

# Purification and Properties of an Enzyme Capable of Degrading the Polysaccharide of the Cyanobacterium, *Nostoc commune*<sup>§</sup>

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*Nostoc commune*, *Paenibacillus glycanilyticus*, Polysaccharide-Degrading Enzyme

A novel *Nostoc commune*-polysaccharide (NPS)-degrading enzyme with a molecular mass of 128.5 kDa was purified from *Paenibacillus glycanilyticus* DS-1. The optimum pH and temperature of the enzyme activity were 5.5 and 35 °C, respectively. The enzyme completely degraded NPS to oligosaccharides, ranging from tetra to hexasaccharides and could degrade the xylan weakly whereas xanthan, gellan, cellulose, curdlan and *p*-nitrophenyl- $\beta$ -D-xylopyranoside were not degraded. Homology analysis of the *N*-terminal amino acid sequence of the NPS-degrading enzyme against the PIR and SWISS-PROT databases indicated that the sequence was not homologous to any other polysaccharide-degrading enzyme.

## Introduction

*Nostoc commune*, a nitrogen-fixing filamentous cyanobacterium, produces copious amounts of viscous extracellular polysaccharide around cells. Although the polysaccharide is thought to play important roles in desiccation tolerance (Hill *et al.*, 1994), and UV tolerance (Scherer *et al.*, 1988) of this cyanobacterium, little is known about its chemical structure, primarily due to its structural complexity. Helm *et al.* have tried to degrade viscous polysaccharides of *N. commune* DRH-1 using conventional enzymes, but their attempt was unsuccessful (Helm *et al.*, 2000). Specific polysaccharide-degrading enzymes are one of the most useful tools for structural study of complex polysaccharides. As recently reported (Dasman *et al.*, 2002), we have isolated a new soil bacterium, *Paenibacillus glycanilyticus* DS-1, with the ability to degrade the *N. commune*-polysaccharide (NPS).

We herein report the purification and characterization of an NPS-degrading enzyme from the bacterium that should become a useful tool in the structural determination of the NPS.

## Materials and Methods

### Preparation of *Nostoc commune*-polysaccharide

*Nostoc commune*-polysaccharide (NPS) was prepared as previously described (Dasman *et al.*, 2002). Field-grown *N. commune* was washed with water and soaked in methanol at room temperature for 3 days. *N. commune* was then dried at room temperature for 3 days, and dry *Nostoc commune* (10 g) was ground in a mortar. The resulting powder was extracted with boiling water for 3 h. The suspension was filtered and a polysaccharide fraction was recovered by ethanol precipitation (final ethanol concentration: 75% v/v). After centrifugation (10,000  $\times$  g, 4 °C for 15 min), the precipitate was dissolved in distilled water, dialyzed and lyophilized to obtain crude NPS (0.72 g).

### Bacterial strain and culture conditions

*Paenibacillus glycanilyticus* DS-1 was isolated from a soil sample of Osaka University Campus as previously described (Dasman *et al.*, 2002). *P. glycanilyticus* DS-1 cells were cultured at 30 °C in LB medium containing 1% tryptone, 0.5% yeast extract and 1% NaCl with 0.2% NPS (NPS medium). Voucher specimen of the strain DS-1 was deposited in IFO 16618<sup>T</sup> = JCM 11221<sup>T</sup> = NRRL B-23455<sup>T</sup>.

<sup>§</sup> This study represents part of a dissertation submitted by Dasman to Osaka University in partial fulfillment of the requirement for a Ph.D. degree.

### Enzyme assays

The activity of NPS-degrading enzyme was measured by determining the amount of reducing sugar released from NPS. The reaction mixture containing 0.1% NPS in 50 mM citrate phosphate buffer (pH 5.5) and the enzyme were incubated for 2 h at 30 °C, the increase in the amount of reducing sugar was determined by the Somogyi-Nelson method (Nelson, 1944). One unit of activity was defined as the amount of enzyme required to release 1  $\mu$ mol of the reducing sugar per min from the NPS. The protein content was measured by the BCA method (Redinbaugh *et al.*, 1986), with a bovine serum albumin as the standard.

