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An α_2-Macroglobulinlike Activity in the Blood of Chelicerate and Mandibulate Arthropods

PETER B. ARMSTRONG, MICHAEL T. ROSSNER, AND JAMES P. QUIGLEY
Marine Biological Laboratory, Woods Hole, MA 02543 (P.B.A., M.T.R., J.P.Q.), Department of Zoology, University of California, Davis, CA 95616 (P.B.A.), Princeton University, Princeton, NJ 08544 (M.T.R.), and Department of Microbiology and Immunology, Downstate Medical Center, SUNY, Brooklyn, NY 11203 (J.P.Q.)

ABSTRACT The α_2-macroglobulins are large molecular weight proteinase-binding proteins that inhibit the ability of proteinases to hydrolyze protein substrates without suppressing activity against amide or ester substrates. They are also able to protect the active site of bound proteinases from active site inhibitors of suitably high molecular weight. The ability to protect the amidolytic activity of trypsin from the macromolecular active site inhibitor, soybean trypsin inhibitor, was used to demonstrate an α_2-macroglobulinlike activity in the blood of the horseshoe crab, *Limulus polyphemus* and the crustaceans *Libinia emarginata* (the spider crab) and *Cancer borealis* (the Jonah crab). The α_2-macroglobulinlike activities of *L. polyphemus* and *L. emarginata* are sensitive to methylamine, but that of *C. borealis* is relatively insensitive. The molecular weights (mw) of the trypsin-protecting proteins in *L. emarginata* and *C. borealis*, estimated from gelfiltration studies, are, respectively, 480×10^3 and 460×10^3, and are significantly smaller than that of *L. polyphemus* (Mr = 570×10^3).

Although proteinases play essential roles in a variety of biological processes (Neurath, '84; Neurath and Walsh, '76), unregulated proteolytic enzymes in the wrong place have the potential for destructive actions prejudicial to the survival of the organism. Similarly, the proteinases involved in tumor dissemination (Quigley, '79) and the invasion of pathogenic organisms contribute to the progress of disease states. To counter these possibilities, a variety of inhibitory molecules have evolved that serve to inactivate and regulate proteinases. In one of the best studied systems, the blood of mammals, two basic categories of inhibitors can be distinguished: the active site inhibitors and the α_2-macroglobulins (Laskowski and Kato, '80; Travis and Salvesen, '83). The former bind to and inactivate the active site of the target proteinase whereas the latter bind the proteinase without inactivating the active site (for reviews see Barrett, '81; Starkey and Barrett, '77; Van Leuven, '82). Proteinases bound to α_2-macroglobulin are inhibited from hydrolyzing proteins but retain activity against low mw ester and amide substrates (for review, see Starkey and Barrett, '77). A rationale for the action of α_2-macroglobulins has been provided by the "trap" hypothesis of Barrett and colleagues (Barrett and Starkey, '73), which envisions the interaction of α_2-macroglobulin and proteinases as being initiated by proteolytic attack at a defined "bait" region of the α_2-macroglobulin molecule (Barrett and Starkey, '73; Barrett et al., '79; Hall and Roberts, '78; Harpel, '73; Roberts and Hall, '83; Salvesen and Barrett, '80; Sottrup-Jensen et al., '81). Hydrolysis of the "bait" region is followed by a rapid change in configuration that physically entraps the proteinase molecule in the interior of the α_2-macroglobulin "cage". The inhibition of proteolytic activity is thought to be a consequence of steric hinderance: the active site of the proteinase is shielded by virtue of enclosure by the α_2-macroglobulin, whereas substrate molecules small enough to diffuse into

Address reprint requests to Dr. Peter B. Armstrong, Department of Zoology, University of California, Davis, CA 95616.

the α_2-macroglobulin cage are hydrolyzed. This hypothesis also accounts for a second unique feature of the α_2-macroglobulin-proteinase interaction: the ability of α_2-macroglobulin to protect the active site of bound proteinase molecules from inactivation by high mw active site inhibitors (Beatty et al., '82; Berthillier et al., '68; Bieth et al., '70; Ganrot, '66; Harpel, '70; Harpel and Mosesson, '73). Protection can be demonstrated by showing that proteinases bound to α_2-macroglobulin retain activity against amide and ester substrates even in the presence of saturating levels of a macromolecular active site inhibitor.

