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Production and Characterization of Acid-stable Pectin Lyase from *Bacillus* sp. PN33

KIM, JONG-CHON, HWA-YOUNG KIM, AND YONG-JIN CHOI*

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

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Abstract A bacterial strain PN33 producing large amounts of extracellular pectin lyase (PNL, EC 4.2.2.10) was isolated from soil. The isolated bacterium was identified as a strain of *Bacillus* sp. Production of PNL by the strain was induced only by pectins, with a higher degree of esterification, which had been added to the culture medium as a sole carbon source. The optimal medium for PNL production was determined to consist of 10 g pectin, 2 g yeast extract, 4 g $K_2HPO_4 \cdot 3H_2O$, 0.6 g $MgSO_4$, and 0.11 g $CaCl_2$ per liter (pH 7.0). The PNL activity in the culture supernatant reached the highest level of 132 mU/ml after 32 h cultivation at 37°C in the optimal medium. The PNL produced was purified to homogeneity by ammonium sulfate fractionation (50~80%), and cation exchange and size exclusion chromatographies. The molecular mass of the enzyme was estimated to be approximately 52 kDa by SDS-PAGE. Almost the same mass was determined by nondenaturing PAGE, indicating that the functional enzyme had a monomeric structure. As expected, the PNL exhibited higher activities on the highly esterified pectins whereas it gave no detectable activity on polygalacturonic acid. The enzyme showed the highest activity at the acidic pH of 6.0, exceptional for a bacterial PNL. Maximum activity was measured at 40°C, although the stability of the purified enzyme was poor at this temperature. Calcium (1 mM) was found to activate the PNL activity by 50%, and also remarkably increased the thermal stability of the enzyme. Phenylmethylsulfonylfluoride (PMSF) and diethylpyrocarbonate (DEPC) inhibited the PNL activity almost completely at the concentration of 5 mM. This result indicates that some serine and histidine residues of the enzyme may play an essential role for catalytic function of the enzyme.

Key words: Pectin lyase, pectin, acidophilic, *Bacillus* sp.

*Corresponding author
Phone: 82-2-3290-3417; Fax: 82-2-923-9923;
E-mail: choiyj@kuccnx.korea.ac.kr

Pectic substances are primarily composed of linear polymeric chains of D-galacturonic acid linked as an α-1, 4 glycoside and containing carboxyl groups either not esterified (pectic acid) or esterified to different degrees with methanol (pectin). Pectin, a major component of pectic substances, is an important polysaccharide with applications in foods, pharmaceuticals, and a number of other industries [8]. About 80% of the world production of high-methoxyl pectin, mainly from apple pomace and orange peel, is reported to be used as a gelling agent to give a gelled texture to foods [8]. On the other hand, in fruit juice technology, degrading pectin, and thereby clarifying juices by the pectinolytic enzymes, is an essential step for processing concentrated fruit juices [2, 7]. The commercial enzyme preparations currently used for this application generally contain a mixture of pectin esterase (PE), polygalacturonase (PG), and pectin lyase (PNL). However, PNL is the only enzyme known to be able to cleave, without the prior action of the other enzymes, highly esterified pectins such as fruit pectins.

The use of isolated PNL is, therefore, thought to be advantageous for several reasons. First, no methanol can be formed in the course of the enzyme action. Second, the specific aroma of the fruits is not damaged. Third, the use of PG- and PE-containing enzyme complexes decreases fruit juice stability because of the coagulant processes caused by the interaction of the de-esterified pectin derivatives with the endogenous Ca^{2+} ions. Then, the isolated PNL is expected to improve greatly the stability and the quality of the fruit juice products. Bacterial PNL has been detected only in a few species including *Erwinia carotovora* [1], *E. chrysanthermi* [14], *E. aroideae* [4], *Pseudomonas fluorescens* [10], and *Bacillus subtilis* [9]. But, the optimum pH for the PNLs from the bacteria listed above are around pH 8~10 at which pHs, saponification can occur in a nonenzymatic manner and then methanol can be formed in the course of enzyme reaction.

This paper describes the isolation and identification of a bacterial strain producing acid-stable PNL, production

of the enzyme by the isolated strain, and purification and characterization of PNL from the bacterium.

