A calcium-stimulated serine peptidase from a true-branching cyanobacterium, *Westiellopsis ramosa* sp. nov.

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Abstract Unbranched heterocytous cyanobacteria produce a number of serine peptidases. We have characterized several peptidases in the cell-free extracts of a true-branched N₂-fixing cyanobacterium, *Westiellopsis ramosa* sp. nov. Upon substrate-gel zymography of intact filaments and heterocytes, five peptidase bands were resolved, whereas in vegetative cells, a single band was discernible. No band was detected in NO₃⁻/NH₄⁺-grown cultures suggesting that the peptidases were present under diazotrophic conditions with much of them confined to heterocytes. Using salt precipitation and chromatography, a caseinolytic peptidase, called Wrp49, was purified which also demonstrated fibrinolytic activity. In SDS-PAGE, the purified peptidase was resolved into 17 and 27 kDa fragments. The enzyme in its native state exhibited $M_r \approx 49$ kDa, and digested gelatin in a substrate gel at a corresponding position. The enzyme showed amidolytic activity on a plasmin specific substrate, D-Val-Leu-Lys *p*-nitroanilide. Moreover, a trypsin specific substrate, *N*-benzoyl-DL-Arg *p*-nitroanilide was hydrolyzed at an apparent $K_m = 0.195$ mM and $V_{max} = 5 \times 10^{-7}$ M s⁻¹. The enzyme was stable in a wide pH and temperature range. While Ca²⁺ stimulated the activity; phenylmethane

sulfonyl fluoride, leupeptin, EDTA and chelants were inhibitory. The activity of the EDTA-inactivated enzyme was completely restored upon adding Ca²⁺, suggesting that both compounds competed with each other in modulating the enzyme activity. The enzyme showed similarities with a Ca²⁺ stimulated subtilisin-like serine peptidase of *Anabaena variabilis* ATCC 29413, but also presented several unique features of metallopeptidases, such as the chelant's response. Moreover, the N-terminal sequence (MTVEN-LARTGVGPGWR) did not match with any of the known peptidases.

Keywords Ca²⁺-stimulation · Fibrinolytic activity · Nitrogen fixation · Serine peptidase · *Westiellopsis ramosa*

Introduction

Upon N-step down, several filamentous cyanobacteria fix molecular nitrogen in specialized cells called heterocytes. Gln is the principal product transported to adjoining vegetative cells. In exchange, vegetative cells reportedly transfer sucrose (Nürnberg et al. 2015) and Glu (Thomas et al. 1977) to heterocytes. Heterocytes also conspicuously accumulate the nitrogen-rich polymer cyanophycin in polar nodules, which is degraded by an enzyme cyanophycinase to β-aspartyl-arginine (Richter et al. 1999). This dipeptide is eventually transported to the adjacent vegetative cells in which another enzyme, isoaspartyl dipeptidase hydrolyzes it into Asp and Arg (Burnat et al. 2014).

Further studies revealed that apart from the above stated amino acids, Ser, Gly, Cys, Thr and Pro were actively produced inside heterocytes (Park et al. 2013). Since relative to vegetative cells, the amino acid biosynthesis genes in heterocytes were not up-regulated, and also the

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corresponding amino acid transcript size did not increase, it became intriguing to find the source of the amino acids. Apart from these amino acids, several hydrophobic amino acids, notably the Ala analogue α -amino isobutyric acid, were also found to be produced inside heterocytes of *Anabaena* sp. strain PCC 7120 (*Anabaena* 7120) in the absence of combined nitrogen (Pernil et al. 2015). Since N_2 -fixation or cyanophycin degradation cannot directly account for the accumulation of these amino acids, it was speculated that some hitherto unknown peptidase(s) hydrolyze ecto- or endogenous proteins under diazotrophic conditions and contribute to these many amino acids.

Numerous proteases and peptidases were detected that hydrolyze β -casein in the extracellular matrix of *Anabaena* 7120 (Oliveira et al. 2015) and *Nostoc punctiforme* PCC 73102 (*N. punctiforme* 73102) (Vilhauer et al. 2014). The *N. punctiforme* 73102 ecto-proteases exhibited sequence similarities with serralyisin-like metallopeptidases. Proteins that were identified in the exoproteome of *Anabaena* 7120 are the calcium-dependent peptidase, Alr1381 and prolyl endopeptidase, All2533 (Oliveira et al. 2015). Apart from these proteins from the external milieu, peptidases from within the cells are also known to hydrolyze casein and other substrates. In *Anabaena fertilissima* CCC 597 (*A. fertilissima* 597), a peptidase was shown to catalyze hydrolytic breakdown of casein and fibrinogen (Banerjee et al. 2013). Moreover, a calcium-dependent trypsin-like peptidase, a calcium-independent peptidase and a prolyl endopeptidase extracted from the cultures of *Anabaena variabilis* ATCC 29413 (*A. variabilis* 29413) have been shown to carry out proteolytic degradation of a number of natural and synthetic peptidase substrates (Lockau et al. 1988; Strohmeier et al. 1994; Maldener et al. 1991), which suggests that extensive proteolytic activity inside the cells of heterocytous cyanobacteria does occur. A calcium-dependent peptidase from *A. variabilis* 29413 (Lockau et al. 1988) which was also found in *Anabaena* 7120 was later characterized to be a subtilisin-like serine peptidase, PrcA, Ava_4009 (Baier et al. 1996). With the advent of a full genome sequence of *Anabaena* sp. 90, it became clear that at least two subtilisin-like peptidases, AcyA, ANA_C13603 and AcyG, ANA_C13594 are reportedly responsible for cleavage and/or cyclization of linear precursor peptides that lead to the biosynthesis of the cyclic peptides, cyanobactins (Leikoski et al. 2010).

In this communication, a Ca^{2+} -stimulated serine peptidase was purified and characterized from the whole trichomes of a true-branching cyanobacterium, *Westiellopsis ramosa* sp. nov., which was recently isolated from a freshwater habitat of Jabalpur, India (Singh et al. 2017).

