A Novel Protease in the Pupal Yellow Body of Sarcophaga peregrina (Flesh Fly) 

ITS PURIFICATION AND cDNA CLONING*

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We purified a novel serine protease with a molecular mass of 26 kDa from Sarcophaga pupae. This protease appeared almost exclusively in the yellow body, an organ that develops temporarily in the pupae of dipteran insects and expands to form the adult midgut by engulfing the larval midgut. cDNA analysis revealed that this protease consists of 239 amino acid residues and has significant structural similarity with bovine trypsin (about 40% sequence identity). The 26-kDa protease gene was transiently activated in 1-day-old pupae. The protease was found to cross-react immunologically with antibody against sarcotoxin IA, an antibacterial protein produced by this insect. It is suggested that this protease participates in the decomposition of the larval midgut in the yellow body during metamorphosis.

In holometabolous insects, most larval tissues disintegrate during the pupal stage, and new adult structures develop from imaginal discs (1). However, little is known about the molecular mechanism underlying the selective decomposition of larval tissues (2). Recently, we suggested that hemocyte cathepsin B is responsible for decomposition of the fat body in Sarcophaga peregrina (flesh fly) (3, 4). We purified this enzyme and isolated its cDNA (5, 6). Sarcophaga cathepsin B differs from its mammalian counterparts in several respects. It does not seem to be a typical lysosomal enzyme, as its optimum pH is higher (pH 6.0) (5). Moreover, production of this enzyme was shown to be regulated at the translational level, since larval hemocytes contained a significant amount of cathepsin B mRNA but not the enzyme itself (7). The mRNA started to be translated on pupation, and cathepsin B then accumulated in the hemocytes. When pupal hemocytes containing cathepsin B interacted with the fat body, the enzyme was released and digested the basement membrane of the fat body, resulting in its decomposition (4).

This paper reports the purification, cDNA cloning, and some characteristics of a novel serine protease that is likely to participate in the decomposition of the larval midgut during metamorphosis. This enzyme was discovered incidentally during a study of sarcotoxin IA, a cecropin-type antibacterial protein produced by Sarcophaga (8). We purified a protein that reacted immunologically with anti-sarcotoxin IA antibody from an extract of Sarcophaga pupae. This protein was revealed to be a novel serine protease, which was preferentially induced in the yellow body, in which disintegration of the larval midgut takes place during metamorphosis. During remodeling of the midgut, the yellow body is known to be formed as a first step (1). It surrounds the larval midgut at the pupal stage. A morphological change of the yellow body is induced to form the adult midgut simultaneously with the disintegration of the larval midgut. Therefore, we assume that this novel protease is responsible for disintegration of the larval midgut within the yellow body. Taking into account our previous results (3, 4), it is suggested that several proteases participate in tissue remodeling during insect metamorphosis.

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solution (10 mM Tris-HCl buffer (pH 7.9) containing 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton X-100, 0.01% (v/v) sodium azide, and 0.25% skim milk), they were dialyzed extensively against the same buffer. The dialyze was applied to a CM-cellulose column (1.5 x 6 cm) that had been equilibrated with the same buffer. The column was washed well, then the adsorbed materials were eluted stepwise with 10 mM sodium phosphate buffers containing 120 and 350 mM NaCl, respectively. The 26-kDa protein was eluted by the latter buffer. The fraction was concentrated to reverse-phase high performance liquid chromatography (HPLC)3 with a Synchropak RP-P C18 column. The 26-kDa protein was eluted with 37% (v/v) acetonitrile when the column was developed with a linear gradient of 0-100% acetonitrile in 0.05%(v/v) trifluoroacetic acid solution. The 26-kDa protein was almost pure at this stage and gave a single band when analyzed by SDS-polyacrylamide gel electrophoresis.

