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# Identification of Natural Target Proteins Indicates Functions of a Serralysin-Type Metalloprotease, PrtA, in Anti-Immune Mechanisms[▽]

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**Serralysins are generally thought to function as pathogenicity factors of bacteria, but so far no hard evidence of this (e.g., specific substrate proteins that are sensitive to the cleavage by these proteases) has been found. We have looked for substrate proteins to a serralysin-type proteinase, PrtA, in a natural host-pathogen molecular interaction system involving *Manduca sexta* and *Photorhabdus luminescens*. The exposure in vitro of hemolymph to PrtA digestion resulted in selective cleavage of 16 proteins, provisionally termed PAT (*PrtA target*) proteins. We could obtain sequence information for nine of these PrtA sensitive proteins, and by searching databases, we could identify six of them. Each has immune-related function involving every aspect of the immune defense: β-1,3 glucan recognition protein 2 (immune recognition), hemocyte aggregation inhibitor protein (HAIP), serine proteinase homolog 3, six serpin-1 variants, including serpin-1I (immune signaling and regulation), and scolexins A and B (coagulation cascade effector function). The functions of the identified PrtA substrate proteins shed new light on a possible participation of a serralysin in the virulence mechanism of a pathogen. Provided these proteins are targets of PrtA in vivo, this might represent, among others, a complex suppressive role on the innate immune response via interference with both the recognition and the elimination of the pathogen during the first, infective stage of the host-pathogen interaction. Our results also raise the possibility that the natural substrate proteins of serralysins of vertebrate pathogens might be found among the components of the innate immune system.**

The outcome of a microbial infection depends on the complex molecular interactions between the host and the pathogen. In this battle, secreted enzymes are employed by the pathogen, of which proteinases are of particular utility as they can efficiently interfere with the function of the proteinaceous immune molecules or promote penetration in the tissues of the host. To understand the function of a proteinase and assess its role in determining virulence, knowledge of its proteolytic system is necessary: i.e., mapping of its target protein(s) and inhibitor(s). Unraveling such systems of pathogen-secreted proteinases also can reveal new components and/or functions of the host's defense mechanisms. Surprisingly, despite the number and importance of proteases of pathogens, only a few of their substrate proteins are known, and the proteolytic system of none of these enzymes has been explored.

Here we investigate the target (PAT) proteins in the proteolytic system of PrtA, a bacterial metallo-endopeptidase in the M10B subfamily of the MA clan of proteinases (31). There are a number of enzymes in M10B, also called serralysins, which are secreted by a wide range of microorganisms, including plant and human pathogens. Serralysin-type proteases are generally supposed to be nonspecific enzymes, involved in, e.g., bioconversion of the host tissues, because they cleave with a relaxed residue preference in a variety of synthetic peptide substrates and denatured oligopeptides of biological origin (1, 2, 9, 22, 23, 25, 30). However, serralysins, like most of the proteases, encounter substrate proteins under physiological conditions, where they are in native conformation. The cleavage of such proteins is substantially restricted by the difficulty of disrupting the native structure around the cleavage site in a 6- to 8-amino-acid-long segment, which is needed to achieve proper substrate binding to the active site of the protease. Therefore, the observations on artificial substrates are poorly informative about the function of proteases and do not exclude the possibility that they can have specific target proteins of special function(s). Except for some inhibitors that are secreted by the bacteria together with the proteases, the proteolytic system of neither of the serralysins has been explored, and natural substrate proteins are hardly known. Due to this, the generally supposed virulence factor role of serralysins has still not been confirmed experimentally. The only observation in this regard is the in vitro cleavage of immunoglobulin A and G proteins and several human defensins, as well as some cell matrix and interconnecting filament proteins, by ZapA of *Proteus mirabilis* (1, 2, 35, 38) and serralysin of *Serratia marcescens* (28, 29). However, the in vivo significance of these reactions, too, remains to be established because the applied conditions, a large enzyme/substrate molar ratio and the very long incubation time (1:10 to 1:6,000 and 3 to 24 h, respectively), do not indicate the sensitive cleavage which might be expected in the case of specific target proteins.

