Purification and Characterization of a 34-kDa Chitobiosidase from *Aeromonas* sp. GJ-18

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Abstract Chitobiosidase was purified and characterized from *Aeromonas* sp. GJ-18 by ammonium sulfate precipitation, anion-exchange chromatography, and gel filtration chromatography. The purified enzyme has a molecular weight of 34 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme showed an optimum pH and temperature of 6.0 and 30–50°C, respectively. The enzyme was stable at pH 5–8 and 50°C and was completely inhibited in the presence of 10 mM Zn²⁺ ions. The enzyme could efficiently hydrolyze colloidal chitin into N,N'-diacetylchitobiose as the major product, indicating that the purified enzyme is a chitobiosidase. When colloidal chitin was used as the substrate, the *K*ₘ and *V*ₘₐₓ of this enzyme were established as 3.45 mg/mL and 2.91 µmol/min, respectively.

Keywords *Aeromonas* sp. GJ-18 · chitin · chitinolytic enzyme · chitobiose · chitobiosidase

Introduction

Chitin, a homopolymer of β-1,4-linked N-acetylglucosamine, is one of the most abundant, easily obtained, and renewable natural polymers. This polysaccharide is a recalcitrant substrate for degradation, because in nature it exists in crystalline and complex forms conjugated with proteins, salts, and other carbohydrates. Chitinases, which hydrolyze chitin, occur in a wide range of organisms including viruses, bacteria, fungi, insects, higher plants, and animals (Duchet et al., 2011). The roles of chitinases in these organisms are diverse. In bacteria, chitinases are synthesized primarily to hydrolyze chitin as a carbon and nitrogen sources (Tsujibo et al., 1998). Chitinases can be classified into two major categories: endochitinases (EC 3.2.1.14) and exochitinases (EC 3.2.1.29), among which exochitinases can be divided into two subcategories: chitobiosidase (EC 3.2.1.29), which catalyzes the progressive release of N,N'-diacetylchitobiose [(GlcNAc)₂] starting at the nonreducing end of chitin microfibril, and N-acetyl-D-glucosaminidase (EC 3.2.1.30), which cleaves the oligomeric products of endochitinases and chitobiosidase, finally generating monomers of GlcNAc. Generally, for efficient chitin degradation, bacterium contains a complex chitinolytic system composed of multiple chitinases, chitobiases, and/or chitin-binding proteins (Sahai and Manocha, 1993).

Previously, we reported a chitinase-producing bacterium, which was isolated from coastal soil and identified as *Aeromonas* sp. GJ-18 (Kuk et al., 2005a). By controlling the reaction temperature, GlcNAc and (GlcNAc)₂ could be selectively produced from chitin using a crude enzyme preparation from this bacterium, indicating the presence of N-acetyl-D-glucosaminidase and chitobiosidase in the crude enzyme preparation (Kuk et al., 2005b). However, the biochemical properties of both enzymes are unknown. In addition, enzymatic production of (GlcNAc)₂ has been regarded to be important, because (GlcNAc)₂ have a significant economical value and an important biotechnological application. In particular, (GlcNAc)₂, the smallest repeating unit of chitin, has been widely used as an important building block for synthesis of various complicated oligosaccharides and polysaccharides (Usui et al., 1990; Yoon, 2005).

Therefore, a detailed understanding of chitobiosidase, key enzyme in the production of (GlcNAc)₂ from chitin, is necessary. Herein, the purification and characterization of a 34-kDa chitobiosidase from *Aeromonas* sp. GJ-18 are reported.

Materials and Methods

Materials. Chitin was purchased from Kitto Life (Seoul, Korea). Swollen chitin was prepared as described by Monreal and Reese.
Purification of chitobiosidase. For chitinase production, *Aeromonas* sp. GJ-18 was cultured in a 250-mL Erlenmeyer’s flask using 100 mL of medium containing 1% swollen chitin, 1% yeast extract, and 1% NaCl, at 30°C for 4 days with shaking (180 rpm). After centrifuging the culture broth at 6,000 x g for 20 min, the supernatant was incubated at 50°C for 1 h. Thereafter, ammonium sulfate was added to the chitobiosidase solution at 60% saturation, and the mixture was left overnight at 4°C. After additional centrifugation, the precipitate was dissolved in 50 mM sodium acetate buffer at pH 5.0, and dialyzed against the same buffer for 24 h. The dialyze was applied on a DEAE Sephadex column (2.5 cm x 70 cm), which was pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The enzyme was eluted with a linear gradient of 0.0 to 0.5 M NaCl at a flow rate of 2.0 mL/min. The pooled fractions containing chitinase activity was dialyzed and concentrated using an Amicon membrane (Merck, Darmstadt, Germany) with a 10,000 molecular weight cut-off (MWCO). The concentrated sample was then applied on a Sephacryl S-300 chromatograph, which was pre-equilibrated with 50 mM sodium acetate buffer at pH 5.0 containing 0.15 M sodium chloride. The fractions containing chitinase activity were collected together for further experiment. The protein concentration was measured with a UV monitor at 280 nm.