Materials and Methods

Isolation of Bacterial Strains and Culture Conditions

To isolate a bacterial strain which is capable of producing acid-stable PNL from natural environments, 1 g of soil sample was suspended in 5 ml of sterile saline and 100 µl of the supernatant was spread on the selective medium. Composition of the selective medium was as follows: 10 g pectin (from apples, approximately 70% esterified), 1 g yeast extract, 1.5 g $(NH_4)_2SO_4$, 3.5 g $K_2HPO_4 \cdot 3H_2O$, 80 mg $MgSO_4 \cdot 7H_2O$, 8 mg $ZnSO_4 \cdot 7H_2O$, and 20 g agar per liter, pH 4.0. After incubation at 37°C for 2 days, colonies grown on the medium were isolated and subcultured on the same medium 10 times serially to ascertain the acidophilic character of the isolates. The surviving cells were again inoculated on the selective medium supplemented with 1 g/l of Congo-red. The bacterial colonies which had formed a clear-zone around them after 3 days incubation at 37°C were selected, and cultured in the liquid selective medium supplemented with 1% apple pectin as the sole carbon source on a rotary shaker (200 rpm) at 37°C for 24 h.

The supernatant obtained by centrifuging the culture broth was used as the crude enzyme solution. The bacterium giving the highest PNL activity but not producing PE was finally selected and identified on the basis of Bergey's Manual of Systematic Bacteriology [11] and Api 20 identification system (Biomerieus Co.).

Enzyme Assays

PNL activity was measured by using a reaction mixture consisting of 0.8 ml of 50 mM acetate buffer (pH 5.0) containing 0.25% apple pectin, and 0.2 ml of the crude enzyme solution. The mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 4 ml of 50 mM sodium acetate buffer (pH 3.6) and analyzed for unsaturated compounds at 235 nm. One unit of the enzyme activity was defined as the amount of enzyme which produced an increase of one unit of A_{235} per min.

PG activity was assayed by determining the reducing groups released in the reaction mixture by the Nelson-copper reduction method described by Somogyi [12]. The reaction mixture contained the following: 50 µl of 1% polygalacturonic acid, 1.9 ml of 50 mM acetate buffer (pH 5.0), and 50 µl of crude enzyme. The reaction was carried out at 37°C for 1 h and stopped by adding the copper reagent. One unit of PG activity was the amount of enzyme which released 1 µmol of galacturonic acid per min.

PE activity was assayed by measuring the pH decrease during the reaction at room temperature. The reaction mixture (pH 7.0) contained 1 ml of crude enzyme, 3 ml of distilled water, and 5 ml of 0.5% pectin with esterification of 93%. One unit of PE activity was the amount of enzyme which decreased 0.1 unit of pH of the reaction mixture in 30 min. PE activity was confirmed by determining the amount of methanol formed by gas chromatographic assay.

Enzyme Purification

Crude enzyme solution was concentrated by ammonium sulfate precipitation (50~80% saturation) followed by dialysis at 4°C overnight against 50 mM sodium phosphate buffer (pH 7.0). The dialyzed enzyme solution was loaded on a CM-Sepharose CL-6B column (2.6×20 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The adsorbed proteins were eluted with 400 ml of linear gradient of 0 to 0.5 M NaCl at a flow rate of 20 ml/h (5 ml/tube). The fractions containing PNL activity were gathered and concentrated by ultrafiltration, and dialyzed against 50 mM sodium phosphate buffer (pH 7.0). The concentrated protein was loaded on a Sephacryl S-200 column (1.6×90 cm) equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and eluted with the same buffer at a flow rate of 15 ml/h. The active fractions were pooled and concentrated to 30 ml by centroplus™10 concentrator (Amicon Co. U.S.A.).

Electrophoresis and Zymogram

SDS-PAGE was performed in an 8% gel containing 0.1% SDS according to the method of Laemmli [6]. Native-PAGE was performed with the same SDS-PAGE gel system but in the absence of SDS and without sample pretreatment. For the zymogram, the native polyacrylamide gel was made in the customary manner except that water was replaced by 2% pectin solution. After electrophoresis, the native gel was washed once with 50 mM phosphate buffer (pH 7.0) for 30 min. The washed gels were then incubated in the same buffer for 2 h at 37°C followed by staining in a 0.1% Ruthenium red solution. The enzymatic activity bands appeared as transparent bands on the red-stained gel.