Materials and methods

Chemicals

All reagents like Hammerstein's casein, gelatin, phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI), leupeptin, iodoacetamide, chymostatin, trans-epoxysuccinyl-L-leucylamide (4-guanidine) butane (E-64), elastatinal, antipain, benzamidine-HCl, antithrombin III, CM-Sepharose, Sephadex G-100, Blue Sepharose, acrylamide, *N*-*N'* methylene bisacrylamide, D-Val-Leu-Lys *p*-nitroanilide (D-VLKpNA), *N*-benzoyl-DL-Arg *p*-nitroanilide (BApNA), *N*-succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide (SA₂PPpNA), *N*-succinyl-Ala-Ala-Ala *p*-nitroanilide (SA₃pNA), *N*-(*p*-tosyl)-Gly-Pro-Arg *p*-nitroanilide (GPRpNA), bovine thrombin and fibrinogen and human plasmin were purchased from Sigma Life Sciences (USA). α_2 -Antiplasmin and iodoacetamide were purchased from Merck, Germany. Dipyriddy and 1,10-phenanthroline were of analytical grade and purchased from Hi-Media, India.

Culture conditions for *W. ramosa*

A *W. ramosa* culture was raised from a single colony grown on agar (1.5%)-solidified BG-11₀ medium (Singh et al. 2017). The cyanobacterium was routinely grown at 28 ± 2 °C in 200 mL of BG-11₀ liquid medium in 500 mL Erlenmeyer flasks placed under constant illumination of 50 μ mol photons $m^{-2}s^{-1}$ using cool fluorescent lamps (Philips, India) with regular shaking at 120 rpm on an orbital shaker (Remi, India). Purity of the culture was routinely checked by phase contrast and bright field microscopy, and by plating culture aliquots on Luria-Bertani agar (1.5%) medium and incubating them at 37 °C for 24 h. A culture of *W. ramosa* was deposited with the Microbial Culture Collection, National Centre for Cell Science, Pune, India, with the accession number MCC 3176.

For experiments using different nitrogen sources, the cultures were grown as above for at least eight generations each of 15–18 days in BG-11₀ medium (Rippka et al. 1979) supplemented with 10 mM of NaNO₃ or NH₄Cl. In parallel, cultures were grown in a “modified” BG-11₀ medium, in which the counter-ions (Na⁺ and Cl[−]) were compensated by adding 10 mM of NaCl as also in the regular BG-11₀ medium without added NaCl. The pH, except for the regular BG-11₀ medium, was adjusted to 7.5 with 5 mM of HEPES-NaOH. Fresh weight of the cultures was determined on glass microfibre filters (GF/C 4.25 cm diameter, Whatman). For dry weight determination, cells on filter paper were dried in a hot air oven at 105 ± 5 °C until

constant weight. Chlorophyll *a* content in culture aliquots was determined according to Mackinney (1941).

Protein extraction from whole filaments, heterocytes and vegetative cells

In batches, 28 L of regular BG-11₀ cultures were harvested by centrifugation (10,000×g) for 10 min and pellets were frozen at −21 °C for 4–6 h in glass lyophilization tubes using a lyophilizer (NSW, India). Unless otherwise stated, all steps were carried out at 4 °C. About 8 g dry weight of frozen pellet was suspended in 60 mL of extraction buffer containing 50 mM Tris–HCl, pH 8.0, 0.1% (v/v) Triton X-100 and 1 mM MgCl₂ and after vortexing on a vortex cyclomixer for 1 h, the culture was incubated overnight at 4 °C in standing position. Cell lysis was achieved by sonication for 4 cycles. After every 5 min sonication at 30 W amplitude, the sample was incubated for 5 min at 4 °C (Ultrasonic Homogenizer; Inkarp, USA). The suspension was centrifuged at 12,000×g for 20 min and the supernatant was collected as crude extract. Protein content was determined (Lowry et al. 1951) with bovine serum albumin as standard.

The method of Strohmeier et al. (1994) was adapted for the preparation of cell-specific extracts. From about 5 L cultures, cells equivalent to 1–2 g fresh weight were harvested by centrifugation (20 min at 4000×g) and suspended in 10 mL extraction buffer. The cell suspension was incubated for 30 min at 37 °C under constant shaking with 1 mg mL^{−1} of lysozyme. This was followed by vegetative cell lysis using sonication for 20 min at 4 °C at 7.0 watts amplitude. Microscopic examination revealed that approx. 95% of vegetative cells had lysed. Heterocytes were pelleted at 300×g for 20 min and the resultant supernatant after another 12,000×g centrifugation for 10 min was treated as broken vegetative cell extract. Pelleted heterocytes were washed twice and raised in 10 mL of above extraction buffer and an extract was prepared as described for the whole trichome.

Determination of caseinolytic activity

Caseinolytic activity was determined according to Ghosh et al. (2008) as follows: in 1 mL assay mixture, 200 µL 1% Hammerstein's casein in 50 mM Tris–HCl at pH 8.0 and cyanobacterial extract equivalent to 50–100 µg protein were incubated at 37 °C for 10 min. 600 µL of 5% (w/v) trichloroacetic acid was added, vortexed and allowed to stand for 30 min at room temperature. The mixture was centrifuged at 10,000×g for 10 min and the absorbance of released tyrosine was recorded at 280 nm (DU800, Beckman).

Enrichment of enzymes using chromatography

The proteins that were extracted from whole trichomes were precipitated by 30 and 70% (NH₄)₂SO₄ cuts. After centrifugation at 12,000×g for 12 min, the precipitant was resuspended in 50 mM Tris–HCl, pH 8.0 to a final protein concentration of 0.15 g mL^{−1}. For further purification, 15 mg protein was applied to a Sephadex G-100 column (1 × 25 cm, Sigma, USA) previously equilibrated with 50 mM Tris–HCl buffer, pH 8.0. Proteins were separated at a flow rate of 2 mL min^{−1}. Protein content and caseinolytic activity in each of the 2 mL fractions eluted in a BioLogic LP system (Bio-Rad, USA) was determined. The fractions with caseinolytic activity were combined and further purified using a CM-Sepharose column (1 × 5 cm, Sigma, USA). The total protein amount loaded was 12.5 mg. After an initial step of washing for 20 min using the buffer described above, a linear gradient of 0.5–2.0 M NaCl was applied to elute the bound proteins. Further purification of ca. 5 mg protein was achieved by affinity chromatography on a Blue Sepharose column (1 × 5 cm Sigma, USA) with a continuous gradient of 0–0.5 M NaCl after having washed the unbound proteins for 10 min. Proteins were concentrated using a protein concentrator of 10 kDa cut-off (Millipore, India).

