Propeptide of the metalloprotease of *Brevibacillus brevis* 7882 is a strong inhibitor of the mature enzyme

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Abstract A metalloprotease gene of *Brevibacillus brevis* (*npr*) was expressed in *Escherichia coli* in a soluble form as native Npr precursor. A significant fraction of the precursor was spontaneously processed, producing the N-terminal propeptide and the mature enzyme. A strong inhibition of the mature Npr by its own propeptide in the crude lysate was observed even in the absence of the covalent linkage between them. Pure precursor, propeptide and the mature Npr were isolated and kinetic parameters of the mature enzyme inhibition by the propeptide were determined. The inhibition is of the tight-binding competitive type with K_i 0.17 nM. Inhibition of metalloproteases from *Brevibacillus megaterium* and thermolysine by the heterologous propeptide of the Npr from *B. brevis* was much weaker or none.

Key words: Proenzyme; Tight-binding inhibition; *Brevibacillus brevis*

1. Introduction

Many proteases in various organisms are synthesized as inactive precursors, preproproteins or zymogens. Generally it is almost impossible to isolate a bacillar protease precursor from a natural strain, since it appears in the cultural medium as a processed mature form. When expressed in *E. coli* or other Gram-negative bacteria, these proteins are accumulated in insoluble inclusion bodies formed by non-active precursor, as found for subtilisin E [1], α-lytic protease from *Lysobacter enzymogenes* [2] and yeast carboxypeptidases Y [3], or they get rapidly processed producing mature form, as in the case of elastase from *P. aeruginosa* [4] and α-lytic protease from *L. enzymogenes* [2]. Therefore protease expression in a form of the non-active precursor is a challenging task. For that reason protease precursors and mechanisms of there activation were not thoroughly studied.

The presequences usually serve as signal peptides for a transport through the plasma membrane. A functional role of the propeptides is less clear. Several functions were proposed for the propeptides. The propeptide may be required for productive folding and/or maintaining the protease in an inactive state inside the cell, as well as for interaction with the transport machinery of the cell which is important for effective secretion of these proteins [5].

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Abbreviations: Dnp, dinitrophenyl moiety; FPLC, fast protein liquid chromatography; Npr, neutral protease (metalloendopeptidase); PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate

The role of protease prosequences is most extensively studied in the serine proteases: subtilisin E from *B. subtilis* [6,7], α-lytic protease from *L. enzymogenes* [8] and the vacuolar carboxypeptidase Y from yeast *S. cerevisiae* [3]. In all mentioned examples the propeptide assists the folding of the respective enzymes in vivo and in vitro, and blocks the enzymatic activity when covalently attached to the 'mature' moiety of the enzyme. In subtilisin E and α-lytic protease their propeptides appear as tight-binding inhibitors of mature enzymes in trans. A propeptide of carboxypeptidase Y has a low affinity for its mature enzyme in vitro. The propeptides of cystein proteases papain and cathepsin B are found to inhibit their respective mature enzymes [9,10]. In a cystein vacuolar protease cathepsin L, a propeptide was shown to be essential for folding [11]. A high affinity binding between propeptides and mature enzymes is shown for aspartic proteases – pepsin and cathepsin D [12], and for metallo-dependent proteases – mammalian carboxypeptidase A [13] and thermolysin from *B. thermoprotheolyticus* [5].

The extracellular metalloprotease *npr* gene of a thermophilic bacterium *B. brevis* encodes for a 528 amino acid preproenzyme including a mature protease of 304 residues and an extensive propeptide of 195 residues [14]. Here we present results on production of this protease, and on studying of the propeptide-mature enzyme interactions.

2. Materials and methods

2.1. Materials

The metalloendopeptidase from *B. megaterium* and *B. brevis* were purified as described previously [15,16]. The thermolysin with an activity of 5000 U mg^{-1} was purchased from Serva.

2.2. Bacterial strains and growth conditions

The full-length *npr* gene encoding for the preproenzyme was cloned in a pUC-derived plasmid vector under the control of a Pr promoter from the λ phage, supplemented with the C1857 thermosensitive repressor gene. The resulting plasmid was designated pCPS1 [17]. The plasmid was introduced into TG1 strain of *E. coli* [18].

The transformants were grown in the liquid LB medium with 200 mg l^{-1} ampicillin at 28°C. The overnight culture was used as a 3% inoculum for the generation of the final culture in the same medium at 250 rpm/28°C for 3 h. The thermoinduction was performed at 37°C for 2 h.