### Preparation of crude NPS-degrading enzyme

*P. glycanilyticus* DS-1 (Dasman *et al.*, 2002) was cultured for 72 h at 30 °C in 600 ml of the NPS medium (150 ml/flask) and harvested by centrifugation at  $10,000 \times g$  at 4 °C for 30 min. The supernatant was used as an extracellular enzyme source and was concentrated by ultrafiltration (molecular weight 20 kDa cutoff, 76 mm, Toyo Roshi Kaisha, Ltd, Tokyo, Japan). The concentrated solution was dialyzed against 20 mM potassium phosphate buffer (pH 7.0). After centrifugation at  $10,000 \times g$  at 4 °C for 10 min, the supernatant was subjected to further purification.

### Ion-exchange chromatography

The crude enzyme solution obtained above was filtered through a 0.20  $\mu$ m sterile filter (DISMIC-25cs, Toyo Roshi Kaisha, Ltd, Tokyo, Japan) and applied to a RESOURCE Q, anion-exchange chromatography (AEC) column, (Amersham Pharmacia Biotech Co., Uppsala, Sweden) previously equilibrated with 20 mM potassium phosphate buffer (pH 7.0). The enzyme was eluted with a linear gradient of 0 to 1 M NaCl in 20 mM phosphate buffer (pH 7.0) at a flow rate of 1 ml/min, and fractions were collected every 10 ml. The active fractions were combined and dialyzed in 20 mM of 2-amino-2-hydroxymethyl-1, 3-propanediol-hydrogen chloride (Tris-HCl) buffer (pH 7.0). The enzyme was applied to RESOURCE Q, AEC column, previously equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The enzyme was eluted with a linear gradient of 0 to 0.5 M NaCl in 20 mM Tris-

HCl buffer (pH 7.0) at a flow rate of 1 ml/min, and fractions were collected every 5 ml. The partially purified enzyme solutions were combined and dialyzed in 20 mM Tris-HCl buffer (pH 7.0), containing 0.15 M NaCl.

### Gel filtration chromatography

The partially purified enzyme was applied to a Superdex 75 HR 10/30, gel filtration chromatography column (Amersham Pharmacia Biotech Co., Uppsala, Sweden) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.0) containing 0.15 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 0.3 ml/min, and fractions were collected every 1 ml.

### Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to the method of Laemmli (1970) by using a Hoeffer Mighty Small system (Amersham Pharmacia Biotech Co., Uppsala, Sweden). Gels were stained with Coomassie Brilliant blue.

### N-terminal amino acid sequence of the purified enzyme

The N-terminal sequences analysis was outsourced (Takara, Shuzo Co., Ltd, Kyoto, Japan) after the band of the purified enzyme by SDS-PAGE was cut.

### Apparent molecular mass

The molecular mass of the enzyme was estimated by SDS-PAGE with commercial marker proteins.

### pH and temperature

The effect of pH on the activity of the purified enzyme was determined in 50 mM citrate phosphate buffer (pH 3.0 to 7.0), potassium phosphate buffer (pH 5.0 to 7.0), Tris-HCl buffer (pH 7.5 to 9.0), and glycine-sodium hydroxide buffer (pH 9.0 to 11.0) using 0.1% NPS as substrate. Effect of pH on the stability was performed by pre-incubation of the enzyme overnight at various pHs (pH 3.0 to 11.0). Optimal temperature was determined by incubating the enzyme solution with NPS in 50 mM citrate phosphate buffer (pH 5.5) for 2 h at various

temperatures. Thermal stability was determined by incubating the enzyme solution for 10 min at various temperatures. After incubation, the remaining activity was measured at 30 °C for 2 h.

#### Substrate specificity

Substrate specificity of the purified enzyme was assayed using a set of polysaccharides, i.e. xylan, xanthan, gellan, cellulose, curdlan and *p*-nitrophenyl- $\beta$ -D-xylopyranoside. The degradation products were analyzed by silica gel TLC (No. 5554 aluminum plate, Merck, Darmstadt, Germany) with a solvent system of 1-butanol-acetic acid-water (2:1:1, v/v) together with oligosaccharide standards. The spots were visualized by heating at 110 °C for 5 min after spraying with orcinol reagent.

