A zinc metalloprotease inhibitor, Inh, from the insect pathogen *Photorhabdus luminescens*

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The entomopathogen *Photorhabdus luminescens* secretes many proteins during the late stages of insect larvae infection and during *in vitro* laboratory culture. The authors have previously characterized and purified a 55 kDa zinc metalloprotease, PrtA, from culture supernatants of *P. luminescens*. PrtA is secreted via a classical type I secretory pathway and is encoded within the operon *prtA-inh-prtBCD*. The 405 bp *inh* gene encodes a 14·8 kDa pre-protein that is translocated to the periplasm by the classical signal-peptide-dependent *sec* pathway, yielding the mature 11·9 kDa inhibitor Inh. Inh is a specific inhibitor of the protease PrtA. This study describes the purification of Inh and the initial characterization of its *in vitro* protease inhibition properties.

Keywords: protease inhibitor, entomopathogen, protein purification

INTRODUCTION

Pathogenic bacteria employ many virulence factors for the invasion, colonization and maintenance of infection within a host organism. An aspect of virulence that has been studied extensively involves the modulation of host matrix metalloprotease (MMP) and of host MMP inhibitor (TIMP) activities. The MMP and TIMP families are involved in the regulation of connective-tissue degradation (Herouy, 2001) and the modulation of their activities has been implicated in the virulence of, for example, *Pseudomonas aeruginosa* and *Escherichia coli* (de Bentzmann *et al.*, 2000), *Borrelia burgdorferi* (Gebbia *et al.*, 2001) and *Chlamydia pneumoniae* (Vehmaan-Kreula *et al.*, 2001). The increase in the degradation of connective tissue produced by an increase in the activities of MMPs and TIMPs is thought to enhance bacterial invasion. However, pathogenic bacteria also secrete their own proteases that are involved in several processes. These include the maturation of other enzymes. For example, *Listeria monocytogenes* phospholipase C requires a zinc metalloprotease for its activation (Poyart *et al.*, 1993; Raveneau *et al.*, 1992) and intrinsic toxin activity, as does the enterotoxin of *Bacteroides fragilis* (Kling *et al.*, 1997; Moncrief *et al.*, 1995), the *Bacillus anthracis* lethal toxin (LeTx) (Hammond & Hanna, 1998; Hanna, 1999) and the *Clostridium* spp. neurotoxin (Tonello *et al.*, 1996). Further-

more, bacterial proteases have been implicated in the direct degradation of host connective tissues. This degradative activity is typically associated with the activity of a zinc metalloprotease, such as that seen in *Pseudomonas aeruginosa* (Olson & Ohman, 1992) and in *Clostridium histolyticum* (Yoshihara *et al.*, 1994).

A class of metzincin metalloendopeptidases that have been studied extensively are those that belong to the repeats-in-toxin (RTX) family (Welch, 1991). This family of zinc metalloproteases are secreted to the external medium via a Type I pathway, and members of this family have been identified in *Erwinia chrysanthemi* (Dahler *et al.*, 1990; Letoffe *et al.*, 1990), *Erwinia amylovora* (Zhang *et al.*, 1999), *Erwinia carotovora* (Marits *et al.*, 1999), *Pseudomonas aeruginosa* (Duong *et al.*, 1992; Guzzo *et al.*, 1990, 1991a, b), *Pseudomonas fluorescens* (Ahn *et al.*, 1999; Kawai *et al.*, 1999; Liao & McCallus, 1998), *Serratia marcescens* (Braunagel & Benedik, 1990; Letoffe *et al.*, 1991; Nakahama *et al.*, 1986), *Pseudomonas brassicacearum* (Chabeaud *et al.*, 2001) and *Photorhabdus luminescens* (M. Valens, A.-C. Broutelle, M. Lefebvre, M. A. Blight, D. Bowen and R. ffrench-Constant, unpublished data). The genetic organization of the operons of members of the RTX family varies between organisms, but essentially includes the structural gene for the RTX zinc metalloprotease(s) and an associated type I secretion apparatus comprising an inner-membrane ATP-binding cassette, a membrane-fusion protein and an outer-membrane pro-

Abbreviations: APR, alkaline protease; APRin, alkaline protease inhibitor; Inh, inhibitor protein.

tein. Moreover, in all cases a gene encoding a specific protease inhibitor is also present. The majority of investigations into these operons have concentrated either on the protease or on its secretion mechanism. Few studies have reported data for the protease inhibitor. The *Erwinia chrysanthemi inh* gene has been cloned and expressed in *Escherichia coli* (Letoffe *et al.*, 1989), and the inhibitor protein (Inh) has been shown to be a heat-stable, low-molecular-mass periplasmic enzyme. Furthermore, the inhibitor of *Pseudomonas aeruginosa* alkaline protease (APR), APRin, has also been characterized with respect to its binding to APR, and it has been shown to require the N-terminal five amino acids for its inhibition activity (Feltzer *et al.*, 2000). In addition, a 12 kDa broad-spectrum protease inhibitor has been described in *Photobacterium luminescens* (Wee *et al.*, 2000), which was secreted into the culture medium by phase II phenotypic-variant cells.

