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Trypsin inhibitors in mesogastropod foot-muscle extracts

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Abstract

Foot-muscle extract of *Semisulcospira libertina* was tested for the presence or absence of a number of enzymes. One of these tests was for trypsin-like esterase. It was found that this particular esterase was not present and that the extract had potent trypsin inhibitory activity. The presence or absence of inhibitors was determined for the following Asian species: *Sinotaia histrica, Oncomelania hupensis nosophora, O. minima, Semisulcospira libertina, S. "trachea," S. niponica, Melanoides tuberculatus, Brotia costula episcopalis.*

We found that all but *B. c. episcopalis, S. niponica* and *S. histrica* had potent inhibitors; i.e., the ratio of protein (μg) in the snail extract to the μg trypsin inhibited was over 160 for these three species but less than 100 for the other species. The distribution of the inhibitors did not appear related to phylogenetic relationships or to the ability of the taxa to transmit human helminths such as *Schistosoma japonicum* or *Paragonimus westermani.*

Ammonium sulfate fractionation of foot-muscle extract of *S. libertina* indicated that there was no separation of a heterogeneous inhibitor component. The greatest activity occurred with the 50% ammonium sulfate cut. Gel filtration (Sephadex G-50) of the extract yielded three important inhibitor components. By comparison with standards, the inhibitor components were found to have molecular weights of 15,200, 11,800 and 10,700. Kinetic studies using the Lineweaver-Burk plot indicated that the inhibitors were non-competitive. Studies of extracts of embryo and oviduct as well as hemolymph indicated that the inhibitors were ubiquitous in the snail and were most likely blood components.

Introduction

Semisulcospira libertina in Japan is under intensive study because of its role as first intermediate host of the human lung fluke, *Paragonimus westermani.* The genus *Semisulcospira* in Japan is comprised of two species groups (Burch & Davis, 1967; Burch, 1968; Davis, 1968a, 1969a,b; Radke & Davis, 1969). These are the *S. libertina* and *S. niponica* groups, where cytologically the haploid chromosome number (n) is 18 to 20 in the former and 7 to 14 in the latter. Other definitive traits are numbers of embryos brooded by the female and basal cords on the adult shell.

Studies were initiated to compare electrophoretic profiles of proteins from foot-muscle extracts, and antigen-antibody systems of *S. libertina* and *S. niponica* in order to assess the degree of genetic relationship between these species. Such a comparative study in the Hydrobiidae was previously reported (Davis, 1968b). Initial results failed to indicate that there were unique profiles or antigen-antibody systems which could be used for species discrimination. While the above mentioned studies were continued, it was decided to initiate investigations of greater refinement in order to determine if the two species were truly indistinguishable in terms of other biochemical properties of foot-muscle

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extract in spite of vast differences in cytology and external phenotype. Accordingly, extracts (freshly prepared and lyophilized) were tested for the presence and activity of a number of enzymes. One of these tests was to determine the presence or absence of trypsin-like esterase.

We soon determined that extracts of *Semisulcospira libertina* did not have a trypsin-like esterase but, on the contrary had potent trypsin inhibitory properties. The purpose of this paper is to present data on: 1) the presence or absence of inhibitors in extracts from seven taxa of mesogastropods; 2) inhibitory properties of extracts from body tissues or components other than foot muscle; 3) isolation of the inhibitor(s) by ammonium sulfate fractionation; 4) characterization of the inhibitor(s) from *S. libertina* foot-muscle extract in terms of molecular weight(s) and selected kinetic properties; 5) the correlation between the possession of trypsin inhibitors and the ability to transmit human helminths.

