Characterization and Functional Analysis of 12 Naturally Occurring Reactive Site Variants of Serpin-1 from *Manduca sexta*

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Serpin gene-1 from the tobacco hornworm, *Manduca sexta*, encodes, through alternative exon usage, 12 reactive site variants (Jiang, H., Wang, Y. and Kanost, M. R., (1994) *J. Biol. Chem.* 269, 55–58; Jiang, H., Wang, Y., Huang, Y., Mulnix, A. B., Kadel, J., Cole, K., and Kanost, M. R. (1996) *J. Biol. Chem.* 271, 28017–28023). These 43-kDa proteins differ from each other only in their COOH-terminal 39–46 residues, which include the reactive site. To test the hypothesis that these proteins are proteinase inhibitors of diverse selectivities and to begin to elucidate their physiological functions, we expressed the 12 serpin-1 variants in *Escherichia coli*. Seven of the variants inhibited mammalian serine proteinases, with association rate constants comparable with those of human serpins. Serpin-1A, with a P1 Arg residue, inhibited both trypsin and plasmin. Serpin-1B (P1 Ala) and serpin-1F (P1 Val) inhibited porcine pancreatic elastase and human neutrophil elastase. Serpin-1H, -1K, and -1Z, all with a Tyr residue at the P1 position, inhibited chymotrypsin and cathepsin G. Serpin-1I (P1 Leu) inhibited both elastase and chymotrypsin. Nine of the serpin variants were active as inhibitors of microbial serine proteinases, including subtilisin *Carlsberg*, proteinase K, and two proteinases secreted by an entomopathogenic fungus, *Metarhizium anisopliae*. In addition, one of the serpin variants, serpin-1J, strongly inhibited activation of *M. sexta* hemolymph phenoloxidase, a pathway involving a serine proteinase cascade. This pathway is a component of the defensive response of insects to microbial infection. These results suggest that the products of *M. sexta* serpin gene-1 may be important in regulating both exogenous and endogenous serine proteinases in hemolymph.

The serpin superfamily is composed of a large number of proteins, most of which function as serine proteinase inhibitors (1). Serpins have been identified in animals, plants, and viruses (2) and therefore appear to have evolved from a common ancestor before divergence of the animal and plant kingdoms. Individual mammalian species have a large number of serpin genes, each encoding a protein with a unique reactive site sequence and physiological function (3). Members of the serpin superfamily are involved in the regulation of a wide variety of physiological processes, including blood clotting, complement activation, inflammatory responses, hormone transport, and tumor suppression (1).

Serpins are typically 370–390 amino acid residues long, with a reactive site loop near the carboxyl terminus. This loop, exposed on the surface of the molecule, is the site at which a serpin interacts with a target serine proteinase (1). During reaction with a proteinase, the P1 residue (4) in the reactive site loop forms a bond with the active site serine residue in the enzyme. The amino acid sequence and conformation of the reactive site loop largely determine the specificity of inhibition. Altering the loop sequence, especially at the P1 position, can cause dramatic changes in the inhibitory selectivity of a serpin (3).

Serpins have been found in the hemolymph of invertebrates, including three groups of arthropods: insects, crayfish, and horseshoe crabs (5). These invertebrate serpins have 12–30% amino acid sequence identity with various mammalian serpins. Functions of invertebrate serpins are largely unknown, although they may regulate proteinases released from blood cells during inflammation-like processes and may regulate proteinase cascades that ultimately activate proteins involved in blood coagulation and melanization.

Study of serpins from hemolymph of a lepidopteran insect, *Manduca sexta* (tobacco hornworm), has revealed a novel genetic mechanism for generating diversity in the serpin reactive site loop (6–8). Analysis of more than 50 *M. sexta* serpin cDNAs and their corresponding genomic sequence has shown that 12 serpin variants (named serpin-1A through -1K and -1Z) are produced from a single gene. These serpins are identical in the amino-terminal 336 residues and differ in their carboxyl-terminal 39–46 residues. This variable region includes the reactive site loop. The variable region is encoded by the ninth exon of the gene, which is present in 12 alternate forms between exons 8 and 10 (8). Alternative splicing to allow only one exon 9/molecule of mature serpin-1 mRNA agrees well with observed structures of *M. sexta* serpin-1 cDNAs (7) and with analogous primary structures found in two silkworm serpins (9).

