

Serine Proteases in the Spiny Lobster Olfactory Organ: Their Functional Expression along a Developmental Axis, and the Contribution of a CUB-Serine Protease

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ABSTRACT: Several serine proteases and protease inhibitors have been identified in the crustacean olfactory organ, which is comprised of the lateral flagellum of the antennule and its aesthetascs sensilla that house olfactory receptor neurons and their supporting cells. The function of these proteases in the olfactory organ is unknown, but may include a role in perireception (e.g., odor activation or inactivation) or in the development or survival of olfactory receptor neurons. To examine directly the function of proteases in the olfactory organ of the Caribbean spiny lobster *Panulirus argus*, we used different tissue fractions from the lateral flagellum in an enzyme activity assay with a variety of protease substrates and inhibitors. Trypsin-like serine protease activity occurs throughout the lateral flagellum but is enriched in the cell membranes from aesthetascs. Cysteine- and metalloprotease activities also occur in olfactory

tissue, but are more abundant in tissue fractions other than aesthetascs. To assess the contribution of one of the olfactory serine proteases—CUB-serine protease (Csp)—Csp was immunoprecipitated using an antibody; results with the remaining fraction suggest that Csp accounts for at least 40% of the total serine protease activity in the olfactory organ. The amount of total serine protease activity follows a developmental axis in the lateral flagellum. Total protease activity is lowest in the proximal zone, which lacks aesthetascs, and the proliferation zone, where olfactory receptor neurons and associated cells are born, and highest in aesthetascs of the distally-located senescence zone, which has the oldest olfactory tissue. © 2004 Wiley Periodicals, Inc. *J Neurobiol* 61: 377–391, 2004

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INTRODUCTION

The role of proteases is as diverse and dynamic as the number of proteases themselves. Four major protease classes are recognized, distinguished by their catalytic site. These are serine-, cysteine-, aspartic-, and me-

tallo-proteases. Each class retains two amino acid residues (histidine and aspartate) to stabilize the third active center residue (serine, cysteine, aspartate, or a free metal ion); together, these form a catalytic triad that acts on substrates (Barrett, 1994). Functions of the proteases include digestion of foods, regulation of other proteins and hormones by catalyzing inactive forms into active forms, modifying extracellular matrix, initiating intracellular and extracellular cascades, blood clotting, and regulation of various functions of nervous systems including development and repair (Barrett, 1994; Yoshida and Shiosaka, 1999; Gingrich and Traynelis, 2000).

Serine proteases, which include trypsin-like and chymotrypsin-like proteases distinguished based on differences in their catalytic effects, are known to act

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in nervous systems by regulating developmental processes such as cellular migration, differentiation, signaling, process extension, apoptosis, plasticity, and survival of neurons and glia (Nakayama, 1997; Marcinkiewicz et al., 1998; Shimizu et al., 1998; Hooper et al., 2001; Lee et al., 2001). Serine proteases and their inhibitors have been identified in several olfactory systems. For example, mRNA for one serine protease, tissue-plasminogen activator, is expressed during development in the olfactory bulb of mammals (Thewke and Seeds, 1996). Another serine protease, the kexin family pro-protein convertase PACE4, is expressed in the adult rat olfactory bulb (Akamatsu et al., 1997). Serine protease inhibitors, such as gliaderived nexin, have also been identified throughout the olfactory bulb (Reinhard et al., 1988, 1994). These may regulate neurogenesis, axogenesis, or apoptosis in the olfactory organ and olfactory bulb. Thus, serine proteases in the olfactory system might play a role in development.

Additionally, serine proteases in olfactory organs might play a role in perireception. This could include the enzymatic degradation of odorant molecules following receptor activation, as has been shown for an ATPase/phosphatase in spiny lobsters (Carr et al., 1990; Gleeson et al., 1992). Serine proteases might also function in olfactory perireception through the production of active odorants from inactive precursors (Rittschof, 1990, 1993).

The olfactory organ of the Caribbean spiny lobster *Panulirus argus* has continuous postembryonic turnover of olfactory receptor neurons (ORNs) and their associated auxiliary cells throughout the animal's life (Steullet et al., 2000; Harrison et al., 2001a,b, 2003). The ORNs innervate cuticular sensors called aesthetasc sensilla, which are located on the distal half of the lateral flagellum of the antennule (Fig. 1). New ORNs are continuously added to the proximal end of the aesthetasc-bearing region; this is called the proliferation zone. With each molt, the new aesthetasc sensilla innervated by these newly proliferated ORNs are added to the exoskeleton in the proliferation zone, and old aesthetascs on the distal end of the aesthetasc-bearing region are lost. This creates a horizontal axis of developmental stages in the aesthetascs and their ORNs. These developmental zones, from proximal to distal, include: the proximal zone located proximal to the proliferation zone; the proliferation zone; a maturation zone where the new cells differentiate into their functional forms and connect to the CNS; a mature zone where the cells are functional; and a senescence zone where the old cells die and lose their functional properties. Altogether, these cellular processes—neuronal proliferation, axonal growth into the central nervous system, functional maturation, and

cell death—must be well regulated for the olfactory organ to maintain its function through the considerable lifespan of spiny lobsters, which can be decades long (Steullet et al., 2000; Harrison et al., 2001a,b, 2003).

We have previously discovered and cloned a CUB-serine protease (*csp*) from the aesthetascs of the olfactory organ of spiny lobsters (Levine et al., 2001). CUB-serine proteases have a domain for a serine protease, a CUB domain (named for Complement subcomponents C1r/C1s, *Uegf*, Bone morphogenetic protein 1), and a signal peptide. CUB-serine proteases are typically secreted (via the signal peptide), bind to the extracellular surface of the membrane (via the CUB domain), and have catalytic activity in the extracellular matrix (via the serine protease) (Hecht and Anderson, 1992; Bork and Beckmann, 1993; Gschwend et al., 1997; Misra et al., 1998). They regulate cell development (e.g., proliferation, axonal growth, apoptosis) in a variety of tissues and species (Bork and Deckmann, 1993). In the spiny lobster, the full-length cDNA sequence of *csp* is 1802 bp, and it encodes a 50.25-kD Csp protein containing all three domains characteristic of CUP-serine proteases. Csp is secreted by secretory cells and associates with the dendrites of the ORNs (Levine et al., 2001; Stoss et al., 2004). Csp is expressed in aesthetascs across the developmental zones. A serine protease with low sequence similarity to Csp but with a similar distribution has been found in the clawed lobster *Homarus americanus* (OET-3) (Hollins et al., 2003).

Other serine proteases and protease inhibitors are present in the spiny lobster olfactory organ and are enriched in the proliferation zone, the site of postembryonic proliferation of ORNs in *P. argus* (Stoss et al., 2003). One of these, a putative protease regulator, is expressed in epithelial cells in the proliferation zone, and its expression increases following injury that causes enhancement of cell proliferation (Stoss et al., 2003). These results suggest that these proteases and protease inhibitors or regulators may be involved in cell proliferation or differentiation.

The goals of this study are to functionally characterize proteases in the olfactory organ of spiny lobsters, to identify the relative contribution of Csp to the total activity of serine proteases, and to compare serine protease activity across the developmental zones of the olfactory organ. We used an enzyme activity assay, with protease substrates and inhibitors to characterize the nature of the proteases in the lateral flagellum and the aesthetascs. A variety of substrates and inhibitors were used because although each has a preference for different classes and subclasses of proteases, none is absolute in its specificity. Thus, using a set of substrates and inhibitors is necessary to es-

