Purification and Characterization of $\beta$-1,6-Glucanase of *Streptomyces rochei* Application in the Study of Yeast Cell Wall Proteins

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A $\beta$-1,6-glucanase was purified to apparent homogeneity from a commercial yeast digestive enzyme prepared from *Streptomyces rochei* by a series of column chromatographies. The molecular mass of the purified enzyme was 60 kDa by SDS-PAGE. The purified enzyme had an optimum pH range from 4.0 to 6.0 and was stable in the same pH range. The enzyme was stable under 50°C but lost almost all activity at 60°C. The enzyme was specific to $\beta$-1,6-glucan and had little activity towards $\beta$-1, 3-glucan and $\beta$-1, 4-glucan. When the $\beta$-1,6-glucan was hydrolyzed with the purified enzyme for 5 h, the reaction products contained 20% glucose, 36% gentiobiose, and 44% other oligosaccharides, suggesting that the enzyme is an endo-type glucanase. When the purified enzyme was used for the digestion of the cell wall of *Saccharomyces cerevisiae*, cell-wall proteins covalently bound to the cell-wall glucan were recovered as soluble forms, suggesting that this enzyme is useful for analysis of yeast-cell wall proteins.

Key words: yeast cell wall; $\beta$-1,6-glucanase; hydrolysis; cell-wall proteins

The cell wall of *Saccharomyces cerevisiae* mainly consists of three components: glucan, chitin, and cell-wall proteins. In addition to governing the shape of the cell, the cell wall supports the mechanical strength of the cell, and takes part in the exchange of materials and information between the cell and the environment. The cell wall contains many species of proteins. However, their functions and biosynthesis pathways are still not clear. The cell-wall proteins (CWPs) of *S. cerevisiae* can be classified into two groups based on whether they can be extracted by SDS or not. The latter SDS-insoluble CWPs are synthesized as GPI-anchored proteins (GPI-CWPs) and are linked to the cell wall through $\beta$-1,6-glucan and $\beta$-1,3-glucan. Therefore, these proteins can be extracted from the cell wall by treatment with either a $\beta$-1,3-glucanase or a $\beta$-1,6-glucanase. Because $\beta$-1,6-glucan is the bridge component between the GPI-CWPs and $\beta$-1,3-glucan, digestion of the cell wall with $\beta$-1,6-glucanase is preferable for preparation of GPI-CWPs without $\beta$-1,3-glucan, which makes identification of the proteins difficult with its large molecular mass and heterogeneity. However, there are a few reports on $\beta$-1,6-glucanases for cell-wall analysis. As far as we know, there is no commercially available purified $\beta$-1,6-glucanase. Thus, we have attempted to purify a $\beta$-1,6-glucanase from a commercial enzyme preparation. We chose Westase (Takara Shuzo Co., Ltd., Shiga, Japan), which is a yeast cell-wall digestive enzyme prepared from the culture supernatant of *Streptomyces rochei* DB34, because the enzyme contains highly active $\beta$-1,6-glucanase as well as $\beta$-1,3-glucanase. Moreover, when yeast cell walls were treated with Westase in preliminary experiments, CWPs were identified in the soluble fraction (not shown). In this paper, we report the purification and characterization of the $\beta$-1,6-glucanase from Westase and that the purified enzyme is useful for analysis of yeast cell-wall proteins.

During the purification process, $\beta$-1,6-glucanase activity was routinely measured by incubation of 10 μl of enzyme in 90 μl of 50 mM sodium acetate buffer (pH 5.0) containing 5 mg/ml pustulan as a substrate (from *Umbilicaria papullose*). Pustulan was dissolved in hot buffer, cooled, then, the precipitate was removed by centrifugation at 2,000 × g for 2 min. After the reaction mixture was incubated at 30°C for 30 min, the reducing sugar released was measured by a method with 3, 5-dinitrosalicylic acid reagent as described by Yu et al. An enzyme unit was defined as the amount of enzyme that catalyzes the release of reducing sugars equivalent to 1 μmol of glucose per minute under the assay conditions. Protein concentrations were measured with a BCA protein assay reagent kit (Pierce Chemical Company, Rockford, IL., U. S. A).

