Purification and Properties of a Xylanase from Cellvibrio gilvus That Hydrolyzes p-Nitrophenyl Cellooligosaccharides

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An enzyme component that hydrolyzes pNP-G2 but not CMC has been isolated from a culture broth of Cellvibrio gilvus by a multi-step procedure involving Butyl-Toyopearl, DEAE-Toyopearl, and CM-Toyopearl chromatographies. The purified enzyme gave a single protein band on native, SDS-, and IEF-PAGE. The enzyme had a molecular weight of 40,000, an isoelectric point of 5.0, an optimum pH of 6.5, and an optimum temperature of 55°C. It was stable from pH 4.0 to 9.0 at 37°C for 1 hr and below 50°C for 30 min. It hydrolyzed agluconic bonds not only of pNP-G2 but also of pNP-G3, pNP-G4, and pNP-G5. Cellooligosaccharides with D.P. of 3 to 5 were not hydrolyzed at all. Instead, the enzyme hydrolyzed xylan 4 times as fast as pNP-G2. Both HgCl2 and p-chloromercuribenzoic acid inhibited the two activities completely. Xylan inhibited the hydrolysis of pNP-G2 competitively. From these results, the purified enzyme was considered to be a unique xylanase that hydrolyzed the agluconic bonds of pNP-Gn.

Cellvibrio gilvus has a cellulase system that produces cellobiose from cellulose1) as a main product, while the cellulase systems of most other microorganisms hydrolyze cellulose to glucose,2) eventually. It is easily anticipated that this bacterium produces an exo-type enzyme(s) that attacks the penultimate linkage of cellulose chains to produce cellobiose exclusively. p-Nitrophenyl-β-cellobioside (pNP-G2) has been used as a specific substrate for this type of enzyme. In the hope of obtaining such an exo-cellulase we examined the cellulase system of C. gilvus using pNP-G2 as a substrate. On hydrophobic chromatography using Butyl-Toyopearl, we found three components which hydrolyzed pNP-G2 but not carboxymethylcellulose (CMC) in a culture supernatant of this bacterium. This paper deals with the purification and characterization of the main component of this group of enzymes.

Materials and Methods

Materials. CMC with D.S. of 0.51 was supplied by Daiichi Industrial Pharmaceutical Co., Ltd. Cellooligosaccharides (G2–G6) were obtained from Seikagaku Kogyo Co., Ltd. pNP-Gn (n = 2–5) were obtained from Nakarai Chemical Co., Ltd., 4-methylumbelliferyl-β-D-cellobioside (MU-G2) was obtained from Sigma Chemical Co. Larch wood xylan was obtained from Aldrich Chemical Co. Inc. Xylooligosaccharides (X3–X6) and xylotetraitol (X4H) were prepared in our laboratory. SDS-PAGE Standard (Low), Combithek ( Calibration proteins for gel chromatography) and an Isoelectric Focusing Calibration Kit were purchased from Bio-Rad Laboratories, Boehringer-Mannheim, and Pharmacia LKB Biotechnology Inc., respectively. All other chemicals were commercial products of analytical grade.

Enzyme source. C. gilvus was cultivated at 30°C for 3 days in a medium containing 0.5% Avicel, 0.5% Casamino acids, 0.5% Yeast extract, 0.05% NaCl, 0.025% KCl, and 0.025% MgSO4·7H2O. Large scale cultivation was done by Meiji Seika Kaisha, Ltd. using a 10-kl fermentor. Culture broth was passed through a UF membrane and the resultant filtrate was dialyzed and lyophilized to obtain crude enzyme powder. This powder was supplied from the

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Enzyme assays. CMCase and xylanase activities were assayed in the reaction mixtures containing 0.05 ml of suitably diluted enzyme solution, 0.25 ml of 1% (w/v) CMC or xylan, and 0.2 ml of 0.1 M phosphate buffer (pH 6.5). Reaction mixtures were incubated for 10 min at 37°C and the reducing sugars produced were measured by the method of Somogyi3) and Nelson4) using glucose or xylose as a standard. One unit of the enzyme activity was defined as the amount that liberated 1 μmol of glucose or xylose equivalent in 1 min under the above conditions. Activity towards PNP-Gn was measured by incubating 0.1 ml of enzyme solution with 0.9 ml of 10 mM phosphate buffer (pH 6.5) containing 1.1 mM each substrate at 37°C for 10 min. The reaction was stopped by adding 0.5 ml of 1 M Na2CO3, and the released p-nitrophenol was measured by the absorbance at 400 nm. One unit of the enzyme activity was defined as the amount that liberated 1 μmol of p-nitrophenol in 1 min under the above conditions.