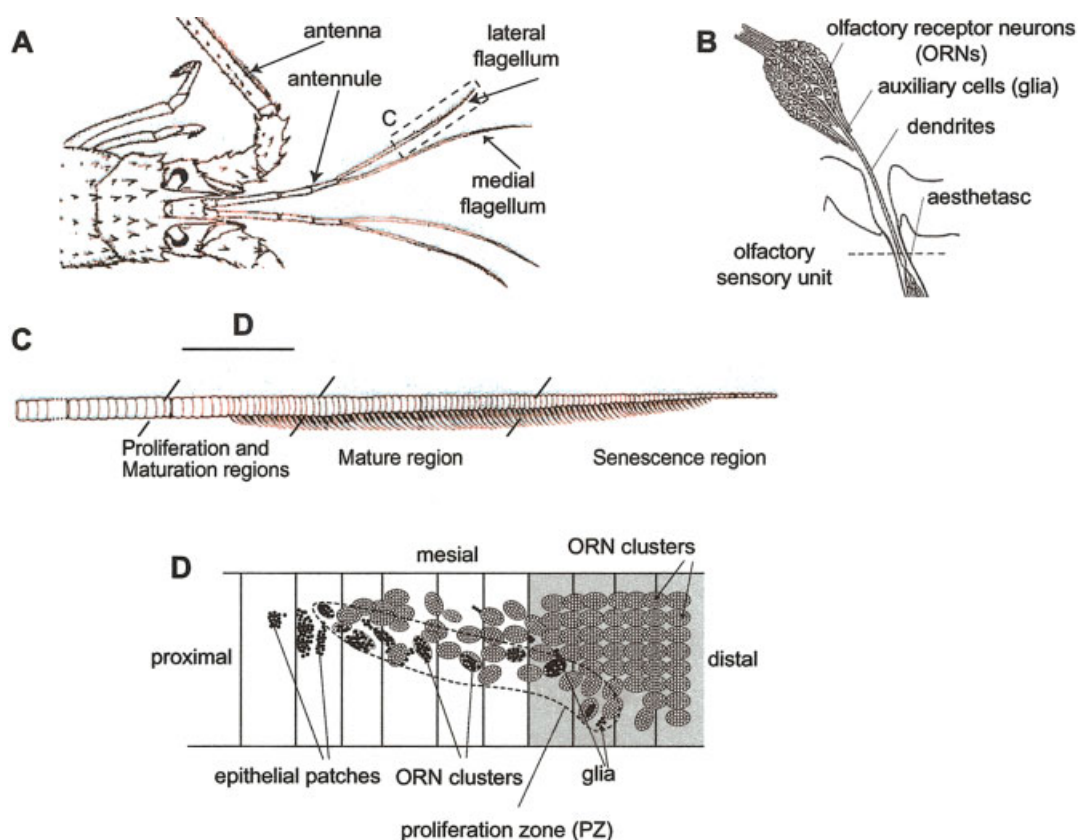


Figure 1 Olfactory organ of the spiny lobster. (A) The olfactory organ is the paired first antennae, or antennules. Each antennule has two flagella—a lateral and a medial. Both flagella are composed of segments called annuli. The lateral flagellum is distinctive in having aesthetascs on its distal region. (B) Enlarged view of longitudinal section of one aesthetasc. The proportions of the structure inside the aesthetasc shaft are not to scale. Each aesthetasc is innervated by ca. 300 ORNs. Cell bodies associated with each aesthetasc form a cluster under the base of the next proximal row of aesthetascs, the dendrites fill the lumen of the cuticular shaft of the aesthetasc, and the axons project to the olfactory lobes in the brain. Each ORN dendrite has inner and outer dendritic segments, with a transitional zone in between. (C) Developmental zones of the lateral flagellum. See Introduction for description of these developmental zones. (D) Model showing the proliferation of cells that occurs during the intermolt stage in the proximal proliferation zone of the lateral flagellum. Shaded boxes represent annuli that possess aesthetascs, and white boxes represent annuli without aesthetascs but with developing clusters (i.e., this is a similar region to that shown in (C)). Cells proliferate (indicated by black dots) along the proximo-lateral margin of the existing ORN population (postmitotic cells indicated by gray dots). The aesthetasc sensilla associated with newly formed clusters (indicated by area within dashed line) develop late in the molt cycle (i.e., during premolt). (Modified from Harrison et al., 2003).

establish a case for the presence of particular types of proteases. Our results show that trypsin-like serine protease activity is associated with membranes in the aesthetascs of the olfactory organ. Cysteine- and metalloprotease activity also occurs in the olfactory organ, but it is more abundant in tissue other than the aesthetascs. Csp activity accounts for at least 40% of the total serine protease activity. The amount of serine protease activity follows a developmental axis in the olfactory organ.

MATERIALS AND METHODS

Tissue Preparation

Antennular lateral flagella were collected from live spiny lobsters (*Panulirus argus*) in the Florida Keys and immediately frozen on dry ice. The antennules were stored on dry ice or at -80°C until used in assays.

Various tissue fractions from the aesthetasc region of the antennular lateral flagella were prepared, to identify the subcellular location of protease activity (Fig. 2). The aes-

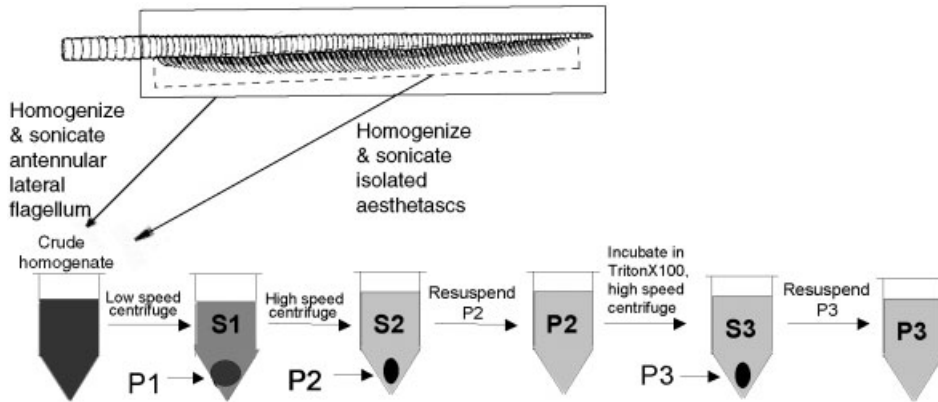


Figure 2 Schematic of tissue fractions collected from the olfactory organ of lobsters. See text for details. P, pellet; S, supernatant.

thetasc-bearing annuli from 50–90 antennules were thawed and minced in 10 mL of *P. argus* saline (PAS) (458 mM NaCl, 13.4 mM KCl, 13.6 mM CaCl₂, 9.8 mM MgCl₂, 14.1 mM NaSO₄, 3 mM HEPES, 1.9 mM glucose, 1.2 mM NaOH, pH 7.4). The solution was homogenized with a mechanical homogenizer (PT 3100 Polytron) at 5000 rpm for 1–2 min on ice and then sonicated (Fisher Scientific 50 Sonic Dismembrator) five times for 5 sec. The homogenate was separated into 1-mL fractions and centrifuged for 10 min at 8000 × *g* at 4°C (Jouan MR 18 22). The supernatant (S1) was collected, and the pellet (P1) was discarded (Fig. 2). The S1 was then centrifuged in 1 mL fractions for 30 min at 42,000 rpm in 4°C (Beckman Optima TL Ultracentrifuge, TLA 120.2 rotor). The supernatants were combined to give the soluble fraction S2, and the pellets were resuspended in PAS to give the P2.

To determine if the enzyme activity is located in the aesthetascs, aesthetascs were shaved from the lateral flagella using a scalpel blade, collected, and then homogenized in a 1-mL glass mortar and pestle in PAS. The pure aesthetasc homogenate was then treated in a similar fashion as the aesthetasc region homogenate, as described above, to produce the S1, P2, and S2 fractions (Fig. 2).

Preparations for developmental regions of the antennular lateral flagellum were similar as above. The olfactory organ was separated into its developmental regions on ice. The proximal zone (PX) included 15–20 annuli proximal to the proliferation zone (PZ). The PZ included 5–10 annuli proximal to the aesthetasc-bearing region and 10–15 annuli in the adjacent aesthetasc-bearing region. The mature zone (MZ) included annuli that were 10–15 annuli distal to the start of the aesthetasc bearing region and 25–30 annuli proximal to the distal tip of the flagellum. The senescence zone (SZ) included the 25–30 most distal annuli. After separation of the developmental zones, the aesthetascs were shaved and collected for each region, yielding three additional tissues: PZ-A (aesthetascs from the proliferation zone), MZ-A (aesthetascs from the mature zone), and SZ-A (aesthetascs from the senescence zone). The aesthetascs and the denuded flagellum from each region were then homogenized separately in 1 mL of PAS buffer with a glass mortar

and pestle, sonicated, and centrifuged to produce the S1 homogenate, as described before.

In preliminary experiments, PAS, Tris, and HEPES buffers at pH 7.4 were all compared for effective buffering capabilities in the enzyme assay; 100 mM Tris, 50 mM HEPES, 50 mM HEPES + 500 mM sucrose, 250 mM HEPES, 500 mM HEPES, and PAS were all tested as homogenizing buffer and reaction buffer. PAS produced the strongest and most reliable signal, probably because it is closest to the normal physiological solution for the enzymes *in situ*. Thus, PAS was used in all subsequent experiments.

