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Serpin-1 splicing isoform J inhibits the proSpätzle-activating proteinase HP8 to regulate expression of antimicrobial hemolymph proteins in *Manduca sexta*

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Abstract

The innate immune system of insects include the Toll pathway, which is mediated by an extracellular serine proteinase cascade. In the tobacco hornworm, *Manduca sexta*, hemolymph proteinase 8 (HP8) promotes the synthesis of antimicrobial proteins by cleaving proSpätzle, the putative ligand of *M. sexta* Toll. HP8 has been observed to form a complex in hemolymph with *M. sexta* serpin-1, which has multiple alternative splicing isoforms. To investigate the regulation of HP8 and its processing of proSpätzle, we characterized the interaction of recombinant HP8 with serpin-1 isoform J (serpin-1J). Recombinant serpin-1J formed an SDS-stable complex with HP8 *in vitro*. The association rate constant of serpin-1J and HP8 was $1.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$, with a stoichiometry of inhibition of 5.4. Serpin-1J inhibited the cleavage of proSpätzle by HP8. Injection of serpin-1J into *M. sexta* larvae resulted in decreased bacteria-induced antimicrobial activity in hemolymph and reduced expression of cecropin, attacin and hemolin mRNA in fat body. Altogether, these results suggest that serpin-1J functions to inhibit HP8 and thereby modulates the concentration of active Spätzle to regulate the Toll pathway response in *M. sexta*.

Keywords

serpin; clip domain serine proteinase; Toll signaling pathway; hemolymph; immunity

1. Introduction

The innate immune system in three different of insect orders (flies, beetles, and moths) has been demonstrated to include a serine proteinase cascade that results in proteolytic activation of a cytokine, Spätzle, the ligand which activates a transmembrane receptor, Toll [1-9]. Activation of Toll stimulates an intracellular signal transduction pathway, which activates rel family transcription factors, promoting expression of antimicrobial proteins [1,6,9]. Such extracellular serine proteinase cascades are often regulated by proteinase inhibitors from the serpin protein superfamily [10-12]. Most serpins are 40-60 kDa proteins, with a reactive center loop (RCL) located 30 to 40 residues from the carboxyl terminus that is exposed at the surface of the serpin. A target proteinase attacks the scissile bond (designated P1-P1') in the serpin

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RCL, forming a covalent acyl-intermediate and triggering a large conformational rearrangement, in which the RCL inserts into a β -sheet in the serpin and results in distortion of the proteinase active site [11,13]. The amino acid sequence of the RCL and particularly the identity of the P1 residue determine the selectivity of inhibition [11].

Serpins have a role in regulating the Toll pathway in innate immune responses of *Drosophila melanogaster* [14-16]; and a mosquito, *Aedes aegypti* [17], but inhibition of a specific proteinase by serpins in these pathways has not yet been demonstrated. In a beetle, *Tenebrio molitor*, *in vitro* experiments in a reconstituted Spätzle activation pathway have demonstrated that three serpins can selectively inhibit the three proteinases of this cascade [18].

In the tobacco hornworm, *Manduca sexta*, a proteinase cascade initiated in response to microbial infection includes hemolymph proteinase-6 (HP6), which activates hemolymph proteinase-8 (HP8), which then cleaves and activates proSpätzle, stimulating expression of several antimicrobial hemolymph proteins [8,9]. Both HP6 and HP8 contain an amino-terminal clip domain and a carboxyl terminal serine proteinase domain, an architecture common to many hemolymph proteinases that function in immune responses of arthropods [19]. HP6 is a putative ortholog of *D. melanogaster* Persephone, and HP8 is most similar to *D. melanogaster* Easter and Spätzle processing enzyme, all clip-domain proteinases that function in the Toll pathway [8].

