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Purification of a Novel, Heat-Stable Serine Protease Inhibitor Protein from Ovaries of the Desert Locust, *Schistocerca gregaria*

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A protease-inhibitor was isolated from mature ovaries of *Schistocerca gregaria* by a combination of trypsin-affinity chromatography and reverse-phase high performance liquid chromatography. It was characterized by aminoterminal amino acid sequencing using Edman degradation based automated microsequencing and by MALDI-TOF mass spectrometry. The N-terminal sequence (Y)XAEXDELA(A)EEY(Y)Q(Q)X(I)(L)M (X being a Cys, an irregular or modified amino acid) revealed no similarities with any other protease inhibitors isolated from invertebrate or vertebrate source. The 14 kDa inhibitor was found to be heat-stable. It shows potent inhibitory activity toward bovine trypsin and chymotrypsin, but not toward pancreatic elastase. It is likely that the characterized inhibitor will serve as an important tool for understanding its role in insect development. © 1997 Academic Press

Since Kunitz and Northrop (1936) purified a trypsin inhibitor from the pancreas (1), various protease inhibitors have been found in animals, plants and microorganisms. Protease inhibitors have recently attracted the attention of biologists as modulators playing key roles in the metabolism of proteins. Insect haemolymph, like vertebrate serum, contains several serine protease inhibitors (2). These can be grouped into two families based on their amino acid sequence and their proteinase inhibition characteristics: the Kunitz-type family (3) and the serpin-type family (4). Details on the function of these inhibitors are as yet not available, but they are thought to play a role in insect defense mechanisms, digestion, metamorphosis and development (5).

Recently, we found that the ovary of the desert locust, *Schistocerca gregaria* contains multiple inhibitors of serine proteases. Five serine protease inhibitors, designated as SGPI-1-5 (*Schistocerca gregaria* protease inhibitors) were purified from acid methanolic extracts of mature ovaries and analyzed by MALDI-TOF mass spectrometry and amino acid sequencing (6). The discovery of SGPI 1-5, in addition to three serine protease inhibitors (7, 8, 9) identified in the haemolymph of *Locusta migratoria* (HI, PMP-D2 and PMP-C), indicated the existence of a new family of 4 kDa serine protease inhibitors, incorporating a cysteine knot. They adopt a tertiary fold (10) hitherto unobserved in the large group of small "canonical" proteinase inhibitors (Kunitz-type).

Besides these, the ovary of *Schistocerca* was found to contain another serine protease inhibitor, which could be extracted in water but not in acidic methanol. This implies the possibility of a fundamental role of the serine proteases in the insect ovary. This paper reports the purification and partial identification of this novel serine protease inhibitor, which does not belong to any of the above described families.

MATERIALS AND METHODS

Purification. Ovaries of 50 females were collected in 0.15 M NaCl and 2 mM $CaCl_2$. After homogenization and centrifugation (15000 rpm) for 30 min, the supernatant was immersed in boiling water for 10 min. After centrifugation, the supernatant was dialyzed against Tris-HCl (50 mM, pH 8) containing 0.5 M NaCl and 10mM $CaCl_2$. After equilibration in the same buffer, the solution of heat-stable proteins was loaded on a trypsin-Sepharose affinity column (50 ml of CNBr-activated Sepharose-4B (Pharmacia) was derivatized with

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1g of bovine trypsin according to the manufacturer's instructions). The column was then washed with 10 column volumes of Tris-HCl buffer. The proteins binding to trypsin were eluted with 50 mM glycine-HCl buffer (pH 3.0) containing 0.5 M NaCl. The fractions with inhibitory activity against bovine trypsin were pooled and dialyzed against 0.2M Tris-HCl (pH 8.0). The pooled fraction was then further purified on a Gilson HPLC system (detector set at 214 nm). Columns and operating conditions were : (i) Biosep-Sec-S-2000 column (7.8 x 300 mm) (Phenomenex, Ca), solvent A: 95% CH_3CN in 0.01% aqueous TFA (trifluoracetic acid); solvent B: 50% CH_3CN in 0.01% aqueous TFA. Column conditions: 50% B for 10 min, followed by a linear gradient to 100% B in 60 min; flow rate: 1.5 ml/min; detector range: 0.2 absorbance units full scale (Aufs); (ii) Genesis C18 (4.6 x 250 mm) (Jones Chromatography), solvent A: 0.1% aqueous TFA; solvent B: 50% CH_3CN in 0.1% aqueous TFA. Column conditions: 20% B for 10 min, followed by a linear gradient to 100% B in 40 min; flow rate: 1.0 ml/min; detector range: 0.2 Aufs.

