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Full Paper

Novel thermostable glycosidases in the extracellular matrix of the terrestrial cyanobacterium *Nostoc commune*

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The cyanobacterium *Nostoc commune* is adapted to the terrestrial environment and forms a visible colony in which the cells are embedded in extracellular polysaccharides (EPSs), which play a crucial role in the extreme desiccation tolerance of this organism. When natural colonies were immersed in water, degradation of the colonies occurred within 2 days and *N. commune* cells were released into the water. The activities that hydrolyze glycoside bonds in various *N. commune* fractions were examined using artificial nitrophenyl-linked sugars as substrates. A β-D-glucosidase purified from the water-soluble fraction was resistant to 20 min of boiling. The β-D-glucosidase, with a molecular mass of 20 kDa, was identified as a cyanobacterial fasciclin protein based on its N-terminal amino-acid sequence. The 36-kDa major protein in the water-soluble fraction was purified, and the N-terminal amino-acid sequence of the protein was found to be identical to that of the water-stress protein (WspA) of *N. commune*. This WspA protein also showed heat-resistant β-D-galactosidase activity. The fasciclin protein and WspA in the extracellular matrix may play a role in the hydrolysis of the EPSs surrounding the cells, possibly as an aid in the dispersal of cells, thus expanding the colonies of this cyanobacterium.

Key Words——desiccation tolerance; extracellular polysaccharide; fasciclin; WspA

Introduction

The terrestrial cyanobacterium *Nostoc commune* has a marked capacity for desiccation tolerance and can survive long-term in a desiccated state (Lipman, 1941). *Nostoc commune* has a biochemically complex extracellular matrix that is important in its tolerance of extreme desiccation (Potts, 1994, 2000). To elucidate the mechanism by which the extracellular matrix protects *N. commune* cells from extreme desiccation, the structure and biophysical properties of the extracellular matrix and the proteins distributed within it have been studied.

The bulk of the *N. commune* extracellular matrix is made up of extracellular polysaccharides (EPSs), mainly glycans (Helm et al., 2000; Shaw et al., 2003). EPSs in *N. commune* colonies amount to over 60% of the dry weight (Hill et al., 1997) and are constituted of various sugars including glucose, galactose, xylose, and uronic acids (Helm et al., 2000; Kajiyama and Kobayashi, 2003). A water-stress protein (WspA) (Hill et al., 1994b; Scherer and Potts, 1989; Wright et al., 2005) and a highly stable and active superoxide dismutase

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(SodF) (Shirkey et al., 2000) have been reported to be distributed within the EPSs, along with the UV-absorbing pigments mycosporine amino acids (Ehling-Schulz et al., 1997) and scytonemin (Hill et al.,1994a; Hunsucker et al., 2001; Wright et al., 2005).

Multiple roles for EPSs have been suggested, including stress tolerance and as a sink for excess energy (Otero and Vincenzini, 2004). It is believed that the EPSs of *Nostoc commune* play a role in the stabilization of cells in the air-dried state, because the glycans in the EPSs inhibit the fusion of membrane vesicles during desiccation and freeze-drying (Hill et al., 1997) and act as an immobilization matrix for secreted enzymes, which remain fully active after long-term air-dried storage (Hill et al., 1994a; Scherer and Potts, 1989; Shirkey et al., 2000). We have demonstrated that the desiccation tolerance of photosynthesis in *N. commune* is strongly associated with EPSs, and that EPS-depleted cells are highly sensitive to desiccation (Tamaru et al., 2005).

During a series of studies of *N. commune*, we observed that natural *N. commune* colonies immersed in water degraded within 2 days; we then investigated the EPS-hydrolyzing enzymes of this organism based on this observation. In the present study, we purified and characterized a β-D-glucosidase and a 36-kDa WspA with β-D-galactosidase activity from the extracellular matrix of the terrestrial cyanobacterium *N. commune*. Since nitrophenyl-linked sugars were used as substrates, we tested whether these enzymes were inactivated by heat to rule out the possibility that the substrates were hydrolyzed non-enzymatically. As a consequence, both the β-D-glucosidase and β-D-galactosidase enzymes were shown to be heat-resistant.