Photobacterium luminescens is a Gram-negative entomopathogenic member of the *Enterobacteriaceae* (Fischer-Le Saux *et al.*, 1999). It exists in a symbiotic relationship with entomophagous nematodes of the family *Heterorhabditidae* [see reviews by Forst *et al.* (1997) and Forst & Nealson (1996)]. The bacteria occupy the intestinal tract of the infective stage of the nematode (the infective juvenile). On finding a host, the nematode enters the larva and releases the bacteria into the insect haemocoel (body cavity). The bacteria rapidly divide, producing a wide range of toxins and hydrolytic exoenzymes that are responsible for the death and subsequent bioconversion of the insect larva. These conditions are ideal for nematode growth and development.

Here, we describe the cloning and expression of the *Photobacterium luminescens* W14 *inh* gene in *Escherichia coli*, together with the purification of Inh from the periplasm of *Escherichia coli*. We also demonstrate the *in vitro* inhibition activity of Inh on its purified cognate protease, PrtA.

METHODS

Bacterial strains and culture conditions. *Photobacterium luminescens* wild-type strain W14 was used as the source of *inh* and of PrtA. The expression and purification of Inh was performed in *Escherichia coli* DH5 α (F ϕ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 deoR recA1 endA1 hsdR17(r $_{K}^{-}$, m $_{K}^{-}$) *phoA supE44 l⁻ thi-1 gyrA96 relA1*) (Life Technologies). *Escherichia coli* and *Photobacterium luminescens* strains were cultured on Luria-Bertani (LB) medium at 30 °C.

Molecular biology techniques. All molecular biology techniques were done as described by Maniatis *et al.* (1982), unless stated otherwise. Restriction endonuclease (Promega) digestions were performed according to the manufacturer's instructions. *Photobacterium luminescens* W14 genomic DNA was isolated as follows. A sample (1.5 ml) of a stationary phase culture of *Photobacterium luminescens* W14, grown in LB medium at 30 °C with agitation, was centrifuged at 8000 g (4 °C); the cell pellet was resuspended in 567 μ l TE (10 mM Tris/HCl, 0.1 mM EDTA, pH 8.0). Following the addition of 30 μ l of 10% (w/v) SDS and 3 μ l proteinase K (20 mg ml $^{-1}$),

the cells were incubated at 37 °C for 1 h. The lysate was mixed thoroughly with 100 μ l of 5 M NaCl and 80 μ l CTAB buffer [10% (w/v) hexadecyltrimethyl ammonium bromide (Sigma) in 0.7 M NaCl] and incubated at 65 °C for 10 min. Upon completion of the incubation, an equal volume of chloroform/isoamyl alcohol (24:1, v/v) was added to the solution and it was mixed thoroughly. The mixture was centrifuged at 8000 g (4 °C) for 5 min. The aqueous phase was extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and centrifuged as before. The aqueous supernatant was then mixed with 0.6 vols of 2-propanol; the precipitated genomic DNA was spooled and washed with 70% (v/v) ethanol prior to air-drying and resuspension in 100 μ l TE buffer.

PCR amplification of *inh* was achieved by using two oligonucleotide primers (Inh-W14-5', 5'-ATATCATATGGTTT-TGCAGCTTGGTATCTG-3', and Inh-W14-3', 5'-ATAT-AAGCTTTTATTCATTTTCTTATTAGTC-3') that provided 5' and 3' *NdeI* and *HindIII* restriction sites (shown in bold), respectively, and which amplified the *Photobacterium luminescens* W14 *inh* gene from its initiation to termination codons. Amplification was performed with the following protocol: 5 cycles at 96 °C for 1 min, 40 °C for 1 min and 72 °C for 30 s, followed by 25 cycles at 95 °C for 1 min, 55 °C for 1 min and 72 °C for 30 s. This amplification resulted in a single PCR product of 422 bp in size. Overexpression of Inh was achieved by cloning the PCR product into the plasmid vector pBAD33-GFPuv (Dr H. Benabdelhak, University Paris Sud, France), which consists of pBAD33 containing the gene encoding GFPuv (Clontech) on an *NdeI*-*HindIII* restriction fragment. Both the vector pBAD33-GFPuv and the *inh* PCR product were digested with *NdeI* and *HindIII*. Digested plasmid DNA yielded four fragments of 5338, 540, 125 and 109 bp in size. The 5338 bp *NdeI*-*HindIII* fragment was isolated following electrophoresis of the fragments through a 0.7% agarose/TAE (40 mM Tris acetate, 2 mM EDTA) gel. It was then ligated with the 407 bp digested PCR product using T4 DNA ligase (Promega). Following their transformation with the ligation product, cells of electrocompetent *Escherichia coli* DH5 α were plated onto LB agar containing 12.5 μ g chloramphenicol ml $^{-1}$ and incubated at 30 °C. The sequence of the resulting recombinant clone, pINH-1, was confirmed by using an ABI model 373 automated DNA sequencer (Applied Biosystems).

