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Purification and Characterization of an Extracellular Protease from *Xenorhabdus nematophila* Involved in Insect Immunosuppression

C. Caldas, A. Cherqui,† A. Pereira, and N. Simões*

Centro de Investigação de Recursos Naturais and Departamento de Biologia, Universidade dos Açores, 9501-801 Ponta Delgada, Açores, Portugal

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***Xenorhabdus nematophila*, a bacterium pathogenic for insects associated with the nematode *Steinernema carpocapsae*, releases high quantities of proteases, which may participate in the virulence against insects. Zymogram assays and cross-reactions of antibodies suggested that two distinct proteases were present. The major one, protease II, was purified and shown to have a molecular mass of 60 kDa and an estimated isoelectric point of 8.5. Protease II digested the chromogenic substrate *N*-tosyl-Gly-Pro-Arg-paranitroanilide (pNA) with V_{max} and K_m values of 0.0551 μM/min and 234 μM, respectively, and the substrate DL-Val-Leu-Arg-pNA with V_{max} and K_m values of 0.3830 μM/min and 429 μM, respectively. Protease II activity was inhibited 93% by Pefabloc SC and 45% by chymostatin. The optimum pH for protease II was 7, and the optimum temperature was 23°C. Proteolytic activity was reduced by 90% at 60°C for 10 min. Sequence analysis was performed on four internal peptides that resulted from the digestion of protease II. Fragments 29 and 45 are 75 and 68% identical to alkaline metalloproteinase produced by *Pseudomonas aeruginosa*. Fragment 29 is 79% identical to a metalloprotease of *Erwinia amylovora* and 75% identical to the protease C precursor of *Erwinia chrysanthemi*. Protease II showed no toxicity to hemocytes but destroyed antibacterial activity on the hemolymph of inoculated insects' larvae and reduced 97% of the cecropin A bacteriolytic activity.**

The bacterium *Xenorhabdus nematophila* (*Enterobacteriaceae*) establishes a symbiotic relationship with *Steinernema carpocapsae* (Nemata: Steinernematidae), building a complex that is pathogenic to a large range of insects, and is currently used as a biological control agent against Lepidoptera (11, 31, 34, 45), Coleoptera (10, 28, 37, 41), and Diptera (40).

X. nematophila is carried by the infective juvenile nematode in a diverticulum of the gut, from an insect cadaver to a new host (1). The infective juvenile releases the bacterium in the insect hemocoel within 5 h of invasion. This complex causes an acute disease, which kills insects within 48 h (2). The injection of a few *X. nematophila* organisms in a susceptible insect larva causes growth inhibition and the death of the insect. During the pathogenic phase, *X. nematophila* is able to survive the vigorous attack of the insect immune system, proliferate in the hemolymph, and kill the larva. Because the number of organisms in the insect hemolymph is very low before insect death, Forst and Nealson (20) hypothesized that *X. nematophila* entered in an intraphagosomal phase and that during this phase the bacteria secrete some factors toxic to the insect. Since the bacterial proliferation does not occur in the hemocoel before insect death it is suggested that the secretions of these pathogens are highly potent virulence factors in insects. Furthermore, *X. nematophila* appears to be generally resistant to the attack of nonspecific antibacterial enzymes of insect hemolymph (13). Also, lipopolysaccharides of *X. nematophila* have been shown to prevent the process of the activation of prophenoloxidase into phenoloxidase (12, 14). The set of mechanisms by which the *X. nematophila* bacteria are able to circumvent the host defense systems and cause insect death, as well as the benefits provided by the bacteria to their symbiotic nematodes, is frequently associated with the extracellular molecules produced by *Xenorhabdus* spp. (4, 14, 21). *Photorhabdus luminescens*, a bacterium associated with the entomopathogenic nematode *Heterorhabditis bacteriophora*, also has a high secretory activity. The virulence of the strain W14 is partially clarified by the characterization of high-molecular-weight toxin complexes. These proteins are secreted and present an oral toxicity to the Lepidoptera (7). However, differences in the genomic organization have been reported when the toxic loci found in *P. luminescens* are compared to their homologues in *X. nematophila* (18). Recently, two overlapping cosmid clones were shown to encode an insecticidal protein DNA region of a highly pathogenic isolate of *X. nematophila* in *Escherichia coli* (35).

Among the extracellular molecules produced by *X. nematophila*, we can find some with proteolytic activities. Although those molecules may play a role in insect toxicity, their importance is highly conflicting. Certain literature acknowledges that proteases might have a role in insect toxicity by analogy with proteases produced by other insect pathogens (23, 39), whereas other authors claim that these proteases are not toxic to the insects, neither by injection nor by oral feeding (6). In this work, we report the identification of two proteases produced by *X. nematophila* and the characterization of one of these. In addition, we show that this protease suppresses antibacterial peptides involved in the insect immune response, thereby providing a role for it in the pathogenic process.

* Corresponding author. Mailing address: Departamento de Biologia, Universidade dos Açores, 9501-801 Ponta Delgada, Açores, Portugal. Phone: 351 296650119. Fax: 351 296650100. E-mail: simoes@notes.uac.pt.

† Present address: Faculté des Sciences, Laboratoire de Biologie des Entomophages, Université de Picardie Jules Verne, Amiens 80039, France.

