好アルカリ性Nocardiopsis sp.TOA-1株の生産するペクチン酸リアーゼの精製と諸性質

誌名	応用糖質科学 : oyo toshitsu kagaku = Journal of applied glycoscience
ISSN	13403494
著者名	満生,慎二 江口,博美 原,洋子 境,正志 森山,康司 後藤,正利 古川,謙介
発行元	日本応用糖質科学会
巻/号	49巻4号
掲載ページ	p. 497-500
発行年月	2002年10月

[*J. Appl. Glycosci.*, Vol. 49, No. 4, p. 497–500 (2002)]

Note

Purification and Some Properties of Pectate Lyase from Alkaliphilic *Nocardiopsis* sp. TOA-1

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An extracellular pectate lyase of alkaliphilic *Nocardiopsis* sp. TOA-1, designated NPLase, was purified to homogeneity. The molecular mass of NPLase was estimated to be 54 kDa. Highest activity of this enzyme was obtained at pH 10.0 and 40°C and the isoelectric point (p*I*) was 4.7. NPLase was an endo type pectate lyase which degrades polygalacturonic acid in a random manner.

Pectate lyases play an important role in the decomposition of plant residues and are widely distributed among soil microorganisms such as actinomycetes.[1,2] Some pectate lyases from *Streptomyces* sp.,[3–6] from *Thermonospora fusca*,[7] and from *Amycolata* sp.[2] have been isolated and characterized. However, there are no reports on pectinolytic enzymes from alkaliphilic actinomycetes. We have previously isolated an alkaliphilic actinomycetes strain *Nocardiopsis* sp. TOA-1 that produces a variety of alkaliphilic enzymes including pectinolytic enzyme.[8] Here we report purification and some properties of an extracellular pectate lyase from an alkaliphilic *Nocardiopsis* strain.

Nocardiopsis sp. strain TOA-1 was originally isolated from tile-joints of a bathroom.[8] Strain TOA-1 was cultivated for 48 h at 30°C in an alkaline pectin medium containing 0.5% pectin and 0.5% yeast extract in the presence of 1% Na_2CO_3. The culture was carried out at 30°C with 200 rpm agitation and 1 vvm airation in a 5L jar fermenter with 1% inoculum.

The NPLase was assayed as described by Kobayashi.[9] The reaction mixture (3.0 mL in total) contained 0.1 mL of diluted enzyme solution, 0.6 mL of 1.0% (w/v) polygalacturonic acid, 0.3 mL of 0.5 M glycine-NaCl-NaOH buffer (pH 10.0), 0.18 mL of 10 mM $CaCl_2$, and 1.92 mL of distilled water. The reaction was incubated at 30°C for 10 min and stopped by addition of 3.0 mL of 50 mM HCl. The production of unsaturated oligogalacturonides was measured at 235 nm. One unit of enzymatic activity was defined as the amount of enzyme which forms 1 μmol of a product per min with a molar extinction coefficient of 4600 M^{-1} cm^{-1}.[10] The protein content was determined by the Lowry method[11] using bovine serum albumin as a standard.

The highest level of pectate lyase was reached after 36 h of cultivation. The proteins of the culture broth were precipitated by adding $(NH_4)_2SO_4$ to get 80% saturation and the precipitate was dialyzed against 20 mM Tris-HCl buffer plus 1 mM $CaCl_2$ (pH 8.0, buffer A), and used as the crude enzyme. The crude enzyme solution containing

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Table 1. Summary of purification of NPLase.

Step	Volume (mL)	Activity (U/mL)	Protein (mg)	Specific activity (U/mg-protein)	Recovery (%)
Culture fluid	7000	0.5	5600	0.6	100
$(NH_4)_2SO_4$	190	15	292	9.8	81
Butyl-Toyopearl	34	48	18.5	88	47
DEAE-Toyopearl	3.3	350	7.8	148	33
Superdex 75	10.0	103	5.9	173	29

30% of $(NH_4)_2SO_4$ was then applied to a column of Butyl-Toyopearl 650M (ϕ2.5 cm×10 cm) that had been equilibrated with buffer A containing 30% of $(NH_4)_2SO_4$. After the column was washed with the same buffer, proteins were eluted using a 300-mL gradient of 30% to 0% $(NH_4)_2SO_4$ in buffer A. Fractions containing the pectate lyase activity were collected and dialyzed against buffer A. The dialyzate was applied to a column of DEAE-Toyopearl (ϕ1.0 cm×5.0 cm) that had been equilibrated with buffer A. After the column was washed with the same buffer, proteins were eluted using a 100-mL gradient of 0 to 1 M NaCl in the same buffer. Fractions containing the pectate lyase activity were collected and applied to a Superdex 75 column (ϕ2.6 cm×60 cm) that had been equilibrated with buffer A containing 0.15 M NaCl. The enzyme thus obtained was used as a purified NPLase.

