



Enzymatic characterization of a serralysin-like metalloprotease from the entomopathogen bacterium, *Xenorhabdus*

Mustafa K. Massoud, Judit Marokházi, István Venekei *

Department of Biochemistry, Eötvös Loránd University, Budapest, 1117 Pázmány, Péter sétány 1/C, Hungary

ARTICLE INFO

Article history:

Received 27 January 2011
Received in revised form 27 April 2011
Accepted 11 May 2011
Available online 31 May 2011

Keywords:

Serralysin
PrtA peptidase
Metalloprotease
Zn-inhibition
Xenorhabdus
Photorhabdus

ABSTRACT

We investigated the enzymatic properties of a serralysin-type metalloenzyme, provisionally named as protease B, which is secreted by *Xenorhabdus* bacterium, and probably is the ortholog of PrtA peptidase of *Photorhabdus* bacterium. Testing the activity on twenty-two oligopeptide substrates we found that protease B requires at least three amino acids N-terminal to the scissile bond for detectable hydrolysis. On such substrate protease B was clearly specific for positively charged residues (Arg and Lys) at the P1 substrate position and was rather permissive in the others. Interestingly however, it preferred Ser at P1 in the oligopeptide substrate which contained amino acids also C-terminal to the scissile bond, and was cleaved with the highest k_{cat}/K_M value. The pH profile of activity, similarly to other serralysins, has a wide peak with high values between pH 6.5 and 8.0. The activity was slightly increased by Cu^{2+} and Co^{2+} ions, it was not sensitive for serine protease inhibitors, but it was inhibited by 1,10-phenanthroline, features shared by many Zn-metalloproteases. At the same time, EDTA inhibited the activity only partially even either after long incubation or in excess amount, and Zn^{2+} was inhibitory (both are unusual among serralysins). The 1,10-phenanthroline inhibited activity could be restored with the addition of Mn^{2+} , Cu^{2+} and Co^{2+} up to 90–200% of its original value, while Zn^{2+} was inefficient. We propose that both the Zn inhibition of protease B activity and its resistance to EDTA inhibition might be caused by an Asp in position 191 where most of the serralysins contain Asn.

Crown Copyright © 2011 Published by Elsevier B.V. All rights reserved.

1. Introduction

It is a widely accepted view that secreted proteolytic enzymes of pathogens can play a significant role in virulence by helping the tissue penetration and/or the suppression of immune response. However, such functions are documented in only few cases and neither ones are fully explored because the natural substrates of these enzymes have hardly been found so far [1,2]. Serralysins exemplify such proteases. They belong to the bacterial subfamily (M10B) in the M10 family (the interstitial collagenases) of zinc-metallo endopeptidases (MEROPS Clan MA, [3]). These enzymes are secreted by a large number of bacteria including plant and human pathogens. Due to their relaxed side chain specificity on oligopeptide substrates or denatured polypeptides [4–9] it is supposed that they function as non-specific proteases during infection. However, the serralysins that were tested on native proteins could cleave only some specific ones [10–17] but not e.g. albumin, fibrinogen and several collagen types [18]. These

findings do not support a general proteolytic function but, instead, suggest defined target protein selectivity.

A good model to investigate the roles of secreted proteolytic enzymes in the pathomechanism is the infection process of two closely related insect pathogen bacteria, *Xenorhabdus* and *Photorhabdus* (Enterobacteriaceae) [19–21]. They provide an easily accessible experimental system of host–pathogen interactions with the advantage of very high pathogenicity due to the extreme virulence of these microorganisms in insects: when injected into the hemocoel, less than 50 cells are capable of establishing infection and kill the host.

To study the role of secreted proteases during *Photorhabdus* and *Xenorhabdus* infection, we have screened a large number of strains of both genera with five different detection methods [22,23]. We have found two different activities in *Photorhabdus* and six in *Xenorhabdus* strains, and could ascribe functions to three of them [22–24]. One of the enzymes found in *Xenorhabdus* (which we distinguished with the name “protease B”) appears to be a serralysin and an ortholog of PrtA, a serralysin-type enzyme of *Photorhabdus*, on the basis of several properties: molar mass, N-terminal sequence, requirement for a metal ion cofactor, early secretion and very similar target protein preference. Therefore it was puzzling that—unlike PrtA from *Photorhabdus*—the putative ortholog from *Xenorhabdus* was less sensitive for the metal complexing compound, EDTA, an inhibitor of serralysins, when tested with zymography [23]. Similar observation has been reported for protease II from the *Xenorhabdus* strain, *Xenorhabdus nematophila* [25].

Abbreviations: pNA, paranitroanilide; AMC, aminomethylcoumarine; Dabcyl, 4-[4'-(dimethylamino)phenylazo]benzoyl; Edans, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid; Succ, succinyl; SBzl, thiobenzyl; BOC, butyloxycarbonyl; CBZ, carbobenzoxy; DTT, dithiotreitol; Fua, furylacryloyl; pNA, para-nitroanilide; SBTI, soy bean trypsin inhibitor; PMSF, phenylmethanesulfonyl fluoride; CPS, count per second

* Corresponding author. Tel.: +36 1 381 2171; fax: +36 1 381 2172.

E-mail addresses: Mutkhalea@yahoo.com (M.K. Massoud), juttka@gmail.com (J. Marokházi), venekei@elte.hu (I. Venekei).

Protease II seems to be the same enzyme as protease B (from *X. kozodoii* var. Morocco) because of its very similar N-terminal sequence and molar mass (~55 kDa).

Here we investigate the enzymatic properties of protease B to compare them to those of Photorhabdus PrtA, the putative ortholog, and to the properties of other serralyins.

2. Materials and methods

2.1. Substrates

The substrates were purchased from Sigma-Aldrich (St. Louis, USA) (His-Ser-4-methoxy-naphthylamide, DL-Val-Leu-Arg-p-nitroanilide, Succ.-Ala-Ala-Pro-Phe-thiobenzylester, L-Ser-AMC, Boc-Val-Pro-Arg-AMC, D-Ala-Leu-Lys-AMC, Boc-Leu-Ser-Thr-Arg-AMC, Z-Gly-Gly-Arg-AMC, His-Ser-4-methoxy-β-naphthylamide) and from Bachem (Bubendorf, Switzerland) (Fua-Leu-Gly-Pro-Ala, Boc-Val-Leu-Lys-AMC, Boc-Gln-Ala-Arg-AMC and hepatitis A virus 3C protease substrate), or prepared as described (Succ.-Ala-Ala-Pro-Xaa-AMC [26], Fua-Ala-Leu-Val-Tyr [7], and DabcyL-Glu-Val-Tyr-Ala-Val-Glu-Ser-Edans [18]). For the preparation of stock solutions, the substrates were dissolved in dimethylformamide.