2.3. Protein purification

The cells were harvested by centrifugation, resuspended in buffer A (50 mM Tris-HCl pH 7.2, containing 1 mM $CaCl_2$) and sonicated on ice. Insoluble and soluble fractions were separated by centrifugation for 20 min at 3750×*g*. The soluble fraction of the crude lysate was loaded onto a 1.5×5 cm column with gramicidin-agarose equilibrated with buffer A. The column was subsequently washed with buffer A and buffer B (buffer A supplemented with 1 M NaCl and 25% isopropanol).

Further purification of the Npr precursor and its derivatives was performed by gel-filtration on 1×30 cm Superdex 75HR (FPLC). The

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column was equilibrated with buffer B and the elution was carried out by the same buffer at a flow rate of 0.5 ml min^{-1}. The fractions were analyzed by SDS-PAGE in a 12.5% gel and Western-blotting with specific rabbit antibodies against Npr purified from cultural medium of *B. brevis*.

2.4. Protein characterization and activity measurement

Samples for PAGE were precipitated with 7% TCA and dissolved in the Laemmli sample buffer [18]. The nitrocellulose filters were developed with a specific antiserum and alkaline phosphatase conjugate/substrate kit (BioRad).

The N-terminal sequencing was performed by gas-phase method with proteins blotted on the PVDF membrane.

The activity was assessed toward Dnp-Ala-Ala-Leu-Arg-NH_2 chromophorous substrate synthesized in our lab. One unit of the Npr enzymatic activity was defined as a quantity of the enzyme cleaving 1 μmol of the substrate per minute under the conditions shown above [19].

2.5. Measurement of the inhibition parameters

The kinetics of the propeptide inhibition of the mature Npr were assessed after preincubation of the propeptide with the mature enzyme for 5 min at 37°C. The enzyme was 1.35 mM, the propeptide was 0.625, 1.25 nM and 2.5 nM. Then the substrate was added in a final concentration of 0.25, 0.5, 0.75 and 1.0 nM. The proteolytic activity was quantified as described above. Reaction rates were calculated from the slope of the product versus time graphs, which were linear in all cases within the experimental error. To determine the inhibition type, all rate values were measured as a function of substrate and inhibitor concentrations and were simultaneously fit, with the program SCIENTIST (MicroMath Inc.), to the following equation set:

$$[E][S]/[ES] = K_m$$

$$[E][I]/[EI] = K_{i1}$$

$$[ES][I]/[ESI] = K_{i2}$$

$$[I]_0 = [I] + [EI] + [ESI]$$

$$[E]_0 = [E] + [ES] + [EI] + [ESI]$$

$$\text{rate} = k_{cat}[ES]$$

where [E], [S], [ES], [I], [EI] and [ESI] were the concentrations of the free enzyme, substrate, enzyme-substrate complex, free inhibitor, enzyme-inhibitor complex and enzyme-substrate-inhibitor complex, respectively, and k_{cat} was the catalytic constant.

The IC_{50}[1] values for heterologous enzymes were determined by addition of the *B. brevis* Npr propeptide to the concentration of 7.5, 12.5, 25, and 50 nM to 0.26 nM metalloproteinase from *B. megaterium*, or by addition of 50 nM *B. brevis* Npr propeptide to 0.15 nM thermolysin.

3. Results and discussion

3.1. Purification of products of the npr gene expression in E. coli

The plasmid pCPS1 encoding a full-length Npr from *B. brevis* was introduced to *E. coli* cells and expressed under the thermoinduction conditions. In contrast to other known protease precursors, like pro-Npr from *Aspergillus fumigatus* [20], prosubtilisin E [1] and the procarboxypeptidase Y [3] expressed in *E. coli* as inclusion bodies, almost all recombinant Npr of *B. brevis* was found in the water-soluble fraction when analyzed by Western-blotting (Fig. 1). Two discrete bands that presumably appeared to correspond to the Npr

