Biochemical Properties of Pectate Lyases Produced by Three Different 
Bacillus Strains Isolated from Fermenting Cocoa Beans and 
Characterization of Their Cloned Genes

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Pectinolytic enzymes play an important role in cocoa fermentation. In this study, we characterized three extracellular pectate lyases (Pels) produced by bacilli isolated from fermenting cocoa beans. These enzymes, named Pel-22, Pel-66, and Pel-90, were synthesized by Bacillus pumilus BS22, Bacillus subtilis BS66, and Bacillus fusiformis BS90, respectively. The three Pels were produced under their natural conditions and purified from the supernatants using a one-step chromatography method. The purified enzymes exhibited optimum activity at 60°C, and the half-time of thermoactivation at this temperature was approximately 30 min. Pel-22 had a low specific activity compared with the other two enzymes. However, it displayed high affinity for the substrate, about 2.5-fold higher than those of Pel-66 and Pel-90. The optimum pHs were 7.5 for Pel-22 and 8.0 for Pel-66 and Pel-90. The three enzymes trans-eliminated galacturionate in a random manner to generate two long oligogalacturonides, as well as trimers and dimers. A synergistic effect was observed between Pel-22 and Pel-66 and between Pel-22 and Pel-90, but not between Pel-90 and Pel-66. The Pels were also strongly active on highly methylated pectins (up to 60% for Pel-66 and Pel-90 and up to 75% for Pel-22). Fe²⁺ was found to be a better cofactor than Ca²⁺ for Pel-22 activity, while Ca²⁺ was the best cofactor for Pel-66 and Pel-90. The amino acid sequences deduced from the cloned genes showed the characteristics of Pels belonging to Family I. The pel-66 and pel-90 genes appear to be very similar, but they are different from the pel-22 gene. The characterized enzymes form two groups, Pel-66/Pel-90 and Pel-22; members of the different groups might cooperate to depolymerize pectin during the fermentation of cocoa beans.

Cocoa fermentation is a key step in the technological transformation of cocoa into chocolate (6, 33, 35). The fermentation of cocoa beans occurs at two levels: the first level involves reactions that take place in the pulp, in the outer part of the beans, and the second-level reactions are located deep within the cotyledons.

Reactions occurring in the pulp mainly concern the transformation of carbohydrates into ethanol and organic acids by a microflora essentially composed of yeast, lactic acid bacteria, acetic acid bacteria, and Bacillus (35). The resulting organic acids produced by the microbial metabolism diffuse into the bean and provoke lowering of the inner pH (16). The low pH, combined with the rise in temperature of the fermenting mass, activates two acidic-pH-dependent enzymes present in the cotyledons: an aspartic endopeptidase and a serine carboxypeptidase (6, 7, 43). The combined actions of these enzymes leads to the transformation of storage proteins into hydrophobic amino acids (5), which are known to be the main precursor molecules of cocoa and the eventual chocolate aroma (4, 35).

The fermentation process is also associated with the actions of various other plant cell wall-degrading enzymes, namely, pectinolytic enzymes. These enzymes, which allow the degradation of the cocoa pulp (34, 35, 36), facilitate the diffusion of microbial metabolites (essentially acetic acid) into the beans. Furthermore, the oligomers generated from the degradation of pectin polymers are used as a carbon source for the growth of the microorganisms. In view of the role they play, pectinolytic enzymes are not only essential for the normal course of cocoa fermentation, they are also key to the good quality of fermented and dried beans (3, 35).

Pectinolytic enzymes are classified into two main groups according to their mode of attack on pectin molecules: de-esterifying enzymes (pectin methyl esterase [EC 3.1.1.11]), which remove the methoxyl group from pectin, and depolymerases, which cleave the β(1,4)glycosidic bonds between galacturonate units, either by hydrolysis (polygalacturonase [EC 3.2.1.15]) or by trans-elimination (pectin lyase [EC 4.2.2.10] and pectate lyase [Pel] [EC 4.2.2.2]). Among these enzymes, the class of pectate lyases is widely distributed in bacteria and fungi, some phytopathogenic (1, 14, 15) and others, such as members of the genus Bacillus (2, 24, 39, 40), nonpathogenic. Pectate lyases are classified into different families according to their primary amino acid sequences (11, 38). The classification can be found on the CAZY (Carbohydrate-Active EnZymes database) server (http://www.cazy.org/) (10).