Thin Layer Chromatography

The TLC was developed twice with a solvent system of n-butanol : acetic acid : water (2:1:1, v/v). After chromatography, the plates were dried, and stained with aniline phthalate solution in ethanol. α-D-Galacturonic acid was used as a standard.

Total Galacturonic Acid Assay

Dische's method [1] was used to measure the content of galacturonic acid in the culture broth. One ml aliquots of

sample were pipetted into each of two test tubes (25× 200 mm) and 0.5 ml of the 0.1% alcoholic carbazole was added to the sample tube and 0.5 ml of purified ethyl alcohol was added to the blank tube. Then, 6 ml of concentrated sulfuric acid was added to each of the two tubes with constant agitation. The mixtures were incubated at room temperature for 15 min, and then the optical density at 525 nm was measured.

Measurement of Clarification of Pectin Solution

To 10 ml of 0.25% pectin from apples (pH 5.0), 1.0 ml of enzyme solution (or water, for standard) was added, and the mixture was incubated at 37°C for 60 min. After heating for 5 min in a boiling water bath, the mixture was centrifuged at 3,000 rpm for 5 min and the transmittance at 660 nm of the supernatant was measured. Relative decrease of the transmittance at 660 nm was calculated and indicated as relative clarification yield (%).

RESULTS AND DISCUSSION

Isolation and Identification of Bacterial Strains Producing Acidic Pectin Lyase

In the first step of screening, 430 acidophilic microorganisms which had formed colonies on the selective medium were isolated after 2 days incubation from 1,500 different soil samples. Next, the isolated bacteria were subcultured serially on the same medium 10 times and then 213 acidophilic strains which grew well on the medium were selected. The strains were again inoculated on the medium supplemented with 0.1% Congo-red and incubated for 3 days to isolate five strains which had formed large haloes around their colonies. The five strains, PN1, PN25, PN33, PN45, and PN59, were found to produce PNL but not PE in pectin medium, and their PNL activities ranged from 34 mU/ml to 104 mU/ml using apple pectin as a substrate. As shown in Fig. 1, PN25 and PN33 showed high PNL activities on high methoxyl pectins. Exceptionally, the PN25 showed high PNL activity on pectin substrates with lower esterification. PNL from the strain PN33 was found to be the most acidic enzyme in a preliminary experiment with crude enzyme preparations. Furthermore, gas chromatographic assay confirmed that the crude enzyme from the strain PN33 did not produce methanol in the course of the enzyme reaction (data not shown). We finally selected the strain PN33 for further studies judging by its substrate specificity of and pH optimum for PNL from the isolate. The PN33 strain was a gram-positive rod-shaped and endospore forming bacterium, and identified as a strain of *Bacillus* sp. by its morphological and biochemical characteristics, shown in Table 1.

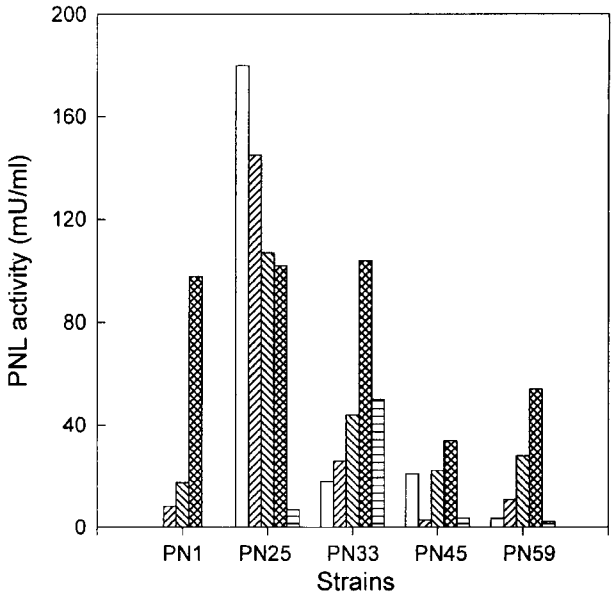

Fig. 1. Substrate specificity of PNL from several bacteria.
The crude enzyme solutions (200 µl) from the isolated strains were incubated with 0.25% different methoxyl pectins (800 µl) in 50 mM sodium phosphate buffer pH 6.0 at 37°C for 1 h. □: Pectic acid; ▨: Pectin with esterification of 28%; ▧: Pectin with esterification of 67%; ▩: Pectin (from apple); ▤: Pectin with esterification of 93%.