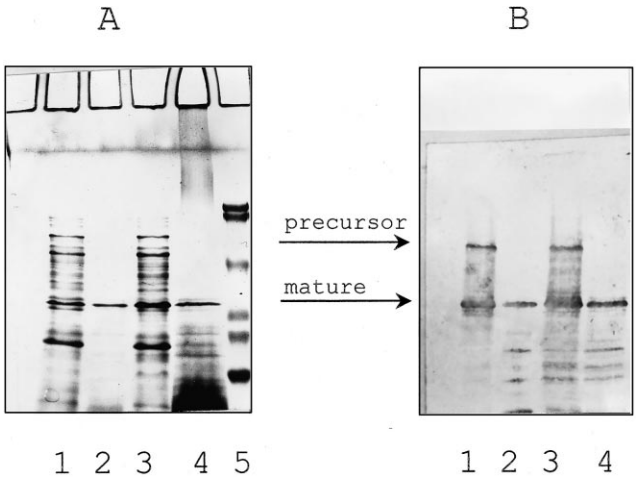

Fig. 1. Electrophoresis (A) and Western-blotting (B) of *E. coli* TG1 (pCPS1) crude lysates. Lanes 1 and 3, soluble fraction; lanes 2 and 4, insoluble fraction. Molecular mass standards are loaded to the slot lane 5 (A) as follows: 67, 45, 36, 29, 25, 18 and 12 kDa. The electrophoresis is performed in 12.5% PAAG in presence of 0.1% SDS. The slab is stained with CBB-R250. The Western-blotting is developed with rabbit anti-Npr antibodies, anti-rabbit conjugate with alkaline phosphatase and BioRad substrate kit.

precursor (60 kDa) and to the mature form (35 kDa) were found in the soluble fraction of cell lysate. The same molecular mass (35 kDa) was previously determined for the natural Npr purified from *B. brevis* [16].

An analysis of the insoluble fraction showed a presence of the mature Npr in the amount of about 3% compared to the soluble form, whereas no traces of the precursor. This observation did not allow a conclusion whether true inclusion bodies had been formed in the cells, or a small fraction of the mature enzyme had got associated with a cell debris.

A purification of the proenzyme and mature Npr was carried out using gramicidin-agarose affinity column as previously described for the isolation of this enzyme from *B. brevis* [16]. The host-cell proteins were easily eluted with buffer A. The mature enzyme was also partially displaced from the column by buffer A. The proenzyme and the residual mature Npr were eluted with buffer B (Fig. 2). Unexpected strong binding of the precursor may be explained by ionic interactions of the propeptide moiety with the positively charged amino groups of the gramicidin. Fractions containing essentially pure mature enzyme or a mixture of the mature enzyme, precursor and a peptide of 25 kDa were obtained (Fig. 2). A substantial difference of the molecular masses of these components allowed to separate them by gel-filtration using Superdex 75HR column (Fig. 2). Fractions containing pure mature enzyme, precursor and 25 kDa peptide respectively were obtained as assessed by PAGE with staining by Coomassie R-250 or Western-blotting. The fraction containing the mature Npr revealed an activity toward the synthetic substrate.

3.2. Identification of the Npr precursor components

The determined N-terminal sequence (Fig. 3) of the mature form coincided with the one previously determined for the mature natural enzyme from *B. brevis* [14]. This result indicated a correct positioning of the propeptide processing site,

[1] IC_{50} is defined as a concentration of the inhibitor reducing the rate of the enzymatic reaction to 50%.