Amino acid sequencing. Following the C18 reverse-phase chromatography, a protein sample (6 pmol) was loaded on a precycled Biobrene Plus-coated glass filter. N-terminal amino acid sequence analysis was carried out on a Perkin Elmer/Applied Biosystems Procise 492 microsequencer running in pulsed-liquid mode.

Mass spectrometry analysis. Mass analysis was performed on a Micromass (Manchester, UK) Tofspec matrix assisted laser desorption time of flight mass spectrometer (MALDI-TOF MS). The instrument was operated in the linear positive ion mode with an accelerating potential in the source of 24 kV. Ionization was accomplished with the 337 nm beam from a N_2 laser with the laser energy precisely tuned for an optimal signal to noise ratio. Data from 20 to 30 shots were averaged to obtain the final spectrum. Calibration was done externally with a mixture of two standard compounds (cytochrome C and horse heart myoglobin).

An aliquot (1.5 μl) of the final volume (3 ml) of the HPLC purified peak containing the protease inhibiting activity was mixed with an equal volume of matrix solution (50 mM α-cyano 4-hydroxycinnamic acid in 30 % CH_3CN containing 0.1% TFA). One microliter of this mixture was deposited on the metal target, allowed to air-dry and introduced into the MALDI source.

Enzyme assays. The purified inhibitor was tested on serine proteases of known specificity as follows. Except for azocasein, all substrate stock solutions were prepared in dimethylformamide (DMF). A suitable sample of inhibitor was incubated for 45 min at 25° C with bovine trypsin (1.6 μM), bovine chymotrypsin (0.08 μM) or porcine pancreatic elastase (0.8 μM) in 0.2M Tris-HCl buffer (pH, 8.0). Residual activities were recorded with the respective substrates, N-α-Benzoyl-DL-Arg-pNitroanilide (0.36 mM), N-Succinyl-Ala-Ala-Pro-Phe-pNitroanilide or N-succinyl-Ala-Ala-Ala-pNitroanilide (0.31 mM) with a Beckman spectrophotometer (quartz was used routinely). All enzymes and substrates were from Sigma Chemical Co.

Measurement of insect gut protease inhibitory activity. Aliquots of 5 μl of gut homogenate (1 equivalent/ml water (0.15 M NaCl, 1 mM $CaCl_2$) were incubated for 45 min in 45 μl Tris-HCl buffer (pH 8.0) with about 10 μg of inhibitor. Control gut homogenates were incubated with Tris-HCl buffer instead of the *Schistocerca* protease inhibitors. Twenty μl of each solution was used for native PAGE-electrophoresis according to Laemmli (11) to separate the gut enzymes. Gut protease bands were revealed by placing the acrylamide gel bands onto slides with a film of agar-azocasein-gel prepared with 0.2 M Tris-HCl buffer (pH 8.0, 10 mM $CaCl_2$, 1 % agar and 0.1 % azocasein). To reveal the site of azocasein degradation, the gel was stained in a Coomassie blue R250 (0.1 %), 25% methanol, 10% acetic acid solution.

RESULTS AND DISCUSSION

Aqueous homogenates of mature ovaries of *Schistocerca gregaria* still retain inhibitory activity towards bovine trypsin after boiling for 20 min. This heat-stable serine protease inhibitor was isolated by a three-step chromatography procedure from an aqueous extract of mature ovaries. After a trypsin-Sepharose affinity column and a second gel filtration column run in normal phase, a single fraction was obtained that inhibited bovine trypsin (Fig. 1a). A final HPLC chromatography step on a reverse-phase C18 column yielded a single homogenous peak (Fig 1b) that was submitted to sequencing and mass spectrometry (Fig. 2). The results confirmed the purity of the inhibitor and gave the following N-terminal sequence, (Y)XAE**X**DELA(A)EEY (Y)Q(Q)X(I)(L)M. At position 2, 4 and 17, no amino acid (X) could be determined. This is compatible with the presence of a Cys, an irregular or a modified amino acid, resulting in the presence of a gap in the sequence data when standard protocols are applied. Amino acids which could not be identified unambiguously are pre-

