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Purification and Characterization of Two Chitosanase Isoforms from the Sheaths of Bamboo Shoots

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ABSTRACT: Two thermally stable chitosanase isoforms were purified from the sheaths of chitosan-treated bamboo shoots. Isoforms A and B had molecular masses of 24.5 and 16.4 kDa and isoelectric points of 4.30 and 9.22, respectively. Using chitosan as the substrate, both isoforms functioned optimally between pH 3 and 4, and the optimum temperatures for the activities of isoforms A and B were 70 and 60 °C, respectively. The kinetic parameters K_m and V_{max} for isoform A were 0.539 mg/mL and 0.262 μmol/min/mg, respectively, and for isoform B were 0.183 mg/mL and 0.092 μmol/min/mg, respectively. Chitosans were susceptible to degradation by both enzymes and could be converted to low molecular weight chitosans between 28.2 and 11.7 kDa. Furthermore, the most susceptible chitosan substrates were 50−70 and 40−80% deacetylated for isoforms A and B, respectively. Both enzymes could also degrade chitin substrates with lower efficacy. *N*-Bromosuccinimide and Woodward's reagent K strongly inhibited both enzymes.

KEYWORDS: *Bambusa oldhamii, purification, characterization, chitosanase, low molecular weight chitosan*

INTRODUCTION

Chitosan is a linear polysaccharide containing β-1,4-linked D-glucosamine (GlcN) and *N*-acetyl-D-glucosamine (GlcNAc) residues. Chitosan is located within the cell walls of some fungi[1−4] and within certain green algae, such as *Chlorella*.[5] Artificial chitosan is commercially produced by the alkaline deacetylation of shellfish (usually crab or shrimp) chitin. Furthermore, research has demonstrated that chitosan and chitosan oligosaccharides can induce plant defense mechanisms by promoting callose and chitosanase synthesis.[6−8]

Chitosanase (EC 3.2.1.132), a hydrolase, acts on chitosan. Most chitosanase isoforms can be distinguished from chitinases by their lower apparent molecular mass and different substrate specificity.[9] Until recently, chitosanases have been studied primarily in bacteria and fungi.[10] Microbial chitosanases are divided into the following three subclasses on the basis of their cleavage position specificity:[11,12] subclass I (e.g., *Streptomyces* sp. N174 chitosanase) cleaves GlcN−GlcN and GlcNAc−GlcN linkages;[13] subclass II (e.g., *Bacillus* sp. No.7-M chitosanase) cleaves only GlcN−GlcN linkages;[14] and subclass III (e.g., *Bacillus circulans* MH-K1 chitosanase) cleaves both GlcN−GlcN and GlcN−GlcNAc linkages.[15] Microbial chitosanase can be used for nutritional purposes or for the cell-wall degradation of certain fungi.[2]

In many plant species, chitosanases were identified as pathogenic proteins. Chitosanase activity has been detected in vesicular-arbuscular mycorrhizal (VAM)-colonized leek (*Allium porrum*) and onion (*Allium cepa*) root as well as chemical- or pathogen-stressed leaves of various plant species.[9,16] Plant chitosanases, chitinases, and β-1,3 glucanases, working separately or in concert, may be involved in defending host plants by degrading the cell walls of fungal invaders.[16,9] In addition, chitosanase activity has been detected in different parts of several healthy plant species.[17,18] The endogenous function of plant chitosanases, however, has not yet been elucidated.

Chitosanases are useful for the production of many biomedical and biotechnology products. They can be used to produce low molecular weight chitosan and chitosan oligomers, which have various biological activities including antifungal, antibacterial, antitumor, and immune-enhancing activities.[19,20] Other applications such as fungal protoplast technology, cytochemical chitosan localization using chitosanase-gold complexes, and fungial control agents have also been investigated.[10,21,22] Like most enzymes, the practical applications of chitosanase depend strongly on the enzyme's characteristics. Therefore, the identification of new chitosanase isoforms would benefit the expansion of chitosanase applications.