Over the last 3 decades, polygalacturonase secreted by yeasts has been the sole pectinolytic enzyme identified in cocoa fer-
mentation. However, we recently reported the involvement of pectate lyases produced by *Bacillus* strains in the cocoa fermentation process (26).

Here, we report the biochemical and molecular properties of purified pectate lyases from three different *Bacillus* strains isolated from fermenting cocoa beans and the characterization of their cloned genes.

**MATERIALS AND METHODS**

Organisms, culture conditions, and enzyme production. The three strains used here had been previously screened as Pel producers from a large population of *Bacillus* strains isolated from fermenting cocoa beans (26) and identified as *Bacillus faxiformis* (BS90), *Bacillus subtilis* (BS66), and *Bacillus pumilus* (BS22). For enzyme production, the strains were grown in 500 ml of medium prepared as follows: 450 ml basal mineral medium containing 2.8 g/liter (NH₄)₂SO₄, 6 g/liter K₂HPO₄, 2 g/liter KH₂PO₄, 0.8 g/liter citrate, 0.2 g/liter yeast extract, 0.1 g/liter MgSO₄·7H₂O, and 1 g/liter CaCl₂, plus 50 ml of LB medium to promote bacterial growth. This medium was supplemented with polygalacturonic acid (PGA) (2 g/liter) unless otherwise indicated and adjusted to pH 6.8. Cultures were incubated at 30°C for 48 h with shaking at 150 rpm. After centrifugation at 6,000 × g for 10 min, the cell supernatants were used for enzyme purification.

Enzyme purification and protein analysis. All steps for purification were performed at a temperature below 5°C. The cell supernatants were adjusted to 1 M diithiothreitol (DTT), and the resulting extracts were subjected to ammonium sulfate fractionation. Solid ammonium sulfate was gradually added to the clarified supernatant fluid at 20, 40, 55, 70, 90, and 100% saturation, with gentle stirring in an ice bath. The resulting fractions were collected by centrifugation at 12,000 × g for 15 min and dissolved in small volumes of buffer A (20 mM Tris-HCl, pH 7.2, 1 mM EDTA, and 1 mM DTT). The 90% and 100% ammonium sulfate fractions containing Pel activity were pooled and dialyzed overnight against buffer A, using a 14,000-Da filtration membrane. The preparations were extensively with the same buffer, and the retained proteins were eluted at 14 ml/min with an increasing NaCl linear gradient, from 0 to 1 M, using a Water high-pressure liquid chromatography (HPLC) system. Fractions (0.7 ml each) were collected and assayed for Pel activity. The purity of the fractions containing Pel activity was estimated by SDS-PAGE (22). Protein bands were detected by Coomassie blue staining. Protein concentration determination was carried out as proposed by Bradford (8).

Pel assay and thin-layer chromatography (TLC). Pel activity was determined spectrophotometrically (UVikon spectrophotometer; Kontron) by the release of polygalacturonate or pectin of unsaturated oligogalacturonides that absorb at 230 nm. One unit of Pel activity was defined as the amount of enzyme that liberated 1 micromole of product per min at 37°C under assay conditions. Specific activity was expressed as enzyme units per milligram of protein. Unless otherwise specified, the standard assay mixture consisted of 100 mM Tris-HCl (pH 8.0 for Pel-66 and Pel-90 and pH 7.5 for Pel-22), 0.1 mM CaCl₂, and 1 g/liter of polygalacturonate in a total volume of 1 ml. The products released from polygalacturonate were continuously monitored at 37°C for 2 min. The molar extinction coefficient of unsaturated oligogalacturonides was assumed to be 5,200 (23). The influences of divalent cations (Ba²⁺, Ca²⁺, Cu²⁺, Fe²⁺, Mg²⁺, Mn²⁺, Ni²⁺, Sr²⁺, and Zn²⁺) on Pel activity were determined with 0.1 mM concentrations of the corresponding chloride or sulfate form. The influence of Ca²⁺ and Fe²⁺ concentrations on Pel activity was investigated by the addition of CaCl₂ and FeSO₄ at concentrations ranging from 0 to 1 mM. To verify the Ca²⁺ requirement, EDTA was added to a final concentration of 1 mM to chelate the endogenous cations. The optimum pH was determined by using 100 mM (each) of the following buffers: acetate (pH 3.0 to 6.0), PIPES (piperazine-N,N'-bis(2-ethanesulfonic acid)-HCl) (pH 6.0 to 7.5), Tris-HCl (pH 6.5 to 9.5), and glycine-NaOH (pH 8.0 to 10.5). The effect of temperature was monitored within the range of 30 to 70°C. The influence of substrate methoxylation on Pel activity was tested by evaluating the activities of the purified enzyme on polygalacturonic acid and pectins at increasing degrees of esterification (from Copenhagen Pectin).