We recently identified complexes of HP8 with serpin-1 in hemolymph samples from *M. sexta* [20]. The *M. sexta* serpin-1 gene encodes 12 variants, which differ in the sequence of the RCL due to mutually exclusive alternative splicing of 12 different versions of exon 9, encoding the carboxyl-terminal ~40 residues of the protein [21,22]. In 2D-PAGE analysis of hemolymph samples, HP8 was present in several spots that also contained serpin-1, at masses consistent with their identification as SDS-stable HP8-serpin-1 complexes [20], suggesting that that serpin-1 maybe regulate HP8 *in vivo*. One HP8-serpin-1 complex contained serpin-1 isoform J (serpin-1J). HP8 cleaves its substrate proSpätzle after Arg¹⁶⁹, and it hydrolyzes an artificial peptide substrate that has Arg as its P1 residue [8,9]. In the RCL of serpin-1J, Arg³⁴³ is the predicted P1 residue [22].

To further investigate the regulation of HP8 by serpin-1J, we examined the inhibitory activity of serpin-1J for HP8, and demonstrated that injection of recombinant serpin-1J into *M. sexta* larvae diminished the production of antimicrobial peptides in response to microbial exposure. Our results are consistent with a hypothesis that serpin-1J regulates HP8 and the production of active Spätzle to modulate the innate immune response.

2. Materials and methods

2.1. Insect rearing

M. sexta eggs originally purchased from Carolina Biological Supplies were used to establish a laboratory colony and reared as described previously [23].

2.2. Preparation of recombinant proteins

M. sexta serpin-1J was expressed in *Escherichia coli* strain XL1-blue using vector H6pQE-60, which encodes an amino-terminal 6×Histidine tag, and they were purified by nickel-affinity chromatography, as described previously [22]. They were then dialyzed against 20 mM Tris-HCl, pH 8.0, and applied to a pre-equilibrated Q-Sepharose™ Fast Flow column (1 ml) (Amersham Biosciences). After washing the column with the dialysis buffer, proteins were eluted with a step gradient of 50 mM, 150 mM, and 1 M NaCl in 20 mM Tris-HCl, pH 8.0. Fractions were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and those containing serpin-1J were pooled and stored at -80°C.

A recombinant form of proHP8 (proHP8_{Xa}), in which the cleavage activation site NNDR⁹⁰ was replaced with IEGR⁹⁰ to permit its activation by bovine Factor Xa, was expressed using *Drosophila* expression system, purified, and activated by Factor Xa as described previously [8,9]. From 350 mL of S2 cell culture medium, 60 µg of purified proHP8_{Xa} zymogen was obtained. This preparation contains a predominant band at 42 kDa corresponding to the proHP8_{Xa} zymogen and a minor band with an apparent mass of 37 kDa, which is an inactive truncated form of proHP8 (Fig. 1B) [8,9]. The full length proHP8_{Xa} accounted for approximately 80% of the total protein in the sample, based on band intensity in SDS-PAGE analysis, and this was taken into account in calculation of proHP8_{Xa} concentration for use in experiments.

2.3. Protein analysis

Protein concentrations were determined using Coomassie PlusTM Protein Assay Reagent (Pierce) with bovine serum albumin (BSA) (Sigma) as a standard. For SDS-PAGE and immunoblot analysis, protein samples were treated with SDS sample buffer containing β-mercaptoethanol at 95°C for 5 min and then separated using 4-12% NuPAGE Bis-Tris gels (Invitrogen). Proteins were detected by staining with Coomassie Brilliant Blue or silver nitrate. For immunoblot detection, proteins were transferred onto a nitrocellulose membrane and detected with rabbit antisera to *M. sexta* HP8 or serpin-1 (each diluted 1:2000) or Spätzle (diluted 1:1000). Antibody binding was visualized using alkaline phosphate-conjugated goat anti-rabbit IgG and an alkaline phosphate substrate kit (Bio-Rad).

2.4. Formation of SDS-stable complexes between HP8 and serpin-1J

Factor Xa-activated HP8_{Xa} (50 ng) was mixed with serpin-1J at a molar ratio of 10:1 (serpin-1J/proHP8_{Xa}). In control samples, proHP8_{Xa} or factor Xa were omitted from the mixture. After incubation at room temperature for 10 min, the reaction mixtures were analyzed by immunoblotting using antiserum against serpin-1 or HP8.

