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ORIGINAL ARTICLE

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 Zn^{2+} 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.

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Materials and Methods

Materials. Chitin was purchased from Kitto Life (Seoul, Korea). Swollen chitin was prepared as described by Monreal and Reese

(1969). Chitin oligosaccharides $(GlcNAc)_n$ (n=1–3) were purchased from Seikagaku Co. (Tokyo, Japan). All others used were of reagent grade.

Purificatioin 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 × 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 dialyzate was applied on a DEAE Sephadex column (2.5 cm × 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. The following buffer systems were used at 50 mM: citrate buffer with pH 2.0–3.0, acetate buffer with pH 4.0–5.0, phosphate buffer with pH 6.0–8.0, and carbonate buffer with pH 9.0–10.0.

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 (K_m) and maximum velocity (V_{max}) 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 $60F_{254}$ plates (Merck) using *n*-propanol/water/NH_4OH (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

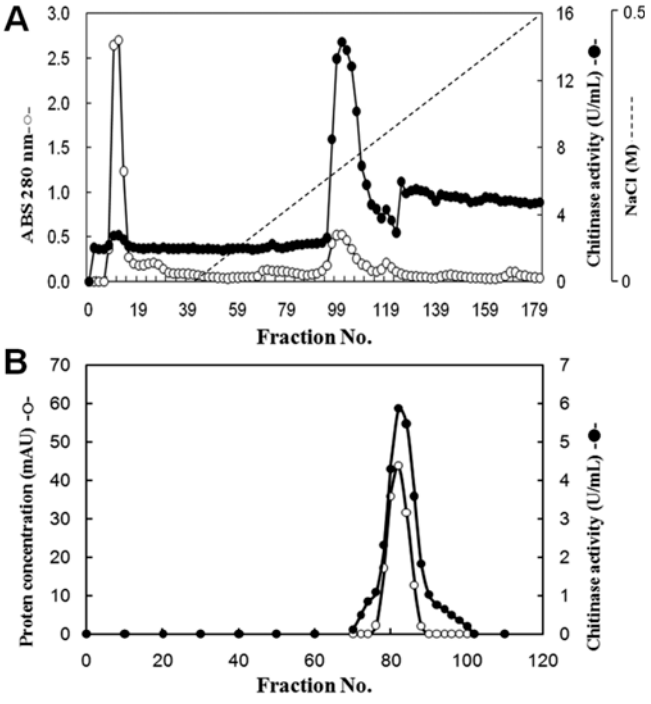

Fig. 1 Purification of chitobiosidase from *Aeromonas* sp. GJ-18 on DEAE Sephadex chromatography (A) and Sephacryl S-300 chromatography (B).

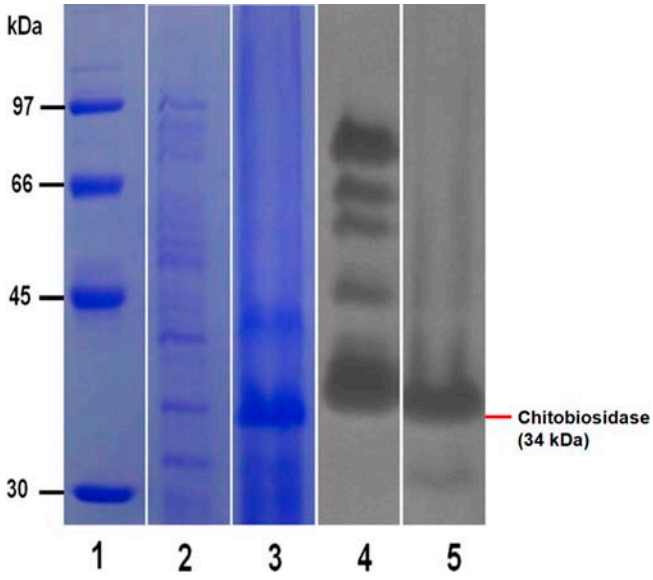

Fig. 2 SDS-PAGE of the purified chitobiosidase from *Aeromonas* sp. GJ-18. Lane 1, protein markers, using low molecular weight markers (Amersham Biosciences); lane 2, crude enzyme; lane 3, purified chitobiosidase from Sephacryl S-300 chromatography; lane 4, activity staining of the same protein sample with lane 2; lane 5, activity staining of the same protein sample with lane 3.

at 50°C for 1 h to inactivate *N*-acetyl-D-glucosaminidase. Subsequently, 60% ammonium sulfate precipitation was applied for enzyme purification. Fig. 1 shows the results of DEAE-Sephadex chromatography (Fig. 1A) and Sephacryl S-300 chromatography (Fig. 1B). All purification procedures yielded 5.1% of chitobiosidase, with a 5.5-purification fold factor and a specific activity of 553.1 U/mg (Table 1).