Bamboo shoots (*Bambusa oldhamii*) are a popular food material in Taiwan. They are harvested in the early summer (May−July) and are usually either supplied fresh or used for processing frozen, canned, dehydrated, or pickled products. Recently, we found that the activities of chitinase and chitosanase in fresh-cut bamboo shoots were significantly amplified during room temperature storage. The activity increase was most notable in the outer sheaths of chitosan-treated bamboo shoots. In this study, we purified two isoforms of chitosanase along with chitinase activity from the sheaths of chitosan-treated bamboo shoots. The purified isoforms were characterized by their enzymatic properties, including molecular mass, isoelectric point, kinetic parameters, thermal stability, effectors, and substrate specificity. Furthermore, these enzymes were used to degrade chitosans for the production of low molecular weight chitosans (10−30 kDa).

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MATERIALS AND METHODS

Plant Material. Fresh shoots of *B. oldhamii* (Munro) were harvested from a bamboo farm in Taipei, Taiwan. Etiolated shoots (15 cm tall on average) were collected. After washings with water and then with a 0.02% sodium hypochlorite solution, the shoots were divided into seven groups of two shoots each. Shoots for four control groups were stored at room temperature (24 ± 2 °C) for 0, 12, 24, or 48 h and were named C0, C12, C24, or C48, respectively. Shoots from the other three groups were coated with 0.1% chitosan (in 0.1% acetic acid) and stored at room temperature (24 ± 2 °C) for 12, 24, or 48 h; these shoots were named CS12, CS24, or CS48, respectively. After the outer sheaths of fresh or stored bamboo shoots were removed, the edible portion of each shoot was divided into the upper node and lower basal sections. The sheaths and edible sections were lyophilized, ground into powder, and stored separately at −80 °C.

Chemicals. Imidazole, tris(hydroxymethyl)aminomethane (Trizma base; Tris), acrylamide, sodium dodecyl sulfate (SDS), sucrose, Coomassie brilliant blue R-250 (CBR), ethyl acetimidate (EAM), sodium *p*-hydroxymercuribenzoate (*p*HMB), diethyl pyrocarbonate (DEPC), *N*-ethyl-5-phenylisoazoline-3′-sulfonate (Woodward's reagent K; WRK), phenylmethanesulfonyl fluoride (PMSF), *N*-acetylimidazole (NAI), 1,2-cyclohexanedione (CHD), 2,4-dinitro-1-fluorobenzene (DNFB), D-glucosamine (GlcN), *N*-acetyl-D-glucosamine (GlcNAc), 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH), and calcofluor white M2R (fluorescent brightener 28) were purchased from Sigma (St. Louis, MO). *N*-Bromosuccinimide (NBS) was purchased from Merck (Darmstadt, Germany). Sephacryl S-100 HR, PhastGel IEF 3−9, isoelectric focusing protein markers (p*I* 3−10), a low molecular weight calibration kit for SDS electrophoresis, and a low molecular weight calibration kit for gel filtration were obtained from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). BCA and Micro BCA protein assay kits were purchased from Pierce Biotechnology Co. (Rockford, IL). All other chemicals were of reagent grade.

Preparation of Crude Enzyme from Lyophilized Bamboo Samples. One gram of the lyophilized sheaths, upper node section or lower basal section, was mixed with 20 mL of 0.1 M sodium acetate buffer (pH 4.5) and stirred for 15 min. Insoluble substances were pelleted by centrifugation (16128*g*, 10 min). The supernatants were collected and assayed for chitinase and chitosanase activities.

Measurement of Chitosanase Activity. A 0.4 mL mixture containing 0.1 mL of 0.5% chitosan in 0.1 M sodium acetate buffer (pH 4), 0.25 mL of 0.06 M sodium acetate buffer (pH 4), and 0.05 mL of a diluted enzyme solution was incubated at 50 °C for 30 min. The reaction was stopped by adding 0.8 mL of reagent A (alkaline copper reagent) and 0.8 mL of reagent B (neocuproine color reagent). The mixture was then incubated at 100 °C for 14 min to assay for reducing sugars, as described by Dygert et al.[23] After the mixture had cooled and 2 mL of H_2O had been added, the quantity of the neocuproine complex was determined by measuring the absorbance at 450 nm. One unit of chitosanase was defined as the amount of enzyme required to liberate 1 μmol of GlcN per minute.

