-sections of tissues were incubated with antibody to *Ascaris* trypsin inhibitor 1 and antibody to TLCK-porcine trypsin developed in rabbits. Antirabbit IgG developed in goats and conjugated to horseradish peroxidase was used to localize the antigens. Immunoperoxidase staining of the primary antigen is shown as dark deposits. In frames 1 to 4 (controls) the primary antibody was immunabsorbed TLCK-porcine trypsin. In frames la to 4a, the primary antibody was developed against *Ascaris* trypsin inhibitor 1 and in frames 1b to 4b, the primary antibody was developed against TLCK-porcine trypsin. Series 1 is body wall muscle, series 2 is intestine (L indicates the lumen of the intestine), series 3 is eggs and series 4 is sperm. Camera magnification is 400 X. Immunoperoxidase staining for *Ascaris* trypsin inhibitor 1 and for porcine trypsin is in the same place in each tissue.
Immunoperoxidase staining shows that trypsin inhibitor is associated with body muscle, intestine and is in the eggs and sperm of Ascaris (Fig. 2, frames 1a to 4a). Porcine trypsin is associated with the same tissues and in the same places, the body muscle, intestine and in the eggs and sperm (Fig. 2, frames 1b to 4b). This is the first demonstration that a host protein has been able to enter the parasite, Ascaris. Direct analysis of whole homogenates of Ascaris or extracts of Ascaris tissues has never revealed the presence of trypsin because free trypsin inhibitor has always been found in every homogenate or extract. The presumptive conclusion is that the host enzyme is present as the trypsin-inhibitor complex.

Examination of the luminal side of the intestinal tissue (Fig. 2, frames 2a and 2b) shows a ridge of dense particles at the brush border for both trypsin inhibitor Ascaris by forming a complex in the brush border of the worm's intestine. In a few experiments, papain, a plant protease that can digest the worm, was incubated in a mixed salt medium with live Ascaris. Digestion of the worm always began at the intestine; it was the first organ or tissue to show signs of disintegration.

Some years ago, it was speculated that since the inhibitors are not secreted by the worm and since host enzyme disappears from the environment only in the presence of a live worm (Peanasky & Abu-Erreish, 1971; Juhász & Matskáš, 1979) the parasite may take up host proteases as an inactive complex with the inhibitors (Peanasky et al., 1974). This is the first demonstration that this may in fact be true. What role the inhibitor-trypsin complex plays in survival of the worm is not clear. We are impressed with the dense accumulation of particles associated with the eggs. Experiments are underway to follow the inhibitor and host enzyme in developing eggs.

We thank Joyce Koenig and Betty Hogan for technical assistance, and Dr. Daniel Neufeld and the Anatomy Department for use of the ultramicrotome and the photomicrographic system.

Some of the data are taken from dissertations submitted by R.B.G. and M.R.M. to the Graduate Faculty of the University of South Dakota in partial fulfillment of requirements for their Master of Arts degrees.

It is an honor for R.J.P. to contribute to this memorial to M. Laskowski, Sr. He was my mentor, ultimate critic, and lifetime confessor.

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**INHIBITORY TRYSYNOWE ASCARIS: REAKTYWNE WIĄZANIE PEPTYDOWE P_{1} INHIBITORA (SPROSTOWANIE) ORAZ LOKALIZACJA INHIBITORÓW I TRYSYNY GOSPODARZA W PRZEKROJACH POPRZECZNYCH ASCARIS**

**Streszczenie**

Inhibitory trysyny 1, 2, 3 u Ascaris należą do rodziny inhibitorów zawierających arginę w reaktywnym wiązaniu peptydowym P_{1}. Jest to sprostowanie wcześniejszych danych, według których w tym miejscu inhibitora lokalizowano lizynę (Peansky et al., 1974, Bayer Symposium V: Proteinase Inhibitors, str. 649 - 666). Dane nasze wskazują również, że przy stosowaniu modyfikacji reszt wg Fritz i wsp. (Z. Physiol. Chem., 1969, 350, 933 - 944) istnieje możliwość mylnej interpretacji wyników, gdy zawartość lizyny wynosi ponad 12% masy cząsteczkowej inhibitory trysyny nowego. W związku z tym sugerujemy stosowanie bezwodnika maleinowego najpierw, a następnie butanedionu i usunięcie grup maleilowych w środowisku kwasnym.

Reakcja barwna z immunoperoksydazą w przekrojach poprzecznych Askaris wykazuje, że przeciwciała w stosunku do inhibitory trysynowego 1 Ascaris wiąże się z mięśnikiem, jelitami, jajami i spermą. Przeciwciała dla TLCK-trysyny świniowej wiąże się z tym samym tkankami i w tych samych miejscach co przeciwciała inhibitory trysyny 1 Ascaris. Jest to pierwsza obserwacja, że białko wytworzone w ciele gospodarza znajduje się w pasyście — Ascaris. Analiza homogenatów i wyciągów z poszczególnych tkank wykazało nadmiar wolnego inhibitory i brak czynnej trysyny. Bialko gospodarza znajduje się wewnątrz pasożyta prawdopodobnie jako kompleks trysyna-inhibitor.

Received 31 January, 1983.