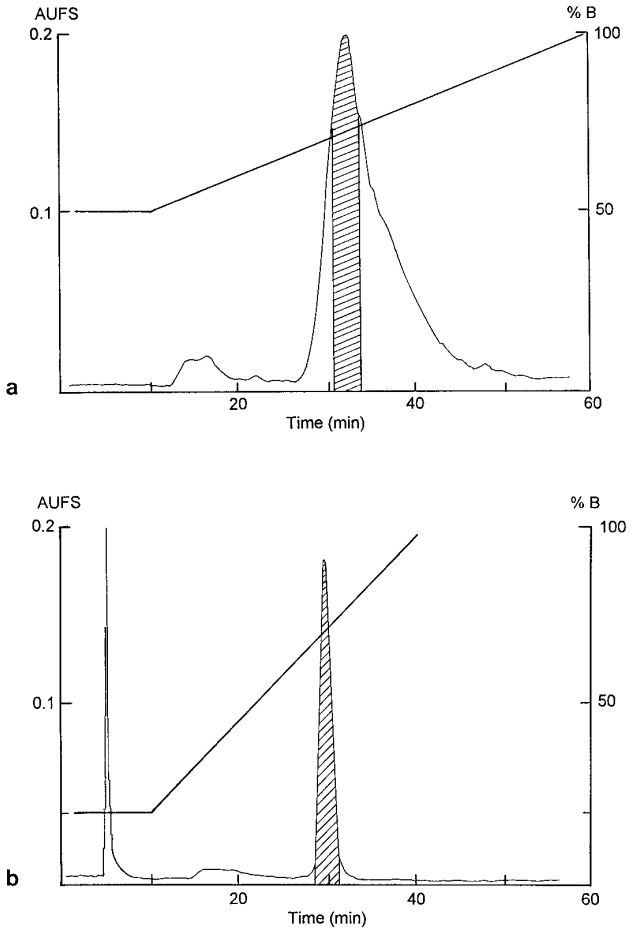

FIG. 1. Chromatogram indicating UV absorbance (absorbance units full scale, AUFS, detected at 214 nm) of the eluate collected from the trypsin-Sepharose column separated (1a) on a Biosep-Sec-S-2000 column run in normal phase and further on a C18 column (1b) separation to homogeneity of the peak eluting at 31-34 min.

