Antibacterial activity of a novel 26-kDa serine protease in the yellow body of *Sarcophaga peregrina* (flesh fly) pupae

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Abstract We have reported a novel serine protease produced by *Sarcophaga peregrina* (Nakajima et al., J. Biol. Chem. 272 (1997) 23805–23810). This 26-kDa protease showed antibacterial activity against several bacteria. This activity was an intrinsic characteristic of the enzyme protein and not directly related to its protease activity, because treating the 26-kDa protease with diisopropyl fluorophosphate had no appreciable effect on its antibacterial activity. Unlike bovine trypsin, the 26-kDa protease interacted with acidic phospholipids, suggesting that its antibacterial activity is attributable to interaction with bacterial membranes.

Key words: Serine protease; Antibacterial activity; Metamorphosis; Insect; Yellow body

1. Introduction

In a previous study, we purified a novel serine protease with a molecular mass of 26 kDa and significant sequence similarity to bovine trypsin from *Sarcophaga peregrina* (flesh fly) pupae [1]. We purified this protease as a protein that cross-reacted immunologically with an anti-sarcotoxin IA antibody. Sarcotoxin IA is a cecropin-type antibacterial protein produced by this insect [2].

Most larval tissues of holometabolous insects are known to disintegrate during the pupal stage and new adult structures develop from imaginal discs [3]. In dipteran insects such as *Sarcophaga*, the larval mid-gut disintegrates and the adult mid-gut develops during the pupal stage. This process proceeds in a temporary organ called the yellow body. Adult mid-gut cells derived from imaginal islands on the larval mid-gut surround the larval mid-gut to form a primordial adult mid-gut, which swells to form the yellow body. The larval mid-gut encapsulated in the yellow body disintegrates in situ and the yellow body itself extends to form an adult mid-gut with development of adult structure. As the 26-kDa protease gene was activated transiently during the pupal stage and the protease was detected selectively in the yellow body, this protease probably participates in larval mid-gut disintegration [1].

During metamorphic mid-gut remodeling, the normal flora living in the larval mid-gut is believed to be killed and eventually replaced by the adult flora [4]. As an antibody against sarcotoxin IA cross-reacted with the 26-kDa protease, we expected the 26-kDa protease to possess both antibacterial and protease activities. We found that the 26-kDa protease exerted significant bactericidal activity against certain bacteria, suggesting that this novel protease is responsible for larval mid-gut disintegration and, at the same time, kills the larval normal flora that are dispersed in the yellow body when the larval mid-gut disintegrates.

2. Materials and methods

2.1. Preparation of the 26-kDa protease and treatment with diisopropyl fluorophosphate (DFP)

The 26-kDa protease was purified to homogeneity from *Sarcophaga* pupae, as described previously [1]. In order to inactivate the 26-kDa protease with DFP, purified enzyme (14.2 μg) was treated for 10 min at 27°C in 250 μl 100 mM Tris-HCl buffer, pH 7.9, containing 1 mM EDTA and 63 mM DFP. Control samples were treated identically, but without DFP.

2.2. Assay of the antibacterial activity of the 26-kDa protease

Staphylococcus aureus (Cowan strain) was grown in tryptic soy broth at 37°C. Bacteria at the logarithmic phase were collected and suspended at a density of 1×10^4 CFU/ml in 10 mM phosphate buffer, pH 5.5. Bacterial suspension (180–360 μl) and protease samples dissolved in the same buffer (20–40 μl) were mixed and incubated for 1 h at 37°C. Then, 100 μl each reaction mixture was plated onto a nutrient agar plate, incubated overnight at 37°C, and the resulting bacterial colonies were counted. The antimicrobial specificity of the 26-kDa protease was assayed essentially as described previously using various bacteria and fungi [2]. The antimicrobial activity of the 26-kDa protease was assessed by measuring the A_{650} value of the culture medium in which each microorganism was growing.

2.3. Interactions of the 26-kDa protease with phospholipids

Multilamellar liposomes were prepared using the methods of Kinsky et al. [5]. Binding of the 26-kDa protease to liposomes was assessed by incubating the mixtures of liposome suspension and the 26-kDa protease (1 μg) at 27°C for 10 min in 100 mM Tris-HCl buffer, pH 7.9, containing 5 μg/ml leupeptin. Then, each mixture was centrifuged at $100\,000\times g$ for 10 min and the top 4/5 (supernatant fraction) and the bottom 1/5 (precipitate fraction) were subjected to SDS-polyacrylamide gel electrophoresis to examine the free and liposome-bound 26-kDa protease. Over 80% of each phospholipid was recovered in the precipitate fraction.