Measurement of Chitinase Activity. A 0.4 mL mixture containing 0.1 mL of 0.5% ethylene glycol chitin in 0.1 M sodium acetate buffer (pH 4.5), 0.25 mL of 0.06 M sodium acetate buffer (pH 4.5), and 0.05 mL of diluted enzyme solution was incubated at 50 °C for 30 min. The quantity of reducing sugars liberated from the reaction was measured as described above. One unit of chitinase was defined as the amount of enzyme required to liberate 1 μmol of GlcNAc per minute.

Protein Determination. Protein content was quantified using a bicinchoninic acid (BCA) or micro BCA protein assay kit according to the method of Smith et al.[24] Bovine serum albumin served as the protein standard.

Synthesis of Glycol Chitin, Carboxymethyl Chitin, and Aminoethyl Chitosan. Glycol chitin (ethylene glycol chitin) was prepared by reacting chitin with ethylene chlorohydrin (2-chloroethanol) as described by Yamada and Imoto.[25] Carboxymethyl chitin was prepared by reacting chitin with chloroacetic acid according to the method of Hirano.[26] Aminoethyl chitosan was prepared by reacting chitosan with 2-chloroethylamine hydrochloride according to the methods of Clifford and Naoyuki.[27,28]

Chitosanase Purification. Five grams of lyophilized bamboo sheaths obtained from 48-h-treated bamboo shoots was dissolved in 100 mL of 0.1 M sodium acetate buffer (pH 4.5), and the mixture was stirred for 15 min. Insoluble substances were removed by centrifugation (16128*g*, 10 min). Ammonium sulfate was added to the supernatant, and the precipitate formed upon 40−80% saturation was collected by centrifugation (16128*g*, 10 min) and dissolved in 0.1 M sodium acetate buffer (pH 4.5). After centrifugation (16128*g*, 10 min), the supernatant was concentrated to a volume of 5 mL with an Amicon ultra-15 ultrafiltration unit with a 10 kDa molecular weight cutoff (MWCO) membrane (Millipore Co., Bedford, MA). The concentrated supernatant was then applied to a Sephacryl S-100 HR column (2.6 × 70 cm) that was pre-equilibrated with 0.1 M sodium acetate buffer (pH 4.5). Samples were eluted with the equilibrium buffer at a flow rate of 30 mL/h. Five milliliter fractions were collected and assayed for chitosanase activity; absorbance values were measured at 280 nm. Fractions containing chitosanase activity were pooled.

The chitosanase obtained from the Sephacryl S-100 HR column was brought to a total volume of 58 mL with H_2O. Ampholyte (40%, 1.53 mL) was added to the enzyme solution, bringing the final concentration to 1%. The mixture was loaded into the Rotofor cell chamber. After 5 h of isoelectric focusing under 12 W of constant power, fractions were gathered according to the manufacturer's instructions (Bio-Rad). The absorbance at 280 nm, pH, and chitosanase activity were measured for each fraction. The fractions from two peaks of chitosanase activity were individually pooled and designated isoform A (pH 2.7−4.1; fractions 1−4) and isoform B (pH 8.0−9.5; fractions 17−20). After dialysis against 0.025 M imidazole−HCl buffer (pH 7.4), the purified protein for isoforms A and B was stored at 4 °C.

Isoelectric Focusing Electrophoresis (IEF). The p*I* value of each chitosanase isoform was determined by IEF using PhastGel IEF 3−9 gels. The Pharmacia PhastSystem Separation Technique File No. 100 describes a detailed procedure for IEF. After electrophoresis, the gels were stained for protein content with CBR. An aliquot of the standard p*I* marker protein mixture from the Pharmacia p*I* calibration kit was run beside the chitosanase sample. This standard protein mixture included trypsinogen (p*I* = 9.3), lentil lectin-basic band (p*I* = 8.65), lentil lectin-middle band (p*I* = 8.45), lentil lectin-acidic band (p*I* = 8.15), myoglobin-basic band (p*I* = 7.35), myoglobin-acidic band (p*I* = 6.85), human carbonic anhydrase B (p*I* = 6.55), bovine carbonic anhydrase B (p*I* = 5.85), β-lactoglobulin A (p*I* = 5.2), soybean trypsin inhibitor (p*I* = 4.55), and amyloglucosidase (p*I* = 3.55). The R_f values measured directly from the gel were used to construct the p*I* calibration curve. The R_f value of the chitosanase protein band was used to estimate the corresponding p*I* value from the calibration curve.

