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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).
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 CaCl₂ (pH 7.0; buffer A), and the retentate (94 ml) was loaded onto a column (2.5×22 cm) of DEAE-Toyopearl 650 M (Toso, 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 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 CaCl₂. One unit of enzymatic activity was defined as the amount of protein that produced 1 mmol of unsaturated oligogalacturonides equivalent to 1 mmol 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).

Table 1. Purification of Pel-4B

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

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-