Analytical methods. Protein was measured by the method of Bradford.5) The absorbance at 280 nm was monitored for the measurement of protein in column eluents. Thin-layer chromatography was done using Kieselgel 60 F254 plate (5 x 7.5 cm) (E. Merck). The plate was developed with the solvent system of 80% acetonitrile solution at a room temperature. p-Nitrophenol and PNP-containing compounds on the plate were detected under UV light and then the sulfuric acid-baking method was used to detect sugars. High-performance liquid chromatography (HPLC) was done using a column of LiChrosorb NH2 (Cica Merck) and a carrier of 65% acetonitrile solution. PNP-containing sugars and sugars were detected by an UV spectrophotometer and an RI detector, respectively. For separation of anomeric forms of oligosaccharides, a LiChrosorb RP-18 (Cica Merck) column was used with water as the solvent.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE) in the absence (native) or presence of sodium dodecyl sulfate (SDS) and isoelectric focusing (IEF) PAGE were done on a Phastsystem of Pharmacia LKB Biotechnology according to their Manual. Phast Gel (Gradient 8-25) was used for native and SDS-PAGE. PhastGel IEF pH 4-6.5 was used for IEF-PAGE. For activity staining of PNP-G2 hydrolyzing activity, the electrophoresed gel plate was immediately dipped in 20 ml of 50 mM phosphate buffer (pH 6.5) containing 1 mM MU-G2 for 5 min and then the plate was exposed to UV light to detect released 4-methylumbelliferon. For activity staining of xylanase, the electrophoresed gel plate was kept in contact with an agar plate (1 mm thick) containing 1% xylan for 5 min at 60°C. Then the agar plate was removed and stained with Congo Red solution. 6)

Amino-terminal sequence analysis. About 20 μg (500 pmol) of the purified enzyme was used for SDS-PAGE using SDS-PAG PLATE 4/20 of Daiichi Chemicals according to their Manual and the protein was blotted on Immobilon Transfer Membranes (Millipore Co). using the semi-dry blotting system of Sartorius. After blotting, the membrane was stained with Coomassie Brilliant Blue to detect protein bands. The area containing protein was cut out and put on an automated protein sequencer/PTH analyzer system (Model 477A/120A) from Applied Biosystems.

Results

Purification of the enzyme

All procedures except where indicated otherwise were done at 4°C.

Step 1: Butyl-Toyopearl 650S column chromatography. This procedure was done at room temperature. Crude enzyme powder (40 g) was dissolved in 500 ml of 10 mM phosphate buffer (pH 6.0) and the turbid solution obtained was centrifuged at 10,000 rpm for 20 min to remove insoluble materials. To the resultant supernatant, ammonium sulfate was added to 30% saturation. The suspension was left for 1 hr and filtered with a Celite bed to remove insoluble materials. The filtrate was put on a Butyl-Toyopearl 650S column (2.3 x 20 cm) pre-equilibrated with 10 mM phosphate buffer (pH 6.0) containing 30% saturated ammonium sulfate and the protein was eluted by stepwise decreases in ammonium sulfate concentration. As shown in Fig. 1, three peaks (P-A, P-B, and P-E) having PNP-G2 hydrolyzing activity but not CMCase activity were obtained. The fractions belonging to the major peak (P-A) were combined and ammonium sulfate was added to 80% saturation. After centrifugation, the precipitated proteins were dissolved in a small amount of 10 mM phosphate buffer (pH 6.0) and desalted by passing through a Bio-Gel P-6 column (2.3 x 45 cm).

Step 2: 1st DEAE-Toyopearl 650S column chromatography. The desalted solution was put on a DEAE-Toyopearl 650S column (1.5 x 20 cm) previously equilibrated with 10 mM phosphate buffer (pH 6.0). Elution was done with a linear gradient of NaCl from 0 to 0.2 M in the same buffer. Fractions having PNP-G2 hydrolyzing activity were pooled and dialyzed...
Fig. 1. Elution Patterns of C. gilvus Cellulase System.
---, absorbance at 280 nm; ○, pNP-G2 hydrolyzing activity; ●, Xylan hydrolyzing activity; ■, CMC hydrolyzing activity.