Purification of Inh from the periplasm of *Escherichia coli* DH5 α . A portion of an overnight culture of *Escherichia coli* DH5 α (pINH-1) that had been grown in LB broth supplemented with 12.5 μ g chloramphenicol ml $^{-1}$ was inoculated into 1 l fresh medium to an OD $_{450}$ of 0.1. This new culture was incubated at 37 °C until growth reached an OD $_{450}$ of 0.6. Induction of *inh* expression, from the pBAD promoter, was achieved by the addition of L-arabinose (to a final concentration of 0.1%, w/v) to the culture and a further 2 h incubation. After this time, the bacterial cells were pelleted by centrifugation at 6000 g (4 °C) for 15 min and the cell pellets were resuspended in 10 ml of 30 mM Tris/HCl containing 20% (w/v) sucrose (pH 8.0). Following the addition of 20 μ l of 0.5 M EDTA (1 mM final concentration) to the suspension, the cells were incubated at room temperature for 10 min, with gentle stirring. The cells were pelleted by centrifugation at 8000 g (4 °C) for 10 min and resuspended in 10 ml ice-cold 5 mM MgSO $_4$. They were then incubated on ice for 10 min, with gentle stirring. After this time, the cells were centrifuged as above; the supernatant containing the osmotically shocked periplasmic fraction was retained.

Inh was purified from the periplasmic fraction of the super-

Fig. 1. Purification of overexpressed Inh from the periplasm of *Escherichia coli* DH5 α (pINH-1). (a) Chromatogram produced following anion-exchange chromatography (MonoQ) of the ammonium-sulphate-precipitated periplasmic fraction. Protein was detected by measuring the absorbance at 280 nm (solid line). The elution gradient (dashed line) and the conductivity (broken line) are also shown. Collected fractions are indicated by solid boxes above the graph. (b) Chromatogram produced following size-exclusion chromatography (Superdex HR75). The protein and conductivity measurements are as for (a). Fraction 10 (indicated by a solid box above the graph) represents fractions 3+4 from (a) which had been subjected to further purification by size-exclusion chromatography. Fractions 1 and 2 are not shown on the chromatograms in (a) and (b) because no Inh was detected in them. Units of absorbance in both chromatograms are arbitrary. (c) SDS-polyacrylamide gel (11% acrylamide) of proteins from un-induced *Escherichia coli* DH5 α (pINH-1), induced *Escherichia coli* DH5 α (pINH-1) and the fractions shown in (a) and (b). Lanes: A, un-induced *Escherichia coli* DH5 α (pINH-1) whole-cell proteins; B, induced *Escherichia coli* DH5 α (pINH-1) whole-cell proteins (induced with 0.1%, w/v, arabinose; 2 h incubation); C, cell pellet following periplasmic extraction; D, periplasmic fraction; E, molecular mass markers. Lanes 1–10 refer to the fractions indicated on the chromatograms in (a) and (b).

nant by anion-exchange and size-exclusion chromatography using an ÄKTA FPLC system (Amersham-Pharmacia Biotech) at 10 °C as follows. A MonoQ HR 5/5 anion-exchange column (Amersham-Pharmacia Biotech) that had been equilibrated in Buffer A (20 mM Tris/HCl, pH 7.5) was loaded with

the periplasmic fraction (approx. 40–70 μ g total protein ml⁻¹) at a flow rate of 1.0 ml min⁻¹. Following washing of the column with Buffer A for 2 column volumes, proteins were eluted with a linear gradient of Buffer B (0–35%; Buffer A + 1 M NaCl) over 20 column volumes at a flow rate of 1.0 ml min⁻¹. Inh eluted at approximately 250 mM NaCl and was further purified by size-exclusion chromatography on a Superdex HR75 column (Amersham-Pharmacia Biotech) using Buffer A as the mobile phase. Eluted Inh was supplemented with 10% (v/v) glycerol and stored at –20 °C prior to further analysis.