3. Results

The 26-kDa protease was originally purified from an extract of *Sarcophaga* pupae as a protein that cross-reacted immunologically with an antibody against sarcotoxin IA [1]. Although no significant sequence similarity between sarcotoxin IA and the 26-kDa protease was found [1], we examined whether the 26-kDa protease possesses antibacterial activity. As respective positive and negative controls, we used sarcotoxin IA and bovine trypsin; the latter shows about 40% sequence identity to the 26-kDa protease. As shown in Fig. 1, the 26-kDa protease, but not trypsin, exhibited significant bactericidal activity, like sarcotoxin IA, when *S. aureus* (Cowan strain) was

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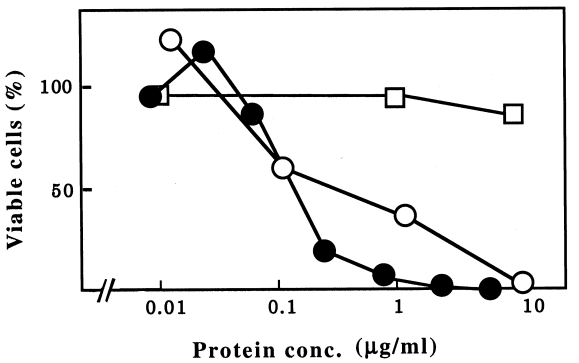

Fig. 1. Bactericidal activities of the 26-kDa protease, sarcotoxin IA and bovine trypsin. Increasing amounts of test sample were incubated with a fixed CFU of *S. aureus* (Cowan strain) and the number of viable cells in each relative to that of the control (treated with buffer alone) was plotted. ○, 26-kDa protease; □, bovine trypsin; ●, sarcotoxin IA.

used as an indicator bacterium and its ED_{50} value was about 0.2 μg/ml under our assay conditions.

We examined the antibacterial specificity of the 26-kDa protease by assessing inhibition of the growth of various microorganisms. As summarized in Table 1, *Corynebacterium bovis* was very sensitive to this enzyme, but *Escherichia coli* was resistant and the minimum inhibitory concentration (MIC) was estimated to be over 75.5 μg/ml under our assay conditions. *Candida albicans* and *Candida pseudotropicalis* were also resistant to this protease, suggesting that generally, bacteria are more susceptible to it than fungi.

Although bovine trypsin was found to have no appreciable bactericidal activity, the possibility remained that the bactericidal activity of the 26-kDa protease was due to its protease activity, i.e. the surface protein(s) of sensitive bacteria was digested by the 26-kDa protease, resulting in cell death. Therefore, we pretreated the 26-kDa protease with DFP to inhibit the protease activity and then examined its bactericidal activity. As shown in Fig. 2, both the DFP-treated and intact 26-kDa protease showed virtually identical bactericidal activities, although the protease activity of the former had been abolished (inset). These results suggest that the antibacterial activity of the 26-kDa protease is not due to its protease

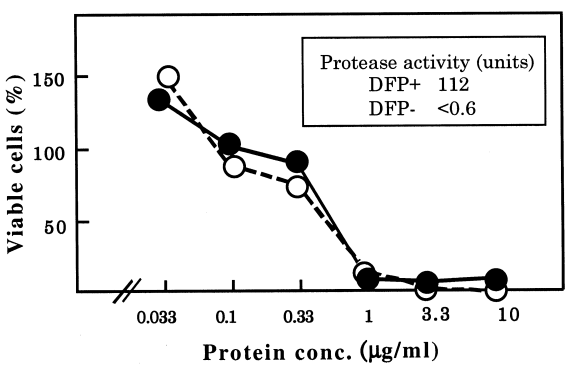

Fig. 2. Bactericidal activity of the DFP-treated 26-kDa protease. Purified 26-kDa protease was treated with DFP and the bactericidal activity was examined. DFP alone had no appreciable bactericidal activity under these assay conditions. Inset: protease activity of 1 μg intact and DFP-treated 26-kDa protease in units (1 unit is the amount that released 1 μmol MCA). ●, DFP-treated 26-kDa protease; ○, intact 26-kDa protease.

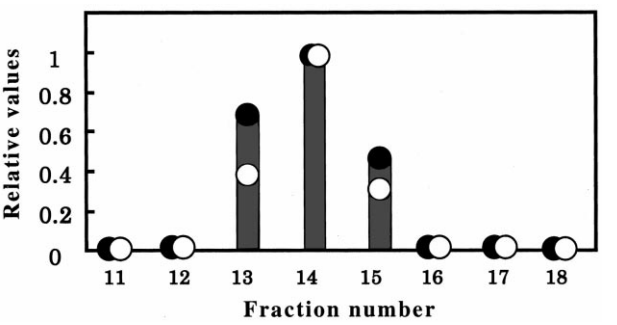

Fig. 3. Reverse-phase HPLC of purified 26-kDa protease. Purified 26-kDa protease was subjected to reverse-phase HPLC using a C_{18} column, the amount of protein, protease activity and antibacterial activity in each fraction were determined and their values relative to those of fraction 14 were plotted. ○, Protease activity; ●, antibacterial activity; column, amount of protein.

activity, but is an intrinsic characteristic of the protein molecule. In this series of experiments, we used purified 26-kDa protease, but it is possible that the antibacterial activity was due to some other substance(s) contaminating the protease fraction. In order to exclude this possibility as far as possible, we subjected the purified 26-kDa protease to reverse-phase HPLC and assayed the protease and antibacterial activities of each fraction to see whether they could be separated, which, as shown in Fig. 3, they could not. The relative values of the amount of protein, antibacterial activity and protease activity in each fraction agreed well. Moreover, as reported previously, only one amino-terminal residue was identified when purified 26-kDa protease was subjected to amino acid sequencing [1]. Therefore, we concluded that the 26-kDa protease itself possesses antibacterial activity.