All purification procedures were done at 4°C except for chromatographies, which were done at room temperature. As a starting material, 1 g of Westase was dissolved in 100 ml of distilled water. This crude enzyme was centrifuged for 20 min at 3,000 × g and

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Abbreviations: CWP, cell-wall protein; GPI, glycosylphosphatidylinositol; HPLC, high performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride
the supernatant was desalted and concentrated to 20 ml with Ultrafree-15 ultrafiltration cartridge, the molecular cut off of which is 10 kDa (Millipore). The solution was put on a column of DEAE-5PW anion-exchange chromatography (21.5 × 150 mm, Tosoh Co., Tokyo, Japan) equilibrated with 20 mM Tris-HCl buffer (pH 7.0). The column was eluted with a continuous linear gradient from 0 to 0.4 M sodium chloride in 20 mM sodium acetate buffer (pH 4.0) at a flow rate of 1 ml/min. The active fractions (about 30 ml) were collected and concentrated by centrifugation with Ultrafree-15 ultrafiltration cartridge, the molecular cut off of which is 10 kDa (Millipore). Then, the enzyme was dialyzed against 50 mM acetate buffer (pH 5) with 1 mM phenylmethylsulfonyl fluoride (PMSF) and stored at 4°C.

The purification process is summarized in Table 1. The β-1,6-glucanase activity was purified 203-fold (giving a specific activity of 83.3 U/mg) with a yield of 28.5%. Homogeneity of the final preparation was checked by SDS-PAGE and Coomassie brilliant blue staining. There was only a single band in the purified enzyme with an apparent molecular mass of 60 kDa, based on comparison with the standard proteins (Fig. 1). The N-terminal amino acid sequence of the purified enzyme was identified as AGEQVTAWLTTTDDSA with an automated protein sequencer (491 Procise Perkin Elmer). This sequence has little similarity with any sequence in the databases, suggesting that the primary structure of the enzyme is different from those of other proteins that have similar activities.

To examine the effects of pH on the enzyme activity, 100 mM McIlvaine buffer (pH 2.0), 20 mM acetate buffer (pH 3.0–6.0), and 20 mM Tris buffer (from pH 7.0 to 10.0) were used as reaction buffers. The purified β-1,6-glucanase, diluted to a suitable concentration, was added to the buffers with pHs from 2.0 to 10.0 containing 5 mg/ml pustulan, and assayed for the β-1,6-glucanase activity. These buffers were also used to investigate the pH stability of the β-1,6-glucanase. The purified enzyme was diluted in suitable concentrations in 50 mM acetate buffer, pH 5.0, and assayed for 15 min at temperatures from 10°C to 80°C, then the
Table 2. Substrate Specificity of the Purified β-1,6-Glucanase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Main linkage type (monomer)</th>
<th>Activity (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chitin</td>
<td>β-1,4</td>
<td>0.0</td>
</tr>
<tr>
<td>Curdlan</td>
<td>β-1,3</td>
<td>0.2</td>
</tr>
<tr>
<td>Dextran</td>
<td>α-1,6</td>
<td>0.0</td>
</tr>
<tr>
<td>Starch</td>
<td>α-1,4; α-1,6</td>
<td>0.4</td>
</tr>
<tr>
<td>Laminarin</td>
<td>β-1,3</td>
<td>0.4</td>
</tr>
<tr>
<td>Pustulan</td>
<td>β-1,6</td>
<td>86.1</td>
</tr>
</tbody>
</table>

Since repetitive freezing and thawing severely damaged the enzyme activity of β-1,6-glucanase, the purified enzyme was stored at 4°C.

The substrate specificity of the purified β-1,6-glucanase was assayed with a variety of glucan substrates (Table 2). The purified enzyme was diluted in a suitable concentration in the 50 mM sodium acetate buffers (pH 5.0) containing 5 mg/ml glucan substrate. After the reaction mixture was incubated at 30°C for 30 min, the reducing sugar released was measured. The maximal activity was detected when substrate was pustulan, which is a linear β-1,6-glucan. When curdlan and laminarín (β-1,3-glucan) were used as substrates, the activities were very low (0.25% and 0.5% compared with pustulan respectively). No activities were detected when chitin (β-1,4-glucan), dextran (α-1,6-glucan), or starch (α-1,4, α-1,6-glucan) were used as substrates. Because the enzyme digested the pustulan only, we concluded that the purified enzyme cleaved the β-1,6 linkage specifically.

