Dissecting capture and twisting of aureolysin and pseudolysin: functional amino acids of the Dispase autolysis-inducing protein

David Fiebig1,2, Anita Anderl1, Sahra Al Djaizani1, Harald Kolmar2, Hans-Lothar Fuchsbauer1*

From the 1Department of Chemical Engineering and Biotechnology, University of Applied Sciences of Darmstadt, Stephanstraße 7, 64295 Darmstadt, Germany; 2Department of Chemistry, Technische Universität Darmstadt, Alarich-Weiss-Straße 4, 64287 Darmstadt, Germany.

*To whom correspondence should be addressed: Hans-Lothar Fuchsbauer: Department of Chemical Engineering and Biotechnology, University of Applied Sciences of Darmstadt, Stephanstraße 7, 64295 Darmstadt; Germany; hans-lothar.fuchsbauer@h-da.de; Tel. +49-6151-1638181.

Running title
Dispase autolysis-inducing protein

Abbreviations
AL, aureolysin; BL, bacillolysin; CTD, C-terminal domain; DAIP, Dispase autolysis-inducing protein; FTP, fungalysin/thermolysin propeptide; LasB, pseudolysin; NTD, N-terminal domain; TL, thermolysin.

Enzymes
Aureolysin, EC 3.4.24.29; bacillolysin (Dispase, Gentlyase), EC 3.4.24.28; pseudolysin (lasB, elastase), EC 3.4.24.4; thermolysin, EC 3.4.24.27; trypsin, EC 3.4.21.4.

Keywords
Aureolysin; autolysis; dispase autolysis-inducing protein; inhibition; pseudolysin; thermolysin
Abstract

The Dispase autolysis-inducing protein (DAIP) from *Streptomyces mobaraensis* attracts M4 metalloproteases, which results in inhibition and autolysis of bacillolysin (BL) and thermolysin (TL). The present study shows that aureolysin (AL) from *Staphylococcus aureus* and pseudolysin (LasB) from *Pseudomonas aeruginosa* are likewise impaired by DAIP. Complete inhibition occurred when DAIP significantly exceeded the amount of the target protease. At low DAIP concentrations, AL and BL performed autolysis, while LasB and TL degradation required reductants or detergents that break intramolecular disulfide bonds or change the protein structure. Site directed mutagenesis of DAIP and removal of an exposed protein loop either influenced binding or inhibition of AL and TL but had no effect on LasB and BL. The Y170A and Δ239-248 variants had completely lost affinity for TL and AL. The exchange of Asn-275 also impaired the interaction of DAIP with AL. In contrast, DAIP Phe-297 substitution abolished inhibition and autolysis of both target proteases but still allowed complex formation. Our results give rise to the conclusion that other, yet unknown DAIP amino acids inactivate LasB and BL. Obviously, various bacteria in the same habitat caused *Streptomyces mobaraensis* to continuously optimize DAIP in inactivating the tackling metalloproteases.
**Introduction**

*Staphylococcus aureus* and *Pseudomonas aeruginosa* are human pathogens causing severe infections such as pneumonia or cystic fibrosis [1,2]. Many other diseases are associated with both bacteria that may be especially fatal for hospitalized patients due to the wide distribution of antibiotic-resistant clinical strains [3]. A series of virulence factors have been recognized, among them metallopeptases of the thermolysin family M4, which are thought to play a major role in subverting the defence systems of the host [4]. Aureolysin (AL, EC 3.4.25.29, UniProt P81177, PDB 1BQB), the metalloprotease from *S. aureus*, contributes to immunoglobulin degradation by activation of the endogenous cysteine protease V8 [5], blood coagulation by prothrombin cleavage [6], septicemia or staphylococcal immune evasion by inactivation of the α1-antichymotrypsin, α1-proteinase and antimicrobial LL-37 peptide inhibitors [7-9]. It further modulates T and B lymphocytes [10], the complement system [11], the production of immunoglobulins [10], and the killing of phagocytosed bacterial cells [12]. Pseudomonal elastase (pseudolysin, LasB, EC 3.4.24.26, UniProt P14756, PDB 1EZM) is the major secreted protease of *P. aeruginosa* and an acknowledged virulence factor in animal infection models due to its ability of tissue destruction and escape from the host immune system [13,14]. Targets of LasB are many proteins such as elastin [15], collagen type III/IV [16], elastin [17], fibronectin [18], immunoglobulin G [19], pulmonary surfactant protein A [20], α1-proteinase inhibitor [21], and components of the complement system [22]. Thermolysin-like metallopeptases have been further characterized in other bacterial pathogens of the genera *Bacillus* [23,24], *Clostridium* [25], *Enterococcus* [26], *Helicobacter* [27], *Legionella* [28], *Serratia* [29], and *Vibrio* [30,31].