## Results and Discussion

#### Purification of the NPS-degrading enzyme

The result of the purification process is shown in Table I. The NPS-degrading enzyme was purified from NPS containing culture fluid in which *P. glycanilyticus* DS-1 cells were grown. The culture fluid was concentrated by ultrafiltration. The major part of the active peak of the NPS-degrading enzyme was eluted by 0.2–0.5 M NaCl solution during the first RESOURCE Q column chromatography (Fig. 1a). Fractions 3 to 5 were combined and further purified. The most effective step was the second RESOURCE Q column chromatography (Table I. and Fig. 1b). The NPS-degrading enzyme was eluted by 0.35–0.4 M NaCl solution. Fractions 16 to 19 were pooled and applied to Superdex 75 HR 10/30, gel filtration column chromatography (Fig. 1c). The NPS-degrading enzyme (fraction 6 in Fig. 1c) was purified 26.4-fold with

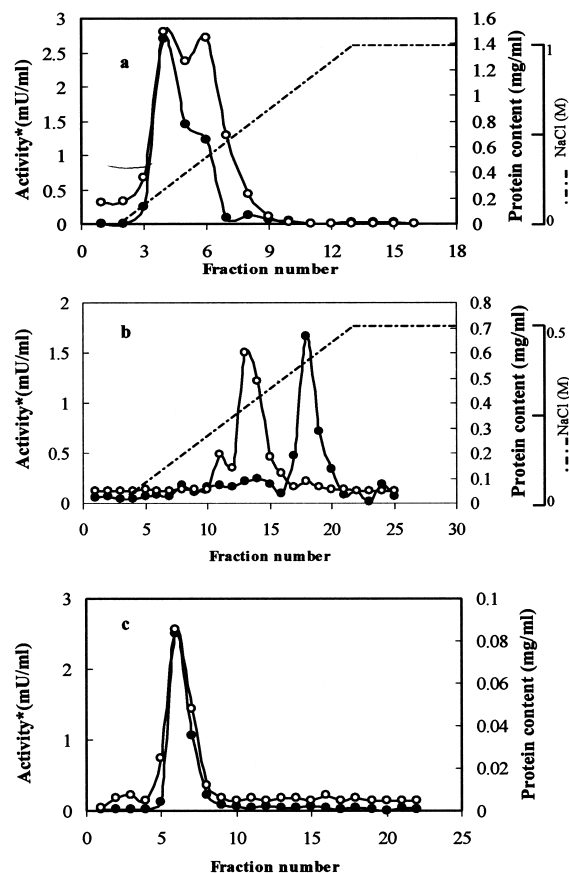


Fig. 1. (a) First RESOURCE Q column chromatogram. Conditions: flow rate, 1 ml/min; temperature 4 °C. (b) Second RESOURCE Q column chromatogram. Conditions: flow rate, 1 ml/min; temperature 4 °C. (c) Superdex 75 HR column chromatogram. Conditions: flow rate, 0.3 ml/min; temperature 4 °C. Symbols: closed circles, activity (mU/ml); open circles, protein content (mg/ml); dashed line, NaCl concentration (M). \* One unit (U) of activity was defined as the amount of enzyme required to release 1  $\mu$ mol of the reducing sugar per min from the NPS.

Purification steps	Total activity [mg]	Total activity* [U]	Specific activity [U/mg]	Purification (fold)	Recovery (%)
Culture fluid	534.79	0.589	0.0011	1	100
	81.35	0.124	0.0015	1.36	21
Ultra filtration					
1st RESOURCE Q	14.91	0.027	0.0024	2.18	4.6
2nd RESOURCE Q	0.436	0.0083	0.0189	17.18	1.4
Superdex 75 HR 10/30	0.085	0.0025	0.0290	26.40	0.43

Table I. Purification of the *Nostoc commune*-polysaccharide-degrading enzyme from *Paenibacillus glycanilyticus* DS-1.

\* One unit (U) of activity was defined as the amount of enzyme required to release 1  $\mu$ mol of the reducing sugar per min from the NPS.