Molecular weight determination using size-exclusion chromatography, native and SDS-PAGE and zymography

Purified enzyme equivalent to 0.25 mg protein and standard proteins (0.5 mg): bovine serum albumin (66.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa) and phosphorylase b (97.4 kDa) was applied to a Sephadex G-100 column (1 × 30 cm, Sigma, USA) and size-exclusion was performed according to Andrews (1965). Caseinolytic activity and protein content were determined in all fractions. Blue Native and SDS-PAGE was carried out according to Laemmli (1970) on 10% acrylamide gels.

Gelatin zymography was performed according to Kim et al. (1998). The resolving 10% SDS–polyacrylamide gel solution contained 0.1% gelatin. The protein extracts corresponding to 10 µg of protein were mixed with an equal volume of loading buffer containing 250 mM Tris–HCl at pH 6.8, 4% SDS, 20% glycerol, and 0.002% bromophenol blue. The gels were washed for 40 min at room temperature with 50 mM Tris–HCl at pH 7.0 containing 2.5% (v/v) Triton X-100, followed by distilled water for 30 min. Subsequent incubation at 37 °C for 12 h with developing buffer containing 10 mM Tris–HCl at pH 7.6, 200 mM NaCl and 0.02% (v/v) Triton X-100 followed by Coomassie Brilliant Blue R250 staining for 2 h and destaining produced clear bands against dark background.

Amino acid sequencing

The partial amino acid sequence was determined at the School of Life Sciences, Hyderabad University, Hyderabad, India, by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) of trypsin-digested purified protein. Homologous sequence search was done using the MASCOT search engine (http://www.matrixscience.com/cgi/search_form.pl?FORMVER=2&SEARCH=PMF). The N-terminal sequences were matched using UniProt/BLAST (www.uniprot.org/uniprot).

Determination of fibrinolytic activity

Hydrolytic cleavage of fibrin clot and resolution of the peptide products was carried out according to Datta et al. (1995). Briefly, fibrin clot (0.5 mL) was prepared in Eppendorf tubes by mixing 10 mL of 0.4% fibrinogen in 50 mM sodium phosphate buffer at pH 7.4 and 0.1 mL of thrombin (10 NIH U). 20 µg protein in 10 µL extract and 0.1 unit of plasmin was added onto the fibrin layer. After 4 h the dissolved and the hard portion of the clot were homogenized in 40 µL of a denaturing solution containing 10 M urea, 4% SDS, 4% mercaptoethanol and 0.002% bromophenol blue. After boiling for 5 min the peptides were resolved on a 10% SDS-PAGE gel.

Determination of amidase activity

Amidolytic activity was determined according to Agrawal et al. (2005) using synthetic chromogenic substrates. The reaction mixture contained: 50 µL substrate—6.5 mM D-VLKpNA, 2.5 mM SA₃pNA or 39 mM GPRpNA in assay buffer; 1.4 mM BApNA or 0.95 mM SA₂PPpNA in 7.5% (v/v) dimethylsulphoxide, and 50 µL extract equivalent to 40 µg protein in 0.1 M Tris–HCl, pH 8.0. The reaction was carried out for 10 min at room temperature. In parallel, controls with boiled extract (100 °C, 5 min) were run. By using a pNA calibration curve, the release of *p*-nitroaniline was monitored as $\Delta_{405\text{nm}}$.

Purified enzyme equivalent to 25 µg protein was incubated for 1 h at 37 °C at given pH values in 50 mM acetate buffer pH 4.0–5.0, 50 mM potassium phosphate buffer pH 6.0–7.0, 50 mM Tris–HCl buffer pH 8.0–9.0, and 50 mM glycine–NaOH buffer pH 10.0–12.0. The enzyme was incubated in the reaction buffer (pH 8.0) for 1 h at temperatures ranging from 10 to 80 °C. The activity of the enzyme was determined using chromogenic substrates. To test the effect of metal ions and animal protease inhibitors, following incubation was set up in reaction buffer (pH 8.0) with purified enzyme for 1 h at 37 °C: 5 mM each of KCl, CaCl₂, ZnSO₄, Co(NO₃)₂, MnCl₂, FeCl₃, CuSO₄, MgCl₂, FeCl₂, or 5 mM each of STI, PMSF, leupeptin,

iodoacetamide, E-64, benzamidine–HCl, antithrombin III and elastatinal, 50 µM antipain, 2 mM Na₂EDTA, and 0.1 mM α_2 -antiplasmin (Merck, Germany) in water, 0.5 mM dipyridyl in ethanol, 5 mM chymostatin in 7.5% (v/v) dimethyl sulphoxide, and 1 mM 1,10-phenanthroline in methanol, prior to start of the assays. Controls received equivalent volumes of the solvents which is < 1% of the assay mixture volume.

Determination of kinetic parameters

The kinetic parameters such as substrate saturation constant (K_m), maximum reaction velocity (V_{max}), catalytic constant (K_{cat}), and catalytic efficiency (K_{cat}/K_m) were measured by assessing enzymatic activity with BApNA at concentrations between 0 and 50 µM. Reactions were initiated by the addition of 40 µg of purified peptidase in a glass cuvette on a spectrophotometer (DU800, Beckman) equipped with thermostated cell compartment. The kinetic parameters were obtained from the initial rate measurements of substrate hydrolysis by purified peptidase and the release of *p*-nitroaniline was monitored at 405 nm, and their values were calculated using a Lineweaver–Burk linear plot.

Results

Growth and proteolytic activity in the presence and absence of combined nitrogen

Westiellopsis ramosa was cultivated in regular BG-11₀ medium and transferred to BG11₀ + NO₃[−], BG11₀ + NH₄⁺ and “modified” BG11₀ (see “Materials and methods” section) for seven generations. Once the culture was acclimatized to the given nitrogen source, in the eighth generation, growth of the organism was recorded in terms of increase in dry weight and chlorophyll *a* content. As depicted in Fig. 1a, regardless of the given nitrogen source, it took 20 days to attain peak biomass (dry weight). However, the time-kinetics of growth and the absolute growth on day 20 was the highest in “modified” BG11₀, followed by regular BG11₀ and NO₃[−]- and NH₄⁺- supplemented media. In regular BG11₀ medium (without NaCl), growth was slightly lower than “modified” BG11₀, though the difference was not significant. Quantifying the growth in terms of chlorophyll *a* content did not change the overall trend (Fig. 1b).