2.5. Inhibition of HP8_{Xa} amidase activity by serpin-1J

Preliminary experiments carried out to identify a suitable colorimetric substrate for detecting activity of HP8_{Xa} led to selection of acetyl-Ile-Glu-Ala-Arg-*p*-nitroanilide (IEAR_pNa), which was hydrolyzed by HP8_{Xa} with the least interfering activity arising from inclusion of factor Xa in the mixture (Table S1). ProHP8_{Xa} (50 ng) was activated by treatment for 6 h with bovine factor Xa [9] and then mixed with 200 µl of 50 µM IEAR_pNa in 0.1 M Tris-HCl, 0.1 M NaCl, 5 mM CaCl₂, pH 8.0. Absorbance at 405 nm was measured continuously for 20 min. One unit of activity was defined as ΔA₄₀₅ = 0.001/min. In control assays, proHP8_{Xa} was omitted from the mixtures. Amidase activity of HP8_{Xa} was defined as the activity of HP8_{Xa} in the presence of Factor Xa minus the activity of Factor Xa alone. There was no significant inhibition of amidase activity of factor Xa by serpin-1J (Fig. S1).

For inhibition assays, serpin-1J was incubated with factor Xa-activated HP8_{Xa} at different molar ratios in 25 µl of 20 mM Tris-HCl and 50 mM NaCl, pH 8.0. In control reactions, proHP8_{Xa} was omitted from the mixture. After incubation at room temperature for 10 min, residual amidase activity was measured as described above.

The association rate constant for inhibition of HP8 by serpin-1J was determined under pseudo-first order conditions using the progress curve method [24]. Activated HP8_{Xa} (1.3 pmol) was mixed with different concentrations of serpin-1J and 0.3 mM IEAR_pNa. Product formation was monitored as absorbance at 405 nm using a PowerWave XS microplate reader (Bio-Tek Instrument, Inc.). Progressive curves were analyzed and second-order associate rate constant (*k_a*) was calculated as described previously [24,25].

2.6. Inhibition of HP8_{Xa} by serpin-1J with proSpätzle as substrate

Factor Xa-activated HP8_{Xa} (25 ng) was mixed with serpin-1J at different concentrations. After incubation at room temperature for 10 min, the reaction mixtures were incubated with recombinant proSpätzle [8,9] at 37°C for 60 min. The mixtures were then analyzed by SDS-PAGE and immunoblotting using antiserum against *M. sexta* Spätzle.

2.7. Effects of serpin-1J on expression of bacteria-induced hemolymph proteins

Day 0, fifth instar larvae were injected with serpin-1J (200 µl/larva, 1 µg/µl) or with BSA (200 µl/larva, 1 µg/µl) as a control. After 30 min, a subset of these larvae was injected again with *Micrococcus luteus* ATCC 4698 (Sigma, 50 µl/larva, 2 ng/µl). Twenty h later, fat body and hemolymph samples were collected. Total RNA samples were prepared from fat body, and cDNA was prepared as described previously [8]. Cell-free hemolymph samples were heated at 95°C for 5 min to remove most high molecular weight proteins and then centrifuged at 10,000×g for 5 min. The supernatant was stored at -20°C. Assay of antimicrobial activity, identification of plasma proteins by mass spectrometry, and quantitative real-time PCR were carried out as described previously [8].