Materials and Methods

1. Source of snails—Species of snails utilized in this study are listed by systematic arrangement in Table 1 along with their localities and date of extract preparation and lyophilization. Four populations of *Semisulcospira libertina* are compared in Table 3. *Oncomelania hupensis nosophora* transmits the Japanese strain of *Schistosoma japonicum*. The parasite does not mature in *O. minima*. *S. libertina* from Nahari was found infected with *P. westermani*. *Semisulcospira niponica* has not been found infected with *P. westermani*. *Brotia costula episcopalis* was from a population transmitting *Paragonimus* sp. in Malaya. *Melanoides* does not transmit *Paragonimus*.

2. Preparation of extract—Foot-muscle extracts were prepared (Davis, 1968a,b; 1969a) by homogenizing 300 mg of blotted wet weight of foot muscle (and some columellar muscle, the epidermis removed) in 2.0 ml of Carriker's (1946) saline. The tissue was first broken up using a motor-driven tissue grinder with teflon-tipped pestle; then this homogenate was transferred to a 5 ml microcup and further homogenized, using a microblade, at 50,000 rpm (Sorvall microhomogenizer) for 60 seconds. All operations were carried out at 2-3°C attained by means of ice baths. The homogenate was centrifuged at 3,000 rpm (1,500×g) for five minutes and the supernatant decanted; the sediment alone was centrifuged again at 4,000 rpm (2,600×g) for five minutes. The supernatants were combined.

The protein content of the extract was determined using the Biuret reaction (fide Kabat & Mayer, 1961). A standard curve was made using crystalline bovine albumin (clinical pathology standard). The spectrophotometer used was a Bausch and Lomb Spectronic-20. The extract was lyophilized in 1 ml units and stored at -20°C until used.

Prior to an experiment the extract was reconstituted with distilled water (except where noted otherwise), centrifuged at 1,000 rpm (160×g) for 10 minutes, and the protein conient was redetermined using the Folin Reagent Test (Daughaday *et al.*, 1952). The redetermination of protein was done to find out if protein was lost upon reconstitution of the lyophilized sample due to denaturization and removal by centrifugation.

For the preparation of embryo extracts, the entire female pallial brood chamber was cut out using iridectomy scissors. Excess tissue was trimmed away and that section of the pallial oviduct containing eggs and embryos with shells was homogenized (300 mg blotted wet weight per 2.5 ml Carriker's saline). Extracts were prepared as was foot-muscle extract.

Hemolymph from *S. libertina* was collected by carefully cracking off the apical end of the shell and allowing the blue hemolymph to flow into a test tube. The pooled hemolymph was centrifuged at 4,000 rpm(2,600×g) for five minutes, the protein content was determined using the Folin test and the remaining hemolymph lyophilized in units of 0.5 ml per ampule. Lyophilized hemolymph was reconstituted with 0.4% NaCl.

3. Ammonium sulfate fractionation—Two units of extract were reconstituted in 15 ml distilled water. Ammonium sulfate was slowly added to the reconsituted extract over a 15-minute period following the schedule in Table 1 of Green & Hughes (1955). After each measured amount of salt was added, the precipitate was centrifuged down, the supernatant decanted and the residue dissolved in 1 ml of tris[3] buffer (0.1 M, pH 7.6).

4. Trypsin inhibitor assay—The method described for determining trypsin esterase activity in human serum and plasma (Rutkowski, 1966) was modified to include a trypsin inhibitor measurement. The assay was performed in a total volume of 3.2 ml containing : (1) 0.6 mg α N benzoyl-L-arginine ethyl ester hydrochloride (BAEE) as the substrate, (2) 2.0 μg purified trypsin (3) a volume of 100 μl of extract or column eluate (or dilutions therefrom), and (4) 0.12 M tris buffer, pH 8.0. A trypsin control was simultaneously performed by omitting (3). A reagent blank was always included omitting (2) and (3) and an extract or eluate control by omitting (2).

The assay was performed at 37°C in square 1.0 cm silica quartz spectrophotometric cuvettes using the Gilford Model 2400 recording spectrophotometer. The increase in absorbance per minute (ΔA/min) at 254 mμ was calculated from the absorbance curves recorded for a minimum of 10 minutes reaction time. After taking into account the influence of the blank and extract or eluate control, the percent inhibition was calculated using the trypsin control as an index of total (completely uninhibited) activity.

