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Purification and characterization of a thermophilic chitinase produced by *Aeromonas* sp. DYU-Too7

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Abstract−An extracellular chitinase, produced by *Aeromonas* sp. DYU-Too7, was purified in the following procedures: ammonium sulfate precipitation, ultrafiltration, chromatographic separation of DEAE-sepharose CL-6B and sephacryl S-100HR. The resulting chitinase has a molecular mass of 36 kDa, an optimal reaction pH of 5.0, and an optimal reaction temperature of 70 °C. It retains almost 100% activity in the pH range of 5.0-8.0. This chitinase has a high thermal tolerance and retained 90% of its activity at 50 °C and 75% at 60 °C. Enzyme activity was inhibited by Ba^{2+}, Hg^{2+}, Mg^{2+} and Ag^{+} cations, but was not substantially inhibited by the K^{+} cation nor the chelating agent EDTA. The K_m and V_{max}, using colloidal chitin as a substrate, are 6.3 g/L and 18.69 μmol/min/mg-protein, respectively. The 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 is an exo-type enzyme, because chitobiose was the main hydrolysate in hydrolysis of colloidal chitin.

Key words: Chitin, Chitinase, *Aeromonas* sp. DYU-Too7

INTRODUCTION

Chitin, a poly-β-1,4-N-acetyl-D-glucosamine (GlcNAc), is the second most abundant polymer in nature, next to cellulose. It is a major component of insect exoskeletons, crustacean shells, as well as the cell walls of most fungi and some algae [1,2]. Chitin and its derivatives, chitosan, in monomer or oligomer forms, have potential applications in many functional physiological processes, including antimicrobial [3], immune-enhancing [4] and anti-tumor activities [5]. Monomer (N-acetylglucosamine) or oligomers (N-acetylchitooligosaccharides), hydrolysates of chitin, can be prepared through either chemical [6] or enzymatic [7] methods. Microbial enzymatic hydrolysis is generally considered better than chemical hydrolysis because of high product specificity, simplicity of preparation procedures, and low environmental contamination.

Chitinases, hydrolyzing chitin into oligomers or monomers, are produced by a wide variety of organisms, including bacteria, fungi, plants, invertebrates and some vertebrates [1,8,9]. Based on hydrolysis pattern, chitinases are classified into two categories: endochitinases and exochitinases [8]. Endochitinases randomly cleave chitin to produce soluble, low molecular mass multimers of N-acetylglucosamine $(GlcNAc)_n$. Exochitinases can be divided into two subcategories: 1) chitobiosidases which remove diacetylchitobioses from the non-reducing end of chitin, and 2) β-D-acetylglucosaminidases which remove N-acetylglucosamine residues from the non-reducing end of chitin [8,10].

Most chitinases produced by *Aeromonas* sp. have molecular masses greater than 50 kDa. For instance, *Aeromonas caviae* and *Aeromonas* sp. 10S-24 produce chitinases with masses of 94 kDa and 70 kDa, respectively [11,12]. The 36 kDa chitinase produced in this study seems to be different from the chitinases produced by other *Aeromonas* sp. This study describes the purification of an extracellular chitinase of *Aeromonas* sp. DYU-Too7 through ammonium sulfate precipitation, ultrafiltration, and chromatographic methods. This study also describes other salient characteristics of the purified chitinase, including inherent enzymatic properties (e.g., molecular mass, optimization and stability in pH and temperature, and metal ion inhibition) and enzymatic kinetics.

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MATERIALS AND METHODS

1. Microorganism and Cultivation Conditions

The tested strain was isolated from beach sand found in Hsinchu, Taiwan. This strain was identified to be *Aeromonas* sp. DYU-Too7 by using 16S rDNA sequencing analysis and the Biolog identification system [13]. The microbes were cultivated in the Luria-Bertani medium until the exponential phase, at which point the microbes were transferred to a chitin broth (CB) medium in a shaker (150 rpm) at 30 °C. The chitin broth (CB) medium contained (all in % w/v) KH_2PO_4 (0.03), K_2HPO_4 (0.07), $MgSO_4$ (0.05), peptone (0.03), yeast extract (0.03) and chitin powder (2.0). The chitin used in this study was bought from Charming & Beauty Co., Ltd. in Taiwan. The chitin powder is pale yellow, is more than 99% pure, and has its particle sizes ranging from 25 to 133 μm.

