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ORIGINAL PAPER

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Enzymatic properties of the highly thermophilic and alkaline pectate lyase Pel-4B from alkaliphilic *Bacillus* sp. strain P-4-N and the entire nucleotide and amino acid sequences

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Abstract We cloned two genes for alkaline pectate lyase, *pel*-4A and *pel*-4B, from alkaline pectinase-producing alkaliphilic *Bacillus* sp. strain P-4-N. The *pel*-4B gene product Pel-4B was purified to homogeneity and characterized. The purified enzyme had an isoelectric point of pH 9.6 and a molecular mass of 35 kDa, values close to those of the *pel*-4A gene product Pel-4A. The pH and temperature optima for activity were as high as 11.5 and 70°C, respectively, which are the highest among the pectate lyases reported to date. The mature Pel-4B (304 amino acids; 33,868 Da) was structurally related to the enzymes in the polysaccharide lyase family 1 and showed 35.6% identity with Pel-4A on the amino acid level. It showed significant homology to other pectate lyases in the same family, such as the enzymes from alkaliphilic *Bacillus* sp. strains KSM-P7 and KSM-P103 and the fungi *Aspergillus nidulans* and *Colletotrichum gloeosporioides* f. sp. *malvae*

Key words Pectin · Pectate lyase · Cloning · Alkaliphile · *Bacillus*

Introduction

Pectinolytic enzymes are produced by plant pathogenic microorganisms and cause leaf spot, sift rot, and wilt (Collmer and Keen 1986). On the other hand, they are very useful enzymes in the food and fabric industries to extract, clarify, and liquefy fruit juices and wines and to ret plant fibers, respectively (Alkorta et al. 1998; Sakai et al. 1993). Pectate lyase (Pel; pectate transeliminase, EC 4.2.2.2) is the enzyme that cleaves α-1,4-galacturonosidic linkages of polygalacturonic acid (PGA) by a *trans*-eliminative mechanism (Rombouts and Pilnik 1980). Many genes for microbial Pels have been cloned and sequenced, and the enzymes form a superfamily based on their deduced amino acid (aa) sequences (Nasser et al. 1993; Shevchik et al. 1997; Brühlmann and Keen 1997). The tertiary structures of PelC (Yoder et al. 1993) and PelE (Lietzke et al. 1994) from *Erwinia chrysanthemi* EC16 and BsPel from *Bacillus subtilis* SO113 (Pickersgill et al. 1994) have been solved, and they have a structural topology of parallel β-strands with a large right-handed coil (Heffron et al. 1998).

We have characterized highly alkaline Pels and sequenced their genes from alkaliphilic bacilli (Kobayashi et al. 1999a, 1999b; Hatada et al. 1999, 2000; Ogawa et al. 2000; Sawada et al. 2000). We also isolated two different genes for Pels, designated *pel*-4A and *pel*-4B, from alkaliphilic *Bacillus* sp. strain P-4-N, which produces an alkaline pectinase (Horikoshi 1972). In a previous paper, we identified the enzymatic properties and gene sequence of the *pel*-4A gene product salt-dependent Pel-4A (Kobayashi et al. 2000). Here we characterize the enzymatic and genetic properties of the *pel*-4B gene product Pel-4B and compare its deduced aa sequence with those of Pel-4A and other enzymes belonging to the large Pel superfamily (Henrissat et al. 1995).

Materials and methods

Bacterial strains and propagation

The source of the gene examined in this study was alkaliphilic *Bacillus* sp. strain P-4-N (Horikoshi 1972). The organism was propagated in an alkaline liquid medium as described by Kobayashi et al. (2000).

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Purification of the enzyme

All purification steps were performed at temperatures not exceeding 5°C. Ammonium sulfate was added to the centrifugal supernatant of the culture broth (470 ml) up to 70% saturation. The precipitate formed was dialyzed against 10 mM Tris-HCl buffer plus 1 mM $CaCl_2$ (pH 7.0; buffer A), and the retentate (94 ml) was loaded onto a column (2.5×22 cm) of DEAE-Toyopearl 650 M (Tosoh, Tokyo, Japan) equilibrated with buffer A. Pel activity was passed through the column by elution with buffer A. The nonadsorbed fractions were pooled (230 ml) and concentrated to 75 ml by ultrafiltration on a YM-3 membrane (Amicon, Beverly, MA, USA). The solution was put on a column (2.5 ×12 cm) of CM-Bio-Gel A (Bio-Rad, Hercules, CA, USA) equilibrated with buffer A, and the column was initially washed with 200 ml of buffer A. Proteins were eluted with a 600-ml linear gradient of 0 to 120 mM KCl in the same buffer. The active fractions eluted between 60 and 70 mM KCl were combined (165 ml) and concentrated to 11 ml by ultrafiltration. The concentrate was put on a column (1.5 × 28 cm) of CM-Bio-Gel A (Bio-Rad) equilibrated with 10 mM borate buffer plus 1 mM $CaCl_2$ (pH 9.5), and proteins were eluted with a 600-ml linear gradient of 0 to 80 mM KCl. The active fractions eluted between 30 and 35 mM KCl were pooled and concentrated and stored in 20% (v/v) glycerol until use.