Materials and Methods

Organisms. Naturally growing colonies of *N. commune* were collected from the field of the Kakuma Campus of Kanazawa University in Japan. The field-collected samples were washed with tap water, air-dried, and stored at room temperature for later use in experiments.

Separation of EPSs, water-soluble fraction, and EPS-depleted cells. Dry colonies (165 g) were rehydrated, suspended in 1.7 L of 0.75 M potassium phosphate buffer (pH 7.0), and homogenized in a blender three times at medium speed for 10 s each. After stirring overnight at room temperature, the suspension was further homogenized in a blender three times at medium speed for 10 s each and left to stand for 10 min at room temperature. The upper EPS layer was removed using a spatula, and the lower aqueous layer was centrifuged at 6,000 × *g* for 10 min at 20°C. The EPS-depleted *N. commune* cells collected in the pellet were stored at −30°C. The supernatant, which retained the water-soluble fraction of the extracellular matrix, was used for the purification of thermostable glycosidases.

Polysaccharide measurements. EPSs were subjected to acid hydrolysis in 4 M HCl at 100°C for 2 h. The total sugars released from the acid hydrolysis were determined using the anthrone sulphuric acid method as described by Fales (1951) with modifications. The anthrone sulphuric acid reagent consisted of 0.2 g anthrone, 8 ml ethanol, 30 ml distilled water, and 100 ml concentrated H_2SO_4, each freshly prepared, which were successively mixed in a conical flask under continuous cooling. A sample of the hydrolyzed EPS solution (200 μl) was mixed with 10 ml of anthrone reagent and heated in a boiling water bath for 7 min, followed by cooling with tap water. The absorbance of the blue-green color that developed was measured at 620 nm. The sugar concentrations were determined from a standard curve constructed using known amounts of glucose.

Determination of chlorophyll a content. Chlorophyll *a* (Chl *a*) was extracted from cells using methanol. The concentrations of Chl *a* were calculated from the absorbance at 665 nm values using an extinction coefficient of 78.741 L g^{-1} cm^{-1} (Meeks and Castenholz, 1971).

Measurement of O_2 evolution and O_2 consumption. Photosynthetic O_2 evolution was measured using a Clark-type oxygen electrode (Rank Brothers, Ltd., Cambridge, UK) with 10 mM $NaHCO_3$ as a final electron acceptor under saturated actinic light of 1,600 μE m^{-2} s^{-1} at 30°C. Using the same apparatus, O_2 consumption was measured under dark conditions.

Purification of a thermostable β-D-glucosidase. The water-soluble fraction of *N. commune* retained in 0.75 M potassium phosphate buffer (pH 7.0) was subjected to centrifugation at 28,000 × *g* for 30 min at 4°C. The supernatant was fractionated by the addition of ammonium sulphate to 40% saturation followed by centrifugation at 28,000 × *g* at 4°C for 30 min. The pellet was discarded and ammonium sulphate was added

to the supernatant to increase its saturation to 80%. The solution was centrifuged at 28,000×*g* at 4°C for 30 min, and the pellet was dissolved in 50 mM Tris-HCl (pH 7.5). After dialysis of the sample overnight against 50 mM Tris-HCl (pH 7.5), the dialysate was boiled in a water bath at 100°C for 20 min and cooled to room temperature. The boiled dialysate was applied to a DEAE-cellulose column (3.5×25 cm) equilibrated with 50 mM Tris-HCl (pH 7.5). After washing the column with five column volumes of 50 mM Tris-HCl (pH 7.5), proteins were eluted stepwise with 150 mM NaCl in 50 mM Tris-HCl (pH 7.5). After overnight dialysis of the eluted proteins, the dialysate was concentrated using a Microcon YM-10 unit (Millipore Corporation, Bedford, USA).

