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Two serine proteinase inhibitors from the larval hemolymph of *Heliothis zea**

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It has been known for a number of years that insect hemolymph, like vertebrate sera, contains polypeptides which are able to inhibit catalytic function of proteolytic enzymes, however, studies on proteins possessing this capability have been limited to a relatively few species. Inhibitors of serine proteinases with specificities primarily towards trypsin and/or chymotrypsin have been isolated and characterized in larvae of the moth *Bombyx mori, Anticarsia gammatalis, Manduca sexta* and fruit fly *Drosophila melanogaster* [1, 2]. These inhibitors fall into two classes: low molecular mass proteins (7–9 kDa) related to vertebrate Kunitz-type inhibitors, and proteins of 45–50 kDa which are members of the serpine super-family of serine proteinase inhibitors. Recently, by studying the inhibition of human leukocyte elastase (HLE), cathepsin G, trypsin, chymotrypsin, and porcine pancreatic elastase (PPE) by the hemolymph of fourteen insect species we found that the hemolymph of corn earnworm (*Heliothis zea*) contained not only a considerable total serine proteinase inhibitory activity but also showed the highest anti-HLE activity among all the species investigated. Therefore, the aim of the present study was to purify and characterize the protein(s) responsible for this activity.

Purification of the inhibitors. Hemolymph from fifth instar larvae was fractionated on a Sephacryl S-300 column in 0.1 M phosphate buffer, 0.15 M NaCl, pH 7.4, at 4–6°C. Fractions which contained the serine proteinase inhibitor activity were pooled and concentrated with a PM-10 membrane (Amicon). Since both the concentrate and the ultrafiltrate showed anti-proteolytic activity, though of different specificity, proteins from these solutions were separately purified. The purification procedure involved gel filtration on Sephadex G-75, ion exchange chromatography on Mono Q column followed by chromatofocusing of the preparations with Mono P HR 5/20 FPLC column over the pH interval 7.0–4.0. In this way two inhibitors were separated and purified.

Characterization of the inhibitors. The inhibitor separated from the concentrate appeared to be a single chain, slightly acidic protein (isoelectric point = 5.6) of M_m 18 kDa, consisting of 170 amino-acid residues, but among them no phenylalanine or tryptophan. N-Terminal amino-acid sequence of 29 residues suggest a strong homology of the inhibitor to Kazal-type inhibitors family [4] (Fig. 1). The inhibitor strongly inhibited human neutrophile elastase ($K_i = 10^{-11}$ M), porcine trypsin ($K_i = 2.1 \times 10^{-10}$ M), and bovine chymotrypsin ($K_i = 10^{-9}$ M) forming 1:1 complexes with each enzymes (Fig. 2). The inhibitor did not inactivate human lung tryptase or PPE. The inhibitor reacted independently with HLE and trypsin at 1:1 molar

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Fig. 1. Alignment of N-terminal sequence of the 18 kDa inhibitor from Heliothis zea with the sequence of some selected Kazal-type inhibitors.