Enzyme assays

Pel activity was routinely measured at 30°C and pH 10.5 in 50 mM glycine-NaOH buffer containing 0.6 mM $CaCl_2$. One unit of enzymatic activity was defined as the amount of protein that produced 1 µmol of unsaturated oligogalacturonides equivalent to 1 µmol of unsaturated digalacturonide per minute (Hasegawa and Nagel 1966). Protein was determined with a DC-protein assay kit (Bio-Rad) with bovine serum albumin as the standard protein.

Electrophoresis and N-terminal amino acid sequence

Polyacrylamide gel electrophoresis (PAGE) was done as described by Taber and Scherman (1964) with 7.5% (w/v) acrylamide slab gels with 25 mM Tris-192 mM glycine (pH 8.3) as the electrode buffer. Sodium dodecyl sulfate-(SDS) PAGE was done with 7.5% acrylamide slab gels by the method of Laemmli (1970) with an SDS-PAGE molecular weight standard kit (Pharmacia, Uppsala, Sweden) as marker proteins. Isoelectric focusing (IEF) of proteins was done as described by Kobayashi et al. (2000), with IEF standards (Sigma, St. Louis, MO, USA) as isoelectric point markers. The N-terminal sequence of the protein was determined using a pulsed liquid-phase protein sequencer (model 476A; Applied Biosystems, Foster City, CA, USA).

DNA isolation and sequencing

Preparation of genomic DNA from *Bacillus* sp. strain P-4-N, restriction digestion, and ligation were done as described by Kobayashi et al. (2000). Gene sequencing was done using a DNA sequencing kit-dye terminator cycle sequencing ready reaction (Perkin-Elmer) and an automated DNA sequencer (model 377; Perkin-Elmer). The *pel*-4B gene was cloned and sequenced using a Takara LA PCR in vitro Cloning kit (Takara, Otsu, Japan), according to the manufacturer's instructions. The entire nucleotide (nt) sequence was finally determined using the synthetic primers shown below the sequence in Fig. 4 (see later in this article).

Nucleotide sequence submission

The nucleotide (nt) sequence data published here have been deposited in the DDBJ, EMBL, and GenBank data banks under accession number AB042100.

Results and discussion

Purification and some properties of Pel-4B

Pel-4B was purified 37-fold to an overall yield of 11.7% with the specific activity toward PGA of 87 units (mg/protein) (Table 1). The purified enzyme was homogeneous as judged by both PAGE and SDS-PAGE. The molecular mass of Pel-4B was approximately 35 kDa by SDS-PAGE. The isoelectric point was around pH 9.6 as estimated by IEF PAGE. The N-terminal aa sequence of the purified Pel-4B was Asn-Thr-Pro-Asn-Phe-Asn-Leu-Gln-Gly-Phe-Ala-

Table 1. Purification of Pel-4B

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Fold
Culture supernatant	1,230	2,930	2.38	100	1.0
Ammonium sulfate	237	1,960	8.27	67	3.5
DEAE Toyopearl	70.2	1,220	17.4	42	7.3
First Bio-Gel A	19.8	1,170	59.1	40	25
Second Bio-Gel A	3.95	343	86.8	12	37

Thr-Leu-Asn-Gly-Gly-Thr-Thr-Gly-Gly. The activity of Pel-4B was abolished by incubation at 30°C for 5–10 min with 1.0 mM ethylenediaminetetraacetic acid (EDTA) in 25 mM Tris-HCl buffer (pH 8.0). The activity of the EDTA-treated enzyme was completely restored by addition of $CaCl_2$ at 0.4 mM. Fe^{3+}, Mn^{2+}, Co^{2+}, and Pd^{2+} ions at 0.4 mM each also restored the activity to 20%, 9.1%, 8.6%, and 4.1% of the original activity, respectively. Sr^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+}, and Fe^{2+} ions could not substitute for Ca^{2+} ions. Unlike that of Pel-4A, the activity of Pel-4B was not enhanced by NaCl.