Determination of Optimum pH. The optimum pH values for the purified chitosanase isoforms were assayed in universal buffer (Britton and Robinson type)[29] at pH values between 2 and 10 using chitosan as the substrate at 50 °C. Results were expressed as relative activity percentages, which were calculated by dividing the specific activity at each pH value by the maximum activity.

Determination of Optimum Temperature and Thermal Stability. The optimum temperature for the activities of the purified isoforms were assayed at pH 4 using a chitosan substrate at temperatures between 30 and 80 °C. The results were expressed as the relative activity percentage, which was calculated by computing the ratio of the specific activity at each temperature to the maximum activity identified within the temperature range studied. To assess thermal stability, aliquots of enzyme solutions (pH 7.4) were incubated in a thermostatic water bath set to various temperatures between 30 and 80 °C. After various time intervals, samples were withdrawn, and the enzyme activity remaining in each sample was measured at 50 °C and pH 4 as described above. The chitosanase activity level from the unheated enzyme solutions represented the initial activity (100%) for each isoform. The portion of activity remaining after incubation at various temperatures for different time intervals was calculated. The thermal inactivation of the purified enzymes was simulated with first-order kinetics as

$$A = A_0 \times e^{-kt} \quad (1)$$

$$\log\frac{A}{A_0} = \left(\frac{-k}{2.303}\right)t \quad (2)$$

where A is the remaining chitosanase activity after t (min) of heat treatment, A_0 is the initial chitosanase activity, and k is the reaction rate constant of first-order kinetics. The reaction rate constant was obtained from a plot of the log of activity remaining versus time.

Determination of Storage Stability. To assess storage stability, enzyme solutions were stored at 4 °C for 56 days. The enzyme activity remaining after storage for different durations was measured. The results were expressed as a plot of chitosanase activity remaining versus time.

Effects of Metal Ions and EDTA. Purified enzymes were concentrated by ultrafiltration using an Amicon Centricon Plus-20 (NMWL 10000) filter. After concentration, a 0.05 mL aliquot of the enzyme was incubated at 30 °C for 30 min with either 0.05 mL of deionized water as the control or 0.05 mL of diluted metal ions. The following metal ion compounds and concentrations were tested: 10 mM $CuSO_4$, $ZnCl_2$, $MgSO_4$, $CaCl_2$, NaCl, KCl, and EDTA; 2 and 0.5 mM $AgNO_3$; and 0.5 and 0.2 mM $HgCl_2$. The remaining enzyme activity in each sample was measured after treatment. The relative activity percentage was calculated by computing the ratio of the specific activity of the treated to untreated enzyme samples.

Effects of Chemical Modification Reagents. Purified chitosanase isoforms were concentrated as described above. After concentration, a 0.05 mL enzyme aliquot was incubated at 30 °C for 30 min with 0.25 mL of reaction buffer[30] as the control or 0.25 mL of a chemical modifier. The following chemical modification reagents were tested: NBS (2 and 20 mM), EAM (1 mM), NAI (10 mM), *p*HMB (2 mM), DEPC (10 mM), DNFB (10 mM), PMSF (10 mM), WRK (200 mM), and CHD (10 mM). After dialysis against 0.05 M sodium acetate buffer (pH 4.5), the enzyme activity in each sample was measured. The relative activity percentage was calculated using the ratio of the specific activity of each chitosanase isoform treated with chemical modification reagents to the activity of the untreated sample.