Purification of a 36-kDa WspA protein with thermostable β-D-galactosidase activity. The water-soluble fraction of *N. commune* retained in 0.75 M phosphate buffer (pH 7.0) was fractionated by the addition of ammonium sulphate to 15% saturation followed by centrifugation at 28,000×*g* at 4°C for 30 min. The pellet was discarded and ammonium sulphate was added to the supernatant to increase its saturation to 50%. The solution was centrifuged at 28,000×*g* at 4°C for 30 min, and the pellet was dissolved in 10 mM Tris-HCl (pH 7.5). After dialysis of the sample overnight against 10 mM Tris-HCl (pH 7.5), the dialysate was subjected to ultracentrifugation at 148,000×*g* at 4°C for 1 h. The buffer concentration of the supernatant was adjusted to 50 mM Tris-HCl (pH 7.5), and the solution was applied to a DEAE-cellulose column (3.5×25 cm) equilibrated with 50 mM Tris-HCl (pH 7.5). The WspA protein collected in the breakthrough was concentrated using a Microcon YM-30 unit (Millipore Corporation).

Glycosidase assay. β-D-Glucosidase activity was measured by the hydrolysis of the artificial chromogenic substrate *p*-nitrophenyl β-D-glucopyranoside (N7006, Sigma). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 5 mM *p*-nitrophenyl β-D-glucopyranoside, and 100 µl of a β-D-glucosidase-containing solution in a total volume of 1 ml. The reaction mixture was incubated at 37°C for 12 h, and the increase in the absorbance at 405 nm was measured using a spectrophotometer (Model U-2800, Hitachi). An extinction coefficient of 54 μM^{-1} cm^{-1} at 405 nm was used to calculate the *p*-nitrophenol concentration. One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of *p*-nitrophenyl β-D-glucopyranoside to release 1 µmol of *p*-nitrophenol per min. Substrate specificity was characterized using the *p*-nitrophenyl-linked sugars *p*-nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl.-α-D-glucopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, *p*-nitrophenyl-α-D-mannopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, and *p*-nitrophenyl-α-D-galactopyranoside, which were purchased from Sigma (Tokyo, Japan) or Nacalai (Kyoto, Japan).

Protein determination. Protein concentrations were determined using the Coomassie Brilliant Blue (CBB) G-250 dye-binding method described by Bradford (1976), with bovine serum albumin (BSA) as a standard.

SDS-PAGE. SDS-PAGE was carried out according to the method of Laemmli (1970), except that the acrylamide concentration was 12.5% (Morsy et al., 2008). The samples were mixed with reducing loading buffer and heated in a boiling water bath for 5 min, followed by immediate cooling on ice. Electrophoresis was carried out at a constant current of 5 mA while the proteins were in the stacking gel and 10 mA while the proteins were in the separating gel, using vertical electrophoresis glass plates. Proteins were stained using CBB R-250 and destained in a solution of 7.5% acetic acid and 25% methanol. Molecular masses were estimated using a molecular size marker set (Dalton Mark VII-L, Sigma) composed of bovine serum albumin (66 kDa), egg albumin (45 kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and bovine α-lactalbumin (12 kDa).

Non-denaturing polyacrylamide gel electrophoresis. Non-denaturing PAGE was carried out according to the method described by Nakajima et al. (2002) with modifications (Morsy et al., 2008). The separating gels contained 7.5% acrylamide, 0.2% bis-acrylamide, and 0.375 M Tris-HCl (pH 8.6), and the stacking gels contained 3.5% acrylamide, 0.093% bis-acrylamide, and 0.25 M Tris-HCl (pH 6.8). The running buffer contained 25 mM Tris and 192 mM glycine. Electrophoresis was carried out using vertical electrophoresis glass plates at a constant voltage of 50 V while the proteins were in the stacking gel and 100 V while the proteins were in the separating gel.

Determination of N-terminal amino-acid sequences of purified proteins. Following SDS-PAGE, the proteins were electrophoretically transferred to PVDF membrane (0.45-µm pore size, Millipore) using semi-

dry blotting with an ATTO Horizeblot AE-6670 at a constant current of 1 mA cm^{-2} for 90 min. N-terminal amino-acid sequencing of the protein was performed using automated Edman degradation at the Division of Biological Sciences Protein Sequencing Facility at the University of California at San Diego, California, USA.