Substrates and Inhibitors Used to Determine Specificity of Proteases

Enzyme specificity was determined using different substrates and inhibitors (Table 1). Two different substrates were used: *N*-alpha benzoyl-L-arginine *p*-nitroanilide (BAPNA), which has specificity for serine protease activity; and L-arginine *p*-nitroanilide (LAPNA), which has specificity for metalloprotease as well as cysteine protease activity (Barrett, 1994; Rawlings and Barrett, 1994).

Several inhibitors for different classes of proteases were used (Table 1). Phenylmethylsulfonyl fluoride (PMSF) was used as a general serine protease inhibitor. Tosyl-L-lysine chloromethyl ketone (TLCK) was used as trypsin-like serine protease inhibitor. Tosyl-L-phenylalanine chloromethyl ketone (TPCK) was used as chymotrypsin-like serine protease inhibitor. *N*-ethylmaleimide (NEM) was used as cysteine protease inhibitors. E64, antipain, and leupeptin were used as mixed cysteine/serine protease inhibitors. The chelators EDTA and 1,10-phenanthroline were used to inhibit metalloprotease activity.

It is important to stress that although the substrates and inhibitors for proteases are targeted for specific catalytic activity, there can sometimes be nonspecific crossreactivity between proteases and their target substrates and inhibitor (Barrett, 1994; Rawlings and Barrett, 1994). For example, although BAPNA and LAPNA are thought of as serine protease and cysteine/metalloprotease substrates, respec-

Table 1 Substrates and Inhibitors, and Their Activity

Substrates	Activity
N- α benzoyl-L-arginine <i>p</i> -nitroanilide (BAPNA)	Serine protease substrate
L-arginine <i>p</i> -nitroanilide (LAPNA)	Metallo- and cysteine protease substrate
Inhibitors	
Serine protease type inhibitors	
Phenylmethylsulfonyl fluoride (PMSF)	General serine proteases
Tosyl-L-lysine chloromethyl ketone (TLCK)	Trypsin-like serine proteases
Tosyl-L-phenylalanine chloromethylketone (TPCK)	Chymotrypsin-like serine proteases
Cysteine and serine protease type inhibitors	
Antipain	Cysteine and serine proteases
Leupeptin	Cysteine and serine proteases
Trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E64)	Cysteine and serine proteases
Cysteine protease type inhibitors	
<i>N</i> -Ethylmaleimide (NEM)	Cysteine proteases
Metalloprotease type inhibitors	
Ethylenediaminetetraacetic acid (EDTA)	Chelator
1,10-Phenanthroline	Chelator

tively, they still can sometimes react with nonspecific, non-targeted proteases. E64 is primarily thought of as pure cysteine protease inhibitor that disrupts the cysteine thiol group in the protease's catalytic domain or disulfide bonds in the protease's tertiary structure. However, E64 can also competitively inhibit trypsin by interfering with the catalytic site (Sreedharan et al., 1996). Antipain and leupeptin are generally categorized as serine/cysteine protease inhibitors, but they can also inhibit trypsin-like serine proteases. PMSF, primarily thought of as a general serine protease inhibitor, can also inhibit activity of cysteine proteases. Because of this lack of specificity in some cases, conclusions as to the activity of proteases should be based on using multiple substrates and inhibitors and by identifying general patterns from the results.

Substrates were prepared fresh as stock solutions in 100% DMSO at 100 mM. They were then diluted in PAS to make a working solution of 2 mM. TLCK, TPCK, and 1,10-phenanthroline stock solutions were dissolved in DMSO, 10% for TLCK, and 100% for TPCK and 1,10-phenanthroline. 200 mM PMSF and 1 mM NEM stock solutions were dissolved in 100% ethanol. E64 and EDTA stock solutions were made in deionized water. Working solutions of all inhibitors were diluted in PAS to a concentration 2.5 times greater than the final reaction concentration. Controls included DMSO or ethanol at appropriate concentrations, to determine if the higher concentrations of the solvents hindered the enzymatic reactions. As mentioned later, TPCK, NEM, and EDTA did not completely dissolve in the PAS, presumably due to the high salt concentration of the PAS buffer.

Assay of Protease Activity

A spectrophotometric assay was used to quantify protease activity. The assay is based on the enzymatic hydrolysis of a substrate, which generates the fluorescent group *p*-nitroanilide (pNA). pNA was detected at 405 nm using a Beck-

man Biomek Plate Reader spectrophotometer. Enzyme activity assays were conducted by transferring 20 μ L of homogenate in PAS to a 96-well plate. Eighty microliters of PAS buffer or a 2.5 times working solution of inhibitor in PAS buffer were added to the homogenate. To start the reaction, 100 μ L of a 2-mM solution of substrate in PAS was added to the reaction volume, giving a final concentration of 1 mM. The initial time ($t = 0$) was measured. Subsequent measurements were read at 10, 20, 40, 60, 80, and 120 min. This was to ensure that the final measurement at 120 min was due to the increase of the production of pNA by the hydrolysis of either the BAPNA or LAPNA substrate.

Data Analysis

The absorbance measurement for each reaction sample was compared to a curve of pNA from 0–300 μ M. Protease specific activity was expressed relative to the amount of protein in the homogenate fractions (nmol pNA/min \cdot mg protein). Protein concentration was determined according to the Bradford Assay. The Bio-Rad protein assay (Bio-Rad Laboratories) was used against a standard curve produced by 0–25 ng/ μ L bovine serum albumin. The specific activity measured at $t = 0$ was subtracted from values taken at subsequent times to eliminate background activity. In some analyses (e.g., Figs. 4–9), values were normalized to the maximum value in each experiment.

Evaluation of the Serine Protease Activity of Csp

The contribution of Csp to the total serine protease activity in the olfactory organ was determined by quantifying the effect of removing Csp from solubilized tissue homogenates using antibody precipitation. The Csp antibody used in our study (CUB 99-6), which targets the CUB domain of Csp, was previously generated and used for immunocytochemi-

cal localization of Csp in the olfactory organ (Levine et al., 2001).

Purification of the Csp Antibody. CUB 99-6 serum was stirred on ice while adding crushed ammonium sulfate to 40% (w/v) over 1 h. The solution was then incubated for 3 h on ice with stirring, followed by centrifugation at $18,000 \times g$ for 30 min at 4°C. The supernatant and pellet were separated, and pellet resuspended in 10 mL of 10 mM sodium phosphate buffer at pH 7.2 with 1 mM EDTA. The solution was then dialyzed twice in this same buffer, and the dialyzed solution was then purified with 30 mL of DEAE on a Q column equilibrated in this buffer.

Preliminary experiments with purified CUB 99-6 were not successful, probably due to presence of EDTA in the sodium phosphate buffer, because EDTA is an effective inhibitor of BApNA and LAPNA hydrolysis (see Results). To correct for this, CUB 99-6 was then dialyzed twice in 10 mM HEPES at pH 7.4 with 0.02% sodium azide, and 3 mL of the antibody solution was purified with both sepharose SP and Q columns, each equilibrated in 10 mM HEPES at pH 7.4. CUB 99-6 was again dialyzed twice in 10 mM HEPES at pH 7.4 with 0.02% sodium azide. The resulting concentration of the antibody solution was 14 $\mu\text{g/mL}$. Dilutions of the antibody were expressed relative to this concentration.

Solubilization of Membrane Proteins from Isolated Aesthetascs. The P2 fraction of isolated aesthetascs (Fig. 2) was collected and solubilized by incubating for 1 h in electrophoresis grade Triton X-100 at a concentration range of 0.001 to 1.0%, in PAS. This solution was then centrifuged at 42,000 rpm for 30 min at 4°C (Beckman Optima TL Ultracentrifuge, TLA 120.2 rotor). The resulting supernatant (S3) and pellet (P3) (Fig. 2) were then assayed for BApNA and LAPNA activities.