Determination of Kinetic Parameters. The Michaelis constant (K_m) and maximal velocity (V_{max}) of chitosan hydrolysis were determined at substrate concentrations between 0.025 and 2 mg/mL. The K_m and V_{max} of each isozyme were calculated from double-reciprocal plots according to the method of Lineweaver and Burk.[31]

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out in 12.5% polyacrylamide gels for protein staining or in 12.5% polyacrylamide gels containing 0.16% (w/v) aminoethyl chitosan for activity staining using bromophenol blue as the tracking dye according to the method of Laemmli.[32] For protein staining, samples were boiled for 5 min with 2.5% (w/v) SDS sample buffer containing 2-mercaptoethanol. For activity staining, samples were heated at 90 °C for 5 min with 40% sucrose and 2.5% (w/v) SDS sample buffer without 2-mercaptoethanol. After electrophoresis, the proteins were stained with CBR. Chitosanase activity was detected using calcofluor white M2R staining; aminoethyl chitosan served as the substrate in the gel matrix as described by Ouakfaoui and Asselin.[18,33] Enzyme were renatured after SDS-PAGE in 100 mM sodium acetate buffer (pH 5.0) containing 1% (v/v) Triton X-100 for 18 h at 50 °C with gentle shaking. Chitosanase activity was detected as a dark (nonfluorescent) band against the UV-fluorescent background of intact aminoethyl chitosan stained with calcofluor white M2R.

Hydrolysis of Chitosan. Three grams of chitosan was digested with 4 mL of either purified chitosanase isoform (0.63 units) at 50 °C for 1 h in 66.6 mL of 0.47 N acetic acid. The pH of the hydrolysis reaction was adjusted to 4.0 using 0.45 M $NaHCO_3$. After the addition of 13 mL of H_2O, the reaction proceeded for 8 or 24 h. The hydrolysis reaction was stopped by adding 3.5 mL of concentrated HCl (36.6%) and heating the mixture in a boiling water bath for 10 min. The insoluble substances were removed by centrifugation (5000*g*, 15 min) and the hydrolysate was collected for analysis of the hydrolysis products.

Gel Filtration of Chitosan Hydrolysis Products. The molecular masses of the chitosan hydrolysis products were determined by gel filtration on an FPLC System (Amersham Pharmacia Biotech AB, Uppsala, Sweden) with a Superose 12 HR (10/30) column. The column was equilibrated with 0.1 M sodium acetate buffer (pH 4.5) at a flow rate of 30 mL/h, and then 0.1 mL of chitosan hydrolysate was applied to the column and eluted with the equilibrium buffer. The GlcN content of each fraction (0.5 mL) was analyzed using the MBTH colorimetric method.[34] The column was calibrated with dextran standards of the following molecular masses: 80.9, 48.6, 23.8, 11.6, 3.26, and 1.27 kDa. Each of the collected fractions (0.5 mL) of dextran standards was analyzed for total carbohydrate concentration using the phenol–sulfuric colorimetric method.[35] A calibration curve was obtained by plotting the elution volume of the dextran standards versus the log of the molecular mass. The molecular masses of the chitosan hydrolysis products were calculated from the calibration curve.

Data Analysis. Measurements were performed in triplicate to asses the effects of various metal ions and chemical modification agents on the activity of chitosanase isoforms. Other analytic measurements were performed in duplicate. The data were expressed as the mean ± standard deviation. Analysis of variance was performed by ANOVA using the SAS program (version 9.1, Statistical Analysis System Inc., Cary, NC; 2006). Mean values were compared by Duncan's multiple-range test or Student's t test. A significance level of 5% was adopted for all comparisons.

RESULTS AND DISCUSSION

Chitinase and Chitosanase Activities in Fresh-Cut and Chitosan-Treated Bamboo Shoots. The chitinase and chitosanase activities were rather low both in the edible basal sections and in the outer sheaths of fresh-cut bamboo shoots (Figure 1). Storage at room temperature for 12–48 h significantly increased ($p < 0.05$) chitinase and chitosanase activities in both portions of bamboo shoots but most notably in the outer sheaths of chitosan-treated bamboo shoots. Both chitinase and chitosanase activities were significantly higher in

