Characterization of Cold- and High-Pressure-Active Polygalacturonases from a Deep-Sea Yeast, Cryptococcus liquefaciens Strain N6

Fumiyoshi Abe,1,2 Hiroaki Minegishi,2 Takeshi Miura,1 Takahiko Nagahama,1 Ron Usami,2 and Koki Horikoshi1

1Extremobiosphere Research Center, Japan Agency for Marine-Earth Science and Technology (JAMSTEC), 2-15 Natsushima-cho, Yokosuka 237-0061, Japan
2Department of Applied Chemistry, Faculty of Engineering, Toyo University, 2100 Kujirai, Kawagoe 350-0815, Japan

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A deep-sea yeast, Cryptococcus liquefaciens strain N6, produces two polygalacturonases, p36 and p40 (N6-PGases). These N6-PGases were highly active at 0–10°C in comparison to a PGase from Aspergillus japonicus. The hydrolytic activity of these N6-PGases remained almost unchanged up to a hydrostatic pressure of 100 MPa at 24°C with a very small activation volume of $1.1 \text{ml/mol}$. At 10°C, however, the activation volume increased to 3.3 or 5.4 ml/mol (p36 and p40, respectively), suggesting that the enzyme–substrate complexes can expand at their transition states. We speculate that such a volume expansion upon forming the enzyme–substrate complexes contributes to decreasing the activation energy for hydrolysis. This can account for the high activity of N6-PGases at low-temperature.

Key words: deep-sea yeast; Cryptococcus liquefaciens strain N6; polygalacturonase; high hydrostatic pressure

Pectic compounds are polysaccharides originating in plants, with $\alpha$-1,4-glycoside linkage of polymers of galacturonic acids. Fungal polygalacturonases (PGases) have been used mainly in food industries, especially in the extraction and clarification of fruit juices. Today, the main source of PGases in industry is from the fungus Aspergillus niger. But commercial preparations of fungal origin contain a complex mixture of different enzymes with pectinolytic activity, including endo- and exo-PGase, pectin lyase, and the undesirable pectin esterases and other nonspecific enzymes. In this sense, yeast PGases might have advantages over fungal enzymes and might offer an alternative to fungal ones to provide pure enzymes. Attempts have been made to isolate PGases from yeasts. The biochemical properties of yeast PGases have been reviewed by Blanco et al. In our previous study, we isolated a PGase-producing yeast, Cryptococcus sp. strain N6, from the Japan Trench at a depth of 6,500 m. Two PGases, p36 and p40, were successfully purified, but their biochemical property had not been examined. In this study, we describe the identification of strain N6 and unique properties of p36 and p40 with respect to their kinetic parameters for hydrolysis.

For genomic DNA extraction, cells were grown on YM (0.5% bacto peptone, 0.3% bacto yeast extract, 0.3% bacto malt extract, 1% glucose) agar. The cells were disrupted by homogenization in an extraction buffer (50 mM TrisHCl, 50 mM EDTA, 3% SDS, pH 8.0) in the presence of alumina, followed by ethanol precipitation.

The divergent D1/D2 domain of 26S rDNA was amplified by PCR with primers F63 (5’-GCATATCA- TAAACGGAGAAAAG-3’) and LR3 (5’-GGTCCG- TGGTTCAAGACGG-3’). DNA sequencing was done using an ABI377 DNA sequencer. The accession number, AB217512, for the D1/D2 domain of 26S rDNA of strain N6 was obtained from the DNA Data Bank of Japan (DDBJ).

Strain N6 was identified as Cryptococcus liquefaciens according to a phylogenetic analysis using the neighbor-joining (NJ) method using Clustal X ver. 1.83 and NJ Plot (data not shown). The type strain of C. liquefaciens NBRC0434T showed no measurable PGase activity in the culture supernatant or on ruthenium red staining, suggesting that the production of PGase is specific to strain N6 but is not a general feature of C. liquefaciens (data not shown).

Purification of PGases from strain N6 was performed on HPLC, as described previously, yielding individual single protein bands on SDS–PAGE. The hydrolysis of polygalacturonic acid (PGA) (Sigma, P-3889, St. Louis, MO) was determined by a 2-cyanoacetamide method by measuring absorbance at 276 nm ($A_{276}$). The reaction was carried out for 15, 30, and 60 min in 10 mM sodium acetate buffer (pH 5.0), and was stopped by the addition of cold 0.1 M borate buffer (pH 9.0). One unit (U)
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Fig. 1. Effects of Temperature on PGase Activity.

The temperature optima were determined in 10 mM sodium acetate buffer (pH 5.0). Data are represented as relative values (%) of the maximal activity at 50 °C with 1.7 ± 0.05 U (p36), 0.21 ± 0.01 U (p40), and 1.0 ± 0.03 U (Aj-PGase) obtained from three independent experiments.

Table 1. Kinetic Parameters Associated with PGA Hydrolysis by p36, p40 and Aj-PGase a

<table>
<thead>
<tr>
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<th>(K_m) (mg/ml) b</th>
<th>(V_{max}) (U) b</th>
<th>(\Delta E) (kJ/mol)</th>
<th>(\Delta V^o) (ml/mol) at 24 °C c</th>
<th>(\Delta V^o) (ml/mol) at 10 °C c</th>
</tr>
</thead>
<tbody>
<tr>
<td>p36</td>
<td>1.4 ± 0.25</td>
<td>1.8 ± 0.14</td>
<td>20.6 ± 0.53</td>
<td>−1.1 ± 0.69</td>
<td>5.3 ± 2.7</td>
</tr>
<tr>
<td>p40</td>
<td>0.70 ± 0.23</td>
<td>0.21 ± 0.03</td>
<td>23.7 ± 1.6</td>
<td>−1.1 ± 1.1</td>
<td>5.4 ± 2.3</td>
</tr>
<tr>
<td>Aj-PGase</td>
<td>3.6 ± 0.38</td>
<td>1.6 ± 0.41</td>
<td>31.4 ± 1.4</td>
<td>−7.4 ± 1.7</td>
<td>−8.0 ± 2.6</td>
</tr>
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</table>

a Data are represented as mean values ± standard deviations from three independent experiments.  
b Data were obtained at 0.1 MPa and 40 °C.  
c Data were obtained at 0.1–100 MPa and 24 °C.  
d Data were obtained at 0.1–100 MPa and 10 °C.