Antibody Incubation Assays. To immunoprecipitate Csp from tissue samples, the purified CUB 99-6 antibody was incubated with solubilized membrane fraction from isolated aesthetascs. Aesthetasc P2 membrane fraction was solubilized by incubating in 0.1% electrophoresis grade Triton X-100 on ice for 1 h. To precipitate the antibody-antigen complex, the solubilized P2 was then incubated in CUB 99-6 antibody at 3, 6, 10, and 30% of the full-strength serum level of purified CUB 99-6 antibody (14 $\mu\text{g/mL}$) at 37°C for 30 min and then at 4°C overnight. These samples were then separated into supernatant (S3) and pellet (P3) fractions by centrifuging for 30 min at 42,000 rpm in 4°C (Beckman Optima TL Ultracentrifuge, TLA 120.2 rotor). The S3 fraction was collected, and the P3 fraction (containing the pelleted Csp-antibody complex) was resuspended in PAS. The S3 and P3 fractions were then transferred to a 96-well plate, added to a final BAPNA concentration of 1 mM, and activity was measured every minute over a 10-min time period, and then again at 15 min.

RESULTS

Serine Protease Activity in the Aesthetasc Region of the Lateral Flagellum

Our results suggest that serine protease activity is present in the antennular lateral flagellum of *P. argus*. In the experiments, BApNA, a typical serine protease substrate, was used to detect the enzymatic activity in the combined soluble and membrane fractions (S1 homogenate) of the aesthetasc region of the antennular lateral flagella. The initial BApNA concentration was varied from 30–1000 μM , and at each substrate concentration enzyme activity was measured at 0–120 min time points during the reaction. The results show that the reaction is dependent on substrate concentration [Fig. 3(A)]. The specific activity is 2.13 nmol/min \cdot mg protein using 1 mM BApNA (Table 2A). Enzyme activity increased as homogenate concentration increased between 100–1000 $\mu\text{g/mL}$ [Fig. 3(B)]. At each concentration, enzyme activity was time dependent [Fig. 3(A,B)]. The reaction rate appears to be first order, as evidenced by a linear increase as substrate concentration increases. These results are consistent with the idea that serine proteases are active in the antennular lateral flagellum.

To distinguish the specificity of the serine protease activity in the lateral flagellum, three serine protease inhibitors were examined for their effect on the hydrolysis of the serine protease substrate BApNA. These inhibitors were TLCK, TPCK, and PMSF (Fig. 4), which typically inhibit trypsin-like serine protease activity, chymotrypsin-like serine protease activity, and general serine protease activity, respectively. To examine specificity, inhibitors for metalloproteases (1,10-phenanthroline and EDTA), cysteine proteases (NEM), and cysteine-/metallo-proteases (E64) were also tested (Fig. 4). PMSF produced 20% inhibition of BApNA hydrolysis, TLCK inhibited by 75%, but TPCK had no effect (though the absence of a TPCK effect might be a function of its poor solubility in PAS buffer). 1,10-Phenanthroline and NEM had no effects on BApNase activity. E64 and EDTA inhibited BApNase activity at 40 and 20%, respectively (Fig. 4). These results support the idea that the BApNase activity in the antennular lateral flagellum is principally due to trypsin-like serine proteases.

LAPNA, which is a typical substrate for metallo- and cysteine-protease activity, was used with the protease inhibitors to detect the metallo- and cysteine-protease activity in the aesthetasc region (Fig. 4). LAPNase activity was detected with a specific activity of 8.64 nmol/min \cdot mg protein at a LAPNA concentration of 1 μM (Table 2A). As inhibitors, E64 and

S1 from Antennular Lateral Flagellum

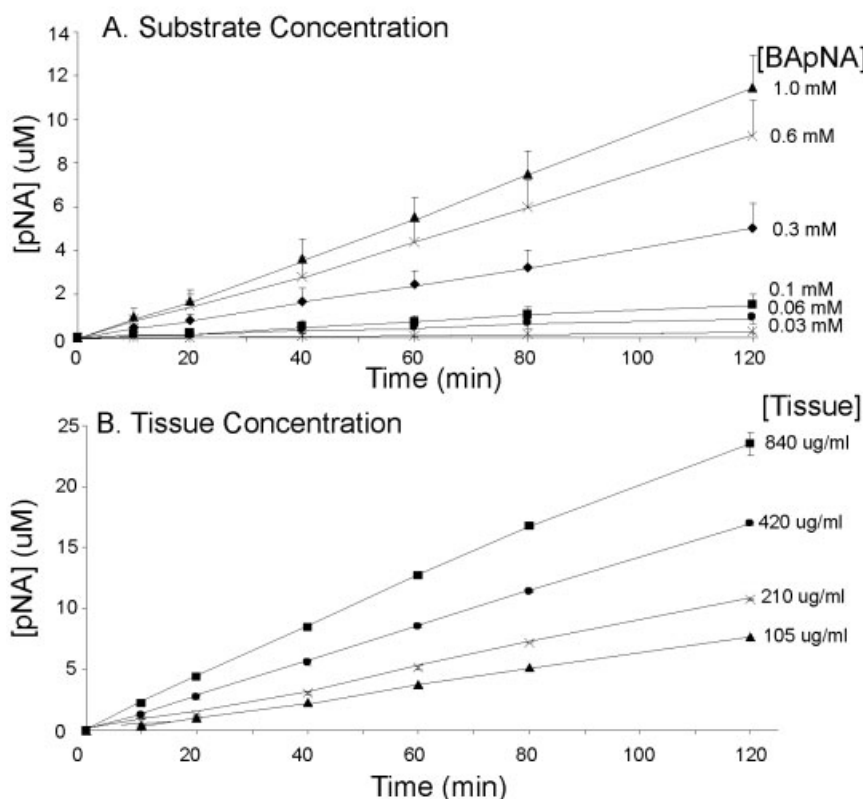


Figure 3 Serine protease activity in the S1 tissue fraction of the antennular lateral flagellum. (A) Effect of varying starting BApNA concentration from 0–1 mM. Results show a concentration-dependent response to the hydrolysis of BApNA. (B) Effect of varying S1 tissue concentration from 105–840 μg protein/ml. Results show that BApNA hydrolysis increases as tissue concentration increases. Incubations were at room temperature. Values are means \pm S.E.M for three homogenates [in (A)] and 1 homogenate [in (B)], each run in triplicate.

NEM were used for cysteine-type inhibition, and the metal chelators EDTA and 1,10-phenanthroline were used as metalloprotease inhibitors. EDTA and 1,10-phenanthroline decreased LAPnase activity by 50 and 95%, respectively, while cysteine protease and serine protease inhibitors did not inhibit by more than 20% (Fig. 4). These results suggest that LAPnase hydrolysis is mainly due to metalloproteases in the aesthetasc region of the lateral antennule flagellum.

A previous study (Levine et al., 2001) indicated that one type of serine protease—a CUB-domain serine protease—is present in aesthetascs, where it is secreted, migrates to the extracellular space of the aesthetascs, and anchors to the extracellular face of the dendritic membrane of olfactory receptor neurons. Consequently, we expect that most of the protease activity would be in the membrane fraction when the lateral flagellum S1 homogenate is fractionated into a soluble fraction (S2) and a membrane fraction (P2).

The lateral flagellum P2 in fact did show higher

specific activity for both BApNA and LAPnase hydrolysis compared to the lateral flagellum S2 (for BApNA, specific activity 16.6 nmol/min \cdot mg protein in P2 vs. 1.77 in S2; for LAPnase, 17.2 nmol/min \cdot mg protein in P2 vs. 13.1 in S2) (Table 2A). This suggests that the serine protease activity is concentrated in the membrane of the aesthetasc region of the antennular lateral flagellum.