Two categories of α_2-macroglobulin have been described in vertebrates: the thiol ester class, which possesses an internal thiol ester bond that is hydrolyzed concomitant with reaction with proteinases (Sottrup-Jensen et al., '80, '81; Swensen and Howard, '80; Tack et al., '80), and the ovostatin class, which lacks the thiol ester bond (Nagase and Harris, '83; Nagase et al., '83). Both bind proteinases without inhibiting the active site and show marked amino acid sequence homology. The α_2-macroglobulins of the blood of vertebrates are of the thiol ester class whereas that of the avian egg is of the ovostatin class (Nagase and Harris, '83; Nagase et al., '83). A diagnostic feature of the thiol ester class of α_2-macroglobulin is susceptibility to inactivation by low mw nucleophilic reagents such as methylamine (Barrett et al., '79; Steinbuch et al., '68; Swensen and Howard, '79; Van Leuven et al., '81a,b; Wang et al., '81), which are thought to attack the thiol ester bond directly (Swensen and Howard, '80; Tack et al., '80).

A knowledge of the phylogenetic distribution of the α_2-macroglobulins should lead to a comparative study of the structure and function of these molecules. The blood of all classes of vertebrates contains an α_2-macroglobulin-like activity (Starkey and Barrett, '82a,b; Starkey et al., '82) and a homologous activity, displaying several of the features listed above, has been detected in the blood of the horseshoe crab, *Limulus* (Quigley and Armstrong, '83; Quigley et al., '82). We have utilized the ability of α_2-macroglobulin to protect trypsin from the active-site inhibitor, soybean trypsin inhibitor, to screen the blood of various arthropods for the presence of an α_2-macroglobulinlike activity. As far as is known, the protection from soybean trypsin inhibitor is unique to α_2-macroglobulinlike molecules. The blood of both chelicerate (i.e., the horseshoe crab *Limulus polyphemus*) and mandibulate (i.e., the crustaceans *Cancer borealis* and *Libinia emarginata*) arthropods contains a trypsin-binding activity that protects trypsin from soybean trypsin inhibitor. In the report that follows, we will refer to the Limulus protein as the Limulus α_2-macroglobulin homolog, since we have been able to show that this protein has extensive functional homology to mammalian α_2-macroglobulin (Quigley and Armstrong, '85), and to the activity present in crustacean blood as CTPP (crustacean trypsin-protecting protein).

MATERIALS AND METHODS

Recently-collected *C. borealis* and *L. emarginata* were chilled at 4°C for 1–3 hr and blood was removed from the leg joints into hypodermic syringes containing a vol of ice cold anticoagulant saline (3% (w/v) endotoxin-free NaCl (Travenol), 50 mM ethylenediamine tetraacetate, final pH 7.0) equal to the vol of blood. The blood cells were removed immediately by centrifugation in a microcentrifuge. In some cases, the pH of the plasma-anticoagulant was then raised to 7.8 by adding Tris buffer.

Blood was obtained from prechilled adult *L. polyphemus* by cardiac puncture under sterile, endotoxin-free conditions and the cells were removed immediately by centrifugation (Armstrong, '85a,b). Hemocyanin was removed by centrifugation of the plasma at 40,000×g for 4 hr and the α_2-macroglobulin homolog precipitated with 12.5% polyethylene glycol. The precipitate was dissolved in 1/10 of the original plasma vol in 0.1 M citrate buffer, pH 6.5. Contaminating proteins were removed by two passes over a Sephacryl S-300 (Pharmacia) column. A low mw active site trypsin and chymotrypsin inhibitor was prepared from washed *Limulus* blood cells by stimulating exocytosis with the ionophore A23187 (Armstrong and Rickles, '82) and collecting the secreted cell products. The inhibitor was partially purified by Sephadex G-50 chromatography (Armstrong and Quigley, '85).