corresponded to changes in \(A_{276}\) in 1 min per microgram of PGase. To examine hydrolytic activity at high pressure, a reaction mixture containing PGase (6.3 ng/ml) and PGA (0.2%) was placed in sterilized tubes in hydrostatic chambers and subjected to pressure of up to 100 MPa (1,000 kgf/cm²) using a hand pump (TP200L, Teramecs, Kyoto, Japan) for up to 60 min. Activity was measured after decompression. For technical reasons, this high-pressure experiment was performed at 24 °C and 10 °C but not at higher temperatures of 40–50 °C. A PGase from Aspergillus japonicus (Aj-PGase; P-3304, Sigma, St. Louis, MO) was used as a comparison.

p36, p40, and Aj-PGase showed the same optimal temperature of 50 °C for PGA hydrolysis (Fig. 1). p36 and p40 still maintained 25–30% of their maximum activity at 0 °C and 40–45% at 10 °C. In contrast, Aj-PGase maintained only 10% of its activity at 0 °C and 18% at 10 °C. Thus, N6-PGases are cold-adapted enzymes in comparison to Aj-PGase. The activation energy \(\Delta E\) for p36, p40, and Aj-PGase activity was 20.6, 23.7, and 31.4 kJ/mol respectively (Table 1).

Because the remaining hydrolytic activity dropped at 50 °C after extended incubation periods, > 60 min, the kinetic parameters \(K_m\) and \(V_{max}\) were determined at 40 °C. p40 had the lowest \(V_{max}\) value, but it had the smallest \(K_m\) value for PGA hydrolysis, indicating that p40 has the highest affinity for PGA (Table 1). The \(K_m\) values for the N6-PGases were in good agreement with those in previous studies, in which \(K_m\) usually fell in the range of 0.1 to 2 mg/ml for yeast PGases.  

Next we examined the effects of high hydrostatic pressure on hydrolysis by PGases. The application of hydrostatic pressure to reactions yields a fundamental physical parameter, i.e., the activation volume \(\Delta V^o\). The activation volume represents the difference in volume between the ground state and the transition state of the enzyme–substrate complex of the reaction, and is derived using the following equation:

\[
\langle \partial \ln k/\partial p \rangle_T = -\Delta V^o /RT,
\]

where \(k\) is the rate constant, \(p\) is pressure (MPa), \(T\) is absolute temperature (Kelvin), and \(R\) is the gas constant (ml MPa/K mol). \(\Delta V^o\) is the apparent volume change of activation (ml/mol). Both p36 and p40 maintained full activity at pressures of up to 100 MPa and 24 °C (Fig. 2A), and thus the activation volume for PGA hydrolysis by p36 and p40 was −1.1 ml/mol at 24 °C (Table 1). This result indicates that the net volume change upon transition of the N6-PGase–PGA complex is very small, at 24 °C. Aj-PGase activity increased as a function of pressure, accompanied by an activation volume of −7.4 ml/mol at 24 °C. This result indicates that the Aj-PGase–PGA complex becomes more compacted at the transition. Interestingly, at 10 °C, N6-PGase activity decreased slightly as a function of pressure, accompanied by small positive values of the
activation volume (3.3 and 5.4 ml/mol for p36 and p40 respectively), whereas the Aj-PGase activity was almost unchanged (Fig. 2B, Table 1). The interpretation of the results is complex, but it can be accounted for by the more expanded structure of the N6-PGase–PGA complex in the transition state than in the ground state at 10°C. In other words, N6-PGases are likely to be flexible and their structures can change differentially at different temperatures to form the transition state. Otherwise, the hydration status of the PGase–PGA complex at the catalytic site might differ between N6-PGases and Aj-PGase. We speculate that this volume expansion upon forming the enzyme–substrate complex contributes to decreasing the activation energy for hydrolysis. This accounts for high activity of N6-PGases at low-temperature. Temperature dependency on the activation volume has been reported in the case of the proteases thermolysin9) and chymotrypsin. 10) We isolated 15 PGase-producing yeast from deep-sea sediment samples. They belonged to the genus Cryptococcus, Rhodosporidium, or Aureobasidium (unpublished data). Although we cannot account for their occurrence in the deep sea, and their PGases have not been purified, deep-sea yeasts might be new resources for the isolation of a variety of PGases. Federici reported the purification and characterization of a 41-kDa PGase from Cryptococcus albidus var. albidus strain IMAT-4735.11) In our preliminary results, the type strain of C. albidus IFO 0378T did not produce a detectable level of PGase (data not shown). Therefore, production of PGase might be a unique property of strain IMAT-4735. To the best of our knowledge, this is the first report to determine the activation volume associated with hydrolysis by PGases, although the effects of higher pressures (> 200–300 MPa) have been examined with respect to high-pressure-induced inactivation of PGases in food materials.12) A systematic determination of the activation volumes of various PGases from fungi to yeasts will be necessary to clarify the mechanism of hydrolysis of α-1,4-glycosidic bonds by PGases.

References
2) Blanco, P., Sieiro, C., and Villa, T. G., Production of