5. Kinetic study—The basic assay for inhibitor activity was altered by using substrate (BAEE) concentrations from 0.3 to 0.7 mg. The inhibitor concentration was varied for each substrate concentration used. The residue obtained after attaining the 50% ammonium sulfate saturation level was redissolved as indicated under method 3 above and used as the inhibitor source. The uninhibited trypsin activity was converted to μ moles of substrate hydrolyzed/min/mg of trypsin (Rutkowski, 1966). The reciprocal of the trypsin activity was plotted against the reciprocal of the substrate concentraction in order to determine the type of inhibition (Lineweaver & Burk, 1934).

6. Gel filtration—Initially, purified ovomucoid, soybean and lima bean trypsin inhibitors (Sigma Chemical Company, St. Louis, Missouri, U.S.A.) having known molecular weights (25,400, 21,500 and 16,200 respectively) were passed through a previously prepared Sephadex G-50 column using 1 ml of a 0.005% solution of each inhibitor. The column dimensions were 33×2.5 cm. A tris buffer (0.1, pH 8.0) was used as the eluting solution.

Two units of snail extract (12.4 mg) were dissolved in a minimum volume of water (1.5 ml) and passed through the column using the same buffer as above. Two ml fractions were collected at a flow rate of 1.0 ml per five minutes until a total collection of 180 ml was reached.

The fractions were tested for inhibitor activity using 100 μl aliquots in the basic assay (method 4 above). Absorbance by each fraction at 280 mμ was also recorded to indicate relative protein concentration.

An estimation of the molecular weights was obtained by first plotting the elution volumes of the standard inhibitors (ovomucoid, soybean and lima bean) against molecular weight (Andrews, 1964). The elution fraction of snail extract showing peak inhibitor activity were then plotted on the curve obtained with the standard inhibitors, thus enabling determination of the molecular weights of the unknowns.

Results

From eight to 58 percent of the protein was lost when lyophilized samples were reconsituted (average loss of 38%). Indication of the loss was the sediment evident after centrifugation.

Initial efforts to isolate the trypsin inhibitor in *S. libertina* foot-muscle extracts involv-

[3]tris=2-amino-2-hydroxymethyl-1, 3-propanediol

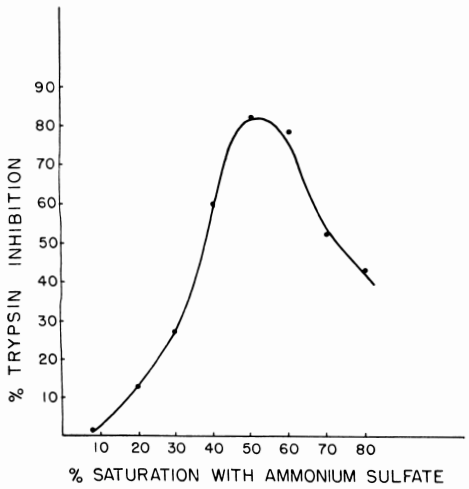

Figure 1. Ammonium sulfate fractionation of *Semisulcospira libertina* foot-muscle extract. Two units of extract (13.2 mg) were used in the experiment. Aliquots of 25 μl of each reconstituted fraction were used in the basic assay test for trypsin inhibition in order to obtain the % inhibition.

ed salt fractionation. The data (Figure 1) indicated a range of inhibitor activity which showed a maximum with the 50% ammonium sulfate cut. Only one inhibitor appeared to be present. Using the partially purified fraction showing peak activity, the kinetics were studied to determine the nature of the inhibition. Figure 2 portrays the results of this study using varying concentrations of substrate and inhibitor. From the curves obtained, using the reciprocal plot method of Lineweaver-Burk, it was evident that the inhibitor(s) was essentially noncompetitive since increase in substrate (decrease in 1/s) did not eliminate inhibitory effect as would be the case with a competitive inhibitor. Although some variation was observed, the curves were generally directed to a common point on the absissa (experimental Km of trypsin).