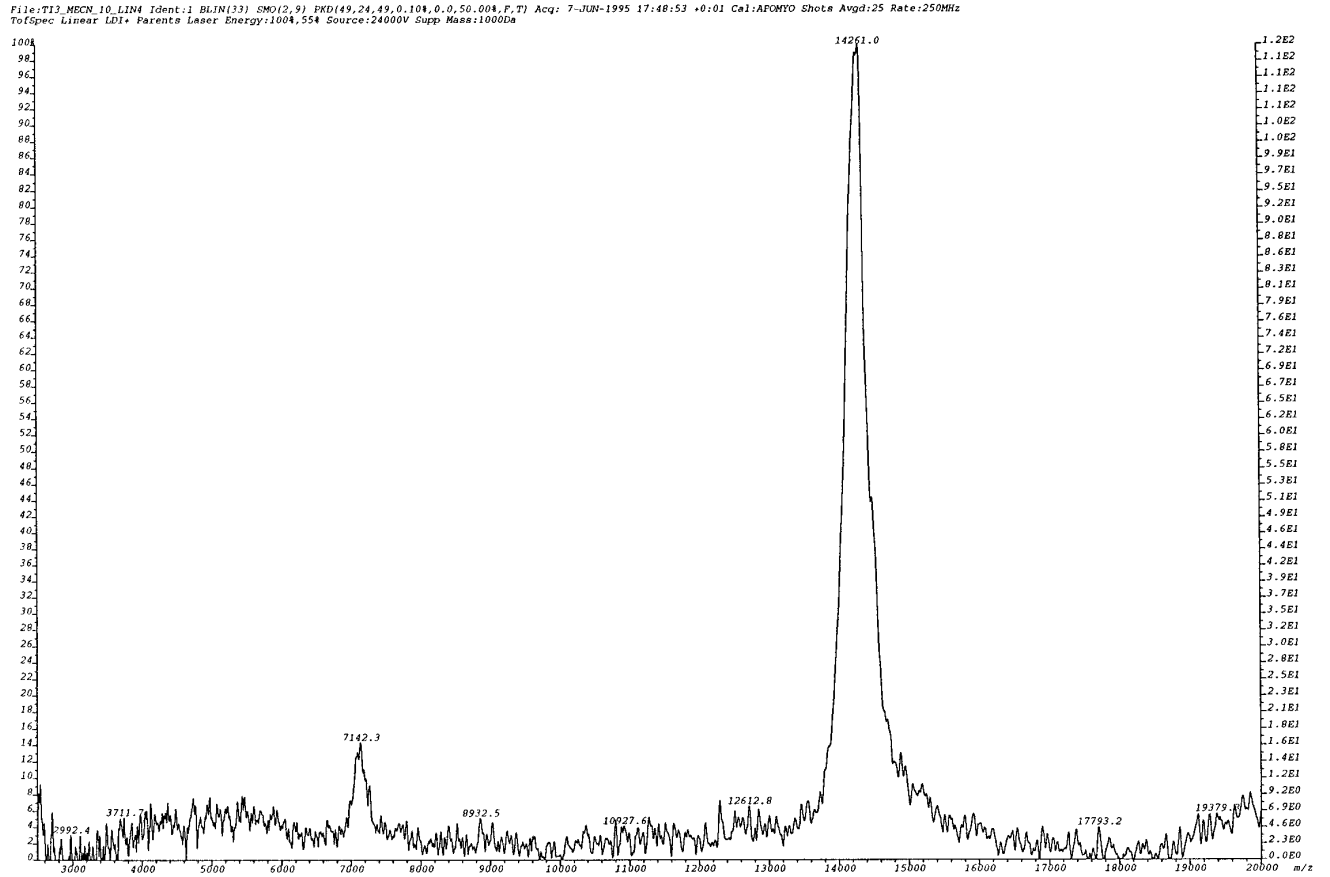

FIG. 2. MALDI-TOF mass spectrometry of SGPIP.

sented between brackets. The molecular mass was determined to be 14260 Da from the peaks at 14261 and 7142, which correspond to the charged ions $[M+H]^+$ and $[M+2H^+/2]$. About 10 nmoles of inhibitor were isolated from 50 ovaries, losses during purification not taken into account. There is no similitude between the partial sequence of this molecule and the protease inhibitors previously isolated from invertebrate and vertebrate source.

The purified inhibitor was tested against representative serine proteases listed in Table 1 and potently inhibited trypsin and chymotrypsin but not pancreatic elastase. It was also tested against intestinal insect proteases. After incubation of a locust gut homogenate with the purified inhibitor (2 μM), 75 % of the total proteolytic activity with azocasein as enzyme substrate, was inhibited. PMSF (phenylmethylsulfonylfluoride, 5 μM final concentration), known for its specific inhibitory activity towards serine proteases, inhibits this intestinal activity for 85%, indicating that the purified heat stable inhibitor is a true serine protease inhibitor, with a very large activity spectrum as indicated in Fig. 3. The zymogram of the gut homogenate shows 9 bands containing proteolytic activity as demonstrated with casein as a general substrate. All of them were inhibited in the presence of the 14 kDa protease inhibitor.

TABLE 1

Protease Inhibitory Activity (%) of SGPIP Toward Three Serine Proteases

Serine proteases (concentration)	Inhibitory activity (%)
Bovine α-trypsin (1.6×10^{-6}M)	100
Bovine α-chymotrypsin (0.8×10^{-7}M)	100
Porcine pancreatic elastase (0.8×10^{-6} M)	0

A number of serine proteases have been purified from insect haemolymph but to date this is the first 14 kDa serine protease inhibitor isolated from insect ovaries. We recently identified 5 serine protease inhibitors (SGPI-1-5 or *Schistocerca gregaria* protease inhibitors) from acid methanolic ovary extracts, all with a molecular weight around 4 kDa and with three disulfide bridges (6). The present inhibitor was isolated from an aqueous ovary extract, and has a molecular weight of 14 kDa. Therefore, it was designated