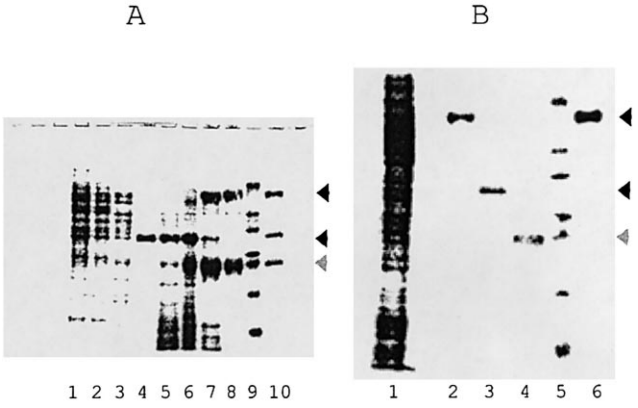

Fig. 2. Electrophoresis of the fractions collected after affinity chromatography over gramicidin-agarose (A) and gel-filtration (FPLC) over Superdex 75HR (B). A: Lane 1, soluble fraction of crude lysate of *E. coli* TG1 (pCPS1); lanes 2–4, fractions desorbed with the loading buffer A; lanes 5–8, fractions desorbed with the eluting buffer B; lane 9, molecular mass standards 67, 45, 36, 29, 25, 18 and 12 kDa; lane 10, mixed preparation, containing pure pro-Npr, mature Npr and the Npr propeptide from *B. brevis*. B: Lane 1, soluble fraction of the crude lysate of *E. coli* TG1 (pCPS1); lanes 2 and 6, pro-Npr; lane 3, mature Npr; lane 4, Npr propeptide; lane 5, molecular mass standards 67, 45, 36, 29, 25, 18 and 12 kDa. Pro-Npr is marked with a black arrow, mature Npr is marked with a grey arrow and the propeptide is marked with a light grey arrow.

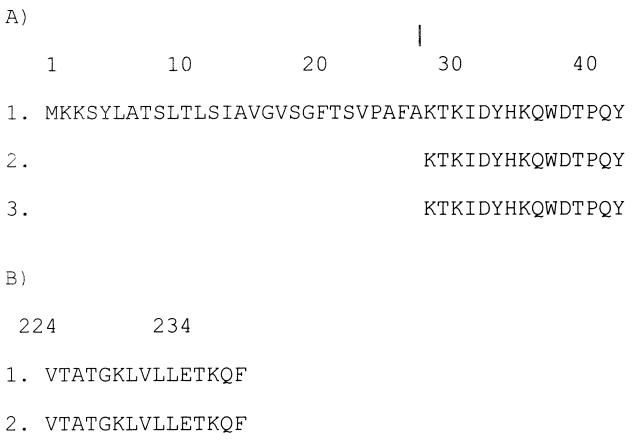

Fig. 3. N-terminal sequences determined by direct sequencing of the Npr derivatives. A: 1: preproenzyme of the Npr of *B. brevis* (predicted from the nucleotide sequence [17]); 2: purified precursor (60 kDa); 3: purified propeptide (25 kDa). B: 1: mature Npr of *B. brevis* (predicted from the nucleotide sequence [17]); 2: purified mature Npr (35 kDa).

undiffering from those in the natural host, even under abnormal conditions.

The N-terminal sequences of the putative precursor and the 25 kDa protein were identical (Fig. 3). This fact allowed to identify the 25 kDa component as the Npr propeptide, as well as to identify the signal peptide processing site at Ala-28-Lys-29. These experiments proved that the putative precursor was the true proenzyme.

3.3. The purified propeptide is a strong inhibitor of the mature enzyme

No proteolytic activity was found in the soluble fraction of *E. coli* (pCPS1) despite the fact that a correctly processed mature Npr was detected there by Western-blotting and amino terminal sequencing. This result can be explained by an inhibitory action of the propeptide produced in the course of the Npr precursor processing.

Heating the cell lysates for 15 min at 65°C led to an increase of the metalloendopeptidase activity and disappearance of the propeptide (Fig. 4). We proposed that presence of the propeptide could be responsible for the inhibition of the Npr. In this case an activation of the mature Npr at the high temperature might be mediated by either dissociation of the propeptide from the complex with the mature enzyme and/or denaturation and further degradation of the propeptide. A similar phenomenon was previously discussed elsewhere for the same Npr of *B. brevis* produced in *B. subtilis*: the proteolytic activity substantially increased after heating the cultural medium to 65°C [21]. The authors [21] suggested another explanation for this observation: a temperature-induced conformational transition of misfolded Npr to the highly active conformation.

Kinetic parameters of the mature enzyme inhibition by the Npr propeptide were examined using the purified components. The Npr K_m value for the oligopeptide substrate was determined to be 0.6 mM, whereas K_m of thermolysin and metalloendopeptidases from *B. megaterium* were 3.2 mM and 0.38 mM respectively [15]. The inhibitory effect of the propeptide was measured in steady-state conditions. Marked inhibition was observed when the propeptide was present at nearly stoichiometric concentrations with respect to Npr (Fig. 5). Therefore, the propeptide is a tight-binding inhibitor [22]. This fact was used in the kinetic analysis and a special model different from Michaelis–Menten's one was applied (see Section 2). The quality of the fit was virtually unaffected when parameter K_{m2}