Fig. 2. Elution Profiles of the Enzyme from CM-Toyopearl 650S Column.
---, absorbance at 280 nm; ○, pNP-G2 hydrolyzing activity; ●, xylan hydrolyzing activity.

against 10 mM phosphate buffer (pH 6.0).

Step 3: 2nd DEAE-Toyopearl 650S column chromatography. The dialyzed solution was put on a DEAE-Toyopearl 650S column (1.5 x 20 cm) pre-equilibrated with 10 mM phosphate buffer (pH 6.0). Elution was done with a linear
**Table I. Summary of Purification**

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg/ml)</th>
<th>Activity (U/ml)</th>
<th>Sp. act. (U/mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pNP-G2</td>
<td>Xylan</td>
<td>pNP-G2</td>
</tr>
<tr>
<td>Crude extracts</td>
<td>2.52</td>
<td>0.04</td>
<td>0.91</td>
<td>0.016</td>
</tr>
<tr>
<td>Butyl-Toyopearl</td>
<td>1.30</td>
<td>0.13</td>
<td>1.39</td>
<td>1.03</td>
</tr>
<tr>
<td>DEAE-Toyopearl, 1st</td>
<td>0.31</td>
<td>0.31</td>
<td>3.10</td>
<td>1.50</td>
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<td>DEAE-Toyopearl, 2nd</td>
<td>0.28</td>
<td>0.42</td>
<td>3.40</td>
<td>1.50</td>
</tr>
<tr>
<td>CM-Toyopearl</td>
<td>0.10</td>
<td>0.70</td>
<td>4.20</td>
<td>7.00</td>
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</tbody>
</table>

**Step 4: CM-Toyopearl 650S column chromatography.** The dialyzed solution (30 ml) was put on a CM-Toyopearl 650S column (1.5 x 20 cm) previously equilibrated with 10 mM phosphate buffer (pH 6.0). Elution was done with a linear gradient of NaCl from 0 to 20 mM in the same buffer. The results are shown in Fig. 2. A sharp symmetrical protein peak coinciding with those of pNP-G2 hydrolyzing activity and xylanase activity was obtained. Peak fractions were pooled, dialyzed against the phosphate buffer, and used as a purified enzyme in the following experiments.

Table I summarizes the result of purification. pNP-G2 hydrolyzing activity was purified 437-fold and xylanase was purified 117-fold. Yield was 4.0% for pNP-G2 hydrolyzing activity and 1.6% for xylanase.

**Physicochemical properties of the purified enzyme**

The purified enzyme showed a single protein band on native, SDS-, and IEF (pH 4.0–6.5)-PAGE as shown in Fig. 3. In another experiment, the purified enzyme was stained for xylanase activity, pNP-G2 hydrolyzing activity, and protein, successively. As is shown in
Fig. 4, the protein band migrated coincidentally with those of pNP-G₂ hydrolyzing activity and xylanase activity. This result conclusively indicated that a single protein has these two activities. The ten amino-terminal amino acids of the purified enzyme were sequenced as follows.

1 2 3 4 5 6 7 8 9 10
Ala-Thr-Thr-Leu-Lys-Glu-Ala-Ala-Asp-Gly.

This is also evidence for the homogeneity of the purified enzyme.

Molecular weight of the purified enzyme was estimated to be 40,000 from SDS-PAGE as
shown in Fig. 3A. The molecular weight of the native enzyme was measured by gel filtration on a Bio-Gel P-200 column (2.6 x 90 cm) in 10 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl. Again a value of 40,000 was obtained, indicating that the purified enzyme has a monomeric structure.

The isoelectric point of this enzyme was estimated to be pH 5.0 from the data shown in Fig. 3C.

Effects of pH and temperature of the purified enzyme

Effects of pH on the activity and stability of the purified enzyme are shown in Fig. 5. Both pNP-G₂ hydrolyzing and xylanase activities were found to be maximum at pH 6.5 and were stable for 1 hr at 37°C between pH 4.0 and 9.0. Effects of temperature on the activity and stability of the purified enzyme are shown in Fig. 6. Both activities were maximal at 55°C and were stable below 50°C for 30 min. Thus, no significant difference was observed between behaviors of the two activities toward pH and temperature.