Purification of PrtA. *Photorhabdus luminescens* W14 phase I cells were grown in 1 l of LB medium at 30 °C (agitation at 250 r.p.m.) to the late stationary phase of growth over 48 h. The culture supernatant was retained following centrifugation of the culture at 8000 g (4 °C) for 30 min. Solid ammonium sulphate was added to the supernatant to a final saturation of 80%, and proteins were precipitated at 4 °C for 2 h with gentle stirring. Precipitated material was collected by centrifugation at 10000 g (4 °C) for 30 min, and the pellets were combined and solubilized in a total volume of 80 ml of a solution containing 1 M ammonium sulphate and 50 mM sodium phosphate (pH 8.0). Chromatographic procedures were performed on an ÄKTA FPLC system at 20 °C. Following a final clarification of the solubilized supernatant by centrifugation at 10000 g (4 °C) for 30 min, the solubilized proteins were loaded onto 30 ml of Octyl Sepharose 4 Fast Flow hydrophobic interaction resin packed in an XK16/20 column (Amersham-Pharmacia Biotech) at a flow rate of 1 ml min⁻¹. Elution of the proteins was performed with a linear gradient over 20 column volumes into 5 mM sodium phosphate (pH 8.0); fractions of 5 ml were collected. The protease activity of the fractions was determined by spectrophotometric determination of the Coomassie blue released at 595 nm from Blue Hide Azure powder (Sigma) as follows. Blue Hide Azure Powder was resuspended to 30 mg ml⁻¹ in a solution containing 20 mM Tris/HCl and 5 mM CaCl₂ (pH 8.0). Aliquots (5 μ l) of each fraction were assayed for 30 min at 37 °C with 0.5 ml of the Blue Hide Azure suspension in 1.5 ml Eppendorf tubes, with constant agitation. Following incubation, the assays were centrifuged and the absorption value at 595 nm was determined for the supernatants. One unit of protease activity was defined as 0.01 A₅₉₅ units released min⁻¹ (ml fraction assayed)⁻¹. Fractions containing protease activity were pooled and applied directly to a MonoQ HR5/5 column (Amersham-Pharmacia Biotech) that had been equilibrated with 5 mM sodium phosphate (pH 8.0). Fractions of 0.5 ml were collected during elution with a solution containing 5 mM sodium phosphate and 1 M NaCl (pH 8.0) over 10 column volumes. Protease-containing fractions were assayed as described above and eluted at approximately 300 mM NaCl. These were then analysed by SDS-PAGE.

Interactions between the protease (PrtA) and the inhibitor (Inh). The demonstration of stoichiometric interactions between PrtA and its cognate inhibitor (Inh) was done by anion-exchange chromatography. Purified PrtA and Inh (both at a concentration of 1 mg ml⁻¹) were diluted in 1 ml of Buffer IEX-A (20 mM Tris/HCl, 1 mM CaCl₂, pH 8.0), either individually or together to a final concentration of 93 μ M – this required 50 μ g of PrtA and 11 μ g of Inh. Samples were analysed by anion-exchange chromatography on a MonoQ HR 10/10 anion-exchange column (Amersham-Pharmacia Biotech) using an ÄKTA FPLC system. Samples were loaded in Buffer IEX-A and eluted with a linear gradient of Buffer IEX-B (Buffer IEX-A + 1 M NaCl) over 10 column volumes to a final concentration of 0.5 M NaCl.

Protein analysis. Protein concentrations were measured using the Coomassie Plus Protein Assay Reagent (Pierce) and denaturing SDS-PAGE (15%, w/v, acrylamide), performed as described by Laemmli (1970). Proteins were stained with Coomassie Brilliant Blue R250 (Sigma).

Protease inhibition assay. Purified *Phototribadus luminescens* W14 PrtA was prepared as described above. The protease activity of PrtA was measured as the increase of BODIPY-FL fluorescence released from cleaved BODIPY-FL-labelled casein using the EnzChek Protease Assay Kit (Molecular Probes). Measurements of fluorescence were made in a Biolumin Microplate Fluorescence Spectrophotometer (Molecular Dynamics), with excitation and emission wavelengths of 485 and 530 nm, respectively. Purified PrtA dissolved in 20 mM Tris/HCl containing 5 mM CaCl₂ (pH 8.0) was assayed in a total volume of 200 µl, with BODIPY-FL casein added to a final concentration of 5 µg ml⁻¹. Alternatively, assays were conducted as described above but in a final volume of 1 ml and using an SFM25 spectrofluorometer (Biotek Kontron) with the same excitation and emission wavelengths. All kinetic experiments were repeated in triplicate and Figs 1 and 4–6 represent the mean data with a typical SE of between 2 and 5%.

RESULTS

Cloning, expression and purification of *Phototribadus luminescens* Inh

The *Phototribadus luminescens* W14 zinc metalloprotease inhibitor gene, *inh*, was obtained as a 422 bp PCR product (see Methods for details). Following digestion of the 422 bp fragment with *Nde*I and *Hind*III, the resulting 407 bp cohesive fragment was ligated to the 5338 bp fragment from pBAD33-GFPuv. This ligation resulted in pINH-1, which expressed *inh* from the arabinose-inducible pBAD promoter.

Inh production by *Escherichia coli* DH5α(pINH-1) was induced by incubating a culture of the cells for 2 h in the presence of 0.1% (w/v) L-arabinose. After this incubation, Inh was purified from the isolated periplasmic fraction of *Escherichia coli* DH5α(pINH-1) in two chromatographic steps. Fig. 1 illustrates the chromatograms produced following anion-exchange (Fig. 1a) and size-exclusion (Fig. 1b) chromatography, together with SDS-PAGE analysis of the fractions produced by these two methods (Fig. 1c).