2. Production and Purification of the Chitinase

All purifying procedures were carried out at 4 °C, except where otherwise noted. After 120 h of cultivation in the CB medium, the broth of *Aeromonas* sp. DYU-Too7 was collected and centrifuged (2,280×g) for 15 min to remove biomass and insoluble solids. The proteins in the supernatant were precipitated by adding an ammonium sulfate solution with 80% saturation [14]. The resulting precipitate was then separated with a centrifuge, re-dissolved in a 50 mM Tris-HCl buffer (pH 7.8), and then dialyzed for 48 h against the same buffer at 4 °C four times. The dialyzed sample was then further concentrated by passing it through an Amicon membrane

with a molecular mass cut-off of 10 kDa. After ultrafiltration to discard small molecules, the sample was poured into a DEAE-sepharose CL-6B column (1.5×25 cm). First, the unadsorbed proteins were washed out with a 50 mM Tris-HCl buffer (pH 7.8). Then, the adsorbed proteins were eluted with the same buffer with a linear gradient of 0-1.0 M NaCl at a flow rate of 0.5 mL/min. The fractions having chitinase activity were combined together and dialyzed in the Tris-HCl buffer, then poured into a column (1×90 cm) of Sephacryl S-100HR, and then eluted with the same buffer at a flow rate of 0.1 mL/min. Each 3 mL fraction was collected and observed for chitinolytic activity on a non-denatured SDS-PAGE.

3. Chitinase Assay

Chitinase activity was determined by using a colorimetric method which estimates reducing end groups in N-acetylglucosamine (NAG) amount degraded from colloidal chitin [15,16]. The assay was performed by mixing 0.2 mL of crude enzyme solution with 1 mL of McIlvaine buffer (pH 5.5) containing 1.0% colloidal chitin. The mixture was incubated at 40 °C for 30 min, and the insoluble substance in the mixture was separated out with a centrifuge (11,428 ×g for 15 min). The supernatant (0.75 mL) was mixed with an equal volume of distilled water and 2 mL of potassium ferricyanide reagent. The mixture was then placed in a boiling water bath for 15 min. The amount of reducing sugars in the mixture was measured with a spectrophotometer (Hitachi U-2000, Tokyo, Japan) at 420 nm. One unit of the chitinase activity is defined as the amount of enzyme required to release 1 μmole of reducing sugars in one minute at 40 °C.

Colloidal chitin was prepared by dissolving chitin powder in concentrated HCl solution, producing a colloidal suspension that could be precipitated at low temperatures (5 to 10 °C). After the above treatment, the structure of colloidal chitin loosened. Therefore, the activity of a chitinase could be measured more accurately if colloidal chitin (with looser structure) was used as a substrate [16,17].

4. Protein Measurement

The protein content in enzyme preparation was monitored by measuring the absorbance at 280 nm during chromatographic separation, and was also determined by the Bradford method [18] using the Bio-Rad protein assay dye reagent with bovine serum albumin as a standard.

5. Determination of Chitinase Activity

To determine the optimal pH for chitinase performance, the chitinase was kept in buffers at various pH levels (3.0-10.0) for 30 min. Depending on pH, the chitinase activity was assayed in either a McIlvaine buffer (pH 3.0-8.0) or a 50 mM Tris-HCl buffer (pH 8.0-10.0), each with 1.0% colloidal chitin. The pH stability of the chitinase was determined by measuring residual chitinase activity after 1 h of incubation at 40 °C at various pH levels.

The optimal temperature for chitinase performance was determined by measuring the chitinase activity level after letting the enzyme react with colloidal chitin in a McIlvaine buffer (pH 5.5) at various temperatures (10-80 °C) for 30 min. The thermal stability of the chitinase was evaluated by incubating the enzyme at various temperatures for 1 h, and then assaying the residual chitinase activity level.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by using the Laemmli method [19] to estimate the molecular mass of a protein. To detect chitinase activity, a zymogram analysis was performed through electrophoresis using a 12% polyacrylamide gel containing 0.1% glycol chitin [20]. After electrophoresis, the gel was incubated in a 0.1 M sodium acetate buffer (pH 5.0) containing 1% Triton X-100 at 37 °C for 2 h, and then stained by coomassie brilliant blue R-250 [21]. Alternatively, the molecular mass of the purified chitinase was also analyzed by ESI-Q-TOF MS (electrospray ionization quadrupole time-of-flight mass spectrometry, Q-TOF, Waters Co., Milford City, MA, USA). This instrument was used to scan the ratio of mass to charge in the range 100-2,500 units (m/z), with each scan of 2 s/step and an inter-scan duration of 0.1 s/step. In the ESI-MS analysis, the quadrupole scan mode was used under a capillary needle at 3 kV, a source block temperature of 80 °C, and a desolvation (solvent removal) temperature of 150 °C. The mass of the desalted form of proteins used for the MS measurements was normally within the range of 5-10 μg.