The gel filtration separation of trypsin inhibitor indicated several components showing activity in *S. libertina* foot-muscle extract. From the peaks of inhibitor activity as seen in Figure 3, essentially three important com-

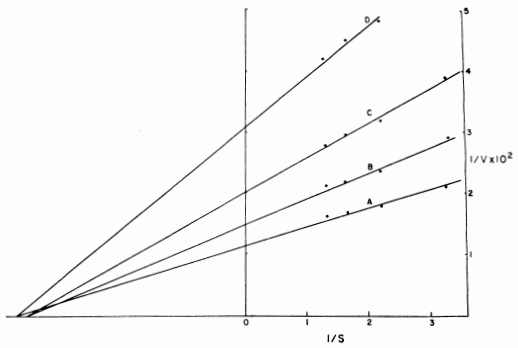

Figure 2. The effect of inhibitor from foot-muscle extract of *Semisulcospira libertina* on trypsin activity using varied substrate (BAEE) concentrations in the assay. The reciprocal plot method of Lineweaver and Burk was used. The inhibitor was contained in the fraction precipitated by 50% saturation with ammonium sulfate. The reconstituted precipitate contained 0.41 g protein protein per ml.

A—uninhibited reaction.

B—10 μl of reconstituted fraction in the reaction mixture.

C—20 μl of reconstituted fraction in the reaction mixture.

D—30 μl of reconstituted fraction in the reaction mixture.

V—μ moles substrate hydrolyzed per minute per mg trypsin.

S—mg substrate (BAEE) in the reaction.

ponents were observed. The peaks obtained immediately after surpassing the column void volume (60 ml) were considered to be associated with the very high total protein eluted and were not interpreted as specific trypsin inhibitors. The preponderance of inhibitor activity was observed to be in the first peak (Peak A, Figure 3) with lesser amounts of activity in two other peaks (Peaks B and C, Figure 3). These latter two peaks may have indicated partial molecular breakdown of the major component. Relative protein values (absorbance at 280 mμ) showed extremely low protein values in the fractions containing large amounts of inhibitor.

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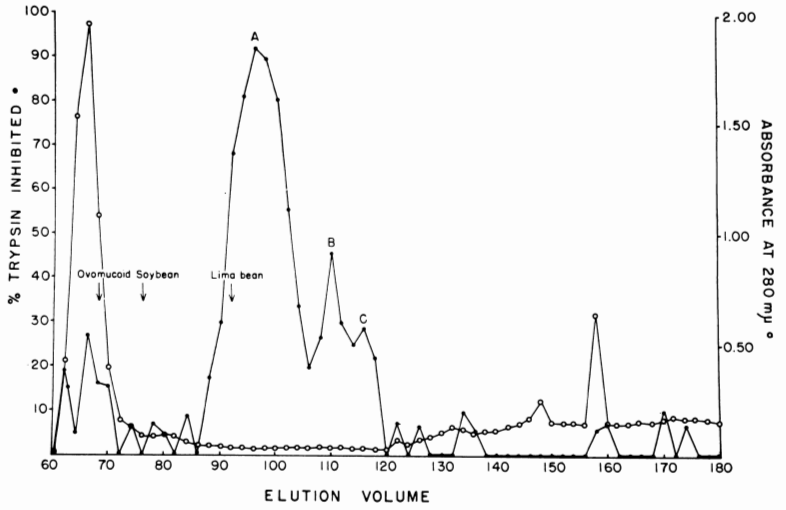

Figure 3. Trypsin inhibition property and protein concentration of fraction of foot-muscle extract of *Semisulcospira libertina* after separation of the extract using a Sephadex G-50 column. Two units of extract (12.4 mg protein) were reconstituted in 1.5 ml of water. After centrifugation the total supernatant was placed on the column. The elution volume of each standard inhibitor is indicated. Volumes with maximum inhibitor activities are indicated by letters A, B and C. No protein or inhibitor activity was detected below the 60 ml elution volume.

rates (Morris, 1948) yielded negative results for these fractions.