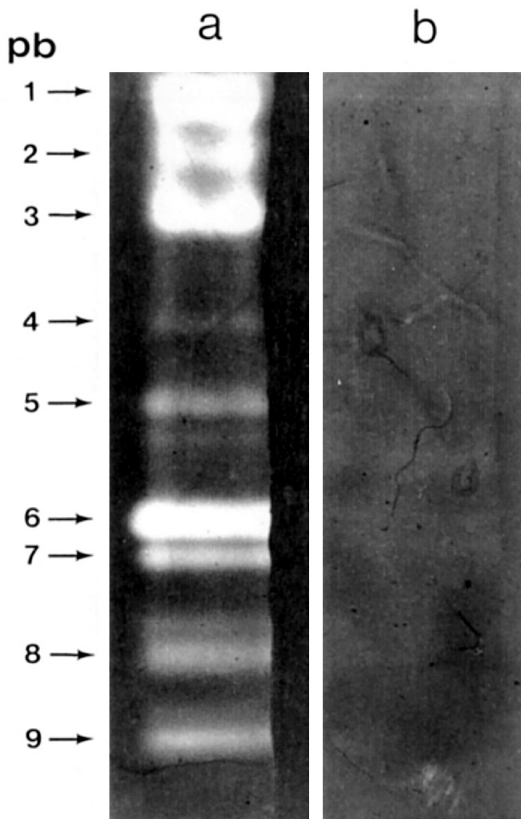

FIG. 3. Inhibitory activity of SGPIP on locust gut proteases after PAGE-electrophoresis and transfer to an agar-casein gel. Gut homogenate (lane a), gut homogenate incubated with SGPIP (lane b). pb 1-9, protease bands.

as SGPIP (*Schistocerca gregaria* protease inhibitor protein) following the agreed nomenclature (12). Two heat stable serine protease inhibitors with a molecular weight of 8000 and 14000 Da have been purified from the haemolymph of *Manduca sexta* (13). The N-terminal sequences of these inhibitors display sequence homologies with bovine pancreatic trypsin inhibitor and other inhibitors of the Kunitz-type. Although the protease inhibitor characteristics of the presently identified SGPIP are similar to the ones of the Kunitz-type serine protease inhibitors, its identified partial sequence does not resemble any of the primary structures of these small canonical protease inhibitors.

The role of serine protease inhibitors in insect ovaries is as yet not known. As the protease inhibitor protein, characterized here, is also an inhibitor of endogenous insect proteases, it probably prevents unwanted proteolysis by serine proteases in the ovary or the developing embryo.

REFERENCES

1. Kunitz, M., and Northrop, J. H. (1936) *J. gen. Physiol.* **19,** 991–1007.
2. Polanowski, A., and Wilusz, T. (1996) *Acta Biochim. Pol.* **43,** 445–453.
3. Laskowski, M. jr. (1986) *Adv. Exp. Med. Biol.* **199,** 1–17.
4. Carrell, R. W., and Boswell, D. R. (1986) *in* Proteinase Inhibitors (Barrett, A. J., and Salvessen, G., Eds.) pp. 375–388 Elsevier, Amsterdam.
5. Eguchi, M. (1993) *Insect Biochem. Physiol.* **105B,** 449–456.
6. Hamdaoui, A., Schoofs, L., Wataleb, S., Devreese, B., Van Beeumen, J., Chiou, S., Vanden Broeck, J., and De Loof A. (1997), submitted for publication.
7. Boigegrain, R.-A., Mattras, H. Brehelin, M., Paroutaud, P., and Colletti-Previero, M.-A. (1992) *Biochem. Biophys. Res. Commun.* **2,** 790–793
8. Nakakura, N., Hietter, H., Van Dorsselaer, A., and Luu, B. (1992) *Eur. J. Biochem.* **204,** 147–153
9. Kellenberger, C., Boudier, C., Bermudez, I., Biet, J. G., Luu, B., and Hietter, H. (1995) *J. Biol. Chem.* **270,** 25514–25519
10. Mer, G., Hietter, H., Kellenberger, C., Renatus, M., Luu, B., and Lefèvre, J. F. (1996) *J. Mol. Biol.* **258,** 158–171
11. Laemmli U. K. (1970) *Nature* **277,** 680–685
12. Wieczorek, M., Otlewski, J. U., Park, K., Leluk, J., Wilimowska–Pelc, A., Polanowski, A., Wilusz, T., and Laskowski, M. (1985) *Biochem. Biophys. Res. Commun.* **126,** 646–652.
13. Ramesh, N., Sugumaran, M., and Mole, J. E. (1988) *J. Biol. Chem.* **263,** 11523–11527.