Substrate specificity

Pel-4B depolymerizes pectins. When the PGA degradation rate at pH 10.5 was taken as 100%, the relative rates toward citrus pectins with degrees of methylation of 31%, 63%, and 93% (Sigma) were 42%, 33%, and 0%, respectively. The viscosity of a PGA solution was decreased gradually by adding Pel-4B and, after 1-h incubation at 30°C, it was reduced as much as 33% of the initial viscosity, whereas the extent of the cleavage was only 3.7% at that time (Fig. 1). These results suggested that Pel-4B is an *endo*-Pel.

Effects of temperature and pH

The effect of temperature on the Pel-4B activity was examined at pH 10.5 in 50 mM glycine-NaOH buffer. The optimal temperature for activity was as high as 70°C (Fig. 2A). Pel-4B was incubated at various temperatures for 15 min in 50 mM glycine-NaOH buffer (pH 10) plus 2 mM $CaCl_2$. The enzyme was stable up to 55°C without $CaCl_2$ and to 60°C with $CaCl_2$ (Fig. 2B).

The optimal pH of Pel-4B was 11.5 in 50 mM glycine-NaOH buffer (Fig. 3A). The observed optima for activity at 70°C and pH 11.5 are the highest among the Pels reported to date. When the enzyme was kept at 40°C for 1 h, the pH stability curve had two peaks around pH 10 and pH 8 (Fig. 3B). The pH instability between the two pH peaks was restored by addition of 2 mM $CaCl_2$. In the presence of 100 mM NaCl, the enzyme was stable during incubation at the pH range from 6 to 11. As in the case of Pel-4A, the reason for stabilization by NaCl of Pel-4B has not yet been clarified.

Nucleotide and deduced amino acid sequences

The nt sequence of the *pel*-4B gene, extending from nt 1 to nt 1,743, is shown in Fig. 4. Starting from an ATG initiation codon at nt 455, there is a long open reading frame (ORF) of 1,002 bp that ends in a TAA stop codon at nt 1,456. The G+C content of the ORF was 42.6%. The putative ribosome-binding site, with the sequence 5′-AAAG-GAAGTGA-3′, was found 5 bp upstream of this ORF. The sequence of the ribosome-binding site had a free energy value of –62.4 kJ/mol. There was a putative sequence of

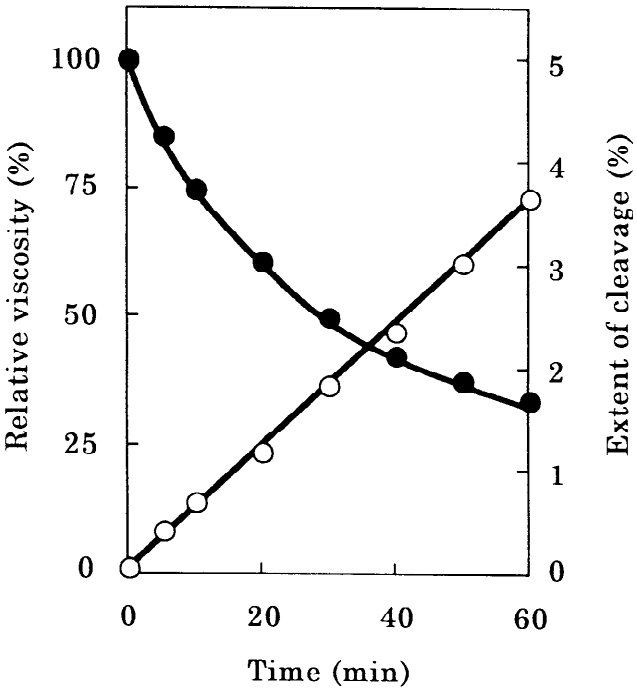

Fig. 1. Ratio of viscosity reduction and galacturonoside bond cleavage. Pectate lyase (Pel-4B) (4.6 μg) was added to the reaction mixture in a final volume of 10 ml. The viscosity in the solution was measured at 30°C at timed intervals. The initial viscosity measured by adding 1.0 ml of 50 mM Tris-HCl buffer (pH 7.5), instead of enzyme, was taken as 100%. At timed intervals, samples (1.0 ml) were withdrawn, and 2.0 ml of 50 mM HCl was added, followed by measurement of absorbance at 235 nm. The percentage of galacturonoside cleavage was calculated based on the total galacturonic acid in the substrate. *Solid circles*, degree of viscosity reduction; *open circles*, extent of galacturonoside bond cleavage