Following the induction of its expression, Inh could be seen as a low-molecular-mass species (11 kDa) that accumulated in whole cells of *Escherichia coli* DH5α (pINH-1) (Fig. 1c, lane B). Fractionation of the cells into a periplasmic fraction (Fig. 1c, lane D) and a residual whole-cell fraction (Fig. 1c, lane C) demonstrated that the majority of Inh was localized in the periplasmic fraction, as expected. This resulted in a high enrichment of Inh, with Inh representing approximately 65% of the total periplasmic protein as estimated from the peak area in the anion-exchange chromatogram (Fig. 1a). SDS-PAGE analysis of the chromatographic fractions clearly showed that all of the Inh bound to the MonoQ resin (absence of Inh in flow-through fractions 1 and 2, Fig. 1c) and that it eluted in a compact peak,

with minor contaminating proteins appearing at approximately 42 and 47 kDa (Fig. 1c, lane 3+4). The material from fractions 3+4 was further purified by size-exclusion chromatography on a Superdex HR75 column (Fig. 1b), resulting in a single migrating 11 kDa form of Inh that was estimated to be 99% pure (Fig. 1c, lane 10).

Inhibition of metzincin metalloendopeptidases by the Inh family minimally requires the N-terminal five amino acid residues of the Inh proteins (Feltzer *et al.*, 2000). Consequently, it was important to determine whether the N terminus of the recombinant Inh purified from the periplasm of *Escherichia coli* DH5α(pINH-1) was identical to that of wild-type Inh from *Phototribadus luminescens* W14. Hence, we determined the N-terminal-amino-acid sequence of the wild-type Inh purified from *Phototribadus luminescens* W14 and of the recombinant Inh purified from *Escherichia coli* DH5α(pINH-1). For both the wild-type and the recombinant protein the N-terminal sequence was SSLVL, indicating that leader peptidase processing was identical in both organisms. Furthermore, matrix-assisted laser desorption ionization/time of flight (MALDI/TOF) mass spectrometry indicated a molecular mass of 11951 Da for Inh, in excellent agreement with the predicted mass of 11953 Da for mature Inh. Therefore, recombinant Inh, which could be purified in substantial amounts from *Escherichia coli* DH5α(pINH-1), was used for PrtA inhibition studies.

Comparison of *Phototribadus luminescens* W14 Inh with other zinc metalloprotease inhibitors

A multiple-amino-acid-sequence alignment of the 13 available Inh proteins (Fig. 2) showed that there are two highly conserved domains between residues 25 and 41 and residues 67 and 100 (*Phototribadus luminescens* W14 Inh co-ordinates) of the Inh proteins. Domain 1 encompasses the predicted leader peptidase cleavage site with the consensus sequence MA/SSL, resulting in the mature polypeptide possessing a strictly conserved Ser at position 2. The crystal structure of the complex between the *S. marcescens* metalloprotease PrtSM and the *Erwinia chrysanthemi* inhibitor (Baumann *et al.*, 1995) (Protein database accession no. 1SMP; <http://www.rcsb.org/pdb/>) and of the complex between *Pseudomonas aeruginosa* APR and APRin (Hege *et al.*, 2001) indicates that both Inh and APRin have a compact globular structure with an unstructured N terminus in direct interaction with the zinc-binding pocket of the protease active site. The first amino acid of Inh is not strictly conserved as it is the main chain atoms that form the inhibitory hydrogen bonds, although Ser₁ of the *Pseudomonas aeruginosa* APRin forms a co-ordinate bond with the zinc ion (Hege *et al.*, 2001). The strictly conserved Ser₂ residue bonds the catalytic Glu of the protease and Leu₃ is accommodated in a hydrophobic pocket. The second highly conserved domain within the Inh family – Domain 2 – is characterized by a β-sheet being bent into a loop by the strictly conserved Pro₆₂

Fig. 2. Multiple-sequence alignment of 13 members of the Inh family. The signal peptide cleavage site is indicated by a vertical bar (MA/SSL), the conserved Domains, 1 and 2, are indicated by solid horizontal bars and the conserved Cys residues involved in a disulphide bond are marked by a double asterisk. E.c-B374, *Erwinia chrysanthemi* B374; E.c-E16, *Erwinia chrysanthemi* E16; E.cr-car, *Erwinia carotovora* sub. *carotovora*; P.l-W14, *Photobacterium luminescens* W14; E.a-E9, *Erwinia amylovora* E9; S.m-27117, *S. marcescens* ATCC 27117; S.m-SM6, *S. marcescens* SM6; P.a-PA01, *Pseudomonas aeruginosa* PA01; P.f-CY091, *Pseudomonas fluorescens* CY091; P.f-SIK-W1, *Pseudomonas fluorescens* SIK-W1; P.f-33, *Pseudomonas fluorescens* 33; P.t-1116S, *Pseudomonas tolaasii* 1116S; P.b-NFM421, *Pseudomonas brassicacearum* NFM421.