For *Km* and maximum rate of metabolism (*V_{max}*), determinations, enzymes were incubated at 60°C with polygalacturonate or pectin (45% methylation) at concentrations ranging from 0.025 to 1 g/liter. TLC was performed as described by Soriano et al. (40) using an aluminum chromatogram sheet (Merck, France) and concentrations of 5 μl/ml of the different enzymes.

**RESULTS**

Enzyme purification. Pels were precipitated at between 90 and 100% ammonium sulfate saturation and further purified by chromatography on a cation-exchange column. The amount of Pel recovered from 500 ml of culture varied between 0.1 and 0.4 mg depending on the production level and the final optical density at 600 nm (OD₆₀₀) of the culture. SDS-PAGE analysis showed satisfactory purity, and the apparent molecular masses were estimated to be 42 kDa for Pel-66 and Pel-90 and 34 kDa for Pel-22 (data not shown).

Influence of pH and temperature. All the Pels studied showed activity over a wide range of temperatures, from 30°C (15% activity) to 70°C (50% activity), with maximum activity at 60°C (Fig. 1). These enzymes proved to be very stable at room temperature, where they lost only 5% of their activity after a 15-day incubation in their respective optimum pH buffers without PGA. Under standard conditions, the half-times of thermostabilization at 60°C were approximately 30 min for Pel-22 and at least 27 min for Pel-66 and Pel-90. The three enzymes rapidly lost their stability above 70°C. At that temperature, Pel-22 lost 67% of its activity within 5 min, while in the same period, Pel-66 and Pel-90 lost 73 and 78% of their activities, respectively.

Pel-22 showed an optimum level of activity at pH 7.5, while Pel-66 and Pel-90 displayed their highest activities at pH 8.0 (Fig. 1). Although the pH range from 3.0 to 10.0 did not significantly influence the stability of enzymes, these enzymes seem to be more stable at their optimum pHs.

Kinetic parameters. The initial velocity for enzymatic reactions was determined using PGA and 45% esterified pectin, at different concentrations, as substrates. Pel-22 showed maximum activity at 0.5 g/liter of PGA and 0.1 g/liter of pectin. For Pel-66 and Pel-90, maximum activity occurred at 0.5 g/liter for both PGA and pectin. The kinetic parameters of purified Pels were deposited in DDBJ GenBank and appear under the following accession numbers: pel-90 gene, GU576909; pel-66 gene, GU576910; and pel-22 gene, GU576911.
on 45% esterified pectin and PGA were determined, and the apparent $V_{\text{max}}$ and $K_m$ values are given in Table 1. The affinity of Pel-22 for both substrates was approximately 2.5-fold higher than those of Pel-66 and Pel-90. In contrast, Pel-66 and Pel-90 degraded both substrates more efficiently than Pel-22.

Effects of divalent cations. Most of the characterized pectate lyases are known to have an absolute requirement for Ca$^{2+}$ as the cofactor. The addition of 1 mM EDTA to the reaction mixture totally inhibited the three purified Pels, demonstrating their absolute requirement for cations as cofactors. The influence of Ca$^{2+}$ was next investigated by adding CaCl$_2$ at concentrations ranging from 0 to 1 mM at the optimal pHs of the different enzymes.

In the presence of PGA as a substrate, similar results were obtained for the three enzymes. The maximal activity was observed at Ca$^{2+}$ concentrations of about 0.1 mM, which provided an increase of enzyme activity of more than 100%. In the range of 0.05 to 0.2 mM Ca$^{2+}$ concentrations, the rise in Pel activity was more than 75%, but enzyme activity dropped sharply below 0.05 mM and above 0.2 mM Ca$^{2+}$ (Table 2).