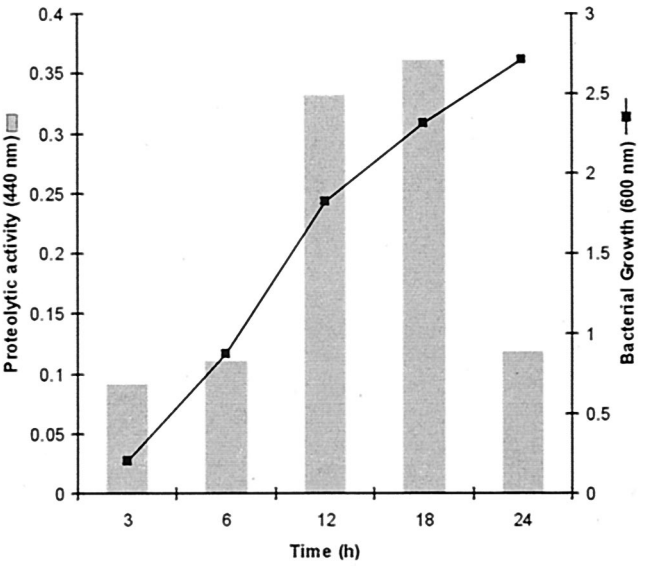

FIG. 1. Release of proteolytic activity during the growth of *X. nematophila* in TSB broth. Samples of culture medium supernatants were collected at different times to determine the release of proteolytic activity measured at 440 nm using azocasein as a substrate.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Stock inoculum of *X. nematophila* was obtained according to the method of Akhurst and Dunphy (2). Ten infective juveniles of *S. carpocapsae* Breton strain were surface disinfected in 1% sodium hypochlorite, transferred to a petri dish with 2 ml of tryptic soy broth (TSB) (Difco, Detroit, Mich.) liquid medium, and bisected at the esophageal bulb level. This medium was incubated for 24 h at 28°C and then spread in nutrient bromothymol agar (NBTA) (nutrient broth 0.0025%, bromothymol blue, and 0.004% 2,3,5-triphenyltetrazolium) solid medium plates. The plates were incubated for 48 h at 30°C. Bacterial growth was achieved by inoculation of 5 ml of TSB liquid medium with a colony from the stock inoculum. After a 24-h incubation period at 28°C and with shaking at 150 rpm, 1 ml of medium was transferred into fresh TSB medium in 500-ml flasks (100 ml of medium/flask). The culture was incubated for 24 h as in the previous stage. Following incubation, the broth was centrifuged at 12,000 × *g* for 10 min at 4°C and filtered through a 0.2-μm-pore-size membrane. The cell supernatant containing proteolytic activity was collected and stocked at −20°C.

Protease II purification. All experiments were performed at room temperature unless otherwise stated. The isolation protocol entailed starting with 800 ml of broth which was then concentrated to 15 ml through an Amicon ultrafiltration system (molecular mass cutoff, 50 kDa). The retentate was filtered with Swinnex (membrane pore size, 0.45 μm) and then loaded at 1 ml/min onto a DEAE-Sepharose column (2.5 by 20 cm) equilibrated with 10 mM cacodylate buffer, pH 7.6 (buffer A), connected to a Pharmacia fast-performance liquid chromatography system. Bound proteins were eluted in a linear gradient of 0 to 1 M of NaCl in buffer A over 60 min, and 1-ml fractions were collected. The proteolytically active fractions from this initial separation were subsequently pooled, desalted by gel filtration PD10 (Pharmacia Biotech, Uppsala, Sweden) in buffer A, and loaded (1 ml/min) onto a 5-ml HiTrap Q Sepharose column (Pharmacia Biotech), equilibrated with buffer A. The bound proteins were eluted in a step (0.1 M) gradient from 0 to 0.5 M NaCl in buffer A. The active fractions from HiTrap Q Sepharose were further separated using a Mono Q column (Pharmacia Biotech) and a linear gradient of 0.35 to 0.45 M of NaCl in buffer A over 120 min at 1 ml/min. Protein elution was monitored at 280 nm.

Enzyme activities. The crude extract and chromatographic samples were assayed for proteolytic activity using azocasein (Sigma, St. Louis, Mo.) as a substrate (42). Aliquots (50 μl) of samples were added to 50 μl of 2% azocasein in Tris-HCl, pH 8, containing 0.2 M NaCl. Fractions were then incubated at 37°C for 1 h. Nondigested azocasein was precipitated by adding 130 μl of 10% trichloroacetic acid (TCA) (Merck, Darmstadt, Germany) to the incubations and centrifuged at 10,000 × *g* for 10 min. The supernatants (100 μl) were transferred to a 96-well microtitration plate containing 200 μl of 1 M NaOH. The absorbance values of the resulting supernatants were measured with a microplate reader (Bio-Rad Laboratories, Richmond, Calif.) with a 440-nm absorbance filter. Increased absorbance indicates the presence of proteolytic activity. The blank was obtained by precipitating the substrate plus the sample in TCA without incubation.

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Mini-PROTEAN II gel system (Bio-Rad Laboratories), using slab gels, 0.5 mm thick, of 10% polyacrylamide, according to the method of Laemmli (32), and the proteins were stained with Coomassie blue G-250. Silver nitrate staining was performed according to the method of Morrissey (36). A prestained SDS-PAGE protein kit containing phosphorylase *b* (106.0 kDa), bovine serum albumin (77. 0 kDa), ovalbumin (50.8 kDa), carbonic anhydrase (35.6 kDa), soybean trypsin inhibitor (28.1 kDa), and lysozyme (20.9 kDa) (Bio-Rad Laboratories, Hercules, Calif.) was used as a source of molecular mass standards.

For the Western blotting, the purified protein was separated in gel electrophoresis under reduction and denaturing conditions. A Laemmli buffer, containing 5% β-mercaptoethanol and heated at 95°C during 5 min, was used to achieve these conditions. The protein was then transferred onto a nitrocellulose membrane (Millipore Corp., Bedford, Mass.) using a liquid transfer apparatus in a continuous buffer system at pH 11, containing Tris-glycine and 20% methanol, applying 0.8 mA/cm^2 for 1 h according to the method of Towbin et al. (43). The membrane was then incubated in Tris-buffered saline (TBS) (0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl) containing 5% (wt/vol) dehydrated milk and 0.05% (vol/vol) Tween 20 for 30 min at room temperature. Subsequently, the membrane was incubated with primary antibody at the appropriate concentration (1:1,000 dilution) in TBS containing 5% (wt/vol) dehydrated milk and 0.05% (vol/vol) Tween 20 for 2 h at room temperature. The membrane was washed three times for 10 min in TBS containing 0.05% (vol/vol) Tween 20, followed by incubation with goat anti-rabbit immunoglobulin G peroxidase conjugates (Sigma) diluted 1:4,000 in TBS. Immunoreactivity was detected by incubating the membrane in TBS containing 0.06% (wt/vol) diaminobenzidine, 0.018% $NiCl_2$ (wt/vol), and 0.3% H_2O_2 (vol/vol).