Table 1. Morphological and biochemical characteristics of the isolated strain PN33.

Factor	Characteristics
Morphological characteristics	
Gram staining	+
Shape of cell	rod
Spore formation	+
Biochemical characteristics	
Catalase	+
Voges-Proskauer test	+
Oxidase	+
Urease	-
β-Galactosidase	+
Arginine dihydrolase	-
Lysine decarboxylation	-
Cytochrome oxidase	+
Indole production	-
Cultural characteristics	
Anaerobic growth	-
Gas from nitrate	-
Growth at 30~50°C	+

Culture Conditions for PNL Production

The effect of carbon sources on the production of PNL by *Bacillus* sp. PN33 was examined using the selective medium as the basal medium. As shown in Table 2, PNL synthesized by the strain could be induced only by

Table 2. Effect of carbon sources on production of PNL.

Carbon source (1%)	Growth (A_{590})	PNL activity (mU/ml)
Pectin (90% esterified)	0.96	107
Pectin (from apple)	1.01	123
Pectin (60% esterified)	0.46	45
Pectin (30% esterified)	0.31	0
Pectate	0.90	0
Galacturonic acid	0.50	0
Fructose	2.90	20
Glucose	2.80	0
Arabinose	0.81	18
Galactose	0.37	5
Maltose	1.45	0
Sucrose	1.37	4
Lactose	0.27	0
Starch	0.37	0
Xylan	2.13	10
None	0.19	0

Cultivation was carried out as described under Material and Methods at 37°C for 30 h except that the indicated nutrients replaced the carbon source in the PNL production medium.

high methoxyl pectins. Glucose, fructose, and xylan supported high cell densities but produced very little PNL. PNL induction by mitomycin reported with *Pseudomonas marginalis* [13], by nalidixic acid with *Erwinia aroideae* [4] and by glycerol with *Pseudomonas fluorescens* [10] could not be detected with *Bacillus* sp. PN33 (data not shown). On the other hand, the cell growth was increased with increasing pectin concentrations of up to 20 g/l. However, the highest PNL production was observed at the pectin concentration of 10 g/l, and at the concentrations higher than this, the enzyme activity was drastically decreased (data not shown).

As shown in Table 3, organic nitrogen sources were found to be more effective for the PNL production as well as for the cell growth than the inorganic nitrogen

Table 3. Effect of nitrogen sources on PNL production.

Nitrogen sources (0.2%, w/v)	Growth (A_{590})	PNL activity (mU/ml)
None	0.20	2
Peptone	1.23	83
Tryptone	1.30	95
Yeast extract	1.35	125
C.S.S[a]	1.04	34
Malt extract	1.06	45
Ammonium phosphate	0.22	25
Ammonium sulfate	0.23	28
Ammonium nitrate	0.25	25

Cultivation was carried out 37°C for 30 h as described under Material and Methods exept that the indicated nutrients replaced the nitrogen source in the PNL production medium containing 1% pectin (from apple). a: Corn steep solids.

compounds examined. The highest activity was obtained when the cells were grown in basal medium supplemented with 0.2% yeast extract as a nitrogen source. Potassium phosphate (dibasic) was also found to remarkably increase the production of PNL. The optimum concentration of K_2HPO_4 for the enzyme production was 0.4%, although maximum cell density was observed at 0.8% (data not shown). This result is well consistent with the work with *Bacillus subtilis* IFO 3134 reported by Sakamoto *et al.* [9]. Finally, the effects on the PNL production of some metal ions added at a 5 mM concentration were examined. The enzyme production and cell growth were greatly stimulated by Mg^{2+} and Ca^{2+}. The most effective concentrations of Mg^{2+} and Ca^{2+} were 5 mM and 1 mM, respectively (data not shown).

Taken together, the optimum medium for the PNL production by *Bacillus* sp. PN33 was determined to consist of 10 g pectin, 2 g yeast extract, 4 g $K_2HPO_4 \cdot 3H_2O$, 0.6 g $MgSO_4$, and 0.11 g $CaCl_2$ per liter, and this medium was employed in the experiments hereafter.