When 45% methylated pectin was used as the substrate, inhibition of enzyme activity due to high Ca$^{2+}$ concentration did not occur and the maximal activity was observed within a large range of Ca$^{2+}$ (from 0.2 to 1 mM) for the three Pels (Table 2).

The influences of several other cations (Fe$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, and Se$^{2+}$) were tested using a concentration of 0.1 mM. It appeared that all the tested cations could activate Pel-22 (Table 3). However, the best cofactors for this enzyme were Fe$^{2+}$ and Ca$^{2+}$, with relative activities on PGA of 231 and 208%, respectively. The best concentration of Fe$^{2+}$ for Pel-22 was 0.2 mM, with 45% methylated pectin as a substrate.

In contrast, Fe$^{2+}$ and Cu$^{2+}$ did not significantly affect the activities of Pel-66 and Pel-90. Mg$^{2+}$, Ni$^{2+}$, Ba$^{2+}$, and Se$^{2+}$ all moderately inhibited these two Pels, whereas stronger inhibition of Pel-66 and Pel-90 was observed with Mn$^{2+}$ and Zn$^{2+}$. Among these cations, Zn$^{2+}$ was the strongest inhibitor of Pel-66 and Pel-90, since it almost completely inhibited the activities of the enzymes (Table 3).

Influence of substrate methoxylation on Pel activity. Pels were strongly active on pectin, with a degree of methoxylation up to 60% (Fig. 2). With 75% pectin methoxylation, Pel-66 and Pel-90 retained approximately 40% of their activity while Pel-22 activity remained higher (more than 75% activity). On 90% pectin esterification, the residual activities of Pel-66 and Pel-90 were around 5% and that of Pel-22 was 14%.

Analysis of reaction products. To determine precisely the modes of action of the Pels, the reaction products obtained from PGA were characterized by TLC; enzymes were used at 5 U/ml. All the Pels studied were found to release a mixture of reaction products composed of dimers, trimers, and two unsaturated oligogalacturonic acid compounds. The major products liberated by Pel-22 were trimers, while Pel-66 and Pel-90 released relatively large amounts of both oligogalacturonic acid compounds and trimers (Fig. 3). Dimers remained the minor product liberated by each Pel. However, the amount of

<table>
<thead>
<tr>
<th>Pel</th>
<th>PGA</th>
<th>Pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$</td>
<td>$V_{\text{max}}$</td>
</tr>
<tr>
<td>Pel-22</td>
<td>0.045</td>
<td>1.41</td>
</tr>
<tr>
<td>Pel-66</td>
<td>0.1</td>
<td>667</td>
</tr>
<tr>
<td>Pel-90</td>
<td>0.125</td>
<td>909.9</td>
</tr>
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</table>

*The initial velocity for the reactions was determined at different polygalacturonate and pectin concentrations under standard conditions. Lineweaver-Burk transformation of the data gave $K_m$ (g/liter) and $V_{\text{max}}$ ($\mu$mol/min/mg) values for each enzyme.
dimers liberated by Pel-22 was extremely small compared with those produced by Pel-66 and Pel-90.

A combination of Pel-22 and Pel-66 (each at 2.5 U/ml) provoked the degradation of the long unsaturated oligogalacturonic acid compounds, leading to an increase of dimers in the reaction product mixture compared with that observed in the presence of each enzyme individually (Fig. 3). A similar result was obtained with a combination of Pel-22 and Pel-90. The profile of the chromatogram obtained from the combined actions of Pel-90 and Pel-66 did not significantly change compared with profiles of the individual enzymes.