The serralysin PrtA investigated in this study is one of the secreted proteinases of the insect-pathogenic bacterium *Photorhabdus luminescens* (*Enterobacteriaceae*), which lives in symbiosis with the entomopathogenic nematode *Heterorhabditis bacteriophora*. Once in the hemocoel, *Photorhabdus* is ex-

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tremely pathogenic for most of the insects. (In the case of, e.g., *Galleria mellonella*, even a single bacterial cell can establish infection which eventually kills the insect [10].) The mechanism of this pathogenicity, the way the bacterium can evade the immune defense, is unknown, but PrtA might take part in it. Supporting this assumption are the facts that *Photorhabdus* starts producing PrtA early during infection (4, 5, 27, 34) and that PrtA does not exhibit activity on native proteins (fibrinogen, albumin, and collagen types I and IV) (27), which might have been expected if it participates in the bioconversion of host tissues as a nonspecific protease (5, 6) or if it is involved in the degradation of extracellular matrix (36). The contribution of PrtA to pathogenicity does not include a direct toxic effect either (5), at variance with several other metalloproteases which are lethal toxins. Here we investigate the possibility that PrtA is a virulence factor with, e.g., an immune suppression function through the specific cleavage of immune proteins by searching for target proteins in the hemolymph of the host insect, *Manduca sexta* (tobacco hornworm; Lepidoptera).

## MATERIALS AND METHODS

**Enzymes.** PrtA was purified from *Photorhabdus luminescens* strain Brecon as described previously (27). Recombinant PrtA was purified from *Escherichia coli* Hb101 strains transformed with pUC19 plasmid containing *Photorhabdus* PrtA (a generous gift from Richard ffrench-Constant, Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom). One hundred milliliters of LB medium was inoculated with several colonies from a fresh LB plate and incubated for 24 h at 37°C. The culture supernatant was dialyzed (cutoff, 12 kDa) for 4 h against 2× 5 liters of buffer A (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM $CaCl_2$). The dialysate was centrifuged at 7,155 × *g* for 15 min and loaded onto a 1.6-cm by 3-cm PAE 300 column (Matrex silica PAE 300; Millipore) equilibrated with buffer A. The elution was performed with a 20-ml 0 to 1.0 M linear NaCl gradient in buffer A with a flow rate of 0.5 ml/min. The fractions containing PrtA were clean of other proteins and protease(s) as checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and zymography with gelatin and casein substrates (26).

The purification of the 12 expressed serpin-1 proteins was conducted on HIS-Select nickel affinity gel (Sigma-Aldrich, St. Louis, MO) according to Jiang and Kanost (14) from 30-ml cultures of the *E. coli* XL1 Blue strain, which was transformed with Bluescript plasmids containing the serpin-1 variants (generous gift form Mike Kanost, Department of Biochemistry, Kansas State University).

Bovine pancreatic trypsin, chymotrypsin, and *Clostridium hystolyticum* collagenase were purchased from Sigma-Aldrich.

**Insects.** *M. sexta* eggs (kindly provided by Stuart Reynolds, Department of Biology and Biochemistry, University of Bath, Bath, United Kingdom) were hatched, and the larvae were reared individually on a wheat germ-based artificial diet according to Reynolds et al. (32).

**Hemolymph samples.** Newly molted, day zero, fifth-stage *M. sexta* larvae were bled, and their hemolymph was immediately diluted into 4 volumes of prechilled sterile phosphate-buffered saline (137.9 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$ [pH 6.5]) containing 1.0 mM phenylthiourea. The samples were sedimented at 2,795 × *g* for 15 min to obtain cell-free hemolymph plasma.

**Fractionation of hemolymph proteins.** The pH of a 5.0-ml hemolymph sample (above) was adjusted to pH 9.0 using 10 M NaOH. After centrifugation (at 36,223 × *g* for 15 min), the clear supernatant was loaded onto a 15.8-cm by 1.0-cm DEAE Sephadex anion-exchange column, equilibrated with a solution of 50 mM Tris-HCl (pH 9.0), 1.0 mM benzamidine, and 1.0 mM phenylthiourea. The proteins were eluted with a linear 0 to 0.9 M NaCl gradient in the equilibrating solution at a flow rate of 0.5 ml/min. Fractions (1.2 ml) were collected and analyzed by SDS-PAGE (described below).

**Partial purification of PAT proteins.** Fifty milliliters of 5× diluted hemolymph was precipitated by the addition of saturated $(NH_4)_2SO_4$ solution (pH 8.0) in two steps so as to obtain 46% and 67% final saturation in the resulting solution. The precipitates were resuspended in 3.0 ml gel filtration buffer (20 mM Tris-HCl [pH 8.0], 1.0 mM benzamidine, 1.0 mM phenylthiourea, 0.3 M sodium acetate [pH 8.0]). Before gel filtration, the samples were dialyzed against the gel filtration buffer (cutoff, 12 kDa), and then the insoluble material was sedimented by centrifugation. Chromatography was performed on a 16/60 Sephacryl-S200 gel-filtration column and on a Superdex-75 analytical gel-filtration column (Amersham Biosciences, Piscataway, NJ) at a flow rate of 0.5 ml/min. The protein content of the effluent was monitored at 280 nm.