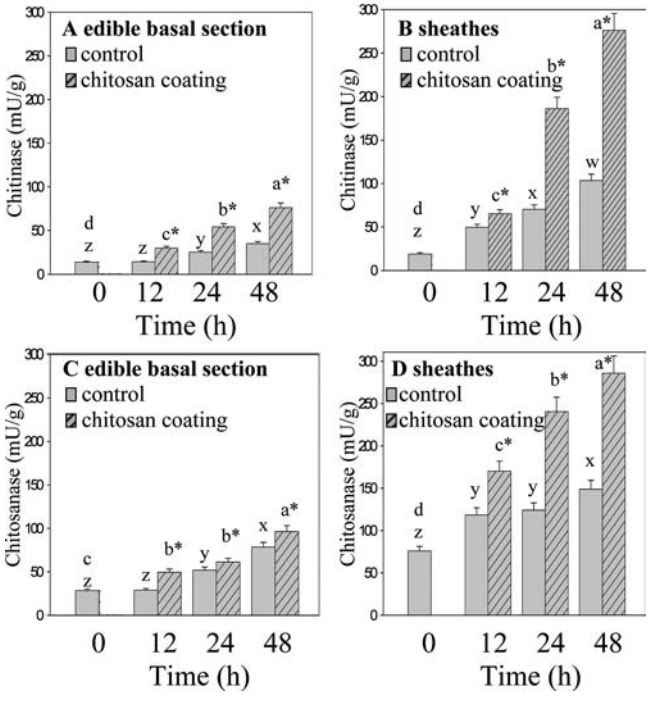

Figure 1. Changes in chitinase and chitosanase activities in the fresh-cut bamboo shoots during room temperature storage: (A, B) changes of chitinase activity in the edible basal section (A) and outer sheaths (B) of bamboo shoots; (C, D) changes of chitosanase activity in the edible basal section (C) and outer sheaths (D) of bamboo shoots. Each box represents the mean value of two replicates of enzyme extract for activity measurements; the range is depicted with a vertical bar. Bars with different letters (w–z for control group; a–d for chitosan coating group) are significantly different ($p < 0.05$). *, significant difference between the control and treated groups at $p < 0.05$ using Student's t test.

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the outer sheaths of chitosan-treated bamboo shoots than in the sheaths of untreated shoots ($p < 0.05$). These results indicate that chitosan may induce chitinase and chitosanase synthesis in bamboo shoots.

Chitosanase Purification. Using the protocols summarized in Table 1 to purify chitosanase from the sheaths of bamboo sheaths, we were able to isolate two isoforms of chitosanase. Ammonium sulfate fractionation was initially used to precipitate chitosanase. The precipitate formed between 40 and 80% ammonium sulfate saturation, and most of the chitosanase activity was recovered. This step successfully removed non-chitosanase-related proteins. The next stage in the purification scheme was gel filtration using Sephacryl S-100 HR, and proteins without chitosanase activity were removed. Finally, the chitosanase was purified by preparative IEF in a Rotofor cell. As shown in Figure 2, this step divided

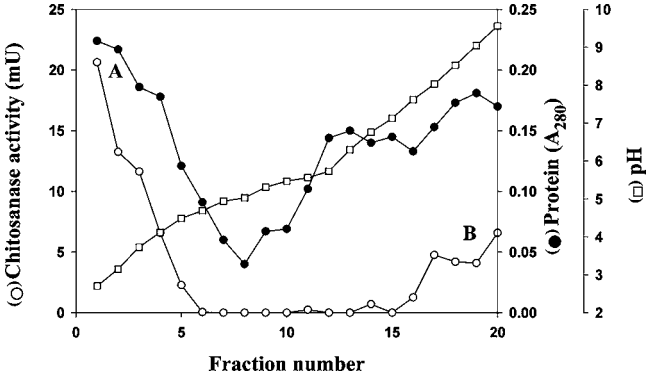

Figure 2. Fractionation of chitosanase isoforms from the sheaths of bamboo shoots by preparative isoelectric focusing electrophoresis in a Rotofor cell.

the chitosanase into two activity peaks. Peak A was focused between pH 2.7 and 4.1, indicating that the isoform in peak A was acidic, whereas peak B was focused between pH 8.0 and 9.5, indicating that the isoform in peak B was basic. Both peaks A and B were collected separately and designed as isoforms A and B, respectively. The specific activities of isoforms A and B were increased 115.1- and 37.1-fold, respectively, and the total yield was 22.4% (Table 1).