TABLE 2. Influence of calcium and iron concentrations on Pel activities using PGA and 45% esterified pectin as substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cation concn (mM)</th>
<th>Enzyme activity (%)</th>
<th>Pel-22</th>
<th>Pel-66 (Ca²⁺)</th>
<th>Pel-90 (Ca²⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>0</td>
<td>100 (9.86)</td>
<td>100 (6.33)</td>
<td>100 (16.18)</td>
<td>100 (12.03)</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>161.66 (8.33)</td>
<td>163.79 (15.9)</td>
<td>149.2 (7.19)</td>
<td>155.47 (20.39)</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>168.33 (10.10)</td>
<td>180.33 (5.18)</td>
<td>143.45 (5.6)</td>
<td>176.58 (0.82)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>185 (14.50)</td>
<td>193.62 (8.78)</td>
<td>202.79 (7.32)</td>
<td>210.33 (8.31)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>213 (11)</td>
<td>235.75 (6.34)</td>
<td>231.38 (1.15)</td>
<td>261.38 (13.61)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>178.33 (13.16)</td>
<td>185.63 (6.42)</td>
<td>187.99 (2.53)</td>
<td>193.5 (15.29)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>141.66 (8.80)</td>
<td>143.31 (12.26)</td>
<td>138.42 (1.21)</td>
<td>108.54 (12.11)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>121.66 (4.80)</td>
<td>121.36 (9.52)</td>
<td>38.13 (1.5)</td>
<td>32.98 (3.96)</td>
</tr>
<tr>
<td>45% esterified pectin</td>
<td>0</td>
<td>100 (5.89)</td>
<td>100 (12.56)</td>
<td>100 (14.32)</td>
<td>100 (8.12)</td>
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<tr>
<td></td>
<td>0.01</td>
<td>149.76 (12.73)</td>
<td>158.12 (7.32)</td>
<td>142.36 (3.4)</td>
<td>141.79 (2.94)</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>178.35 (15.39)</td>
<td>185.61 (6.1)</td>
<td>162.31 (3.8)</td>
<td>178.64 (18.32)</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>213.54 (13.54)</td>
<td>228.18 (8.18)</td>
<td>211.58 (2.4)</td>
<td>215.54 (1.91)</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>266.8 (9.02)</td>
<td>279.67 (4.87)</td>
<td>272.28 (7.99)</td>
<td>251.42 (11.27)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>271.01 (10.64)</td>
<td>281.32 (4.12)</td>
<td>344.71 (5.28)</td>
<td>283.67 (1.93)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>274.25 (15.74)</td>
<td>272 (4.8)</td>
<td>361.13 (7.88)</td>
<td>304.59 (14.44)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>274.34 (9.49)</td>
<td>259.65 (1.25)</td>
<td>375.84 (12.46)</td>
<td>363.33 (14.79)</td>
</tr>
</tbody>
</table>

Activity under standard conditions (100 mM Tris-HCl, 1 g/liter PGA, and pH 7.5 or 8) without any added cation was used as a reference (100%). The influence of the cation concentration was determined by using the corresponding chloride or sulfate salt from 0 to 1 mM. Each result reported is the average of three independent experiments, and the standard deviations (±) are indicated in parentheses.

TABLE 3. Effects of divalent cations on Pel activities

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>Enzyme activity (%)</th>
<th>Pel-22</th>
<th>Pel-66</th>
<th>Pel-90</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 (5.53)</td>
<td>100 (3.16)</td>
<td>100 (4.72)</td>
<td></td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>208.48 (0.28)</td>
<td>237.16 (16.64)</td>
<td>262.59 (24.52)</td>
<td></td>
</tr>
<tr>
<td>Fe²⁺</td>
<td>231.42 (1.91)</td>
<td>94.58 (4.1)</td>
<td>100.3 (0.30)</td>
<td></td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>193.27 (1.46)</td>
<td>99.84 (3.41)</td>
<td>119.87 (12.51)</td>
<td></td>
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<tr>
<td>Zn²⁺</td>
<td>132.18 (2.6)</td>
<td>1.9 (0.71)</td>
<td>2.69 (0.98)</td>
<td></td>
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<tr>
<td>Mg²⁺</td>
<td>190.39 (16.62)</td>
<td>77.22 (0.75)</td>
<td>91.59 (3.86)</td>
<td></td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>135.74 (4.6)</td>
<td>15.96 (1.68)</td>
<td>17.48 (2.14)</td>
<td></td>
</tr>
<tr>
<td>Ni²⁺</td>
<td>190.92 (7.31)</td>
<td>68.71 (3.83)</td>
<td>100.61 (15.95)</td>
<td></td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>180.97 (13.76)</td>
<td>62.17 (2.7)</td>
<td>79.04 (7.88)</td>
<td></td>
</tr>
<tr>
<td>Sc²⁺</td>
<td>157.52 (3.13)</td>
<td>82.26 (13.81)</td>
<td>89.92 (6.39)</td>
<td></td>
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</table>