Before anion-exchange chromatographies, the protein solutions were dialyzed against an anion-exchange buffer (20 mM Tris-HCl [pH 8.0], 1.0 mM EDTA, 1.0 mM phenylthiourea, 1.0 mM benzamidine) and the insoluble material was precipitated with centrifugation. The flow rate in anion-exchange chromatographies was 0.5 ml/min. The PAE 300 column (9.0 by 1.0 cm) was eluted with a 0 to 0.5 M NaCl linear gradient in the anion-exchange buffer, whereas during chromatography on a MonoQ 5/50 GL fast protein liquid chromatography column (Amersham Biosciences), the following NaCl gradient was used in the anion-exchange buffer: 0 to 0.4 M within 35 min and then 0.4 to 0.5 M within 5 min. The protein content of the effluent was monitored at 280 nm.

**Blotting and N-terminal sequencing.** The (partially) purified PAT protein-containing samples were run in 10% acrylamide–SDS gels under reducing conditions. The gels were soaked for 10 min in transfer buffer, consisting of 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid; Sigma), pH 11.0, plus 10% methanol, and blotted onto Immobilon-P transfer membrane (Millipore, Bedford, MA) at 200 mA for 2 h. The protein bands on the membrane were visualized by Coomassie brilliant blue R-250 staining. The bands of PAT proteins were cut out and subjected to Edman sequencing in a Microtec protein sequencer (Applied Biosystems, Foster City, CA) by András Patthy at the ELTE-MTA Biotechnology Research Group. Identification through NCBI database search for similar sequences was made with BLAST algorithms.

**Digestion of hemolymph and PAT proteins with PrtA.** During the initial search for PrtA substrate proteins, volumes of hemolymph fractions (described above), which contained 0.5 to 5.0 μg protein, were exposed to digestion with 0.3 ng PrtA (~0.3 nM final concentration) or 30 ng chymotrypsin, trypsin, and *Clostridium* collagenase (~60 nM final concentration) at room temperature in the presence of 50 mM Tris-HCl (pH 8.0), 10 mM $CaCl_2$, and 0.1 M NaCl (a reaction buffer in which all of the applied enzymes could exhibit their highest activity on synthetic substrates). Samples were withdrawn at 45 and 90 min of incubation. In order to find the PAT protein-containing fractions after the various isolation steps, samples of appropriate volumes were digested with 0.3 nM PrtA in a final volume of 20 μl at room temperature for 90 min. The purified serpin-1 variants were subjected to PrtA cleavage at a ratio of 1 to 4 μM serpin-1 to 30 nM PrtA (1.0 to 3.7 μg serpin-1 to 30 ng PrtA) in the reaction buffer at room temperature for 90 min. All samples from the digestions were analyzed by SDS-PAGE.

**Gel electrophoresis.** To monitor purification steps, to detect the PrtA cleavage of hemolymph proteins, and to determine the mass of the PAT proteins, standard SDS-PAGE was carried out in 10% (wt/vol) polyacrylamide gels. The samples were treated with dithiothreitol and boiled for 5 min. The gels were stained with Coomassie brilliant blue R-250.

## RESULTS

As an initial step in finding the substrate proteins of *Photorhabdus* PrtA, we analyzed hemolymph samples by SDS-PAGE during infection or after PrtA injection, but we could detect, in the former case, only two slight changes, which were not reliably reproducible. We thought this negative result could be misleading for a protease that—as we supposed—is target protein specific because the number of proteins in the hemolymph is large and some are present in high concentrations, so it is hard to detect the cleavage of those substrate proteins which are present in small amounts. For the same reason, the exposure of *M. sexta* hemolymph to in vitro digestion with PrtA did not allow sensitive detection of such target proteins. Therefore, we first performed protein separation on a DEAE anion exchanger (see Materials and Methods). By SDS-PAGE analysis, we distinguished four groups of the fractions that differed in at least one protein band from each other. Fractions 36, 41, 49, and 67 represent these groups (Fig. 1). We used these four hemolymph fractions to treat with PrtA at a ratio of 0.5 to 5.0 μg hemolymph protein to 0.3 ng PrtA (~0.3 nM). In order to determine the specificity and sensitivity of

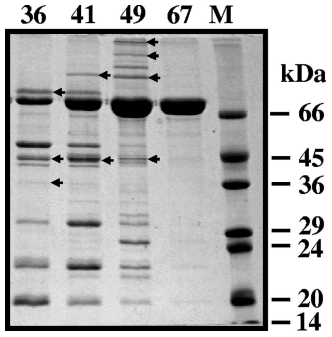


FIG. 1. SDS-PAGE analysis of hemolymph fractions generated by DEAE Sephadex chromatography. Each of the four groups of fractions with a distinct protein composition is represented with one fraction in the figure. The fraction numbers are shown above the lanes. Arrows point to those proteins which were selectively degraded by PrtA (Fig. 2). M, molecular mass standard.

