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Biochemical characterization of a novel chitotriosidase from suspension-cultured bamboo (*Bambusa oldhamii*) cells

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ABSTRACT. Chitinase was induced in suspension-cultured bamboo cells in the presence of 2,4-dichloro phenoxyacetic acid (2,4-D) and secreted into the medium during cultivation. A novel chitinase, designated chitotriosidase, was purified from the medium of the suspension-cultured cells by sequentially applying $(NH_4)_2SO_4$ (40-80% saturation) fractionation, hydrophobic chromatography, DEAE-Sephacel ion-exchange chromatography, and preparative polyacrylamide gel electrophoresis. The purified chitotriosidase was active toward chitin oligomer substrates but almost inactive toward chitin polymer. The optimal pH for 4-methylumbelliferyll-β-D-*N*, *N'*, *N''*-triacetylchitotrioside (4-MU-$GlcNAc_3$) hydrolysis of the enzyme was 3. The optimal temperature was 70°C, and the K_m was 4.07 μM. The molecular mass was 90.5 kDa, as estimated by both gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The isoelectric point was 5, as estimated using two dimensional electrophoresis and gel activity staining. The chitotriosidase was thermally stable, as it retained almost all of its activity after incubation at 60°C for 30 min or storage at 4°C for a year. Mercuric ion (0.5 mM) significantly inhibited the enzyme's activity. The end products of *N*-acetylglucosamine oligomers ($GlcNAc_n$, n = 3~6) hydrolysis catalyzed by the enzyme were $GlcNAc_{1\sim2}$ or $GlcNA_{1\sim3}$, as analyzed using thin-layer chromatography. The smallest of the chitin oligomer substrates for the enzyme action was a chitin trimer.

Keywords: Bamboo (*Bambusa oldhamii*) suspension cells; Biochemical characterization; Chitinase; Chitotriosidase; 4-MU−$GlcNAc_3$.

INTRODUCTION

Chitin is a structural polysaccharide composed of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residue, found in the exoskeletons of most arthropods and in the cell walls of fungi. The insoluble chitin polymer was converted to its monomer by two enzymes, a chitinase (EC 3.2.1.14) and a β-*N*-acetylhexosaminidase (EC 3.2.1.52). Chitinases, which catalyze the chitin hydrolysis, are found in a wide range of organisms, including bacteria, fungi, higher plants, crustaceans, and some vertebrates. Chitinases have a variety of roles in different organisms. For example, bacteria produce these enzymes to assimilate chitin as a carbon or nitrogen source. In yeast, chitinase is associated with cell division and growth (Kuranda and Robbins, 1991). In fish, chitinase performs a digestive function in the stomach and intestines (Jeuniaux, 1961; Lindsay, 1984; Clark et al., 1988). In higher plants, chitinase is regarded as a pathogen-related protein protecting plants from fungal infection (Taira et al., 2002). Plant chitinases are generally expressed constitutively at low levels. However, synthesis of these enzymes increases upon viral, bacteria, or fungal infection of the plant. In addition to pathogen stimulation, chitinase synthesis can be induced by other environmental factors. These factors include wounding, heat shock, the phyto-hormone ethylene, and chemicals such as salicylic acid (Park et al., 2004). Based on the amino acid sequence, chitinases are largely classified into two families, glycosyl hydrolase 18 and 19 (Collinge et al., 1993; Henrissat and Bairoch, 1993). Plant chitinases are classified into seven classes (class I through VII) (Neuhaus et al., 1996; Neuhaus, 1999). Most plant chitinases, however, belong to classes I through IV, and each of the remaining three classes currently have only one known member. Class I chitinase consists of a chitin-binding domain (CBD) and a catalytic domain. Class II chitinase shows sequence similarity to the class I chitinase but lacks the CBD. Class

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III chitinase shows no sequence similarity to class I or II but has a distant sequence similarity to bacterial and fungal chitinases. Class IV chitinase shows sequence similarity to the class I chitinase, but it is smaller due to four deletions. Class V chitinase (nettle lectin precursor) shows two CBD but no catalytic activity because both catalytic residues are mutated (Lerner and Raikhel, 1992). Class VI chitinase (from sugar beet) shows a truncated CBD and possesses by far the longest known spacer sequence (Berglund et al., 1995). Class VII chitinase is a rice chitinase without CBD but with a catalytic domain highly homologous to the class IV chitinase domain (Neuhaus, 1999). Insect chitinases, most microorganism chitinases and class III plant chitinase are classified into family 18. Plant chitinase class I, II and IV are classified into family 19 (Berglund et al., 1995). Class III plant chitinase was found to exhibit a stable $(\beta\alpha)_8$ barrel fold with the intra-molecular disulfide bonds and a higher level of thermal stability (up to 60°C) (Terwisscha van Scheltinga et al., 1996; Hamel et al., 1997; Hollis et al., 2000) than other classes. Until now many chitinases have been isolated from plant organs such as roots, leaves, fruits, and seeds (Graham and Sticklen, 1994). However, some, such as that of barely (Kragh et al., 1991), carrot (De Jong et al., 1992), and rice (Masuta et al., 1991) have been isolated from cell suspension cultures as well. These have the advantage of strictly controllable growth conditions, which prevents the experiments from being complicated by differentiation processes. Suspension plant cell cultures are suitable systems for a variety of physiological studies and metabolic investigations (Masuda et al., 1988). Recently, we found that at least two chitinase isoforms were induced in suspension-cultured bamboo cells in the presence of 2,4-dichlorophenoxyacetic acid and secreted into the medium during cultivation. Here, we report on the purification and characterization of a novel bamboo chitotriosidase, which can hydrolyze the synthetic substrate 4-MU–$GlcNAc_3$ but hardly degrades chitin polymer substrates.