2.2. Purification of protease B

Four liters of LB medium was inoculated from an overnight LB culture of *X. kozodoii* Morocco strain such that the OD₆₀₀ of the resulting cell suspension was ~0.05. It was grown at 30 °C until the OD₆₀₀ was ~0.3 (20–22 hours, late logarithmic phase). The pH of the culture supernatant was set to 8.0 with NaOH then 150 mL slurry of QAE Sephadex A-50 resin (Pharmacia) was added to it, which had previously been equilibrated with buffer A (50 mM Tris-HCl, pH 8.0, 10 mM CaCl₂). After shaking for 2–3 hours in the cold room, the resin was filtered off and washed three times with 600 mL of buffer A. Before elution of proteins, the resin was filled into a column and was washed with a column volume of buffer A. The elution was performed with 200 mL of a linear NaCl gradient (0–1.0 M) in buffer A at 1.6 mL/min flow rate. The zymographically active fractions were pooled and dialyzed against buffer S (50 mM Tris-HCl, pH 8.0, 1.0 mM CaCl₂). The dialysate was centrifuged (71 × g for 15 min), then applied to a Matrex Silica PAE 300 (Millipore) anion exchanger column (1.6 × 2.0 cm) equilibrated with buffer S. After loading, the column was washed with 20 mL buffer S, and then the proteins were eluted with 40 mL of a linear NaCl gradient (0–0.1 M) in buffers S at 0.2 mL/min flow rate. The protein composition and purity of chromatography fractions and enzyme preparations were checked with SDS-PAGE. The occurrence of degraded or variant molecular forms was investigated also with zymography following native or SDS-PAGE as described [22,23]. The enzyme preparations were stabilized with the addition of 50 g/mL bovine serum albumin, and they were stored at –20 °C.

2.3. Measurement and calculation of enzyme activity

The measurements were performed in 1.0 mL final volume, at 30 °C in an enzyme assay buffer (50 mM Tris-HCl (pH 8.0), 10 mM CaCl₂, 0.1 M NaCl and 50 µg/mL bovine serum albumin) at 5–30 nM enzyme concentration. The activities on fluorometric substrates were measured at 380 nm excitation and 460 nm emission wavelengths (substrates with AMC chromophore), or at 335 nm excitation and 410 nm emission wavelengths (substrate with 4-methoxy-naphthylamide chromophore), or at 340 nm excitation and 495 nm emission wavelengths (substrates with DabcyL quencher and Edans fluorophore groups). The activities on photometric substrates with p-nitroanilide leaving group were measured at 410 nm light, whereas the 2-furylacryloyl group blocked substrates and the substrate with thiobenzyl leaving group were measured at 324 nm light. In the case of the latter, the enzyme assay

buffer was supplemented with 25 mM 4,4-dithiodipyridine, an SH reagent. The reactions were started with the addition of the appropriate substrate. For the calculation of the activities on substrates with DabcyL quencher and Edans fluorophore the same procedure was applied as described in Ref. 18. The determination of the molar fluorescence gave 4.36×10^{11} ΔCPS/M for the hepatitis A protease C substrate, which is essentially the same value as that of the substrate of PrtA [18]. The activities on substrates containing AMC chromophore were calculated with 1.2×10^{13} ΔCPS/M molar fluorescence, which was determined with AMC calibration. In the case of the substrate with pNA chromophore the $\epsilon = 8100 \text{ M}^{-1} \text{ cm}^{-1}$ absorption coefficient was used in the calculations.

2.4. Measuring the effect of inhibitors and metal ions

20 nM enzyme was incubated with inhibitors at room temperature for 20 min in the assay buffer (in the case of EDTA, Ca²⁺ ion was omitted from the assay buffer) before measuring the remaining activity on Boc-Val-Leu-Lys-AMC substrate (at 40 µM final concentration, two orders of magnitude below K_M). As a control, protease B was incubated under the same conditions, but without the presence of inhibitor. To test the effect of metal ions, they were added to the 1,10-phenanthroline or EDTA inhibited enzyme and incubated for 5.0 min prior to activity measurement. The activities were calculated from the initial substrate hydrolysis rates (the first, linear part of reaction curves) where it is proportional to k_{cat}/K_M (because of the substrate concentration well below K_M).

2.5. Determination of the pH profile of activity

The activity was measured at 40 µM substrate (Boc-Val-Leu-Lys-AMC) and 50 nM enzyme concentration (as above), in the following buffer solutions: sodium acetate (pH 4.5, 5.0, 5.5), MES-HCl (pH 6.0, 6.5), MOPS-HCl (pH 7.0, 7.5), HEPES-HCl (pH 8.0), Tris-HCl (pH 8.5, 9.0), CAPS-HCl (pH 10.0 and 11). The concentration of the buffer systems was 50 mM containing 50 µg/mL bovine serum albumin 10 mM CaCl₂ and 0.1 M NaCl. The enzyme activities were calculated as above. Data points were fitted with Origin 5.0 software using the following equation:

$$k_{\text{cat}}/K_M = L_1 \left(\frac{1}{1 + \frac{10^{-\text{pH}}}{10^{-\text{pK}_1}}} \right) \left(\frac{1}{1 + \frac{10^{-\text{pK}_2}}{10^{-\text{pH}}}} \right) + L_2 \left(\frac{1}{1 + \frac{10^{-\text{pH}}}{10^{-\text{pK}_3}}} \right) \left(\frac{1}{1 + \frac{10^{-\text{pK}_4}}{10^{-\text{pH}}}} \right) \quad (1)$$

Where L_1 and L_2 are amplitude factors, $(k_{\text{cat}}/K_M)_{\text{lim}1}$ and $(k_{\text{cat}}/K_M)_{\text{lim}2}$, respectively.

2.6. Analysis of substrate hydrolysis products

200 µmol DabcyL-Glu-Val-Tyr-Ala-Val-Glu-Ser-Edans or hepatitis A virus 3C protease substrate were incubated with 6.0 nM enzyme in 120 µL final volume of enzyme assay buffer (without bovine serum albumin—above) until at least 90% of the substrate was hydrolyzed (~200 min). The reaction products were separated on a Zorbax 300 SB C18 (250 × 4.6 mm) HPLC column and analyzed with electron-spray-ionization mass spectrometry as described [18].

3. Results and discussion

3.1. Purification procedure of protease B

We developed a short, two step procedure for the purification of protease B from *X. kozodoii* var. Morocco. The steps of purification are summarized in Table 1, and the procedure is described in the Materials and methods. We note that—despite the huge loss in total protein—there was a substantial increase in the total enzyme activity after the first step, similarly to what we had observed during the

Table 1
Summary of purification of protease B.

| Step | Vol. (ml) | Total protein (mg) | Spec. activity ($\text{Ms}^{-1} \text{mg}^{-1}$) | Total activity (Ms^{-1}) | Yield (%) |
|-------------------------|-----------|--------------------|--|-------------------------------------|-----------|
| Culture supernatant | 3000 | ~100 | 9.2×10^{-9} | 9.2×10^{-7} | 100 |
| QAE Seph. ion exchange | 35 | 7.8 | 3.3×10^{-6} | 2.6×10^{-5} | 283 |
| PEI silica ion exchange | 8.4 | 1.9 | 8.3×10^{-6} | 1.6×10^{-5} | 174 |

The activities were measured on hepatitis A virus protease C substrate (Dabcyl-Gly-Leu-Arg-Thr-Gln-Ser-Phe-Ser-Edans).

purification of *Photobacterium* PrtA [18]. We suppose the explanation is the same: during QAE ion exchange chromatography the preparation gets rid of an inhibitor, as in the case of PrtA. The second purification step on PAE Silica anion exchanger (see **Materials and methods**) eliminated all the contaminating proteins present in the fractions from the QAE Sepharose column. On testing the purity of the preparation with SDS-PAGE after the second step, one single band of a ~55 kDa protein was visible with coomassie staining, protease B (Fig. 1A). When we checked the preparations with the sensitive and non-specific zymographic method following SDS-PAGE or native PAGE, using two different substrates, we found one activity band, that of protease B (Fig. 1B and C). Therefore we concluded that our preparations were pure enough for enzymatic characterization since they did not contain detectable amount of contaminating protein(s) or protease(s). The single, sharp band of protease B also excluded both the degradation by (self) cleavage and the presence of alternative form(s). (PrtA peptidase of *Photobacterium* is produced in several variant forms [18,22].) The protease B preparations remained stable up to one year on storage at -20°C without signs of degradation or loss of activity. Therefore we supposed that no inactivation occurred during preparation either and so we considered our preparations pure and fully active during enzyme activity calculations.