PrtA cleavage, we also subjected the hemolymph fractions to digestion with pancreatic trypsin, chymotrypsin, and *Clostridium histolyticum* collagenase (60 nM final concentration). Figure 2 shows that PrtA hydrolyzed 10 proteins in hemolymph fractions 36, 41, and 49. (No cleavage was observed in fraction 67.) Since one of these proteins was also cleaved by another protease (collagenase; ~110-kDa protein in Fig. 2b), we concluded that nine proteins might be specific substrates to *Photorhabdus* PrtA. We provisionally distinguished these proteins with the name "PAT-*n*," where "PAT" is the acronym for *Prt*A *t*arget and *n* shows the molar mass estimated by the protein's relative mobility in SDS-PAGE.

The purification of the two relatively abundant PAT proteins PAT-52 and -110 could be accomplished at a very low yield. Therefore, in the case of the other PAT proteins, which were present in the hemolymph in lower concentrations, we aimed at a purity only to reach a separation which allowed N-terminal sequencing after SDS-PAGE and blotting. During this procedure, we obtained further information about the cleavage of these PAT proteins and, more importantly, we detected six new ones. These were present in the hemolymph in such a small amount that they became detectable by Coomassie staining of acrylamide gels only after they were concentrated by ion-exchange chromatography.

The scheme of PAT protein isolation is summarized in Fig. 3 (for technical details, see Materials and Methods). The PAT proteins separated into two fractions during $(NH_4)_2SO_4$ precipitation: the 46% precipitate contained all of them, except for PAT-52, which remained in the supernatant and was precipitated at 67% $(NH_4)_2SO_4$. Pure PAT-52 could be obtained by Sephacryl-S200 chromatography followed by analytical gel filtration on a smaller-capacity but higher-separation-power Superdex-75 column. Sephacryl-S200 gel-filtration of proteins in the 46% $(NH_4)_2SO_4$ precipitate separated PAT-41 from PAT-90 and -110. The fractions from the Sephacryl-S200 column containing PAT-90 and -110 were further purified on a PAE 300 column, while those containing PAT-41 were purified on a MonoQ column. (The weak hydrophobic interaction effect during the anion-exchange chromatography on PAE 300 made the purification very effective for PAT-90 and -110, which did not work as well in the case of other PAT proteins.)