6. Kinetic Parameters

Kinetic experiments were performed with colloidal chitin (0-22.5 g/L) dissolved in McIlvaine buffer (pH 5.5) at 40 °C. The Michaelis-Menten constant (K_m) and maximal velocity (V_{max}) were determined by Lineweaver-Burk double reciprocal plot.

7. Product Analysis

The final products of the enzymatic hydrolysis of colloidal chitin were analyzed with an HPLC. The assay was performed by mixing 0.2 mL of pure enzyme solution and 1 mL of the McIlvaine buffer (pH 5.5) containing 1.0% colloidal chitin. First, the mixture was incubated at 40 °C for a pre-determined time, and then the insoluble substance in the mixture was separated out with a centrifuge (11,428×g) for 15 min. The supernatant (0.5 mL) was mixed with an equal volume of dilute acetonitrile solution (46% acetonitrile containing 0.008 N H_2SO_4), and centrifuged (11,428×g) for 5 min to remove insoluble solids. The remaining supernatant was analyzed with an HPLC (Hitachi L-7100, Tokyo, Japan) using a LiChrospher 100-NH_2 column (5 μm, 4×250 mm, Merck Co., Darmstadt, Germany) and a UV detector (Diode array detector, Hitachi L-4500, Tokyo, Japan) at a wavelength of 205 nm [6]. The mobile phase was a solution of acetonitrile/water (70/30, v/v) at a flow rate of 1 mL/min.

RESULTS AND DISCUSSION

1. Purification of a Chitinase Produced by *Aeromonas* sp. DYU-Too7

When *Aeromonas* sp. DYU-Too7 was cultivated in the CB medium, most of the chitinases secreted by the microbe were detected during the stationary growth phase. The proteins were isolated from the culture supernatant by adding ammonium sulfate solution with 80% saturation. The precipitate was re-dissolved, and then dialyzed. The dialyzed sample was subjected to ultrafiltration and then chromatographic separation through DEAE-sepharose CL-6B and Sephacryl S-100HR. From the anion exchange chromatographic (DEAE-sepharose CL-6B) diagram, several fractions of proteins (denoted as Peaks 1-7) showed chitinase activities and were further analyzed with an SDS-PAGE (Fig. 1). The results indicated that Peaks 1-3 had simpler protein spectrums and had more significant amount of the 36 kDa protein than others. The in-gel chitinase (zymogram) assay also showed that three proteins with molecular masses of 36, 60 and 70 kDa (data not shown) possessed chitinase activity, and

that the 36 kDa protein had highest chitinase activity among the three. Therefore, in this study, the 36 kDa protein was the target chitinase selected for further study. The proteins in Peaks 1-3 were combined for further purification by gel filtration chromatography (Sephacryl S-100HR). One peak showing chitinase activity was clearly observed (data not shown). The molecular masses and the hydrolytic activity of the proteins (reacted with glycol chitin) were determined from those simple bands produced with zymogram analysis at 37 °C for 4 h, Fig. 2. Furthermore, the molecular mass of the target chitinase was also confirmed by the ESI-Q-TOF MS measurement which showed a peak of 36,007 Da (Fig. 3). Both results indicated that the protein of the simple peak was a 36 kDa chitinase.

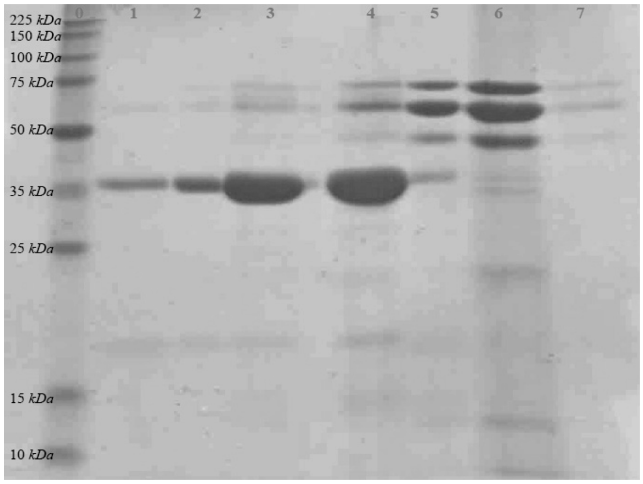

Fig. 1. SDS-PAGE analysis of proteins (denoted as Peaks 1-7), produced by *Aeromonas* sp. DYU-Too7, from ion exchange (DEAE-sepharose CL-6B) chromatography. Lane 0 represents the molecular mass marker, and Lane i is for Peak i (i=1, 2, …, 7).