3.2. Investigation of cleavage site preference of protease B

3.2.1. Testing the activity on short oligopeptide substrates

With the aim of finding good substrate(s) for enzymatic characterization we initially screened twenty relatively simple oligopeptide substrates containing chromophores as leaving group on the N-terminus of the scissile bond. Seventeen of these substrates, the

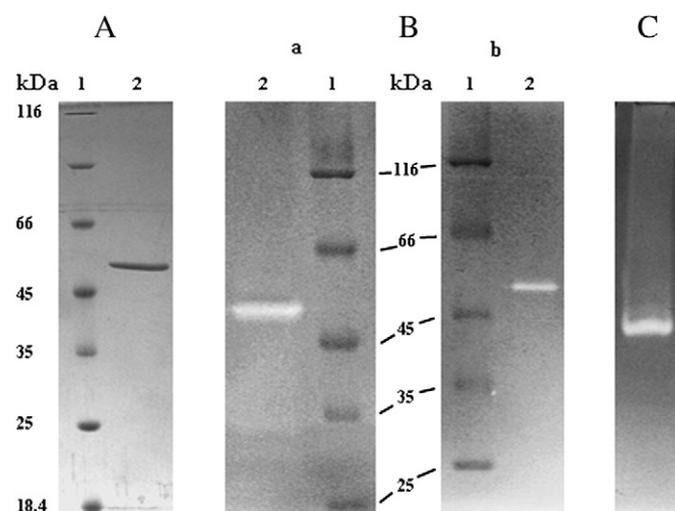


Fig. 1. Testing the purity of protease B preparations. A) SDS-PAGE with coomassie staining; B) zymography following SDS-PAGE using casein (a panel) and gelatin (b panel) as substrate; C) zymography following native-PAGE using gelatin as substrate.

fluorescent ones (containing aminomethyl-coumarine (AMC) or β -methoxy-naphthylamine leaving group) and the ones with SBzl chromophore, permitted sensitive measurement. A less sensitive, photometric substrate, DL-Val-Leu-Arg-pNA, was also tested because it was reported to be cleaved by the *X. nematophila* protease, protease II [25], which we supposed to be related to (or the same as) our enzyme (see **Introduction**). Because this substrate contains a basic residue at the P1 position (on the C-terminus of the scissile bond [27]), and serralyisins were found to prefer such residues in this position, we selected a variety of substrates that contained Arg, or Lys at the P1 site (which is N-terminal to the chromophore group in these substrates).

Being different only in the P1 residue (the Xaa site), the Succ-Ala-Ala-Pro-Xaa-AMC substrates made possible an investigation of the P1 residue discrimination of *Xenorhabdus* protease B. (Only the hydrolyzed ones of these substrates are shown in **Table 2**). The activity data clearly showed that the positively charged basic residues were the preferred ones because only the substrates containing Lys and Arg at this site (substrates #2 and 3) were cleaved, and those that contained aliphatic (Ala, Leu), aromatic (Phe) or negatively charged (Asp) amino acids were not (data not shown). The absence of basic amino acids might be a reason also why protease B did not cleave further two oligopeptide substrates, Fua-Leu-Gly-Pro-Ala and Fua-Ala-Leu-Val-Tyr (these substrates were hydrolyzed by unidentified enzyme(s) in the culture supernatant of *Xenorhabdus* strains [23]). This P1 residue preference of protease B was the same as that of protease II [25], which also cleaved substrate #12, but differed from our earlier findings on *Photobacterium* PrtA, which preferably cleaves in hydrophobic peptide segments, C-terminal to mostly polar but not charged P1 residues (therefore it did not cleave substrates #2 and 3 [18]). At the same time, the (P1) Lys and Arg specificity of protease B matched that of other serralyisins (serralyisin of *S. marcescens* [5], alkaline proteinase of *P. aeruginosa* [4–7] and ZapA of *Proteus mirabilis* [9,28]) which were found to cleave with the highest rate next to (P1) Arg when tested on a number of biological and synthetic substrates.

The performance of synthetic substrates is influenced by both their sequence and length. The number of substrates in our set was small for a detailed analysis of these effects but permitted few conclusions. The shortest substrates, N-benzoyl-Arg-AMC, N-CBZ-Lys-SBzl, L-Ser-AMC and His-Ser- β -naphthylamide (#4–7), are able to interact with the enzyme mainly through the P1 residues and much less by the flanking groups. Since the thioester bond in substrate #5 is much easier to hydrolyze than the amide bond in the other substrates, the inactivity of protease B on this substrate clearly demonstrates that the restriction of enzyme–substrate interactions mainly to those between merely the P1 substrate and the S1 enzyme sites is not sufficient for catalysis by this enzyme. The number of enzyme substrate interactions is extended in the other, easy-to-hydrolyze substrate, Succ-Ala-Ala-Pro-Phe-SBzl (#1 in **Table 2**), but protease B was inactive on this also, illustrating how much the bulky aromatic residue is unfavorable in the P1 position. The P1 residue is basic in substrate #9 and this substrate is longer than substrates #4–7 thus permits further interactions, including positions (P4)P3-P1. The inactivity of protease B on this substrate can probably be explained with unfavorable interactions of the bulky aromatic groups at sites P3 (benzyl) and P4 (benzoyl). This explanation is supported by the fact that the other simple substrates of the same length (substrates #10–14), which have various amino acid sequence but do not contain aromatic ring in the P1-P3 positions, were cleaved by protease B.

The specificity constant (k_{cat}/K_M) was similar on all of the hydrolyzed simple substrates (#2 and 3 and #10–15), the differences remained within one order of magnitude, not allowing any conclusion regarding the residue discrimination of protease B at the P2 and P3 substrate sites. However, the differences in K_M and k_{cat} values were larger, up to two and four orders of magnitude, respectively. The 10^{-4} to 10^{-3} s^{-1} values for k_{cat} generally show a

Table 2
The kinetic parameters of *Xenorhabdus* protease B on 19 synthetic substrates.