The hydrolysis product of pustulan was analyzed

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Fig. 2. HPLC of the Action of Purified β-1,6-Glucanase on Pustulan.

Pustulan (5 mg/ml) was incubated with the purified β-1,6-glucanase (with a final concentration of 160 mU/ml) at 37°C for the indicated times. Gn refers to the glucose oligomer (n = degree of polymerization).
on a column equilibrated with 100 mM NaOH, and Dionex) at room temperature. The samples were put into a mixture of oligosaccharides (from n = 20). The large oligosaccharides were then degraded into smaller ones. After 5 h of digestion, there were 20% glucose, 36% gentiobiose, and other small oligosaccharides in the hydrolysis products. These results suggest that the purified enzyme acts as an endo-glucanase.

Trichoderma harzianum produces two endo-β-1,6-glucanases, BGN16.1 and BGN16.2.8,9 They released a series of large β-1,6-oligosaccharides from pustulan in the early hydrolysis products and released gentiobiose as the major final product. BGN16.1 did not produce glucose in the hydrolysis products while BGN16.2 did. Similar to BGN16.1 and BGN16.2, the purified β-1,6-glucanase from S. rochei was unable to cleave gentiobiose to glucose, as shown in Fig. 2. Gentiotriose was not found in the hydrolysis products with the purified β-1,6-glucanase from S. rochei but it was found in those with BGN16.1 and BGN16.2. Although we cannot know why the enzyme did not release gentiotriose and other small odd-numbered glucose oligomers (n = 3, 7, ... ) in the final hydrolysis product of pustulan, we can estimate that the mode of action of the purified β-1,6-glucanase from S. rochei to pustulan is different from those of BGN16.1 and BGN16.2.

To confirm that the purified enzyme is useful to characterize cell wall proteins of yeasts, the cell walls of S. cerevisiae YPH49915) were digested with the purified enzyme and released proteins were analyzed. The yeast cells were grown at 30°C with shaking for two days in YPAD medium (1 % yeast extract, 2 % bactopeptone, 2 % glucose, 0.04 % adenine sulfate). The cells were harvested by centrifugation at 1500 × g for 5 min, and suspended in fresh YPAD medium. After grown in the medium at 30°C with shaking for 24 h, the cell walls were prepared as described previously.10) The yeast cells were harvested by the centrifugation, and suspended in 20 mM Tris-Cl buffer (pH 8.0) containing 0.1 mM PMSF. The cells were disrupted with glass beads (diameter, 0.45 – 0.50 mm) by a homogenizer (B. Braun) for 5 × 1 min. After this was washed with 1 mm PMSF twice and 5 mM LiCl 3 times, the cell walls were extracted with 2 % SDS at 100°C for 10 min, and then washed with 1 mM PMSF 5 times. The cell walls obtained thus were free from proteins that were bound non-covalently. The cell walls were suspended in 50 mM acetate buffer containing 1 mM PMSF (pH 5.0). After addition of the purified β-1,6-glucanase at a final concentration of 100 mU/ml, the reaction mixture was incubated at 30°C for 24 h. After centrifugation at 12,000 × g for 20 min, the supernatant containing cell wall proteins was put through SDS-PAGE and stained with Coomassie brilliant blue. Three major proteins with molecular masses of about 180, 120, and 48 kDa were released from the cell walls of S. cerevisiae YPH499 (Fig. 3). The proteins were also electrophoresed and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, England). Immunodetection of proteins with anti-Cwp1p16) was done according to the ECL immunodetection procedure (Amersham Pharmacia Biotech). There was one band with a molecular mass of about 48 kDa, indicating that the 48-kDa protein was Cwp1p (Fig. 3).
Cwp1p is one of major cell wall proteins that are covalently bound to the cell wall through β-1,6-glucan.4,16 This result confirmed that the purified β-1,6-glucanase is useful to the study of yeast cell wall proteins.

References