The *Bacillus* strain PN33 produced high levels of PNL under neutral conditions, and the optimal initial pH of the culture medium was pH 7.0. The optimum culture temperature was determined to be 37°C for enzyme synthesis. However, the enzyme production as well as cell growth was greatly inhibited at temperatures higher than 42°C.

Time Course of the PNL Production

The time course of the PNL production was determined under the cultural conditions optimal for the enzyme production. As shown in Fig. 2, kinetics of the PNL production during the culture revealed a similar pattern

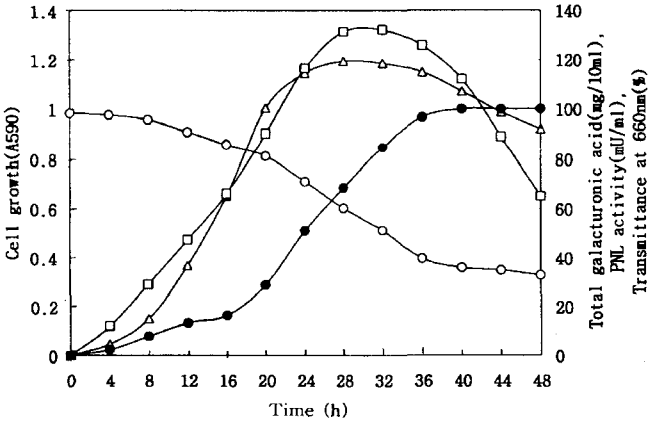

Fig. 2. Time course of PNL production by *Bacillus* sp. PN33.
Cultivation was carried out as described under Materials and Methods at 37°C for 48 h in a 250 ml optimal production medium in a 2-l flask. ○: total galacturonic acid (mg/10 ml); △: cell growth (A_{590}); □: PNL activity (mU/ml); ●: transmittance at 660 nm (relative clarification yield: %).

to that of the cell growth and the maximum levels of cell growth and enzyme activity were observed after 28 h cultivation. Thereafter, the PNL activity decreased sharply, indicating that the enzyme was fairly unstable. In contrast, with PNL activity, the clarification activity continued to increase even after 28 h of culture although at a declining rate. This unanticipated disagreement can not be explained clearly at present, but some intracellular components released by the cell lysis might cause coagulation of suspended materials in the reaction mixture. However, as was expected, the total amount of galacturonic acid was remarkably decreased during the exponential phase of cell growth.

Enzyme Purification and Estimation of Molecular Mass

The starting material for the purification was 2 l of the crude enzyme preparation containing 0.5 mg of protein ml^{-1} with a specific pectin lyase activity of 0.24 U/mg. The enzyme solution was concentrated to 15 ml by ammonium sulfate precipitation (50~80% saturation). The concentrated enzyme solution was applied on a CM-Sepharose CL-6B column after dialysis overnight. The PNL was eluted at approximately 0.25 M NaCl. The concentrated PNL was loaded onto a Sephacryl S-200 column. Chromatography on this column resulted in a single symmetrical peak of PNL activity. A 47.9-fold increase in specific activity and a recovery yield of 12.9% were calculated for the purified PNL (Table 4). Peak fractions from the Sephacryl S-200 column yield a single protein band on Coomassie Brilliant blue-stained SDS-PAGE gel (Fig. 3A). The size of the PNL as determined by SDS-PAGE was 52 kDa, and almost the same molecular mass was estimated by nondenaturing gel electrophoresis as shown in Fig. 3B. These results indicate that the functional PNL has a monomeric structure. Pectin lyase activity was confirmed to be attributed to the purified protein by zymogram technique (Fig. 3C).

General Properties

To identify the mode of enzyme action, products of the pectin cleavage reaction catalyzed by PNL from *Bacillus*

Table 4. Summary of purification of the PNL from *Bacillus* sp. PN33.