Electrophoresis and activity staining. The crude enzyme from *Aeromonas* sp. GJ-18 and the purified one were subjected to SDS-PAGE, followed by an in-gel activity staining (Fig. 2). In the crude enzyme preparation, there were at least five chitinases, and their molecular weights were, based on its R_f value, calculated to be 34, 48, 56, 65, and 90 kDa (Fig. 2, lanes 2 and 4). Bacteria, including *Aeromonas* sp., produce a complex chitinolytic system composed of multiple chitinases. All these chitinases act synergistically to degrade chitin. For instance, a flake-chitin degradation marine bacterium, *Aeromonas hydrophila* H-2330, secreted five chitinases and a β-*N*-acetylglucosaminidase into the culture medium. Among the six chitinases, the main chitinase was purified, and its molecular weight was determined to be 62 kDa (Hiraga et al., 1997). Similarly, Ueda *et al*. (Ueda et al., 1995) reported that the bacterium, *Aeromonas* sp. No. 10S-24, produced six chitinases with their molecular weights in the range of 89-120 kDa. However, all these chitinases may be encoded by one single gene. Furthermore, *Aeromonas caviae* CB101 secreted four chitinases (approximately 92, 82, 70, and 55 kDa) encoded by a single gene *chi1* (Mehmood et al., 2010). Multiple chitinases could be produced from one parent chitinase, via post-translational proteolytic cleavages.

The purified chitobiosidase was further purified to homogeneity on SDS-PAGE, and its molecular weight was determined to be 34 kDa on SDS-PAGE (Fig. 2, lanes 3 and 5). The molecular weight of this enzyme was determined to be around 36 kDa by gel filtration chromatography, suggesting the enzyme is monomeric (data not shown). The molecular weight is higher than that of

Table 1 Purification of chitobiosidase from *Aeromonas* sp. GJ-18

Purification Step[a]	Total protein (mg)[b]	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude enzyme	164	16530	100.8	100	1.0
$(NH_4)_2SO_4$	57.9	10395	179.6	35.3	1.8
DEAE Sephadex	27.3	7525	275.8	16.6	2.7
Sephacryl S-300	8.4	4646	553.1	5.1	5.5

[a]All operations were carried out at 4°C.
[b]Protein concentrations were estimated by the Bradford method.

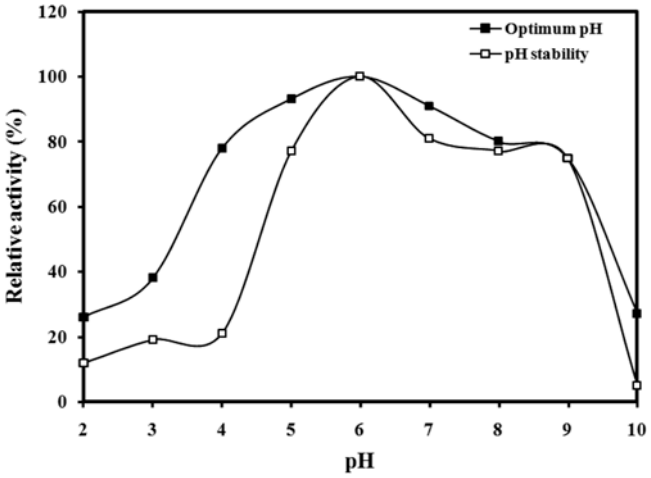

Fig. 3 Effects of pH on the activity (-■-) and stability (-□-) of chitobiosidase from *Aeromonas* sp. GJ-18.