Zymography. SDS-PAGE zymograms were performed as described by Schmidt et al. (39) with minor modifications. Ten percent polyacrylamide gels were copolymerized with 0.05% gelatin. Samples were dissolved in nonreducing Laemmli sample buffer without heat denaturation and run at 100 V. Following electrophoresis, the gels were washed for 30 min in 50 mM Tris-HCl buffer, pH 7.6, containing 2.5% Triton X-100 (Merck), with gentle agitation, in order to remove the excess of SDS. Then, the gels were incubated for an additional 4 h with several changes in a solution of 50 mM Tris-HCl, pH 7.6, containing 0.2 M NaCl and 5 mM $CaCl_2$. Zones of proteolysis were detected by overnight Coomassie blue staining.

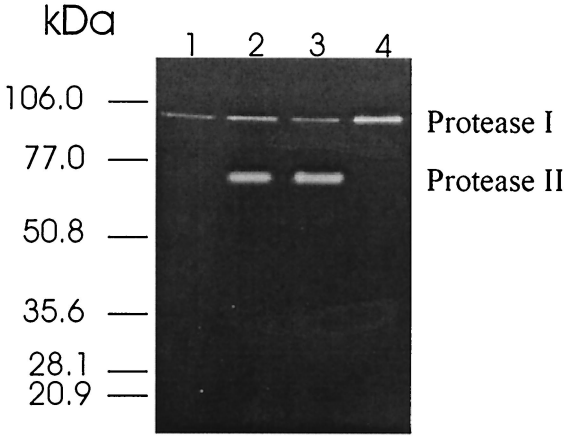

FIG. 2. Zymography analysis of the *X. nematophila* proteases released during 24 h of growth. Samples from the *X. nematophila* culture medium supernatants were collected at different times, dissolved in nonreducing Laemmli sample buffer, and applied to a 10% polyacrylamide gel containing 0.05% gelatin. The incubation and staining of the postrun gel were carried out as described in Materials and Methods. Shown are samples at 6 (lane 1), 12 (lane 2), 18 (lane 3), and 24 (lane 4) h of growth.

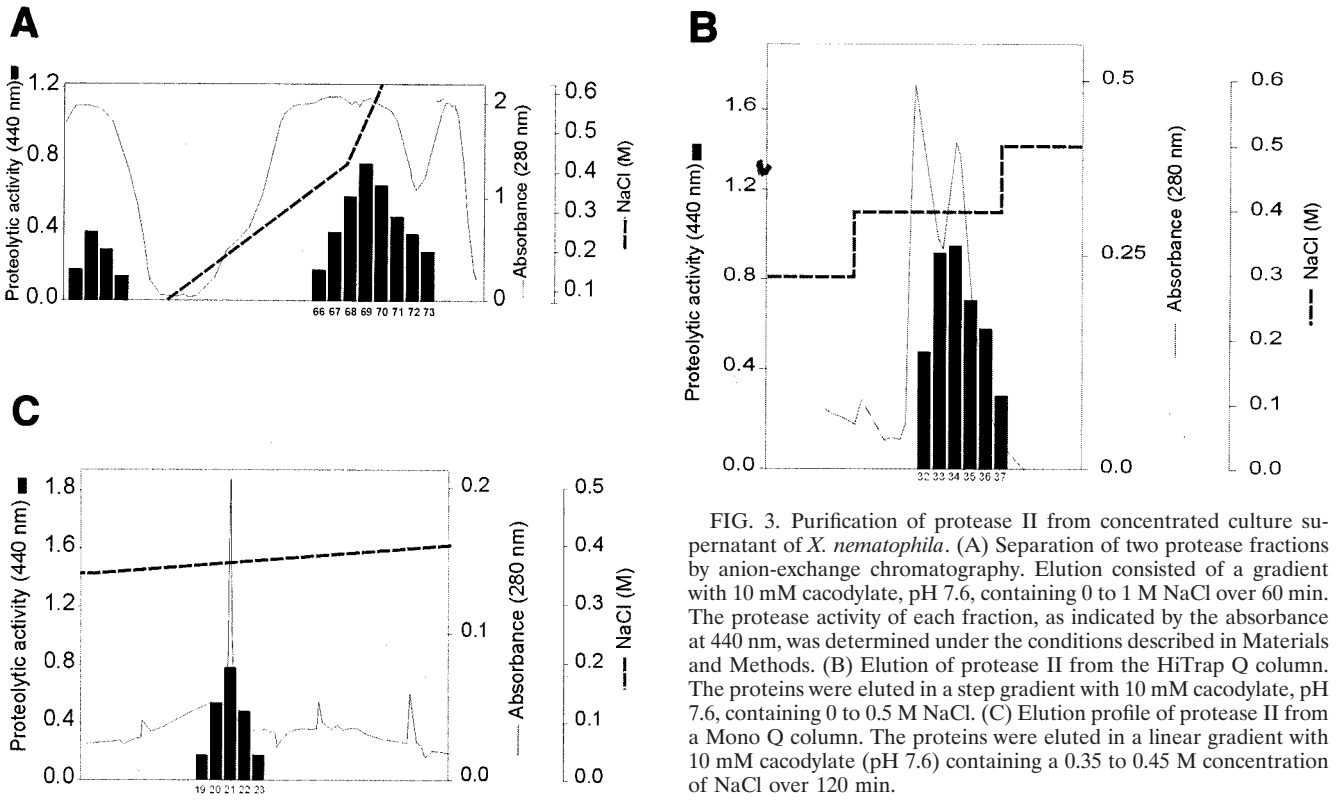

FIG. 3. Purification of protease II from concentrated culture supernatant of *X. nematophila*. (A) Separation of two protease fractions by anion-exchange chromatography. Elution consisted of a gradient with 10 mM cacodylate, pH 7.6, containing 0 to 1 M NaCl over 60 min. The protease activity of each fraction, as indicated by the absorbance at 440 nm, was determined under the conditions described in Materials and Methods. (B) Elution of protease II from the HiTrap Q column. The proteins were eluted in a step gradient with 10 mM cacodylate, pH 7.6, containing 0 to 0.5 M NaCl. (C) Elution profile of protease II from a Mono Q column. The proteins were eluted in a linear gradient with 10 mM cacodylate (pH 7.6) containing a 0.35 to 0.45 M concentration of NaCl over 120 min.