Effects of chemical reagents

Effects of metals and other chemical reagents on two activities were studied as shown in Table II. In these experiments, enzymatic activities were assayed in the standard conditions including 1 mM of each reagent. Both HgCl₂ and p-chloromercuribenzoic acid (PCMB) inhibited the two activities completely under the above conditions. On the other hand, CaCl₂ slightly stimulated the two activities. Other metals and reagents had neither inhibitory nor stimulative effects toward the two activities. It could be pointed out that the two activities behaved in a similar way toward the chemical

<table>
<thead>
<tr>
<th>Reagents (1 mM)</th>
<th>Activity (%) to Xylan</th>
<th>Activity (%) to pNP-G₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>130</td>
<td>120</td>
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<td>MgCl₂</td>
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<tr>
<td>HgCl₂</td>
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</tr>
<tr>
<td>EDTA</td>
<td>104</td>
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</tr>
<tr>
<td>IAA</td>
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<tr>
<td>NBS</td>
<td>99</td>
<td>100</td>
</tr>
<tr>
<td>PCMB</td>
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<td>0</td>
</tr>
</tbody>
</table>

EDTA, ethylenediaminetetraacetic acid; IAA, iodoacetic acid; NBS, N-bromosuccinimide.

Fig. 7. Inhibition on pNP-G₂ Hydrolyzing Activity by Xylan.
Initial rates of pNP-G₂ hydrolysis were determined in the absence or presence (0.01% or 0.02%) of xylan and the data were graphed by a double reciprocal plot.
○, without xylan; ○, 0.01% xylan; △, 0.02% xylan.
reagents tested.

Inhibition of pNP-G₂ hydrolyzing activity by xylan

These results suggest that the two activities are catalyzed on the same single site of the enzyme protein. This was further confirmed by an inhibitory action of xylan, one substrate of the enzyme, on the hydrolysis of pNP-G₂, the other substrate. As is shown in Fig. 7, xylan inhibited the hydrolysis of pNP-G₂ and the type of inhibition was competitive. This indicates clearly that the two activities are catalyzed by a single site of the enzyme protein. The Ki calculated from the data shown in this figure was 0.07%, which was quite close to the Km for xylan, 0.064%, as shown in the next section.

Action on pNP-G₂ and xylan

As has been described above, the purified enzyme hydrolyzed both pNP-G₂ and xylan. p-Nitrophenol and G₂ are the only products from the former, and xylose, xylobiose, and xylotriose were produced from the latter. CMC, Avicel, and acid-swollen cellulose were not hydrolyzed at all. Km and k₀ for pNP-G₂ and xylan were 0.51 mM and 0.064%, and 6.7 sec⁻¹ and 26 sec⁻¹, respectively.

Hydrolysis of pNP-Gₙ

In the experiment shown in Fig. 8, the action of the purified enzyme toward pNP-cellobioside, -cellotrioside, -cellotetraoside, and cellopentaoside were examined by TLC. All these compounds were hydrolyzed into p-nitrophenol and the respective cellobio- saccharides. However, further hydrolysis of resultant cellobiosaccharides was not observed. The enzyme could hydrolyze only the agluconic bond of pNP-Gₙ. The hydrolysis rates of these pNP-Gₙ were essentially the same as that of pNP-G₂. Hydrolysis of pNP-glucoside, however, was not observed under these conditions.

Anomeric form of the reaction product

The anomeric form of saccharides produced from X₄H and pNP-G₄ by the action of this enzyme was studied. Reaction mixtures containing 1% of substrate and 40 mU of the enzyme were incubated for 3 min at 37°C and 20 μl of the reaction mixture was used immediately for HPLC analysis. Figure 9 shows that β-X₂ and β-X₃, with a negligible
amount of α-counterparts, were produced from \( X_4H \). Likewise, only β-G4 was produced from \( \text{pNP-G}_4 \) (data not shown). The enzyme retained the anomeric configuration of the sugars produced.

Discussion

Storvik and King\(^1\) suggested a long time ago that \( C. \text{gilvus} \) had cellulase(s) that had an exowise mode of action. In an attempt to isolate cellubiohydrolase (EC 3.2.1.91) from the cellulase system of \( C. \text{gilvus} \), we fractionated the cellulase system of this bacterium on Butyl-Toyopearl. As shown in Fig. 1, three peaks having pNP-G\(_2\) hydrolyzing activity but not CMCase were obtained. We purified the major peak (P-A) of them to an electrophoretically homogeneous state. During purification, we believed that this was a cellubiohydrolase. Upon examination of the substrate specificity of the purified enzyme, however, we found that it hydrolyzed xylan more rapidly than pNP-G\(_2\) and that it did not hydrolyze cellobiooligosaccharides at all. So we repeated the purification to examine the xylanase activity in each step.