Cloning and Sequencing of the 26-kDa Protein cDNA—Purified 26-kDa protein was digested with lysyl endopeptidase, and the resulting peptides were separated by HPLC on a C18 column (Gilion). The fractions containing each peptide were lyophilized and the sequences of the 5 peptides, including the 10 amino-terminal residues, were determined. Using these peptide sequences, oligodeoxyribonucleotides corresponding to MHPQYDPV and DAIVAGWG were synthesized. Their sequences were 5'-ATGCC/TCA/CCICA/GTG/TA/TGCGA/TC/GCGTC/CCGGTCA/-3' and 5'-CCCGAICA/TCA/GGCACA/GTATTGC/AGTGCA/GTC-3', respectively. These oligodeoxyribonucleotides were mixed and labeled with [γ-32P]ATP by the method of Sgaramella and Khonara (14). Total RNA was extracted from homogenates of day-old pupae by the guanidine thiocyanate method of Chirgwin et al. (15); poly(A)-rich RNA was prepared with (dT)30 latex beads. A cDNA library of this poly(A)-rich RNA was constructed with the expression vector Zap (Stratagene). This cDNA library of this poly(A)-rich RNA was constructed with the expression vector Zap (Stratagene). This cDNA library in bacteriophage Uni-Zap XR vector converted into cDNA library in plasmid pBluescript by the method of Short et al. (16). We screened 5 x 106 colonies and obtained nine hybridization-positive clones. We analyzed the two plasmids with the longest insert. These clones yielded identical restriction maps, and their inserts were found to contain one determined amino acid sequence. Therefore, one of these clones was selected for further analysis. For nucleotide sequencing of the cDNA, various deletion derivatives of the DNA fragment were prepared using exonuclease III and mung bean nuclease. Each deletion derivative was sequenced, and the nucleotide sequences of both strands were determined.

Northern Blot Hybridization—Northern blot hybridization was performed in 50% formamide, 5 x SSPE (1 x SSPE equals 15 mM NaCl, 10 mM Na2HPO4, 1 mM EDTA), 1 x Denhardt’s solution (0.02% (v/v) each of Ficoll 400, bovine serum albumin, and polyvinylpyrrolidione), 0.1% SDS, and sonicated salmon sperm DNA solution (200 μg/ml) for 18 h at 42 °C. The filters were then washed for 15 min each time with 0.1 x SS0, 0.1 x SSC equals 0.15 M NaCl, 0.05 M sodium citrate) containing 0.1% SDS at room temperature and 42 °C and then autoradiographed at −80 °C. The DNA used as a probe was a 26-kDa protein cDNA-labeled with [α-32P]dCTP using the BcaBEST random primer labeling kit (Takara, Tokyo).

Assay of Protease Activity—Protease activity was assayed with various peptidylmethylcoumaryl-7-amide (peptidyl-MCA) substrates. The enzyme reaction was performed in 0.25 mM Tris-HCl buffer (pH 7.9) containing 0.1 mM substrate, 1 mM EDTA, and the enzyme sample. After incubation for 10 min at 27 °C, the reaction was terminated by

1 The abbreviations used are: HPLC, high performance liquid chromatography; MCA, methylcoumaryl-7-amide; Boc, butyloxycarbonyl; E-64, trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane.

Fig. 1. Immunoblotting analysis of pupal extract with affinity-purified anti-sarcotoxin IA antibody. Third instar larvae or pupae at various developmental stages were homogenized and centrifuged. The resulting supernatant was subjected to immunoblotting analysis with affinity-purified anti-sarcotoxin IA antibody. Each lane contained 30 μg of protein. Synthetic sarcotoxin IA (1 ng) was analyzed simultaneously. Arrowheads indicate the positions of sarcotoxin IA and a 26-kDa protein that cross-reacts immunologically with anti-sarcotoxin IA antibody, respectively. The lower panels show the sarcotoxin IA regions after autoradiography for 4 days to visualize sarcotoxin IA signals. Lane Toxic, 1 ng of sarcotoxin IA; lane 0, third instar larvae. Pupal extracts were prepared from 1- to 9-day-old pupae and were run in lanes 1-9, respectively. A, sarcotoxin IA antibody; B, sarcotoxin IA antibody treated with silica beads coated with sarcotoxin IA. Adding 0.375 ml of 17% aqueous acetic acid. The florescence was measured at excitation and emission wavelengths of 380 and 460 nm, respectively.

Affinity Purification of an Anti-26-kDa Protein Antibody—An antibody against the 26-kDa protein was raised by injecting the purified protein (15 μg) in complete Freund’s adjuvant into two male albino rabbits with three booster injections of 10 μg of protein and incomplete Freund’s adjuvant at intervals of 14 days. Affinity purification of the antibody was achieved in essentially the same way as the preparation of a specific antibody against regenectin (17).