A stock solution of trypsin (Sigma Cat. No. T-8003, ×2 cryst., 9100 BAEE units/mg) was prepared at 1 mg/ml in 1 mM HCl, and aliquots were stored frozen until used. Trypsin was incubated with samples of blood or blood products in the presence of 10 mM $CaCl_2$ and 10 mg/ml bovine serum albumin in 50 mM Tris buffer, pH 8.1, for 15 or 30 min at room

temperature. A twofold excess by weight of soybean trypsin inhibitor dissolved in 50 mM Tris buffer, pH 8.1 was added and the preparation was incubated for an additional 15 min. The presence of enzymatically-active trypsin was determined by measuring the rate of hydrolysis of the amide substrate, BAPNA (Nα-benzoyl-DL-argenine p-nitroanilide, Sigma Cat. No. B4875; Erlanger et al., '61). In the absence of an α_2-macroglobulinlike activity, the soybean trypsin inhibitor suppressed completely the amidolytic activity of trypsin under these assay conditions. This control was included in every assay. In the experimental samples, the amount of enzymatically active trypsin is a measure of the amount of α_2-macroglobulinlike activity present in the sample (Ganrot, '66).

RESULTS AND DISCUSSION

α_2-Macroglobulin is a high mw proteinase-binding molecule that, unique amongst proteinase inhibitors does not inactivate the active site of the bound enzyme, but instead encloses the bound enzyme, shielding it from contact with macromolecules. We have used the ability of α_2-macroglobulin to shield trypsin from the macromolecular active site inhibitor soybean trypsin inhibitor (Mr = 21,000) (Ganrot, '66) to screen for the presence of an α_2-macroglobulinlike activity in the blood of invertebrates. As far as we are aware, this activity is specific for α_2-macroglobulinlike molecules, and has been reported for both the thiol ester (Beatty et al., '82; Berthillier et al., '68; Bieth et al., '70; Ganrot, '66; Harpel, '70; Harpel and Moresson, '73) and ovostatin (Nagase and Harris, '83) classes of α_2-macroglobulin.

Presence of a trypsin-protecting protein in Limulus plasma

The trypsin protection assay could be used to quantitate the α_2-macroglobulinlike activity in the plasma of the chelicerate arthropod, *Limulus polyphemus* (Fig. 1), a species

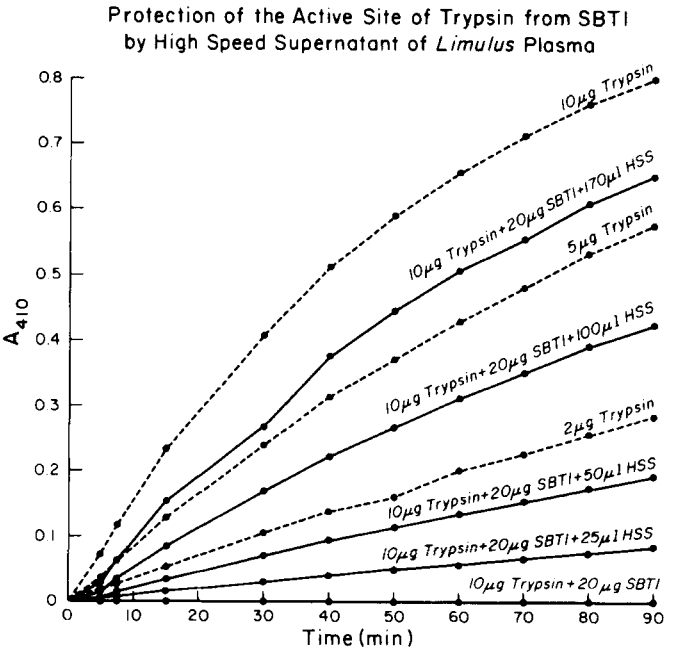

Fig. 1. Trypsin-mediated hydrolysis of BAPNA: protection of trypsin from soybean trypsin inhibitor (SBTI)—inhibition by a fraction of *Limulus* plasma freed of hemocyanin by centrifugation (high speed supernatant, HSS). Trypsin hydrolysis of the low mw amide substrate, BAPNA was carried out as described in Materials and Methods. Hydrolysis of BAPNA, measured by an increase in absorbancy at 410 nm (A_{410}), was approximately linear with time of incubation and trypsin concentration (2–10 μg) and was completely inhibited by 20 μg of SBTI. Preincubation (15 min) of 10 μg of trypsin with the indicated volumes of high speed supernatant (HSS) protected a fraction of the trypsin from SBTI inhibition in a dose-dependent fashion. 170 μl of HSS preincubated with 10 μg of trypsin permitted trypsin hydrolysis of BAPNA in the presence of SBTI equivalent to the hydrolysis rate of about 6–7 μg of trypsin.