MATERIALS AND METHODS

Bamboo suspension cell cultures

Two grams of bamboo (*Bambusa oldhamii*) suspension cells were incubated in Murashige-Skoog (MS) liquid medium supplemented (Murashige and Skoog, 1962) with 3% sucrose and 3 ppm 2,4-dichlorophenoxyacetic acid (2,4-D) in a 125 mL flask. The mixture was agitated in a shaking incubator (125 rpm) at 25°C in the dark. The suspension cell cultures were subcultured every 12 days.

Preparation of crude enzyme

Bamboo suspension cells, subcultured for 3 days, were separated from the medium using vacuum filtration. After filtration, the cells were discarded, and the resulting filtrate was collected and designated as crude chitotriosidase.

Ammonium sulfate fractionation

Crude chitotriosidase was fractionated by adding $(NH_4)_2SO_4$. The precipitate formed between 40 and 80% saturation of $(NH_4)_2SO_4$ was collected by centrifugation (Beckman JA 25.5, 27,000 ×*g*, 30 min) and dissolved in a small amount of 50 mM sodium phosphate buffer, pH 7.4 (PB-7.4) containing 0.5 M $(NH_4)_2SO_4$.

Hydrophobic interaction chromatography

The chitotriosidase obtained from the above $(NH_4)_2SO_4$ fractionation was applied to a Phenyl-Sepharose 6 Fast Flow column (2.6 × 9 cm) pre-equilibrated with 50 mM PB-7.4 containing 0.5 M $(NH_4)_2SO_4$. After sample absorption, the column was washed with equilibrium buffer containing 0.5 M $(NH_4)_2SO_4$ until most of the non-bound protein was eluted. The column was then stepwise eluted with 0.4 M, 0.3 M, 0.2 M, 0.1 M and 0 M $(NH_4)_2SO_4$ in 50 mM PB-7.4 at a flow rate of 60 mL/h. Six mL-fractions were collected. Non-bound protein fractions were discarded and bound protein fractions containing chitotriosidase activity were pooled and concentrated using ultrafiltration in an Amicon YM-10 membrane for the following purification.

Anion exchange chromatography and preparative polyacrylamide gel electrophoresis (PAGE)

The chitotriosidase obtained from the above Phenyl-Sepharose 6 Fast Flow column was dialyzed against 50 mM PB-7.4 and then applied to a DEAE-Sephacel column (2.6 × 9 cm) pre-equilibrated with 50 mM PB-7.4. After sample absorption, the column was washed with equilibrium buffer until most of the non-bound protein was eluted. The column was then stepwise eluted with 0 M, 0.1 M, 0.2 M, 0.3 M, 0.4 M and 1 M NaCl in 50 mM PB-7.4 at a flow rate of 40 mL/h. Six mL-fractions were collected. Non-bound protein fractions containing chitotriosidase activity were pooled, concentrated in an Amicon YM-10 membrane, and loaded on a preparative polyacrylamide gel (12.5% separation gel). After electrophoresis the protein band with chitotriosidase activity was sliced out and placed in an electrophoretic concentrator (ISCO Model 1750) to elute the enzyme.

Estimation of molecular mass

The chitotriosidase obtained from electrophoretic concentrator was applied to a Sephacryl S-100 column (1.6 × 95 cm) pre-equilibrated with 50 mM PB-7.4 containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 30 mL/h and 2.5 mL fractions were collected. The enzyme molecular mass was estimated using gel filtration on a Sephacryl S-100 column according to Whitaker (1963). Thyroglobulin (669 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobulin (17 kDa) and vitamin B 12 (1.58 kDa) served as standards.

Measurement of chitotriosidase activity

Aliquots of 50 μM 4-MU-$GlcNAc_3$ (160 μL), 400

μL of 0.1 M sodium acetate buffer (pH 4.0), 180 μL of deionized water, and 20 μL of diluted enzyme solution in a total volume of 760 μL were incubated at 50°C for 20 min. After the reaction, 1 mL of 1 M glycine/NaOH buffer (pH 10.6) was added to enhance the fluorescence measured at an excitation wavelength of 355 nm and emission wavelength of 460 nm (McCreath and Gooday, 1992). One unit of enzyme activity for 4-MU-$GlcNAc_3$ hydrolysis was defined as the amount of enzyme releasing 1 nmole of 4-methylumbelliferone (MU) per min under assay conditions.

