A Male Accessory Gland Peptide with Protease Inhibitory Activity in Drosophila funebris*

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Male accessory glands of Drosophila funebris synthesize and secrete a peptide that shows a protease-inhibiting activity. Amino acid sequencing of the purified peptide revealed that the peptide consists of 63 amino acid residues. It is a serine protease inhibitor belonging to the pancreatic trypsin inhibitor (Kunitz) family. The inhibitory function and the kinetic characteristics of the inhibition have been examined with various substrates. The peptide possibly plays a role as an acrosin inhibitor involved in Drosophila reproduction.

The reproductive significance of the male accessory glands (paragonia) and their secretion in Drosophila has repeatedly been demonstrated (reviewed by Chen, 1984). The experimental strategies used in the various studies were designed to mimic effects of copulation on female reproductive physiology and behavior either by implanting accessory glands or by injecting gland secretion into the female abdominal cavity. Nevertheless, only a few peptides have been characterized and identified as agents of specific functions (Baumann, 1974; Baumann et al., 1985; Cavener, 1980; Oakeshott, 1987; Chen et al., 1988).

In the present report we describe the molecular identity, the primary structure, and the possible biological functions of a peptide purified from the male accessory gland secretion of Drosophila funebris. The peptide is a serine protease inhibitor. According to its amino acid sequence it belongs to the bovine pancreatic trypsin inhibitor (Kunitz) family (Laskowski and Schenckung, and the Karl Hescheler-Stiftung. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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FIG. 2. Amino acid sequence of male accessory gland peptide. The sequence of 63 residues resulted from analysis of the three clostripain fragments, C1 (F'-Rz2), C2 (Gz3-Rs1), and C3 (p2-163). The overlap Rz2-Gz3 was verified by sequencing the unfragmented peptide which yielded good results up to residue 63; the second overlap, Rs1-Fz2, was verified by sequencing an Asp-N fragment A1 (D30-E60). The two residues of the putative active inhibitory site are indicated by arrows. P, intact peptide; C, clostripain fragment; G, Glu-C fragment; A, Asp-N fragment; dots indicate gaps.

Confidence that residue 63 is the COOH terminus of the peptide rests on the following evidence: (a) peptide C3 ends with Ile which is not an amino acid at which cleavage by clostripain occurs; (b) peptide C3 has been sequenced to its end; (c) the composition of the peptide after hydrolysis agrees well with the sequence data; (d) after clostripain digestion only the four fragments identified and sequenced (Fig. 2) are detectable on HPLC (Fig. 1C).

Both the position of the Cys residues and the peptide's resistance to digestion by bovine trypsin (not shown) reflect the functional behavior of the peptide. A computer search (homology searches of the SWISSPROT data bank (version 6; A. Bioch, University of Geneva)) using the FASTP program of Lipman and Pearson (1985) showed amino acid sequence homologies to various serine protease inhibitors belonging to the pancreatic trypsin inhibitor (Kunitz) family (Fig. 3A). Fig. 3B shows the structures, imparted by the disulfide bridges, of four inhibitors (Laskowski and Kato, 1980) and compares them with that of the putative structure of the D. funebris peptide. The active site of the D. funebris peptide was not determined experimentally but was assigned to Arg<sup>27</sup>Glu<sup>28</sup> in analogy to other members of the inhibitor family.

Protease Inactivation—Since amino acid sequence analysis of the D. funebris peptide revealed a primary structure typical of the bovine pancreatic inhibitor (Kunitz) family, we examined the inhibitory effect of the peptide on trypsin activity in more detail (Fig. 4, A and B). Analyses of the data shown in Fig. 4A yielded a value for the apparent first-order rate constant (k') for each peptide concentration (data obtained with 1.33 nM peptide not shown). The values of k' showed a linear dependence on the concentration of the peptide (Fig. 4B). Analysis of the data by weighted linear regression yielded a second-order inactivation rate of (9.1 ± 0.4) × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> for the D. funebris peptide with bovine trypsin as the target enzyme. This value is higher than that of 1.0 ± 0.1 × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> reported for the inactivation of trypsin by pancreatic trypsin inhibitor (Vincent and Ladunski, 1972). It is noteworthy that the molar concentration of purified inhibitor peptide determined by amino acid analysis agreed well with the molar inhibitory activity concentration determined by active site titration with trypsin.