Figure 4 graphically portrays an estimation of the molecular weight of eluted fractions giving peak inhibitor activity. The elution volumes of known trypsin inhibitors (ovomucoid, soybean and lima bean) were used to prepare the curve. A satisfactory linear relationship was observed with the three standardizing inhibitors. The estimated molecular weight of the major component (Peak A) was 15,200; the other two components (Peaks B and C) were estimated as having molecular weights of 11,800 and 10,700.

The trypsin inhibitor assay was performed on various snail extracts. As shown in Table 2, taxa of *Oncomelania* have extremely potent inhibitors where the ratio of μg protein in the snail extract to μg trypsin inhibited was 5.9 and 8.9. Likewise, *Semisulcospira libertina*, *S. trachea* and *Melanoides tuberculatus* were characterized by potent inhibitors. *S. niponica* and *Brotia* had weak inhibitors while *Sinotaia* did not possess inhibitors, as evidenced by the great quantity of extract protein necessary to inhibit a μg of trypsin (1178.4)

When four population of *Semisulcospira libertina* were tested (repeating the test on *S. libertina* from Shimoda), the ratios of μg protein to μg trypsin inhibited ranged from 28.9 to 31.0 for populations in the Hakone-Izu area. The ratio for *S. libertina* from Shimoda was elevated by 11.3 when compared with results of the firft analysis. With this variation in mind it is evident that an order of magnitude of difference is present when the potency of inhibitor from *S. libertina* from Nahari is considered (ratio of 92.7).

When embryo extract and hemolymph from *S. libertina* were tested, it was evident that potent inhibitors were present in both, with those in hemolymph having about half the potency of inhibitors in the tissue extracts.

Discussion

Trypsin is a proteolytic enzyme (molecular weight, 24,000), an endopeptidase acting on : 1) CO-NH linkages, hydrolyzing the peptide bonds where the carbonyl group is from an L-arginine or L-lysine residue ; 2) ester linkages where the carbonyl group is for an L-arginine or L-lysine residue. As such, the synthetic compound benzoyl arginine ethyl ester is suitable as a substrate for trypsin hydrolysis. For a discussion on the hydrolysis of proteins and synthetic esters one should consult Hill (1965).

None of the gastropod extracts studied contained an enzyme exhibiting trypsin-like esterase activity. On the contrary, extracts from taxa of *Oncomelania* and *Semisulcospira* had potent trypsin inhibitors. The ratios given in Table 2 provide a basis for comparing inhibitor activites of the various extracts tested. The lower the ratio of concentration of inhibitor source to concentration of trypsin inhibited, the greater is the specific inhibitor activity. A wide range of ratios was obtained (5.85 to 1,178.4) : It is interesting to note that the commercially available purified trypsin inhibitors soybean, lima bean and ovomucoid gave ratios of 0.71, 0.89 and 0.77 respectively, using the described assay system. From the protein concentration and inhibitor studies with the column eluate fractions containing inhibitor activity Peaks A, B and C, approximate ratios of 1.2, 2.1 and 2.7 respectively were obtained. The ratios reported in Tables 2 and 3 were made using total protein in the muscle extracts, not protein of purified inhibitor. Thus, ratios ranging from 5.9 to 59.2 indicate relative powerful inhibition activity. Ratios of 100 and over obtained using total protein can be considered the result of using extract with weak inhibition properties while ratios over 1,000 indicate no inhibitor activity. More detailed isolation studies than those reported here are required for more accurate data on purified inhibitor.