The purified enzyme is considered to have enough evidence to be homogeneous. It showed a single protein band on PAGEs at three different conditions, native, SDS-, and IEF-PAGE (Fig. 3). In addition, protein, pNP-G\(_2\) hydrolyzing activity, and xylanase comigrated exactly on IEF-PAGE (Fig. 4). Furthermore, the amino terminal 10 amino acids of the purified enzyme were sequenced. All these results clearly indicate that a single protein with a molecular weight of 40,000 has both pNP-G\(_2\) hydrolyzing activity and xylanase activity. In addition, the two activities behaved in similar ways to pH, temperature, and several chemical reagents as shown in Figs. 5 and 6 and Table II. Xylan, one substrate of the enzyme, competitively inhibited the hydrolysis of pNP-G\(_2\), the other substrate, as shown in Fig. 7. These results conclusively indicate that the two activities are catalyzed on the same single site of the enzyme protein.

Action of the purified enzyme toward xylan was typical, it acted in endo fashion and retained the anomeric configuration of sugars produced. However, its action toward pNP-G\(_n\) was found to be quite unusual. No gluconic bond in these compounds was hydrolyzed. Only the agluconic bond was hydrolyzed (Fig. 8). All agluconic bonds in these compounds were hydrolyzed at essentially the same rate. The enzyme hydrolyzed pNP-G\(_2\) at a rate of 18.7% of that of xylan. In conclusion, it is not a cellulase but a xylanase having a relatively strong capacity to hydrolyze the agluconic bond of pNP-G\(_n\).

Numerous papers\(^7\)\(^8\) have been published on xylanases from various microbes. We also purified two xylanases from \( \text{Robillarda} \text{ sp. Y-20} \),\(^9\) a fungus isolated in our laboratory as a potent cellulase producer. Some of xylanases have been reported to have broad substrate specificity. Xylanases from \( \text{Aspergillus niger} \),\(^10\) \( \text{Talaromyces byssochlamydoides} \),\(^11\) and \( \text{Neurospora crassa} \)\(^12\) hydrolyzed cellulose although at much slower rates than xylan. However, xylanase A purified from \( \text{Clostridium acetobutylicum} \)\(^13\) showed a higher specific activity toward CMC than that to xylan. Some xylanases were also reported to have slight arabinosidase activity. Biely and Vrsanska\(^14\) did an extensive study on xylanase from \( \text{Cryptococcus albidus} \). The enzyme not only hydrolyzed aryl-β-D-xylopyranoside but also transferred the xylose residue from the substrate to cellobiose to form β-D-xylosycellobiose.

Some xylanases cloned in \( \text{Escherichia coli} \) were reported to have pNP-G\(_2\) hydrolyzing activity. Gilbert \textit{et al.}\(^15\) reported that \( \text{Pseudomonas fluorescens} \) subsp. \( \text{cellulosa} \) xylanase cloned in \( \text{E. coli} \) hydrolyzed pNP-G\(_2\). Lüthi \textit{et al.}\(^16\) reported that \( \text{Caldocellum saccharolyticum} \) xylanase cloned in \( \text{E. coli} \) hydrolyzed CMC and pNP-G\(_2\). In these cases, however, recombinant xylanases were not purified and had quite low activity toward pNP-G\(_2\) (less than 1% of the activity toward xylan). Grépinet \textit{et al.}\(^17\) purified xylanase Z produced in \( \text{E. coli} \) TG1 (pCT1208) carrying the \( \text{Clostridium} \)
thermocellum xynZ gene. This enzyme hydrolyzed pNP-G₂ at a rate 8.1% of that of xylan but did not hydrolyzed cellobiooligosaccharides or CMC. Xylanase Z seems to be close to the xylanase of C. gilvus in both its substrate specificity described above and its molecular weight (41,000). However, this xylanase protein lacks more than half of its complete sequence and it is not known whether the intact xylanase Z (M.W. 90,000), produced by recombinant E. coli carrying the complete xyn Z gene or Clostridium thermocellum, can hydrolyze pNP-G₂ or not. Curiously all xylanases reported to have pNP-G₂ hydrolyzing activity were produced by recombinant E. coli. Our enzyme seems to be the first nonrecombinant xylanase having this activity. Gilbert et al.¹⁵) suggested that pNP-G₂ and MU-G₂ could not be used as substrates to test for exoglucanase activity. This paper indicates clearly that it is quite dangerous to use pNP-G₂ as a specific substrate for cellobiohydrolase or cellulase.

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References