sigma A-type promoter of *Bacillus subtilis*, with 5′-ATCACT-3′ as the potential –35 region and 5′-TATAAT-3′ as the potential –10 region, separated by 16 bp. An inverted-repeat (palindromic) sequence from nt 1,457 to nt 1,497 was found immediately downstream of the termination codon. The free energy value of a stem-loop structure was calculated to be –98.1 kJ/mol.

The ORF encoded 333 aa including a putative signal sequence of 29 aa, as shown under the nt sequence in Fig. 4. The aa sequence deduced from the nt sequence of the *pel*-4B gene contained a hydrophilic/hydrophobic sequence from Met1 to Ala29 that is similar to signal peptides of *B. subtilis* (Simonen and Palva 1993). A deduced aa sequence identical to the 20 N-terminal Asn–Gly of Pel-4B was found at aa 30–49. Thus, the calculated molecular mass of the mature enzyme (304 aa; Asn30–Pro333) would be 33,868 Da, a value very close to that of 35 kDa determined for Pel-4B. Similar to Pel-7 from *Bacillus* sp. strain KSM-P7 (Kobayashi et al. 1999a), Pel-103 from *Bacillus* sp. strain KSM-P103 (Hatada et al. 1999), and PelL from *E. chrysanthemi* EC16 (Alfano et al. 1995), Pel-4B is rich in Asn (12.5%).

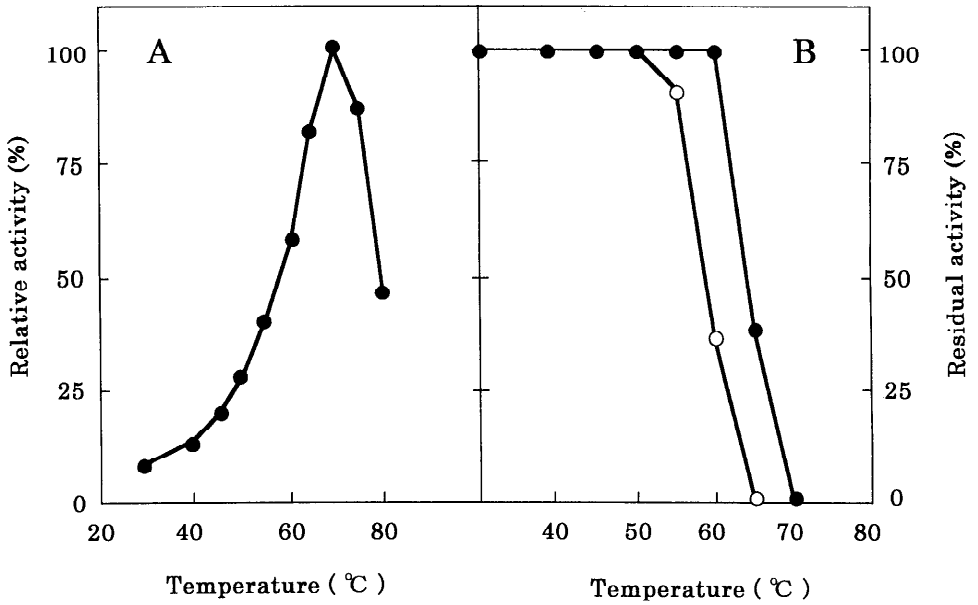

Fig. 2A,B. Effects of temperature. **A** Pel activity was assayed at various temperatures in 50 mM glycine-NaOH buffer (pH 10.5) plus 0.6 mM $CaCl_2$ with 0.13 µg Pel-4B. **B** Thermal stability. Pel-4B (0.64 µg) was incubated at the indicated temperatures for 15 min in 50 mM glycine-NaOH buffer (pH 10) in the absence (*open circles*) or presence (*solid circles*) of 2 mM $CaCl_2$. Portions of the solution were withdrawn, and the residual activity was measured under the standard conditions of the assay. The original activity without heating was taken as 100%