In addition to higher BApnase activity found in the lateral flagellum membrane fraction (P2), protease inhibitors had a greater effect on BApnase activity in the P2 than in either the S2 or S1 (Fig. 5). PMSF, TLCK, E64, and EDTA reduced BApnase activity in the P2 by 40–90% (Fig. 5A). The decrease in BApnase activity by the common metalloprotease inhibitors could be caused by an ion or calcium-dependency for the enzymes (Barrett, 1994; Rawlings and Barrett, 1994) or by an unexpected competitive inhibition of the active site. The chelating inhibitors might also have a nondirect effect on the BApnase

Table 2 Comparison of Total Activity and Specific Activity for Aesthetasc Region of the Lateral Flagellum (A) and Isolated Aesthetascs (B)

Tissue	Total activity (nmol/min)	Specific activity (nmol/min mg protein)
A. Antennular Lateral Flagellum		
BAPnase activity		
S1	0.530 ± 0.030	2.13 ± 0.27
P2	0.069 ± 0.002	16.6 ± 1.0
S2	0.184 ± 0.024	1.77 ± 0.26
LAPnase activity		
S1	2.29 ± 0.048	8.64 ± 0.71
P2	0.072 ± 0.003	17.2 ± 0.8
S2	0.831 ± 0.048	13.1 ± 2.4
B. Isolated Aesthetascs		
BAPnase activity		
S1	0.042 ± 0.005	2.70 ± 0.17
P2	0.005 ± 0.001	5.76 ± 0.79
S2	0.008 ± 0.001	1.58 ± 0.21
LAPnase activity		
S1	0.180 ± 0.017	10.6 ± 0.6
P2	0.005 ± 0.001	5.32 ± 1.12
S2	0.062 ± 0.006	10.7 ± 0.6

The tissue preparations were fractionated into their combined membrane and soluble fraction (S1), membrane fraction following centrifugation (P2), and soluble fraction following centrifugation (S2) (see Fig. 2). Incubations were with 1 mM BAPNA or 1 mM LAPNA for 2 h at room temperature.

activity, such as acting as a noncompetitive inhibitor and changing the overall structure or kinetics of the enzyme.

LAPnase activity in the lateral flagellum P2 was almost completely inhibited by EDTA and 1,10-phenanthroline and partially inhibited by PMSF,

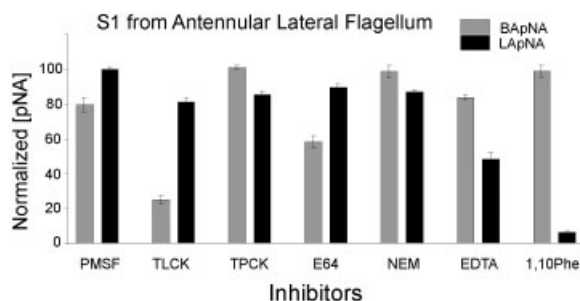


Figure 4 Comparison of 1 mM BAPNA hydrolysis and 1 mM LAPNA hydrolysis using inhibitors in the S1 fraction from antennular lateral flagellum. Incubations were for 2 h at room temperature. Data were normalized to control reactions (saline, without inhibitor). Values are mean ± S.E.M. for three homogenate preparations, each run in triplicate.

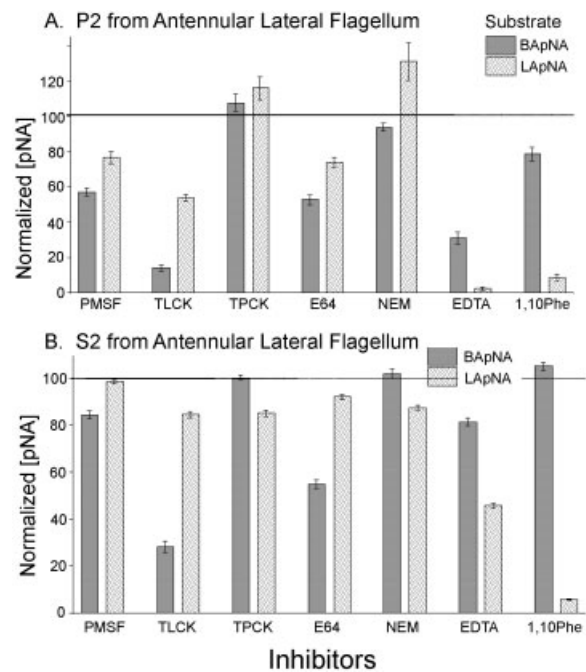


Figure 5 Comparison of 1 mM BAPNA and 1 mM LAPNA hydrolysis with inhibitors in the P2 (A) and S2 (B) fractions of the lateral flagellum. Incubations were for 2 h at room temperature. Data were normalized to the values for the reactions in the absence of any inhibitors. Values are mean ± S.E.M. for three homogenate preparations, each run in triplicate.

TLCK, and E64 (25–50% inhibition) [Fig. 5(A)]. The LAPNA hydrolysis in the S2 was not substantially reduced by serine or cysteine protease inhibitors (PMSF, TLCK, TPCK, NEM, E64), but was inhibited by 50–90% in the presence of metalloprotease inhibitors (EDTA, 1,10-phenanthroline) [Fig. 5(B)]. The inhibitory effect of some serine protease inhibitors on LAPnase activity in the lateral flagellum P2 was not expected, because LAPNA is normally a substrate for metallo- and cysteine proteases rather than serine proteases. LAPnase inhibition by serine protease inhibitors may result from nonspecific interactions with serine proteases that hydrolyze LAPNA, or that they are acting directly on cysteine and metalloproteases. PMSF has demonstrated the ability to reduce activity for some types of cysteine proteases (Barrett, 1994; Rawlings and Barrett, 1994). Therefore, a similar reaction with TLCK could be taking place, either with cysteine or metalloproteases in the membrane, thus diminishing LAPnase activity.

Altogether, our results indicate that metalloproteases are located in the soluble fraction of the lateral flagellum, while the membrane is likely to contain cysteine- and metalloprotease activity. This is summarized in Figure 7(A) and (B).

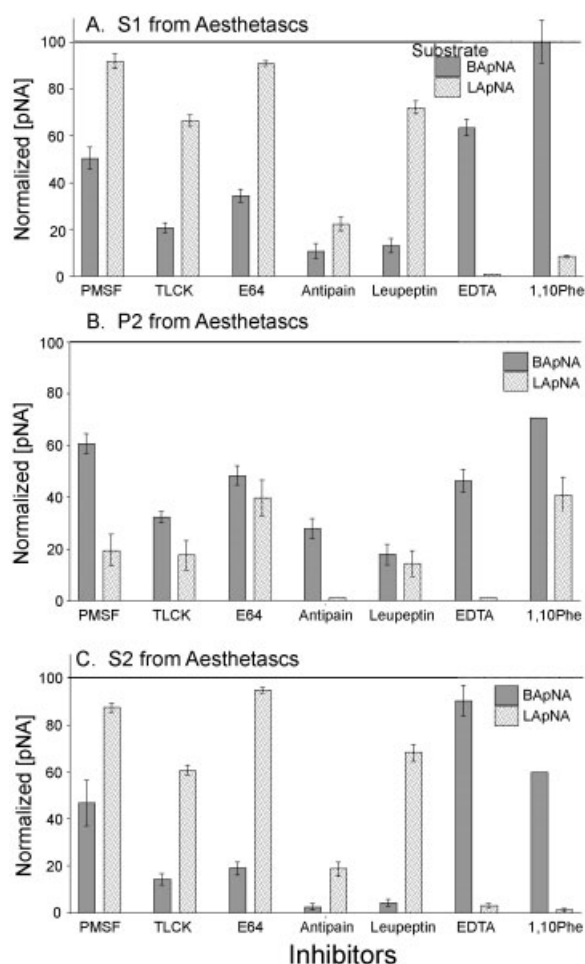


Figure 6 Comparison of 1 mM BApNA and 1 mM LApNA hydrolysis with inhibitors in the S1 (A), P2 (B), and S2 (C) fractions of the isolated aesthetascs. Incubations were for 2 h at room temperature. Data were normalized to the values for the reactions in the absence of any inhibitors. Values are mean \pm S.E.M. for three homogenate preparations, each run in triplicate.

Serine Protease Activity in Isolated Aesthetascs

To examine whether the aesthetascs themselves contain serine protease activity, we isolated aesthetascs from the lateral flagellum, made a homogenated isolated aesthetasc preparation (aesthetasc S1), and characterized protease activity within it. The inhibitors had a greater effect on isolated aesthetasc S1 (Fig. 6) compared to the lateral flagellum S1 (Fig. 4). Proteases in the aesthetasc S1 were also characterized with inhibitors, but NEM and TPCK were replaced with the serine/cysteine protease inhibitors antipain and leupeptin, because of concern about the solubility of TPCK and NEM (see Methods).