Because the *M. sexta* serpin-1 variants differ in the amino acid sequences of their reactive site loops, they were predicted to be inhibitors of diverse selectivity with the potential to regulate proteinases in various physiological processes (7). We report here the inhibitory activities of the 12 serpin-1 variants against a panel of mammalian, fungal, and bacterial proteinases as a first step toward understanding physiological functions of these serpins.

**EXPERIMENTAL PROCEDURES**

Plasmid Construction, Protein Expression, and Affinity Purification of *Manduca Serpins*—A recombinant plasmid expressing *M. sexta* serpin-1B cDNA, serpin-1B/H6pQE-60 (10), was used as a starting point in constructing plasmids for expressing the other 11 serpin-1 variants. A cDNA fragment from the HindIII site upstream of the variable region to another restriction site in the vector was substituted with the corresponding fragment from each donor plasmid. Because vectors and re-
striiction sites for some serpin cDNAs were different, the cloning schemes (Fig. 1) are explained as follows. (i) Serpin-1A, -1D, -1E, -1G, -1J, -1K, and -1Z cDNAs, directionally cloned in EcoRI-XhoII sites of pBluescript (SK) (7), were shifted into the expression plasmid by replacement of a HindIII-PvuII fragment. (ii) Serpin-1P and -1JcDNA (Fig. 1A), located in EcoRI sites of pBluescript (SK) (16), were transferred into the expression plasmid by nondirectional HindIII cloning. (iii) Serpin-1C, -1H, and -1I cDNA fragments were isolated from a polymerase chain reaction-derived serpin-1 cDNA mini-library (8) and then switched into the expression plasmid by replacement of a HindIII-PvuII fragment. Plasmid constructs were confirmed by sequence analysis using a specific primer, 5'-AGCTTAAGTGCAACCTG-3', located at the HindIII site upstream of the variable region. The fusion proteins expressed from these constructs all have an NH2-terminal sequence of Met-His-His-His-His-His-Ala-Met-Ala-Gly-Glu-Thr-Asp. The first 7 residues are encoded by the vector, whereas the rest are from the serpin-1 cDNA, including the last 3 residues of the signal peptide (Ala-Met-Ala) and the complete mature protein sequences. Procedures for expression and N14 affinity purification of the recombinant serpins were described previously (10).

**Inhibitory Activity Assay**—To measure the inhibitory activities, 10 l of a serine proteinase were incubated with a serpin (5 l, 1 mg/ml) in 0.1 M Tris-HCl, pH 8.0, in a cuvette at room temperature for 5 min. Then 0.7 ml of an appropriate chromatographic substrate solution (50 l in 50 mM Tris-HCl buffer, pH 7.8) was added with gentle mixing. After centrifugation at 10,000 x g for 5 min prior to assay of phenoloxidase activity.

**Preparation of Prophenoloxidase (PPO) Activation Fraction from Hemolymph**—Hemolymph from cut prolegs of six day 2 fifth instar larvae was collected (approximately 10 ml/larva) into chilled polypyrrole tubes, each containing 750 ml of anticoagulation saline (14). Hemocytes were pelleted by centrifugation at 10,000 x g for 10 min. The supernatants were combined and an equal volume of saturated ammonium sulfate (pH 7.0) was slowly added with gentle mixing. After centrifugation at 10,000 x g for 10 min, the pellet was dissolved in 900 ml of chilled water (one-third of the initial hemolymph volume) and stored in aliquots at −70°C.

**Activation of Prophenoloxidase**—Ten l of elictor (Micrococcus lysodeikticus ATCC 4698, 1 mg/ml; Sigma) was mixed with 100 l of 1.5 diluted PPO activation fraction. At intervals, an aliquot (10 ml) was assayed for phenoloxidase activity (15). To test for direct inhibition of phenoloxidase activity, phenoloxidase was activated by treatment with the detergent cetylpyridinium chloride as described by Hall et al. (15) and then incubated with serpin-1J at a final concentration of 500 l/ml for 5 min prior to assay of phenoloxidase activity.