NH₄Cl supplemented cultures showed poor growth with highly shrunk cytoplasmic content and typical “T” shaped branching. Cultures supplemented with NaNO₃ showed better growth along with the presence of branching. Only cultures without external supplementation of nitrogen showed heterocytes (Supplementary material Fig. S1).

Fig. 1 Growth in terms of change in dry weight (**a**) and chlorophyll *a* content (**b**) of *W. ramosa* for 24 days in the regular BG11₀ medium (—○—), and in “modified” BG11₀ (—□—), BG11₀ + NO₃[−] (—●—)

and BG11₀ + NH₄⁺ (—□—) media. Prior to the start of the experiment, cells were grown in the respective medium for seven generations. Bar represents mean ± SD (*n* = 3)

In casein plate assays, clearing of casein was detected only in the area surrounding the sonicated cells harvested from BG11₀ and not around the culture filtrates after removal of the cultures or the buffer in which cells were lysed (Supplementary material Fig. S2).

Homogenizing cells harvested from liquid cultures by sonication in Triton X-100 containing buffer yielded the highest casein hydrolytic activity. The average value in regular BG11₀ grown cells was 34.5 ± 2.43 nmol Tyr min^{−1} mg protein^{−1}. No activity was detected in heat-denatured (5 min, 100 °C) extract or upon omission of casein. For testing reliability, caseinolytic activity was determined in triplicate sets of six experiments each (*n* = 18). To establish the variance, the mean value of each of the six experiments was randomly paired. The calculated *F* value upon applying one-way ANOVA ($F_{5,12} = 0.25$, $p < 0.05$) was smaller than the tabulated value indicating that there was no significant difference between the mean values computed from the six experiments. Casein hydrolytic activity was determined for cultures grown under different nitrogen conditions as described above. Upon comparison, the activity in the absence of combined nitrogen was found to be significantly higher than what was detected in the presence of combined nitrogen (Supplementary material Table S1).

In-gel activity staining by substrate-zymography is a reliable method to screen for the presence of proteolytic enzymes. The cultures grown under different nitrogen conditions were subjected to substrate-SDS-PAGE. After Coomassie Brilliant Blue staining, five bands of approx. $M_r = 66$, 49, 36, 30 and 27 kDa were visualized under combined N-free growth, which indicates the existence of multiple gelatinolytic peptidases or their oligomeric forms

Fig. 2 Gelatin (substrate) SDS PAGE zymograms of protein extracts prepared from *W. ramosa* cells grown for eighth generations in “modified” BG11₀ (lane #a), BG11₀ + NO₃[−] (lane #b) and BG11₀ + NH₄⁺ media (lane #c); and in vegetative cells (lane #d) and heterocytes (lane #e). The clearing zone indicates peptidase activity. Representative gels are presented

(Fig. 2 lane #a). No such bands were visible with NO₃[−] and NH₄⁺ (lane #b–c). Zymogram (lane #d) also revealed appearance of a single 30 kDa band in the protein extract of vegetative cells. On the other hand, a complete set of five bands was detected in isolated heterocytes (lane #e).

Partial purification and characterization of peptidase, Wrp49

A caseinolytic peptidase from whole trichomes of *W. ramosa* grown in regular BG11₀ medium, called Wrp49

(*W. ramosa* protease 49 kDa), was purified by combining salt-exclusion and chromatographic steps. The purification results are presented in Fig. 3a–c and Table 1. The enzyme precipitated between 30 and 70% $(\text{NH}_4)_2\text{SO}_4$, and was resolved as one active fraction by Sephadex G-100 gel filtration chromatography using 50 mM Tris–HCl at pH 8.0. Further purification was achieved by using a CM-Sepharose column, in which Wrp49 activity was found in the fractions eluting with 0.5 M NaCl. Further, the enzyme was retained by Blue-Sepharose column and eluted in the fractions of 0.1 M NaCl. After all purification steps, 17% recovery (yield) and 102-fold increase in specific activity of the crude extract was achieved (Table 1).

Native and SDS-PAGE, and size-exclusion chromatography were employed to verify the purity of Wrp49 and determine its molecular mass. Standard proteins of 29–97.4 kDa and Blue-Sepharose purified enzyme preparation were simultaneously applied to the size exclusion column and collectively five protein peaks were discernable. Wrp49 could co-elute with any of the four standard proteins or elute as a separate peak. Size-exclusion chromatography showed a significant caseinolytic activity in peak no. 3 and not in the preceding or succeeding peaks (Fig. 4a). By comparing with standards, the peak no. 3 corresponded to molecular weight of 49 kDa. The

molecular mass was confirmed by native PAGE to be 49 kDa (Fig. 4b). Upon SDS-PAGE of the purified enzyme on 10% gel, two peptide bands of 27 and 17 kDa were clearly visible (Fig. 4c). Wrp49 was resolved by substrate (gelatin)-gel PAGE to ascertain that not more than one enzyme is involved in protein digestion as was revealed for the crude extract (*cf.* Figure 2a). As shown in Fig. 4d, with gelatin as a substrate only one clear band corresponding to a position at 49 kDa was discernible.

The N-terminal sequence (MTVENLARTGVGPGWR) of Wrp49 exhibited 100% similarity with the *S*-adenosyl-L-methionine (SAM)-dependent methyltransferase from *Actinomadura rifamycini*. Using the UniProt database we wanted to compare the sequence with the N-terminal sequences of serine peptidases annotated from the unbranched genera, *Nostoc* and *Anabaena* and the branched ones, *Hapalosiphon* and *Fischerella*. As shown in the Supplementary material Table S2, the Wrp49 N-terminal sequence did not match with any of the cyanobacterial serine peptidases or the uncharacterized peptidases. Upon running BLAST tool, no similar sequence was found in any cyanobacterium, suggesting that the N-terminal sequence of Wrp49 can be considered as “unique” sequence specific to the genus, *Westiellopsis*. Further search revealed occurrences of the “unique” N-terminal sequences in:

Fig. 3 Separation of protein (—◆—) and caseinolytic activity (■) of ammonium sulphate fractionated *W. ramosa* whole trichome extracts through chromatography on **a** Sephadex G-100, **b** CM-Sepharose and **c** Blue Sepharose columns

Table 1 Purification profile of a caseinolytic protease Wrp49 from whole cell extract of *W. ramosa*