Metallopeptases of the thermolysin (M4) family exhibit a multi-domain structure, commonly consisting of fungalysin/thermolysin propeptide (FTP), PepSY propeptide, N-terminal β-sheet (NTD), and C-terminal α-helix domains (CTD) (Figure S1A) [32]. The structure of few variants such as the transglutaminase-activating metalloprotease is completed by an additional convertase domain following CTD [33]. While FTP/PepSY act as internal chaperone supporting the protease to adopt its functional structure, NTD and CTD accommodate the active site amino acids comprising the canonical HEXXH motif and a typically 20aa-remote glutamate [34]. FTP and PepSY inhibit NTD and CTD by
crosswise interaction during folding, thus directing the cleavable peptide bond between propeptide and mature domains to the active site such that auto-catalytical activation can take place. Mature thermolysin is stabilized by four bound calcium ions, referred to as Ca(1-4), which contribute to the extraordinary heat-resistance of the protease (Figure S1A) [35]. Interestingly, removal of a single calcium ion, Ca(3), in the N-terminal β-sheet domain NTD triggers autolysis of thermolysin [36]. In contrast, aureolysin is not enabled to bind Ca(3), and pseudolysin is only equipped with a single calcium ion in proximity to the active site [37,38]. Stability of pseudolysin is ensured by two disulphide bonds, which are absent in the structures of thermolysin and aureolysin. One pseudolysin cross-bridge seems to substitute the missing Ca(3) in the NTD while the other link the C-terminal peptide to the penultimate helix of the CTD (Figure S1B).

Thermolysin has been extensively used as zinc-dependent model protease for the design of site-directed inhibitors, especially due to its close relationship to human angiotensin-converting enzyme and carboxypeptidase A [39]. More than 200 x-ray structures are recorded in the PDB database shedding light on interaction between thermolysin and synthetic hydroxamates [40], carboxylates [41], phosphonamidates [42,43], or mercaptans [44]. Crystal structures of pseudolysin inhibited by a synthetic carboxylate and phosphoramidon, the natural phosphoramidate from *Streptomyces tanashiensis* [45], are reported (PDB 1U4G, 3DBK) as well. Moreover, new approaches were developed regarding the inhibition of pseudolysin as expedient strategy for treating bacterial infections caused by *P. aeruginosa* [46-48].

We have discovered the Dispase autolysis-inducing protein (DAIP, UniProt P84908, PDB 5FZP) from *Streptomyces mobaraensis* that binds M4 metalloproteases with high affinity [49,50]. The interaction of DAIP with bacilloysis (BL, Dispase, Gentlyase, EC 3.4.24.28, UniProt P29148, PDB 4GER) results in the immediate self-degradation of the bound metalloprotease while the more resistant thermolysin is only inhibited by DAIP [50]. DAIP adopts a regular seven-bladed beta-propeller, inappropriate of hydrolysing peptide bonds due to the absence of a fused catalytic domain or respective functional amino acids [51]. The rigid beta-propeller of DAIP is thought to modify the bound metalloprotease in such a way that autolysis-prone peptide bonds are exposed, and the active site is twisted [50]. Action of DAIP particularly affects the α-helical domain of metalloproteases as the
crystallized structure of a C-terminal thermolysin fragment in complex with DAIP (PDB 6FHP) suggests [50]. Here, we studied capture, inhibition and autolysis of aureolysin and pseudolysin to show versatility of DAIP in tackling clinical pathogens. Point mutations and removal of an exposed loop, derived from the DAIP thermolysin CTD complex, further illuminate the role of DAIP in attraction and twisting of M4 metalloproteases.