2D electrophoretic analysis. A 1-ml fraction from the Mono Q purification step was precipitated with TCA, to a 10% final concentration, and centrifuged for 10 min at 10,000 × *g*. The pellet was dried and resuspended with rehydration buffer {9.8 M urea, 0.5% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 10 mM dithiothreitol, 0.5% ampholytes [pH 3 to 10], 0.001% bromophenol blue}. The sample was loaded into 7-cm-long ready strips (Bio-Rad Laboratories), previously rehydrated, with the buffer mentioned above, for 12 h at 20°C, according to the manufacturer's recommendations. Strips were focused in 3 steps: step 1, 250 V for 15 min; step 2, 250 to 4,000 V for 2 h; step 4,000 V for 5 h. Prior to running the second dimension (2D) SDS-PAGE, the strips were equilibrated in a solution of 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 0.130 M dithiothreitol and in a solution of 6 M urea, 2% SDS, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, and 0.135 M iodoacetamide, which were used separately for 15 min to overlay the strip. Following this SDS-PAGE was performed in a 1.5-mm-thick 10% acrylamide gel under the conditions already described.

Protein determination. The soluble protein concentrations were determined at all purification steps by the Coomassie blue dye binding method (8). Bovine serum albumin (1 mg/ml) was used as a standard protein.

Analysis of substrate susceptibility to protease II. Several chromogenic substrates—*N*-acetyl-Ile-Glu-Ala-Arg-paranitroanilide (pNA), *N*-benzoyl-Pro-Phe-Arg-pNA hydrochloride, *N*α-benzoyl-L-arginine-pNA hydrochloride, *N*-benzoyl-Phe-Val-Arg-pNA hydrochloride, *N*-carbobenzyloxy (CBZ)-Gly-Gly-Leu-pNA, D-Ile-Phe-Lys-pNA, *N*-succinyl-Gly-Gly-Phe-pNA, *N*-tosyl-Gly-Pro-Arg-pNA, DL-Val-Leu-Arg-pNA, and *N*-succinyl-Ala-Ala-Pro-Phe-pNA (Sigma)—were prepared at a 10 mM stock solution in methanol.

Reactions began with the addition of 1.76 μg of protease II (80 μl from Mono Q fraction) to 20 μl of 10 mM substrate and 100 μl of Tris-HCl, pH 8. The change in optical density at 410 nm was determined every 5 min for 30 min using a Bio-Rad microplate reader. Negative controls consisted of heat-inactivated protease II mixed with substrate.

Kinetic analysis. Kinetic assays were performed at room temperature using DL-Val-Leu-Arg-pNA and *N*-tosyl-Gly-Pro-Arg-pNA as substrates. The reaction mixture contained 1.76 μg of protease II (80 μl), and substrate concentrations ranged from 0.0001 M to 0.0024 M. Optical density was measured every 5 min over a 60-min interval with a Bio-Rad microplate reader at 410 nm. V_{max} and K_m

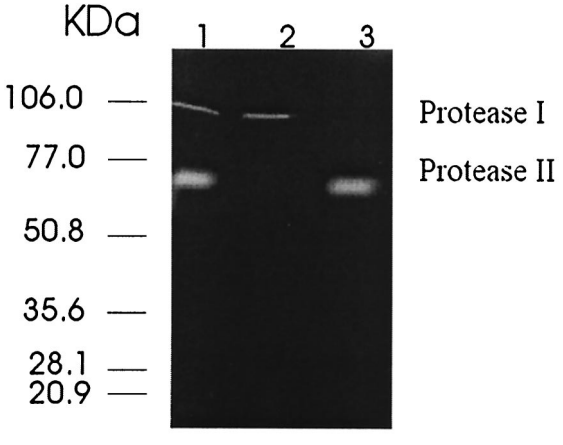

FIG. 4. Zymography analysis of purified proteases separated by DEAE column chromatography. Lanes: 1, crude extract; 2, fraction from exclusion (protease I); 3, fraction from elution (protease II).

TABLE 1. Purification of *X. nematophila* protease II

Sample analyzed	Total vol (ml)	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Yield (%)
Concentrated supernatant	15	18.35	97	5	100
DEAE Sepharose	12	0.62	79	126	81
HiTrap Q Sepharose	8	0.27	37	135	38
Mono Q	5	0.11	19	176	20

[a] One unit of protease activity was defined as the amount of enzyme required to produce an absorbance change of 0.01 under the conditions of the assay.