Steps	Total activity (nmol Tyr per min)	Total protein (mg)	Specific activity (nmol Tyr per min and mg of protein)	Yield (%)	Purification fold
Cell-free extract	8345	430	19	100	1
(NH ₄) ₂ SO ₄ precipitation	5920	150	39	75	2
Sephadex G-100	3424	12	285	41	15
CM- Sepharose	2480	4	620	30	33
Blue Sepharose	1428	0.735	1943	17	102

Fig. 4 Molecular mass of pooled Blue-Sepharose-eluted fractions (cf. Fig. 3c; Table 1) on **a** size exclusion on Sephadex G, **b** native and **c** SDS-PAGE and on **d** in gel activity staining on gelatin. Protein equivalent to 250, 80, 40 and 20 μ g were applied for experiment **a–d**, respectively. For experiment **a**, samples and standards in 50 mM Tris–HCl, pH 8.0 was filtered at 1 mL min^{−1} and caseinolytic activity determined in pooled fractions corresponding to peaks #1–5 (bar)

trypsin-like serine proteases with a C-terminal “post-synaptic density protein–*Drosophila* disc large tumor suppressor-zonula occludens-1 protein” (PDZ) domain (Nos7524_5573, *Nostoc* sp. ATCC 29411), a peptidase S8/S53, subtilisin (NIES19_11680, *Anabaena cylindrica* ATCC 27899), and at least four other serine peptidases (ACX27_00470, *Nostoc piscinale* CENA21; A4S05_12910, *Nostoc* sp. KVIJ20; ANA_C13100, *Anabaena* sp. 90 and NIES592_12145, *Fischerella major* NIES-592).

Amidase activity of Wrp49

Synthetic oligopeptide substrates mimic the protein substrates specific to animal serine peptidases, and are used to determine the nature of the caseinolytic peptidase as being analogous to trypsin-, chymotrypsin- or other serine peptidases. Upon hydrolytic action on the terminal amino acid at P1 position, the product *p*NA is released. It can be deduced from the time-kinetic release of *p*NA from the oligopeptide substrates that Wrp49 most efficiently hydrolyzed VLK*p*NA and BAp*p*NA, specific substrates for plasmin and trypsin (Fig. 5), showing a specific activity of 110 ± 11.2 and 106 ± 10.2 μ mol *p*NA min^{−1}mg^{−1} protein, respectively. The enzyme also hydrolyzed a thrombin- and plasmin-specific substrate, GPR*p*NA, though at much lower rate (14 μ mol *p*NA min^{−1}mg^{−1} protein). No activity was detected with SA₂PP*p*NA and SA₃*p*NA, the substrates specific to chymotrypsin and elastase, respectively.

The kinetic properties of Wrp49 activity were elucidated using BAp*p*NA as substrate. From the Lineweaver–Burk plot (Fig. 6), the apparent substrate saturation constant (K_m) and the maximum reaction velocity (V_{max}) were 0.195 mM and 5×10^{-7} Ms^{−1}, respectively. The turnover number (K_{cat}) and the catalytic efficiency (K_{cat}/K_m) were 0.14 s^{−1} and 7.1×10^2 M^{−1}s^{−1}, respectively.

Effect of pH and temperature on Wrp49 activity

The enzyme preparation, prior to the onset of assays with BAp*p*NA as substrate, was incubated for 1 h under given pH values ranging from 4.0 to 12.0 and at given temperatures ranging from 10 to 80 °C. From Fig. 7a, b the optimal pH was deduced to be 8.0 and the optimal temperature to be 40 °C.

Effects of metal ions and peptidase inhibitors

The effect of metal ions and animal protease inhibitors on the activity of Wrp49 with BAp*p*NA as substrate is summarized in Tables 2 and 3. Under incubation conditions, Ca²⁺ and Co²⁺ significantly enhanced the activity by two-

Fig. 5 Time-kinetic release of pNA from synthetic oligopeptide substrates with purified peptidase, Wrp49 (cf. Fig. 3). Protein extract equivalent to 40 μg protein (—◆—) or corresponding boiled extracts

(—□—) were added to above assays. Inset Specific activity as a function of concentration of protein. Bar represents mean $\pm$ SD of three independent experiments in triplicate analysis ($n = 9$)

Fig. 6 Lineweaver-Burk plot of BA_pNA substrate for purified peptidase, Wrp49. 40 μg protein was assayed with BA_pNA in a concentration range from 10 to 50 μM. A representative plot of triplicate experiments is shown

to three-fold, whereas Cu²⁺ resulted in about 38% inhibition (one-way ANOVA, $F_{3,8} = 3.339$, $p < 0.05$). The

effect of other metal cations was not significant ($p > 0.05$). Wrp49 was almost completely inhibited by serine peptidase inhibitor, PMSF, serine- and cysteine peptidase inhibitor, leupeptin, and metallopeptidase inhibitors, 1,10 phenanthroline, dipyriddy, and EDTA (one-way ANOVA, $F_{3,8} = 0.984$, $p < 0.05$). At the recommended effective doses, the enzyme activity was partially inhibited (36–75%) by trypsin peptidase inhibitors, benzamidine HCl, antipain and STI. The other peptidase inhibitors had no significant effect on the activity ($p > 0.05$).

Since *W. ramosa* was cultivated in a medium containing Ca²⁺, it is likely that the enzyme Wrp49 already has acquired the metal necessary to attain the control values. If the EDTA and Ca²⁺ compete, replenishment of Ca²⁺ should alleviate the EDTA-effect, i.e. should start to increase the activity. To ascertain this, the enzyme was treated with 2 mM EDTA for 1 h. In the amidolytic assay using BA_pNA substrate, EDTA-pre-treated enzyme was incubated with 2, 3 and 5 mM CaCl₂. Without Ca²⁺ the

Fig. 7 Residual activity of purified peptidase, Wrp49, with BApNA substrate as a function of **a** pH and **b** temperature. The enzyme was incubated at stated pH in various buffers (see “Materials and

methods” section) or at different temperature for 1 h before starting the reactions. Data are expressed as mean $\pm$ SD ($n = 3$)

Table 2 Residual activity of purified protease, Wrp49, upon incubation with metal ions