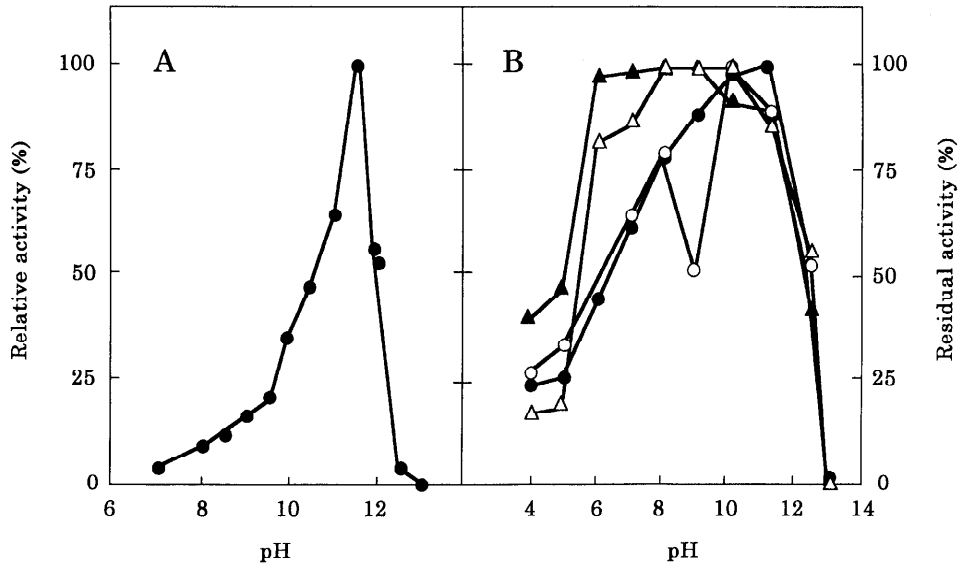

Fig. 3A,B. Effects of pH. **A** Pel activity was assayed at 30°C and at the indicated pH either in 50 mM Tris-HCl buffer (pH 7–9.5), 50 mM glycine-NaOH buffer (pH 8–12), or in 50 mM KCl-NaOH buffer (pH 12–12.8) with 0.26 µg Pel-4B. **B** Effect on stability. Pel-4B (1.6 µg) was incubated for 1 h at 40°C and at the indicated pH in 20 mM each of various buffers in the absence (*open circles*) or presence (*solid triangles*) of 100 mM NaCl, 2 mM $CaCl_2$ (*solid circles*), or both additives (*open triangles*). Portions of the solution were withdrawn, and the residual activity was measured under the standard conditions of the assay. The original activity at pH 10.5 (without preincubation) was taken as 100%. Buffers used: acetate, pH 4–6; (*N*-2-morpholino) propane sulfonic acid (MOPS), pH 6–8; Tris-HCl, pH 7–9; glycine-NaOH, pH 8–11; KCl-NaOH, pH 12.4–13

Amino acid homology and possible catalytic residues

When suitably aligned, the deduced aa sequence of mature Pel-4B was found to exhibit some homology to those of known Pels belonging to the polysaccharide lyase family 1. Pel-4B showed the highest homology to Pel-103 and to Pel-7 with 68.0% and 67.7% identity, respectively. The enzyme had moderate homology to fungal Pels from *Colletotrichum gloeosporioides* f. sp. *malvae* (37.1% identity; GenBank no. AF156983) and *Aspergillus nidulans* (38.1% identity; Ho et al. 1995). It also shared limited identity with other Pels, such as PelA (23.6%), PelB (30.7%), PelC (30.3%), and PelE (25.8%) from *E. chrysanthemi* EC16 (Keen and Tamaki 1986; Tamaki et al. 1988), BsPel from *B. subtilis*