SDS-PAGE was performed with 12% polyacrylamide.[12] The isoelectric point (p*I*) was determined using a mini IEF Cell (Bio-Rad) with 5% (w/v) acrylamide gel and 0.5% (w/v) ampholine (pH 3–10; Bio-Rad). Cytochrome c (Oriental yeast) was used as isoelectric point (p*I*) markers. The N-terminal amino acids were determined by using a protein sequencer (PPSQ-21, Shimadzu).

The purification of NPLase is summarized in Table 1. NPLase was purified to homogeneity on the criterion of SDS-PAGE (Fig. 1). A 288-fold purification was obtained with a recovery of 29%. The specific activity of NPLase toward polygalacturonic acid was determined to be 173 U/mg protein. The p*I* was determined to be 4.7.

The optimal temperature of NPLase was 40°C and optimal pH was 10.0 (Fig. 2A and 2C). NPLase was stable in pH ranges from 8.0 to 11.0 when incubated at 30°C for 10 min (Fig. 2B).

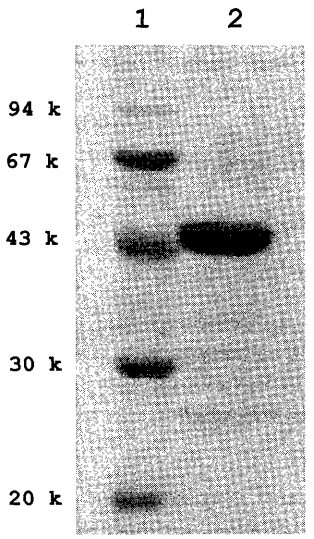

Fig. 1. SDS-PAGE of the purified NPLase.

Lane 1, molecular mass marker proteins: phosphorylase b, 94 kDa; bovine serum albumin, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrorase, 30 kDa; trypsin inhibitor, 20.1 kDa. Lane 2, purified NPLase.

NPLase was found to be stable below 30°C when incubated at various temperatures for 10 min (Fig. 2D). In the presence of 1 mM $CaCl_2$, NPLase became more stable in higher pH ranges (pH from 7.0 to 11.0) (Fig. 2B) and higher temperature at 40°C (Fig. 2D).

Pectate lyases are classified as either exo type or endo type.[3] These two types of digestion are differentiated by the rate of the decrease of substrate viscosity relative to the product accumulation. NPLase decreased the viscosity to 60% by cleavage of only 1.8% of the substrate bonds. This result suggests that NPLase is an endo type pectate lyase.

The N-terminal 15 amino acid was determined to be SDEPVGYASMNGGTT. The result of a

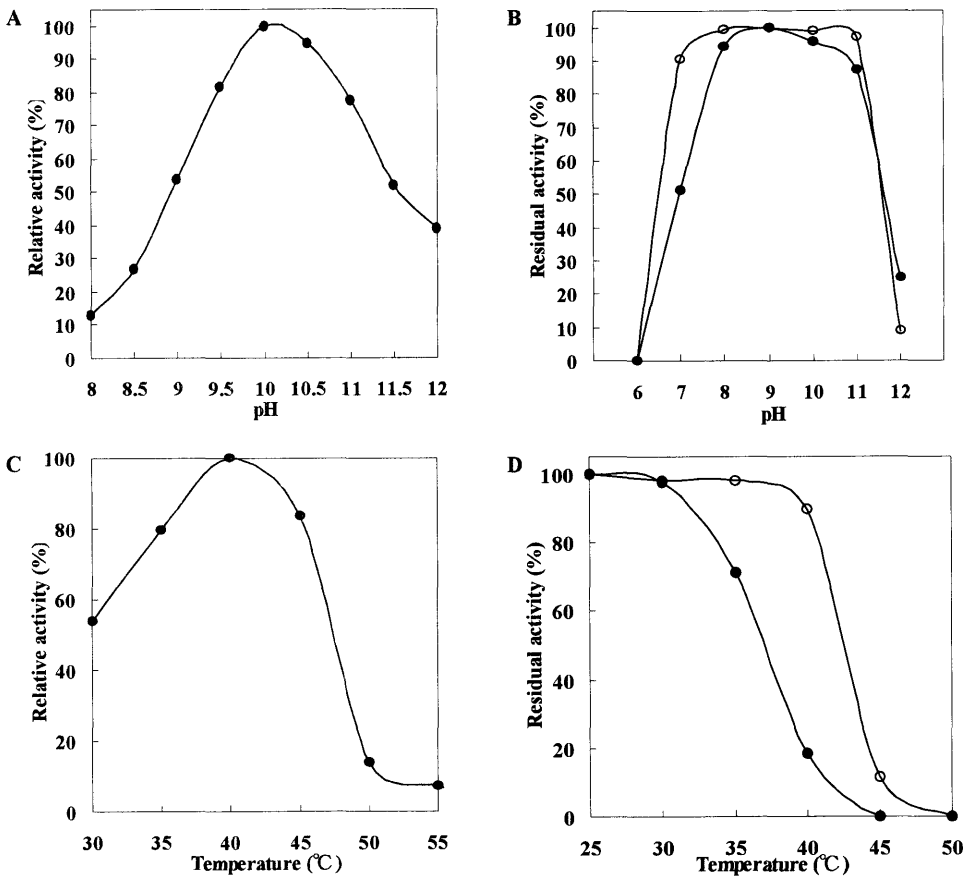

Fig. 2. Effects of pH and temperature on the activity and stability of NPLase.