Metal ions	Concentration (mM)	Relative activity (%)
Control	–	100 $\pm$ 3.8
K ⁺	5	92 $\pm$ 3.3
Cu ²⁺	5	38 $\pm$ 2.2*
Co ²⁺	5	189 $\pm$ 4.4*
Zn ²⁺	5	90 $\pm$ 3.2
Mn ²⁺	5	97 $\pm$ 3.5
Ca ²⁺	5	245 $\pm$ 7.2*
Fe ²⁺	5	88 $\pm$ 3.2
Mg ²⁺	5	94 $\pm$ 3.8
Fe ³⁺	5	98 $\pm$ 3.4

The enzyme equivalent to 40 μg protein was incubated with/without (controls) metal salts at 37 $^{\circ}\text{C}$ at pH 8.0 for 1 h. Amidolytic activity was determined using BApNA substrate. 100% activity equals to 75 $\mu\text{mol pNA produced min}^{-1} \text{mg}^{-1} \text{protein}$. Data expressed as mean $\pm$ SD ($n = 3$)

* $p < 0.05$ (significantly different values from controls; one-way ANOVA, LSD)

activity was negligible, but with Ca^{2+} supplementation there was a concentration-dependent increase in the activity attaining the respective values of 46, 52 and 64 $\mu\text{mol pNA min}^{-1} \text{mg}^{-1} \text{protein}$.

Since different composition of peptidases in the crude enzyme preparations was observed in intact heterocyte- and vegetative cell zymograms (cf. Fig. 2d, e), it was tested which peptidases were inhibited by PMSF and EDTA. Heterocyte- and vegetative cell-free extracts were pre-incubated with both the inhibitors and resolved on substrate-gels. As shown in Fig. 8a, b, controls revealed 5 peptidase bands in a molecular mass range of 27–66 kDa in

Table 3 Residual activity of purified protease, Wrp49, upon incubation with metabolic inhibitors and chelators

Inhibitors	Concentration (mM)	Relative activity (%)
Control	–	100 $\pm$ 5.2
STI	5	64 $\pm$ 3.4*
PMSF	5	0
Leupeptin	5	0
Iodoacetamide	5	92.2 $\pm$ 3.4
Chymostatin	5	89 $\pm$ 4.2
E-64	5	96 $\pm$ 3.1
Elastatinal	5	91 $\pm$ 5.2
Antipain	0.05	25 $\pm$ 2.1*
Benzamidine HCl	5	32 $\pm$ 2.5*
1,10-Phenanthroline	1	0
EDTA	2	0
Dipyridyl	0.5	0
Antithrombin III	5	79 $\pm$ 4.6
α_2 -Antiplasmin	0.1	92 $\pm$ 5.6

The enzyme equivalent to 40 μg protein was incubated in presence of inhibitors/solvents (controls). 100% activity denotes 78 $\mu\text{mol pNA produced min}^{-1} \text{mg}^{-1} \text{protein}$

* $p < 0.05$ (significantly different values from controls; one-way ANOVA, LSD)

heterocyte- and just one band corresponding to 30 kDa in vegetative cell extracts. The treatments resulted in the disappearance of bands of around 66 and 49 kDa masses in heterocytes only. To demonstrate that Ca^{2+} alleviated the EDTA-effect on peptidase activities, the previously EDTA treated heterocyte extracts were first run on a gel to confirm that the activity at positions 66 and 49 kDa masses was missing. The extracts were incubated further with the given

concentrations of Ca^{2+} for about an hour and then applied to the gel. As demonstrated in Fig. 8c, the activities at the two band positions increased as a function of increase in concentrations of Ca^{2+} .

Fibrinolytic activity of purified Wrp49

Since Wrp49 hydrolyzed a plasmin-specific substrate VLKpNA, it was examined if the three fibrin proteins, namely α - and β -chains, and γ -dimer were hydrolyzed by the enzyme. As shown in Fig. 9a, incubation with the enzyme demonstrated that within 1 h, the γ -dimer bands disappeared while the α - and β -chains remained present. No effect on the α -chain and β -chain fibrins could be seen after 4 h. When Wrp49 was replaced with plasmin, the γ -dimer was degraded in less than 1 h and the α -chain after another 3 h of incubation. However the β -chain was not substantially affected even after prolonged treatment (Fig. 9b).

Discussion

In this communication, we report the presence of several peptidases inside heterocytes and vegetative cells of *W. ramosa*, of which at least two are Ca^{2+} -dependent peptidases. Negative bacterial growth indicated that the peptidase activity was of cyanobacterial origin.

The peptidase isolated in this work showed similarity with serine peptidases of *A. variabilis* 29413 and *Anabaena* 7120 (Strohmeier et al. 1994; Maldener et al. 1991). These enzymes hydrolytically cleaved casein and the synthetic oligopeptide substrates with -Arg or -Lys at P1 position. Based on substrate and inhibitor specificity, the enzyme Wrp49 appears to be a trypsin-like serine peptidase. Other

common properties between Wrp49 and *Anabaena* peptidases were: a native molecular weight of 50–55 kDa, a broad pH activity range with an optimal pH of 8.0, Ca^{2+} stimulation, and a strong inhibition effect by chelants, PMSF, leupeptin, antipain and benzamidine-HCl while relatively weak inhibition by STI. These characteristics made us to conclude that Wrp49 has similarity with a subtilisin-like serine peptidase (subtilase), PrcA, of *A. variabilis* 29413 (Baier et al. 1996). There were some differences with regard to Ca^{2+} response though. Ca^{2+} was not absolutely required for Wrp49 activity, yet its activity with EDTA was completely lost in the absence of exogenous Ca^{2+} . Notwithstanding, upon adding increments of Ca^{2+} the activity started to reappear, circumventing the EDTA-effect. It is possible that the required Ca^{2+} for optimal activity was available from the growth media, and exogenous Ca^{2+} only stimulated the activity by two-fold by filling the binding sites that were left unoccupied. In comparison, adding Ca^{2+} activated the *A. variabilis* 29413 subtilase by over 15-fold, which was an absolute requirement for optimal activity. Even in presence of Ca^{2+} in *trans*, EDTA was able to suppress the enzyme's activity, suggesting that the EDTA-effect was irrevocable. Another prominent difference was that substrates with -Pro at P2 position such as GPRpNA were hydrolyzed more efficiently than BApNA by the *A. variabilis* 29413 peptidase while Wrp49 could hardly react on GPRpNA.