To further characterize and localize the BApNA

activity in the isolated aesthetascs, the aesthetasc S1 was fractionated into membrane (P2) and soluble (S2) components, as well as subjected to protease inhibitors, including the serine/cysteine protease inhibitors antipain and leupeptin. BApNase specific activity was 2.7 nmol/min \cdot mg protein for aesthetasc S1, 5.8 nmol/min \cdot mg protein for aesthetasc P2, and 1.6 nmol/min \cdot mg protein for aesthetasc S2. In aesthetasc S1 [Fig. 6(A)], aesthetasc P2 [Fig. 6(B)], and aesthetasc S2 [Fig. 6(C)], PMSF, TLCK, antipain, and leupeptin were equally effective at inhibiting BApNase activity (Fig. 6). In aesthetasc P2, where the CUB-serine protease is likely to be located (Levine et al., 2001), the four serine protease inhibitors decreased activity by ca. 40–80% [Fig. 6(B)]. This supports the idea that the isolated aesthetasc P2 contained serine protease activity from a trypsin-like protease. However, TLCK and EDTA did not have as much effect on BApNA hydrolysis in the aesthetasc P2 [Fig. 6(B)] as they did on the aesthetasc S2 [Fig. 6(C)], or even the P2 fraction of the lateral flagellum [Fig. 5(A)].

LApNA, a typical substrate for metallo- and cysteine amino-protease activity, and various protease inhibitors were used to detect the metallo- and cysteine-protease activity in the aesthetascs. High LApNase activity was detected in the aesthetasc tissue fractions: specific activity was 10.6 nmol/min \cdot mg protein in the aesthetasc S1, 5.3 nmol/min \cdot mg in the aesthetasc P2, and 10.7 nmol/min \cdot mg protein in the aesthetasc S2 (Table 2B).

Antipain (a serine/cysteine inhibitor) and 1,10-phenanthroline and EDTA (metalloprotease inhibitors) were effective at inhibiting LApNase activity in the aesthetasc soluble and membrane fractions (Fig. 6), as expected. However, serine protease inhibitors were surprisingly effective at inhibiting the metallo- and cysteine protease activity in the membrane fraction. TLCK and PMSF inhibited LApNase activity in the aesthetasc P2 by 80% [Fig. 6(B)]; these inhibitors may affect LApNA hydrolysis as they did in the lateral flagellum (Fig. 5). E64 and leupeptin (serine/cysteine protease inhibitors) had little effect on LApNase activity in the aesthetasc S2 [Fig. 6(C)], but were inhibitory in the aesthetasc P2 [Fig. 6(B)].

These results suggest that metalloprotease and cysteine protease activities are found in the soluble fraction of the isolated aesthetascs. Membrane proteolytic activity that is characterized by LApNA is more difficult to characterize, possibly because of possible crossreactivity either due to the substrate hydrolysis or inhibitor interactions. Metalloproteases appear to be present because activity is inhibited by EDTA and 1,10-phenanthroline. Cysteine proteases can hydrolyze LApNA, which could be contributing to the

Summary of inhibition of proteases in lobster olfactory tissue

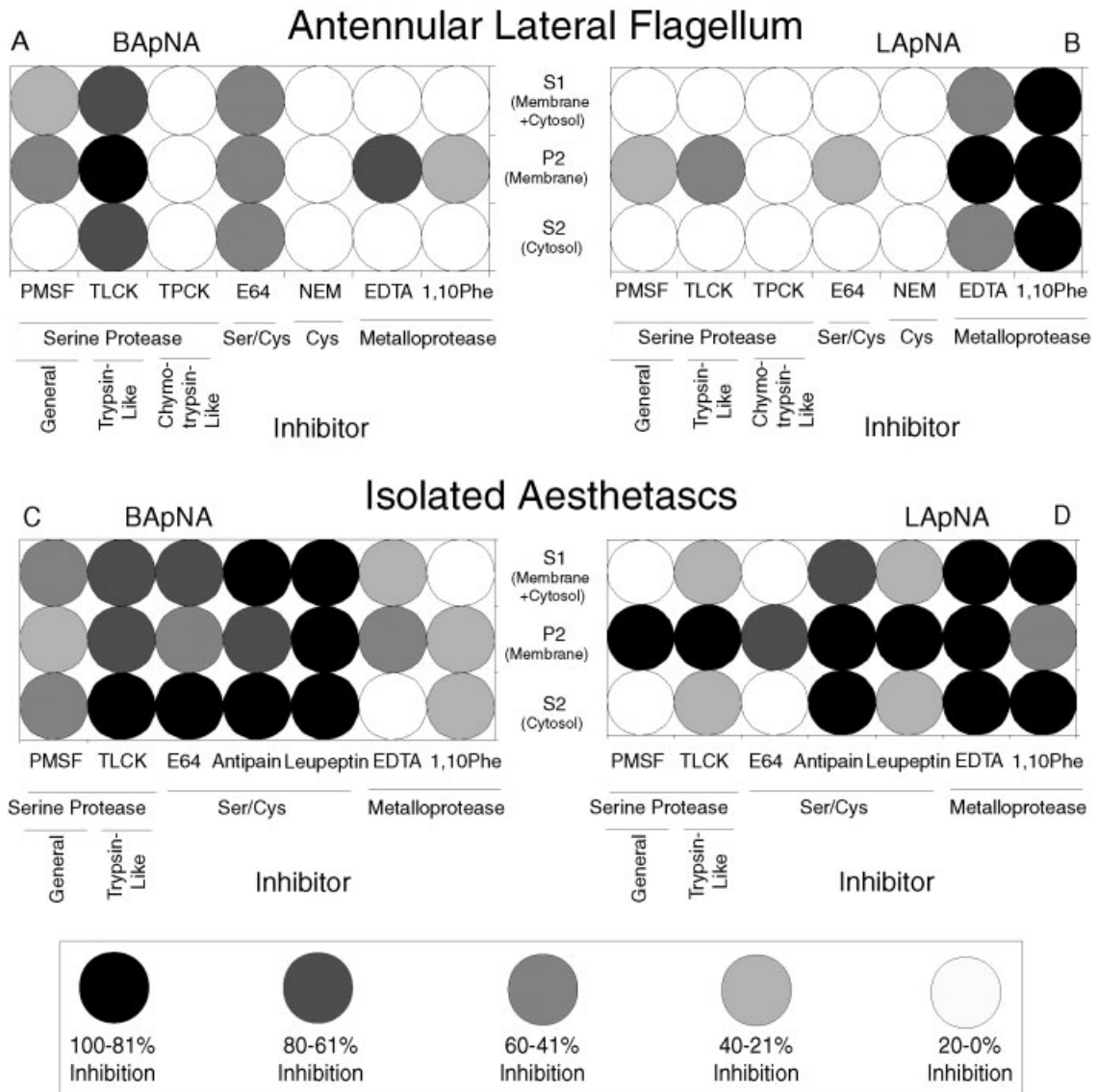


Figure 7 Summary of the effects of protease inhibitors on the hydrolysis of BApNA (A,C) and LApNA (B,D) by proteases in different tissue fractions (S1 = membrane + cytosol; P2 = membrane; S2 = cytosol) of the antennular lateral flagellum (A,C) and isolated aesthetascs (B,D). The efficacies of the protease inhibitors are derived from the data in Figures 4–6, and are expressed as % inhibition values in a gray scale with ranges of 100–81, 80–61, 60–41, 40–21, and 20–0% inhibition.

reduction of activity with the addition of E64, antipain, and leupeptin, while PMSF and TLCK could be crossreacting with these cysteine proteases. Conversely, LApNA may be crossreacting with serine proteases in the membrane, which would explain decreased activity with the addition of PMSF and TLCK, as well as E64, antipain, and leupeptin.

A summary of the activities of different protease inhibitors against the protease substrates BApNA and

LApNA and for aesthetasc tissue fraction is given in Figure 7(C) and (D).