Materials and methods

Production of proteins used in the study

The Dispase autolysis-inducing protein (DAIP), bacillolysin (BL), and non-functional E138A-BL were produced in E. coli as described [50,51]. The same procedures, resulting in highly purified BL and E138A-BL, were applied to prepare thermolysin (TL), non-functional E143A-TL, and non-functional E141A-pseudolysin (E141A-LasB) using codon-optimized synthetic genes (Genscript, New Jersey, USA). Proteolytically active pseudolysin was a generous gift from Dr. Jesko Köhnke from the Helmholtz Institute for Pharmaceutical Research of the Saarland (HIPS, Saarbrücken, Germany).

The production of aureolysin (AL) was performed according to the procedure of Nickerson et al. [52]. In brief, a codon-optimized gene (Genscript) encoding His<sub>6</sub>-proaureolysin in pET22b(+) (Merck, Darmstadt, Germany) was used to transform E. coli BL21(DE3)RIL (Agilent Technologies, Waldbronn, Germany). Upon culture in auto-induction medium (10 g/l pepton, 5 g/l yeast extract, 10 g/l NaCl, 0.45 g/l glucose, 4.5 g/l glycerol, 1.2 g/l lactose, 22 mM KH<sub>2</sub>PO<sub>4</sub>, 17.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.8 mM MgSO<sub>4</sub>) for 22 h at 28 °C, the supernatant of 6 l disrupted cells in 50 mM phosphate, 500 mM NaCl, and 6 M urea pH 7.4 was applied onto a 5 ml His Trap HP column (GE Healthcare, Darmstadt, Germany) using a flow rate of 5 ml/min. Protein folding, self-activation and elution of active AL was then induced at flow rates of 3 ml/min by linear decreasing urea from 6 M to 1 M (50 min) and from 1 M to 0 M (15 min). The combined AL fractions were concentrated by acetone precipitation (4 °C, 2.5 volumes of -20 °C solvent) and Fractogel EMD TMAE (bed volume of 3 ml, Merck) binding in 20 mM 5 mM CaCl<sub>2</sub> and Tris/HCl pH 7.5 containing 0 M (AL binding) or 1 M NaCl (AL elution).

Modification of DAIP was carried out by size overlap extension PCR following standard protocols.
Protease assays

The activity of the metalloproteases was determined according the method of Weimer et al. [53]. In brief, intrinsically quenched dabsFans (39.2 µM) in 2 mM CaCl₂ and 50 mM Tris/HCl pH 7.5 was hydrolyzed by 1.2 µM metalloprotease at 30 °C (final volume of 200 µl). Fluorescence of cleaved Phe-EDANS was continuously monitored at 520 nm (λ_exc of 340 nm).

Protease degradation was determined according to Fiebig et al. [50] by continuous increase in fluorescence at 580 nm (λ_exc of 485 nm) upon addition of 2-10 µM DAIP to a mixture of 0.5-8 µM metalloprotease and SYPRO orange (twofold concentration, S5692, Merck-Sigma-Aldrich) in 2 mM CaCl₂ and 50 mM Tris/HCl pH 7.5 at 30 °C. The mixtures of nonfunctional proteases additionally contained 0.08-8 µM bovine trypsin.

CD spectroscopy

Circular dichroism spectroscopy was carried out with 6 µM DAIP, 6 µM E138A-bacillolysin or 6 µM thermolysin in 5 mM MOPS, 2 mM CaCl₂ pH 6.5 using Jasco j-815 CD spectrometer (Jasco, Pfungstadt, Germany). Each sample was multiply analyzed at 195-260 nm, and averaged ellipticity was plotted against the wavelength.

Size exclusion chromatography

A mixture of 10 µl 26.7 µM DAIP and 10 µL 26.7 µM metalloprotease in 2 mM CaCl₂, 300 mM NaCl, and 50 mM Tris/HCl pH 8.0 was separated by a 2.4 ml Superdex 200 3.2/300 increase column (GE Healthcare) at flow rates of 30 µl/min. The single proteins were used as controls.