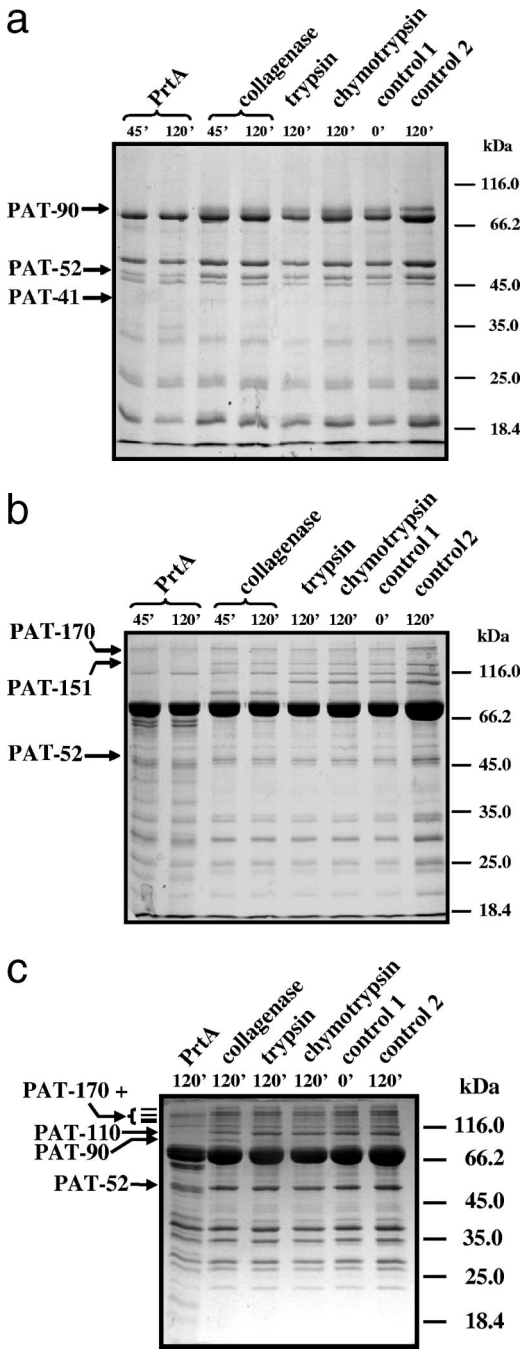


FIG. 2. Selective PrtA cleavage of proteins in three DEAE fractions of distinct hemolymph protein compositions. Panels a, b, and c show the analysis of DEAE fractions 36, 41, and 49, respectively (Fig. 1). The conditions of the reactions shown above the lanes are the type of the protease applied (control is without protease) and incubation time. (For further details, see Materials and Methods.) Arrows point to PAT proteins, which are selectively cleaved by PrtA. The numbers refer to the estimated molecular masses of PAT proteins. These are not shown for three proteins which are larger than PAT-170 in DEAE fraction 49 (c) because of the imprecision of estimation.

The MonoQ chromatography made new PAT proteins detectable. It separated two PAT-41 (PAT-41a and -b) and three PAT-54 (PAT-54a, -b, and -c) proteins, as well as PAT-35 and -63. Together with these, the number of proteins that were

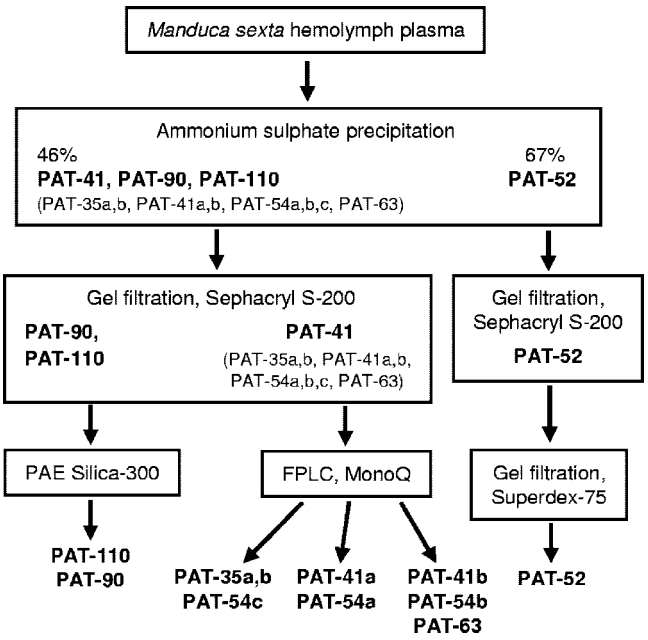


FIG. 3. Procedure for the separation of PAT proteins. PAT protein names in boldface letters in a purification step indicate those proteins that were detectable following that step.

cleaved by PrtA increased to 15. (The further purification of PAT-110, -90, and -52 [not shown] did not reveal other proteins sensitive to PrtA cleavage.)

As PAT proteins became more concentrated and better isolated from other proteins, some features of their cleavage could be observed. For example, PAT-52 was never hydrolyzed completely (Fig. 2b and c and Fig. 4c), even after a longer (120 min) exposure to PrtA. In contrast, PAT-110 and -90 proved to be very sensitive: they were completely cleaved in the presence of even 0.03 nM PrtA (instead of the usual 0.3 nM) within less than 40 min. Importantly, when pure PAT-110 was exposed to trypsin or chymotrypsin digestion under the same conditions as those with PrtA, it remained intact, showing that this protein (like other PAT proteins) is not generally sensitive for proteolysis (data not shown).

The appearance of cleavage products was mostly temporary. This suggests that they might be further degraded by either PrtA or contaminating proteinases which were present in the hemolymph and started acting on PAT proteins only after an initial cleavage by PrtA. However, the cleavage products of PAT-170, -54a, -41a, -35a and -b, and PAT-52 have longer half-lives, suggesting that these might not be degraded further by PrtA. In the case of the latter, the cleavage product was just a little smaller than the intact PAT-52 (Fig. 2b and c and Fig. 4c), showing that PrtA clipped only 10 to 15 amino acids from one of the molecular termini.