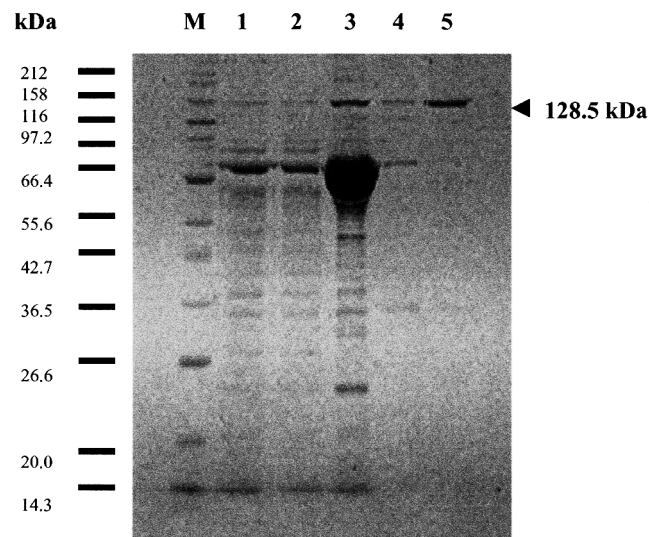


Fig. 2. Electrophoretic profiles of the *Nostoc commune*-polysaccharide-degrading enzyme. The purified NPS-degrading enzyme was subjected to SDS-PAGE and stained with Coomassie Brilliant blue. Lane M, molecular weight standards consisting of myosin (212 kDa), MBP- $\beta$ -galactosidase (158 kDa),  $\beta$ -galactosidase (116 kDa), phosphorylase *b* (97.2 kDa), serum albumin (66.4 kDa), glutamic dehydrogenase (55.6 kDa), MBP2 (42.7 kDa), lactate dehydrogenase (36.5 kDa), triose phosphate isomerase (26.6 kDa), trypsin inhibitor (20.0 kDa), and lysozyme (14.3 kDa). Lane 1, culture fluid pool; lane 2, ultra filtration pool, lane 3, 1st RESOURCE Q (AEC); lane 4, 2nd RESOURCE Q (AEC); lane 5, purified enzyme (128.5 kDa) from *P. glycanilyticus* DS-1 after Superdex 75 HR 10/30. Arrows indicate the positions of the purified NPS-degrading enzyme.

0.43% retention of total activity. The purified enzyme was found to be homogeneous by SDS-PAGE (Fig. 2).

#### Properties of the NPS-degrading enzyme

The molecular mass of the enzyme was estimated to be 128.5 kDa by SDS-PAGE (Fig. 2). The *N*-terminal amino acid sequence of the NPS-degrading enzyme was determined to be AAAISQNHTFET. The maximum activity was observed at pH 5.5 is near that of xanthan lyase from the *Paenibacillus alginolyticus* XL-1 (pH 6.0) (Ruijsenaars *et al.*, 1999) and the enzyme was stable over a wide pH range at 30 °C (pH 5.0 to 8.0). The maximal enzyme activity was at 35 °C at pH 5.5. Incubation of the enzyme at 45 °C for 10 min caused a loss of over 73% activity, and incubation at 55 °C completely inactivated the enzyme. However there is a difference: this enzyme was stable below 40 °C at pH 5.5 whereas the *Paenibacillus alginolyticus* XL-1 is stable below 45 °C (Ruijsenaars *et al.*, 1999).

#### Substrate specificity

The NPS-degrading enzyme was assayed with various substrates to study the substrate specificity. The NPS-degrading enzyme completely degraded the NPS to oligosaccharides, ranging from

tetra to hexasaccharides (data not shown). The following substrates were checked to determine the specificity of the NPS-degrading enzyme: xylan, xanthan, gellan, cellulose, curdlan and *p*-nitrophenyl- $\beta$ -D-xylopyranoside. The enzyme could weakly degrade xylan (49.5%), whereas the xanthan, gellan, cellulose, curdlan and *p*-nitrophenyl- $\beta$ -D-xylopyranoside were not degraded. Homology analysis of the *N*-terminal amino acid sequence of the NPS-degrading enzyme against the PIR and SWISS-PROT databases indicated that the sequence was not homologous to any other known polysaccharide degrading-enzyme.

As a result, this purified NPS-degrading enzyme is a novel polysaccharide-degrading enzyme that should be a useful tool in the structural determination of cyanobacterial polysaccharide, which should play an important role in desiccation and UV tolerance. We also found NPS-degrading activity in the cell free extract of *Paenibacillus glycanilyticus* cells according to the method of Hashimoto *et al.*, (Hashimoto *et al.*, 1999). The characterization and properties of intracellular enzyme is under investigation.

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