RESULTS

Detection of a 26-kDa Protein That Cross-reacts Immunologically with Anti-sarcotoxin IA Antibody in the Pupal Extract—Previously, we demonstrated that the sarcotoxin IA gene was transiently activated during the early pupal stage in Sarcophaga (18). To prove that sarcotoxin IA is in fact present in the pupae, we performed immunoblotting using pupal extracts prepared from pupae harvested at various developmental stages. As shown in the lower panel of Fig. 1A, sarcotoxin IA was detected in 3-6-day-old pupae, and the content was estimated to be approximately 10 ng/mg of protein. Synthesis of sarcotoxin IA corresponded with expression of the sarcotoxin IA gene that we reported previously (18).

In addition to sarcotoxin IA, the antibody was found to cross-react with a protein with a molecular mass of 26 kDa (26-kDa protein) (top panel). When the antibody was treated with sarcotoxin IA-coated silica beads to absorb specific antibody, the remaining antibody reacted with neither sarcotoxin IA nor with the 26-kDa protein (Fig. 1B), indicating that the 26-kDa protein cross-reacted immunologically with anti-sarcotoxin IA antibody. Other signals in the high molecular mass region are nonspecific background ones, since they were independent of the first antibody. The 26-kDa protein was detected exclusively in 3-7-day-old pupae; thereafter it disappeared rapidly. The 26-kDa protein signal was very strong, and its content did not seem to change throughout this period. This protein is not a precursor of sarcotoxin IA, because cDNA analysis has shown that there is no precursor protein for sarcotoxin IA (19).
the 26-kDa protein, we purified the 26-kDa protein from pupal extracts. Purification was monitored by immunoblotting followed by densitometric scanning of the 26-kDa protein band using anti-sarcotoxin IA antibody. 1 unit of 26-kDa protein was defined as the amount that gave the same immunoreactivity as 0.25 ng of sarcotoxin IA on immunoblotting.

The typical purification is summarized in Table I, and HPLC, SDS-polyacrylamide gel electrophoresis, and immunoblotting profiles of the sample during the final purification step are shown in Fig. 2. About 250 µg of pure protein was obtained from the extracts of about 200 pupae.

**cDNA Cloning of the 26-kDa Protein**—To determine the amino acid sequence of the 26-kDa protein, we isolated its cDNA. For this, we first determined the amino acid sequences of five peptides derived from the 26-kDa protein, including its amino-terminal sequence. These were VIMHPQYDPVHITND-VALLR, DAIYAVGWLFK, FLDWIHNSR, YPWTIAQVLK, and IVGTVQVRQV (amino terminus). Having obtained this sequence information, we synthesized DNA probes, used them to screen the cDNA library of *Sarcophaga* pupae, and obtained nine hybridization-positive clones.

The nucleotide sequence of the insert and the deduced amino acid sequence of one of these clones are shown in Fig. 3. The amino acid sequence contained all the sequences determined for the five peptides derived from the 26-kDa protein, indicating that it was a cDNA clone for the 26-kDa protein. We designated the Ile at position 87 as its amino-terminal residue starting from the first Met residue. Therefore, the 26-kDa protein consists of 239 amino acid residues. The 86 residues from the first Met are thought to be a prosegment that includes a signal sequence, possibly consisting of 18 residues.

No significant sequence similarity was found between the 26-kDa protein and sarcotoxin IA. Therefore, it is uncertain why the antibody against sarcotoxin IA cross-reacted with the 26-kDa protein. However, as shown in Fig. 4, the 26-kDa protein was found to have significant sequence similarity with bovine trypsin, and the His, Asp, and Ser residues conserved in many serine proteases are also present in the 26-kDa protein, suggesting that the 26-kDa protein is a serine protease. The maximal sequence identity between the 26-kDa protein and bovine trypsin was about 40%.

**Protease Activity of the 26-kDa Protein**—As it became evident that the 26-kDa protein was likely to be a novel serine protease, we assayed its protease activity using various peptidyl-MCA substrates. As shown in Table II, purified 26-kDa protein clearly hydrolyzed substrates with Arg or Lys at their carboxyl termini. Therefore, the substrate specificity of this protease is very similar to that of trypsin. Other substrates, including those of chymotrypsin and prollyl endopeptidase, were not hydrolyzed by this enzyme.

We examined the characteristics of this protease using Boc-Gln-Ala-Arg-MCA as a substrate. The optimum pH range for this protease was rather broad, between pH 6 and 8. However, no enzyme activity was detected above pH 10 or below pH 4, suggesting that it is not a lysosomal enzyme. This protease was sensitive to diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, leupeptin, and antipain but not to E-64, indicating that it is a typical serine protease.