Measurement of chitinase activity

Aliquots of 0.2% ethylene glycol chitin (100 μL), 90 μL of 0.1 M sodium acetate buffer (pH 4.0) and 10 μL of diluted enzyme solution in a total volume of 200 μL were incubated at 45°C for 30 min. The reducing sugar produced was measured colorimetrically (decrease of absorbance of 405 nm) using ferri-ferrocyanide reagent as described by Imoto and Yagishita (1971).

Determination of Michaelis constant and maximal velocity

The Michaelis constant (K_m) and maximal velocity (V_{max}) of 4-MU-$GlcNAc_3$ and 4-MU-$GlcNAc_2$ hydrolysis were determined at substrate concentrations ranging from 1 to 25 μM under the optimal buffer pH (3.0) and temperature (70°C) of the enzyme. The K_m and V_{max} of chitotriosidase for both substrates were calculated using the Lineweaver-Burk reciprocal plot graphic method (Lineweaver and Burk, 1934).

Determination of optimal pH and optimal temperature

The optimal pH for chitotriosidase was assayed in a universal buffer (Britton and Robinson type) from pH 2 to 10 at 50°C using 4-MU-$GlcNAc_3$ as the substrate. The optimal temperature for chitotriosidase was assayed at pH 4 from 20 to 90°C.

Determination of thermal stability

To assess thermal stability, the enzyme solutions were immersed in a thermostatic water bath at various temperatures from 20 to 90°C for 30 min, and the remaining activities were measured as described above.

Sodium dodecyl sulfate (SDS)-PAGE and gel activity staining

SDS-PAGE was carried out in 12.5% polyacrylamide gel containing 50 μM 4-MU-$GlcNAc_3$ for activity staining. The proteins mixed with the sample buffer [125 mM Tris (pH 6.8), 2 mM EDTA·2 Na, 2% SDS, 10% glycerol] without 5% (v/v) β-mercaptoethanol were boiled for 5 min. Following electrophoresis, the gel was incubated at 37°C in 0.1 M sodium acetate buffer (pH 4.0) containing 1% (v/v) Triton X-100 for 1 h. Chitotriosidase activity was detected by exposing the gel to a fluorescent lamp at 365 nm against a dark background (Tronosmo and Harman, 1993).

Thin-layer chromatography of hydrolysis products

Seventy five μL of diluted enzyme was incubated with 25 μL of 0.1 M sodium acetate buffer (pH 4.0) and 100 μL of 5 mM $(GlcNAc)_{3\sim6}$ in a thermostatic water bath at 50°C for 48 h. The enzymatic hydrolysis products were subjected to thin-layer chromatography (TLC) on a silica gel plate 60 F_{254} in a solvent system composed of n-propanol-water-ammonia water (70:30:1 v/v). The TLC plates were developed by first dipping them in acetone saturated with silver nitrate and sprinkling them with ethanol containing 0.5 M NaOH.

Two-dimensional electrophoresis (2-DE) and isoelectric point estimation

Ice-cold acetone (50 mL) was added to the purified chitotriosidase (5 mL) and stored at -20°C for 16 h. The protein precipitates were harvested by centrifugation at 14,000 ×*g* for 10 min at 4°C. The precipitates were then washed with ice-cold acetone, and the resulting suspension was centrifuged at 14,000 ×*g* for 10 min at 4°C. The wash-step was repeated 10 times to remove the salts. After washing, the precipitates were resuspended with 200 μL sample buffer (6 M urea, 2 M thiourea, 0.5% Triton X-100, 0.5% immobilized pH gradient strips (IPG) buffer) and vortexed at 4°C for 16 h. The resulting suspension was centrifuged at 14,000 ×*g* for 10 min at 4°C. After centrifugation, the precipitates were discarded, and the resulting protein solutions were collected and pippeted into the IPG strip holder channels. Eleven centimeter pH 3-10 immobilized pH gradient strips were lowered into the protein solutions with the gel side down and overlayered with the dry-strip fluid. The holders were placed onto IPGphor platform electrode plates, and IPG-IEF was run on the IPGphor isoelectric focusing (IEF) system (Amersham Pharmacia Biotech). Following IEF separation, the gel strips were equilibrated in an equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS and a trace of bromophenol blue. The equilibrated gel strips were then applied onto 0.75-mm thick 12.5% SDS polyacrylamide gels and sealed with 0.5% agarose. The SDS-PAGE was run at 4°C. After 2-DE, the protein spot was visualized as described above using activity staining. The isoelectric point (*p*I) of the enzyme was estimated according to the scale shown on the immobilized pH gradient strips.

Effect of metal ions

Purified chitotriosidase in 20 μL of Tris-acetate buffer (10 mM, pH 4.0) was incubated with 10 μL of H_2O containing various metals (Li^+, Na^+, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Hg^{2+}) at concentrations from 1 to 50 mM. The enzyme activity was then measured using 4-MU-$GlcNAc_3$

as the substrate. All of these metals were chloride salts. The relative activity was expressed as the percentage ratio of the specific activity of the enzyme in the presence of a metal to that in the absence of the metal.