Results

Degradation of colonies by rehydration

While washing *N. commune* colonies harvested from the field, we have observed the degradation of colonies when they were immersed in water for a few days. This process of colony degradation was subsequently examined under laboratory conditions (Fig. 1). When desiccated *N. commune* colonies were rehydrated in 25 mM HEPES–NaOH (pH 7.0) with continuous stirring using a magnetic stirrer for 3 days at room temperature, some degradation of the colonies was observed after 1 day (Fig. 1A). After 2 days, almost all of the colonies were degraded and homogenized (Fig. 1A). Microscopic examination during the experiment revealed filaments of cells in the homogenate (Fig. 1C). The chlorophyll content and the levels of photosynthetic O_2 evolution and O_2 consumption in the homogenate were essentially similar to those of freshly rehydrated colonies when normalized to dry mass. The homogenate contained 2.0±0.3 mg Chl *a* g^{-1} dry mass, and approximately 210 μmol O_2 g^{-1} dry mass h^{-1} of photosynthetic O_2-evolving activity and approximately 130 μmol O_2 g^{-1} dry mass h^{-1} of O_2 consumption were detected. These results suggest that vital cells with intact biological activities are released into the water during the degradation of the colonies. Since the degradation of the colonies was inhibited by incubation at 4°C or treatment in a boiling-water bath in the presence of 1% SDS, we postulated that EPS-hydrolyzing enzymes are involved in this phenomenon. Thus, the activities that hydrolyze glycosidic bonds in the extracellular matrix were examined using artificial nitrophenyl-linked sugars as substrates.

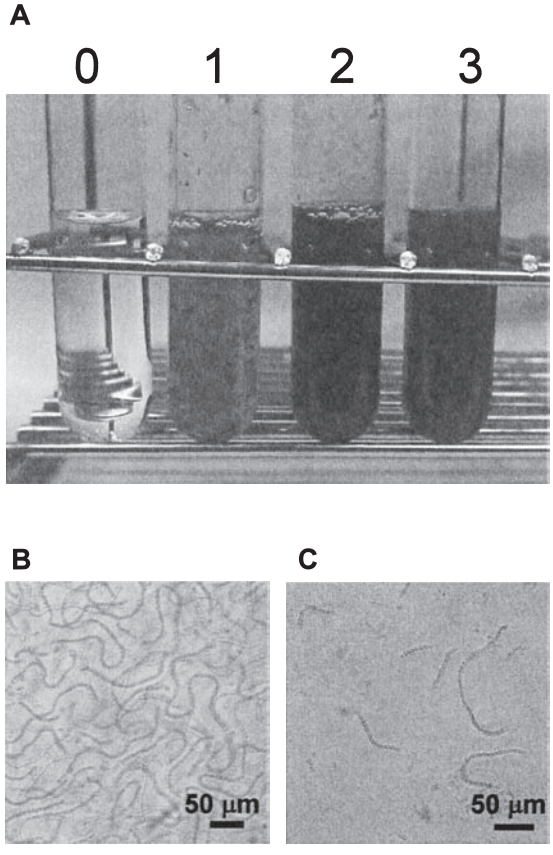

Fig. 1. Degradation of natural *Nostoc commune* colonies by rehydration.

A. Desiccated *N. commune* colonies were rehydrated in 25 mM HEPES–NaOH (pH 7.0) and continuously stirred using a magnetic stirrer for 3 days at room temperature. The homogenate was transferred to a test tube and photographed. 0, after 10 min of rehydration; 1, after 1 day; 2, after 2 days; 3, after 3 days. B. Cells in a natural *N. commune* colony with EPSs. C. Cells in the homogenate after 2 days of rehydration.

Separation of EPSs, water-soluble fraction, and EPS-depleted N. commune cells

A method for the separation of EPSs, the water-soluble fraction of the extracellular matrix, and EPS-depleted cells of *N. commune* colonies was developed. Table 1 shows the quantitative analysis of the EPSs, the water-soluble fraction, and the EPS-depleted cells of *N. commune* separated using this method. The total recovered EPSs represented 80% of the total dry mass, and the cells represented only 6%, indicating that EPSs are the major component of *N. commune* colonies. Approximately 96% of the total proteins were recovered from EPS-depleted cells, and chlorophyll was found to be especially abundant, indicating that almost all of the cells of the *N. commune* colonies were recovered with this method. Figure 2 shows the protein profiles of crude extracts of the EPS-depleted *N. commune* cells (Fig. 2, lane 1) and the water-soluble fraction retained in the 0.75 M phosphate buffer (Fig. 2, lane 2). The major protein band in the water-soluble fraction had a molecular mass of 36 kDa (Fig. 2, lane 2), and this protein was further characterized as de-

Table 1. Separation of extracellular polysaccharides (EPSs), the water-soluble fraction, and EPS-depleted cells of the terrestrial cyanobacterium *Nostoc commune*.