lacking other proteinase inhibitors in the plasma (Quigley and Armstrong, '83). Two potential problems confronting this assay might result in artifactually low estimates of α_2-macroglobulin activity: 1) endogenous, low mw trypsin inhibitors might, if they were small enough, enter the α_2-macroglobulin "cage" and inactivate the bound trypsin (Eddeland and Ohlsson, '78); and 2) endogenous proteinases released from blood cells, or activated from the zymogen form during bleeding, could interact with, and inactivate, α_2-macroglobulin so that none was available to react with the exogenously added trypsin. If these endogenous proteinases were relatively unstable or were incapable of hydrolyzing BAPNA, the α_2-macroglobulin activity would not be recognized.

In order to examine the potential applicability of these problems to the assay of arthropod blood, we used a purified preparation of the α_2-macroglobulin homolog from the arthropod, *Limulus polyphemus*. The endogenous, low mw active site trypsin and chymotrypsin inhibitor released from Limulus blood cells stimulated to undergo exocytosis with ionophore (Armstrong and Quigley, '85) was capable of reducing the amidolytic activity of trypsin already bound to Limulus α_2-macroglobulin. In a typical experiment, a sample of purified Limulus α_2-macroglobulin homolog was incubated with excess trypsin; then an aliquot was incubated with excess blood cell-derived active site inhibitor present in a partially purified preparation. This treatment resulted in a 70% reduction in the subsequent hydrolysis of BAPNA by the α_2-macroglobulin-bound trypsin in the standard assay containing soybean trypsin inhibitor.

The mw of the acid-stable active-site inhibitor obtained from washed Limulus blood cells is 6100 (Armstrong and Quigley, '85), significantly smaller than soybean trypsin inhibitor (Mr = 21,000), which appears to display little or no ability to inactivate trypsin that has bound to α_2-macroglobulin.

As a model for the ability of endogenous proteinases to react irreversibly with the Limulus α_2-macroglobulin homolog and prevent subsequent interaction with trypsin, the Limulus α_2-macroglobulin homolog was reacted with the metallo-proteinase thermolysin prior to its reaction with trypsin. Thermolysin does not hydrolyze BAPNA. In a typical experiment, an amount of thermolysin (2 μg) just capable of saturating the amount of purified Limulus α_2-macroglobulin homolog present (calculated from data on the hydrolysis of [^{14}C]-methyl casein by the thermolysin preparation: Quigley and Armstrong, '83) caused an 85% reduction in the amount of subsequent binding of trypsin to the α_2-macroglobulin homolog.