Data analysis

For analysis of cell growth and enzyme activity of suspension-cultured bamboo cells, analytic measurements were performed in triplicate. For purification and characterization of chitotriosidase, analytic measurements were performed in duplicate.

RESULTS

Purification of chitotriosidase

During the bamboo suspension cell incubation period, cells and culture filtratres were collected every 3 days for a period of one month for chitotriosidase activity determination. The chitotriosidase activity in the medium was much higher than that in the cells during cultivation (data not shown). As shown in Figure 1, chitotriosidase in the medium reached the highest level in activity and specific activity at day 3. Therefore, the bamboo suspension-cultured cell filtrate was collected for chitotriosidase purification at day 3. Chitotriosidase was precipitated from the culture filtrate using ammonium sulfate fractionation (40-80% saturation) and further purified through the sequential steps of Phenyl-Sepharose hydrophobic interaction chromatography, DEAE-Sephacel ion exchange chromatography, and preparative PAGE.

As shown in Figure 2, chitotriosidase was bound to the Phenyl-Sepharose column at 0.5 M $(NH_4)_2SO_4$ and eluted by phosphate buffer using a stepwise $(NH_4)_2SO_4$ decreasing gradient (0.5-0 M). Most proteins without chitotriosidase activity were removed using this effective step. After hydrophobic chromatography, chitotriosidase was further purified by DEAE-Sephacel ion-exchange chromatography, during which a protein peak containing chitotriosidase activity was not bound to the column. However, several protein peaks without chitotriosidase activity were bound and eluted using a stepwise NaCl gradient (0.1-1.0 M) (Figure 3). Final enzyme purification was performed using preparative PAGE and electrophoretic elution. After this step, the purified enzyme could be stored in sodium phosphate buffer (50 mM, pH 7.4) or Tris-acetate buffer (10 mM, pH 7.4) at 4°C for a year without significant loss of activity. The purification results are summarized in Table 1.

SDS-PAGE and gel activity staining

The chitotriosidase preparations at each stage of the purification were treated with SDS without 2-mercaptoethanol and analyzed by SDS-PAGE and gel activity staining. As shown in Figure 4, chitotriosidase activity was detected as a fluorescent light band against a dark background, and all chitotriosidase preparations contained a chitotriosidase activity band with a molecular

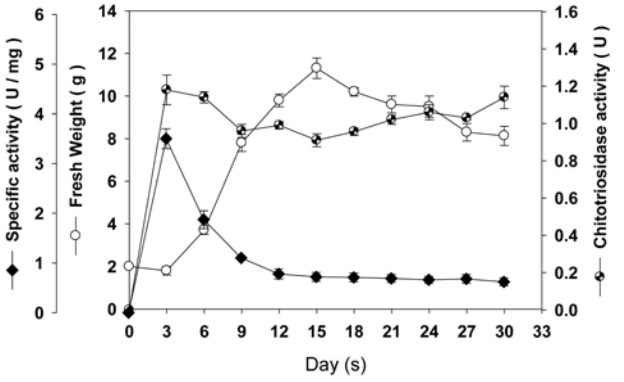

Figure 1. Changes in fresh weight of cells and chitotriosidase activity in the medium of bamboo cell suspension cultures. Each experimental point is the mean of three replicates (± SD).

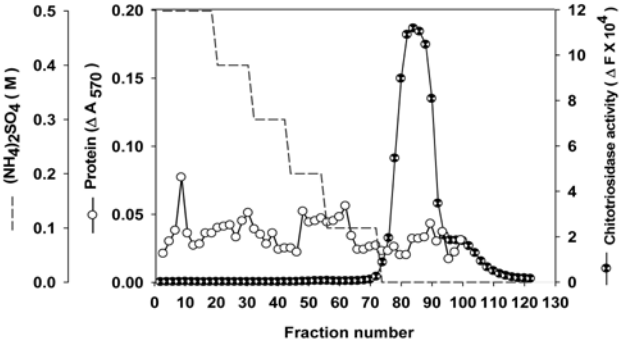

Figure 2. Hydrophobic chromatography of chitotriosidase on a Phenyl-Sepharose 6 Fast Flow column. The column (2.6 × 9 cm) was equilibrated with 50 mM sodium phosphate buffer (pH 7.4) containing 0.5 M $(NH_4)_2SO_4$, after which proteins precipitated with 40~100% saturated $(NH_4)_2SO_4$ solution were applied. Proteins bound to the column were eluted with a stepwise gradient of ammonium sulfate (0.5 ~ 0 M) in equilibrium buffer at a flow rate of 60 mL/h; 6 mL- fractions were collected.