Chitinase activity assay. To determine chitinase activity, each reaction mixture containing 900 µL of 1% swollen chitin in 50 mM sodium acetate buffer (pH 5.0) and 100 µL of chitobiosidase solution was incubated at 37°C for 2 h. The reaction was stopped by addition of 200 µL of 1 N NaOH. Enzyme activity was determined by measuring the amount of reducing sugar released via the Schales’ method (Imoto and Yagishita, 1971) using a standard curve for GlcNAc. One unit of chitinase activity was defined as the amount of enzyme required to release 1 µmol of GlcNAc per hour.

Electrophoresis and activity staining. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970). After SDS-PAGE, chitinase activity was detected on the gel by the method described by Trudel and Asselin (1989). Glycoprotein staining after SDS-PAGE was carried out by the Periodic acid/Schiff base method (Gander, 1984). For 2-D PAGE, the purified protein was separated using Immobiline DryStrip (13 cm, linear 3–10 pH gradient; GE Healthcare, Munich, Germany). Isoelectric focusing (IEF) was performed using a Multiphor II electrophoresis unit obtained from GE Healthcare (Piscataway, NJ) following the manufacturer’s instructions. The protein concentration was determined by the Bradford method (Bradford, 1976).

Effects of pH and temperature on chitobiosidase activity. To determine the optimum pH, the enzyme activity was measured at pH 2.0–10.0 at 37°C for 2 h using swollen chitin as substrate. The pH stability of the enzyme was determined by pre-incubating the enzyme in buffer solutions of various pH values for 24 h at 4°C. Residual enzyme activity was measured, and the relative activity was calculated. To determine the optimum temperature, the chitobiosidase was incubated with the substrate of swollen chitin at various temperatures (20–70°C) at pH 5.0 for 2 h. Thermal stability was determined by incubating the enzyme at various temperatures for 2 h. Residual enzyme activity was measured, and the relative activity was calculated.

Effect of metal ions on chitobiosidase activity. To specify the effect of metal ions on enzyme activity, enzyme activity was measured under standard conditions in the presence of 1 or 10 mM various metal ions. Enzyme activity assay in the absence of metal ion was considered to be 100%.

Substrate specificity. To determine the substrate specificity of chitobiosidase, 1% each of various chitinous substrates were incubated with the enzyme solution under the standard enzyme reaction condition. The substrates applied in the enzyme reaction were colloidal chitin, water-soluble chitin (with a deacetylation degree of 50%), α-swollen chitin, glycol chitin, α-power chitin, β-power chitin, colloidal chitosan, and swollen chitosan.

Kinetic parameters. To determine the kinetic parameters of the chitobiosidase, a series of enzyme reactions were performed using colloidal chitin as a substrate at various concentrations ranging from 0.5 to 2.5 mg/mL at 37°C for 30 min. Michaelis constant (Km) and maximum velocity (Vmax) were determined based on the Lineweaver-Burk curve.

Analytical methods. GlcNAc and (GlcNAc)2, obtained from enzymatic hydrolysis of chitin were also analyzed by TLC and HPLC. TLC analysis was performed on silica gel 60F254 plates (Merck) using n-propanol/water/NH4OH (70:30:1, v/v/v) as a developing solvent. Amino sugars were shown by spraying the TLC plate with aniline/diphenylamine reagent followed by heating the plate at 100°C for 3 min (Tanaka et al., 1999). HPLC (Shimadzu Model 10AD, Tokyo, Japan) analysis was performed under the following conditions: column, NH2P-50 4E (Shodex, Tokyo, Japan); mobile phase, acetonitrile/water (70:30, v/v); flow rate, 1.0 mL/min; UV detector, 210 nm.

Results and Discussion

Purification of chitobiosidase from *Aeromonas* sp. GJ-18. According to the previous reports, the crude enzyme preparation from *Aeromonas* sp. GJ-18 contains two types of exochitinases: N-acetyl-d-glucosaminidase and chitobiosidase (Kuk et al., 2006). N-Acetyl-d-glucosaminidase from *Aeromonas* sp. GJ-18 was inactive at temperature over 50°C, possibly due to its denaturation, whereas chitobiosidase was active at the same temperature. Therefore, before purification, the crude enzyme was pre-heated.