3. Results

3.1. Serpin-1J can inhibit HP8

We isolated recombinant serpin-1J and proHP8_{Xa} (Fig. 1A and 1B) for use in experiments to investigate their interactions. The formation of an SDS-stable complex of a serpin with a proteinase it inhibits is a characteristic feature of a serpin-proteinase reaction [26]. To test whether serpin-1J form such a complex with HP8, we mixed recombinant serpin-1J with factor Xa-activated HP8_{Xa} and detected the appearance of a higher molecular weight complex by immunoblotting, using antisera to HP8 and serpin-1 (Fig. 1C and 1D). In the absence of factor Xa, anti-HP8 antibodies recognized the 42-kDa proHP8_{Xa} zymogen and a 37-kDa truncated inactive HP8 present in the recombinant preparation [9]. After activation with factor Xa, the proHP8_{Xa} zymogen band disappeared, and the 34 kDa HP8 catalytic domain was detected by the HP8 antibody. When serpin-1J was mixed with active HP8_{Xa}, the 34-kDa catalytic domain was not detected, but a new immunoreactive band at ~66-kDa (the expected mass of a serpin-1J/HP8_{Xa} complex) was observed (Fig. 1C). This complex was also recognized by antibody to serpin-1 (Fig. 1D). This result indicates that serpin-1J can form a covalent complex with HP8_{Xa}.

To further investigate the inhibition of HP8_{Xa} by serpin-1J, we tested its ability to inhibit hydrolysis of a colorimetric peptide substrate by HP8_{Xa}. HP8_{Xa} activity decreased linearly as serpin-1J concentration increased (Fig. 2A). The stoichiometry of inhibition [11,24] was 5.4, indicating that under the conditions tested, serpin-1J preferentially acts as a substrate rather than an inhibitor of HP8.

3.2. Kinetics of the interaction between HP8_{Xa} and serpin-1J

We determined the apparent second order association rate constant (k_a) for the inhibition of HP8_{Xa} by serpin-1J to be $1.3 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 2B). The second order rate constant for association of serpin and proteinase to form the acyl enzyme complex, corrected with consideration of the stoichiometry of inhibition [11], is $7.0 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. In a time-course analysis to detect the formation of the SDS-stable complex between HP8_{Xa} and serpin-1J (Fig. 3), we observed that a significant amount of the complex could be detected as a 66-kDa band reacting with HP8 antibody by one minute after mixing the serpin and proteinase. This band increased in intensity by three min and then remained unchanged through ten min of incubation. The 34-kDa band representing the catalytic domain of HP8 decreased in abundance by one

min and had disappeared by three min. These results are consistent with the complete conversion of active HP8 to an inhibited complex with serpin-1J within three min under the tested conditions (1.7 μ M serpin-1J).

3.3. Serpin-1J blocks the activation of proSpätzle by HP8 and inhibits microbe-induced expression of antimicrobial proteins

HP8 cleaves and activates the cytokine proSpätzle, leading to induced expression of antimicrobial proteins that are secreted into the hemolymph [8,9]. To investigate the inhibition of HP8 activity against this natural substrate, we incubated proSpätzle with active HP8_{Xa} in the absence or presence serpin-1J (Fig. 4). As observed previously [9], active HP8_{Xa} efficiently cleaved recombinant proSpätzle, converting the 38-kDa proSpätzle to a 12-kDa product corresponding to the active carboxyl-terminal fragment, Spätzle-C108. Serpin-1J inhibited this processing of proSpätzle in a concentration-dependent manner (Fig. 4).

If serpin-1J regulates HP8 activity *in vivo*, increased serpin-1J concentration in hemolymph would be predicted to result in decreased activation of proSpätzle during an immune response, resulting in diminished up-regulation of acute phase response genes activated by the Toll pathway. To test this hypothesis, we injected larvae with serpin-1J or with BSA as a control and then observed the subsequent innate immune response to an injection of a Gram-positive bacterium, *M. luteus* (Fig. 5). Injection of bacteria stimulated a strong increase in hemolymph antimicrobial activity, assayed against *E. coli*. Pre-injection of larvae with serpin-1J significantly decreased this innate immune response (Fig. 5A).