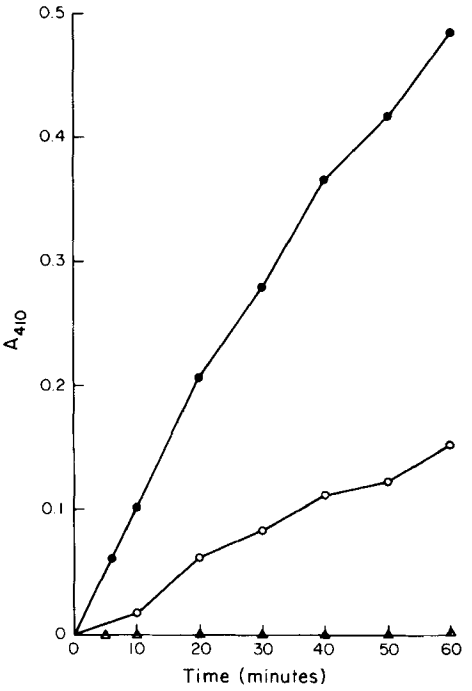

Fig. 2. Ability of a factor present in *C. borealis* plasma collected in the presence of EDTA to protect the amidolytic activity of trypsin from the macromolecular active site inhibitor, soybean trypsin inhibitor. The activity against the substrate BAPNA was monitored as absorbance at 410 nm. In the absence of plasma, the amidolytic activity was inhibited completely by soybean trypsin inhibitor. ●——●, 10 μg trypsin; ○——○, 10 μg trypsin, 20 μg soybean trypsin inhibitor, 100 μl C. borealis plasma; △——△, 10 μg trypsin, 20 μg soybean trypsin inhibitor.

Trypsin-protecting activity (CTPP) in the blood of crustacea

Blood collected from C. borealis and L. emarginata under conditions where clotting had occurred before the blood cells were centrifuged from the plasma, was consistently negative for a trypsin-protecting activity by our assay. However, a trypsin-protecting activity could be demonstrated if animals were bled into syringes containing a solution of trypsin. Under these conditions, the trypsin would be available at the moment of extra-

TABLE 1. Individual variation in trypsin-protecting and active site inhibitory activities in the plasma

Individual No.[1]	Trypsin-protecting activity[2]			Active-site inhibitory activity[3]	
	L. polyphemus	*L. emarginata*	*C. borealis*	*L. emarginata*	*C. borealis*
1	79	2.0	10.2	0.84	0.74
2	44	0.0	5.7	0.64	0.79
3	21	3.6	9.1	0.79	0.63
4	73	0.0	8.2	0.82	0.65
5	8	0.0	0.7	0.81	0.58
6	29	18.4	1.3	0.71	0.56
7	39		8.8		0.72
8	40		0.6		0.05
9	69		6.0		0.50
10	53		10.1		0.69

[1]Protocol numbers for separate individuals.
[2]μg trypsin activity protected/ml plasma.
[3]Fractional inhibition of the BAPNA hydrolytic activity of 10 μg of trypsin by 50 μl of plasma.

vasation and could compete effectively for any CTTP that was present. Trypsin-protecting activity was also detectable if blood was collected in the presence of EDTA, which reduces exocytosis of blood cells and other Ca^{++} dependent processes (Fig. 2). Since the latter technique allowed us to prepare unreacted CTPP, it was adopted for the studies that follow.

The blood prepared in this manner showed considerable variability from individual to individual in the amount of trypsin-protecting activity that was detected (Table 1). The amount was less than that observed in most of the horseshoe crabs (Table 1). In addition to the α_2-macroglobulinlike trypsin-protecting activity, both C. borealis and L. emarginata possessed an active site trypsin inhibitory activity in the blood (Table 1, Fig. 3). At least a portion of this was present in the blood cells and was released when washed cells were stimulated to undergo exocytosis by exposure to the ionophore A23187. In a sample experiment, the cells contained in 0.3 ml of Libinia blood released an active site inhibitory activity adequate to half inhibit 10 μg of trypsin. Since crustacean blood cells are notably unstable following bleeding (Dall, '64; Hardy, 1892; Ratcliffe and Rowley, '79; Ravindranath, '80; Tait, '10,'11,'18; Tait and Gunn, '18), we could not be certain, even with the precautions taken, that exocytosis was completely suppressed during collection of blood. Thus, active site inhibitors may be absent from the plasma of intact animals, but are released from the blood cells upon bleeding. This appears to be the case in Lim-