<i>E. chrysanthemi</i> -B374	----	89.2	70.0	49.3	50.8	44.0	37.2	45.8	43.0	41.4	40.8	39.1	31.9
<i>E. chrysanthemi</i> -E16	85.8	----	68.3	51.5	53.3	44.8	38.6	44.3	41.4	42.2	39.2	35.9	29.8
<i>E. carotovora</i> -sub- <i>carotovora</i>	59.2	57.5	----	52.2	47.5	41.6	37.2	44.3	35.9	36.7	33.8	33.6	28.4
<i>P. luminescens</i> -W14	40.3	41.0	45.5	----	41.0	38.1	34.7	43.8	35.6	37.0	34.6	33.3	27.9
<i>E. amylovora</i> -E9	37.7	38.5	35.2	30.6	----	43.7	39.3	45.0	32.6	33.3	31.3	33.3	26.2
<i>S. marcescens</i> -ATCC27117	33.6	30.4	27.2	25.4	31.7	----	69.7	39.7	34.4	34.4	32.3	28.9	24.1
<i>S. marcescens</i> -SM6	27.6	25.5	26.9	26.0	28.3	65.5	----	33.1	30.1	29.5	28.1	23.3	20.8
<i>P. aeruginosa</i> -PA01	30.5	28.2	30.5	28.5	32.1	34.4	26.4	----	38.2	42.0	38.3	38.2	32.6
<i>P. fluorescens</i> -CY091	28.1	28.1	25.8	24.4	26.4	22.7	20.5	29.0	----	90.2	81.5	73.0	40.4
<i>P. fluorescens</i> -SIK-W1	25.8	26.6	26.6	23.7	25.6	20.3	18.5	30.5	82.8	----	83.9	70.5	39.7
<i>P. fluorescens</i> -33	28.5	26.9	26.2	23.5	23.7	23.8	20.5	30.1	68.5	70.2	----	71.8	37.1
<i>P. tolaasii</i> -1116S	25.8	22.7	24.2	20.7	21.7	18.0	14.4	28.2	62.3	63.1	58.9	----	35.5
<i>P. brassicacearum</i> -NFM421	23.4	22.0	19.1	19.0	19.1	21.3	17.5	26.2	31.9	30.5	29.4	26.2	----

Fig. 3. Similarity matrix for proteins belonging to the Inh family.

(mature protein co-ordinates). This loop is in close proximity to the extended N terminus, with a closest distance of 3.52 Å between the Pro₆₂ and Arg₄ side chains. Domain 2 may stabilize the N-terminal of Domain 1 via the Pro₆₂ loop, enabling efficient interaction and insertion of the Inh N terminus into the protease active site. Other than Domains 1 and 2, the intervening sequences of Inh are poorly conserved and result in percentage identity and similarity values that vary from 89.2 and 85.8% identity and similarity, respectively, between *Erwinia chrysanthemi* B374 and *Erwinia chrysanthemi* E16 to only 20.8% identity between *Pseudomonas brassicacearum* NFM421 and *S. marcescens* SM6 and 18.0% similarity between *Pseudo-*

monas tolaasii 1116S and *S. marcescens* ATCC 27117 (Fig. 3).

Interaction of PrtA with Inh

The complexes formed between the *S. marcescens* PrtSM and the *Erwinia chrysanthemi* inhibitor (Baumann *et al.*, 1995) and between APR and APRin (Feltzer *et al.*, 2000) both indicate a 1:1 molar stoichiometry for inhibition. To demonstrate an interaction between the purified *Photobacterium luminescens* W14 proteins, PrtA and Inh were analysed by anion-exchange chromatography (see Methods) either individually or following mixing (see Methods) and incubation at 4 °C for 15 min.

Fig. 4. Anion-exchange chromatography of PrtA-Inh complexes. Equimolar (93 μ M) amounts of Inh (11 μ g) and PrtA (50 μ g) were either analysed individually or following mixing and incubation on ice for 15 min upon a MonoQ HR10/10 anion-exchange column at a flow rate of 1 ml min⁻¹ and linear elution with NaCl. Peaks are as labelled. Units of absorbance are arbitrary.

The theoretical charges of PrtA, Inh and PrtA + Inh at pH 8.0 were obtained from the titration curves calculated at http://www.up.univ-mrs.fr/~wabim/d_labim/compo-p.html and were -4.88, -30.69 and -37.39, respectively. Therefore, one would expect that the PrtA-Inh complex would be retained more strongly upon an anion-exchange resin than either Inh or PrtA alone. Fig. 4 illustrates the chromatograms obtained following analysis of PrtA, Inh and PrtA + Inh on a MonoQ HR 10/10 column. Equimolar loadings (93 μ M) of each protein were analysed and the curve for PrtA + Inh showed no residual PrtA or Inh peaks, indicating that all of the PrtA and Inh were combined as a bimolecular species, eluting at 318 mM NaCl rather than at 253 mM (Inh alone) and 298 mM NaCl (PrtA alone). Similar experiments (data not shown) using 2:1 PrtA/Inh or Inh/PrtA showed residual peaks for PrtA and Inh, respectively. Therefore, these data demonstrate a 1:1 molecular stoichiometry between PrtA and Inh when they are in solution together.