To evaluate the effect of serpin-1J injection on expression of specific antimicrobial peptides, we analyzed heat-stable plasma proteins by SDS-PAGE and peptide mass fingerprinting using MALDI-TOF mass spectrometry (Fig. 5B, 5C, and Table S1). Bands at approximately 24 kDa and 4 kDa (identified respectively as attacin-1 and a mixture of cecropin A and B) were present at significantly greater intensity in hemolymph from larvae injected with bacteria than in controls. In larvae pre-injected with serpin-1J before treatment with bacteria, the intensity of these bands for attacin and cecropins was significantly decreased.

Injection of *M. luteus* stimulated a strong increase in mRNA level for several genes encoding hemolymph proteins that function in the antibacterial response, including the antimicrobial proteins attacin-1, cecropin-6, lysozyme, and a pattern recognition protein, hemolin (Fig. 5D). Pre-injection of larvae with serpin-1J significantly decreased this response for attacin-1, cecropin-6, and hemolin, but not for lysozyme. These results indicate that increasing the concentration of serpin-1J in hemolymph decreases the production of cecropin, attacin, and hemolin after immune challenge in *M. sexta*, consistent with a role for serpin-1J in regulating the activity of HP8 and processing of proSpätzle during immune responses in *M. sexta*.

4. Discussion

Serpins in blood plasma regulate serine proteinase cascades in vertebrate animals and in arthropods [10,27-29]. Genetic analysis has demonstrated that three serpins (Spn43Ac, also known as Necrotic, Spn77Ba, Spn5) function in regulating the Toll pathway in innate immune responses of *D. melanogaster* ([14-16]. Mutations in these serpin genes result in constitutive expression of Drosomycin, an antimicrobial peptide whose gene is normally up-regulated through proteolytic activation of Spätzle and signaling through the Toll pathway. However, the proteinases inhibited by these serpins have not yet been identified. In a mosquito, *A. aegypti*, depletion of serpin-2 by RNA interference can increase expression of Toll pathway-dependent immune genes [17]. Yeast two-hybrid experiments suggest that *A. aegypti* serpin-2 can interact with proteinase CLIPB9, which may have a role in the Toll pathway [30], but inhibitory activity of serpin-2 for this proteinase has not yet been demonstrated. A proteinase

cascade pathway for activation of Spätzle in the immune response of a beetle, *T. molitor*, has been well characterized biochemically [5,6,31]. Three *T. molitor* serpins have been identified that each can inhibit one of the three proteinases of this cascade [18]. These serpins blocked activation of Spätzle in a reconstituted pathway *in vitro* [18]. However, injection of the serpins into larvae did not affect the expression of antimicrobial peptides induced after injection of bacteria.

In several insect species and in nematodes, serpin genes have been identified that employ mutually exclusive alternative splicing to produce serpin isoforms with a common body but variable sequences in the RCL, resulting in inhibitors of diverse inhibitory selectivity from a single gene [21,32-36]. The *M. sexta* serpin-1 gene produces 12 such variants, which differ in sequence of the RCL and have a broad array of inhibitory activities [20-22]. Immune challenge does not stimulate increased transcript levels for any of the variants [20]. However, although *M. sexta* serpin-1 has been quite useful as a model serpin for structural studies [37,38], the physiological functions of the serpin-1 splicing variants are not well understood. Serpin-1J, which has Arg as its P1 residue, has previously been shown to inhibit prophenoloxidase-activating proteinase-3 [39] and to form a covalent complex in hemolymph with HP8 [20].

In this work, we have investigated *M. sexta* serpin-1J as an inhibitor of HP8, a proteinase that activates Spätzle as an immune response. In *M. sexta*, microbial exposure stimulates activation of proHP6 (an ortholog of *Drosophila* Persephone), and HP6 then cleaves and activates proHP8 [8]. HP8 cleaves proSpätzle to liberate the active C-terminal C-108 fragment, which exists as a homodimer and stimulates expression of several antimicrobial protein genes [9], presumably through binding to a Toll receptor in fat body. It is likely that the proteinases in this pathway are regulated by serpins present in *M. sexta* plasma. Seven serpins have been identified in *M. sexta* hemolymph so far [28]. Immuno-affinity purification of serpin-proteinase complexes from hemolymph resulted in detection of complexes of HP6 with serpin-4 and serpin-5 [40]. Similar experiments have demonstrated complexes of HP8 with serpin-6 [25] and with and with serpin-1 [20].