The isolation and concentration of eight proteins permitted N-terminal sequence determination. One of them, PAT-35, gave double signal in each sequencing cycle indicative of two proteins (distinguished as PAT-35a and PAT-35b), which increased the total number of PAT proteins to 16. The N-terminal sequences are shown in Table 1. PAT-110 and -90 had very similar sequences. With the exception of PAT-41b, -90, and -110, the interrogation of the Protein Data Bank yielded matches with high confidence (low E value of BLAST). In the case of PAT-35a and -b, we generated all of the possible sequence variants ($2^{10}$ = 1,024 sequences) with the help of a C-script and then loaded them on the BLAST server. When we restricted the search to *M. sexta* sequences with an upper limit of E value at 0.1, the search resulted in two proteins, scolexins

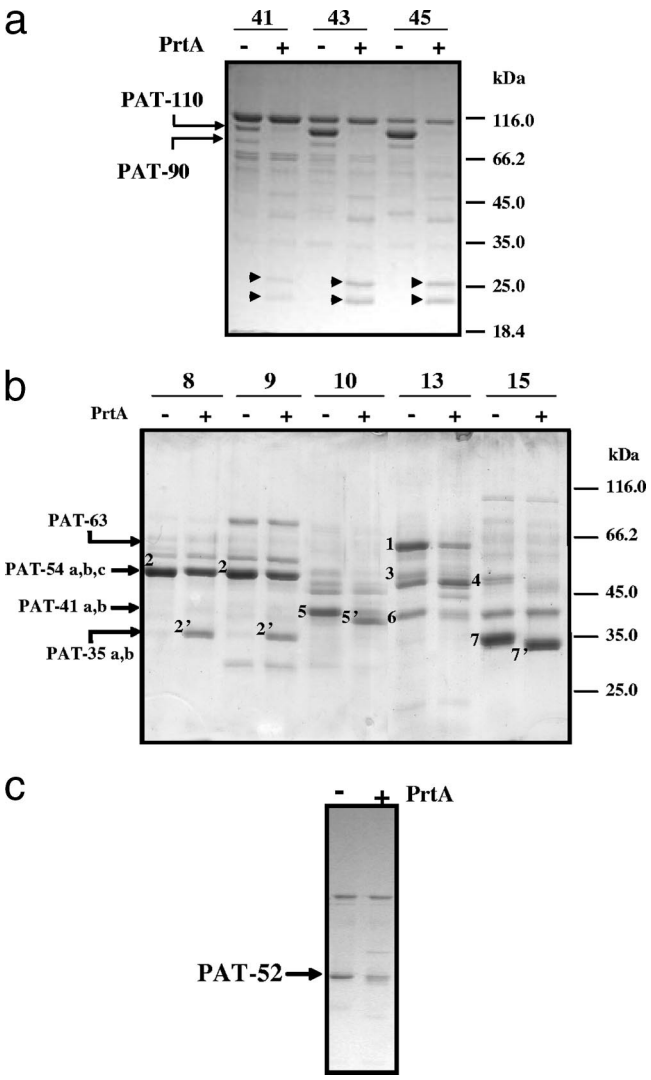


FIG. 4. PrtA digestion and SDS-PAGE analysis for finding PAT proteins after the various purification steps. The PAT protein-containing fractions of only three purification steps (shown in Fig. 3) are shown. Fraction numbers of the respective purification step and the incubation in the presence (+) or absence (−) of PrtA are indicated above the lanes. (For further details, see Materials and Methods.) (a) Finding PAT proteins after PAE 300 chromatography of the PAT-90- and -PAT-110-containing fractions from Sephacryl S-200 chromatography. Arrowheads indicate PAT-110 cleavage products. (b) Analysis of fractions following MonoQ fast protein liquid chromatography of the PAT-41-containing fractions from the Sephacryl S-200 chromatography. The bands of PAT proteins are labeled with numbers as follows: 1, PAT-63; 2, PAT-54a; 3, PAT-54b; 4, PAT-54c; 5, PAT-41a; 6, PAT-41b; and 7, PAT-35a and b. Numbers with primes indicate putative degradation products of PAT proteins of the corresponding number. (c) Detection of PAT-52 by PrtA digestion in a fraction of Sephacryl S-200 chromatography of proteins in the 40% $(NH_4)_2SO_4$ precipitate.