Isothermal titration calorimetry

Aliquots (2 µl) of 100-200 µM DAIP were titrated to a stirred solution of 200 µl 10-20 µM metalloprotease in 10 mM CaCl₂, 300 mM NaCl, and 50 mM Tris/HCl pH 8.0 at 30 °C using MicroCal PEAQ-ITC (Malvern Instruments, Malvern, UK). The thermodynamic data were calculated by the internal software via peak integration and data fitting.
Other procedures

Determination of protein content by bicinchoninic assay and protein melting points was performed as described previously [50,51].

Results and discussion

Production of the proteins

The crystal structure of DAIP in complex with the C-terminal thermolysin fragment [50] was used to uncover functional amino acids that are involved in binding and twisting of M4 metalloproteases. Mutations were introduced by size-overlap extension PCR, and all DAIP variants were obtained in good yields (Table 1). Moreover, we underwent the attempt to prepare aureolysin (AL), non-functional E144A-AL, non-functional E141A-pseudolysin (E141A-LasB), thermolysin (TL) and E143A-TL in E. coli according to our published procedures for bacillolysin (BL) and E138A-BL [50] to further examine DAIP-mediated inactivation of metalloproteases from pathogens. The preceding signal peptides allowed secretion into the periplasmic space, and a C-terminal oligohistidine tag enabled the purification by IMAC. The preparation of non-functional proteases required the split production of pro-enzyme and non-functional mature domains [50] and, upon metal affinity chromatography, degradation of the pro-peptide by trypsin that did not impair the mature protease domains NTD and CTD in general. Other purification steps were acetone precipitation, Fractogel EMD TMAE, phenyl-Sepharose, Gly-\(d\)-Phe affinity or size exclusion chromatography. While highly purified E141A-LasB, TL, and E143A-TL were obtained in excellent yields (Figure S2), the amount of active AL remained low, only indicated by slow hydrolysis of the \(dabSF_{ans}\) dipeptide. A strong protein band at 60 kDa indicated presence of the unprocessed precursor molecule as result of misfolding and failed self-activation.

We then decided to reproduce the intra-cellular production procedure of Nickerson and colleagues comprising urea-mediated dissolution of His_{6} pro-AL and folding on immobilized metal affinity resins [52]. AL then adopts the functional structure by reduction of urea and detaches from the metal-bound pro-peptide by auto-activation. Although yields were slightly enhanced by this procedure, mature AL
was strongly contaminated by other proteins, most likely by large amounts of AL fragments (Figure S3). Any further purification was associated with severe loss of the protease, even revealing significance of Ca(3) and cysteine cross-bridges in TL and LasB stabilisation that are absent in AL. The respective IMAC fractions of AL were combined, concentrated by acetone precipitation and DEAE resin binding, and stored at -80 °C.

**Capture of M4 metalloproteases by DAIP**

We next studied the interaction of the produced metalloproteases with DAIP. Active and non-functional bacillolysin, BL or E138A-BL, were used as controls. Size exclusion chromatography indicated high affinity of DAIP for each metalloprotease by the reduced retention times of binary complexes and the complete disappearance of single proteins at equimolar concentrations (Figure 1A). The strong interaction of DAIP with the metalloproteases was confirmed by isothermal titration calorimetry (ITC) (Figure 2). While, like BL and E138A-BL, binding of E141A-LasB by DAIP was an endothermic process driven by an increase in entropy, the capture of recombinant TL enhanced enthalpy and entropy as was already shown for the commercial thermolysin [50]. Low dissociation constants of 5-50 nM revealed the detection limit of ITC. As reported earlier [50], DAIP binds two bacillolysin molecules under ITC conditions, which we believe that they form a piggyback complex, one protease molecule in complex with DAIP, the other in complex with the bound protease molecule. It should be further noted that the obtained amounts of active aureolysin were too small to perform similar SEC or ITC experiments.

The metalloproteases E138A-BL and TL were further studied by CD spectroscopy to observe structural modification by DAIP-mediated complex formation (Figure 1B). The DAIP ellipticity spectrum was characterized by a broad minimum at 215 nm as may be expected for propeller proteins shaped by β-strands (blue dots). The NTD (β-strands) and CTD domains (α-helices) of both metalloproteases caused two distinct minima around 210 nm and 220 nm (red dots) that almost disappeared in the equimolar complex with DAIP (green dots). A comparison of added individual and complex spectra (green and purple dots) only showed modified ellipticity between 195 nm and 198 nm, thus suggesting that few structural elements were changed in the DAIP complex of E138A-BL.
and TL. As β-propellers usually possess a rigid structure, changes in the metalloprotease structures appear to be more likely.