Table 1
Inhibition of the mature enzymes by their own propeptides

Enzyme	Constant of inhibition (nM)	Type of inhibition	References
Subtilisin E	2000	competitive	[7]
Protease IV from papaya	860	n.d.	[10]
Thermolysin	6.0	mixed	[5]
Npr from *A. fumigatus*	3.0	n.d.	[20]
Carboxypeptidase A	2.0	competitive	[13]
Papain	1.89	n.d.	[10]
Subtilisin BPN′	1	competitive	[7]
Cathepsin B	0.4	n.d.	[9]
Npr from *B. brevis*	0.17	competitive	present work
α-Lytic protease	0.1	competitive	[8]

n.d., no data available.

was set to infinity, indicating that the inhibition was of a purely competitive type. The following parameter values were obtained: $K_m = 0.6 \pm 0.2$ mM, $K_{il} = 0.17 \pm 0.03$ nM, $k_{cat} = 3800 \pm 500$ min^{-1} (63 ± 9 s^{-1}). Therefore the propeptide of the Npr of *B. brevis* is one of the strongest propeptide inhibitors studied so far. Only the propeptide of the α-lytic protease has a lower K_i (Table 1). The competitive type of inhibition with the homologous propeptide is found for the majority of studied proteases (Table 1), though a mixed/non-competitive type was reported for thermolysine [5].

3.4. Enzyme specificity of the inhibition by the propeptide of the Npr from B. brevis

The inhibition by their own propeptides was previously shown for a number of Npr's from various sources. We compared an action of the *B. brevis* Npr propeptide upon heterologous metalloproteases. It turned out that the inhibitory effect of Npr propeptide was very specific. The IC_{50} for the Npr from *B. megaterium* is 16-fold higher than determined for the homologous enzyme (12.5 nM and 0.8 nM respectively). There was no inhibition of the thermolysin even in the presence of 50 nM concentration of *B. brevis* Npr propeptide.

Heterologous propeptide inhibition was studied for the chymotrypsin-like proteases SGPB from *S. griseus* and pancreatic elastase using the α-lytic protease propeptide [8], as well as the inhibition of the Npr from *B. stearothermophilus* and Npr 24.11 with the propeptide of the thermolysin [5]. Inhibition by the heterologous propeptides was always weaker or none.

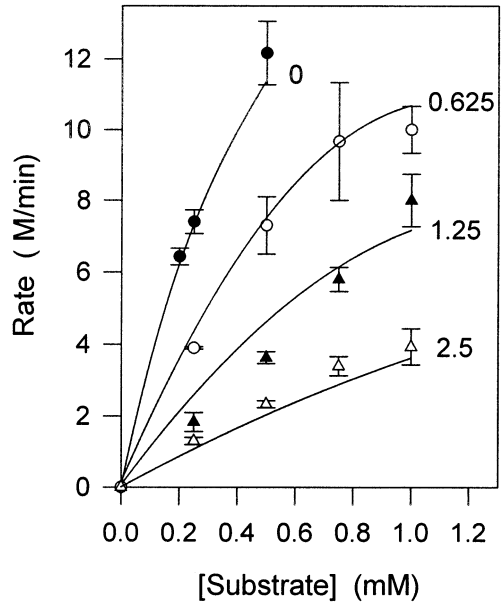

Fig. 5. A function of the Npr-catalyzed reaction rate from the substrate concentrations under varying concentrations of the propeptide inhibitor. Total propeptide concentrations (nM) are shown near the curves. The curves are calculated by SCIENTIST package for the competitive inhibition mechanism, using the parameter values, given in the text.

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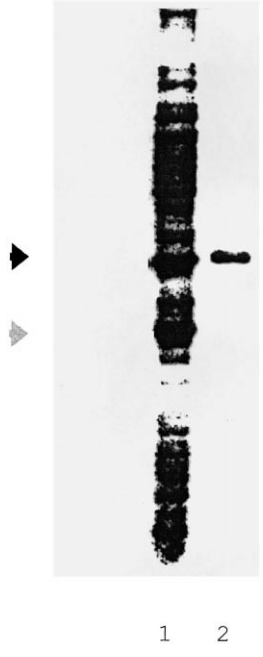

Fig. 4. Electrophoresis of the cell lysate treated by heating at 65°C. Lane 1, intact soluble fraction of *E. coli* TG1 (pCPS1) crude lysate; lane 2, soluble fraction of *E. coli* TG1 (pCPS1) crude lysate after heating for 15 min at 65°C. The mature Npr is shown with a grey arrow, the propeptide is shown with a light grey arrow.

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