TABLE 1. N-terminal sequences of nine PAT proteins and identification of six of them by database sequence

| PAT protein | N-terminal sequence | Name | BLAST E value | Supposed function | Reference(s) |
|---|---|---|---|---|---|
| PAT-35ab | DANDIDIQQN<br>GPGANQLNLK | | | | |
| | DPGANDIQLN[a] | Scolexin A[b] | $4 \times 10^{-2}$ | Immune | 11, 20 |
| | GANDIQLNQK[a] | Scolexin B[b] | $6 \times 10^{-2}$ | Immune | |
| PAT-41a | DQGSPDLPLATEPPT | | | | |
| | DQGSPDLPLATEPPT[a] | SPH-3[b] | $3 \times 10^{-4}$ | Immune | 33 |
| PAT-41b | GVIKEESI | Unknown | | Unknown | |
| PAT-52 | ETDLQKILRESNDQFTA | | | | |
| | ETDLQKILRESNDQFTA[a] | Serpin-1[b] | $1 \times 10^{-5}$ | Immune | 14, 15, 17 |
| PAT-54a | TPASVPRKVLXYYDSK | | | | |
| | TPASVPXKVLXYYDSK[a] | HAIP[b] | $2 \times 10^{-3}$ | Immune | 18 |
| PAT-63 | LEVPDALKEAIYPKG | | | | |
| | LEVPDALKEAIYPKG[a] | β1,3-GRP-2[b] | $2 \times 10^{-2}$ | Immune | 19, 24, 39 |
| PAT-90 | QXNESLVII | | | | |
| PAT-110 | QRXESLVIIIVEDXGXAAI | Unknown | | Unknown | |

[a] Database sequence.
[b] Database name.

A and B. Their polypeptide chains are of the same length and exhibit more than 90% sequence identity, explaining why PAT-35a and -b did not separate during the isolation steps or in SDS-PAGE. We performed a search with the PAT-41b, -90, and -110 sequences in *M. sexta* expressed sequence tag libraries obtained from fat body and hemocyte (NCBI database) as well as among the recently published sequences of 123 *M. sexta* hemolymph proteins (12). However, we did not find matches. This is very surprising in the case of PAT-110, which is a relatively abundant, constitutive component of the hemolymph. Thus, we could identify six PAT proteins altogether. All of these had been known, supposed, or proven later (as for PAT-41a; see Discussion) to have immune-related functions.

We investigated the cleavage of PAT-52 (*Manduca* serpin-1) further to explain the fact that a fraction of this protein always remained uncleaved by PrtA (described above). This protein is present in the hemolymph in 12 C-terminal variants (32), and even the purified fraction might contain several of them. Therefore, the most likely explanation for our observation was that only some of the variants are sensitive for PrtA cleavage. To verify this and to establish which ones are cleaved, we expressed and purified all of the variants (see Materials and Methods) and subjected them to PrtA digestion. Several variants (B, E, F, J, and Z) were, indeed, not digested, and the others in turn were hydrolyzed with an occasional accumulation of 1.0- to 1.5-kDa-smaller, short-lived cleavage products (data not shown). PrtA must discriminate serpin-1 variants through their differential, 40- to 50-amino-acid-long C-terminal end, which contains the reactive site loop that confers the protease selectivity (14) and—as revealed by their different PrtA sensitivity—also the susceptibility to proteolytic cleavage by a noncognate protease (i.e., not a serine protease). The N-terminal analysis of serpin-1 preparations from *M. sexta* hemolymph after exposure to PrtA digestion (which contained a mixture of cleaved and not cleaved molecules) supported this assumption (PrtA cleavage on the C terminus) because we got a single sequence identical to that of the known N terminus of (the uncleaved) *Manduca* serpin-1 (data not shown).

## DISCUSSION

We searched for target proteins in the proteolytic system of a serralysin, PrtA, using the hemolymph of *M. sexta*. The presence of only a small number of hemolymph proteins we found to be cleaved by PrtA does not support the view that, as generally serralysins are thought to be, PrtA is a nonspecific protease having solely a role in, e.g., bioconversion. This is in accord with our former observation that PrtA did not hydrolyze native-state collagen, fibrin, and albumin (27). We found altogether 16 PAT proteins, most of which are minor components of the hemolymph. PrtA readily and selectively cleaved these and, as judged by the high substrate/proteinase ratios and the relatively short reaction times, their sensitivity to PrtA was substantially higher than those of several, partly immune-related proteins to other serralysin-type enzymes, ZapA of *Proteus mirabilis* (1, 2, 35, 38) and serralysin of *S. marcescens* (28, 29). These features and the fact that *M. sexta* is a potential host for the deadly insect pathogen *Photorhabdus luminescens*, which secrets PrtA early during infection, suggest that the cleavage of PAT proteins might have a role in virulence. The identities (and functions) of 10 PAT proteins are still unknown. Thus, the exploration of the substrate side in the proteolytic system of PrtA in *M. sexta* is not finished yet, and, therefore, a full assessment of the roles of PrtA is still not possible. Nevertheless, the known functions of the 6 PAT proteins identified, regardless of the functions of the other 10, do indicate already that, as one of its possible roles, PrtA

might participate in the immunosuppressive mechanisms of *Photorhabdus*.