**DAIP-mediated inhibition and autolysis of M4 metalloproteases**

We then compared inhibition and autolysis of the metalloproteases as a function of DAIP concentration. The most remarkable result was that autolysis of bacillolysins (BL), the most susceptible metalloprotease, was completely inhibited by high amounts of DAIP (Figure 3A). In other words, at high concentrations, DAIP alters BL in such a way that autoproteolytic degradation cannot take place. Absence of proteolytic activity was either observed by the persistence of the BL electrophoresis band (Figure S4A) but, more importantly, by the absence of BL fragments, which increase SYPRO orange fluorescence [50]. A similar result was obtained with active AL, TL and LasB (Figure 3B-D, Figure S4B-D). However, it should be noted that inhibited TL still allowed dabSFans access to the active site, thus keeping residual activity of about 10 percent (Figure 3C). The inhibition of all studied metalloproteases substantiates our former conclusion [50] that DAIP most likely changes the structure near the active site of the proteolytic enzymes.

Complete reduction of BL activity prompted us to investigate the effect of preincubation on DAIP-mediated inhibition and autolysis. Preincubation of BL with DAIP for 30 min resulted in rapid decomposition of BL if the amount of DAIP was too small to inhibit the protease completely. Hence, the effective concentration allowing DAIP to inactivate BL by half (EC₅₀) was correspondingly reduced by one order of magnitude (Figure 3A). A similar result was obtained with aureolysin (Figure 3B). In contrast, as LasB and TL resisted autolysis within the analyzed time span, the EC₅₀ of DAIP were not affected by preincubation (Figure 3C/D). Former experiments have shown that detergents accelerate the decomposition of thermolysin by DAIP [50]. Similarly, the reduction of intramolecular disulfide bonds, lacking in BL, AL, and TL, caused pseudolysin to perform autolysis in the presence of DAIP (Figure S5).

**Functional amino acids of DAIP influencing capture and twisting of M4 metalloproteases**

The CTD fragment of thermolysin (from Gly-257 to Val-315) in complex with DAIP (PDB 6FHP) superposed well with full-length TL (PDB 2TLX), but the TL loop region WNGS and amino...
acids in close vicinity severely clash with DAIP (Figure 4A/B, red box) [50]. Importantly, access of protease substrate molecules seems to be possible as the TL active site is not covered by DAIP. The nearly unchanged structure of DAIP suggests that the reduced activity of TL is the result of active site twisting. Especially, Phe-297 of the clashing DAIP β-turn seems to collide with β-sheet 9 and α-helix 2 of thermolysin that accommodates the Zn$^{2+}$-binding Glu-143 and His-146 of the active site. (Figure 4C, Figure S6, Figure S7). Moreover, several DAIP amino acids in close contact with thermolysin or the thermolysin CTD fragment were considered excellent candidates for site-directed mutagenesis (Figure 4D).

Initially, binding sites for two Ca$^{2+}$ ions, which cross-link two DAIP molecules in the crystal (PDB 5FZP), were substituted by alanine. However, substitution of Glu-247, Asp-267, Glu-272, and Glu-343 had no effect on DAIP activity (Figure S6). Only AL inhibition was impaired by the exchange of Asp-322 (Table 1, Figure 4C/D). The result was consistent with earlier observations that transfer of proteinase-stabilizing calcium ions is not the reason for autolysis [49].