Fraction	Dry mass		Total polysaccharides[c]	Total protein		Chl[a]	
	g	%	g	mg	%	mg	%
Homogenate of *N. commune* colonies	165	100	NM[a]	4,300	100	79	100
EPS-depleted cells	10	6	NM	4,150	96	78	99
Water-soluble fraction[b]	20[d]	12	19.5	74	1.7	ND[e]	0
EPS	134[d]	81	132	22	0.5	ND	0

[a]NM, not measured.

[b]Solubilized in 0.75 M phosphate buffer (pH 7.0) as described in MATERIALS AND METHODS.

[c]The polysaccharide content was estimated as the glucose equivalent after acid hydrolysis, as described in MATERIALS AND METHODS.

[d]After dialysis against distilled water, the samples were dried at 70°C.

[e]ND, not detectable.

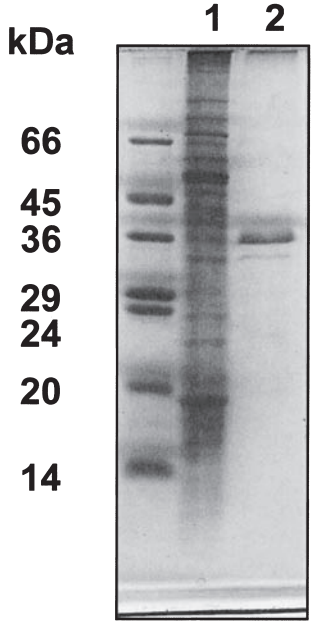

Fig. 2. Protein profile of the terrestrial cyanobacterium *N. commune*.

A crude extract of EPS-depleted cells of *N. commune* (lane 1) and the water-soluble fraction retained in 0.75 M phosphate buffer (pH 7.0) prepared as described in MATERIALS AND METHODS (lane 2) were fractionated by SDS-PAGE and stained with Coomassie Brilliant Blue (CBB). A molecular size marker (Dalton Mark VII-L, Sigma, SDS-7) was used as a standard.

scribed below.

Purification and characterization of thermostable β-D-glucosidase

The cyanobacterium *N. commune* contained glycosidase activities that hydrolyzed different substrates (Table 2). Most of the glycosidase activities were released from *N. commune* colonies in 0.75 M phosphate buffer and were recovered in the water-soluble fraction (Table 2). The water-soluble fraction contained 71% of the total β-D-glucosidase activity. After boiling for 20 min, 97% of the β-D-glucosidase activity in the water-soluble fraction was still present. The crude extract of EPS-depleted cells contained 19% of the total β-D-glucosidase, but this activity was not heat-resistant. These results indicate that the thermostable β-D-glucosidase is localized in the extracellular matrix and is solubilized by 0.75 M phosphate buffer.

The thermostable β-D-glucosidase was purified to homogeneity with a purification factor of 700-fold (Table 3). Following DEAE-cellulose chromatography, the purified protein appeared as a single band with an apparent molecular mass of 20 kDa in SDS-PAGE (Fig. 3A), and also as a single band in non-denaturing PAGE (Fig. 3B). The single protein band was eluted from the non-denaturing polyacrylamide gel to examine its β-D-glucosidase activity, and almost all of the activity that was applied was recovered, demonstrating that the purified protein is a β-D-glucosidase. Table 4 shows the substrate specificity of the enzyme. The enzyme specifically hydrolyzed β-D-glucopyranoside, but showed low activities toward other β- and α-linked glycosides.

The N-terminal amino-acid sequence of the purified 20-kDa thermostable β-D-glucosidase was ANIVDTAVNNGSFNTLVAAI, which is similar to the N-terminal amino-acid sequences of hypothetical proteins anno-

Table 2. Glycosidase activities in *N. commune*.