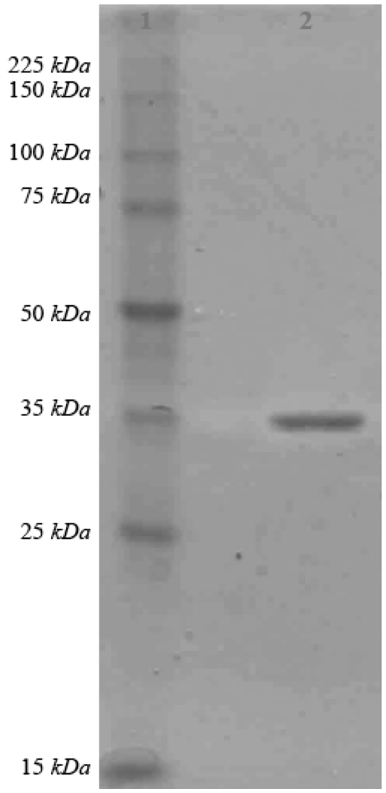

Fig. 2. Zymogram analysis of the proteins combined in Peaks 1-3 (shown in Fig. 1) from gel (Sephacryl S-100HR) filtration. Lane 1 represents the molecular mass marker, and Lane 2 is for the combined Peaks 1-3.

The 36 kDa chitinase produced in this study seemed to be different from the chitinases produced by other *Aeromonas* sp. Most chitinases produced by *Aeromonas* sp. have molecular masses greater than 50 kDa. For instance, *Aeromonas caviae* and *Aeromonas* sp. 10S-24 produced a 94 kDa chitinase and a 70 kDa chitinase, respectively [11,12]. The *Aeromonas hydrophila* strain SUWA-9 secreted an exochitinase (90 kDa) and three endochitinases (60, 70, and 90 kDa) [22]. *Aeromonas schubertii* produced a 30 kDa chitinase and a 38 kDa chitinase, which was confirmed by a zymogram system and had not been isolated or purified [21]. No other 36 kDa chitinases produced by *Aeromonas* sp. were reported.

2. Characterization of the 36 kDa Chitinase

2-1. Optimal pH and pH Stability

The optimal pH for 36 kDa chitinase activity was determined to

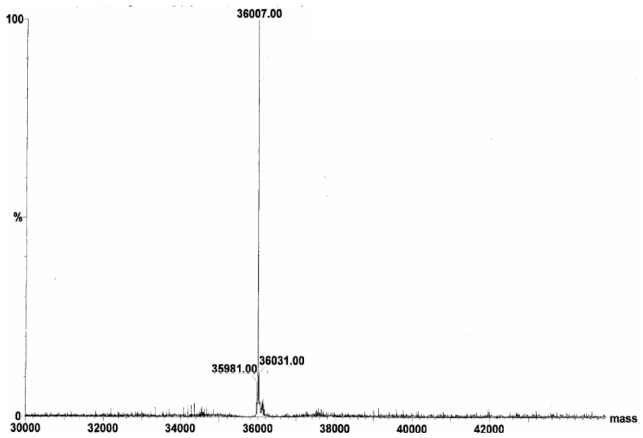

Fig. 3. ESI-Q-TOF MS spectrogram of the protein with chitinase activity from the gel filtration (shown in Fig. 2).

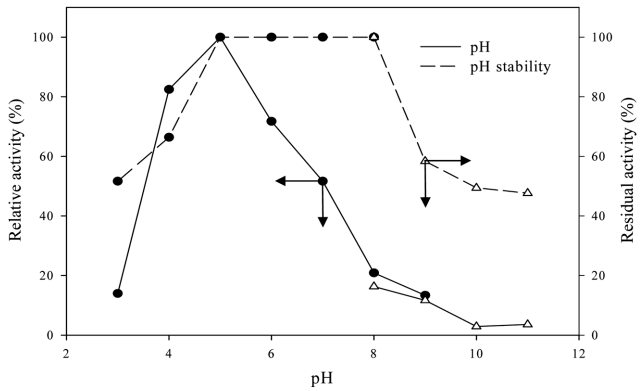

Fig. 4. Effect of pH on the activity of the 36 kDa chitinase and its pH stability. Circles (●) are for McI buffer (pH 3-9), and triangles (△) for Tris-HCl buffer (pH 8-11).

be 5.0 (Fig. 4). In terms of pH stability, the chitinase retained almost 100% activity in the range of pH 5.-8.0 for 1 h, which performed better than other chitinases. The 90 kDa chitinase of *Aeromonas hydrophila* strain SUWA-9 has an optimal pH at 7.0 [22]. The recombinant chitinase (100 kDa) of *Aeromonas caviae* had an optimal pH of 6.5 and retained more than 60% activity in pH 5.0-8.0 for 30 min [23]. The 62 kDa chitinase produced by *Aeromonas hydrophila* H-2330 had an optimal pH of 5.0-8.0 [24]. Three recombinant chitinases (47, 49 and 52 kDa) of *Aeromonas* sp. 10S-24 each had two optimal pHs, 4.0 and 7.0. When incubated with ethylene glycol chitin as the substrate, these three chitinases were stable over a wide pH range (3.0-9.0) [25]. Nawani et al. [26] reported that the chitinase of *Microbispora* sp. V2 retained 61% activity in pH 3.0 for 1 h.