Activity under standard conditions (100 mM Tris-HCl, 1 g/liter PGA, and pH 7.5 or 8) without any added cation was used as a reference (100%). The influence of each divalent cation was determined by using the corresponding chloride or sulfate salt at 0.1 mM. Each result reported is the average of three independent experiments, and the standard deviations (±) are indicated in parentheses.

pGN66, contained a DNA fragment insert of 3.8 kb. Restriction mapping of the insert enabled us to subclone the 1.8-kb HindIII-HindIII fragment harboring the pel gene (data not shown) into a pBluescript plasmid to give pGN66-1. The complete nucleotide sequence of this fragment was then determined. The sequence contains an open reading frame (ORF) of 1,260 bp that starts with an ATG codon at nucleotide 402 and ends with a TAA termination codon at nucleotide 1664. At 8 nucleotides upstream of the start codon, there is a purine-rich sequence (AGAAAA) that could be a ribosome binding site (RBS). Putative -35 (TTGCTA) and -10 (TGATAA ATT) promoter signals were found upstream of the Pel-66

Cloning and characterization of the gene encoding Pel-66.

The strategy used to isolate the B. subtilis BS66 pel gene was the selection of recombinant plasmids exhibiting Pel activity on the polygalacturonate agar plates, as described by Keen et al. (18). Among at least 4,000 E. coli transformants, one clone showing Pel activity was detected. This plasmid, named pGN66, contained a DNA fragment insert of 3.8 kb. Restriction mapping of the insert enabled us to subclone the 1.8-kb HindIII-HindIII fragment harboring the pel gene (data not shown) into a pBluescript plasmid to give pGN66-1. The complete nucleotide sequence of this fragment was then determined. The sequence contains an open reading frame (ORF) of 1,260 bp that starts with an ATG codon at nucleotide 402 and ends with a TAA termination codon at nucleotide 1664. At 8 nucleotides upstream of the start codon, there is a purine-rich sequence (AGAAAA) that could be a ribosome binding site (RBS). Putative -35 (TTGCTA) and -10 (TGATAA ATT) promoter signals were found upstream of the Pel-66

FIG. 2. Influence of the degree of pectin esterification on Pel activity. The different pectins were added to the standard reaction mixture at 1 g/liter. Enzyme activities were measured at 37°C. Assays were done in triplicate, and the error bars indicate the standard deviations. Zero percent methoxylation corresponds to PGA.

