Purification and Properties of Chitinase from *Arthrobacter* sp. NHB-10

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A chitinase was purified from the culture filtrate of nigeran-degrading *Arthrobacter* sp. NHB-10 by precipitation with ammonium sulfate and column chromatographies on DEAE-Sephadex A-50 and Superose 12. The final preparation was homogenous in polyacrylamide gel electrophoresis. The molecular weight of the purified enzyme was 30,000 and its isoelectric point was 6.8. The optimum pH and temperature for the enzyme activity were 5.0 and 45°C, respectively. The enzyme was stable from pH 3 to 7 and up to 55°C. The enzyme activity was inhibited by Hg²⁺ and p-chloromercuribenzoic acid. Two internal amino acid sequences of the enzyme were AGPQLLTGY and IG-GVMT.

**Key words:** chitinase; *Arthrobacter*; chitin

Chitin, an insoluble linear β-1,4-linked polymer of N-acetylglucosamine (GlcNAc), is one of the most abundant polysaccharides in nature. In our previous paper, we isolated nigeran (alternating α-1,3- and α-1,4-linked D-glucan found in the hyphal wall of fungi such as *Aspergillus niger*)-degrading *Arthrobacter* sp. (strain NHB-10). Recently, we also found that NHB-10 strain has lytic activity on *A. niger* and *A. japonicus* cells. As chitin is the main structural component of fungal cell walls, the chitinase (EC 3.2.1.14) seems to be a key enzyme in the lysis of the cell wall. This paper describes the properties and internal amino acid sequences of the chitinase purified from *Arthrobacter* sp. NHB-10. N,N',N''-Triacetylchitotriose ([GlcNAc]₃) was used as a substrate for chitinase activity. In a standard assay, 25 μl of appropriately diluted enzyme solution was incubated with 50 μl of 0.4% substrate solution and 25 μl of 0.2 M phosphate buffer (pH 5.5) at 40°C for 30 min. After the reaction was stopped by boiling for 30 sec, liberated GlcNAc was measured by the method of Reissig et al. One unit of enzyme was defined as the amount that liberated 1 μmol of GlcNAc equivalent per min under the standard assay conditions. Protein was measured by the method of Lowry et al.⁶

The strain NHB-10 was grown at 28°C in a medium consisting of 1.0% powdered chitin, 0.1% yeast extract, 0.5% polypeptone, 0.2% KH₂PO₄, 0.1% MgSO₄·7H₂O, and 0.1% NaCl (pH 7.0). The cultivation was continued in a 500-ml shaking flask on a reciprocating shaker. After 6 days of cultivation, the chitinase activity was highest and the powdered chitin in the medium was completely solubilized. Therefore, we obtained crude enzyme from a 6-day culture filtrate (695 ml) of NHB-10 by precipitation with ammonium sulfate (80% saturation). The crude enzyme solution (145 ml) was freeze-dried and the resulting residue was dissolved in 50 mM Tris-HCl buffer (pH 8.0). The enzyme solution was put on a column (2.3 × 36 cm) of DEAE-Sephadex A-50 (Pharmacia Biotech) and the column was eluted with a linear gradient of NaCl (0 to 0.5 M) in the same buffer. Chitinase activity was detected in the non-adsorbed fraction and in the fraction eluted with 0.15-0.30 M NaCl. In this study, we further purified the enzyme from the non-adsorbed fraction. The enzyme solution (132 ml) was concentrated by lyophilization and then buffered with 50 mM sodium phosphate buffer (pH 7.0). The solution was put on a Superose 12 column (10 × 300 mm, Pharmacia Biotech) and the column was eluted with the same buffer at a flow rate of 0.5 ml/min. Chitinase activity was detected at a retention time of about 25 min and enzyme solution (6.7 ml) was obtained. As shown in Table, the overall purification was 21-fold with a recovery of 8%

### Table: Purification of Chitinase from *Arthrobacter* sp. NHB-10

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NH₄)₂SO₄ ppt</td>
<td>84.3</td>
<td>36.2</td>
<td>0.43</td>
<td>1.0</td>
<td>100</td>
</tr>
<tr>
<td>DEAE-Sephadex A-50</td>
<td>7.02</td>
<td>10.5</td>
<td>1.50</td>
<td>3.5</td>
<td>29</td>
</tr>
<tr>
<td>Superose 12 FPLC</td>
<td>0.32</td>
<td>2.92</td>
<td>9.13</td>
<td>21.2</td>
<td>8.1</td>
</tr>
</tbody>
</table>

FPLC, Fast protein liquid chromatography.