In contrast, the 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 in this study had an optimal pH of 5.0 and remained steadily stable (retained almost 100% activity) in the pH 5.0-8.0 for 1 h, and is better than the other chitinases produced by *Aeromonas* sp. This indicates that the 36 kDa chitinase produced by *Aeromonas* sp. DYU-Too7 could have high chitolytic activity in the degradation of chitinous wastes near a pH neutral condition.

2-2. Optimal Temperature and Thermal Stability

The effect of temperature on the chitinase activity was assayed between 10 and 80 °C at 10 °C intervals (Fig. 5). The optimal temperature for the 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 was determined to be 70 °C. This temperature was higher than the optimal temperatures of chitinases produced by other *Aeromonas* microbes. The optimal temperature for the 90 kDa chitinase of *Aeromonas hydrophila* strain SUWA-9 and the 89-120 kDa chitinase of *Aeromonas* sp. no. 10S-24 is 50 [22,27].

The 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 was more stable than other chitinases of *Aeromonas* sp. in a range of temperatures. The 36 kDa chitinase of DYU-Too7 retained 90% of its original activity at 50 °C and 75% at 60 °C for 1 h. However, incubating the chitinase at 70 °C for 1 h resulted in a 95% loss of its original activity. The recombinant chitinases of *Aeromonas caviae* and *Aeromonas* sp. 10S-24 were thermally stable up to 42.5 °C and 50 °C for 30 min, respectively, but activity levels for both decreased rapidly when the temperature was raised above 50 °C [23,25]. The 58 kDa chitinase of *Pseudomonas aeruginosa* strain 385 had an optimal temperature of 45 °C, and retained 90% of its original activity up to 50 °C. Above 50 °C, stability fell rapidly, with no activity detected after 1 h of incubation at 60 °C [28]. Unlike other chitinases, the 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 has the potential to be applied in the degradation of chitinous wastes at higher temperatures (above 60 °C). The 36 kDa chitinase has an optimal temperature of 70 °C for activity, and thermal stability up to 60 °C. Sutrisno et al. [25] reported a similar result for the three recombinant chitinases of *Aeromonas* sp. 10S-24 which had an optimal temperature of 60 °C, but thermal stability only up to 50 °C. This was due to the fact that the enzyme-substrate complex was more thermal resistant than the free enzyme. The formation of the enzyme-substrate complex protects the enzyme activation site from denaturation by heat.

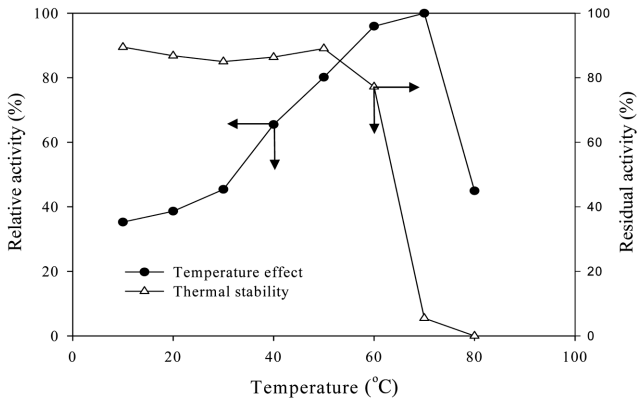

Fig. 5. Effect of temperature on the activity of the 36 kDa chitinase and its thermal stability.

2-3. Effect of Metal Ions and EDTA

The effect of metal ions and EDTA was determined by measuring the activity level of the 36 kDa chitinase after it was pre-incubated in a solution each with 10 mM metal cation or EDTA at 40 °C for 1 h. Experimental results showed that the 36 kDa chitinase was inhibited by Ba^{2+}, Hg^{2+}, Mg^{2+} and Ag^{+} cations, but was not significantly affected by K^{+} ion nor the chelating agent EDTA (Table 1). These results are similar to those of the chitinases produced by *Microbispora* sp. V2 [26], *Vibrio alginolyticus* H-8 [29] and *Aspergillus fumigatus* YJ-407 [30]. Lin et al. [23] also reported that a recombinant chitinase (100 kDa) of *Aeromonas caviae* was inhibited significantly by Hg^{2+} cation. The sulphydryl groups in the active site, which contributed to chitinase activity, were chelated with the Hg^{2+} cation which inhibited chitinase activity [26,31]. The chitinase of *Microbispora* sp. V2 lost 90% of its original activity after reacting with 1 mM Hg^{2+} for 1 h [26]. The Hg^{2+} cation is a common inhibitor of most chitinases. However, the activity level of the purified 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 was reduced by only 45% after reacting with 10 mM Hg^{2+} cation for 1 h. The result indicates that the 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 had better resistance to Hg^{2+} inhibition than other chitinases.