The classical reciprocal plot method of

Table 1 Systematic arrangement of the gastropods (Prosoybranchia : Mesogastropoda) utilized in these studies, the locality where they were collected and the dates of extract preparation and lyophilization

Systematic list	Locality and date
Super-family Architaenioglossa	
family Viviparidae	
Sinotaia histrica (Gould, 1859)	Japan, Honshu, Saitama Pref., Yagyu Town, 12 September 1968
Super-family Rissoacea	
family Hydrobiidae	
Oncomelania hupensis nosophora (Robson, 1915)	Japan, Honshu, Yamanashi Pref, Kofu Valley, 28 April 1967
O. minima (Bartsch, 1936)	Japan, Honshu, Niigata Pref., Sado Is., 29 June 1968
Super-family Cerithiacea	
family Pleuroceridae	
Semisulcospira libertina (Gould, (1859)	Japan, Honshu, Shizuoka Pref., Shimoda Town, 1 June 1969
S. niponica (Smith, 1876)	Japan, Honshu, Shiga Pref., Otsu City, Lake Biwa, 3 August 1967
family Thiaridae	
Melanoides tuberculatus (Müller, 1774)	Japan, Okinawa, Chinen-son, Yamozato, 1 April 1968
Brotia costula episcopalis (Lea, 1850)	Malaysia, W. Malaysia, Selangor, 31 May 1969

Table 2 Snail taxa ranked by decreasing potency of trypsin inhibitors. The protein content (mg/ml) of the extract both prior to lyophilization and after centrifugation prior to testing the inhibition phenomena is given

Snail taxa	Protein prior to lyophilization	Protein prior to tests	Protein* / inhibition
Oncomelania hupensis nosophora	6.9	2.9	5.9
O. minima	4.6	2.7	8.9
Melanoides tuberculatus	6.9	4.7	21.3
Semisulcospira libertina			
-foot-	4.7	3.1	27.7
-embryo-	2.8	1.7	26.2
-hemolymph-	10.5	9.3	59.2
Brotia costula episcopalis	4.5	3.3	161.2
S. niponica	5.4	3.6	202.9
Sinotaia histrica	4.5	2.9	1178.4

* $\frac{\mu g \text{ protein in extract}}{\mu g \text{ trypsin inhibited}}$

Table 3 The potency of trypsin inhibitors in different populations of *Semisulcospira libertina* from Japan

Locality and date of lyophilization	Protein (mg/ml) prior to lyophilization	Protein (mg/ml) prior to tests	Protein* / inhibition
Honshu, Izu Peninsula, Shizuoka Pref., Shimoda, June, 1968	6.2	3.8	39.0
Honshu, Izu Peninsula, Shizuoka Pref., Iwashina, December, 1969	6.4	4.6	35.3
Honshu, Kanagawa Pref., Hakone Mts., Ashino Lake (synonym=*S. trachea*), 9 May 1967	5.2	2.5	28.9
Shikoku, Kochi Pref., Nahari, 11 May, 1968	6.6	4.1	92.7

* $\frac{\mu g \text{ protein in extract}}{\mu g \text{ trypsin inhibited}}$

Lineweaver-Burk was used to determine the type of inhibtion. If the inhibitor was of a competitive type (competition of inhibitor and enzyme for the substrate), the amount of inhibition would decrease as more substrate (lower 1/s) is added. As substrate concentration approaches infinity (1/s approaches zero), inhibitor effect is completely obviated. This would mean that assays performed at infinite substrate concentration would have the same activity (or 1/v) no matter what concentration of inhibitor was present. All linear reciprocal plots would thus meet at a point on the vertical axis where 1/s is zero or substrate concentration is at infinity. In the case of noncompetitive inhibition the addition of more substrate does not have an effect on the inhibition. This is probably due to a stoichiometric binding of the inhibitor to the active site of the enzyme. The linear plots would therefore not meet at the vertical axis since even an infinite concentration of enzyme would not relieve the inhibition. In the case of non-competitive inhibition, the linear plots meet at a common point on the base line (1/v=0) at this point 1/s=1/Km where Km

is the experimental Michaelis constant of the enzyme (Dixon & Webb, 1968).