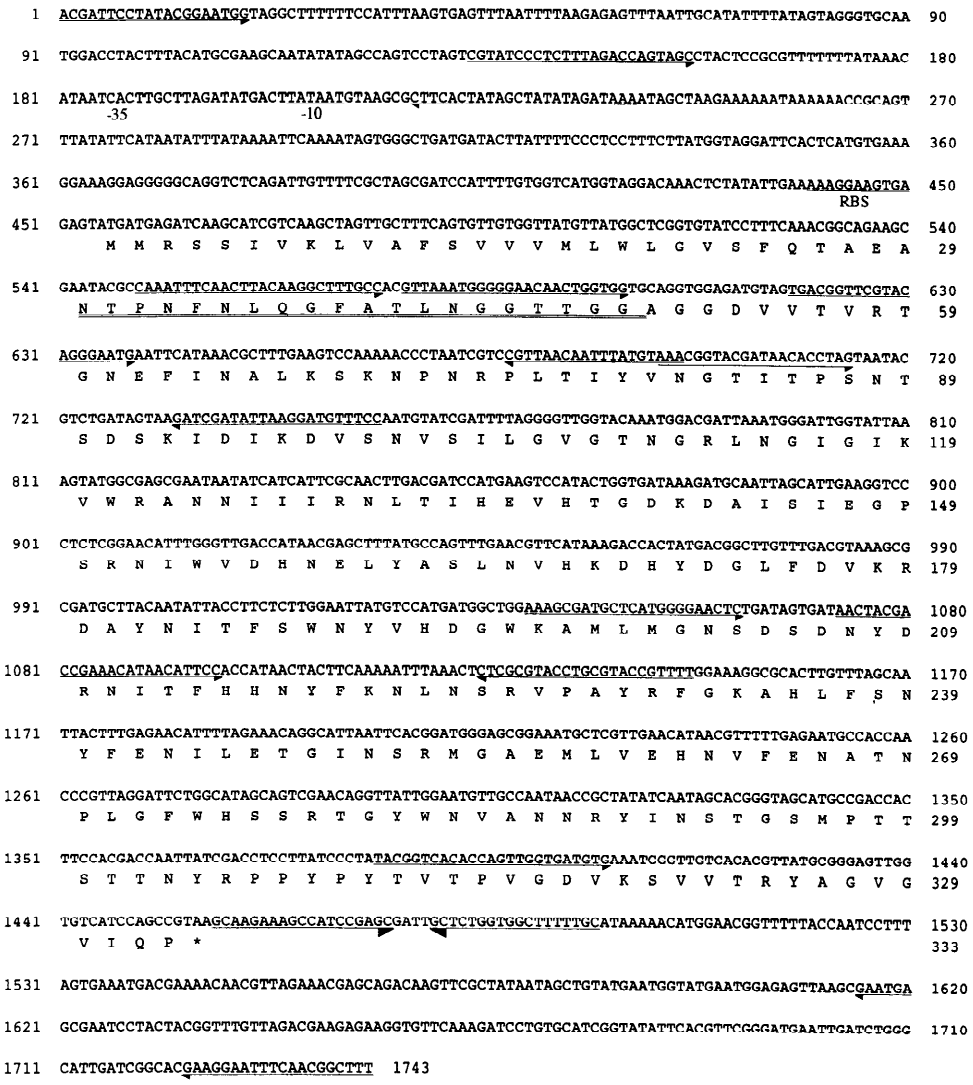

Fig. 4. Nucleotide sequence and the deduced amino acid sequence of the entire *pel*-4B gene and its flanking regions. *Numbers* on either side of the sequences denote nt and aa positions, respectively. The nt sequence similar to –35 and –10 consensus promoters of *B. subtilis* is *underlined*. The possible ribosome-binding site is indicated by *RBS*. The *pel*-4B ORF extends from Met1 to Pro333. The *double-underlined* aa sequence refers to the N-terminal end of the extracellular, mature enzyme produced by *Bacillus* sp. strain P-4-N. Inverted repeats downstream of the stop codon TAA (*) are indicated by *convergent arrows*. The nt sequences indicated by *arrows* are the primers used for determination of the entire *pel*-4B gene

SO113 (25.0%; Nasser et al. 1993), and a Pel from *Pseudomonas marginalis* (25.1%; Nikaidou et al. 1993). The homology of Pel-4B with Pel-4A was only 35.6% identity.

The deduced aa sequence of mature Pel-4B was suitably aligned with those of Pel-4A and PelC from *E. chrysanthemi* EC16 (Tamaki et al. 1988; Henrissat et al. 1995) (Fig. 5). Lys190, Arg218, and Arg223 are suggested to form catalytic residues in the *Erwinia* PelC after site-directed mutagenesis (Kita et al. 1996). Scavetta et al. (1999) identified Lys190 and Arg218 for catalysis and Asp at aa 129, 131, 160, and 162, Glu166, and Asp170 for substrate or calcium binding in PelC by the crystallographic analysis of an enzyme–plant cell wall complex. Therefore, Asp at aa 140, 142, 169, 172, and 176, Lys196, and Arg225 appear to play indispensable roles in the *trans*-eliminative cleavage by Pel-4B, despite the limited overall sequence identity and difference in aa usage with Pel-4A and PelC. However, the catalytic properties of Pel-4B are significantly different from those of Pel-4A and PelC. Pel-4B is very unusual in that it has very high pH and temperature optima for activity.