2-4. Kinetic Parameters

The 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 catalyzes reaction, using colloidal chitin as a substrate. There was a first-order reaction at a substrate concentration less than 5 g/L, followed by a zero-order reaction at the range of 15.0-22.5 g/L. The values of K_m and V_{max} for the 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 were 6.3 g/L and 18.69 μmol/min/mg-protein, respectively (Fig. 6). When the 90 kDa chitinase secreted by the *Aeromonas hydrophila* strain SUWA-9 was used to hydrolyze *p*-NP-GlcNAc (*p*-nitrophenyl-N-

Table 1. Effect of metal ion or EDTA on the activity of the 36 kDa chitinase

Ion (10 mM)	Relative activity (%)
None	100.0
Ba^{2+}	69.8
Hg^{2+}	54.8
Mg^{2+}	73.2
Ag^{+}	62.3
K^{+}	103.7
EDTA	106.3

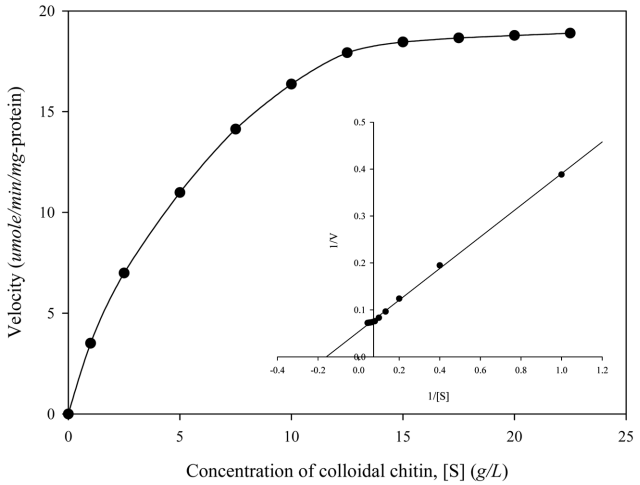

Fig. 6. Determination of K_m and V_{max} of 36 kDa chitinase using colloidal chitin as a substrate. Inset: the corresponding Lineweaver-Burk double reciprocal plot. The fitted linear regression gives an equation with r^2=0.998.

acetyl-β-D-glucosamine) and *p*-NP-GalNAc (*p*-nitrophenyl-N-acetyl-galactosamine), both K_m values were the same at 0.50 mM, but V_{max} values were 115 and 8.0 μmol/min/mg-protein, respectively [22]. The recombinant chitinase (100 kDa) of *Aeromonas caviae* with 4-MU-$(GlcNAc)_2$ (4-methylumbelliferyl-β-D-N,N'-diacetylchitobioside) had a K_m value of 21.55 μM and a V_{max} value of 4.36 nmol/min/mg protein [23]. The K_m value was 0.27 mM for the β-GlcNAcase secreted by *Aeromonas* sp. 10S-24 in hydrolysis of *p*-NP-GlcNAc [32]. The K_m value was 2.9 mM for the 75 kDa chitinase secreted by *Aeromonas schubertii* with colloidal chitin azure as a substrate [21]. The 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 has lower V_{max} values than other chitinases of *Aeromonas* sp. due to the selection of model compounds for hydrolysis. The colloidal chitin used in this study was a large, loose molecule and had fewer non-reducing ends for the chitinase to hydrolyze simultaneously. Therefore, the maximum reaction rate decreased.