Steps	Total protein (mg)	Total activity (Unit)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Culture broth	995.4	240	0.24	100	1.0
Ammonium sulfate fractionation (50~80%)	369.7	100.5	0.27	41.9	1.12
CM-Sepharose CL-6B	16.3	45	2.76	18.7	11.5
Sephacryl S-200	2.69	31	11.5	12.9	47.9

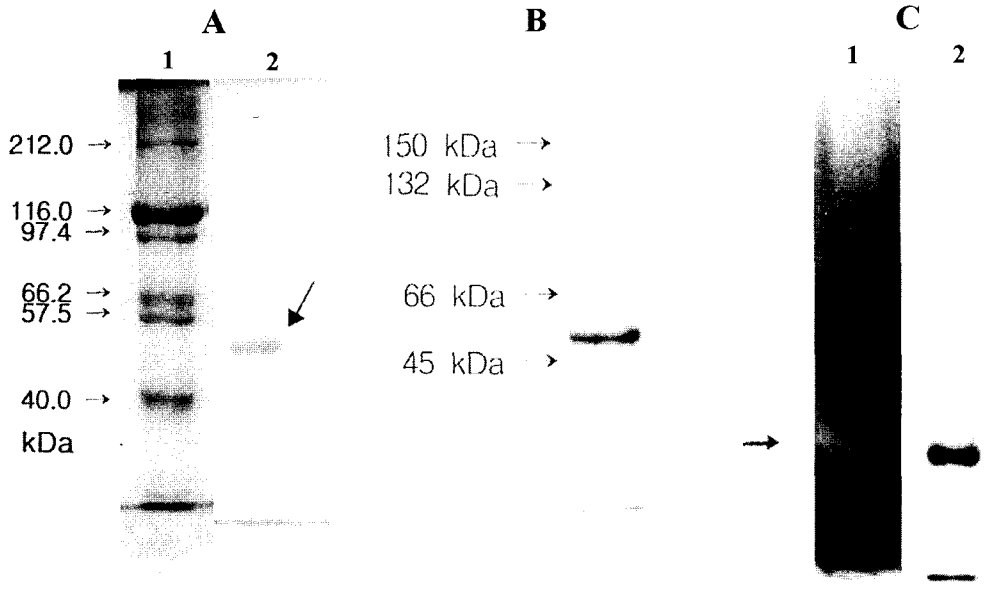

Fig. 3. SDS-PAGE, nondenaturing PAGE of the purified PNL and its zymogram.

A. Aliquot of protein solutions from Sephacryl S-200 column were electrophoresed on the 8% SDS-PAGE. Lane 1: protein molecular weight size markers; lane 2: purified PNL. B. Gel electrophoresis was carried out on the 8% nondenaturing polyacrylamide gel. C. Lane 1: the PNL activity band (arrow) on the zymogram; lane 2: the PNL protein band.

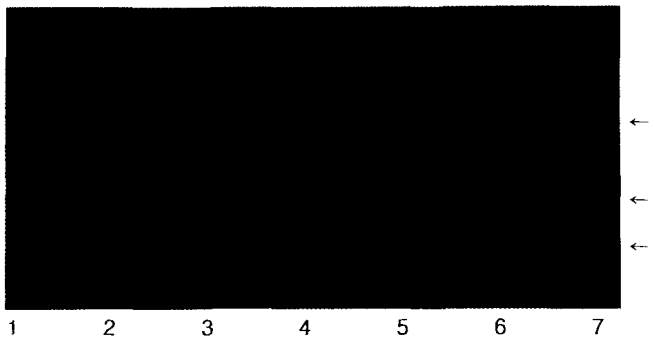

Fig. 4. TLC of the reaction product of pectin by purified PNL.

Lane 1: α-D-galacturonic acid; lane 2: reaction products incubated with *Aspergillus niger* pectin lyase purchased from Sigma Co. for 2 h; lanes 3-7: reaction products incubated with the purified PNL for 0, 0.5, 1, 2, 3 h, respectively. Arrows indicate the spots thought to be by monomer, dimer, trimer of methylgalacturonide (from upper to lower).