**RESULTS**

**Plasmid Construction, Expression, and Affinity Purification of the 12 Serpins—cDNAs for all 12 of the cDNA constructs were used to reconstitute all 12 of the cDNA variants by substituting an equivalent restriction fragment from each variant cDNA (see “Experimental Procedures”). Open bar, plasmid vector; filled bar, constant regions of Manduca sexta serpin-1 cDNA; shaded bar, the region of cDNA corresponding to exon 9; cross-hatched bar, vector sequence that differs, depending on how the original variant cDNA was cloned. X represents an EcoRI site for clones expressing serpin-1 variants B, F, and J; Y represents an Xhol site for clones expressing serpin-1 variants A, C, D, E, G, H, J, K, and Z.

![FIG. 1. Recombinant plasmids for expressing M. sexta serpin-1 variants. A cDNA for serpin-1B in expression vector H6pQE-60 was used to reconstitute all 12 of the cDNA variants by substituting an equivalent restriction fragment from each variant cDNA (see “Experimental Procedures”). Open bar, plasmid vector; filled bar, constant regions of Manduca sexta serpin-1 cDNA; shaded bar, the region of cDNA corresponding to exon 9; cross-hatched bar, vector sequence that differs, depending on how the original variant cDNA was cloned. X represents an EcoRI site for clones expressing serpin-1 variants B, F, and J; Y represents an Xhol site for clones expressing serpin-1 variants A, C, D, E, G, H, J, K, and Z.](image-url)
reactive site loop. Coexpression of chaperones GroEL and GroES did not increase solubility of serpin-1A, which suggests that the solubility is affected by properties of the reactive site loop rather than by global folding failure. Factors such as expression rate or serpin polymer formation may have effects on the solubility of these recombinant serpins (16).

The soluble fusion proteins extracted from E. coli were recovered in one step to over 80% purity by a Ni²⁺ affinity batch method, as illustrated for serpin-1B (Fig. 2). The amino-terminal sequences of the recombinant serpins were determined by Edman degradation to be identical to the expected sequences.

Inhibitory Activity of the Recombinant Serpins—To screen the inhibitory activity of the M. sexta serpins, we tested for the inhibition of mammalian digestive enzymes (trypsin, chymotrypsin, and elastase) and several serine proteinases from human blood (plasmin, cathepsin G, and neutrophil elastase). The results, summarized in Table I, showed that seven of the serpin-1 variants can inhibit one or more of these enzymes. Both porcine pancreatic and human neutrophil elastases were inhibited by M. anisopliae.

Some of the serpin-1 variants are active toward serine proteinases secreted by microorganisms (Table I). PR2, a fungal serine proteinase from the chymotrypsin family (17), with specificity for cleaving after Arg residues, was inhibited by serpin-1A, -1J, and -1E. PR1, proteinase K, and subtilisin are serine proteinases from a family that is unrelated in sequence to the chymotrypsin family (18). Proteinase K, favoring Phe and Tyr residues, was inhibited by serpin-1B, -1C, -1H, and -1K. Subtilisin Carlsberg, preferring Leu to aromatic residues at the P₁ position, was inhibited by serpin-1B, -1G, and -1F. Serpin-1B and -1C inhibited chymoelastase PR1 from the entomopathogenic fungus M. anisopliae.

A characteristic feature of a serpin-enzyme reaction is the formation of a complex that is stable in SDS (1). We found that all of the enzyme-serpin pairs exhibiting inhibition form SDS-stable complexes (data not shown). This includes complexes with the following serine proteinases from the subtilisin family: subtilisin Carlsberg, proteinase K, and PR1.

Scissile Bond Determination—To identify the position of the scissile bond in M. anisopliae serpin-1 variants, we purified the COOH-terminal peptide released from a serpin-enzyme reaction and determined the sequence of the amino-terminal 5 amino acid residues by Edman degradation (Table I). After reaction of serpin-1B with elastase, a peptide with an NH₂-terminal sequence of Ser-Leu-Ile-Leu-Tyr was released. This result is consistent with that reported earlier using a different method (10). From the amino acid sequence deduced from the serpin-1B cDNA, the P₁ residue of serpin-1B is an alanine at position 343, which is consistent with the specificity of elastases.