2-5. Hydrolysis Pattern

The hydrolysis of colloidal chitin by the 36 kDa chitinase was incubated with a McIlvaine buffer (pH 5.5) containing 1.0% colloidal chitin at 40 °C. The insoluble substance in the mixture was removed by centrifugation before the HPLC analysis. Fig. 7 shows that chitobiose was the main hydrolysate and its concentration was 1.40 g/L at 4 h and 2.21 g/L at 24 h. The major hydrolytic product being chitobiose indicated that the 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 is likely an exo-type enzyme. The 90 kDa chitinase secreted by *Aeromonas hydrophila* strain SUWA-9 could hydrolyze N-acetylchitooligomers into GlcNAc, but did not hydrolyze colloidal chitin well [22]. Among the three recombinant chitinases (47, 49 and 52 kDa) of *Aeromonas* sp. 10S-24, the 52 kDa chitinase exhibited the highest rate of hydrolysis, and hydrolyzed colloidal chitin into $(GlcNAc)_2$ and small amounts of $(GlcNAc)_3$ and GlcNAc [25]. Lin *et al.* [23] reported that the recombinant chitinase (100 kDa) of *Aeromonas caviae* could hydrolyze chitin into GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$ and $(GlcNAc)_4$ within 24 h at 42.5 °C, with $(GlcNAc)_3$ being the major (661.6 mg/L) product. The chitinases of *Aeromonas* sp. 10S-24 and *Aeromonas caviae* are endo-type enzymes. The chitinases secreted by *Bacillus cereus* 6E1 [33] and *Bacillus thuringiengiensis* HD-1 [34] can hydrolyze either *p*-nitrophenyl-$(N$-acetyl-β-D-glucosaminide$)_2$ or 4-MU$(GlnAc)_2$ into chitobiose. Similarly, the chitinase secreted by *Bacillus* sp. NCTU2 can also digest colloidal chitin into chitobiose [35]. In this study, the 36 kDa chitinase of *Aeromonas* sp. DYU-Too7 is an exo-type chitinase that has a potential application to production of chitobiose.

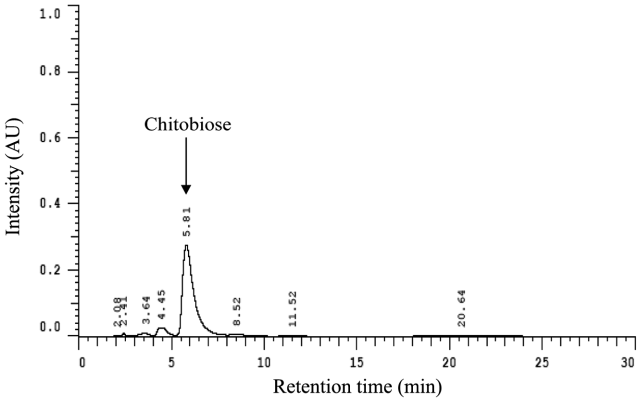

Fig. 7. Chromatogram of hydrolysates from colloidal chitin hydrolyzed by the 36 kDa chitinase.

CONCLUSIONS

A major extracellular chitinase produced by *Aeromonas* sp. DYU-Too7 was isolated and identified to be an exo-type enzyme, which hydrolyzed colloidal chitin into chitobiose. The apparent molecular mass of the chitinase was confirmed to be 36 kDa by the SDS-PAGE and the ESI-Q-TOF MS. This chitinase is one of the few known chitinases produced by *Aeromonas* sp. with a molecular mass less than 50 kDa. The 36 kDa chitinase had an optimal pH of 5.0 and an optimal temperature of 70 °C. The chitinase retained almost 100% activity in the range of pH 5.0-8.0. Compared with other chitinases produced by *Aeromonas* sp., the 36 kDa chitinase had a high thermal tolerance and retained 90% of its activity at 50 °C and 75% at 60 °C. This result suggests that the enzyme-substrate complex protected the enzyme from denaturation by heat. The 36 kDa chitinase activity was inhibited significantly by Hg^{2+}. Using colloidal chitin as a substrate, the 36 kDa chitinase followed a first-order reaction at a substrate concentration less than 5 g/L and a zero-order reaction at a concentration in the range of 15.0-22.5 g/L. The K_m and V_{max} values were 6.3 g/L and 18.69 μmol/min/mg-protein, respectively, when colloidal chitin was used as the substrate. Since the colloidal chitin has fewer non-reducing ends for the chitinase to hydrolyze simultaneously, the V_{max} value obtained in this study is lower than other V_{max} values for other chitinases produced by *Aeromonas* sp.

REFERENCES

1. J. Flach, P. E. Pilet and P. Jolles, *Experientia.*, **48**, 701 (1992).
2. L. Y. Zheng and Y. L. Xiao, *Korean J. Chem. Eng.*, **21**, 201 (2004).
3. G. J. Tsai, Z. Y. Wu and W. H. Su, *J. Food Prot.*, **63**, 747 (2000).
4. A. Tokoro, M. Kobayashi, N. Tatewaki, K. Suzuki, Y. Okawa, T.