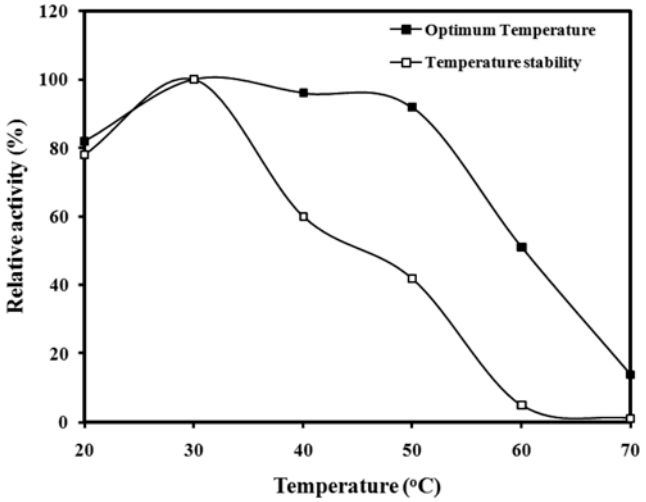

Fig. 4 Effecst of temperature on the activity (-■-) and stability (-□-) of chitobiosidase from *Aeromonas* sp. GJ-18.

chitobiosidase from *Bacillus licheniformis* strain JS (22 kDa) (Waghmare and Ghosh, 2010) but lower than the 97-kDa chitobiosidase from *Vibrio harveyi* (Rarael et al., 1989) and 96-kDa chitobiosidase from *Serratia marcescens* 2170 (Tadayuki et al., 2008)

After SDS-PAGE, the gel was stained with the Periodic acid/Schiff base reagent, and the result indicated chitobiosidase is not a glycoprotein (data not shown). The enzyme was also subject to 2D-electrophoresis, and its pI value was determined be in the range of 7.0-7.5 (data not shown). The pI value of this enzyme is slightly lower than that of chitinase from *Aeromonas* sp. 10S-24 (pI 7.9–8.1) (Ueda et al., 1995), but higher than that of the main chitinase purified from *A. hydrophila* H-2330 (pI 4.0) (Hiraga et al., 1997).

Effect of pH and temperature on chitobiosidase activity. The optimum pH of the purified chitobiosidase from *Aeromonas* sp. GJ-18 was determined to be 6.0, and the enzyme was relatively stable at pH 5.0–9.0 (Fig. 3). The enzyme could hydrolyze swollen chitin at all tested temperatures (20–70°C), and its optimum temperature was determined to be 30–50°C (Fig. 4), which was within those of previously reported chitobiosidases (45–55°C) (Waghmare and Ghosh, 2010). The enzyme was unstable at over 40°C and extremely unstable over 50°C (Fig. 4).

Effect of metal ions on chitobiosidase activity. The effect of various metal ions on chitobiosidase activity is shown in Table 2. Zn^{2+} ions showed complete inhibition of the enzyme at 10 mM; however, 7% of activation was observed in the presence of 1 mM K^{+}. Hg^{2+} is a strong inhibitor for several chitobiosidase activities (Waghmare and Ghosh, 2010). In the present study, similar result was obtained in that 78% of inhibition was observed with 10 mM Hg^{2+} ions (Table 2).

Substrate specificity of chitobiosidase and kinetic parameters. The activity of chitobiosidase on several chitinous substrates was compared. The enzyme showed a high activity on colloidal chitin, water-soluble chitin (DD 50%), and swollen chitin; however, a low activity was observed on glycol chitin and chitin powder, and very low activity was found on substrate of colloidal chitosan and swollen chitosan (Table 3).

The kinetic parameters of this enzyme on substrate of colloidal chitin were determined by Lineweaver-Burk plotting. K_m and V_{max} were established to be 3.45 mg/mL and 2.91 μmol/min, respectively (Fig. 5).

Table 2 Effect of metal ions on chitobiosidase from *Aeromonas* sp. GJ-18

Metal ions	Relative activity (%)	
	1 mM	10 mM
Control	100	100
Ag^{+}	51	41
K^{+}	107	101
Co^{2+}	92	85
Cu^{2+}	101	69
Hg^{2+}	36	22
Mg^{2+}	102	99
Mn^{2+}	88	87
Pb^{2+}	68	43
Zn^{2+}	77	0

Table 3 Comparison of chitobiosidase activity on the modified substrates

Substrate	Relative activity (%)
Colloidal chitin	100
Water soluble chitin	76.7
Swollen chitin	71.5
Glycol chitin	15.5
α-Chitin power	14.2
β-Chitin power	6.1
Colloidal chitosan	1.9
Swollen chitosan	2.8