PAT-63 is β-1,3 glucan recognition protein 2 (β-1,3-GRP-2) of *M. sexta* (24). Similar proteins have been found in different families of invertebrates (3, 19, 21, 24, 39). By binding β-1,3-glucans (e.g., laminarin and crudlan) and/or lipopolysaccharides, such pattern recognition proteins function as immune receptors, which trigger a proteolytic cascade leading to the activation of prophenoloxidase (3, 13, 21, 24, 37, 39) and an unknown signaling pathway for the production of immune-inducible antibacterial peptides and proteins (21). PAT-54a, hemocyte aggregation inhibitor protein, had been found through its effect on hemocytes in vitro (18). It might be involved in cellular immune responses like nodulation and encapsulation of pathogens. PAT-35a and -b, scolexins A and B, are chymotrypsin-like proteinases (11, 20) which are thought to induce coagulation reactions during nodulation and encapsulation. PAT-52, *Manduca* serpin-1, was found to inhibit in vitro various serine proteinases (14, 17). Of the 12 C-terminal sequence variants, generated via alternative splicing (14, 15), functions were found only for serpin-1I (one of the PrtA cleaved variants [see Results]) and serpin-1J, which inhibit hemolymph proteinase 14 (37) and prophenoloxidase-activating proteinase-3 (16), respectively. Thus, these are supposedly involved in the regulation of one of the most important instant immune responses of insects, melanization, which is caused by phenoloxidase activation. The 12 serpin-1 variants, which differ in their 40- to 50-amino-acid-long C-terminal sequences, were cleaved at different rates. This specifically shows a differential sensitivity to PrtA and indicates generally that the C-terminal segment, which is responsible for the protease selectivity of serpin-1, may also be the determinant of their proteolytic stability. PAT-41, serine proteinase homolog 3 (SPH-3), belongs to a large group of proteins that are similar to serine proteinases in their amino acid sequences but are catalytically inactive due to the replacement of the catalytic residue(s) (33). Members of the group are also found in vertebrates, and some of them contain an N-terminal, disulfide knotted extension called a "clip-domain." With the exception of several clip-domain SPH proteins, their function is unknown. SPH-3, a non-clip-domain SPH, has been known only as an immune-inducible protein (40). However, knockdown studies using RNA interference revealed that SPH-3 plays a central role in the signaling pathway of *M. sexta* that regulates the gene expression of antimicrobial effector proteins and peptides but not that of immune receptors (G. Felföldi, S. Reynolds, et al., submitted for publication).

Supposing that PAT proteins are cleaved by PrtA also in vivo, the functions of the identified proteins suggest a virulence strategy of *Photorhabdus* to enhance its survival during the first, decisive phase of infection and how the activity of a serralysin-type proteinase like PrtA might be part of this strategy: the strategy is a concerted attack on both the recognition and response functions of the innate defense system of the host by using a protease which has a relaxed specificity (of cleavage site) (27) and yet is able to hydrolyze proteins in their native state. This activity can support well the other immunosuppressive effects, such as the antiphagocytic (hemocyte function suppressive [7, 36]) and phenoloxidase-inhibiting (8) effects. The lowest detectable concentration of PrtA in vivo, 14 h postinfection, is ~5 pM (calculated using the data in reference 27). This might produce sufficient cleavage of PrtA-sensitive (target) proteins to interfere with the immune processes of a host if we consider that the in vitro cleavage of some PAT proteins was complete within 40 min at a PrtA concentration of 30 to 300 pM and that a substantially longer incubation time is available in vivo. The degree of contribution of this activity to immune suppression and virulence is hard to assess. It is also hard to investigate because in most of the cases, as is known from systems biology studies, the absence of one component of a system does not cause detectable change. In the very complex system of molecular interactions between a pathogen and a host, during the establishment of infection, both sides fight for survival, which forces them not to rely on only several mechanisms. Indeed, hosts use multiple defense mechanisms, while *Photorhabdus* employs "overkilling" (8). This might be the explanation for the seemingly contradictory observations with wild-type *Photorhabdus* strains (26) and a PrtA knockout mutant (R. ffrench-Constant and J. Marokházi unpublished results) that pathogenicity and PrtA production did not correlate in a simple survival test. Nonetheless, our in vitro observations raise the possibility that PrtA has a role in the virulence mechanism of *Photorhabdus* for the suppression of the innate immune response of insects. Since this mechanism can be advantageous for pathogens also in hosts with adaptive immune systems, target proteins to serralysins of pathogens of such organisms might also be found among the molecules of their innate immune system.

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