Next, the prominent loop between Glu-239 and Gly-248 of DAIP (Δ239-248) was removed, and the clashing Phe-297 within the rigid β-turn between Thr-295 and Gly-301 was exchanged for alanine (Figure 4A-C; Table 1). Moreover, amino acids putatively interacting with thermolysin via hydrogen bonds (Glu-99, Asn-101, Asp-122) or the protein backbone (Tyr-170, Asn-275) were likewise replaced (Figure 4D). Although melting points of the N275A, D322A and Δ239-248 proteins were markedly reduced, similar inhibition of LasB and BL suggested proper folding and unimpaired function (Table 1). Indeed, none of the introduced mutations could decisively weaken DAIP-mediated capture and inhibition in LasB and BL. On the contrary, BL seemed to perform autolysis faster in the presence of the F297A and Δ239-248 variants. In any case, the introduced mutations clearly discriminated between DAIP amino acids that either contribute to the attachment of aureolysin and thermolysin or to conformational changes, which affect the proteolytic activity. Absence of heat release, depicted by ITC, suggested that substitution of Tyr-170 completely abolishes the ability of DAIP to capture both metalloproteases. AL and TL inhibition was likewise disrupted by the replacement of Tyr-170 (Table 1, Figure 5). Furthermore, the Δ239-248 loop of DAIP seems to act like a “bumping post” or an orientation guide for the proper docking of the target metalloprotease. Erasing
the loop impeded the attachment of thermolysin to DAIP that considerably higher amounts of DAIP (more than two orders of magnitude) were required to reduce the proteolytic activity. Loop removal was still more effective in aureolysin as proteolytic activity remained unchanged in the presence of Δ^{239-248}-DAIP. Erasing DAIP activity for aureolysin by Asn-275 substitution further proved that specific amino acids at the DAIP surface might individually control the attachment of a target protease (Figure 5).

The amino acid Phe-297 within the prominent β-turn of DAIP works completely different from Tyr-170, Asn-275 and the loop between Glu-239 and Gly-248. Removal of the aromatic ring in F297A further allowed DAIP to attract thermolysin and aureolysin but abolished completely the inhibitory activity (Figure 5). The hydrolysis rate of TL was even considerably enhanced by the F297A variant, thus shedding light on the β-turn of DAIP. This β-turn protrudes the propeller surface like a thorn and includes between Gly-294 and Gly-301 (^{294}GTYFQAYG^{301}) several space-demanding residues. While the side-chain of Phe-297 impairs activity of thermolysin and aureolysin, other bulky DAIP amino acids such as Tyr-296, Tyr-300, Gln-298, and even the methyl group of Ala-299 may influence twisting and autolysis of other metalloproteases. The close vicinity to the β-turn and the involvement in AL inhibition further suggest that Asp-322 could contribute to the structural changes. In any case, amino acids at the upper surface of the rigid DAIP propeller have two different functions, both target molecule binding and structure transformation, that seem to be exerted independently but in a cooperative manner. Specific, even not yet identified, capture amino acids could provide for the destruction of a large number of harmful metalloproteases in the habitat of *S. mobaraensis*.

The study confirms the recently postulated three-step mechanism for the DAIP catalysis consisting of (1) capture of the M4 metalloproteases, (2) inhibition as result of twisting, and (3) autolysis [50]. Protein association and twisting are obviously separate processes, albeit functional amino acids influencing subsistence of bacillolysin and pseudolysin remain to be determined.

**Conclusions**

The Dispase autolysis-inducing protein (DAIP) is a powerful tool to control proteolytic activity of neutral M4 metalloproteases in the habitat of *Streptomyces mobaraensis*. As shown here, aureolysin
(AL) and pseudolysin (LasB) from the pathogenic bacteria *Staphylococcus aureus* and *Pseudomonas aeruginosa* are targets of DAIP as was already proven for bacillolysin (BL) from *Paenibacillus polymyxa* and thermolysin (TL) from *Bacillus thermoproteolyticus* rokko [50]. These enzymes are captured and twisted by DAIP in a way that their proteolytic activity is inhibited at low protease concentration. As the amount of target protease increases, unbound (active) protease molecules hydrolyze the autolysis-prone captives in complex with DAIP. Degradation of BL and AL occurs rapidly while TL and LasB need support by detergents or reductants that modify the structure and open the cysteine cross-bridges, respectively. Binding of the target proteases and twisting are mediated by different amino acids of DAIP as was shown here. A DAIP tyrosine, Tyr-170, seems to be essential for the attachment of thermolysin and aureolysin, possibly by π-π-stacking with Tyr-274 or Tyr-261, conserved in TL and AL (Figure S6, Figure S7A/B), while the DAIP loop between Glu-239 and Gly-248 may orientate the captured enzymes properly. The obvious involvement of Asn-275 and Asp-322 (possibly repulsing AL Asp-120 (Figure S7D)) in aureolysin binding or twisting suggests that other DAIP amino acids take part in complex formation and inhibition. Indeed, the functional amino acids, determined here, are unlikely involved in BL or LasB attraction. On the other hand, the exposed DAIP β-turn between Tyr-296 and Tyr-300 most likely acts like a spike that transforms the bound target proteases into their autolysis-prone structures. Substitution of Phe-297 (in collision with TL Tyr-93 or AL Tyr-90 (Figure S7C/D)) by alanine clearly abolished DAIP-mediated inhibition of AL and TL.