The most distinguishing feature was that, the N-terminal sequences of the two enzymes, PrcA and Wrp49 had no similarity. Interestingly, many serine peptidases of unbranched- and branched heterocytous cyanobacteria, including Wrp49, have been shown to possess “unique” N-terminal sequences, not found in other cyanobacteria. For the moment, we think that more peptidase sequences from diverse cyanobacterial taxa should be submitted to the

Fig. 8 Zymograms of protein extracts of **a** broken vegetative cells and **b** heterocytes representing control (lane #1), PMSF (lane #2) and EDTA (lane #3) treatments; and **c** EDTA-pre-treated heterocytes without (lane #1) or with 2, 3 and 5 mM Ca^{2+} (lane #2–4) and the control heterocytes with solely 5 mM Ca^{2+} (lane #5) incubations. Representative gels are presented

Fig. 9 SDS-PAGE of fibrin clot upon treatment with **a** 20 μ g protein equivalent of purified Wrp49 and **b** 0.1 units of plasmin (2 μ g protein) for the given periods indicated. The fibrinopeptides thus generated were identified from relative M_r values. Representative gels of three independent experiments are shown

UniProt database so that there is a better chance of getting analogous sequences. It should be noted that, for comparison no peptidase sequence from *Westiellopsis* is available.

SDS-PAGE of Wrp49 revealed separation of two peptides at 27 and 17 kDa which arose from a native protein with M_r 49 kDa. Either the peptidase is a dimer or one of the peptides co-eluted with the putative peptidase. A dimeric state of serine peptidases is a common observation in N_2 fixing cyanobacteria. Two bands were observed for the Ca^{2+} dependent peptidase and the trypsin-like specificity endopeptidase from *A. variabilis* 29413 (Lockau et al. 1988; Strohmeier et al. 1994), the serine peptidase from *A. fertilissima* 597 (Banerjee et al. 2013), *A. variabilis* 29413 subtilase (Baier et al. 1996) and a HetR peptidase from *Anabaena* 7120 (Zhou et al. 1998).

Upon comparing the kinetics data of *W. ramosa* and *A. variabilis* 29413 peptidases (Strohmeier et al. 1994), it becomes apparent that K_m values for both enzymes with BApNA as substrate are in a range of 0.05–0.2, suggesting that there exists a difference in the substrate affinity as well. The catalytic constant (K_{cat}) represents the number of mols of product per mol of enzyme in unit time, which in case of Wrp49 is 0.14 mol of pNA per mol of the enzyme. Upon comparing the index or value with bovine trypsin (Kristjánsson, 1991) it appears that the enzyme is about 10-times less efficient in hydrolysis. Comparing catalytic efficiency (K_{cat}/K_m), the Wrp49 index is about 5-times less relative to trypsin, suggesting that the rate of the reaction rather than the substrate affinity is a major determinant of low catalytic efficiency of Wrp49.

Similar to a serine peptidase that was isolated from the rice-field cyanobacterium *A. fertilissima* 597 (Banerjee et al. 2013), Wrp49 had a native molecular weight of 49 kDa and showed a 27 kDa and a 17 kDa peptide band on SDS-PAGE gels. Another similarity is that, apart from

BApNA both amidolytically cleaved a plasmin-specific substrate VLKpNA. In fact, both enzymes hydrolyzed the plasmin γ -dimer to fibrinopeptides of identical masses. The effects of metabolic inhibitors and metal ions are also similar, viz., activation by Ca^{2+} and Co^{2+} and inhibition by inhibitors common for serine- and metallopeptidases. The question why 1,10-phenanthroline, a type-II metal chelant strongly inactivates the enzyme cannot be answered with certainty. It is speculated that the serine peptidases may have domains that can bind to the type-II metals such as Co^{2+} , and perhaps this in turn would modulate the active sites. In *N. punctiforme* 73102 exoproteome, Ca^{2+} binding RTX proteins have metallopeptidase-serralyisin motifs (Vilhauer et al. 2014). A composite peptidase of a marine *Synechococcus* sp. strain WH7805 (Christie-Oleza et al. 2015) was constituted of a Zn-metallopeptidase, a serralyisin, and a subtilisin-like serine peptidase. In an Antarctic isolate, *Janthinobacterium* strain AU11, an extracellular peptidase acquired from cyanobacteria by horizontal gene transfer resembled a serralyisin-like metallopeptidase, but the inhibitor profile was similar to subtilisin (Martinez-Rosales et al. 2015). The dual nature of peptidases can be extended to fungal and bacterial fibrinases (Wu et al. 2009) and ketatinases (Tork et al. 2013), which were termed as “chymotrypsin/subtilisin-like serine metallopeptidases” as these enzymes aligned with serine peptidases, yet the metal/chelant-response was similar to metallopeptidase. The evolutionary and functional relevance of such composite peptidases is at the moment not clear, but would be interesting to unravel in future.

To sum up, a serine peptidase with metallopeptidase-like features, namely inhibition by type-II metal chelants, was characterized in a true-branching *Westiellopsis ramosa*. It can be assumed that proteolysis of ecto- and endogenous proteins may have a distinct advantage for

these soil-dwelling and aerophytic cyanobacteria, which owing dry habitat may seek additional N nutrients from surrounding plant exudates for propagation and cellular differentiation.

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Author's contribution SNB and PS designed the experiments. ND and PS performed the experiments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