| | Substrate | | | | | k_{cat} (s^{-1}) | K_{M} (M) | $k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \text{M}^{-1}$) |
|----|-----------|----|----|----|-----|---|-------------------------------|--|
| | P4 | P3 | P2 | P1 | P1' | | | |
| 1 | | | | | | | No activity | |
| 2 | | | | | | $7.7 \pm 1.70 \times 10^{-3}$ | $1.1 \pm 0.4 \times 10^{-4}$ | 7.7×10^1 |
| 3 | | | | | | $1.4 \pm 0.46 \times 10^{-3}$ | $1.7 \pm 0.6 \times 10^{-5}$ | 8.2×10^1 |
| 4 | | | | | | | No activity | |
| 5 | | | | | | | No activity | |
| 6 | | | | | | | No activity | |
| 7 | | | | | | | No activity | |
| 8 | | | | | | | No activity | |
| 9 | | | | | | | No activity | |
| 10 | | | | | | $2.4 \pm 0.91 \times 10^{-4}$ | $4.0 \pm 1.5 \times 10^{-4}$ | 5.9×10^1 |
| 11 | | | | | | $6.7 \pm 0.22 \times 10^{-3}$ | $1.5 \pm 0.3 \times 10^{-4}$ | 4.5×10^1 |
| 12 | | | | | | $1.1 \pm 0.19 \times 10^0$ | $3.8 \pm 0.4 \times 10^{-3}$ | 2.9×10^2 |
| 13 | | | | | | $1.8 \pm 0.53 \times 10^{-3}$ | $8.9 \pm 1.6 \times 10^{-5}$ | 2.0×10^1 |
| 14 | | | | | | $7.1 \pm 0.98 \times 10^{-3}$ | $6.5 \pm 1.6 \times 10^{-5}$ | 1.1×10^2 |
| 15 | | | | | | $5.7 \pm 0.37 \times 10^{-3}$ | $6.2 \pm 0.7 \times 10^{-5}$ | 9.2×10^1 |
| 16 | | | | | | | No activity | |
| 17 | | | | | | | No activity | |
| 18 | | | | | | $3.3 \pm 0.2 \times 10^{-1}$ | $3.3 \pm 1.0 \times 10^{-5}$ | 1.1×10^4 |
| | | | | | | $2.1 \pm 0.3 \times 10^{2a}$ | $9.0 \pm 0.2 \times 10^{-5a}$ | 2.3×10^{6a} |
| 19 | | | | | | $1.1 \pm 0.2 \times 10^0$ | $2.1 \pm 0.4 \times 10^{-5}$ | 5.1×10^4 |

The values (\pm standard deviation) are averages of three measurements on two different enzyme preparations. The arrow indicates the site of cleavage.

^a *Photorhabdus* PrtA activity from Ref. [18].

positioning of the scissile bond, which is not good enough for efficient catalysis. But substrate #12, exhibiting a relatively high k_{cat} value, was substantially better in this regard, which can probably be ascribed to the contribution of (P2) Leu and (P3) Val residues to substrate positioning. This might be seriously compromised in substrate #11 by the bulky tert-butoxycarbonyl group at the P4 position. The acceptance by protease B of non-polar aliphatic side chains at the P2 and P3 positions is a feature which was observed with most of the substrates of serralyisins [4–9,18,28].

3.2.2. Testing the activity on long oligopeptide substrates

Generally, a significant enhancement of proteolytic activity can be reached if the enzyme can bind the substrate also C-terminal to the scissile bond (sites P1'–P4'). We have observed this effect earlier on *Photorhabdus* PrtA activity [18]. Here we tested it using two longer, fluorescence-quenching-type substrates (#18 and #19—Table 2) that allow such extended enzyme–substrate interactions. Indeed, the specificity constant of protease B on substrate #18 increased almost a hundred-fold compared to the best of the simple substrates (substrate #12), through a hundred-fold improvement in K_{M} , indicative of a stronger substrate binding. The catalytic efficiency was also more than two orders of magnitude higher relative to those on the simple substrates except for the best simple substrate, #12, compared to which it was slightly lower. A possible explanation for this might be that substrate #18—unlike the others—did not contain Arg or Lys residue, found to be the needed ones in P1 site (see above). Substrate #19 eliminated this problem, too, but there was only a modest increase (less than one order of magnitude) in both the catalytic efficiency and in the specificity constant (relative to that on substrate #18). The latter is still two orders of magnitude less than this value of proteases on their best substrates (typically in the range of $10^6 \text{ s}^{-1} \text{ M}^{-1}$), including *Photorhabdus* PrtA on substrate #18 (Table 2). To confirm the significance of basic residue in substrate #19 we performed a mass spectrometric analysis of the reaction products. Unexpectedly, however, the result proved that the single bond hydrolyzed in this substrate was the Ser–Phe and not the Arg–Thr bond. Cleavage at Ser in P1 site has been observed so far in two rare cases among many reported cleavage sites of serralyisins: in α 1-antichymotrypsin by serralyisin [29] and in the antimicrobial peptide, LL-37, by ZapA [9]. We note that a preference for (P1) Ser is an interesting agreement with the occurrence of this residue in the P1 position of almost all of the known serralyisin inhibitors of prokaryotic

origin [3]. Unfortunately, we were unable to do cleavage site analysis on the hydrolysis products of substrate #18 because too many fragments formed from cleavages at probably three or more sites, i.e. protease B did not prefer any of the sites over the others in this substrate.

If no P-site – S-site interaction(s) dominate(s) in the alignment of a substrate to the active site of a protease then even subtle sequence differences can cause a large shift in the alignment and, concomitantly, in the site of cleavage. This type of enzyme–substrate interaction can produce a relaxed cleavage site preference typical for serralyisins, and might be the explanation for the difference in S1 site preference of protease B between the short and long substrates (substrates #1–7 and #18, 19, respectively). For example, although a cleavage of substrate #19 after Arg would mean the same alignment of P1 and P2 residues as in the case of substrate #12 it might be (energetically) unfavorable if the bulky DABCYL chromophore following Gly) would disrupt the binding at P3 and P4 sites. At the same time, the alignment with the observed cleavage site (P1 Ser) allows the binding of an aromatic P1' residue (Phe), which might be better energetically than the binding of the smaller and more polar Thr.

Although substrate #19 proved suboptimal for protease B we tested its enzyme selectivity using two general digestive enzymes, trypsin and chymotrypsin. The high $k_{\text{cat}}/K_{\text{M}}$ values, 1.6×10^7 and $2.8 \times 10^6 \text{ s}^{-1} \text{ M}^{-1}$ that we obtained for pancreatic trypsin and chymotrypsin respectively, showed that substrate #19 is not selective. As a consequence, unlike substrate #18, which proved very selective and sensitive for the activity measurements of PrtA in biological samples [18], substrate #19 was not good enough for such measurements of protease B activity (in e.g. insect hemolymph) due to high background activity (data not shown).

3.2.3. Comparison of the activities of *Xenorhabdus* protease B and *Photorhabdus* PrtA

Finally we compared the kinetic parameters of PrtA and protease B on each other's best substrate (substrates #18 and #19, respectively) to test how these two, supposedly very closely related serralyisin-type enzymes discriminate between cleavage sites. When compared on substrate #18 their difference in the specificity constant was two orders of magnitude. Since the K_{M} values were the same, the difference arose almost entirely from the different catalytic efficiencies (k_{cat}), which indicated a better scissile bond positioning in PrtA. At the same time, the difference between the specificity constants was extremely

high if the enzymes were compared on substrate #19, as Photorhabdus PrtA did not cleave this substrate at all. Since the sequences of substrates #18 and 19 are very different, they provide substantially dissimilar enzyme–substrate interaction possibilities. While PrtA has earlier been proved to be rather selective for bonds in (P4–P4') peptide segments that contain hydrophobic amino acids [18] (which is missing in substrate #19), protease B seems to tolerate/prefer better a more polar environment of a cleavage site (as it is suggested by the sequence of the substrates in Table 2 that were hydrolyzed by protease B). We suppose this is why protease B could cleave substrate #18, and PrtA was not able to cleave the more polar substrate #19.