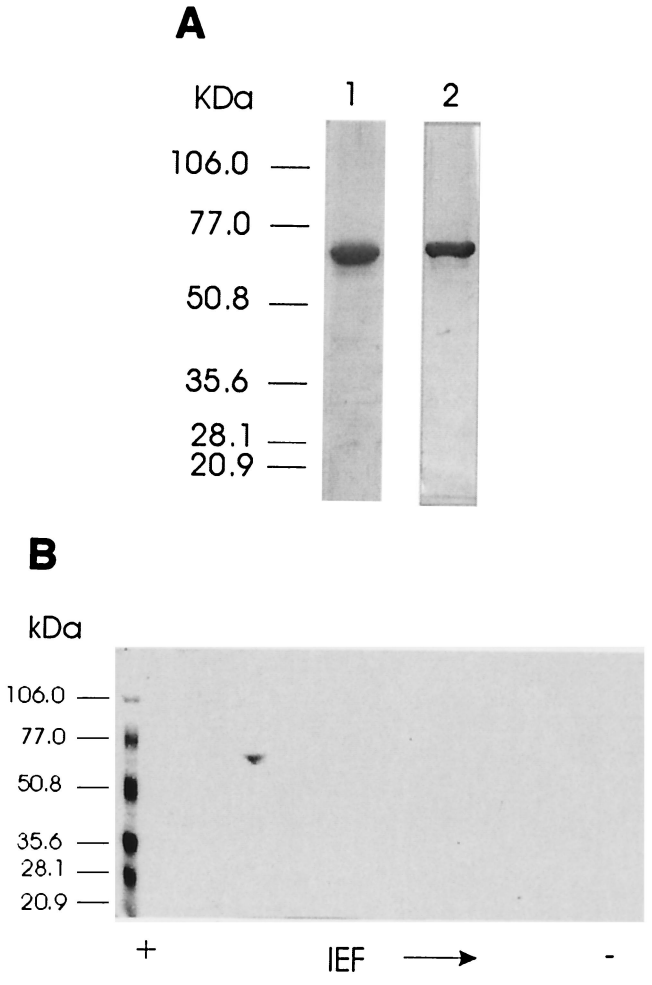

FIG. 5. Analysis of protease II by SDS-PAGE, Western blot, and 2D electrophoresis. (A) SDS-PAGE and Western blot analysis of purified protease II. Lane 1 contains the Mono Q fraction showing purified protease II. Protein (4.4 μg) was applied to a SDS–10% PAGE gel. The electrophoretic run was performed at 100 V, and the gel was subsequently silver stained. Lane 2 shows the results of Western blot analysis of 2 μg of purified fraction using antibodies raised against Prot.II-29 peptide as the primary antibody. (B) 2D analysis of purified protease II. A concentrated sample of protease II was applied to a 7-cm ready strip gel to perform the first-dimension step. The 2D step was performed in an SDS–10% polyacrylamide gel that was silver stained. IEF, isoelectric focusing.

values were determined by using the Lineweaver-Burk presentation. Reactions were run in triplicate.

Determination of optimal pH for protease II activity. The reactions between purified protease II and substrate DL-Val-Leu-Arg-pNA were assayed in triplicate for 30 min in solutions ranging from pH 2 to 11. Controls consisted of substrate at the pH values tested without protease II. The conditions of the assay were those used for substrate susceptibility except for the buffer applied.

Inhibition and heat stability. The chromatographically purified protease II was assayed against azocasein in the presence of protease inhibitors: antipain, chymostatin, leupeptin, phosphoramidon, aprotinin, E-64, pepstatin, bestatin, EDTA, and Pefabloc (Boehringer, Mannheim, Germany). Azocasein hydrolysis was measured as described above after a 30-min incubation of protease II with the inhibitor, at room temperature.

The optimal temperature was determined by protease II and azocasein incubated at different temperatures (4, 10, 23, 37, 45, 55, and 65°C) for 1 h, and this was followed by the determination of the proteolytic activity. The heat stability was determined by incubation of protease II at 55, 60, and 65°C for 10 min, and the remaining proteolytic activity measured has been described above.

TABLE 2. Substrate sensitivity to protease II

Substrate	Optical density at 30 min[a]	Enzyme activity[b]
N-Acetyl-Ile-Glu-Ala-Arg-pNA	0	0
N-Benzoyl-Pro-Phe-Arg-pNA hydrochloride	0	0
*N*α-Benzoyl-L-arginine-pNA hydrochloride	0	0
N-Benzoyl-Phe-Val-Arg-pNA hydrochloride	0	0
N-CBZ-Gly-Gly-Leu-pNA	0	0
D-Ile-Phe-Lys-pNA	0	0
N-Succinyl-Gly-Gly-Phe-pNA	0	0
N-Tosyl-Gly-Pro-Arg-pNA	0.197	1.10
DL-Val-Leu-Arg-pNA	0.416	1.47
N-Succinyl-Ala-Ala-Pro-Phe-pNA	0	0

[a] Reactions were started with the addition of protease II to buffer and substrate. The reaction mixture was immediately placed into a microtiter plate reader, where the optical density at 410 nm was read at 5-min intervals for 30 min and the units of enzyme were calculated from the change in optical density (ΔA) per minute.

[b] One unit of protease II activity was defined as ΔA/min × total assay volume/sample volume × E at 410 nm × light path (cm), where the extinction coefficient (E) of the product (paranitroanilide) at 410 nm was 9.75 and the light path was 0.53 cm (26).

Assays for antibacterial activity inhibition. A slightly modified growth inhibition zone assay described by Hoffmann et al. (25) was used. Hemolymph with antibacterial activity was collected from *Galleria mellonella* and *Pseudaletia unipuncta* (Lepidoptera: Noctuidae) 24 h postinoculation with *E. coli*. Twenty microliters of active hemolymph was incubated with 0.44 μg of protease II in 60 μl of 0.1 M phosphate buffer, pH 6.4, at 37°C for 1 h. Treated and untreated hemolymph was tested for antibacterial activity by placing 30 μl of each in wells prepared on *E. coli* pour plates (10^5 organisms per plate). Plates were then incubated overnight at 37°C. Diameters of the growth inhibition zones were recorded. Assays with 1% cecropin A (Sigma) were performed under the same protocol.

A bacteriolytic assay was performed, with slight modifications, according to the method of Kaaya (29). Log-phase *E. coli* was centrifuged and suspended in ice-cold 0.1 M phosphate buffer, pH 6.4, to give it a density of 0.3 to 0.5 at 595 nm. A volume of 45 μl of 1% cecropin A, incubated for 1 h at 37°C with protease II or with heat-inactivated protease II (control), was added to 200 μl of bacterial suspension. The mixture was then incubated for 30 min at 37°C. After the incubation, the absorbance at 595 nm was measured with a Bio-Rad microplate reader within 1 h.

Susceptibility of hemolymph proteins to protease II. Hemolymph from *G. mellonella* and *P. unipuncta* was collected under ice-cold conditions, in Eppendorf tubes with a few crystals of phenylthiourea (Sigma). The hemolymph was centrifuged for 10 min at 4°C and 10,000 × *g*. The supernatant corresponding to the serum was recovered, and aliquots of various volumes were incubated with 1.90 μg of active and heat-inactivated protease. A final volume of 200 μl was obtained by adding 0.01 M Tris-HCl, pH 8. The mixtures were incubated at 37°C for 1 h and then subjected to SDS-PAGE analysis under the conditions described above.