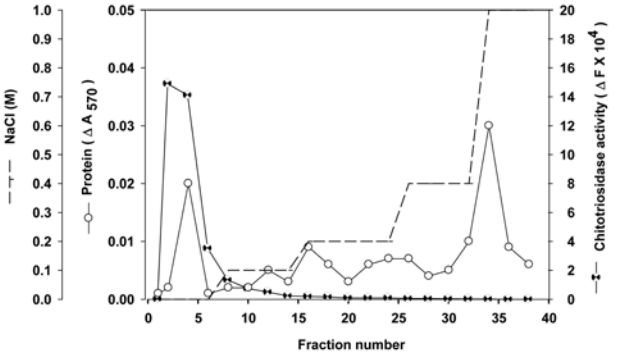

Figure 3. Ion-exchange chromatography of chitotriosidase on DEAE-Sephacel. The column (2.6 × 9 cm) was equilibrated with 50 mM sodium phosphate buffer (pH 7.4), after which chitotriosidase from the Phenyl-Sepharose 6 Fast Flow column was applied. The column was washed with equilibrium buffer and eluted with a stepwise gradient of NaCl (0 ~ 1 M) in equilibrium buffer at a flow rate of 40 mL/h; 6 mL- fractions were collected.

Table 1. Purification of chitotriosidase from bamboo suspension-cultured cells[a].

Procedure	Total activity (units)[b]	Total protein (mg)	Specific activity (units/mg)	Purification (fold)	Yield (%)
Crude enzyme solution	44.5	8.28	5.37	1	100
$(NH_4)_2SO_4$ fractionation (40~80% saturation)	13.72	0.44	31.2	5.81	30.8
Phenyl-Sepharose 6 Fast Flow	4.0	-	-	-	9.0
DEAE-Sephacel	1.92	-	-	-	4.3
Preparative PAGE	1.52	-	-	-	3.4

[a]Data were obtained from 400 mL of medium of bamboo suspension-cultured cells at day 3.
[b]One chitotriosidase unit is defined as the amount of enzyme releasing 1 nmole of 4-methylumbelliferone per min from 4-MU-$(GlcNAc)_3$.

mass of 90.5 kDa. If the purified chitotriosidase was denatured with SDS containing 2-mercaptoethanol and analyzed by SDS-PAGE and gel activity staining, no chitotriosidase activity band was detected (data not shown). These results indicated that the chitotriosidase denatured by SDS alone could be rescued after SDS removal and that disulfide bond reduction of this enzyme by a reducing agent gives rise to irreversible denaturation in this enzyme.

Molecular mass

The enzyme molecular mass was 90.5 kDa, as estimated by Sephacryl S-100 HR gel filtration. This value was close to that estimated by SDS-PAGE and activity staining using 4-MU-$GlcNAc_3$ as the substrate (Figure 4, lane 1). This result indicates chitotriosidase to be a monomeric enzyme.

Isoelectric point (*p*I)

The *p*I value of the enzyme was 5, as analyzed by two-dimensional electrophoresis and activity staining (Figure 5).

Optimal pH, optimal temperature and thermal stability

As shown in Figure 6, the purified chitotriosidase had an optimal pH of 3 (Figure 6A) and an optimal temperature of 70°C (Figure 6B). When the enzyme was incubated at temperatures from 20 to 90°C for 30 min, the enzyme was stable below 60°C, lost about 20% activity at 70°C, and completely lost activity above 80°C (Figure 6C).

Effects of metal ions

Hg^{2+} (0.5-1 mM) almost completely inhibited the activity of the enzyme. However, other metal ions (Li^+, Na^+, Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}) at concentrations from 2.5 to 50 mM had no significant effect on this enzyme's activity (data not shown).

Michaelis constant (K_m) and maximal velocity (V_{max})

The K_m and V_{max} of the enzyme for 4-MU-$GlcNAc_3$

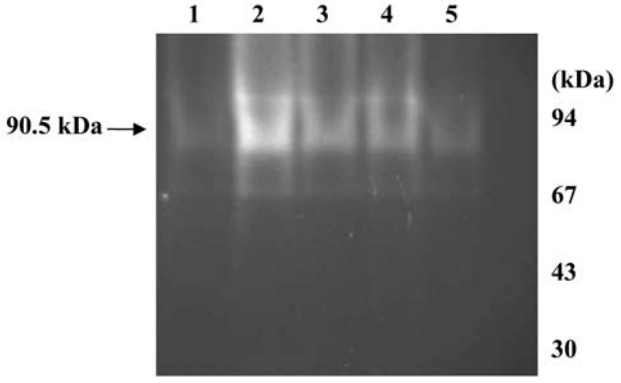

Figure 4. Activity (4-MU-$GlcNAc_3$ hydrolysis) staining after SDS-PAGE of chitotriosidase at different purification stages. Lane 1, Electrophoretic elution (preparative PAGE); Lane 2, Anion-exchange chromatography (DEAE-Sephacel); Lane 3, Hydrophobic interaction chromatography (Phenyl-Sepharose 6 Fast Flow); Lane 4, Ammonium sulfate fractionation (40~100% saturation); Lane 5, Crude enzyme.