3.3. The pH profile of protease B activity

For a further characterization of Xenorhabdus protease B we investigated the pH profile of its activity on substrate #11. As seen in Fig. 2 the pH curve showed high activity between pH 7.0 and 8.0 which range is narrower than that of Photorhabdus PrtA [18]. The data points could be fitted the best with Eq. (1) (see Materials and methods), which indicated four ionization steps. The acidic values, pK_{a1} and pK_{a3} , were ~ 5.5 and ~ 6.5 , respectively. The former supposedly belongs to a glutamic acid and an associated water molecule in the catalytic site, as it has been shown in the case of serralyisins and some other Zn-metalloproteases [30–33], while the latter might show the influence of a histidine residue, three of which are around catalytic zinc. The basic pK_a values, $pK_{a2} \sim 8.5$ and $pK_{a4} \sim 11.1$, are more difficult to interpret. We suppose that the latter belongs to the Lys residue in the substrate, while the former might be related to the ionization state of Tyr216, as it has been proposed for serralyisin [32].

3.4. The effect of inhibitors and metal ions on protease B activity

3.4.1. The inhibitor profile of protease B activity

The inhibitor sensitivity (Table 3A) clearly proved that Xenorhabdus protease B is a metallo- and not a serine-proteinase, as it was inhibited by the metal ion complex forming compound, 1,10-phenanthroline, but not by PMSF, a specific reagent of active serine residue, and by SBTI, an inhibitor of trypsin-like serine proteases. The inhibition by Zn^{2+} (which has not been observed in the case of Photorhabdus PrtA or other serralyisins) was indicative also for the nature of the catalytic metal ion: it is known that some Zn-metalloproteases (e.g. aminopeptidases, thermolysins) are often inhibited by excess amount of Zn^{2+} because of the formation of a zinc monhydroxide bridge [31]. The notion that the catalytic ion is Zn is in agreement with the fact that this is the catalytic ion of most of the metalloproteases (and every serralyisin). The inhibition with thiol

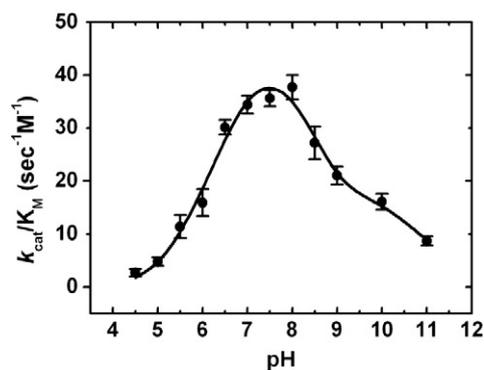


Fig. 2. The pH profile of Xenorhabdus protease B activity. The reaction rates were determined as the slope of the first, linear part of substrate hydrolysis curves which were measured on Z-Gly-Gly-Arg-AMC substrate at 4.0 μ M substrate and 50 nM enzyme concentration. The data are the average of three measurements. For the buffers used and the conditions of measurement see Materials and methods. Data points were fitted with Eq. 1, see Materials and methods.

Table 3A

The effect of various treatments on the activity of Xenorhabdus protease B.^a

| Addition | Final Concentration (mM) | Relative activity (%) ^b |
|---------------------|--------------------------|------------------------------------|
| EDTA | 1.0 | 83 \pm 10 |
| 1,10-phenanthroline | 1.0 | 13 \pm 11 |
| PMSF | 1.0 | 90 \pm 17 |
| SBTI | 10.0 | 92 \pm 12 |
| Thimerosal | 1.0 | 91 \pm 9 |
| DTT | 1.0 | 48 \pm 5 |
| Cystein | 1.0 | 87 \pm 10 |
| Zn^{2+} | 2.0 | 16 \pm 4 |
| Cu^{2+} | 2.0 | 144 \pm 17 |
| Co^{2+} | 2.0 | 224 \pm 36 |

^a The values are the average of at least three measurements on at least three enzyme preparations.

^b Compared to the activity of the enzyme which was not treated with inhibitor (control, at 20 nM enzyme, $4.25 \pm 0.38 \times 10^1 \text{ s}^{-1} \text{ M}^{-1}$).

reagents (DTT, Cys, thimerosal) showed similar tendency as in the case of Photorhabdus PrtA (DTT inhibition was the strongest while thimerosal did not inhibit [18]) but the effects were milder.

EDTA inhibited Xenorhabdus protease B weakly. The 20% inhibition measured at 1.0 mM concentration increased only to 40%, when EDTA treatment extended from 20 to 120 min or if excess amount (5.0 mM) of this chelator was applied. Although serralyisin-type enzymes sometimes show reduced sensitivity to EDTA inhibition, this weakness appears to be unusual. It is unexpected also when it is compared to the closely related enzyme, Photorhabdus PrtA, which is completely inhibited by EDTA [18]. This might show two features: i) the catalytic ion in protease B might be bound tighter or it is less accessible to EDTA than in related metalloproteases (see below), which can be inhibited much stronger or completely even with a milder EDTA treatment (see M10B subfamily enzymes in ref. 34); ii) the stability of protease B molecule against self digestion might probably be higher than that of e.g. Photorhabdus PrtA: While we did not see self-cleavage of protease B, PrtA becomes sensitive to self-cleavage upon EDTA treatment, which removes the catalytic Zn^{2+} somewhat slower than the structural Ca-ions causing a gradual auto-degradation of the structurally destabilized enzyme by its residual activity [35].

3.4.2. The effect of metal ions on protease B activity

The metal ions, Zn^{2+} , Cu^{2+} and Co^{2+} , which are usually able to restore the activity of metalloproteases after the removal of their catalytic ions, could also rescue the activity of protease B except for Zn^{2+} (which was also efficient in the case of Photorhabdus PrtA). Moreover, the addition of Zn^{2+} increased the partial inhibition by EDTA (Table 3B). We suppose that this inhibition was brought again about by the formation of zinc monhydroxide bridge (see above). The addition of Cu^{2+} and Co^{2+} caused “supernormal” activities (not

Table 3B

Rescue of activity with metal ion addition after inhibition with metal ion removal.^a

| Metal ion added (2.0 mM) | Relative activity (%) ^b | |
|--------------------------|------------------------------------|-------------------|
| | 1,10-phenanthroline ^a | EDTA ^a |
| - | 9 \pm 8 | 62 \pm 12 |
| Zn^{2+} | 10 \pm 10 | 18 \pm 10 |
| Cu^{2+} | 156 \pm 31 | 76 \pm 6 |
| Co^{2+} | 187 \pm 35 | 206 \pm 23 |
| Mn^{2+} | 84 \pm 19 | 94 \pm 18 |
| Ca^{2+} | 17 \pm 6 | - |
| Fe^{2+} | 15 \pm 4 | - |

^a The final concentration of the complex forming compound was 1.0 mM. The time of enzyme treatment was 20 and 120 min with 1,10-phenanthroline and EDTA, respectively.