Similarly, we determined that the newly exposed NH₂ termini for serpin-1H, -1K, and -1Z are Val-Glu-Ser-Ile-Asp, Ser-Phe-His-Phe-Val, and Leu-Ser-Ala-Val-Ile, respectively, after incubation with chymotrypsin. Thus, from the deduced amino acid sequences, tyrosine was identified as the P₁ residue in these three chymotrypsin inhibitors at position 341, 343, and 342 of serpin-1H, -1K, and -1Z, respectively.

We determined that serpin-1I was cleaved by porcine pancreatic elastase between Leu³⁴³ and Ser³⁴⁴, releasing a peptide with an NH₂-terminal sequence of Ser-Leu-Glu-Phe-Ser. The molecular mass of this released peptide was determined by

![FIG. 2. SDS-polyacrylamide gel electrophoretic analysis of fractions in the purification of the recombinant serpin-1B. Log phase E. coli cells from a 30-ml culture were induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 5 h, pelleted by centrifugation, and resuspended in 5 ml of sonication buffer. After sonication, the suspension (lane 1, 1 μl) was centrifuged to yield a pellet (lane 2, 1 μl) and a supernatant (lane 3, 1 μl). The supernatant was used for Ni²⁺ affinity purification of the recombinant serpin. Proteins that did not bind to the Ni²⁺-nitrilotriacetic acid resin (lane 4, 1.2 μl) and the bound proteins, eluted with imidazole (400 mM total) (lane 5, 0.25 μl and lane 6, 0.05 μl) were analyzed. In lanes 1-4, the loading was normalized to represent equivalent amounts of E. coli. The molecular masses of standard proteins are marked. The serpin bands on the stained gel are indicated by an arrow.](image)

### Table I

**Inhibitory activities of reactive site variants of M. sexta serpin-1**

Bovine pancreatic trypsin (TRYP), human plasmin (PLAS), bovine pancreatic α-chymotrypsin (CHY), human cathepsin G (CATHG), porcine pancreatic elastase (PPE), human neutrophil elastase (HNE), proteinase K (PROK), subtilisin Carlsberg (SCARL), and M. anisopliae PR1 and PR2 were incubated with purified serpins and assayed for residual enzyme activity. Compared with negative control ovalbumin, inhibition less than 10% is scored as “—.” Percent inhibition represents mean ± S.D. (n = 3). The known P₁ sites are underlined.

| Variant | Predicted reactive site | Proteinase inhibition |
|---------|-------------------------|-----------------------|
|         | TRYP | PLAS | CHY | CATHG | PPE | HNE | PROK | SCARL | PR1 | PR2 |
| Serpin-1A | F I T R Q A R L | 66 ± 1 | 93 ± 1 | — | — | — | — | — | 100 ± 0 |
| Serpin-1B | I V P A S L I L | — | — | 51 ± 2 | 95 ± 1 | 57 ± 2 | 83 ± 0 | 67 ± 0 | 87 ± 3 |
| Serpin-1C | F I I E S Y S S | — | — | 44 ± 2 | 35 ± 0 | — | — | 48 ± 4 |
| Serpin-1D | R G I R P R P S | — | — | — | — | — | — | — | — |
| Serpin-1E | R V K K K F R | — | — | — | 22 ± 1 | 41 ± 2 | 22 ± 1 |
| Serpin-1F | I A V V S S I D | — | — | — | — | — | — | 27 ± 3 |
| Serpin-1G | I V G I T S I Q | — | — | — | 30 ± 0 | — | — |
| Serpin-1H | F I T Y V E S I | — | — | 64 ± 3 | 63 ± 3 | — | 37 ± 11 | — |
| Serpin-1I | I V A L S L E F | — | — | 41 ± 3 | 79 ± 4 | 22 ± 1 | 47 ± 3 |
| Serpin-1J | L T D B C S D | — | — | — | — | — | — | 36 ± 4 |
| Serpin-1K | I T T Y S F H F | — | — | 36 ± 2 | 68 ± 1 | — | 41 ± 4 | — |
| Serpin-1Z | G I A Y L S A V | — | — | 60 ± 6 | 63 ± 3 | — | — | — |
mass spectrometry to be 4239.1 Da. The value calculated from the deduced amino acid sequence is 4248.6 Da, which is slightly larger but within experimental error. Because serpin-1I also inhibited chymotrypsin and cathepsin G, we tried to determine whether there might be more than one scissile bond in serpin-1I. The peptide resulting from the reaction of serpin-1I with cathepsin G eluted at the same retention time on HPLC as the peptide released by elastase, and it had a mass of 4255.3 Da. These data indicate that serpin-1I was cleaved at the same position between Leu343 and Ser344 by elastase and cathepsin G, an enzyme with chymotrypsin-like specificity.