Inhibition of PrtA by Inh

Casein hydrolysis by purified *Photobacterium luminescens* W14 PrtA in the presence of increasing concentrations of Inh was measured. PrtA was diluted to a concentration of 1 nM (see Methods) and Inh was added to give molar ratios of PrtA/Inh ranging from 1:0 to 1:10. Proteins

were incubated for 2 min at 25 °C followed by the addition of the PrtA substrate, BODIPY-FL-labelled casein. The increase in fluorescence was measured (Fig. 5a) and the slope of steady-state hydrolysis was calculated between 120 and 540 s. A bar graph of the substrate-hydrolysis rate for each PrtA/Inh molar ratio (Fig. 5b) clearly demonstrated that little significant inhibition of PrtA by Inh occurred until a molar ratio of 1:0.5 PrtA/Inh was used; total inhibition was observed at molar ratios of 1:1 PrtA/Inh and above. These data are consistent with the unimolecular stoichiometry of inhibition described above and are indicative of a strong interaction between the two proteins that results in the efficient inhibition of PrtA by Inh. To further characterize the interaction between PrtA and Inh, the association constant for the PrtA-Inh complex was determined.

Kinetics of PrtA inhibition by Inh

Many protease(P)-inhibitor(I) interactions are characterized by classical 'slow-binding' kinetics with a typical equilibrium characterized by:

Not all protease-inhibitor interactions result in covalent bonding (i.e. an irreversible reaction), and frequently inhibitor binding is so tight that one can not determine a binding constant. Under these conditions the reaction is classified as 'pseudo-irreversible' and the most relevant quantity to measure is the apparent rate of inhibition (or association), k_{ass} , where:

$$k_{\text{ass}} = \frac{k_1 k_2}{k_{-1}}$$

The inhibition curves for purified *Photobacterium luminescens* W14 PrtA were determined using BODIPY-FL-labelled casein with 1.0 nM PrtA and either a 1:1 or 1:10 PrtA/Inh molar ratio (Fig. 6). Following the addition of Inh to the PrtA/casein-hydrolysis reaction, a rapid decline in PrtA activity was observed as detected by the reduced rate of change of fluorescence due to liberated BODIPY-FL-labelled peptides. At a molar ratio of 1:10 PrtA/Inh (and higher ratios; data not shown) inhibition was sufficiently rapid that a pseudo-first-order kinetic analysis was not possible. Therefore, to determine a reasonable estimate of k_{ass} , a second-order analysis was performed at a molar ratio of 1:1 analysing the data from the addition of Inh ($t = 120$ s) to stable inhibition at $t = 240$ s. A plot of the reciprocal residual enzyme concentration against time (Fig. 6, inset) yielded a straight line with slope $k_{\text{ass}} = 1.34 \times 10^7$ M s⁻¹ and an intercept (9.98×10^8 M⁻¹) equal to the reciprocal of the starting enzyme concentration. The latter is in good agreement with the initial enzyme concentration of 1.0 nM. The large value for k_{ass} indicates that the PrtA-Inh complex is highly stable and that if an intermediate complex exists it is rapidly

Fig. 5. Inhibition of PrtA activity by Inh. (a) PrtA (1 nM) was pre-incubated with Inh in molar ratios ranging from 1:0 to 1:10 PrtA/Inh at 25 °C for 2 min. BODIPY-FL-labelled casein substrate (5 $\mu\text{g ml}^{-1}$) was added to the mixture and the fluorescence generated by the release of labelled peptides was followed with time. (b) Bar graph of the ratios shown in (a) against the steady-state substrate-hydrolysis rate measured as the slope of traces in (a) between 120 and 540 s.

converted to the stable inhibited form. Since PrtA–Inh can be dissociated by the addition of SDS, no covalent modification of the complex occurs; therefore, we can consider the PrtA–Inh interaction to be pseudo-irreversible. Thus, it is possible to calculate the half-life of protease inhibition from:

$$t_{1/2} = \frac{0.693}{k_{\text{ass}} \times [\text{I}]}$$

For the *Photorhabdus luminescens* W14 PrtA–Inh complex, the protease inhibition $t_{1/2} = 51.7$ s. Studies of PrtA

homologues and their kinetic interactions with their cognate Inh homologues will yield comparative inhibition data for that presented above.

DISCUSSION

Proteases and protease inhibitors are important for many biological processes and have been exploited in industrial applications, such as biological detergent production, and in clinical treatments, including HIV therapy (Brechtel *et al.*, 2001). Specific protease inhibitors have been suggested as new treatments in bacterial

Fig. 6. Kinetics of PrtA inhibition by Inh. PrtA (1 nM) was incubated with BODIPY-FL-labelled casein (5 µg ml⁻¹); Inh was added at either a 1:1 or a 1:10 PrtA/Inh molar ratio after 120 s. Fluorescence was monitored over time. The inset graph represents a plot of the reciprocal residual enzyme concentration (1/[E_t]) against time at a PrtA/Inh molar ratio of 1:1 (second-order kinetics), which yields a straight line with slope $k_{\text{ass}} = 1.34 \times 10^7 \text{ M s}^{-1}$ and an intercept ($9.98 \times 10^8 \text{ M}^{-1}$) equal to the reciprocal of the starting enzyme concentration.