Preparation for the N-terminal amino acid sequence and internal sequences. Chromatographic pure fractions of protease II were subjected to SDS-PAGE and electroblotted onto polyvinylidene fluoride (PVDF) membrane (Millipore). Tris-glycine buffer, pH 11, containing 20% methanol was used as electrophoretic buffer in a Mini Trans-Blot Cell (Bio-Rad Laboratories). Protease II on PVDF membrane was stained with a solution of 0.5% Ponceau red (Fluka, Buchs, Switzerland), washed with water, excised, and subjected to analysis.

The N-terminal amino acid sequence analysis was performed by automated Edman degradation (17). Additional internal sequences were obtained after digestion with endo-Lys-C. Peptide fragments produced by the digestion were isolated by reverse-phase high-performance liquid chromatography and four were sequenced. The analysis of the peptide sequences was performed by the Emory University Microchemical Facility (Atlanta, Ga.).

Peptide sequences were compared with entries in various sequence databases

TABLE 3. Inhibitor reactivity with protease II

Inhibitor	Inhibitor class[a]	Solvent	Inhibitor concn	% Activity remaining[b]
None				100
Antipain dihydrochloride	Serine	H_2O	1 mM	79
Chymostatin	Serine	DMSO[c]	100 μM	55
Leupeptin	Ser/Cys	H_2O	1 μM	116
Pefabloc SC	Serine	H_2O	4 mM	7
Aprotinin	Serine	H_2O	0.3 μM	103
E-64	Cysteine	50% Ethanol	5 μM	114
Pepstatin	Aspartic	Methanol	1 μM	94
Bestatin	Metallo	Methanol	130 μM	105
Phosphoramidon	Metallo	H_2O	570 μM	96
EDTA	Metallo	H_2O	1.3 mM	98
EDTA	Metallo	H_2O	20 mM	57

[a] Inhibitor class refers to those proteases known to be susceptible to each compound tested.
[b] Enzyme samples were incubated with an inhibitor for 30 min at 37°C and then the remaining activities in the sample were determined. Each value represents the mean of three experiments and the activity in the reaction mixture containing no inhibitor was considered 100% activity
[c] DMSO, dimethyl sulfoxide.

using the European Bioinformatics Institute FASTA program. A BLOSUM62 matrix was used (38).

Peptide synthesis and production of peptide-specific antibodies. The peptide Prot. II-29 was synthesized according to the amino acids sequence resulted from the digestion of the purified protease. The peptide synthesis was performed according to the manufacturer (Emory University Microchemical Facility). The synthesized peptide was then linked to a carrier protein, keyhole limpet hemocyanin, with the goal of increasing the immunogenicity of the peptide, and then injected intradermally into a rabbit (Eurogentec Laboratories, Seraing, Belgium). The antiserum was collected 6 weeks later.

RESULTS

Bacterial growth and proteolytic activity on the medium. *X. nematophila* was grown aerobically in TSB liquid medium. The proteolytic activity was identified during this growth by a routine detection enzyme activity using either azocasein as a substrate or proteolytic activity in gel electrophoresis containing gelatin. Proteolytic activity in the growth medium increased until reaching its maximum at 18 h of growth, corresponding to the exponential period of bacterial growth (Fig. 1). During growth, the bacteria released two different proteases that were characterized by gel electrophoresis, presenting two different molecular weights as proved by zymography. In the first hours of growth, a protease, designated as protease I, appeared in the growth medium. Protease I had a high mass, approximately 90 kDa, but was low in proteolytic activity. At 12 h of growth, a second proteolytic band appeared in the zymogram with a medium molecular mass but a high activity and was designated as protease II. In the following hours, the proteolytic activity decreased, and only protease I persisted at 24 h (Fig. 2). Apparently, protease II has a higher affinity for azocasein than protease I. In a growth medium supplemented with calcium the release of proteolytic activity did not increase; nevertheless, the release of activity and bacterial growth occurred earlier (data not shown).

Protease II purification and characterization. Culture supernatant (800 ml) of *X. nematophila*, resulting from 18 h of growth in medium, was concentrated by ultrafiltration (Amicon Stirring Cells) to 15 ml. The filtrate formed showed a 3.5- to 4.4-fold increase in specific activity against the substrate azocasein, in comparison to the unconcentrated supernatant. The 15 ml of concentrated supernatant was filtrated and applied to a DEAE column, which was equilibrated with a cacodylate buffer, pH 7.6. The hydrolysis of the azocasein substrate showed two proteolytic peaks of activity (Fig. 3A), and the analysis of the zymogram demonstrated that the exclusion peak of the DEAE contained the active protease band corresponding to protease I, while protease II was eluted at 0.35 to 0.4 M (Fig. 4). The proteolytic active fractions collected from the elution were applied to a HiTrap Q Sepharose column and eluted by isocratic gradients of NaCl. The elution profile revealed a peak of proteolytic activity at 0.4 M NaCl (Fig. 3B). The fractions showing high activity were pooled and then loaded onto a Mono Q column. The elution showed a well-defined single peak of protease activity (Fig. 3C). The total activity recovered, after the different purification steps, was 20%, and a purification factor of 35-fold was obtained for protease II (Table 1).

SDS-PAGE of the purified protein, obtained in the last purification step, exhibited a single band in the silver-stained gel (Fig. 5A, lane 1), indicating that protease II was purified to apparent homogeneity. Based on the electrophoretic mobility, the molecular mass of this protein was determined to be 60 kDa. The 2D electrophoretic analysis showed an estimated isoelectric point of 8.5 (Fig. 5B).

Protease II was tested against numerous substrates. The enzyme was able to hydrolyze azocasein, as well as some synthetic paranitroanilide substrates. Among the chromogenic substrates analyzed for susceptibility to protease II, only *N*-tosyl-Gly-Pro-Arg-pNA and DL-Val-Leu-Arg-pNA were digested (Table 2).