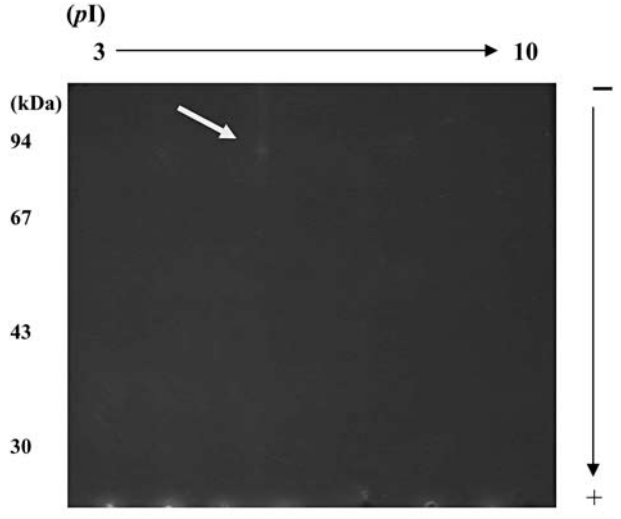

Figure 5. Two-dimensional electrophoresis and activity (4-MU-$GlcNAc_3$ hydrolysis) staining of purified chitotriosidase.

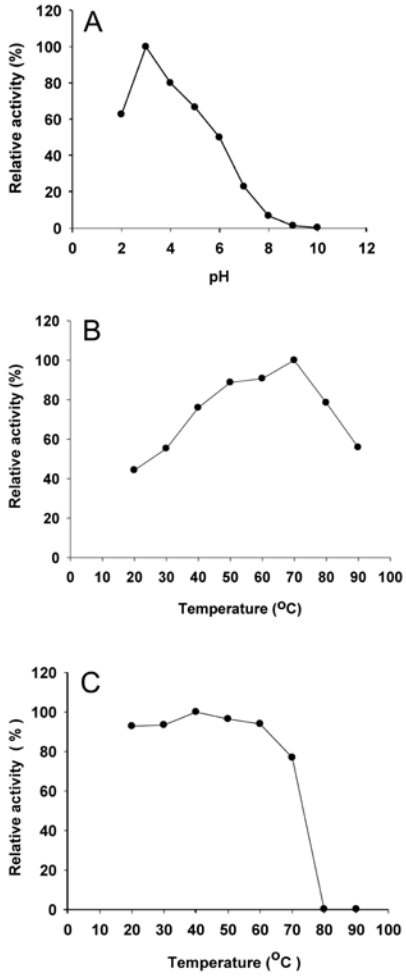

Figure 6. The optimal pH, temperature and thermostability of chitotriosidase from bamboo suspension-cultured cells. The chitotriosidase activity was detected at 45°C for measuring optimal pH (A) or at pH 4 for measuring optimal temperature (B) and thermostability (C) with 4-MU-GlcNAc$_3$ as the substrate.

and 4-MU-GlcNAc$_2$ hydrolysis were calculated from Lineweaver-Burk plots. As shown in Table 2, chitotriosidase showed a lower Michaelis constant (K_m) and higher V_{max} for 4-MU-GlcNAc$_3$ than for 4-MU-GlcNAc$_2$. Comparison of K_m and V_{max} values revealed that chitotriosidase had about a 2-fold higher affinity for 4-MU-GlcNAc$_3$ than for 4-MU-GlcNAc$_2$ and hydrolyzed the former substrate about 3-fold faster than the latter. The specificity constant (V_{max}/K_m) of chitotriosidase for 4-MU-GlcNAc$_3$ was much higher than that for 4-MU-GlcNAc$_2$. These results indicate that 4-MU-GlcNAc$_3$ was clearly the preferred substrate for the chitotriosidase.

Chitin oligosaccharide hydrolysis products

In an attempt to gain more information on the chitotriosidase action, chitin oligomers with chain length from 3 to 6 were used as substrates, and the end-product profiles were examined. As shown in Figure 7, chitin trimer was hydrolyzed into monomer and dimer with some amounts of remaining trimer. Chitin tetramer, pentamer, and hexamer were completely hydrolyzed into monomer, dimer, and trimer. Using chitin dimer as a substrate, no hydrolysis product was observed (data not shown). These results indicated that chitotriosidase was an endo or random type hydrolase and that chitin trimer was the smallest chitin oligomer that could be hydrolyzed by this enzyme.

DISCUSSION

Bamboo cell-suspension cultures offered a convenient starting material for the isolation of chitinases because most of the chitinases induced in the presence of 2,4-D were secreted into the medium. At least two chitinase isoforms were secreted into the medium during cultivation.