^b Compared to the activity of the enzyme which was not treated with inhibitor (control, at 20 nM enzyme, $4.25 \pm 0.38 \times 10^1 \text{ s}^{-1} \text{ M}^{-1}$).

observed in Photorhabdus PrtA) which roughly corresponded to those that we found when we added Cu^{2+} and Co^{2+} ions without prior removal of the catalytic ion (Table 3A). Cu^{2+} and Co^{2+} are known not only to restore the activity of a number of metalloproteases (partially or completely [34]) but also to enhance it. For example, Co^{2+} increases the activity of alkaline proteinases of *P. aeruginosa* [4,5], a serralysin. Similarly, using a tripeptide mimic synthetic substrate, a 20- to 30-fold increase in specificity constant was found in serralysin upon replacement of Zn^{2+} with either Cu^{2+} or Co^{2+} [32]. The mechanism how these ions enhance the activity has not been studied in detail, thus it is a question how and why they can increase the specificity constant (mainly through increasing k_{cat}). It is known that Cu^{2+} and Co^{2+} can activate water molecule, similarly to Zn^{2+} , but we can only suppose that a specific structure of the active site is needed also which is available only in some of the metalloproteases. (As for instance, such a structure might be present in Xenorhabdus protease B but not in Photorhabdus PrtA.) Similarly, it might be a special structure in the active site that makes Zn^{2+} less sensitive to the removal by EDTA (a much weaker chelator of Zn^{2+} than 1,10-phenanthroline). For example, the local sequence and/or network of interactions either gives an unusually important structural role to Zn^{2+} (resulting in a stronger binding of it), or makes the access to Zn^{2+} unfavorable for EDTA.

3.4.3. Structural interpretation of EDTA and Zn^{2+} effect on protease B

To find explanation for the striking difference between the two, apparently closely related enzymes, Xenorhabdus protease B and Photorhabdus PrtA, in the effect of Zn^{2+} and EDTA addition comparison of active site structures would be needed. Due to the absence of these structures and the full length amino acid sequence of protease B we could compare the amino acid sequence of Xenorhab-

dus and Photorhabdus PrtA peptidases and analyzed the 3D structure of three such serralysins that are not so closely related to each other than the PrtA peptidases, on the following basis: First, if the amino acid sequences are so similar as those of protease B and the PrtA peptidases from *Xenorhabdus* in the N-terminal region where these enzymes are the least conserved then supposedly they are very similar or identical in the highly conserved active site region (Fig. 3). Second, since the 3D structure of the serralysins, which are not so closely related to each other (serralysin of *Serratia marcescens*, alkaline proteinase of *Pseudomonas aeruginosa* and PrtC peptidase of *Erwinia chrysanthemi*), were essentially the same in the active site region (r.m.s.d. 1.2–1.8 Å) it is reasonable to suppose that PrtA peptidases have the same structure.

The only sequential difference between Xenorhabdus and Photorhabdus PrtA in the active site is at position 191, where Asp is in the Xenorhabdus and Asn is in the Photorhabdus enzyme (Fig. 3). This position is equivalent to Glu270 in carboxypeptidase A such that the side chain carboxylate is “above” the catalytic Zn^{2+} in ~5.2 distance. This carboxylate group was found to stabilize the Zn-hydroxide bridge, which is a requirement to the inhibitory effect of Zn^{2+} [36]. We suppose that – in addition to the protease B (Xenorhabdus PrtA) specific Zn-ion inhibition of enzyme activity – a carboxylate group at position 191 of protease B might be responsible for the other difference between protease B and Photorhabdus PrtA, that the former is less sensitive to EDTA inhibition. In support of this assumption another 191Asp serralysin enzyme (an insecticidal protease, SMP6.1 from *Serratia* sp.) also exhibited reduced sensitivity to EDTA treatment [37]. (Out of 145 serralysin sequences in the MEROPS database that could reliably be aligned in the active site region only 21 contained amino acid other than Asn in position 191, and only twelve of these were Asp.)

| | (MEROPS ID/Mernum.) | 2 | 3 | 13 | 14 | 15 | 16 | 17 |
|---------------------------------------|---------------------|----------------------|---|------------------|--------|--------------|-------|----------------|
| Serralysin <i>Serratia</i> sp. | (MER001096) | AATTG--YDAVDD-LLHYHE | | YGTQAYAFLPNTI | WQGD | LGGQTWY | NVNQS | --VKHPATEDY |
| Protease C <i>E. chrysanthemi</i> | (MER001099) | ANTS-SAYNSVYD-FLRYHD | | YGTQAYAYYPGNYQGA | --- | GSSWY | NKQSN | --IRNPGSEEEY |
| AprA <i>Pseu. aeruginosa</i> | (MER005068) | GPSS-S-YGLINS-FAHQYD | | --GAFAFAYL | PFDP | MPGSH | --KGS | SWLYLINSYQVNTT |
| PrtA <i>P. luminescens</i> Brecon | | SEDVSGSEKANELLK | | | | | | |
| PrtA <i>P. luminescens</i> (TT01) | (MER029131/#909040) | EDVSGSEKANELLKWLQAYI | | --SYAFAYL | PTPEKT | -Q-V-G-TWYN | AKSRT | FLNNDIDVNGY |
| PrtA <i>P. luminescens</i> (W14) | (MER029131) | KDVSSEKANELLKWLQAYI | | --SYAFAYL | PRPEKT | -Q-L-G-TWYN | AKSRT | FSNNDIDVNGY |
| PrtA <i>P. temperata</i> K122 | (MER029131/#029132) | SELIGSAKANELQTQLQAYV | | --SYAFAYL | PSPEK | -Q-S-G-TWYN | LKSRT | FSENDIGVNGY |
| PrtA <i>P. temperata</i> C1 | (MER029131/#090941) | SELIGSAKANELQTQLQAYV | | --SYAFAYL | PSPEK | -Q-S-G-TWYN | LKSHT | FSENDIGVNGY |
| PrtA <i>P. asymbiotica</i> | (MER029131/#188082) | EEVSGSEKANELLKWLQAYI | | --SYAFAYL | PRPEKT | -Q-L-G-TWYN | AKSRT | FTNNDIDVNGY |
| PrtA <i>Photorhabdus</i> sp. Az29 | (MER029131/#090889) | SELIGSAKANELQTQLQAYV | | --SYAFAYL | PSPEK | -Q-S-G-TWYN | LKSRT | FSENDIGVNGY |
| PrtA <i>X. nematophila</i> | (MER029131/#090977) | VGLSDSHSAQDVNALLTAYV | | --DYAFANL | PQGGK | --M-V-Y-TWYN | AKSHT | FVDNDIDVNGY |
| PrtA <i>X. bovienii</i> | (MER029131/#200534) | VGLSDSKSVQDVNALLTAYV | | --DYAFATL | PKGQN | --T-T-Y-SWY | AESRT | FIDNDIDVNGY |
| Protease B <i>X. kozodoii</i> Morocco | | LSDSSVYQDVNSLLK | | | | | | |