infections where the proteases are second-generation antibiotic targets (Travis & Potempa, 2000). *Photorhabdus luminescens* is a natural pathogen for insect larvae, but it has recently been identified in human infections (Peel *et al.*, 1999). During insect infection, *Photorhabdus luminescens* secretes a wide variety of hydrolytic enzymes to the external medium, including lipase (Wang & Dowds, 1993), chitinase (Chen *et al.*, 1996) and protease (Ong & Chang, 1997). The role of these enzymes is thought to be for the bioconversion of the insect cadaver to provide nutrients for the development of the *Heterorhabditis* nematodes that, in symbiosis with *Photorhabdus luminescens* bacteria, are responsible for the penetration and infection of the insect larva. Many pathogenic bacteria use hydrolytic enzymes to penetrate host tissues during invasion, e.g. hyaluronidase is secreted by group A streptococci (Hynes *et al.*, 2000). It is therefore not inconceivable that the extracellular hydrolytic enzymes of *Photorhabdus luminescens* would provide it with an advantage in human infections for tissue penetration, if this normally psychrophilic bacterium were adapted to growth at a higher temperature. Other bacteria not normally associ-

ated with human infections have also been found to be clinically relevant. For example, *Pseudomonas fluorescens* is a psychrophilic bacterium that is typically associated with food contamination, yet it has been found in nosocomial infections of humans. The origin of these infections is thought to be either directly via deep wounding (Dubey *et al.*, 1988) or via bacterial colonization of medical equipment (Burgos *et al.*, 1996; Hsueh *et al.*, 1998). Therefore, it is important to consider pathogenic bacteria not normally associated with human infection to be potential human pathogens. An understanding of the biology of such organisms may identify potential second-generation antibiotic targets and extracellular-enzyme inhibitors.

The zinc metalloprotease inhibitor of *Photorhabdus luminescens* presented in this work has a high specificity and affinity for the extracellular PrtA protease. Analysis of the complexes formed between the *S. marcescens* metalloprotease (PrtSM) and the *Erwinia chrysanthemi* inhibitor (Baumann *et al.*, 1995) and between *Pseudomonas aeruginosa* APR and APRin (Hege *et al.*, 2001) indicate that the mechanism of inhibition of this class of

proteases is unique and involves the extreme N terminus of the inhibitor molecule. The N-terminal five amino acid residues of the inhibitor protein are an absolute requirement for inhibition, as demonstrated by deletion studies on APRin (Feltzer *et al.*, 2000) and on *S. marcescens* SMPI (Bae *et al.*, 1998), where serial deletion of residues 1–5 of APRin resulted in a progressive reduction of its affinity for APR and deletion of the N-terminal three residues of SMPI resulted in reduced protease inhibition, respectively. However, studies have demonstrated that there are several important differences in the apparent role of the extreme N terminus of inhibition proteins of different bacterial species, and these suggest possible subtle differences in the mechanism of action for the different protein inhibitors. The deletion of Gly₁ and Ser₂ from *S. marcescens* SMPI had little effect upon SMP inhibition, whereas the deletion of Leu₃ resulted in severely impaired function (Bae *et al.*, 1998). Furthermore, mutation of SMPI Leu₃ indicated a requirement for a hydrophobic side chain for efficient inhibition (Bae *et al.*, 1998). Conversely, for both *Pseudomonas aeruginosa* APRin and *Erwinia chrysanthemi* Inh, the extreme N-terminal residues appear to be absolutely necessary for inhibition (Feltzer *et al.*, 2000; Letoffe *et al.*, 1989). Circular dichroism studies of APRin also indicate that there may be structural differences between different inhibitor molecules, when compared with *Erwinia chrysanthemi* Inh (Feltzer *et al.*, 2000). These data may, in part, contribute to the observations of highly varied affinities between inhibitors and their cognate metzincin proteases. APRin binds to APR with a K_D of approximately 4 pM (Feltzer *et al.*, 2000), whereas K_D values for *S. marcescens* SMPI and SMP (Bae *et al.*, 1998) and for *Erwinia chrysanthemi* PrtA, PrtB and PrtC and their cognate Inh (Letoffe *et al.*, 1989) are reported as 0.7 µM and 1–10 µM, respectively. The data presented here for the inhibition of PrtA by *Photothabdus luminescens* Inh also indicate a strong and highly stable interaction ($k_{\text{ass}} = 1.34 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$); they also indicate that if an intermediate complex exists it is rapidly converted to the stable inhibited form. We were unable to estimate a K_D value for the association of *Photothabdus luminescens* PrtA and Inh as (unlike for the *Pseudomonas aeruginosa* and *Erwinia chrysanthemi* proteases) we have not yet been able to demonstrate cleavage of chromogenic substrates and have, therefore, been limited to the use of a multiply-cleaved proteinaceous substrate, BODIPY-FL-labelled casein, in our studies.

Here, we have presented the purification and initial characterization of the interaction of the *Photothabdus luminescens* W14 inhibitor, Inh, with its cognate protease, PrtA. Further studies of the specific interactions between proteases of the repeats-in-toxin (RTX) zinc metalloprotease family and their cognate, or otherwise, inhibitors will provide more information about this unique mode of inhibition. Co-crystallization and structural analyses of the *Photothabdus luminescens* W14 PrtA–Inh complex are currently being performed and these data will be reported elsewhere.

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