Table 2. Chitotriosidase kinetic parameters for hydrolysis of fluorogenic synthetic substrates

Substrate[a]	V_{max} (nmole/min)[b]	K_m (μM)[b]	V_{max}/K_m (nmole/min/μM)
4-MU-GlcNAc$_3$	270	4.07	66.4
4-MU-GlcNAc$_2$	98	9.04	9.96

[a]4-MU-GlcNAc$_2$: 4-methylumbelliferyll-β-D-*N,N'*-diacetylchitobioside, 4-MU-GlcNAc$_3$: 4-methylumbelliferyll-β-D-*N,N',N''*-triacetylchitotrioside.
[b]Equal amounts of purified chitotriosidase (20 μL) were used for both substrates for kinetic parameter determination. Protein concentration of purified chitotriosidase was too low to be determined.

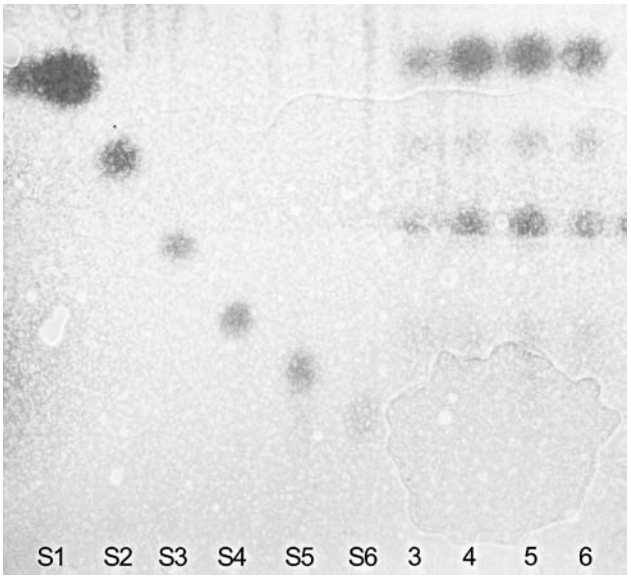

Figure 7. Thin-layer chromatography showing hydrolysis of *N*-acetylglucosamine oligomers by chitotriosidase. Lanes S_1 ~ S_6 denote standard *N*-acetylglucosamine oligomers GlcNAc, (GlcNAc)$_2$, (GlcNAc)$_3$, (GlcNAc)$_4$, (GlcNAc)$_5$ and (GlcNAc)$_6$, respectively. Lanes 3~6 denote hydrolysis of *N*-acetylglucosamine oligomer products from trimer to hexamer, respectively.

Table 3. Comparison of properties of chitodextrinase from marine bacterium *Vibrio furnissii* and chitotriosidase from bamboo (*Bambusa oldhamii*) suspension-cultured cells.

Specificity	*Vibrino furnissi* Chitodextrinase	*Bambusa oldhamii* Chitotriosidase
Chitin polymer	-	-
Chitin oligomer	+	+
Action type	endo	endo
Optimal pH	6.5~7	3
Optimal temperature (°C)	35~37	70
Molecular mass (kDa)	120	90.5
K_m for *p*Np-GlcNAc$_2$ (μM)[a]	170	-
K_m for 4-MU-GlcNAc$_2$ (μM)	-	9.04
K_m for 4-MU-GlcNAc$_3$ (μM)	-	4.07
The shortest chitin oligomer substrate	GlcNAc$_4$	GlcNAc$_3$

[a]*p*Np-GlcNAc$_2$: *p*-Nitrophenyl-*β*-D-*N,N'*-diacetylchitobioside.

The first isoform, designed chitotriosidase, which is specific for chitin oligomer hydrolysis, reached the highest activity at day 3 while the second isoform, capable of degrading chitin polymer, reached the highest activity at day 15 (data not shown). The chitotriosidase activity in the medium was much higher than that in the cells during cultivation, suggesting that most chitotriosidase induced in the cells was secreted into the culture medium. In this report we describe the purification and characterization of a newly discovered chitotriosidase from the medium of bamboo cell suspension cultures. Two properties of the chitotriosidase observed during purification are of interest: (a) the enzyme appears to be highly hydrophobic. It was strongly absorbed to the Phenyl-Sepharose 6 Fast Flow in 0.5 to 0.1 M $(NH_4)_2SO_4$ at pH 7.4. Phosphate buffer without $(NH_4)_2SO_4$ was required to elute the enzyme from the Phenyl-Sepharose 6 Fast Flow. This facilitated enzyme purification using hydrophobic column chromatography because most other proteins were removed in the flow-through and the salt decrease-eluted fractions; (b) the enzyme was a weakly acidic protein with an isoelectric point of 5. However, it was not absorbed to the anion exchanger DEAE-Sephacel at pH 7.4. This discrepancy is probably due to the highly hydrophobic enzyme surface, which influenced the enzyme absorption to the charged solid support groups. The negative charge of phosphate buffer components competing with chitotriosidase is also probable.