**Immunoblotting of the 26-kDa Protease**—To estimate the biological role of the 26-kDa protease, we examined its localization within pupae. For this, we prepared affinity-purified antibody against the 26-kDa protease using purified protein. In contrast to sarcotoxin IA antibody, this antibody reacted only with the 26-kDa protease and not with sarcotoxin IA. Significant fluorescence was detected in the pupal midgut (the so-called “yellow body”) when pupal sections were subjected to an immunofluorescence study. Localization in other regions was not clear because of high background fluorescence (data not shown).

To confirm this observation, we prepared homogenates of the whole body, head, thorax and abdomen, and yellow body of 3-day-old pupae and subjected them to immunoblotting. As shown in Fig. 5A, the 26-kDa protease signal was detectable in homogenates of the whole body, thorax and abdomen, and yellow body but not in that of head. Moreover, several other signals besides that of the 26-kDa protease were detected in the

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**TABLE I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>26-kDa protein</th>
<th>Total protein</th>
<th>Specific content</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate supernatant</td>
<td>87,500</td>
<td>941</td>
<td>93</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulfate, 40–65% saturation</td>
<td>43,200</td>
<td>470</td>
<td>92</td>
<td>49.4</td>
</tr>
<tr>
<td>CM-cellulose</td>
<td>14,400</td>
<td>0.684</td>
<td>21,000</td>
<td>16.5</td>
</tr>
<tr>
<td>Reverse-phase HPLC</td>
<td>10,000</td>
<td>0.245</td>
<td>41,000</td>
<td>11.4</td>
</tr>
</tbody>
</table>

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**FIG. 2.** Chromatographic and electrophoretic profiles during the final purification step of the 26-kDa protein. A, reverse-phase HPLC. Chromatographic conditions were as follows: column, Synchro- pak RP-P(C18) (250 × 4.1 mm); solution A, 0.05% trifluoroacetic acid in *H*2O; solution B, 0.05% trifluoroacetic acid in acetonitrile; elution, linear gradient of 30–60% solution B in solution A; flow rate, 1 ml/min. The absorbance at 220 nm was monitored. B SDS-polyacrylamide gel electrophoresis and immunoblotting of purified 26-kDa protein. Purified 26-kDa protein (1 µg) was subjected to polyacrylamide gel electrophoresis under denaturing conditions and stained with Coomassie Bril- liant Blue (lane 1). Twenty-five ng of purified 26-kDa protein was detected by immunoblotting (lane 2). The gel was calibrated using the following marker proteins: bovine serum albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25 kDa), and cytochrome c (13 kDa).
The deduced amino acid sequence of the 26-kDa protein is shown below: the nucleotide sequence. The numbers of the nucleotides are shown to the left of each line. Chemically determined partial amino acid sequences are underlined, and the putative signal sequence is shown by dashed underlining. The N-terminal residue is indicated by a horizontal arrow. The initiation and termination codons are shown in bold type.

The homogenate of the yellow body, suggesting that various proteases that cross-react immunologically with the anti-26-kDa protease antibody are concentrated in the yellow body. The yellow body appears at the pupal stage and engulfs the larval midgut, finally developing into the adult midgut after the larval midgut has been disintegrated in situ. Therefore, we examined the expression of these proteases in the yellow body in parallel with adult development within the pupal case. As is evident from Fig. 5, no appreciable signal was detectable when the midguts of third instar larvae or the yellow bodies of 1-day-old pupae were examined. However, at least four signals, including that of the 26-kDa protease, became detectable in the yellow bodies of 2-day-old pupae. Many of these persisted for several days but disappeared rapidly during the late pupal stage.