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Downloaded from http://aem.asm.org/ on January 30, 2021 by guest
sequences of B. pumilus. Consequently, the primers F (5'-H11032
amplify the pel-22 X74880). showed high identity with the nucleotide sequence of the Pel
same purine-rich and inverted sequences. These two genes
pel-66
CAACACCTGCATTTG-3', and Rev F (5'-H11032-TTAAGGGTTTACTTTTC
ATGGAATACG) and Rev F (5'-H11032-TACTGCTGACTGTTTCCTGC-3')
downstream of the termination codon of the
control was prepared under the same conditions as the assays, except
66
galacturonic acid; lane B, di- and trigalacturonic acids. The negative
were added to the standard reaction mixture at a concentra-
tion of 5 U/ml, and the preparations were incubated at 45°C for 10 h.
For a combination of two Pels, 2.5 U/ml of each enzyme was used.
Twenty microliters of reaction products was run on the chromatogram
Merck (France). Lanes A and B are the standards. Lane A, galacturonic acid; lane B, di- and trigalacturonic acids. The negative
was prepared under the same conditions as the assays, except
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Characterization of the gene encoding Pel-22. An attempt to
clone the pel-90 gene from the genomic-DNA library of B.
was unsuccessful because of the instability of the recombinant plasmids displaying Pel activity. To avoid this
problem, we PCR amplified the pel-90 gene using genomic DNA of B. fusiformis BS90 as a template and two primers
(Forward, 5'-AAGCTTGGGCATAAAAGCAAGG-3'; Reverse, 5'-TACGTGACTGTTCCTGC-3') designed from the
pel-66 gene. This strategy was retained because Pel-66 and Pel-90 displayed similar biochemical properties and molecular
weights. A 1.55-kb fragment was successfully amplified from the BS90 genome. To verify whether the 1.55-kb amplified
fragment contained the pel-90 gene, the PCR products were ligated into a pGEMT vector (Promega, France), giving rise to the
plasmid pGN90 (data not shown). E. coli NMS22 competent
cells transformed with this plasmid exhibited Pel activity. The nucleotide sequence of the pel-90 gene also revealed an
ORF of 1,260 bp, starting with an ATG codon at position 152
and ending with a TAA codon at position 1414. The
ORF of 1,260 bp, starting with an ATG codon at position 152
and ending with a TAA codon at position 1414. The
and ends with a TGA termination codon at nucleotide 1,206.
The nucleotide sequence of the pel-22 gene displays 90% identity to that of the pel-103 gene from Bacillus sp. KSM-P103
(EMBL accession number AB015044.1) and 80% identity with the pel gene from Bacillus sp. KSM-7 (EMBL accession number
AB015043.1).
Amino acid sequence analysis. The ORFs of the Pel-66 and
Pel-90 genes encode polypeptides of 420 amino acids with calculated molecular masses of 45.81 kDa and 45.52 kDa,
respectively. The mature Pel-66 and Pel-90 both have a molecular
mass of 42 kDa, as shown by SDS-PAGE. The difference between the calculated molecular masses and those found in
SDS-PAGE experiments indicated that the three Pels are produced as precursors with a signal sequence at the NH2 extremity,
which is consistent with their extracellular localization. The two proteins show 97% identity. The pel-22 gene encodes a
polypeptide of 353 amino acids, with a calculated molecular mass of 38.9 kDa, while the mature protein has a molecular
mass of 34 kDa. This protein exhibited 25% and 26% identity with Pel-66 and Pel-90, respectively. The deduced amino acid
sequences of the cloned enzymes were compared with protein sequences in the Swissprot and EMBL databases. All the Pels
studied appeared to have identity with pectate lyases belonging to the polysaccharide lyase Family 1 (see Fig. S1 in the
supplemental material). However, Pel-66 and Pel-90 were closer to the Pel from B. subtilis SO113 (24), with more than 98%
identity, while Pel-22 appeared to be similar to Pel-103 from
Bacillus sp. KSM-P103 (12), with 95% identity, and Pel-7 from
Bacillus sp. KSM-P7 (21), with 82% identity.
Among the conserved regions, Pel-66 and Pel-90 showed residues Asp-185, Asp-224, and Asp-229 in positions identical
to that of the mature Pel from B. subtilis SO113 (21), with 82% identity, while Pel-22 appeared to be similar to Pel-103 from
Bacillus sp. KSM-P103 (12), with 95% identity, and Pel-7 from
Bacillus sp. KSM-P7 (21), with 82% identity.
Characterization of the gene encoding Pel-22. An attempt to
amplify the pel-22 gene from the genomic DNA of B. pumilus
BS22, using the same primers described above, was unsuccessful.
Consequently, the primers F (5'-GCTTCCTAGAAAATC
ATGGAATACG) and Rev F (5'-TAAAGTTATTTTCAACACTGCATTG-3')
designed from the pel gene
sequences of B. pumilus DKS1 and SAFR-032 (EMBL accession
numbers ACD11362 and ABV64163, respectively) and of
Bacillus sp. KSM-P103 (NCBI accession number AB015044.1), retrieved from the EMBL and NCBI databases. A 1,250-kb
DNA fragment was successfully amplified with these two primers. The amplified fragment was ligated into a pGEMT vector
(Promega, France) to give the plasmid pGN22 (data not shown). This construct was subsequently used to transform E.
coli competent cells, leading to clones exhibiting Pel activity. The nucleotide sequence of the pel-22 gene showed an ORF of
1,062 bp, which starts with an ATG codon at nucleotide 142
and ends with a TGA termination codon at nucleotide 1,206.
The nucleotide sequence of the pel-22 gene displays 90% identity to that of the pel-103 gene from Bacillus sp. KSM-P103
(EMBL accession number AB015044.1) and 80% identity with the pel gene from Bacillus sp. KSM-7 (EMBL accession number
AB015043.1).
Amino acid sequence analysis. The ORFs of the Pel-66 and
Pel-90 genes encode polypeptides of 420 amino acids with calculated molecular masses of 45.81 kDa and 45.52 kDa,
respectively. The mature Pel-66 and Pel-90 both have a molecular
mass of 42 kDa, as shown by SDS-PAGE. The difference between the calculated molecular masses and those found in
SDS-PAGE experiments indicated that the three Pels are produced as precursors with a signal sequence at the NH2 extremity,
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identity, while Pel-22 appeared to be similar to Pel-103 from
Bacillus sp. KSM-P103 (12), with 95% identity, and Pel-7 from
Bacillus sp. KSM-P7 (21), with 82% identity.
Among the conserved regions, Pel-66 and Pel-90 showed residues Asp-185, Asp-224, and Asp-229 in positions identical
to that of the mature Pel from B. subtilis SO113. These residues
were shown to be involved in the Ca2+ binding site (27). In
contrast, these Asp residues do not seem to be conserved in
Pel-22, which is consistent with the difference between Pel-66/ Pel-90 and Pel-22 in regard to the cofactors required.
DISCUSSION
Here, we report the biochemical characterization of Pels
from the three main pectinolytic Bacillus strains isolated from
fermenting cocoa beans: B. fusiformis (BS90), B. subtilis
(BS66), and B. pumilus (BS22) (26). The genes encoding these
enzymes were further cloned and characterized. Pel-22, Pel-66,
and Pel-90 proved to be thermophilic, with a high level of
activity at 60°C. This is not surprising, since most of the Pels
from Bacillus have been reported to show maximum activity at
temperatures between 60 and 70°C (12, 17, 21, 40). Furthermore,
the Pels studied present strong stability at room temperature
in a range of pH 3 to 10. Thus, these enzymes appear
to have the same robustness as the Pel from B. subtilis SO113