| | (MEROPS ID/Mernum.) | 17 | 18 | 19 | 20 | 21 | 22 |
|-----------------------------------|---------------------|--------|--------|-------|--------|--------|---------------------------|
| Serralysin <i>Serratia</i> sp. | (MER001096) | RQTFTH | EIGH | ALGL | SHPG | DYNAGE | GN-PTYND-VTYAEDTRQFSLMSY |
| Protease C <i>E. chrysanthemi</i> | (MER001099) | RQTFTH | EIGH | ALGL | AHPG | EYNAGE | GED-PSYND-AVYAEDSYQFSIMSY |
| AprA <i>Pseu. aeruginosa</i> | (MER005068) | RQTLTH | EIGH | VGLS | HDPG | DYNAGE | GN-PTYRD-ATYAQDTRGYSVMSY |
| PrtA <i>P. luminescens</i> (TT01) | (MER029131/#909040) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DKVSP | PTYKNSATYFEDSRAYTVMSY |
| PrtA <i>P. luminescens</i> (W14) | (MER029131) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DVEVSP | PTYKNSATYFEDSRAYTVMSY |
| PrtA <i>P. temperata</i> (K122) | (MER029131/#029132) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DKKER | PTYKNSATYFEDSRAYTVMSY |
| PrtA <i>P. temperata</i> (C1) | (MER029131/#090941) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DKKER | PTYKNSATYFEDSRAYTVMSY |
| PrtA <i>P. asymbiotica</i> | (MER029131/#188082) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DKVSP | PTYKNSATYFEDSRAYTVMSY |
| Photorhabdus sp. AZ29 | (MER029131/#090889) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DKKER | PTYKNSATYFEDSRAYTVMSY |
| PrtA <i>X. nematophila</i> | (MER029131/#090977) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DEIRPN | YINSAEYFEDCRAYTVMSY |
| PrtA <i>X. bovienii</i> | (MER029131/#200534) | RQTFTH | EIGHTL | GLQHP | ADYNAS | DAVRPT | YISVGEYFEDSRAYTVMSY |

Fig. 3. Amino acid sequence alignment of protease B, Xenorhabdus PrtA, several Photorhabdus PrtA enzymes and three other serralysins. Shown are part of the N-terminal region (positions 17–33) and the active site region (positions 130–227) of the catalytic domain. (Position numbering is according to that in serralysin.) Amino acids that constitute the active site (the S4–S4' positions) are in pale grey boxes. Boldface letters indicate conserved amino acids that are characteristic to serralysins. Arrow points to position 191. Striped bars and double arrows designate β -sheet segments and α -helices, respectively. (The N-terminal sequence of PrtA from *P. luminescens* Brecon and protease B from *X. kozodoii* Morocco are from ref. 22 and 23, respectively. To identify the substrate contacting enzyme positions (P4–P4') we compared the 3D structure of serralysin from *Serratia marcescens*, alkaline proteinase from *Pseudomonas aeruginosa* and PrtC from *Erwinia chrysanthemi*, PDB IDs: 1SMP, 1KAP and 1K7G, respectively [38–40].)

4. Conclusions

Taken together, the enzymatic properties of protease B, presented here, support our conclusion from the high similarity of its N-terminal sequence to Xenorhabdus PrtA and the identity of protease B and Photorhabdus PrtA in their target protein discrimination [23] that protease B belongs to serralysins and that it is a Xenorhabdus ortholog of Photorhabdus PrtA (a Xenorhabdus PrtA protease). However, the properties of the Xenorhabdus and Photorhabdus protease are dissimilar in some respects. For example, they seem to have different cleavage site preference and sensitivity to several inhibitors. This is surprising in the case of such close orthologs and considering the fact also that the two enzymes have the same target protein profile and that their producers, *Xenorhabdus* and *Photorhabdus*, are sister bacterium genera following a very similar life strategy (see Introduction). We suppose that small changes in the contacts of amino acids on the second shell of interactions around the catalytic ion can explain these differences. The substitution of Asn191 with Asp in the Xenorhabdus enzyme might be the most significant of these. As their dissimilar dynamics of production [18,23] is probably related to the differences in the role of these of these enzymes particularly in the symbiotic phase of infection, the different enzymatic properties of protease B (*Xenorhabdus* PrtA) and *Photorhabdus* PrtA might also reflect to some degree differences in their function during infection.

Acknowledgements

The authors acknowledge Katalin Kékesi (Proteomics Laboratory, Biology Institute, Eötvös Lorand University) for mass spectrometric analysis. Mustafa K. Massaoud had a Ph.D. fellowship from the Libyan Ministry of Education.