It is likely that various proteases that cross-react immunologically with anti-26-kDa protease antibody are synthesized simultaneously in the pupal yellow body. Therefore, we examined changes in the expression of the 26-kDa protease gene with time after pupation by Northern blotting. As shown in Fig. 6, mRNA for the 26-kDa protease was detected exclusively in RNA extracted from 1-day-old pupae, and no appreciable signal was detected in RNA from pupae at other stages. Thus, it is clear that the 26-kDa protease gene is transiently activated for...
We found and purified a 26-kDa protein that cross-reacts immunologically with anti-sarcotoxin IA antibody in the pupal extracts of *Sarcophaga*. This protein was found to be a novel serine protease with significant sequence similarity to bovine trypsin. Northern blotting and immunoblotting experiments revealed that the 26-kDa protease gene was transiently activated before induction of the enzyme, and that the timing of gene activation seems to depend strictly upon the developmental stage of the adult in the pupal case. The expression of the genes for other proteases that cross-react immunologically with the 26-kDa protease are likely to be regulated in the same way. Although we were able to identify the timing of the activation of the 26-kDa protease gene, the cells synthesizing the mRNA for this enzyme remained to be identified. As it is localized in the yellow body, adult midgut cells are most probable candidate cells.

The 26-kDa protease was first detected immunologically using an antibody against sarcotoxin IA. Sarcotoxin IA is an antibacterial protein produced by *Sarcophaga*, which consists of 39 amino acid residues (19). We raised the antibody using chemically synthesized sarcotoxin IA. No significant sequence similarity was found between sarcotoxin IA and the 26-kDa protease. During remodeling of the midgut from the larval to the adult type during metamorphosis, normal *Sarcophaga* flora living in the larval midgut are killed and replaced with adult flora. It is possible that the 26-kDa protease contains an epitope with a similar secondary or tertiary structure to sarcotoxin IA, and that this epitope has sarcotoxin IA-like antibacterial activity that might kill the normal larval flora that are dispersed in the yellow body when the larval midgut disintegrates in it. Another possibility to be noted is that sarcotoxin IA is a covalent inhibitor of the 26 kDa protease, and the covalent bond sarcotoxin IA reacted with the antibody. This possibility needs further examination.

**REFERENCES**


**Table II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity (μmol of 7-amino-4-methyl coumarin released/mg)</th>
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<tr>
<td>Boc-Gln-Ala-Arg-MCA</td>
<td>258</td>
</tr>
<tr>
<td>Boc-Leu-Thr-Arg-MCA</td>
<td>203</td>
</tr>
<tr>
<td>Boc-Phe-Ser-Arg-MCA</td>
<td>131</td>
</tr>
<tr>
<td>Pro-Phe-Arg-MCA</td>
<td>77</td>
</tr>
<tr>
<td>Carbobenzyoxy-Phe-Arg-MCA</td>
<td>69</td>
</tr>
<tr>
<td>Boc-Val-Leu-Lys-MCA</td>
<td>60</td>
</tr>
<tr>
<td>Pyr-Gly-Arg-MCA</td>
<td>17</td>
</tr>
<tr>
<td>Carbobenzyoxy-Arg-Arg-MCA</td>
<td>7</td>
</tr>
<tr>
<td>Benzoyl-Arg-MCA</td>
<td>3</td>
</tr>
<tr>
<td>Succinyl-Leu-Leu-Val-Tyr-MCA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Succinyl-Ala-Ala-Pro-Phe-MCA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Succinyl-Ala-Pro-Ala-MCA</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Succinyl-Gly-Pro-MCA</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

**FIG. 5. Immunoblot analysis of the 26-kDa protease.** A, homogenates (5 μg/lane) of the whole body, head, thorax and abdomen, and yellow body of 3-day-old pupae were subjected to immunoblotting analysis with an antibody against the 26-kDa protease and a radioiodinated second antibody. Lane 1, purified 26-kDa protease (10 ng); lane 2, larval midgut; lanes 3–8, yellow bodies of 1-, 2-, 3-, 7-, 8-, and 9-day-old pupae, respectively; lane 9, adult midgut. The arrow shows the position of the 26-kDa protease.

**FIG. 6. Northern blot analysis of the 26-kDa protease.** Total RNA was prepared from insects at various developmental stages and subjected to Northern blot hybridization. The probe was full-length 26-kDa protease cDNA. Each lane contained 20 g of total RNA. Lane 1, unfertilized eggs; lane 2, embryos; lane 3, first instar larvae; lane 4, second instar larvae; lane 5, early third instar larvae; lane 6, late third instar larvae; lane 7, white pupae; lane 8, 1-day-old pupae; lane 9, 3-day-old pupae; lane 10, 5-day-old pupae; lane 11, 7-day-old pupae; lane 12, newly emerged adults. Lower panel, staining of rRNA with ethidium bromide used as an internal reference.

**FIG. 7.**
A Novel Yellow Body Protease of Sarcophaga

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