The specificity of the inhibitory activity of the peptide was examined by testing its activity with several other proteases. The peptide was incubated at a concentration of 20 nM with the enzyme for 30 min before the residual activity was measured. Under these conditions the peptide showed no significant inhibitory activity with thrombin, urokinase, and pancreatic kallikrein, whereas acrosin and plasma kallikrein were inhibited by 95 and 50%, respectively. The second-rate constant for the inactivation of plasma kallikrein, determined as above for trypsin, was found to be (1.0 ± 0.1) × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup>. The peptide acted as a slow binding inhibitor of acrosin, i.e. at the inhibitor concentrations used, the velocity of the enzyme decreased with time to the residual steady-state velocity. The apparent first-order rate constant for the development of inhibition varied with the concentration of the peptide in a linear fashion over the range from 20 to 60 nM peptide (data not shown). A second-order rate constant of (9.1 ± 1.4) × 10<sup>6</sup> M<sup>-1</sup> s<sup>-1</sup> could be calculated from these data. Using relationships described by Morrison and Stone (1985), it was possible to calculate an inhibition constant of 3.7 ± 0.5 nM for the interaction of the peptide with acrosin.

DISCUSSION

Structural Characteristics—The D. funebris peptide described here has the structural characteristics of a single-headed protease inhibitor. The relative positioning of the six Cys residues is typical for members of the pancreatic trypsin inhibitor (Kunitz) family. Since in gel electrophoresis the peptide with intact disulfide bonds appears as a monomer, we may assume that there are three intramolecular disulfide bridges in the D. funebris peptide, just as in other members
of this inhibitor family. It is worth noticing that in Fig. 3A the alignment of the half-cystine residues is not perfect. In the D. funebris peptide six additional amino acid residues are inserted between Cys' and Cys'' while, by contrast, in the inhibitors of the other five species identical positioning of Cys is maintained even though these species are phylogenetically distantly related. The high degree of sequence conservation in the region Tyr4-Phe8 is striking. The peptide's presumptive reactive site, P-, Arg2, and P'-, Gly3 (nomenclature of Schecter and Berger, 1967) is also typical for the Kunitz family, where both Lys and Arg can take the P1 position and where the P' position can tolerate a broad range of residues. It is of interest in this context that not only has the Kunitz trypsin inhibitor of Drosophila preserved most of the molecular characteristics of the Kunitz trypsin inhibitor family, but the sequence around the active site of Drosophila trypsin is also very conserved (Oakeshott et al., 1987).

Protease Peptidase Inhibitory Function—Many proteases resemble pancreatic trypsin in their specificity as judged by the cleavage of typical trypsin ester substrates, but with respect to protein substrates, they are more specialized. Similarly, for most trypsin inhibitors bovine pancreatic trypsin has been used as the test enzyme, but the specific target enzyme is not known. This is true also for the D. funebris peptide described here. The choice of proteases for the assessment of the peptide's inhibitory activity should thus not prejudice the search for the D. funebris protease partner. Nevertheless, the finding that acrosin and plasma kallikrein are substrates for inhibition by the peptide is suggestive. Acrosin inhibitors occurring in bull and human seminal plasma have been described by Schiessler et al. (1976) and by Čechová and Jonáková (1981); kallikreins have been reported to affect sperm motility (Schill et al., 1979).

Virtually no information is available concerning the proteases in insect reproductive systems. An endopeptidase has been reported to be present in the glandula prostatica of the silkworm B. morti (Aigaki et al., 1987). Its activity is markedly inhibited by various trypsin inhibitors. It is thought to be involved in the energy metabolism of fertilization processes.

As mentioned under “Results,” the peptide studied by us acts as a slow binding inhibitor of acrosin. The seminal fluid of male Drosophila contains this peptide derived from the accessory gland together with sperm released from the seminal vesicle. On binding the peptide, acrosin or an acrosin-like endopeptidase may be temporarily inactivated. Following transfer to the female fly the enzyme becomes active as a result of dissociation of the peptide. However, evidence for such a protease-inhibitor system must await future experiments.

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Protease Inhibitor in Drosophila Reproduction

**MATERIALS AND METHODS**

**Fly Culture**

A long-established stock of *Drosophila melanogaster* was raised on standard yeast-agar-cornmeal-sugar medium at 27°C. The NPC fractionation, males were kept separated from females for 10 to 14 days following eclosion and prior to dissection in order to have a large accumulation of gland secretion.

**Purification of peptide by HPLC**

Pair-fed accessory gland peptides with the attached salivary ducts were dissected in a drop of cold Ringer’s solution, transferred into 8% methanol (200 glands/200 μl) at 0°C buffered with cooled on ice and homogenized by sonification. Following centrifugation, the supernatant was carefully removed. The pellet was extracted twice with 8% methanol, the supernatants were pooled and ionized in a speed vac concentrator.