Fraction	Total protein	Substrate					
		β-D-Gluco-pyranoside	α-D-Gluco-pyranoside	β-D-Galacto-pyranoside	α-D-Galacto-pyranoside	β-D-Manno-pyranoside	α-D-Manno-pyranoside
	mg (%)	U[a] (%)	U[a] (%)	U[a] (%)	U[a] (%)	U[a] (%)	U[a] (%)
Homogenate of *N. commune* colonies	4,300 (100)	150 (100)	100 (100)	134 (100)	117 (100)	42 (100)	27.5 (100)
EPS-depleted cells	4,150 (96)	28 (19)	16 (16)	18.5 (14)	12 (10)	4.5 (11)	2.5 (9)
Water-soluble fraction[b]	74 (1.7)	107 (71)	68 (68)	98 (73)	89 (76)	30 (72)	19 (70)
EPS	22 (0.5)	13 (9)	15 (15)	16.5 (12)	15 (13)	7 (16)	5 (19)

The experiments were repeated three times and mean values are shown.

[a]One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of *p*-nitrophenyl-linked glycopyranoside to release 1 μmol of *p*-nitrophenol per min.

[b]Solubilized in 0.75 M phosphate buffer (pH 7.0), as described in MATERIALS AND METHODS.

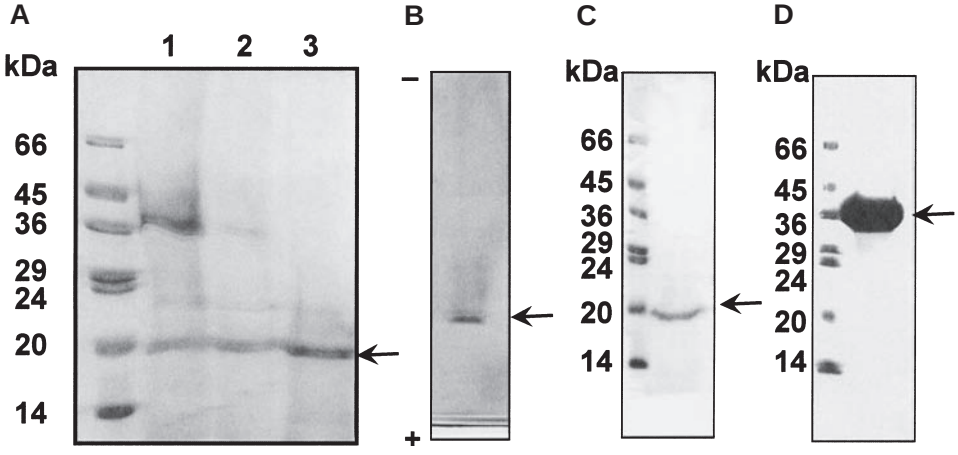

Fig. 3. Purification of a thermostable β-D-glucosidase and a thermostable β-D-galactosidase from *N. commune*.

A. Thermostable β-D-glucosidase. Samples of the water-soluble fraction solubilized in 0.75 M phosphate buffer (pH 7.0) containing 100 mU of the β-D-glucosidase activity (lane 1), the 40–80% ammonium sulphate precipitate fraction containing 100 mU of the thermostable β-D-glucosidase activity (lane 2), and the active fraction after DEAE-cellulose chromatography containing 200 mU of the thermostable β-D-glucosidase activity (lane 3), were fractionated using SDS-PAGE and stained with CBB. B. Non-denaturing PAGE of the thermostable β-D-glucosidase. The purified thermostable β-D-glucosidase (100 mU) was fractionated with PAGE under non-denaturing conditions and stained with CBB. The arrowhead shows the β-D-glucosidase band. C. The β-D-glucosidase band eluted from the non-denaturing gel with 260 mU of activity was fractionated by SDS-PAGE and stained using CBB. D. WspA protein with thermostable β-D-galactosidase activity. The arrowhead shows the 36-kDa WspA protein after DEAE-cellulose chromatography. A molecular size marker (Dalton Mark VII-L, Sigma, SDS-7) was used as a standard.

Table 3. Purification of a thermostable β-D-glucosidase from *N. commune*.