A: Effects of pH on enzyme activity. The enzymatic activity was assayed at 30°C and at the indicated pHs, 50 mM Tris-HCl buffer (pH 8.0) and 50 mM glycine–NaCl–NaOH buffer (pH 9.0–12.0). B: Effects of pHs on enzyme stability with (○) or without (●) 1 mM $CaCl_2$. The reaction mixtures were incubated for 10 min at 30°C and at the indicated pHs: pH 6.0 (50 mM phosphate buffer), pH 7.0 and 8.0 (50 mM Tris-HCl buffer) and pH 9.0–12.0 (50 mM glycine–NaCl–NaOH buffer). C: Effect of temperature on enzyme activity. The enzymatic activity was assayed at temperatures from 30 to 55°C, at pH 10.0. D: Effects of temperatures on enzyme stability with (○) or without (●) 1 mM $CaCl_2$. The enzyme in 20 mM Tris-HCl buffer (pH 8.0) was incubated at temperatures from 25 to 50°C. After 10 min of incubation, the pectate lyase activity was assayed at 30°C and pH 10.0.

Origin	N-terminal amino acid sequence															
NPLase				**S**	**D**	E	P	V	G	Y	A	S	M	N	G	G	T	T	15
A. niger	A	T	V	**S**	**D**	A	A	F	G	Y	A	S	L	N	G	G	T	T	18

Fig. 3. Comparison of N-terminal amino acids of NPLase with pectate lyase from *Aspergillus niger*.

Identical amino acid residues are indicated by bold letters. Numbers of the sequence denote the position of the aligned amino acid sequences.

FASTA search of this sequence showed highest similarity (73%) within 15 amino acids to the N-terminal sequences of the pectate lyase from *Aspergillus niger* (accession no. AJ276331) (Fig. 3).

Table 2 summarizes the general properties of NPLase in comparison with pectate lyases from various actinomycetes. The molecular mass of the NPLase (54 kDa) is similar to that of pectate lyase

Table 2. Comparison of NPLase with pectate lyases from other actinomycetes strains.

Pectate lyase source	Molecular mass (kDa)	p*I*	Optimal pH	Optimal temperature (°C)	Cleavage pattern
Nocardiopsis sp. TOA-1	54	4.7	10.0	40	endo
S. nitrosporeus	41	4.6	10.0	50	endo
S. massasporeus	54	5.5	9.5	40	exo
Amycolata sp.	31	10.0	10.2	70	endo
Thermonospora fusca	55	4.1	10.4	60	endo

from *T. fusca* (55 kDa)[7] and *S. massasporeus* (54 kDa).[5] The p*I* (4.7) is close to that of pectate lyase from *S. nitrosporeus* (4.6).[6] The optimal pH of NPLase (pH 10.0) was similar to that of pectate lyases from other actinomycetes strains (pH 9.5–10.5).[3-7] The relatively higher optimal pHs for pectate lyases from other actinomycetes might be due to the fact that glycosidic bonds of esterified D-galacturonans can be split by a mechanism of β-elimination under alkaline conditions.[13] Thus, NPLase from alkaliphilic *Nocardiopsis* sp. TOA-1 exhibited intermediate properties of hitherto analyzed pectate lyases from various actinomycetes. Gene cloning and further characterization of NPLase are currently underway.

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(Received February 27, 2002; Accepted May 13, 2002)

好アルカリ性 *Nocardiopsis* sp. TOA-1 株の生産するペクチン酸リアーゼの精製と諸性質

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好アルカリ性放線菌 *Nocardiopsis* sp. TOA-1 株の培養上清より，Butyl-Toyopearl, DEAE-Toyopearl, Superdex 75 (HPLC) の各カラムクロマトグラフィーを用いて，電気泳動的に単一なペクチン酸リアーゼ (NPLase) を得た．NPLase の分子質量は 54 kDa，等電点は 4.7，至適温度は 40°C，至適 pH は 10.0 であった．pH 安定性は 8.0 から 11.0，熱安定性は 30°C までであり，カルシウム塩の存在により，それぞれ 7.0 から 11.0，40°C までとなった．NPLase のポリガラクツロン酸に対する分解様式はエンド型であった．