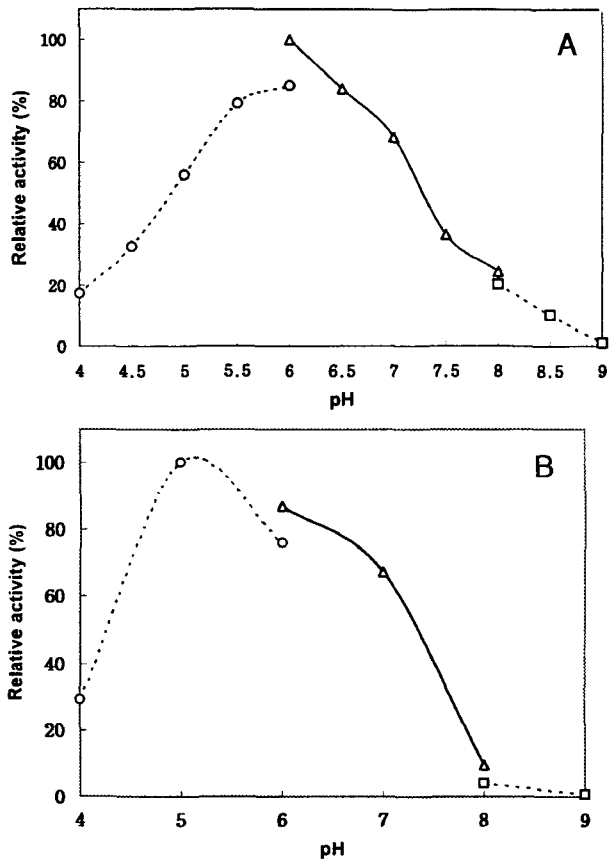

Fig. 5. Effect of pH on the activity (A) and the stability (B) of purified PNL.

A. Enzyme activity was measured in a pH range from 4.0 to 9.0. Enzyme reaction was carried out at 40°C for 30 min. B. Enzyme solution was incubated at 4°C for 24 h in different buffers. After incubation, enzyme reaction was carried out at 40°C for 30 min and then the residual activity was measured. ○: 50 mM sodium acetate buffer; △: 50 mM potassium phosphate buffer; □: 50 mM Tris-Cl buffer.

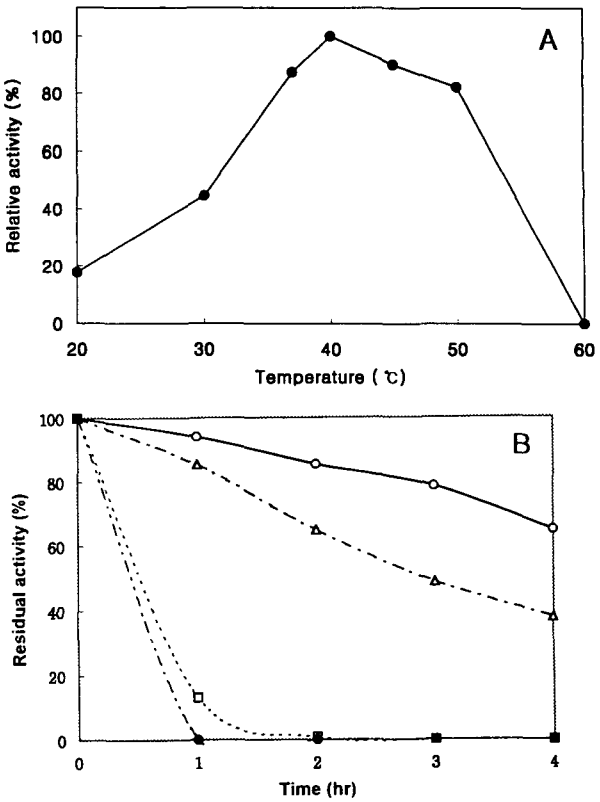

Fig. 6. Effect of temperature on the activity (A) and the stability (B) of purified PNL.

A. Enzyme reaction was carried out for 30 min at various temperatures as in the figure. Relative activity was represented as the percentage of the enzyme activity at 40°C. B. Enzyme activity was measured at 40°C for 30 min allowing the enzyme solution to stand at various temperatures at 1 h intervals for 4 h (○: 30°C; △: 40°C; □: 50°C; ●: 60°C).

Table 5. Effects of metal ions and group-specific modifying reagents on the PNL activity.

Metal ions and chemical modifying reagents	Relative activity (%)
Fe^{2+}	80
Zn^{2+}	56
Mn^{2+}	77
Mg^{2+}	107
Co^{2+}	33
Hg^{2+}	7
Cu^{2+}	3
Ca^{2+}	150
Li^{+}	100
2-Mercaptoethanol	95
p-Chloromercuribenzoate	94
H_2O_2	71
1,2-Cyclohexandion	93
Hydroxylamine	83
N-Bromosuccinimide	93
Phenylmethylsulfurfluoride	4
Diethylpyrocarbonate	1
None	100

Enzyme reaction was carried out at 40°C for 30 min in the presence of various metal ions (1 mM) or chemical modifying reagents (5 mM).