References

- [1] G. Dalhammar, H. Steiner, Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antimicrobial proteins in insects, *Eur. J. Biochem.* 139 (1984) 247–252.
- [2] F. Tonello, S. Morante, O. Rossetto, G. Schiavo, C. Montecucco, Tetanus and Botulism neurotoxins, a novel group of zinc-endopeptidases, in: K. Suzuki, J. Bond (Eds.), *Intracellular Protein Catabolism*, Plenum Press, New York, 1996, pp. 251–260.
- [3] N.D. Rawlings, A.J. Barret and A. Bateman, MEROPS: the peptidase database, *Nucleic Acid Research* 38 (2010), Database Issue, D227–D233.
- [4] K. Morihara, H. Tsuzuki, T. Oka, On the specificity of *Pseudomonas aeruginosa* alkaline proteinase with synthetic substrates, *Biochim. Biophys. Acta* 309 (1973) 414–429.
- [5] H. Maeda, K. Morihara, Serralysin and related bacterial proteinases, in: A.J. Barret (Ed.), *Methods in Enzymology*, 248, 1995, pp. 395–415.
- [6] D. Louis, J. Bernillon, J.M. Wallach, Specificity of *Pseudomonas aeruginosa* serralysin revisited, using biologically active peptides as substrates, *Biochim. Biophys. Acta* 1387 (1998) 378–386.
- [7] D. Louis, J. Bernillon, J.M. Wallach, Use of a 49-peptide library for a quantitative and qualitative determination of pseudomonas serralysin specificity, *Int. J. Biochem. Cell Biol.* 31 (1999) 1435–1441.
- [8] M.A.F. Anéas, F.C.V. Portaro, I. Lebrun, L. Juliano, M.S. Palma, B.L. Fernandes, ZapA, a possible virulence factor from *Proteus mirabilis* exhibits broad protease substrate specificity, *Brazilian Journal of Medical and Biological Research* 43 (2001) 1397–1403.
- [9] R. Belas, J. Manos, R. Suvanasthi, *Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides, *Infect. Immun.* 72 (2004) 5159–5167.
- [10] A. Molla, K. Matsumoto, I. Oyama, T. Katsuki, H. Maeda, Degradation of protease inhibitors, immunoglobulins and other serum proteins by Serratia protease and its toxicity to fibroblast in culture, *Infect. Immun.* 53 (1986) 522–529.
- [11] B.W. Senior, M. Alberchtsen, M.A. Kerr, *Proteus mirabilis* strains of diverse type have IgA protease activity, *J. Med. Microbiol.* 24 (1987) 175–180.
- [12] A. Molla, T. Kagimoto, H. Maeda, Cleavage of immunoglobulin G (IgG) and IgA around the hinge region by proteases from *Serratia Marcescens*, *Infect. Immun.* 56 (1988) 916–920.
- [13] A. Molla, S. Tanase, Y.M. Hong, H. Maeda, Interdomain cleavage of plasma fibronectin by zinc-metalloproteinase from *Serratia marcescens*, *Biochim. Biophys. Acta* 955 (1988) 77–85.
- [14] B.W. Senior, M. Alberchtsen, M.A. Kerr, A survey of IgA protease production among clinical isolates of *Proteaeae*, *J. Med. Microbiol.* 25 (1988) 27–31.
- [15] T. Oda, Y. Kojima, T. Akaike, S. Ijiri, A. Molla, H. Maeda, Inactivation of chemotactic activity of C5a by the serratial 56-kilodalton protease, *Infect. Immun.* 58 (1990) 1269–1272.
- [16] H. Tanaka, T. Yamamoto, Y. Shibuya, N. Nishino, S. Tanase, Y. Miyauchi, T. Kambara, Activation of human plasma prekallikrein by *Pseudomonas elastase* II. Kinetic analysis and identification of scissile bond of prekallikrein in the activation, *Biochim. Biophys. Acta* 1138 (1992) 243–250.
- [17] C. Wasiff, D. Cheek, R. Belas, Molecular analysis of a metalloprotease from *Proteus mirabilis*, *J. Bacteriol.* 177 (1995) 5790–5798.
- [18] J. Marokházi, N. Mihala, F. Hudecz, A. Fodor, L. Gráf, I. Venekei, Cleavage site analysis of a serralysin-like protease, PrtA, from an insect pathogen *Photorhabdus luminescens* and development of a highly sensitive and specific substrate, *FEBS J.* 274 (2007) 1946–1956.
- [19] N.E. Boemare, R.J. Akhurst, R.G. Mourant, DNA Relatedness between *Xenorhabdus* spp. (Enterobacteriaceae), Symbiotic Bacteria of Entomopathogenic Nematodes, and a Proposal To Transfer *Xenorhabdus luminescens* to a New Genus, *Photorhabdus* gen. nov. *Int. J. Syst. Bacteriol.* 43 (1993) 249–255.
- [20] S. Forst, B. Dowds, N. Boemare, E. Stackebrandt, *Xenorhabdus* and *Photorhabdus* spp.: Bugs That Kill Bugs, *Annu. Rev. Microbiol.* 51 (1997) 47–72.
- [21] S. Forst, D. Clarke, Bacteria-nematode symbiosis, in: R. Gaugler (Ed.), *Entomopathogenic Nematology*, CABI Publishing, Wallingford, 2002, pp. 57–77.
- [22] J. Marokházi, K. Lengyel, Sz. Pekár, G. Felföldi, A. Patthy, L. Gráf, A. Fodor and I. Venekei, Comparison of proteolytic activities produced by entomopathogenic *Photorhabdus* bacteria: Strain- and phase-dependent heterogeneity in composition and activity of four enzymes, *Appl. Environ. Microbiol.* 70 (2004) 7311–7320.
- [23] M.K. Massaoud, J. Marokházi, A. Fodor, I. Venekei, Proteolytic enzyme production of insect pathogen *Xenorhabdus* bacterium strains and characterization of an early secreted protease as a potential virulence factor, *Appl. Environ. Microbiol.* 76 (2010) 6901–6909.
- [24] J. Marokházi, Gy. Kóczán, F. Hudecz, L. Gráf, A. Fodor, I. Venekei, Enzymatic characterization with progress curve analysis of a collagen peptidase from an entomopathogenic bacterium, *Photorhabdus luminescens*, *Biochem. J.* 379 (2004) 633–640.
- [25] C. Caldas, A. Cherqui, A. Pereira, N. Simoes, Purification and characterization of an extracellular protease from *Xenorhabdus nematophila* involved in insect immunosuppression, *Appl. Environ. Microbiol.* 68 (2002) 1297–1304.
- [26] L. Gráf, A. Jancsó, L. Szilágyi, Gy. Hegyi, K. Pintér, G. Náray-Szabó, J. Hepp, K. Medzihradsky, W.J. Rutter, Electrostatic complementarity within the substrate-binding pocket of trypsin, *Proc. Natl. Acad. Sci. USA* 85 (1988) 4961–4965.
- [27] I. Schechter, A. Berger, On the size of the active site in proteases. I. Papain, *Biochem. Biophys. Res. Commun.* 27 (1967) 157–162.
- [28] B.L. Fernandes, M.A.F. Anéas, L. Juliano, M.S. Palma, I. Lebrun, F.C.V. Portaro, Development of an operational substrate for ZapA, a metalloprotease secreted by the bacterium *Proteus mirabilis*, *Brazilian Journal of Medical and Biological Research* 33 (2000) 765–770.
- [29] J. Potempa, W. Watorek, J. Travis, The inactivation of human plasma alpha 1-proteinase inhibitor by proteinases from *Staphylococcus aureus*, *J. Biol. Chem.* 261 (1986) 14330–14334.
- [30] W.L. Mock, J. Yao, Kinetic characterization of the serralysins: A divergent catalytic mechanism pertaining to astacin-type metalloproteases, *Biochemistry* 36 (1997) 4949–4958.
- [31] K.S. Larsen, D.S. Auld, Carboxypeptidase A: Mechanism of zinc inhibition, *Biochemistry* 28 (1989) 9620–9625.
- [32] H.I. Park, L.-J. Ming, Mechanistic studies of the astacin-like Serratia metalloprotease serralysin: highly active (>2000%) Co(II) and Cu(II) derivatives for further corroboration of a “metallotriad” mechanism, *J. Biol. Inorg. Chem.* 7 (2002) 600–610.
- [33] J.A. Sigman, S.R. Edwards, A. Pabon, M.J. Glucksman, A.J. Wolfson, pH dependence studies provide insight into the structure and mechanism of thimet oligopeptidase (EC 3.4.24.15), *FEBS Lett.* 545 (2003) 224–228.
- [34] A.J. Barrett, N.D. Rawlings, J.F. Woessner, *Handbook of proteolytic enzymes*, Academic Press, San Diego, London, Boston, New York, Sydney, Tokyo, Toronto, 1998.
- [35] D.J. Bowen, T.A. Rocheleau, C.K. Grutzmacher, L. Meslet, M. Valens, D. Marble, A. Dowling, R. french-Constant and M.A. Blight, Genetic and biochemical characterization of PrtA, an RTX-like metalloprotease from *Photorhabdus*, *Microbiology* 149 (2003) 1581–1591.
- [36] K.S. Larsen, D.S. Auld, Characterization of an inhibitory metal binding site in carboxypeptidase A, *Biochemistry* 30 (1991) 2613–2618.
- [37] Y. Zhang, D.D. Bak, H. Heid, K. Geider, Molecular characterization of a protease secreted by *Erwinia amylovora*, *J. Mol. Biol.* 289 (1999) 1239–1251.
- [38] U. Baumann, M. Bauer, S. Letoffe, P. Deleplaire, C. Wandersmann, Crystal structure of a complex between *Serratia marcescens* metallo-protease and an inhibitor from *Erwinia chrysanthemi*, *J. Mol. Biol.* 248 (1995) 653–661.
- [39] U. Baumann, S. Wu, K.M. Flaherty, D.B. McKay, Three dimensional structure of the alkaline proteinase of *Pseudomonas aeruginosa*: a two domain protein with a calcium binding parallel beta roll motif, *EMBO J.* 12 (1993) 3357–3364.
- [40] T. Hege, U. Baumann, Protease C of *Erwinia chrysanthemi*: the structure and role of amino acids Y228 and E189, *J. Mol. Biol.* 314 (2001) 187–193.