The stoichiometry of inhibition of ~5 for serpin-1J with HP8 suggests that serpin-1J partitions into inhibition rather than the substrate pathway in 1 out of 5 interactions with HP8 under the conditions tested in our experiments. At the concentrations of these proteins in *M. sexta* hemolymph, serpin-1J may be a physiologically relevant regulator of HP8. Total serpin-1 concentration in *M. sexta* plasma varies between 0.2-0.6 mg/ml [41]. We estimate based on two-dimensional gel electrophoresis analysis that serpin-1J accounts for approximately 5% of the total serpin-1 in plasma (E.J. Ragan and M.R. Kanost, unpublished results), or 10-30 µg/ml (0.2 – 0.6 µM). The proHP8 zymogen concentration in larval hemolymph is approximately 10 µg/ml [8]. As it seems unlikely that all of the proHP8 zymogen would be activated during a natural immune response, the concentration of active HP8 would be somewhat lower than 0.2 µM. At a natural concentration of 0.5 µM serpin-1J in larval hemolymph, the *in vivo* half-life of HP8 due to inhibition by serpin-1J, calculated as $1/(k_a \times [I])$, is ~ 2 min, suggesting that serpin-1J could rapidly inhibit HP8 under physiological conditions. This rate of inhibition is similar to the observed formation a covalent complex of purified serpin-1J with HP8 *in vitro* within 1-3 min (Fig. 2B). These results are consistent with the detection of HP8-serpin-1J complex formed in *M. sexta* plasma [20].

Serpin-1J was able to inhibit the action of HP8 in cleaving its natural substrate proSpätzle *in vitro* (Fig. 4). We carried out *in vivo* experiments to test whether increased concentration of serpin-1J would lead to diminished expression of antimicrobial hemolymph proteins, predicted to occur if serpin-1J inhibits HP8 and thereby results in a lower concentration of active Spätzle. Elevation of hemolymph serpin-1J concentration approximately 10-fold through injection of recombinant serpin-1J resulted in significant decreases in hemolymph antimicrobial activity

and in decreased protein and mRNA levels for individual antimicrobial proteins after injection of bacteria (Fig. 5). This result, somewhat analogous to a genetic “overexpression” experiment, provides further evidence that serpin-1J may be a physiologically significant regulator of HP8 during immune responses *in vivo*.

This study provides evidence that serpin-1J may function to regulate HP8 in the proteinase cascade leading to production of active Spätzle. However, target proteinases for the remaining eleven splicing isoforms remain to be discovered. Identification of serpin-1K in a hemolymph complex with a digestive chymotrypsin [20] suggests that some isoforms may play a protective role as inhibitors of such potentially destructive proteinases that may gain access to the hemocoel. Additionally proteinases made by microbial pathogens may be potential targets that could provide a stimulus for evolution of insect hemolymph serpins with a broad range of inhibitory selectivities. Future research is needed to identify the functions of the multiple serpin variants and the proteinases they inhibit under natural conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BSA	bovine serum albumin
HP6	hemolymph proteinase 6
HP8	hemolymph proteinase 8
IEAR_pNa	acetyl-Ile-Glu-Ala-Arg- <i>p</i> -nitroanilide
SDS-PAGE	SDS-polyacrylamide gel electrophoresis

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Fig.1.