Kinetic analysis of the hydrolysis of *N*-tosyl-Gly-Pro-Arg-pNA and DL-Val-Leu-Arg-pNA by protease II was conducted, and the V_{max} values were 0.0551 μM/min and 0.3830 μM/min, respectively, and K_m values were 234 μM and 429 μM, respectively.

The inhibition profile of the purified enzyme was evaluated using several protease inhibitors. The protease II activity on azocasein was not affected by leupeptin, aprotinin, E-64, bestatin, phosphoramidon, or 1.3 mM EDTA (Table 3). The serine protease inhibitor Pefabloc SC significantly inhibited the proteolytic activity of protease II, since only 7% of the residual activity was measured in the presence of a 4 mM concentration

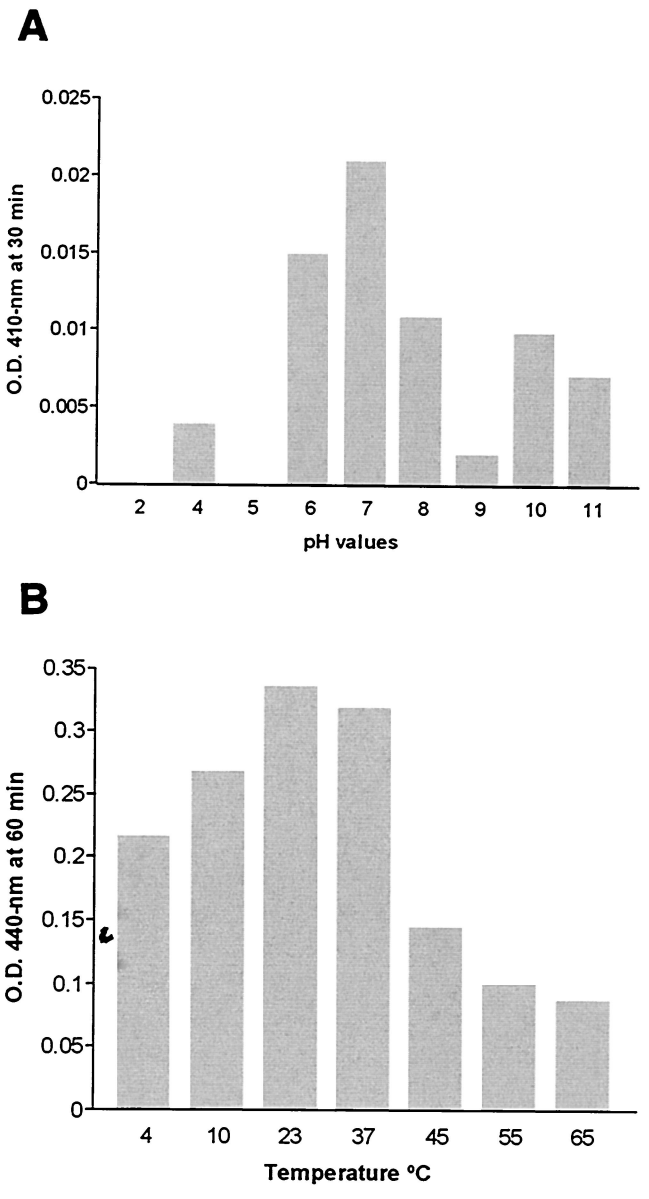

FIG. 6. Proteolytic activity of protease II at different pH values and at different temperatures. (A) The optimum pH was determined by using DL-Val-Leu-Arg-pNA as a substrate in the conditions described in the Materials and Methods. (B) The optimum temperature was determined against azocasein as previously reported. O.D., optical density.

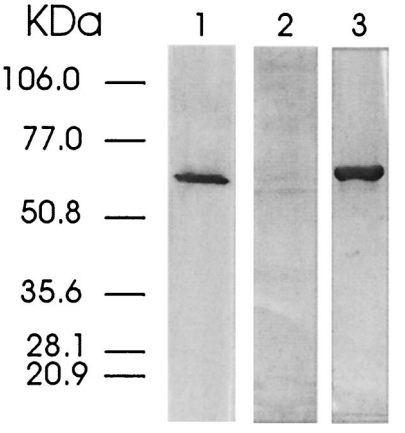

FIG. 7. Analysis of protease II by Western blotting. *X. nematophila* growth media collected at different times and purified protease II were transferred to nitrocellulose membranes. Proteins were detected by Western blotting using antibodies raised against Prot.II-29 peptide as primary antibody. Lanes: 1, 18 h of growth in medium; 2, 24 h of growth in medium; 3, purified fraction.

of inhibitor. Chymostatin, a chymotrypsin inhibitor, inhibited the protease, but less effectively than Pefabloc, since only 55% of the activity was retained. A small inhibition was also detected in the presence of antipain, a serine protease inhibitor. These inhibitions by the serine protease inhibitors suggest that the protease II purified from the *X. nematophila* growth medium is a serine protease. EDTA causes inhibition when it is used at 20 mM.

The optimal pH for the purified protease has a value of 7, determined by DL-Val-Leu-Arg-pNA hydrolysis (Fig. 6A); the optimal temperature for the protease activity was observed at 23°C (Fig. 6B). Protease II was found to be stable for several days in unpurified and purified fractions at −20°C. Nevertheless, the protein was not heat stable. The incubation of the purified sample at 60°C for 10 min induced a 90% reduction of the proteolytic activity.

Microsequencing of protease II. Direct sequencing of protease II did not yield an amino acid sequence, indicating the likelihood of a blocked N terminus. Internal sequence analysis after peptic digestion, with Lys-C proteinase, was performed on the protease II bound to the PVDF membrane. Four peptides resulting from the digestion were fractionated by high-performance liquid chromatography and subjected to Edman degradation. The following sequences were obtained: Prot. II-22, FTEVSSIYK; Prot. II-29, GDTVYGFNSNTDRDFMT ATDANSK; Prot. II-45, YGYSSAPLLNDISAIQELYYANMET; and Prot. II-19, FVDSFSGK. Database searches of these peptides provided several matches with statistical significance (Table 4).