- Agrawal MK, Zitt A, Bagchi D, Weckesser J (2005) Characterization of proteases in gut of *Daphnia magna* and their inhibition by *Microcystis aeruginosa* PCC7806. *Environ Toxicol* 20:314–322. <https://doi.org/10.1002/tox.20123>
- Andrews P (1965) The gel filtration behaviour of proteins related to their molecular weights over a wide range. *Biochem J* 96:595–606
- Baier K, Nicklisch S, Lockau W (1996) Evidence for propeptide-assisted folding of the calcium-dependent protease of the cyanobacterium *Anabaena*. *Eur J Biochem* 241:750–755
- Banerjee S, Prasanna R, Bagchi SN (2013) Purification and characterization of a fibrinolytic protease from cultured natural isolate of a cyanobacterium, *Anabaena fertilissima*. *J Appl Phycol* 25:1111–1122. <https://doi.org/10.1007/s10811-012-9946-6>
- Burnat M, Herrero A, Flores E (2014) Compartmentalized cyanophycin metabolism in the diazotrophic filaments of a heterocyst forming cyanobacterium. *PNAS* 111:3823–3828. <https://doi.org/10.1073/pnas.1318564111>
- Christie-Oleza JA, Armengaud J, Guerin P, Scanlan DJ (2015) Functional distinctness in the exoproteomes of marine *Synechococcus*. *Environ Microbiol* 17(10):3781–3794. <https://doi.org/10.1111/1462-2920.12822>
- Datta G, Dong A, Witt J, Tu AT (1995) Biochemical-characterization of basilase, a fibrinolytic protease from *Crotalus basiliscus basiliscus*. *Arch Biochem Biophys* 317:365–373. <https://doi.org/10.1006/abbi.1995.1176>
- Ghosh SK, Bagchi D, Bagchi SN (2008) Proteolytic activity in *Microcystis aeruginosa* PCC7806 is inhibited by a trypsin inhibitory cyanobacterial peptide with a partial structure of microviridin. *J Appl Phycol* 20:1045–1052. <https://doi.org/10.1007/s10811-007-9304-2>
- Kim SH, Choi NS, Lee WY (1998) Fibrin zymography: a direct analysis of fibrinolytic enzymes on gels. *Anal Biochem* 263:115–116. <https://doi.org/10.1006/abio.1998.2816>
- Kristjánsson MM (1991) Purification and characterization of trypsin from the pyloric caeca of rainbow trout (*Oncorhynchus mykiss*). *J Agric Food Chem* 39:1738–1742. <https://doi.org/10.1021/jf00010a009>
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Leikoski N, Fewer DP, Jokela J, Wahlsten M (2010) Highly diverse cyanobactins in strains of the genus *Anabaena*. *Appl Environ Microbiol* 76(3):701–709. <https://doi.org/10.1128/AEM.01061-09>
- Lockau W, Massalsky B, Dirmeyer A (1988) Purification and partial characterization of a calcium-stimulated protease from the cyanobacterium, *Anabaena variabilis*. *Eur J Biochem* 172:433–438. <https://doi.org/10.1111/j.1432-1033.1988.tb13906.x>
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Mackinney G (1941) Absorption of light by chlorophyll solution. *J Biol Chem* 140:315–322
- Maldener I, Lockau W, Cai Y, Wolk CP (1991) Calcium-dependent protease of the cyanobacterium *Anabaena*: molecular cloning and expression of the gene in *Escherichia coli*, sequencing and site-directed mutagenesis. *Mol Gen Genet* 225:113–120
- Martinez-Rosales C, Marizcurrena JJ, Iriarte A, Fullana N (2015) Characterizing proteases in an Antarctic *Janthinobacterium* sp. isolate: evidence of a protease horizontal gene transfer event. *Adv Polar Sci* 26:88–95. <https://doi.org/10.13679/j.advps.2015.1.00088>
- Nürnberg DJ, Mariscal V, Bornikoe J, Nieves-Morion M (2015) Intercellular diffusion of a fluorescent sucrose analog via the septal junctions in a filamentous cyanobacterium. *M Bio* 6(2):e02109–e02114. <https://doi.org/10.1128/mBio.02109-14>
- Oliveira P, Martins NM, Santos M, Couto NAS (2015) The *Anabaena* sp. PCC 7120 exoproteome: taking a peek outside the box. *Life* 5(1):130–163. <https://doi.org/10.3390/life5010130>
- Park JJ, Lechno-Yossef S, Wolk CP, Vieille C (2013) Cell-specific gene expression in *Anabaena variabilis* grown phototrophically, mixotrophically, and heterotrophically. *BMC Genomics* 14(1):759. <https://doi.org/10.1186/1471-2164-14-759>
- Pernil R, Picossi S, Herrero A, Flores E (2015) Amino acid transporters and release of hydrophobic amino acids in the heterocyst-forming cyanobacterium *Anabaena* sp. strain PCC 7120. *Life* 5:1282–1300. <https://doi.org/10.3390/life5021282>
- Richter R, Hejazi M, Kraft R, Ziegler K, Lockau W (1999) Cyanophycinase, a peptidase degrading the cyanobacterial reserve material multi-L-arginyl-poly-L-aspartic acid (cyanophycin): molecular cloning of the gene of *Synechocystis* sp. PCC 6803, expression in *Escherichia coli*, and biochemical characterization of the purified enzyme. *Eur J Biochem* 263(1):163–169. <https://doi.org/10.1046/j.1432-1327.1999.00479.x>
- Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY (1979) Generic assignments, strain histories and properties of pure culture of cyanobacteria. *J Gen Microbiol* 111:1–61
- Singh P, Dubey N, Bagchi SN (2017) *Westiellopsis ramosa* sp. nov., intensely branched species of *Westiellopsis* (cyanobacteria) isolated from Jabalpur, Madhya Pradesh, India. *Plant Syst Evol* 303:1239–1249. <https://doi.org/10.1007/s00606-017-1434-7>
- Strohmeier U, Gerdes C, Lockau W (1994) Proteolysis in heterocyst-forming cyanobacteria: characterization of a further enzyme with trypsin-like specificity, and of a prolyl endopeptidase from *Anabaena variabilis*. *Z Naturforsch* 49:70–78
- Thomas J, Meeks JC, Wolk CP, Shaffer PW, Austin SM (1977) Formation of glutamine from [¹⁵N] ammonia, [¹³N] dinitrogen, and [¹⁴C] glutamate by heterocysts isolated from *Anabaena cylindrica*. *J Bacteriol* 129:1545–1555
- Tork SE, Shahein YE, El-Hakim AE, Abdel-Aty AM (2013) Production and characterization of thermostable metallo-keratinase from newly isolated *Bacillus subtilis* NRC 3. *Int J Biol Macromol* 55:169–175. <https://doi.org/10.1016/j.ijbiomac.2013.01.002>
- Vilhauer L, Jervis J, Ray WK, Helm RF (2014) The exo-proteome and exo-metabolome of *Nostoc punctiforme* (cyanobacteria) in

- the presence and absence of nitrate. Arch Microbiol 196:357–367. <https://doi.org/10.1007/s00203-014-0974-2>
- Wu B, Wu L, Chen D, Yang Z (2009) Purification and characterization of a novel fibrinolytic protease from *Fusarium* sp. CPCC 480097. J Ind Microbiol Biotechnol 36(3):451–459. <https://doi.org/10.1007/s10295-008-0516-5>
- Zhou R, Wei X, Jiang N, Li H (1998) Evidence that HetR protein is an unusual serine-type protease. PNAS 95:4959–4963