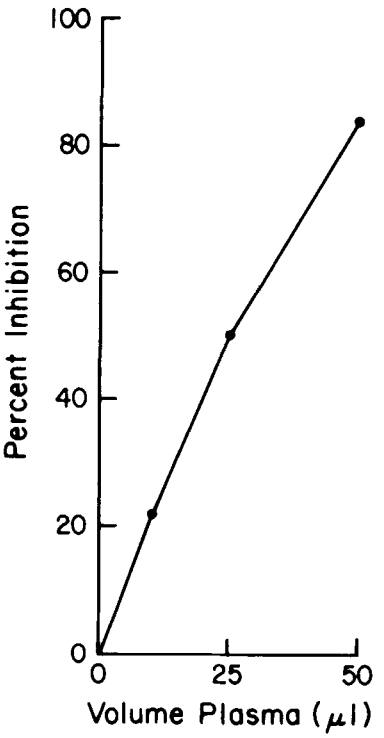

Fig. 3. Inhibition of tryptic activity by *Libinia emarginata* serum, using BAPNA as substrate. Varying vol of serum were preincubated with 10 μg of trypsin for 15 min; then the substrate was added, and hydrolysis was followed spectrophotometrically at 410 nm, room T. The extent of inhibition was calculated as: (absorbance of the sample containing 10 μg trypsin − absorbance of the sample containing 10 μg trypsin + serum)/absorbance of the sample containing 10 g trypsin.

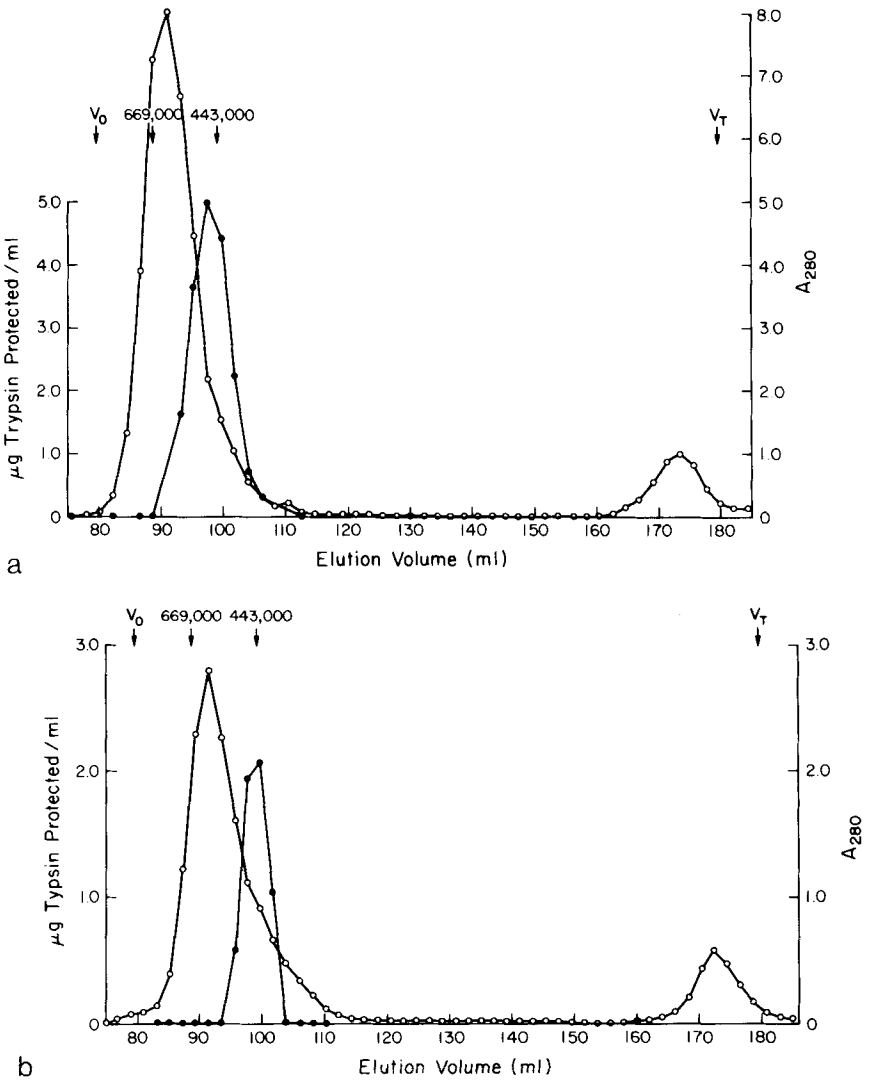

Fig. 4. Gel filtration of plasma of *C. borealis* (Fig. 4a) and *L. emarginata* (Fig. 4b) on Sephacryl S-300 resin; elution with 0.5 M NaCl – 0.025 M Tris, pH 7.75. CTPP was assayed as an activity that protected the amidolytic activity of trypsin from soybean trypsin inhibitor. The mw standards were thyroglobulin (Mr = 669 × 10^3) and ferritin (Mr = 443 × 10^3). Vo = void vol, V_T = included vol. ●——●, trypsin-protecting activity (μg trypsin protected/ml). ○——○, A_{280}.