Steps	Total protein	Total activity	Specific activity	Yield	Purification factor
	mg	U[a]	U mg protein^{-1}	%	fold
Crude extract	4,300	52.5	0.01	100	1
Water-soluble fraction[b]	74	51	0.7	97	70
40–80% ammonium sulphate precipitation	17	40	2.4	76	240
DEAE-cellulose	4	28	7	53	700

[a]One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of *p*-nitrophenyl-linked glucopyranoside to release 1 μmol of *p*-nitrophenol per min.

[b]Solubilized in 0.75 M phosphate buffer (pH 7.0) as described in MATERIALS AND METHODS.

Table 4. Substrate specificity of the purified thermostable β-D-glucosidase from *N. commune*.

Substrate	Activity[a]	
	U mg protein^{-1}	%
p-Nitrophenyl-β-D-glucopyranoside	7	100
p-Nitrophenyl-α-D-glucopyranoside	0.14	2
p-Nitrophenyl-β-D-mannopyranoside	0.28	4
p-Nitrophenyl-α-D-mannopyranoside	0.07	1
p-Nitrophenyl-β-D-galactopyranoside	0.35	5
p-Nitrophenyl-α-D-galactopyranoside	0.17	2

The experiments were repeated three times and mean values are shown.

[a]One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of *p*-nitrophenyl-linked glycopyranoside to release 1 μmol of *p*-nitrophenol per min.

Table 5. Substrate specificity and thermostability of the β-D-galactosidase activity of the purified 36-kDa WspA from *N. commune*.

Substrate	Activity[a]		Thermostable activity[b]	
	U mg protein^{-1}	%	U mg protein^{-1}	%
p-Nitrophenyl-β-D-galactopyranoside	2	100	2	100
p-Nitrophenyl-α-D-galactopyranoside	0	0	0	0
p-Nitrophenyl-β-D-mannopyranoside	0	0	0	0
p-Nitrophenyl-α-D-mannopyranoside	0	0	0	0
p-Nitrophenyl-β-D-glucopyranoside	0.2	10	0.2	10
p-Nitrophenyl-α-D-glucopyranoside	0	0	0	0

The experiments were repeated twice and mean values are shown.

[a]One unit of activity was defined as the amount of enzyme that catalyzes the hydrolysis of *p*-nitrophenyl-linked glycopyranoside to release 1 μmol of *p*-nitrophenol per min.

[b]The purified 36-kDa WspA protein was boiled in a water bath for 20 min and the remaining thermostable activity was measured.

tated as surface and secreted fasciclin proteins from the cyanobacteria *Nostoc punctiforme* PCC 73102 (accession number ZP_00106518), *Anabaena variabilis* ATCC 29413 (accession number YP_322682), and *Nostoc sp*. PCC 7120 (all4894, accession number NP_488934).

Characterization of a WspA with thermostable β-D-galactosidase activity

The major protein in the extracellular matrix, with a molecular mass of 36 kDa (Fig. 2, lane 2), was purified (Fig. 3D). The purified 36-kDa protein specifically hydrolyzed β-D-galactopyranoside but showed little or no activity toward other β- or α-linked glycosides (Table 5). The activity was still detected after boiling for 20 min (Table 5), indicating that it is heat-resistant. The

apparent molecular mass of 36 kDa and the abundance of the protein in the extracellular matrix (Fig. 2) are in good agreement with those of water-stress protein (WspA) (Hill et al., 1994b; Wright et al., 2005). The N-terminal amino-acid sequence of the purified 36-kDa protein was ALYGYTIGEDR, which is identical to that of WspA of *N. commune* (Wright et al., 2005). These results demonstrate that the WspA protein has thermostable β-D-galactosidase activity.

Discussion

The terrestrial cyanobacterium *N. commune* forms a visible colony that consists of EPSs and cellular filaments embedded within the EPSs. *N. commune* colonies are naturally subjected to regular cycles of desiccation and wetting in their native habitats. In this study, we found that *N. commune* colonies degraded within a few days when immersed in continuously stirred water (Fig. 1). This degradation occurred rather slowly and was not evident in desiccating colonies; wet colonies are usually desiccated overnight when air-dried under laboratory conditions. The release of vital cells was accompanied by colony degradation (Fig. 1C), a phenomenon that may have a role in the vegetative reproduction of the cells and possibly the dispersal of cells, to expand the colonies. It remains to be characterized whether the formation of motile hormogonia occurs and disperses the cells during the degradation of the colonies, although the conditions that induce hormogonia formation in this cyanobacterium have not yet been elucidated (Campbell et al., 2007; Potts, 2000).