sp. PN33 were analyzed by TLC. As seen in Fig. 4, spots thought to be by monomer, dimer, and trimer of methylgalacturonide could be detected in the course of the enzyme reaction. These limited products are known to be the sugar derivatives formed by a random endolytic depolymerization of pectin, indicating that the PNL belongs to the endo type of pectin lyase. The pH optimum of PNL for apple pectin was found at 6.0 and stability of the enzyme appeared best around the pH of 5.0 (Fig. 5). In general, pH optima of PNLs of bacterial origins are in the range of pH 8 to 10. Therefore, this property of the *Bacillus* sp. PN33 enzyme can be applied to maceration of vegetable tissues at pH 6 at which saponification can hardly occur. Maximum activity was measured at 40°C, although stability of the purified enzyme was poor at this temperature as shown in Fig. 6. PNL activity was examined in the presence of 1 mM of metal ions or 5 mM of some enzyme modifiers (Table 5). Ca^{2+} increased the enzyme activity by about 50% of its original activity and Mg^{2+} also showed a slight positive effect. The most effective Ca^{2+} concentration was 1 mM and at higher concentrations the activity was decreased gradually. In addition, Ca^{2+} was found to significantly improve thermal stability of the PNL as illustrated in Fig. 7. Among the enzyme modifiers tested, only phenylmethylsulfonylfluoride (PMSF) and diethylpyrocarbonate (DEPC), the target amino acids of which are serine and histidine, respectively, inhibited the enzyme activity almost completely at a 5 mM concentration. This inhibition suggests that some histidine and serine residues of the PNL protein may be directly or indirectly implicated in its catalytic function.

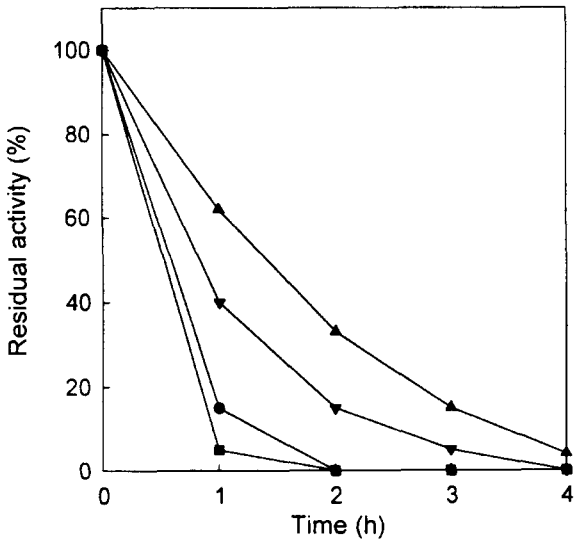

Fig. 7. Effects of Ca^{2+} on the thermal stability of purified PNL.
The reaction mixture (pH 6.0) was incubated at various temperatures in the absence or presence of 2 mM Ca^{2+} ion for indicated times as in the figure and then the residual activity was measured (●: 50°C, $-Ca^{2+}$; ▲: 50°C, $+Ca^{2+}$; ■: 60°C, $-Ca^{2+}$; ▼: 60°C, $+Ca^{2+}$).

Table 6. Substrate specificity of the PNL and its kinetic parameters for the various substrates

Substrate (0.2%)	Specific activity (U/mg)	K_m (mg/ml)	V_{max} (ΔA_{235}/min/mg)
93% esterified pectin	5.11	0.44	6.17
Apple pectin	4.11	0.56	5.32
67% esterified pectin	0.94	0.89	1.35
28% esterified pectin	0.93	1.19	2.00
Polygalacturonic acid	0.00	-	-

Substrate Specificity

The purified PNL showed high activity against pectin from apples and pectin with the esterification degree of 93%. On the other hand, the enzyme gave no detectable activity on polygalacturonic acid as shown in Table 6. These results suggest that the purified enzyme from *Bacillus* sp. PN33 is an endo-pectinlyase (EC 4.2.2.2). The K_m and V_{max} values for some pectin substrates were estimated from Lineweaver-Burk plots. The PNL exhibited higher affinities for substrates with a higher degree of esterification. K_m and V_{max} for pectin with 93% esterifiation were 0.44 mg/ml and 6.17 ΔA_{235}/min/mg, respectively.

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