The reaction of serpin-1F with human neutrophil elastase yielded a peptide of 4542.1 Da, only 7.1 Da less than the calculated mass of the peptide beginning at residue 343 and extending to the carboxyl terminus. This result indicates that the scissile bond of serpin-1F is between Val342 and Asp343.

We previously predicted that the P1 residue of serpin-1A to be Arg342 (7). To examine this directly, we purified the COOH-terminal peptide released from the reaction of serpin-1 with Micrococcus lysodeikticus (9) homologous to serine proteinases from the horseshoe crab clotting pathway (24). However, direct experimental support has been obtained only for serpin-1A. The P1 residue of serpin-1A is indeed Arg342.

### Measurement of Association Rate Constants

Rate constants for the association of serpins with different proteinases (kₐ) are an indication of inhibitor selectivity. The association rate constants for some enzyme-inhibitor pairs are listed in Table II and compared with human α1-proteinase inhibitor and α1-antichymotrypsin. The kₐ of serpin-1B with porcine pancreatic elastase is about 10- and 5-fold greater than those of serpin-1F and -1I, respectively. These three insect serpins react faster with pancreatic elastase than does human α1-proteinase inhibitor. Serpin-1K and -1Z inhibit chymotrypsin at rates similar to that of α1-proteinase inhibitor. Serpin-1H and -1I react with chymotrypsin about 10 times slower than do serpin-1K or -1Z but 10 times faster than does human α1-antichymotrypsin. With a kₐ close to 10⁶ M⁻¹ s⁻¹, serpin-1A functions as an efficient trypsin inhibitor.

### Inhibition of Phenoloxidase Activation

Phenoloxidase is present in insect hemolymph as a zymogen, prophenoloxidase, that is activated by selective proteolysis upon addition of microbial cell wall components such as peptidoglycan or β-1,3-glucans. The fraction of M. sexta hemolymph proteins precipitated by 50% saturated ammonium sulfate (the PPO activation fraction) contains the components necessary for activation of prophenoloxidase, whereas the majority of hemolymph serpins are removed. This is consistent with results previously obtained with Bombyx mori hemolymph proteins (20). We found that phenoloxidase activity appears within 5 min after addition of Micrococcus cells to the PPO activation fraction (Fig. 3). The phenoloxidase activity peaked at about 40 min and gradually decreased to 90% of its maximum after 4 h.

To test whether any of the recombinant serpins affect activation of prophenoloxidase, we incubated the PPO activation fraction with Micrococcus and Manduca serpin-1 variants (at final serpin concentrations of 0.2–1 mg/ml) and measured phenoloxidase activity after 40 min. Serpin-1J and its allelic variant serpin-1J’ (μ) completely blocked phenoloxidase activation at 40 min, whereas none of the other 11 serpin-1 variants had any effect on prophenoloxidase activation. Serpin-1J and -1J’ inhibited prophenoloxidase activation in a concentration-dependent manner, with 50% inhibition at a concentration of 30 μg/ml (Fig. 4). The addition of serpin-1A at 500 μg/ml after activation of prophenoloxidase had already occurred had no effect on phenoloxidase activity (data not shown), indicating that serpin-1J does not inhibit phenoloxidase directly, but instead inhibits activation of its zymogen.

### DISCUSSION

Study of serpins from two lepidopteran insects, M. sexta and B. mori, has revealed an assortment of serpins in their hemolymph. Twelve serpin variants have been identified in M. sexta hemolymph, all encoded by a single gene through alternate exon splicing (7). The specific proteinase targets of these serpins have not been identified. To begin the elucidation of the physiological functions of the M. sexta serpins, we expressed the 12 reactive site variants of M. sexta serpin-1 in E. coli and characterized their inhibitory activities.