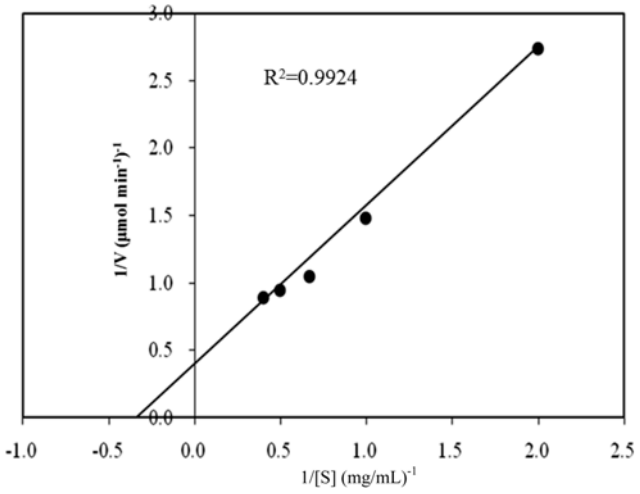

Fig. 5 Effects of colloidal chitin concentration on the activity of chitobiosidase from *Aeromonas* sp. GJ-18.

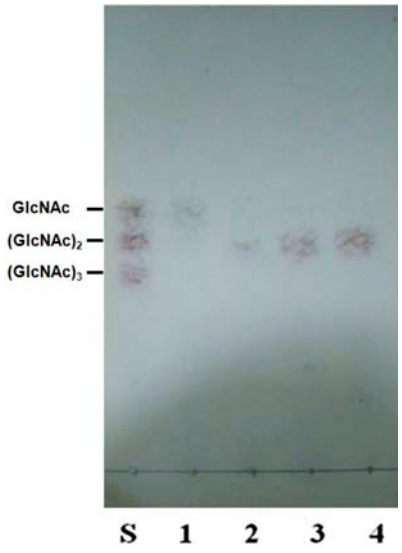

Fig. 6 TLC analysis of the hydrolyzed products. The enzyme solution from *Aeromonas* sp. GJ-18 was incubated with swollen chitin, and the hydrolyzed products were analyzed by TLC using aniline/diphenylamine as the visualizing reagent. Lane S, standards of $(GlcNAc)_{1-3}$; Lane 1, the hydrolyzed products by crude enzyme; lane 2, the hydrolyzed products by crude enzyme pre-heated at 50°C for 1 h; lane 3, the hydrolyzed products by enzyme solution after ammonium sulfate treatment; lane 4, the hydrolyzed products by enzyme solution after Sephacryl S-300 chromatography.

Production of $(GlcNAc)_2$ by purified chitobiosidase. Both TLC and HPLC were used to analyze the reaction products. The products were hydrolyzed by crude enzyme composed of GlcNAc and $(GlcNAc)_2$ (Fig. 6, lane 1); however, after heating of crude enzyme at 50°C for 1 h, only $(GlcNAc)_2$ was detected, indicating that the *N*-acetyl-D-glucosaminidase was inactivated, whereas chitobiosidase was still active (Fig. 6, lane 2). It was not surprising that only $(GlcNAc)_2$ was detected in the hydrolyzed products by the further purified enzyme (Fig. 6, lanes 3 and 4). These results were also confirmed on HPLC (Fig. 7).

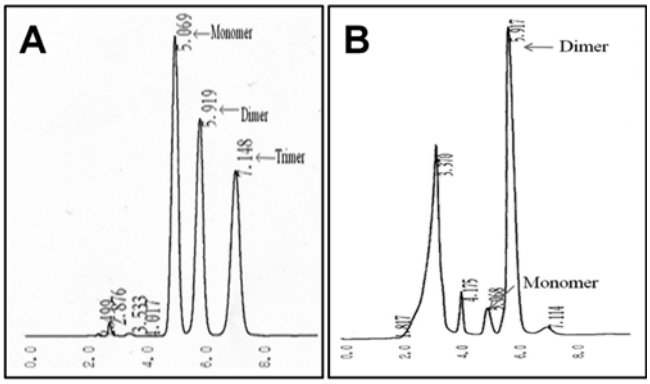

Fig. 7 HPLC analysis of the hydrolyzed product. The purified enzyme from *Aeromonas* sp. GJ-18 was incubated with 1% swollen chitin, and the hydrolyzed products were analyzed by HPLC. A, standards of $(GlcNAc)_{1-3}$; B, products from swollen chitin by purified chitobiosidase.

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