In a previous study, we demonstrated that sarcotoxin IA interacted with acidic phospholipids of bacterial membranes, resulting in membrane disorganization and disruption of the electrochemical membrane potential [6]. Therefore, we examined whether the 26-kDa protease interacted with phospholipids by preparing multilamellar liposomes using phosphatidylglycerol (PG), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and examining binding of the 26-kDa protease to these liposomes. It became clear that the 26-kDa protease bound selectively to the acidic phospholipids, PG, PS and PI, but not to the neutral phospholipids, PC and PE. A typical pattern of interaction of the 26-kDa protease with PS is shown in Fig. 4. No appreciable interactions between bovine trypsin and these phospholipids were detected under the same conditions. These results suggest that the antibacterial activity of the 26-kDa

Table 1
Antimicrobial specificity of the 26-kDa protease

Microorganism	MIC value (μg/ml)
Staphylococcus aureus (Cowan)	18.9
Staphylococcus aureus (Smith)	75.5
Corynebacterium bovis (1810)	2.4
Bacillus subtilis (PCI219)	18.9
Escherichia coli (NIH J)	>75.5
Salmonella typhi (T-63)	18.9
Candida albicans	>75.5
Candida pseudotropicalis	>75.5

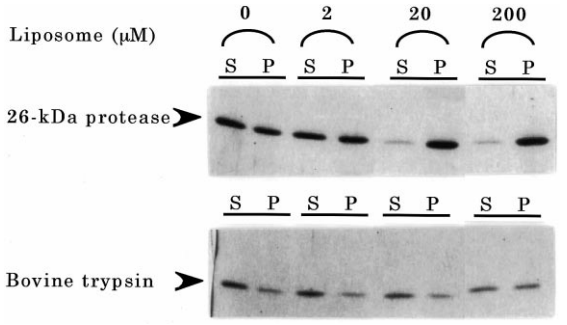

Fig. 4. Binding of purified 26-kDa protease to PS liposomes. Increasing amounts of liposomes were mixed with 1 μg purified 26-kDa protease or bovine trypsin and kept at 27°C for 10 min. After centrifugation, the supernatant (top 4/5) and pellet (bottom 1/5) fractions were subjected to SDS-polyacrylamide gel electrophoresis to estimate the amount of protease recovered in each fraction. S and P indicate the supernatant and pellet fractions, respectively.

protease is due to its interaction with bacterial membranes, as it does not seem to enter bacterial cells.

4. Discussion

We found that the 26-kDa protease purified from *Sarcophaga* pupae possesses antibacterial activity. We expected this would be the case because we found that this protease cross-reacted immunologically with an antibody against sarcotoxin IA. Sarcotoxin IA is an antibacterial protein comprising 39 amino acid residues produced by this insect [7]. However, as reported previously, no significant sequence identity between sarcotoxin IA and the 26-kDa protease was found, suggesting that this antibody recognized an epitope in the 26-kDa protein with a similar secondary and/or tertiary structure to sarcotoxin IA. There seem to be two possible situations: one is that the epitope in the 26-kDa protease that reacts with the antibody against sarcotoxin IA is in fact crucial for its antibacterial activity, and the other is that its antibacterial activity and antibody reactivity are not directly related. As reported previously, sarcotoxin IA is very toxic to *E. coli* and *C. bovis*, with MIC values usually below 1 μg/ml [2]. However, although *C. bovis* was sensitive, *E. coli* was rather resistant to the 26-kDa protease. Therefore, it is too early to conclude that the modes of action of the 26-kDa protease and sarcotoxin IA are the same.

Recently, several serine proteases and proteins structurally related to them were reported to possess bactericidal activity and they were thought to play roles in the host defense mechanisms of various organisms [8,9]. Such molecules have been named serprocidins and cathepsin G, elastase, protease-3 and azurocidin are members of the serprocidin family. The amino acid identity between the 26-kDa protease and cathepsin G was about 31% [10]. We propose that the 26-kDa protease is also a member of the serprocidin family. The 26-kDa protease was found to be synthesized at a specific developmental stage of *Sarcophaga*, when remodeling of the mid-gut takes place in the yellow body, and it was suggested to be involved in disintegration of the larval mid-gut in situ. The 26-kDa protease may play a role in killing the normal flora that is released from the disintegrating larval mid-gut into the yellow body and elimination of the larval flora is a prerequisite for its replacement by the adult flora.

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