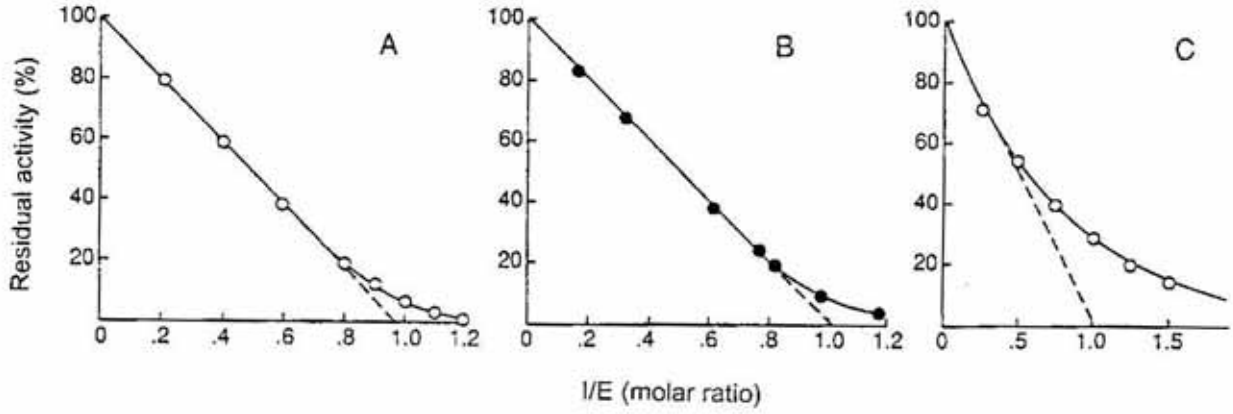

Fig. 2. Stoichiometry of binding of the 18 kDa inhibitor with HLE (A), trypsin (B), and chymotrypsin (C). Each enzyme was incubated for 5 min at room temperature with increasing amounts of inhibitor and residual enzyme activity was measured with specific substrates.

ratio for each, moreover, the complex HLE:inhibitor:trypsin inhibited chymotrypsin (Fig. 3) although this ability was clearly impaired as compared to the free inhibitor. As binding of one proteinase to the inhibitor did not preclude the binding of the other one, it seems likely that this protein has independent binding sites for each enzyme. The effect of chemical modification of the inhibitor on its activity greatly supported this hypothesis. Oxidation with *N*-chlorosuccinimide resulted in a rapid decrease in inhibitory activity towards HLE (not shown). This suggests that a methionine residue is involved in the binding site for this enzyme. Similarly, blocking of arginine residue caused a rapid loss of antitrypsin activity, indicating that this amino-acid residue resides in the trypsin binding sites of the inhibitor.

The second inhibitor which was purified from the ultrafiltrate appeared to be a low molecular protein of 6.3 kDa, consisting of 56 amino-acid residues. It inhibited bovine chymotrypsin ($K_i = 10^{-9}$ M) and human neutrophile cathepsin G ($K_i = 2.6 \times 10^{-9}$ M) but did not affect the activity of human lung tryptase, HLE or PPE. The amino-acid sequence of this inhibitor (Fig. 4) resembles to some extent the basic pancreatic trypsin inhibitor family (alignment not shown).

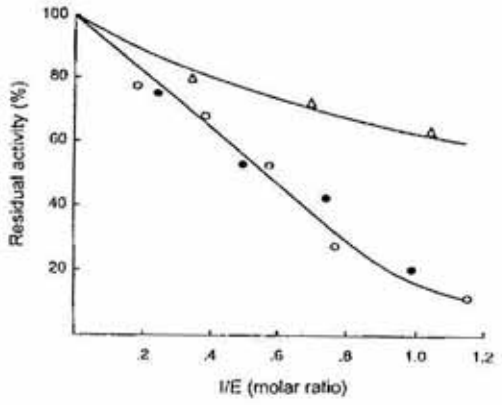

Fig. 3. Inhibition of HLE (○), trypsin (●) and chymotrypsin (Δ) by the complex: INHIBITOR:TRYPSIN, INHIBITOR:HLE and HLE:INHIBITOR:TRYPSIN, respectively.
Equal amounts of each enzyme were mixed with increasing amounts of inhibitor after the latter had been preincubated for 5 min at room temperature with an equal molar concentration of trypsin, HLE or both of them. Samples were incubated in 0.2 M Tris/HCl, 5 mM $CaCl_2$ for 5 min and residual activity of a given proteinase was measured with a specific substrate.

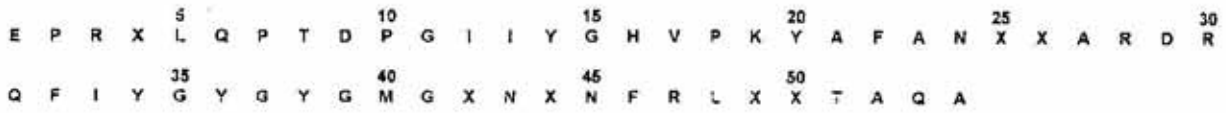

Fig. 4. Amino-acid sequence of the N-terminus of the 6.3 kDa inhibitor from H. zea.

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