The continued binding of thermolysin by the F297A variant further showed that capture and inhibition are two independent steps of the DAIP inactivation mechanism. Unfortunately, the introduction of more than single point mutations destabilizes the DAIP structure, thus limiting the investigation of more amino acids or multiply substituted variants. Only structure of the respective metalloprotease in complex with DAIP will reveal the individual hot spots. However, such expedient approach is a challenge. Even the smallest traces of the active protease will degrade the DAIP-modified enzymes during the crystallization procedure.

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**Declarations of Interest**

The authors declare no conflict of interest.

**Author contribution**

DF, HLF designed research; DF, SAD performed research; DF, SAD, AA, HK, HLF analysed data; DF, HLF wrote the paper with comments from HK.

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Table 1. Stability and activity of DAIP variants.

| Variant | Yield \(^a\) (mg/l) | Melting point \(^b\) (°C) | Effective DAIP concentration \(^b\) EC\textsubscript{50} (mol/mol) | pseudolysin |
|---------|----------------------|---------------------|-------------------------------|-------------|
|         | bacillolysin         | thermolysin         | aureolysin \(^c\)              |             |
| Wildtype| 25                   | 56.5                | 1.18±0.06                      | 1.20±0.04   |
| E99A    | 19                   | 54.0                | 1.33±0.05                      | 1.10±0.12   |
| N101A   | 22                   | 55.4                | 0.90±0.04                      | 1.07±0.10   |
| D122A   | 14                   | 55.4                | 0.98±0.14                      | 1.73±0.14   |
| Y170A   | 11                   | 54.3                | 1.03±0.06 failure              | 11.38±1.33  |
| \(\Delta^{239-248}\) | 12 | 50.9                | 0.57±0.01 failure              | failure     |
| N275A   | 19                   | 48.7                | 0.85±0.06                      | 1.07±0.07   |
| F297A   | 17                   | 54.4                | 0.58±0.01 failure              | >100        |
| D322A   | 11                   | 50.2                | 1.63±0.01                      | >90         |

\(a\) Purified protein per liter culture. \(b\) Upon mixing various concentrations of DAIP with 50 nM BL, 5 nM TL, 4.5 nM AL or 6.3 nM LasB in 50 mM Tris, 5 mM CaCl\(_2\), pH 8.0, proteolytic activity was immediately measured using dabS\textsubscript{cat}. The data are the mean of three independent measurements. \(c\) The used aureolysin, was highly contaminated, most likely by AL fragments.
Figure legends

Figure 1. Complex formation of DAIP with neutral metalloproteases.

(A) Equimolar amounts of DAIP and the indicated protease in 50 mM Tris, 300 mM NaCl and 10 mM CaCl$_2$ were separated at pH 8.0 by Superdex 200 chromatography (green lines). DAIP (blue) and proteases (red) alone were used as controls. Inset: Coomassie-stained 12.5 % polyacrylamide gels of the proteins at top of the peaks. (B) CD spectra of equimolar amounts of DAIP and the indicated protease (green line). The purple line shows the added spectra of individual DAIP (blue) and the protease (red).

Figure 2. Capture of metalloproteases by DAIP.