ulus, where plasma containing the α_2-macroglobulin homolog can be prepared free of active site inhibitor (Quigley and Armstrong, '83). The blood cells of Limulus, prior to exocytosis, contain all of the circulating active site proteinase inhibitory activity, and are notably stable following bleeding; therefore, plasma, uncontaminated by products secreted by blood cells is easily prepared (Armstrong and Quigley, '85).

TABLE 2. Effect of methylamine on the trypsin-protecting activity of arthropods

Species	α_2-Macroglobulin activity[1] (± standard error of the mean)		
	Control[2]	Treated[3]	% Inactivation
Libinia emarginata (whole plasma)	18.4 ± 0.24	4.6 ± 0.36	75
Cancer borealis (whole plasma)	14.7 ± 0.36	15.1 ± 0.48	−3
C. borealis (partially purified)[4]	5.5 ± 0.03	4.4 ± 0.07	20
Limulus polyphemus (hemocyanin-free plasma)[5]	140.5 ± 2.00	5.4 ± 0.82	96
L. polyphemus in *C. borealis* plasma[6]	163.6 ± 3.18	24.5 ± 0.96	85

[1]Micrograms of trypsin protected from soybean trypsin inhibitor per ml of preparation.
[2]Tris-buffered plasma, pH 7.8–8.0 was incubated at 25°C for 16 hr.
[3]Tris-buffered plasma, pH 7.8–8.0 was incubated with 0.2 M methylamine at 25°C for 16 hr.
[4]Peak activity fraction from a Sephacryl S-300 column run of whole plasma.
[5]Whole plasma from which the hemocyanin had been removed by ultracentrifugation.
[6]Equal vol of *L. polyphemus* plasma (hemocyanin-free) and *C. borealis* plasma were mixed, then incubated with methylamine.

The mw of the trypsin-protecting activity present in the blood of the two crustaceans was estimated by gel filtration chromatography using Sephacryl S-300 resin and eluting with 0.5 M NaCl − 0.025 M Tris, pH 7.75. Single peaks of CTPP were observed for C. borealis (Fig. 4a) and L. emarginata (Fig. 4b) with mw of 460×10^3 and 480×10^3, respectively. These are significantly smaller than the Limulus α_2-macroglobulin homologue, Mr = 570×10^3 (Quigley and Armstrong, '85).

An attempt was made to determine the sensitivity to methylamine, as a way of assigning the crustacean α_2-macroglobulinlike trypsin-protecting activities to either the thiol ester or the ovostatin class. Strongly buffered samples (Tris buffer, pH 7.8–8.0) containing 0.2 M methylamine were incubated at room temperature for 18 hr. Table 2 shows that both L. polyphemus and L. emarginata CTPP'S are sensitive to methylamine. That of C. borealis is only moderately sensitive in partially purified preparations prepared by gel filtration chromatography and the activity present in whole plasma is insensitive. This latter result was not a consequence of inadequate control of pH, since this remained above 7.8 throughout the incubation, nor is it a result of the binding of methylamine by some other element in the plasma, since the Limulus α_2-macroglobulin homolog was inactivated by methylamine even in the presence of C. borealis plasma (Table 2). At present we are unable to account for the resistance of C. borealis α_2-macroglobulin to methylamine. The trypsin-protecting protein of L. emarginata clearly conforms to the generalization that the α_2-macroglobulins of the plasma are of the thiol ester class.

ACKNOWLEDGMENTS

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NOTE ADDED IN PROOF

Results similar to ours have recently been reported: Hergenhahn, H.G. and K. Söderhäll (1985) α_2−macroglobulin like activity in plasma of the crayfish *Pacifastacus leniusculus*. Comp. Biochem. Physiol. B, in press.

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