The loss of protein due to lyophilization is attributed to denaturization and clumping of particles when the extracts were reconstituted in distilled water. This is due, in part, to the relatively gentle centrifugation (1500× g) in the initial preparation of extract which leaves particles in suspension. The procedure of determining the protein content prior to lyophilization was established to ascertain the protein content of fresh extract and to determine the amount of protein to be lyophilized in the ampules.

Trypsin inhibitors are widespread in nature : lung tissue, blood, human and bovine colostrum (Fruton & Simmonds, 1958) ; wholewheat flour (Shyamala & Lyman, 1964) ; several plants (Pusztai, 1967) ; egg white proteins from some birds (Nakamura *et al.*, 1966) ; and others reviewed by Laskowski & Laskowski (1954).

The only known report on an invertebrate trypsin inhibitor which also inhibits chymotrypsin was that of Rhodes *et al.* (1963), in which a body wall inhibitor (MW 4,650) and a perienteric fluid inhibitor (MW 7,100) were detected from *Ascaris suum*. Both inhibitors form stable complexes with trypsin in a 1 : 1 molecular ratio indicating non-competitive inhibition. An ovoinhibitor (not to be confused with ovomucoid) has been characterized by Tomimatsu *et al.* (1966) after isolation from chicken egg white. The inhibitor (MW 46,500) combines with trypsin in the ratio of 2 : 1. Greene *et al.* (1960) reported a trypsin inhibitor in bovine pancreatic juice (MW 6,155) which forms a very tightly bound complex with trypsin in a 1 : 1 ratio. An α_1 globulin which non-competitively inhibits plasmin, trypsin, chymotrypsin and thrombin in human serum has been studied by Rimon *et al.* (1966). The inhibitor (MW 47,000) forms a stoichiometric complex with trypsin. A proteolytic inhibitor with anticoagulant activity has been separated from human urine and plasma by Shulman (1955). An irreversible combination of this inhibitor (MW 16,700) with trypsin was reported.

The variability of potency of inhibitors, as determined by the ratio of protein to trypsin inhibited, within a species was great, ranging from 27.7 to 92.7 for populations of *S. libertina*. The Nahari population was exceptional due to the difference in ratio compared with ratios for populations from the Izu-Hakone region. Variation within one population tested at different times was about 11 μg protein per μg trypsin inhibited.

The difference between two species of the same genus (in the case of *Semisulcospira*) was very much greater than the difference between populations of the same species. *S. niponica* differed greatly from *S. libertina* as evidenced by ratio of extract protein to inhibited trypsin (209.9 in the former and 27.7 to 92.7 in the latter). It is important to note that, to date, this is the first biochemical evidence indicating a distinct genetic difference between these species which correlates with phenotypic differences of adult and embryo shell sculpture, other embryo shell characters and cytology. Electrophoretic and immunological comparisons between these species with foot-muscle extract have, to date, yielded no species-specific systems (Davis, unpublished).

Extracts of foot muscle and embryo as well as hemolymph have potent inhibitors which indicates that the molecular species of the inhibitors are widespread throughout the body of the snail and not localized. It is likely that the inhibitors are a blood constituent. The fact that the hemolymph (blood) had half the inhibitor potency of foot or embryo extracts is probably related to great amount of protein in blood relative to protein in the tissue extract (Table 2). Remember that the ratio in Table 2, column 4, is a result of using μg total protein in the extract or blood, not purified inhibitor and as such it is likely that much of the protein in the blood is not involved in inhibiting trypsin (remeber the low concentration of protein eliciting great inhibition of trypsin shown in Figure 3). Further investigations on the inhibitor(s) in hemolymph are planned. As