FIG. 3. Analysis of the different end products of Pel activities. Enzymes were added to the standard reaction mixture at a concentration of 5 U/ml, and the preparations were incubated at 45°C for 10 h. For a combination of two Pels, 2.5 U/ml of each enzyme was used. Twenty microliters of reaction products was run on the chromatogram sheet (Merck, France). Lanes A and B are the standards. Lane A, galacturonic acid; lane B, di- and trigalacturonic acids. The negative control was prepared under the same conditions as the assays, except that no enzyme was added to the reaction mixture. The arrows indicate the end products of the synergistic action. Blacks arrows, individual action; white arrows, combined action.
Paenibacillus barcinonensis results regarding substrate specificity are comparable to those obtained 5% of their activity while Pel-22 retained 14%. These two enzymes showed significant activity on pectin with up to 60% (for Pel-66 and Pel-90) or 75% (for Pel-22). Furthermore, the cytoplasmic enzymes PelW and Ogl from the same bacterium also use various cations (Co²⁺, Mn²⁺, or Ni²⁺) as cofactors (37). However, one of the atypical features of Pel-22 was that it appeared to use a wide range of divalent cations as cofactors, with iron proving to be the best. To the best of our knowledge, this is the first time that iron has acted more efficiently than Ca²⁺ as a cofactor; and they have the same molecular mass. As regards these properties, they behave like most of the previously characterized Pels from the genus Bacillus. On the other hand, Pel-22 has a low specific activity, a high affinity for methylated pectin. Based on their amino acid sequences and their biochemical properties, the three enzymes were clustered into two groups. Pel-66 and Pel-90 are very similar to each other: they both have a relatively low affinity for substrates, a high specific activity, and an alkaline optimum pH; they absolutely require Ca²⁺ as a cofactor; and they have the same molecular mass. As regards these properties, they behave like most of the previously characterized Pels from the genus Bacillus. On the other hand, Pel-22 has a low specific activity, a high affinity for its substrates, and an optimum pH near neutrality, and it uses a large variety of divalent cations as cofactors, among which Fe²⁺ appeared to be the best. This last characteristic makes Pel-22 interesting, since it constitutes the first Pel found to use Fe²⁺ as the preferential cofactor. Furthermore, it appeared that the enzymes from these two groups cooperate to efficiently degrade the substrates. The synergistic effect observed is of great concern in the choice of microbial starter strains for cocoa fermentation improvement. Regarding their biochemical properties, the three Pels should be able to take important places in the process of depectinization of the pulp during cocoa fermentation. Crystallography studies on Pel-22 might give new insights into the mechanisms of activation of a Pel by various divalent cations, including Ca²⁺ and Fe²⁺.

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