In polyacrylamide gel electrophoresis (PAGE) of the purified enzyme, a single protein band was detected (Fig. 1A) and its mobility coincided with that of the band corresponding to chitinase activity detected by the standard assay using the enzyme released from another section of the gel. The molecular weight of enzyme was estimated to be 30,000 by SDS-PAGE⁶ (Fig. 1B, lane 2) and 38,000 by gel filtration of Superose 12 (data not shown), indicating a monomeric structure. Electrophoresis on a thin-layer agarose gel⁷ (Funakoshi Co.) showed that the isoelectric point of the enzyme was 6.8. N-terminal and internal amino acid sequences of the purified chitinase were analyzed on an Applied Biosystems model 491 Procise protein sequencer. Internal amino acid sequences of only two peptides obtained by digestion with lysylendopeptidase were found to be AGPQLLTGY and IG-GVMT.
The enzymatic properties were examined using 1.6 mU of the purified chitinase under the standard conditions. The activity was measured in 50 mM buffers adjusted to various pHs. The optimum pH for the enzyme activity was 5.0. The enzyme was stable from pH 3.0 to 7.0 after incubation at 4°C for 48 hr in various 50 mM buffers. The optimum temperature for the enzyme was 45°C. When the enzyme was kept at various temperatures for 10 min, it was stable up to 55°C and was completely inactivated at 65°C. The enzyme activity was significantly inhibited by Hg²⁺ and p-chloromercuribenzoic acid (1 mM), suggesting some importance of a sulphydryl group for the expression of the enzyme activity. The purified chitinase hydrolyzed colloidal chitin and trimer to pentamer of GlcNAc, but not (GlcNAc)₂, p-nitrophenyl β-D-N-acetylglucosaminide, chitopentaose (chitosan pentamer), or other substrates (carboxymethyl cellulose, laminarin, amylase, maltopentaose, isomalto-pentaose, laminariobiase, maltose, and cellobiose). The Lineweaver-Burk plot demonstrated that the Kₘ (mm) was 2.01 for (GlcNAc)₁, 1.99 for (GlcNAc)₂, 2.13 for (GlcNAc)₃, and 1.53 for (GlcNAc)₆.

After incubation with 0.95 ml of colloidal chitin suspension (1.0 of absorbance at 610 nm) and the chitinase (250 mU) at 40°C, the absorbance of the reaction mixture was reduced about 17% for 3 hr, 22% for 6 hr, 30% for 12 hr, and 40% for 24 hr. High-performance liquid chromatography analysis demonstrated that the hydrolysis products from colloidal chitin were mainly (GlcNAc)₂ with a small amount of GlcNAc in 24 hr of reaction. The purified chitinase (4.8 mU) was reacted with various N-acetylchitoooligosaccharides (0.3 mg) at 40°C for 30 min and then the products were analyzed by thin-layer chromatography (Fig. 2). The enzyme hydrolyzed (GlcNAc)₂ to GlcNAc and (GlcNAc)₆ (Fig. 2, lane 2), and (GlcNAc)₄ to GlcNAc, (GlcNAc)₂, and (GlcNAc)₆ (Fig. 2, lane 3). The (GlcNAc)₂ and (GlcNAc)₆ were hydrolyzed to GlcNAc, (GlcNAc)₂, (GlcNAc)₄, and (GlcNAc)₆ (Fig. 2, lane 4 and 5). However, the enzyme did not hydrolyze (GlcNAc)₄ (Fig. 2, lane 1). After 24 hr of reaction, the GlcNAc and (GlcNAc)₂ were detected as predominant products.

Many bacterial chitinases have been purified and
characterized from the species of Streptomyces,5-8 Bacillus, Aeromonas, Alteromonas, Xanthomonas, Enterobacter, Serratia, and Clostridium. Although three strains of yeast or fungal cell wall lytic Arthrobacter produced a chitinase(s) and used chitin as a carbon source,9-10 there have not been detailed studies on chitinase from Arthrobacter. Properties of our enzyme were similar to those of chitinases from four Streptomyces strains5-8 in low molecular weight, from S. griseus8 in neutral pH, from S. griseus8 and S. erythraeus5 in acidic optimum pH, and from S. orientalis6 in inhibition of enzyme activity by Hg2+. BLAST and FASTA analysis of the internal amino acid sequences found no apparent similarity to any microbial and plant chitinases. We are now studying the cloning and nucleotide sequence of the chitinase using oligonucleotides from the internal amino acid sequences analyzed.

References