Serine Protease Activity Due to Csp

To determine the contribution of Csp to the serine protease activity in aesthetasc tissue, we used an antibody—CUB 99-6 (Levine et al., 2001)—to immu-

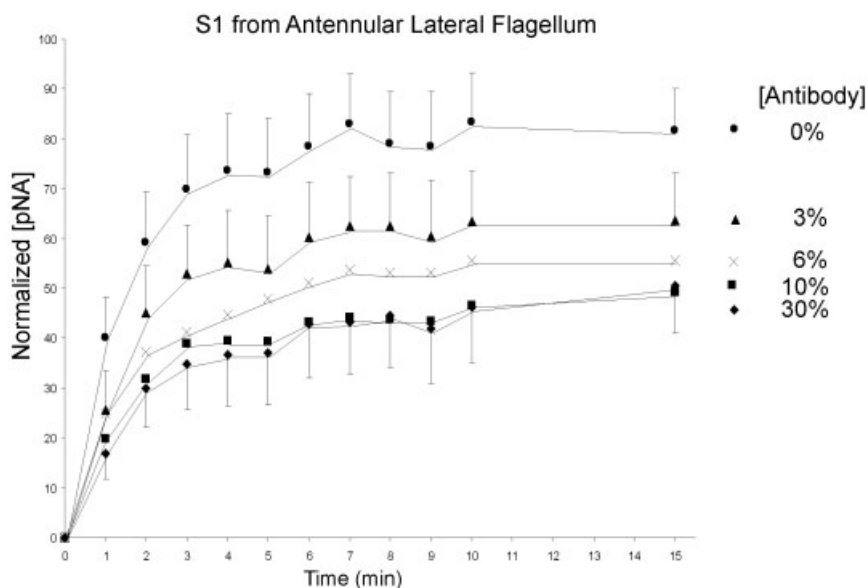


Figure 8 Contribution of Csp to the serine protease total activity for S3 fraction of isolated aesthetascs. The tissue was solubilized in 0.1% Triton X-100, and had added different dilutions of the CUB 99-6 antibody: Antibody dilution is expressed as a percentage of the purified, serum level, which is 14 $\mu\text{g}/\text{mL}$. Values are pNA values following the hydrolysis of 1 mM BApNA; shown are the means \pm S.E.M. for three homogenate preparations, each run in triplicate. All values were normalized to the maximum value within each homogenate.

noprecipitate Csp and then determine its effect on BApNase activity from aesthetasc tissue. Csp is thought to be a secreted protein that is anchored to the extracellular face of the dendritic membrane of ORNs via its CUB domain (Levine et al., 2001). If the CUB domain does in fact serve as a membrane anchor, the CUB domain may be relatively inaccessible to the antibody. This is supported by our preliminary experiments in which exposure of CUB 99-6 to nonsolubilized aesthetasc tissue did not reduce its BApNase activity. In an attempt to circumvent this problem by increasing access of the CUB domain to the antibody, the P2 membrane fraction of aesthetasc tissue was solubilized in Triton X-100. A Triton X-100 concentration series from 0.001 to 1% was tested to establish which Triton concentrations increase BApNase activity in this aesthetasc tissue; 0.01 and 0.1% Triton caused the greatest increase—a fourfold increase in the BApNase activity compared to tissue without Triton; 0.01% Triton caused a much greater increase in the BApNase activity in the P3 relative to the S3, whereas 0.1% caused a much greater increase in the BApNase activity in the S3 relative to the P3. Because in the immunoprecipitation experiment described in the following paragraph, we were interested in the activity remaining in the soluble S3 fraction after immunoprecipitation of Csp, we used 0.1% Triton.

Csp was removed from solubilized aesthetasc tissue via immunoprecipitation. The S2 aesthetasc tissue frac-

tion was solubilized in 0.1% Triton X-100 and exposed to a dilution series of CUB 99-6 with final antibody concentrations of 0, 3, 6, 10, and 30% of the serum levels. Centrifugation yielded a pellet (P3) with the Csp–CUB 99-6 complex and a supernatant (S3) containing any free Csp as well as all other proteases. BApNase activity was assayed for the S3 fraction of this antibody concentration series. BApNase activity was reduced by the Csp antibody in a time- and concentration-dependent manner (Fig. 8). At all antibody concentrations, maximum reduction of BApNase activity was reached near 7 min. When measured at 15 min, the reduction of BApNase was 22, 32, 40, and 38% in 3, 6, 10, and 30% CUB 99-6 levels, respectively, compared to the no-antibody control. Thus, maximum reduction was reached with 10% CUB 99-6, with no further change with 30% antibody. LApNA activity was not affected by the antibody (data not shown), implying that the CUB 99-6 antibody complexed specifically with a serine protease, presumably Csp, and did not affect other types of proteases. This is supported by our previous demonstrations of the antigen specificity of CUB 99-6 using Western gels (Levine et al., 2001). The fact that the reduction of BApNase activity saturated by 10% CUB 99-6 suggests that all Csp was complexed at this dose of antibody, that Csp accounts for at least 40% of the BApNase activity, and that a combination of other proteases account for as much as 60% of the BApNase activity.

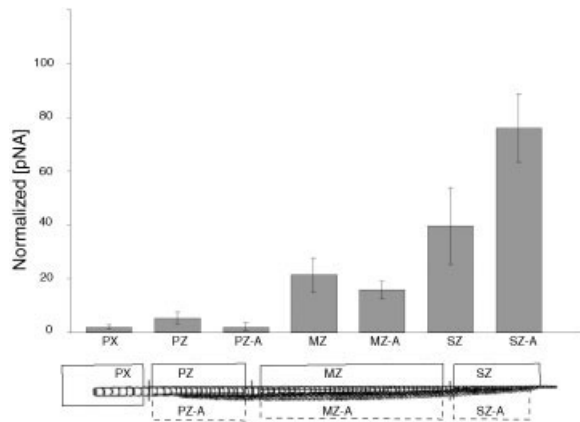


Figure 9 Serine protease specific activity for the S1 fraction of each of the developmental zones. Shown is the hydrolysis of 1 mM BAPNA for 2-h incubations at room temperature. Values are means \pm S.E.M. for three homogenate preparations for each developmental zone, each preparation run in triplicate. All values were normalized to the maximum value within each homogenate. MZ, mature zone; MZ-A, aesthetascs from the mature zone; PX, proximal zone; PZ, proliferation zone; PZ-A, aesthetascs from the proliferation zone; SZ, senescence zone; SZ-A, aesthetascs from the senescence zone.

Serine Protease Activity in the Developmental Regions of the Lateral Flagellum

To distinguish where the serine protease activity is highest in the lateral flagellum, the olfactory organ was separated into its developmental zones. These include: the proximal zone (PX), which is without aesthetascs; a proliferation zone (PZ), where new aesthetascs and their ORNs are added; a mature zone (MZ), where the new ORNs differentiate into functional forms and are odor responsive; and a senescence zone (SZ), which contains the oldest aesthetascs and where aesthetascs and their ORNs are eventually lost at molting (Steullet et al., 2000; Harrison et al., 2001a,b).

After separating the olfactory organ into these developmental zones, the aesthetascs were removed from lateral flagella, and these zones and their corresponding aesthetascs were homogenized separately. The S1 homogenates were assayed with BAPNA to compare serine protease activity in these tissues.

Developmental zones have statistically significantly different levels of BAPNase activity [ANOVA: $F(6, 66) = 12.038, p = 0.0000001$] (Fig. 9). BAPNase activity is lowest in the younger developmental zones and highest in the older zones. SZA has significantly greater values than all zones except the SZ, while SZ is greater than all zones except SZA and MZ (Duncan's post hoc tests, $p < 0.05$).

DISCUSSION

Our results show that functional serine protease activity occurs in the olfactory organ—the antennular lateral flagellum—of the spiny lobster *P. argus*. This serine protease activity includes trypsin-like activity, and is in part due to a CUB-serine protease (Csp) (Fig. 8), as anticipated from a previous demonstration of a Csp that has a trypsin-like protease domain and that associates with the dendritic membranes of olfactory receptor neurons (ORNs) (Levine et al., 2001). Our current results also reveal cysteine- and metalloprotease-like functional activities in the spiny lobster's olfactory organ. The enzymatic activity due to serine proteases present in lateral flagellar tissue is primarily located on the cell membranes, in contrast to metalloprotease activity, which is concentrated in the soluble fractions [Fig. 7(A,B)]. Trypsin-like serine protease activity is also prevalent in isolated aesthetasc (olfactory) sensilla on the lateral flagellum, particularly in the cell membrane (Levine et al., 2001) [Fig. 7(C,D)]. There is a developmental axis to serine protease activity on the lateral flagellum: it increases from a low in the proximal zone (which has no aesthetascs) and proliferation zone (with the youngest ORNs), to highest levels in the aesthetascs of the distal, senescence zone (which has the oldest ORNs) (Fig. 9).