Polyclonal antibodies raised against the synthesized peptide Prot.II-29 were tested against protease II under reducing conditions. The antibodies cross-reacted strongly to the purified and unpurified protease II at a dilution of 1/1,000 when the sample was reduced with β-mercaptoethanol (Fig. 5A, lane 2). On the contrary, the antibodies did not cross-react with the protease I present in the *X. nematophila* growth medium (Fig. 7).

Biological activities of protease II. Protease II was not toxic to hemocytes of either *P. unipuncta* or *G. mellonella.* In contrast, important modifications of the protein profile of humoral fractions of both insects were observed after incubation with protease II.

Protease II was able to reduce as much as 100% of the antibacterial activity on the hemolymph of inoculated *G. mellonella* and *P. unipuncta* larvae, as well as destroy cecropin A activity on the growth inhibition zone assays. Ninety-seven percent of the cecropin A lytic capacity was also reduced by protease II in in vitro assays.

Protease II apparently had no activity on the proteins of the

TABLE 4. Identity determined by FASTA using a BLOSUM62 matrix

Fragment	Organism	Molecule	Identity (%)	ε
Prot. II-29	*E. amylovora*	Metalloprotease	79.2	4.2×10^9
	E. chrysanthemi	Protease C Prec.[a]	75.0	1.3×10^8
	P. aeruginosa	Alkaline proteinase	75.0	1.8×10^8
	P. aeruginosa	Alkaline metalloproteinase	75.0	1.8×10^8
	E. chrysanthemi	Protease B Prec.	75.0	1.8×10^8
Prot. II-45	*Pseudomonas* sp.	Serralysin Prec.	68.0	4.5×10^6
	P. aeruginosa	Alkaline metalloproteinase	68.0	2.1×10^6
	E. amylovora	Metalloprotease	64.0	1.4×10^5
	E. amylovora	Protease B Prec.	64.0	4.3×10^5

[a] Prec., precursor.

extracellular matrix, such as collagen, elastin, and fibrin (data not shown).

DISCUSSION

Among the enzymes secreted by *X. nematophila*, we purified a protease that was able to destroy antibacterial factors present in insect hemolymph. Secretion of this protease could explain why the bacteria released into insect hemocoel by the associated nematode, even at low numbers, can overcome the insect defenses and install themselves in a very efficient way. In the growth medium we identified, on a zymogram, two distinct proteases apparently produced at different times. Protease I persisted in the medium until the stationary phase was achieved, whereas protease II was detected in the medium at 12 h postinoculation and remained for only 6 h. Antibodies raised against an internal peptide of protease II showed a high specificity against the purified and unpurified protease II but did not cross-react with protease I. The limited time that protease II was present suggests that it is different from protease I, which remained within the stationary phase.

Protease II is a 60-kDa protein with a pI of 8.5. This enzyme probably belongs to the serine protease family. Pefabloc SC and chymostatin, two serine protease inhibitors, inhibited protease II, totally and partially, respectively. Two chromogenic substrates, *N*-tosyl-Gly-Pro-Arg-pNA and DL-Val-Leu-Arg-pNA, specific for serine proteases, are positively hydrolyzed by protease II, whereas the inhibition induced by EDTA, a metalloprotease inhibitor, at a significant concentration is negligible.

Protease II is expressed during just a few hours at the beginning of the exponential growth phase of *X. nematophila*. On the other hand, the closely related entomopathogenic bacterium, *P. luminescens*, releases proteases in the stationary growth phase. These proteases have little or no toxic activity in insects, neither by injection nor by oral feeding, but the participation of these molecules on the activation of a toxic complex of proteins secreted by *P. luminescens* was not excluded (6).

Protease II clearly destroyed the antibacterial activity on the hemolymph from immune *G. mellonella* and *P. unipuncta* larvae. Moreover, protease II inhibited cecropin A, an inducible antibacterial peptide that is particularly active against gram-negative bacteria, isolated from several species of Lepidoptera and Diptera (19, 24). In the same way, two hemolymph serine protease inhibitors involved in *P. unipuncta* humoral defense showed no effect on proteolytic activity released by *X. nematophila* (9). The inactivation of antibacterial factors in insects must cause an immunodepression that enhances the ability of *X. nematophila* to establish itself in the hemocoel, causing a septicemia followed by insect death. In addition to showing the destruction of antibacterial factors, we showed that protease II is able to promote the digestion of some proteins of the insect hemolymph. The hydrolysis of these proteins may provide nutritional factors to the associated nematode necessary for its complete development. Axenic *S. carpocapsae* is able to invade and kill *G. mellonella* larvae; however, the progeny of these nematodes, in the absence of the bacteria, is much reduced in relation to nematodes developing in monoaxenic conditions (5, 24). The optimal temperature and pH of protease II were 23°C and 7, which correspond to the optimal conditions for nematode development, either in insects or in in vitro culture (15).

The characterization of this protease indicated a strong relationship to other enzymes isolated from other bacteria, in such areas as molecular weight, substrate specificity, and the internal sequences that presented a high level of homology. Four peptides resulting from protease II internal digestion were sequenced. Amino acid sequences were computed for homologies with other toxic proteases excreted by bacteria. Fragments Prot II-29 and Prot II-45 are 75 and 68% identical to an alkaline metalloproteinase isolated from *P. aeruginosa* (16, 22). *P. aeruginosa* is commonly isolated from insects and is occasionally pathogenic under laboratory conditions (27). The pathogenicity of *P. aeruginosa* was correlated with the release of proteases that are involved in insect hemolymph clotting (30). The alkaline proteases of *P. aeruginosa* destroy the ground substance of supporting structures once they interfere with fibrin formation (33).

The analysis of the fragment Prot II-29 showed it to be 79% identical to a metalloprotease of *Erwinia amylovora* and 75% identical to the protease C precursor of *Erwinia chrysanthemi*. Bacteria of the genus *Erwinia* were reported in association with insects, even though they are not known to be pathogenic agents (3, 44).

Research is being conducted to show to what extent the role of protease II may be relevant to pathogenicity and how it may participate in nematode development inside insect hemolymph.

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