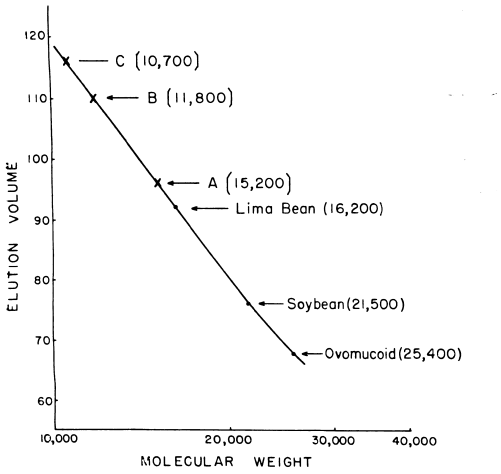

Figure 4. Estimation of molecular weights of trypsin inhibitors in foot-muscle extract from *Semisulcospira liberitina*. The curve was obtained by plotting the elution volumes (from G-50 column) containing the standard trypsin inhibitors of known molecular weight (parentheses). The elution volumes having peak inhibitor activity in Figure 3 were plotted and the molecular weights determined (brackets).

total proteins in hemolymph just prior to assay (after reconstitution of lyophilized material) are three to six times as much as those in tissue extract (Table 2, column 4), it is expected that partially purified hemolymph inhibitor(s) will be more potent than those from tissue extract.

There is apparently no correlation between the possession of trypsin inhibitors and the ability of transmit human helminths. *Oncomelania minima* will not transmit *Schistosoma japonicum* (Japanese strain), while *O. h. nosophora* is the natural first intermediate host. Both taxa have potent inhibitors. Both *Semisulcospira libertina* and *Brotia costula episcopalis* transmit *Paragonimus*. The inhibitors is potent in the former, very weak in the latter.

Acknowledgements

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中腹足目貝類の脚部筋肉抽出液に見られる trypsin inhibitors

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カワニナ (*Semisulcospira libertina*) の脚部筋肉抽出液を用いて，多数の酵素についてその有無を検討した．その内の 1 つが trypsin-like esterase である．この trypsin-like esterase は検出されなかつたが，脚部筋肉抽出液が強い trypsin 抑制作用を示すことが判明した．そこでこの trypsin inhibitors の有無をアジアに分布する中腹足目貝の以下の種類について検討した．すなわち用いた貝の種類は *Sinotai histrica, Oncomelania hupensis nosphora, O. minima, Semisulcospira libertina, S. trachea, S. niponica, Melanoides tuberculatus* および *Brotia costula episcopalis* である．

この結果，*B. c. episcopalis, S. niponica* および *S. histrica* の種を除く総ての貝に trypsin inhibitors を認めた．すなわち抑制された μg trypsin に対する抽出液中の蛋白質 (μg) の比率は上記の 3 種の貝では 160 以上であり，他の貝では 100 以下であつた．貝におけるこの trypsin inhibitors 存在と貝の系統発生学上の類縁関係にも，また inhibitors の存在とある貝が *Schistosoma japonicum* や *Paragonimus westermani* のような人体感染寄生蠕虫の中間宿主となりうるかどうかということにもなんら相関関係はないように思われる．

S. libertina の脚部筋肉抽出液の硫安塩析では，それぞれに異つた inhibitor component を分離することはできなかつたが，trypsin 抑制作用は 50% 飽和硫安分

画において最も顕著であつた. Sephadex G-50 による抽出液のゲル濾過の結果，3つの重要な inhibitor components が得られた. これを standards と比較すると，inhibitor componets の分子量はそれぞれ 5,200, 11,800 および 10,700 であることが解つた. Lineweaver-Burk の方法による速度論的解析を試みたところ，この inhibitors は非競合阻害性に働くことが解つた. 胎生貝および卵管の抽出液，ならびにヘモリンフの実験から，この inhibitors がいろいろの貝に広く認められること，またこのものはおそらく blood components に由来するものであろうことが明らかとなつた.