A Plethora of Serine Proteases and Protease Inhibitors in the Lobster Olfactory Organ

Several serine proteases and serine protease inhibitors have been identified in the olfactory organ of the crustaceans *P. argus* and *Homarus americanus*. The first was a CUB-serine protease (Csp) (Levine et al., 2001), which is located in the aesthetasc sensilla along the entire length of the lateral flagellum of *P. argus*. There, it is expressed only in secretory cells, and it may associate with the dendrites of the ORNs. Other serine proteases and serine protease inhibitors or regulators have since been found in the olfactory organ of *P. argus* and *H. americanus* (Hollins et al., 2003; Stoss et al., 2003). One serine protease in *H. americanus*, OET-03, is a chymotrypsin that has little sequence similarity to Csp of *P. argus*; it has no CUB domain, but does have a signal sequence and it is expressed in secretory cells associated with the aesthetasc sensilla, suggesting that it is a secreted protein (Hollins et al., 2003). Another RNA transcript enriched in olfactory tissue of *H. americanus* is an $\alpha 2$ -macroglobulin (Hollins et al., 2003), which belongs to a family of proteins that regulate protease activity by controlling access of substrate to proteases

and the subcellular location of proteases (Armstrong and Quigley, 1999).

A serine protease (PET-5), a serpin protease inhibitor (PET-4), and a protease inhibitor in the Kazal family (PET-20) are enriched in the proliferation zone of the olfactory organ of *P. argus* where new olfactory sensory units (i.e., aesthetascs and their olfactory receptor neurons and auxiliary glial cells) are produced. Furthermore, PET-20 is upregulated by damage to the olfactory organ that causes cell death followed by enhanced cell proliferation (Stoss et al., 2004). PET-20 is expressed only in epithelial cells, and PET-3 and PET-4 are expressed in several cell types in the ORN lineage (Stoss et al., 2004). These results suggest that there may be a diversity of specific functions for these proteases and their regulators, as described in the next section.

Our observation that trypsin-like serine protease activity is present in isolated aesthetasc sensilla, particularly in the cell membrane fraction, is consistent with our finding that the Csp, a trypsin-like CUB-serine protease, is present in the aesthetascs (Levine et al., 2001). Furthermore, immunoprecipitation of Csp from aesthetasc tissue using a Csp-specific antibody demonstrates that Csp accounts for at least 40% of BApNase activity in aesthetasc tissue (Fig. 8). Csp might even account for more than 40% of the BApNase activity: if some of the Csp molecules in our homogenates had the serine protease domain dissociated from the CUB domain, then the serine protease domain would remain in the supernatant while the CUB domain only would be immunoprecipitated. In any case, Csp appears to make a major contribution to the serine protease activity in the olfactory organ.

Interestingly, solubilization of aesthetasc tissue increased BApNase activity. We believe that this is because treatment with Triton X-100 increases the access of the BApNA to the membrane-bound Csp and other serine proteases. It may do this by some combination of effects. At 0.01% Triton, the increased BApNase activity is in the pellet (containing membrane associated proteases); at this concentration, Triton may be extracting lipids from the cell membrane to which Csp is bound and thus allowing more unobstructed access of the substrate to the membrane-bound Csp. At 0.1% Triton, the increased BApNase activity is in the soluble fraction; at this concentration, Triton may be solubilizing the cytoskeleton such that Csp is no longer membrane-bound and thus resides in the supernatant. This suggests that Csp is normally associated with the cell membrane but its activity can be enhanced when it is free of the membrane. Regardless of the mechanism, this solubilization technique is important in making accessible the CUB to the CUB 99-6 antibody and

thus in immunoprecipitating Csp, thus allowing us to access Csp's contribution to the total serine protease activity.

Possible Functions of Olfactory Serine Proteases

Our characterization of serine protease enzymatic activity in the antennular lateral flagellum, and in aesthetascs in particular, is the first direct analysis of their function in this olfactory organ. Even though our results give some insights into possible functions of these serine proteases, their roles are still uncertain. Several possibilities have been previously suggested (Levine et al., 2001; Hollins et al., 2003), including a role in postembryonic development, such as genesis and differentiation of neurons and auxiliary glial cells, maintenance of function of the olfactory cells, including repair following damage, and apoptosis. These olfactory proteases might also function in perireception.

Serine proteases have been shown to be instrumental in the development of and repair following injury to various parts of the nervous system of vertebrates (Reinhard et al., 1988, 1994; Hogan, 1996; Thewke and Seeds, 1996; Akamatsu et al., 1997; Mehler et al., 1997; Nakayama, 1997; Marcinkiewicz et al., 1998; Shimizu et al., 1998; Yoshida and Shiosaka, 1999; Shou et al., 2000; Hooper et al., 2001; Lee et al., 2001; Sallés and Strickland, 2002). Serine proteases might play a similar role in the development and repair in the olfactory organ of crustaceans. Olfactory sensory units are continuously replaced throughout an animal's life (Steullet et al., 2000; Harrison et al., 2001a,b, 2003). This replacement includes birth of new olfactory sensory units in the proliferation zone, maintenance of olfactory sensory units in the mature zone, and death of olfactory sensory units in the senescence zone. This replacement can be modulated by damage and physiological state, demonstrating the importance of signaling molecules (Steullet et al., 2000; Harrison et al., 2001a,b, 2003, 2004). The nature of these signals is poorly understood, but there is evidence for both cell-cell contact signals and diffusible signals that function at a distance (Harrison et al., 2001a,b, 2003, 2004; Stoss, et al., 2004). Serine proteases could play important roles in such signaling, such as by activating pro-form enzymes or trophic factors, which then bind to receptors on epithelial cells, neuronal or glial precursor cells, or on ORNs and glial cells themselves.

An interesting observation that supports a possible function of serine proteases in regulation of some aspects of development is the developmental axis to serine protease functional activity. Although serine proteases are functionally expressed in aesthetascs along the entire antennular lateral flagellum, their

specific activity is lowest at the proximal end, increases distally, and is highest at the distal tip (Fig. 9). This correlation suggests that serine proteases might function in some developmentally related aspects of olfactory function. This could be birth, maturation, or death of the sensory units. Because we are able to experimentally modulate these developmental processes with damage (Harrison et al., 2001b, 2003, 2004), it should be possible to explore any causal links between serine proteases and olfactory development and function.

This gradient in functional activity of serine proteases in the lobster's olfactory organ might result from any of a number of mechanisms. First, it might be a consequence of a gradient in expression of serine proteases themselves. Some serine proteases are known to be expressed more in some developmental zones parts of the crustacean olfactory organ than others (Stoss et al., 2004), while others, such as Csp (Levine et al., 2001), are expressed all along the aesthetasc region of the olfactory organ. A second possible explanation for the gradient of functional expression along the developmental axis is a gradient in the expression of endogenous serine protease inhibitors. Serine protease inhibitors are known in the crustacean olfactory organ (Hollins et al., 2003), and some are expressed more highly in some developmental regions than in others (Stoss et al., 2004).

An alternative to a role of serine proteases in development is that they might function in perireception. In olfactory perireception, odorant stimuli are processed by molecules located in or around the dendritic membrane of ORNs, thus affecting which odorant molecules are active and how these odorants move in or out of the receptor environment (Getchell et al., 1984). For example, ATPase/phosphatase activity has been demonstrated in aesthetascs of *P. argus*, and these enzymes can modulate the concentrations of odorants such as AMP and ATP. The activity of these ectoenzymes affects responsiveness of ORNs sensitive to these odorants by facilitating recovery and limiting desensitization of ORNs (Carr et al., 1990; Gleeson et al., 1992). Additionally, such enzymes might generate active molecules from inactive odors, as suggested by Rittschof (1990, 1993) from behavioral studies on hermit crabs. The serine proteases examined in our study, which are active extracellularly, might act in a similar fashion.

CONCLUSION

Other work has demonstrated that a diversity of serine proteases and protease inhibitors are expressed in the olfactory organ of crustaceans. These molecules can

differ in their expression profiles, including the cell types that express them. Our study uses an enzyme functional assay to demonstrate serine protease activity in the spiny lobster's olfactory organ. Trypsin-like serine protease activity is particularly enriched in the membrane fraction of the olfactory sensory units (i.e., aesthetasc sensilla and their olfactory receptor neurons). One well-studied trypsin-like serine protease—a CUB-serine protease, Csp (Levine et al., 2001)—makes a substantial contribution to the serine protease activity in aesthetascs. This serine protease activity occurs along the entire olfactory organ, but it varies along the organ's developmental axis, with highest activity in the region of oldest cells where senescence and death of sensory units occur, and is lowest in the area of birth of olfactory sensory units. This finding suggests a role for the serine proteases in the development, maturation, and/or senescence of olfactory sensory units.

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