Aliquots of DAIP (2 µL) were injected at pH 8.0 and 30 °C to stirred 200 µL solutions of 20 µM bacillolysin (A), 15 µM E138A bacillolysin (B), 20 µM E141 pseudolysin (C), and 20 µM thermolysin (D) in 50 mM Tris, 300 mM NaCl and 10 mM CaCl$_2$ using isothermal titration calorimetry (ITC). The released heat was integrated and fitted by the PEAK-ITC software that even calculates the shown thermodynamic data, binding sites, and dissociation constants.

Figure 3. Inhibition of metalloproteases by DAIP.

Mixtures of 50 nM bacillolysin (A), ~4.5 nM aureolysin (B), 5 nM thermolysin (C), and 6.5 nM pseudolysin (D) and the indicated molar ratio of DAIP in 50 mM Tris pH 7.5 and 2 mM CaCl$_2$ were preincubated (green line) for 30 min at 37 °C or not (blue line). Residual activity was determined in triplicates by hydrolysis of dabSFlans and increase in fluorescence at 520 nm ($\lambda_{\text{exc}}$ of 340 nm).

Figure 4. Prediction of functional amino acids of DAIP.

A, protein complex PDB 6FHP depicting DAIP (tan), the DAIP loop from Glu-239 to Gly-248 (golden), and thermolysin CTD from Gly-257 to Val-315 (cornflower blue). B, DAIP complex matched with thermolysin PDB 2TLX (sky blue). The collision between thermolysin $^{115}$WNGS$^{118}$ (cyan) and DAIP $^{295}$TYFQ$^{298}$ is red-framed. Notice the TL Ca$^{2+}$ positions opposite the proteins’ interface. C, Phe-297 of the DAIP $\beta$-turn clashing with TL $\beta$-sheet 9 and $\alpha$-helix 2 that includes Zn$^{2+}$-
binding Glu-143 and His-146 of the active site. D, other DAIP amino acids forming hydrogen bonds with CTD or interacting with the backbone of superposed thermolysin.

**Figure 5.** DAIP amino acids influencing metalloprotease binding and inhibition.

Hydrolysis of *dabSFans* in 2 mM CaCl$_2$ and 50 mM Tris/HCl pH 7.5 was continuously measured at 520 nm ($\lambda_{\text{exc}}$ of 340 nm) after addition of ~4.5 nM aureolysin (A) or 5 nM thermolysin (B) and DAIP in the indicated molar ratio. C, Superdex 200 chromatography of 15 µM thermolysin and 15 µM DAIP in 300 mM NaCl, 10 mM CaCl$_2$, and 50 mM Tris/HCl pH 7.5.
A

![Graph A](https://portlandpress.com/biochemj/article-pdf/doi/10.1042/BCJ20200407/887277/bcj-2020-0407.pdf)

B

![Graph B](https://portlandpress.com/biochemj/article-pdf/doi/10.1042/BCJ20200407/887277/bcj-2020-0407.pdf)

C

![Graph C](https://portlandpress.com/biochemj/article-pdf/doi/10.1042/BCJ20200407/887277/bcj-2020-0407.pdf)

D

![Graph D](https://portlandpress.com/biochemj/article-pdf/doi/10.1042/BCJ20200407/887277/bcj-2020-0407.pdf)

- **A**: Graph showing the relationship between DAIP:BL (mol/mol) and enzyme activity.
- **B**: Graph showing the relationship between DAIP:AL (mol/mol) and enzyme activity.
- **C**: Graph showing the relationship between DAIP:TL (mol/mol) and enzyme activity.
- **D**: Graph showing the relationship between DAIP:LasB (mol/mol) and enzyme activity.

- **Legend**:
  - Blue circles: 30 min pre-incubation
  - Green triangles: w/o pre-incubation

- **Data Points**:
  - DAIP:BL:
    - 0.11 ± 0.01
    - 1.12 ± 0.06
  - DAIP:AL:
    - 4.81 ± 0.18
    - 13.15 ± 0.48
  - DAIP:TL:
    - 1.65 ± 0.17
  - DAIP:LasB:
    - 0.63 ± 0.02
Aureolysin

Thermolysin

DAIP:Protease

Aureolysin

DAIP:protease

C

200

A280 / mAU

22

24

26

28

20

22

24

26

28

time / min

DAIP

thermolysin (TL)

TL + wt

TL + Y170A

TL + Δ239-248

TL + F297A