Alkaliphiles and their alkaline exoenzymes might have evolved specific mechanisms that allow them to survive and retain stability in highly alkaline environments (Ito et al. 1998; Horikoshi 1999). We have cloned and sequenced various genes for high-alkalinity Pels from alkaliphilic *Bacillus* strains, such as a thermophilic enzyme (Pel-4B; this study), a salt-dependent enzyme (Pel-4A; Kobayashi et al. 2000), and a Pel having protopectinase activity (Pel-7; Kobayashi et al. 1999a), all belonging to the polysaccharide lyase family 1, a low molecular weight enzyme (Pel-15; Hatada et al. 2000) belonging to family 3, a high molecular weight enzyme (Pel-15H; Ogawa et al. 2000) belonging to family 9, and a Pel (Pel-15E; Sawada et al. 2000) belonging to the minor family 10. We are now constructing a phylogenetic tree of the highly alkaline Pels and less-alkaline enzymes.

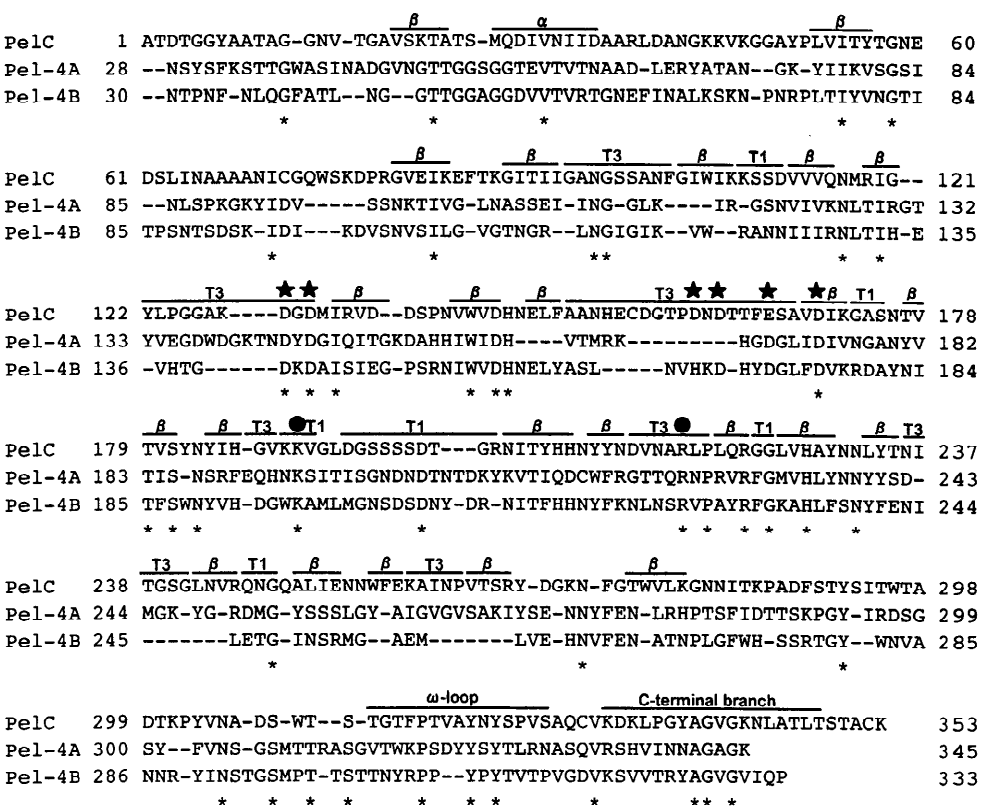

Fig. 5. A multiple alignment of the deduced amino acid sequences of Pel-4B with those of PelC and Pel-4A. The aa sequence alignment was done with the GENETYX program (SDC Software Development, Tokyo, Japan). The putative catalytic residues (*solid circles*) and calcium- or substrate-binding site (*solid stars*) proposed for PelC from *Erwinia chrysanthemi* EC16 (Scavetta et al. 1999) are shown *above* the sequence. The structural motifs of PelC, defined by Henrissat et al. (1995) and Heffron et al. (1998), are *underlined*

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