Enzymatic activities that hydrolyze glycoside bonds were released from the *N. commune* extracellular matrix (Table 2), and these enzymes may be involved in the hydrolysis of the EPSs surrounding the cells. The in-vitro hydrolysis of EPSs by the purified β-D-glucosidase was tested according to methods used for other bacterial enzymes that degrade *N. commune* EPSs (Dasman et al., 2002). The *N. commune* β-D-glucosidase did not degrade EPSs extracted from this organism (data not shown). Since it is known that various microorganisms reside in the extracellular matrix of the natural colonies of *N. commune*, we cannot completely rule out the possibility that enzymes derived from these microorganisms may be involved in the degradation of colonies. The details of the degradation of *N. commune* colonies remain to be characterized in future studies.

The N-terminal sequence of the purified β-D-glucosidase of *N. commune* identified it as a fasciclin protein, which is thought to be a secreted surface protein. Genes encoding fasciclin proteins are found in all cyanobacteria whose genomes have been sequenced. The hypothetical proteins that they encode have deduced molecular masses of about 14 kDa and are composed of a single FAS1 domain, which is easily recognized due to the presence of two conserved sequence motifs, termed H1 and H2 (Kawamoto et al., 1998). Fasciclin I, consisting of four FAS1 domains, was originally identified as a neuronal cell adhesion molecule in insects (Clout et al., 2003; Snow et al., 1988), and FAS1 domains are present in many secreted and membrane-anchored proteins in prokaryotes and eukaryotes (Billings et al., 2002; Borner et al., 2002; Carr et al., 2003; Elkins et al., 1990; Huber and Sumper, 1994; Ito et al., 2005; Johnson et al., 2003; Kim et al., 2000, 2002; Sato et al., 2004). It is worth noting that a fasciclin protein is highly expressed in *Nostoc* symbionts of lichens (Paulsrud and Lindblad, 2002), although the role of this protein in the symbionts has not been characterized. It has been shown that the FAS1 domain is involved in cell adhesion and interaction with the extracellular matrix (Billings et al., 2002; Kim et al., 2000), suggesting that the *N. commune* fasciclin protein with β-D-glucosidase activity also functions on the cell surface or in the extracellular matrix. A molecular-genetic analysis and the creation of a mutant with an inactivated fasciclin gene will be required to directly demonstrate the functions of the fasciclin protein in *N. commune*, although experimental conditions for the transformation of this organism have not yet been established. To examine the functions of fasciclin proteins in cyanobacteria, a molecular-genetic characterization using model cyanobacteria such as *Nostoc* sp. PCC7120 would be a promising avenue for future studies.

It has been reported that water-stress proteins (WspA), which have molecular masses of 30 to 39 kDa, are the most abundant soluble proteins isolated from extracellular glycans of *N. commune* (Hill et al., 1994b; Scherer and Potts, 1989; Wright et al., 2005) and bind the UV-A absorbing pigment scytonemin (Wright et al., 2005). In this study, WspA was purified as a single band with a molecular mass of 36 kDa and no microheterogeneity of WspA was present in our preparation from naturally growing colonies of *N. commune* (Figs. 2 and 3). It has been mentioned that the

rehydration of desiccated colonies leads to structural changes in the extracellular matrix (Shaw et al., 2003), and that WspA associated with a xylanase activity has a role in the modification of the glycan structure through limited hydrolysis of crosslinks (Hill et al., 1994b; Potts, 2000). To test this idea, we investigated whether the purified WspA had any glycosidase activities (Table 5). The purified WspA was colorless to the eye and, therefore, not likely to be associated with the UV-A/B-absorbing pigments scytonemin or mycosporine-like amino acids, suggesting that these pigments are not required for the β-D-galactosidase activity. The modification of EPSs by the glycosidases in the extracellular matrix remains to be characterized in future studies, which should elucidate the mechanisms of the extreme tolerance to desiccation of this terrestrial cyanobacterium.

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