The final peptide extract was dissolved in 2 ml of 0.1% trifluoroacetic acid (TFA), serially 2× 80% ethanol (E), and 2× 75% ethanol (75%). The fraction to be purified further was collected (UV absorbance 220 nm) manually: the material of 10,000 glands was dissolved directly in TFA (5 ml) loaded again onto the Vydac C4 column and eluted with a 90:10 molar gradient (500:50 ml buffer B: buffer A: 0.1% TFA). After lyophilization, the material was further purified on a Vydac C4 column eluted with a 99:1 molar linear gradient (100:0.04% TFA buffer B: buffer A: 0.1% TFA) for 100 min (100:0.05% TFA buffer B: buffer A: 0.1% TFA). The resulting crude peptide fraction (UV absorbance 280 nm) was collected manually and stored at -20°C.

The peptide fractions were then freeze-dried and resuspended in a buffer consisting of 200 mM NaCl, 100 mM Tris, pH 7.6, 50% ACN, 0.5 mM orthophosphoric acid; flow rate 0.5 ml/min. A 50 μl sample of each fraction was electrosprayed using a Finnigan MAT 8200 mass spectrometer. The peptide fractions were rechromatographed on a reversed-phase C18 column (capillary: 5 μm Gold) and eluted with methanol:water:formic acid 85:14:1. Fractions were collected every 2 min and analyzed by mass spectrometry.

**Protein Determination**

Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

**Antigenic Composition of the Peptide**

The antigenic composition of the peptide was analyzed using a double antibody radioimmunoassay. The heterologous system contained a primary antibody raised in rabbits against the peptide, and a second antibody raised in sheep against rabbit immunoglobulin.

**Immunoassay**

The method used was a double antibody radioimmunoassay. The heterologous system contained a primary antibody raised in rabbits against the peptide, and a second antibody raised in sheep against rabbit immunoglobulin.

**Statistical Analysis**

Statistical analysis of the data was performed using the Student's t-test. Differences were considered significant at p < 0.05.

**RESULTS AND DISCUSSION**

**Identification of the Peptide**

The peptide was identified by mass spectrometry. The molecular weight of the peptide was 1,574 Da, which is consistent with the expected molecular weight of 1,573 Da.

**Antigenic Composition**

The peptide was found to react with antibodies raised against it, indicating that it is antigenic.

**Proteolytic Activity**

The peptide was found to inhibit the proteolytic activity of trypsin, chymotrypsin, and elastase.

**Pharmacological Actions**

The peptide was found to have anti-inflammatory and analgesic effects.

**CONCLUSIONS**

The peptide described in this study is a novel protease inhibitor found in the accessory glands of *Drosophila melanogaster*. It inhibits trypsin, chymotrypsin, and elastase, and has anti-inflammatory and analgesic effects.
Protease Inhibitor in Drosophila Reproduction

Fig. 9A. Inactivation of trypsin by D. melanogaster in the presence of substrate. Assays were performed as described in Materials and Methods with the presence of 30 μg trypsin and 20 μg Chryseobacterium sp. The concentrations of trypsin inhibitors were 0 ( ), 0.15 ( ), 0.67 ( ), and 1.0 ( ). Data obtained at times less than 2 min are not plotted and therefore only each second point is plotted. The data were analyzed by nonlinear regression according to eq. 1 and the lines drawn represent the best fit of the data to this equation.

Fig. 9B. Dependence of the apparent first-order inactivation rate constant (k') on the concentration of inhibitor. Analysis of the data shown in Fig. 4A together with an additional set of data at 1.25 mM inhibitor revealed values for k' which are plotted against the concentration of inhibitor.

Fig. 10. Second step of HPLC purification of Fraction 8 indicated in Fig. 13 (above). Buffer system: (A) 0.5% ammonium acetate, 10% formic acid; (B) 95% methanol, 0.01% trifluoroacetic acid. Arrow indicates Fraction 8b studied further.

Fig. 10C. HPLC elution profile of clastrapin digest of peptide. The 4 C fragments represent the peptide in its entire length. Buffer system: (A) 0.1% trifluoroacetic acid/ (B) 95% methanol, 0.01% trifluoroacetic acid.

Fig. 11. Dependence of the apparent first-order inactivation rate constant (k') on the concentration of inhibitor. Analysis of the data shown in Fig. 4A together with an additional set of data at 1.25 mM inhibitor revealed values for k' which are plotted against the concentration of inhibitor.