Mikami, S. Suzuki and M. Suzuki, *Microbiol. Immunol*., **33**, 357 (1989).
5. A. Tokoro, N. Tatewaki, K. Suzuki, T. Mikami, S. Suzuki and M. Suzuki, *Chem. Pharm. Bull*., **36**, 784 (1988).
6. K. B. Chang, J. Lee and W. R. Fu, *J. Food Drug Anal*., **8**, 75 (2000).
7. M. Sakurad, D. P. Morgavi, K. Komatani, Y. Tomita and R. Onodera, *Cur. Microbiol*., **35**, 48 (1997).
8. C. R. Kupiec and I. Chet, *Curr. Opin. Biotechnol*., **9**, 270 (1998).
9. G. W. Gooday, *Biodegradation*, **1**, 177 (1990).
10. G. E. Harman, C. K. Hayes, M. Lorito, R. M. Broadway, A. Dipietro, C. Peterbauer and A. Tronsmo, *Phytopathology*, **83**, 313 (1993).
11. Y. Sitrit, C. E. Vorgias, I. Chet and A. B. Oppenheim, *J. Bacteriol*., **177**, 4178 (1995).
12. M. Ueda, M. Kojima, T. Yoshikawa, N. Mitsuda, K. Araki, T. Kawaguchi, K. Miyatake, M. Arail and T. Fukamizo, *Eur. J. Biochem*., **270**, 2513 (2003).
13. T. S. Lien, J. R. Too, S. T. Wu and S. T. Yu, *J. Food Biochem*., **29**, 422 (2005).
14. S. Pitpreecha and S. Damrongsakkul, *Korean J. Chem. Eng*., **23**, 972 (2006).
15. I. Imoto and K. Yagishita, *Agric. Biol. Chem*., **72**, 1154 (1971).
16. S. C. Hsu and J. L. Lockwood, *Appl. Microbiol*., **29**, 422 (1975).
17. C. Wiwat, P. Siwayapahm and A. Bhumiratana, *Cur. Microbiol*., **39**, 134 (1999).
18. M. M. Bradford, *Anal. Biochem*., **72**, 248 (1976).
19. U. K. Laemmli, *Nature*, **227**, 680 (1970).
20. J. Trudel and A. Asselin, *Anal. Biochem*., **178**, 362 (1989).
21. S. H. Guo, J. K. Chen and W. C. Lee, *Enzyme Microb. Technol*., **35**, 550 (2004).
22. X. Lan, N. Ozawa, N. Nishiwaki, R. Kodaira, M. Okazaki and M. Shimosaka, *Biosci. Biotechnol. Biochem*., **68**, 1082 (2004).
23. C. S. Lin, H. C. Chen and F. P. Lin, *Enzyme Microb. Technol*., **21**, 472 (1997).
24. K. Hiraga, L. Shou, M. Kitazawa, S. Takahashi, M. Shimada, R. Sato and K. Oda, *Biosci. Biotechnol. Biochem*., **61**, 174 (1997).
25. A. Sutrisno, M. Ueda, H. Inui, T. Kawaguchi, Y. Nakano, M. Arai and K. Miyatake, *J. Biosci. Bioeng*., **91**, 599 (2001).
26. N. N. Nawani, B. P. Kapadnis, A. D. Das, A. S. Rao and S. K. Mahajan, *J. Appl. Microbiol*., **93**, 965 (2002).
27. M. Ueda, A. Fujiwara, T. Kawaguchi and M. Arai, *Biosci. Biotechnol. Biochem*., **59**, 2162 (1995).
28. S. E. Thompson, M. Smith, M. C. Wilkinson and K. Peek, *Appl. Environ Microbiol*., **67**, 4001 (2001).
29. K. Ohishi, M. Yamagishi, T. Ohta, M. Suzuki, H. Izumida, H. Sano, M. Nishijima and T. Miwa, *J. Ferment. Technol*., **82**, 598 (1996).
30. G. Xia, C. Jin, J. Zhou, S. Yang, S. Zhang and C. Jin, *Eur. J. Biochem*., **268**, 4079 (2001).
31. R. Gupta, R. K. Saxena, P. Chaturvedi and S. S. Virdi, *J. Appl. Microbiol*., **78**, 378 (1995).
32. M. Ueda and M. Arai, *Biosci. Biotechnol. Biochem*., **56**, 1204 (1992).
33. S. Y. Wang, A. L. Moyne, G. Thottappilly, S. J. Wu, R. D. Locy and N. K. Singh, *Enzyme Microb. Tech*., **28**, 492 (2001).
34. N. Arora, T. Ahmad, R. Rajagopal and R. K. Bhatnagar, *Biochem. Biophys. Res. Commun*, **307**, 620 (2003).
35. C. M. Wen, C. S. Tseng, C. Y. Cheng and Y. K. Li, *Biotechnol. Appl. Biochem*., **35**, 213 (2002).