Although more than 10 serpins have been purified from hemolymph of invertebrates, their physiological functions remain largely unknown (5). The only exceptions are two Limulus intracellular serpins, LICI-1 and LICI-2, which regulate serine proteinases that are components of a cascade leading to coagulation (21, 22). In insects, several biological functions for serpins have been proposed (23). Hemolymph serpins may regulate endogenous proteinases released by hemocytes or fat body in inflammation-like processes. Serpins may also play roles in regulating proteinases involved in tissue remodeling during metamorphosis. Another pathway possibly regulated by insect serpins is the establishment of dorsal-ventral polarity during embryogenesis, which involves proteins (snake and easter) homologous to serine proteinases from the horseshoe crab clotting pathway (24). However, direct experimental sup-

### Table II

Association rate constants of some reactive site variants of M. sexta serpin-1 interacting with trypsin, chymotrypsin, and elastase

| Variant | Trypsin | Chymotrypsin | Elastase |
|---------|---------|--------------|----------|
| Serpin-1A | 7.8 ± 0.8 × 10⁵ | 3.1 ± 0.3 × 10⁶ | 3.1 ± 0.1 × 10⁷ |
| Serpin-1B | 3.4 ± 0.5 × 10⁵ | 1.9 ± 0.1 × 10⁶ | 7.1 ± 0.5 × 10⁷ |
| Serpin-1F | 1.6 ± 0.1 × 10⁶ | 1.0 ± 0.2 × 10⁷ | |
| Serpin-1H | 1.3 ± 0.3 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |
| Serpin-1I | 1.3 ± 0.3 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |
| Serpin-1J | 1.0 ± 0.1 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |
| Serpin-1K | 1.0 ± 0.1 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |
| Serpin-1L | 1.0 ± 0.1 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |
| Serpin-1M | 1.0 ± 0.1 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |
| Serpin-1N | 1.0 ± 0.1 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |
| Serpin-1O | 1.0 ± 0.1 × 10⁴ | 5.9 ± 0.6 × 10⁶ | 1.0 ± 0.1 × 10⁴ |

**Fig. 3.** Activation of PPO in the 0–50% ammonium sulfate fraction of M. sexta hemolymph (PPO activation fraction). Phenoloxidase activity was assayed at different times after the addition of M. lysodeikticus (circles) or water (squares).
In the competition with the inhibition reaction, we were unable to use proteinase samples, and secondary proteolysis of serpins in trypsin for serpin-1H, -1K, -1I, and -1Z. Because of technical difficulties, we were able to demonstrate inhibitory activity against at least one proteinase for 11 of the serpin-1 variants. Each serpin variant has a unique selectivity or spectrum of inhibition of the proteinases in the panel. Each serpin’s selectivity is evidently primarily defined by the P1 residue in the reactive site. We identified the P1 residues of serpin-1A, -1B, -1F, -1H, -1I, -1K, and -1Z to be Arg, Ala, Val, Tyr, Leu, Tyr, and Tyr, respectively. These results are in agreement with the cleavage specificities of their target proteinases: trypsin for serpin-1B, elastases for serpin-1B, -1F, and -1I, and chymotrypsin for serpin-1H, -1K, -1I, and -1Z. Because of technical difficulties associated with low rates of reaction, impurities in proteinase samples, and secondary proteolysis of serpins in competition with the inhibition reaction, we were unable to determine the P1 position in serpin-1C, -1E, -1G, and -1J.

Of the 12 variants, serpin-1B has the broadest selectivity, inhibiting elastases and also cathepsin G, proteinase K, the subtilisin Carlsberg, and PR1, which resemble chymotrypsin in specificity. A site-directed mutant of serpin-1B, replacing the P1 Ala to Phe (A343F), is a fast chymotrypsin inhibitor but also shows slow inhibition of trypsin (10). This broad selectivity of serpin-1B could be related to the Pro residue at the P2 site, which may affect interaction of the P1 residue with the primary binding site of the proteinases. Human α1-proteinase inhibitor, with a Pro at P2 and a Met at P1, inhibits trypsin, chymotrypsin, and elastase. Human heparin cofactor 2 also, with a Pro at P2 and a Leu at P1, inhibits both thrombin and chymotrypsin (25, 26). It was also reported that the P2 residue of human anti-thrombin is very important in optimal presentation of the reactive center to a cognate proteinase (27).