The purified chitotriosidase was an endo or random type hydrolase that cleaved chitooligosaccharides but barely cleaved chitin polymer substrates such as ethylene glycol chitin (data not shown). The enzyme molecular mass (90.5 kDa) was much larger than that of chitinases (~30 kDa) found in most higher plants (Graham and Sticklen, 1994). The enzyme was a monomeric protein and thermally stable, as it retained almost all of its activity after incubation at 60°C for 30 min or prolonged storage at 4°C for a year. The presence of disulfide bonds was essential for rescuing the enzyme's activity after SDS-PAGE. These results indicate that the chitotriosidase is a short substrate preferred endochitinase with remarkable thermal stability. On reviewing the published literatures so far, this study was shown to be the first on plant endochitinase specific for chitin oligomer cleavage. Although an endo-type chitodextrinase, specific for chitooligosaccharide degradation, was cloned and characterized from the marine bacterium *Vibrio furnissi* (Keyhani and Roseman, 1996), some properties of the chitotriosidase identified in this study are different (Table 3). For instance, it had a much lower optimal pH and higher optimal temperature. The molecular mass of the chitotriosidase (90.5 kDa) was smaller than that of the chitodextrinase (120 kDa). The K_m values for 4-MU-GlcNAc$_{2\sim3}$ hydrolysis of the chitotriosidase were much lower than the K_m value for *p*Np-GlcNAc$_2$ hydrolysis of the chitodextrinase. Moreover, the smallest oligomer recognized as a substrate by the chitotriosidase (GlcNAc$_3$) was also different from that of the chitodextrinase (GlcNAc$_4$).

From the effect of metal ions, chitotriosidase activity was completely inhibited by $HgCl_2$. The inhibitory effect of mercuric ion on enzyme activity was probably due to the interaction with sulfhydryl group of the enzyme. $HgCl_2$ and organic mercurials reacted rapidly, and at low concentration (<1 mM) with sulfhydryl groups; however, they also reacted with other groups including the imidazole, carboxyl, and peptide groups. In this study, only Hg^{2+} at concentrations from 0.5 to 1 mM completely inhibited chitotriosidase activity. No dose responses can be observed. It is possible that the concentration of Hg^{2+} is too high. In addition, if inhibition was due to reaction with essential sulfhydryl groups of the enzyme, the enzyme activity could be reversed by addition of an excess of a thiol compound. However, further investigation should be conducted.

In *in vitro* cultures the expression of chitinase and β-1,3-glucanase have been shown to be regulated by plant hormones (Shinish et al., 1987; Sauter and Hager, 1989; Grosset et al., 1990). This supports the idea that chitinase has not only defense-related but endogenous functions as well. In this study, chitotriosidase may play an important role in bamboo cell division because chitotriosidase exhibited the highest activity in the medium at the mid-log phase of suspension-cultured cells shown in Figure 1. The role of chitinase is usually considered to be an active or passive mechanism against pathogens (Hamel et al., 1995; Regalado et al., 2000); specific isoforms may play roles in embryonic development (De Jong et al., 1992), pollination, and sexual reproduction (Leung, 1992). However, little is known on the endogenous role of chitinase especially the newly isolated chitotriosidase in plant cell-suspension cultures.

In conclusion, the chitotriosidase described here is a short substrate preferred endochitinase with remarkable thermal and storage stability. It is a newly discovered plant endochitinase specific for chitin oligomer cleavage. In addition, it is also a valuable enzyme source for chitin monomer and trimer production although further studies on enzyme application such as cloning and over-expression of chitotriosidase and synergestic degradation of chitin by chitotriosidase and other endochitinases remain to be performed.

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綠竹筍懸浮培養細胞一種新型的幾丁三醣酶檢定

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綠竹筍懸浮細胞於含有 2,4-D 激素培養過程誘發幾丁質酶並分泌於培養液中，經由硫酸銨劃分 (40-80% 飽和度)、Phenyl-Sepharose 疏水性層析、DEAE-Sephacel 離子交換層析及製備式聚丙烯醯胺膠體電泳等連續純化步驟，可由懸浮細胞培養濾液分離一種新型幾丁質酶，稱為幾丁三醣酶。此幾丁三醣酶可水解 N- 乙醯幾丁寡醣，但幾乎不水解高分子幾丁質，其水解 N- 乙醯幾丁三醣衍生物 4-MU-$GlcNAc_3$ 之最適 pH 為 3 ，最適溫度為 70°C 而 K_m 值為 4.07 μM。以膠體過濾法及 SDS-PAGE 電泳法測得酵素分子量皆為 90.5 kDa，二維電泳及膠體酵素活性染色測得酵素等電點 (*p*I) 為 5。純化之幾丁三醣酶於 60°C 保溫 30 分鐘或 4°C 貯存一年，幾無活性損失，相當安定，汞離子 (0.5 mM) 顯著抑制酵素活性。以薄層層析法分析顯示酵素水解幾丁質寡醣 ($GlcNAc_n$，n = 3~6) 之產物為 $GlcNAc_{1\sim2}$ 或 $GlcNAc_{1\sim3}$ ，而可作用之最低聚合度幾丁質寡醣則為 $GlcNAc_3$。

關鍵詞 ： 綠竹筍懸浮培養細胞 ； 幾丁質酶 ； 幾丁三醣酶 ； 生化檢定 ；4-MU-$GlcNAc_3$。