(A-B) SDS-PAGE analysis of purified recombinant serpin-1J and proHP8_{Xa}. (A) Purified serpin-1J (1.0 µg) was analyzed by SDS-PAGE under reducing conditions followed by coomassie blue staining. (B) Purified proHP8_{Xa} (75 ng) was analyzed by SDS-PAGE under reducing conditions followed by silver staining. (C-D) SDS-stable complex formation between serpin-1J and HP8. ProHP8_{Xa} (50 ng) was activated by factor Xa and then incubated for 10 min at room temperature with a 10-fold molar excess of serpin-1J. In control reactions, proHP8 or factor Xa were omitted. The samples were analyzed by SDS-PAGE and immunoblotting using antibodies to HP8 (C) or serpin-1 (D). Serpin-1 antibodies recognized intact serpin-1J (~43 kDa) and a lower band (~42 kDa) that is a serpin-1J degradation product (cleaved serpin). HP8 antibody recognized the 42 kDa proHP8_{Xa} zymogen, a 34 kDa catalytic domain produced upon activation, and an inactive, truncated proHP8_{Xa} that is present in the preparation [9]. Both antibodies recognized a band at ~66 kDa, representing a serpin-HP8 complex. Size and positions of molecular mass standards are indicated to the *left* of each blot.

Fig.2.

Analysis of serpin-1J and HP8 interactions. (A) Stoichiometry of inhibition of HP8 by serpin-1J. Recombinant serpin-1J was incubated with Factor Xa-activated HP8_{Xa} at different molar ratios for 10 min at room temperature. The residual amidase activity was measured using IEARpNA as substrate. The intersection of a line generated by linear regression extrapolated to the X axis occurred at a molar ratio of 5.4. (B) Kinetic analysis of serpin-1J and HP8 interactions. Activated HP8_{Xa} (6.5 nM) was mixed with 300 μ M IEARpNa and serpin-1J at 0 (■), 16.3 (□), 32.5 (▼), 65 (▽), 195 (◆), 325 (◇), and 530 (▲) nM. The progress of enzyme inactivation at each concentration of serpin-1J was followed by measuring the ΔA_{405} of the reaction every 49 sec (*inset*). Pseudo-first-order rate constants of inhibition of HP8_{Xa} (k) and the second-order association rate constant (k_a) was calculated as described in Experimental Procedures.

Fig.3.

Rate of formation of an SDS-stable complex between HP8 and serpin-1J. Activated HP8_{Xa} (0.17 μ M) was incubated with serpin-1J (1.7 μ M) at room temperature for 0, 1, 3, 5, 8, and 10 min. The reaction mixtures were separated by SDS-PAGE, followed by immunoblot analysis with antiserum against *M. sexta* HP8. Positions of molecular weight standards are indicated on the *right*.

Fig.4.

Serpin-1J inhibits the activation of purified proSpätzle by HP8_{Xa}. ProHP8_{Xa} (25 ng) was activated by Factor Xa (50 ng), and then incubated for 10 min at room temperature with serpin-1J at molar ratios indicated at the *bottom*, then incubated with proSpätzle (100 ng) at 37°C for 1 h. The mixtures were subjected to SDS-PAGE and immunoblotting using Spätzle antibodies. The sizes and positions of molecular weight standards are indicated on the *left*. Bands representing proSpätzle and the active cysteine-knot domain (Spätzle-C108) are marked by *arrows*.

Fig.5.

Effect of injection of serpin-1J on the expression of bacteria-induced hemolymph proteins. Fifth instar day 0 larvae were injected with 200 μ g serpin-1J or BSA. After 30 min, a subset of these larvae was injected with 100 ng of *M. luteus*. After 20 h, hemolymph was collected and fat body RNA samples were prepared from each insect, for assay of plasma antimicrobial activity and mRNA levels for bacteria-induced hemolymph proteins. (A) Antimicrobial activity of plasma assayed against *E. coli*, (B) Identification of plasma antimicrobial peptides by SDS-PAGE and MALDI-TOF peptide mass fingerprinting, (C) Quantitative analysis of attacin and cecropin band intensity by densitometry, (D) mRNA levels for indicated genes assayed by quantitative RT-PCR relative to ribosomal protein S3 mRNA level as described in Experimental Procedures. In A, C, and D, bars represent mean $\pm$ S.D. (n=3). Bars labeled with different letters are significantly different (one-way ANOVA followed by the Newman-Keuls test, $P < 0.05$).