Serine proteinases from the subtilisin and chymotrypsin families are products of convergent evolution. They have entirely different overall folding patterns but share an essentially superimposable catalytic machinery. Although serpins are well characterized as inhibitors of proteinases from the chymotrypsin family, reports of their activity as inhibitors of proteinases from the subtilisin family have been limited. We found that several of the M. sexta serpins are active against some bacterial and fungal enzymes from the subtilisin family. We observed inhibition of subtilisin Carlsberg, proteinase K, and Metarhizium PR1 by some of the serpin-1 variants and detected SDS-stable complexes between the serpins and these enzymes. These results are consistent with a more detailed study on the interaction of human α1-proteinase inhibitor with subtilisin Carlsberg and proteinase K (19). Our study further supports the conclusion that serpins can inhibit proteinases from both serine proteinase families, presumably by the same mechanism.

M. anisopliae isolate ME1 is a fungal pathogen of M. sexta. To reach the hemocel and establish mycosis, the fungus releases digestive enzymes to penetrate through the insect cuticle, which is composed of cross-linked proteins, chitin, and lipids. Proteinases released by the fungus appear to be important in this process for invasion and subsequent establishment of infection (17, 18). The observation that some of the M. sexta serpins inhibit Metarhizium PR1, PR2, and other microbial proteinases suggests that some of the insect serpins may be involved in host defense against proteinases produced by pathogenic microorganisms.

Phenoloxidase and its activation are involved in a number of insect physiological processes (28, 29). Phenoloxidase catalyzes reactions that produce quinolic substances that either cross-link proteins and chitins in cuticular sclerotization or polymerize to form melanin. Phenoloxidase is also a part of insect defensive responses to wounding and infection. A serine proteinase cascade is thought to be triggered by recognition of bacterial and fungal cell wall components, activating phenoloxidase by limited proteolysis. Although the hemolymph proteinases in this cascade have not been purified and characterized yet, they are probably tightly regulated to prevent excess production of cytotoxic quinones by active phenoloxidase. Injection of antibodies to a mixture of the serpin-1 variants into M. sexta larvae to inactivate hemolymph serpins was shown to result in elevated phenoloxidase activity, suggesting that the prophenoloxidase activating enzyme(s) may be regulated by endogenous serpins (23).

We provide evidence here that serpin-1J and -1J′ (μ), but not the other 11 serpins encoded by the same gene, can efficiently block activation of M. sexta phenoloxidase (Fig. 4). This result strongly suggests that serpin-1J may be a physiological inhibitor of one of the serine proteinases in the proposed activating cascade. Because serpin-1J has Arg at its P1 position, we predict that its target enzyme in hemolymph may have trypsin-like specificity. Serpin-1J at 30 μg/ml inhibited phenoloxidase by 50%. We determined previously that total serpin-1 concentration in M. sexta hemolymph is 0.2–0.6 mg/ml, depending on developmental stage (30). Assuming that each serpin accounts for 1/12 of the total concentration, serpin-1J may be present at a concentration range (17–50 μg/ml) that could effectively regulate phenoloxidase activation in hemolymph.

The pathways for invertebrate phenoloxidase activation and hemolymph clotting in horseshoe crabs are analogous in many ways to the complement system and blood coagulation cascade in mammals, which are regulated by serpins. This resemblance might give insights and prospects for the study of invertebrate serpin functions. It may not be unrealistic to predict the existence of many serine proteinases in insect hemolymph. A serine proteinase has been purified from hemolymph of B. mori and found to be inhibited by a serpin, silkworm antitrypsin, isolated from the same insect (20). The function of this proteinase as a component of the phenoloxidase activating cascade was excluded (20), and thus it may participate in a process that has not yet been discovered. We have recently isolated cDNAs from M. sexta hemocytes, encoding proteins similar to horseshoe crab clotting factors (2). These proteins, whose functions are not yet known, may also be targets for regulation by hemolymph serpins.
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