SDS-PAGE and Activity Staining of Chitosanase. The purified chitosanase isoforms A and B were analyzed by SDS-PAGE and gel activity staining. As shown in Figure 3, isoform A revealed one major protein band with a molecular mass of 24.5 kDa and a minor protein band with a molecular mass of 16.4 kDa; both bands showed chitosanase activity. However, isoform B revealed only one protein band with a molecular mass of 16.4 kDa, which also showed chitosanase activity.

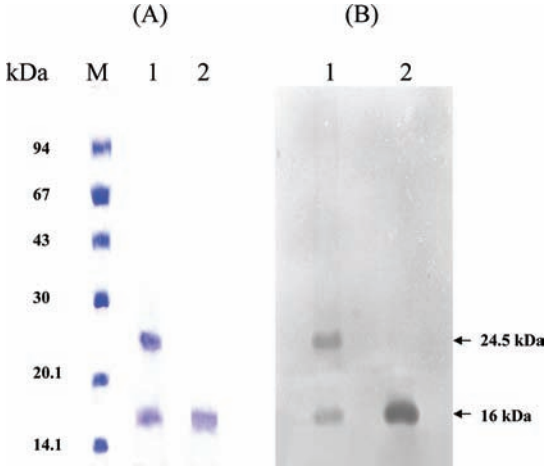

Figure 3. SDS-PAGE (A) and chitosanase activity staining (B) of chitosanase isoforms A and B. Lane M contains marker proteins. Lanes 1 and 2 contain purified chitosanase isoforms A and B, respectively.

These results indicate that the purified isoform A was composed of a major and a minor chitosanase isoform, whereas isoform B was homogeneous. The molecular masses of both isoforms were within the range of values reported for this enzyme from other plant sources. Published sources of chitosanase include VAM fungi-colonized leek (20 kDa) and onion roots (14, 20, and 35 kDa),[16] various organs of the plant *Cucumis sativus* (10, 12, 14, 16, 18, and 20 kDa),[17] chemical-stressed barley leaves (19 kDa),[22] *Citrus sinensis* (29−30.5 kDa),[36] and fig ficin preparations (13 kDa).[37]

Isoelectric Focusing Electrophoresis of the Purified Enzymes. When the purified isoforms A and B were focused in PhastGel IEF 3−9 gels containing wide-range ampholytes (p*I* 3−10) and stained for protein content, they revealed protein bands with isoelectric points of 4.30 and 9.22, respectively (Figure 4). There is little information regarding the isoelectric points of plant chitosanase. A hydrolase with dual chitinase and chitosanase activity in tomato stem had an isoelectric point of pH 8.5.[38] Several acidic hydrolases with chitinase and chitosanase activities from sweet orange callus tissue had isoelectric points from pH 4.5 to 5.4.[36]

Optimum pH, Optimum Temperature, and Thermal Stability. The purified isoforms A and B retained approximately 70−100% relative activity between pH 3 and 6 but rapidly lost activity above pH 6. The optimum pH for both isoforms was pH 3−4 (data not shown). This value was within the range of values reported for this enzyme from other plant sources such as sweet orange callus tissue (pH 5),[36] pineapple stem bromelain (pH 3),[39] and fig ficin (pH 4.5).[37]

Table 1. Purification of Chitosanase from the Sheaths of Bamboo Shoots

procedure	total volume (mL)	total activity (mU)[a]	total protein (mg)	specific activity (mU/mg)	purification (fold)	yield (%)
crude extract[b]	77	1191	182	6.5	1	100
40−80% $(NH_4)_2SO_4$ saturation	12	936	11.3	82.7	12.6	79
Sephacryl S-100 HR	18	446	2.5	206.4	31.5	44
Rotofor cell						
isoform A	8.8	203	0.27	753.4	115.1	17.1
isoform B	9.9	63	0.26	243	37.1	5.3

[a]One chitosanase unit is defined as the amount of enzyme releasing 1 μmol of glucosamine per minute at 50 °C and pH 4.5; 1 mU is 1×10^{-3} unit. [b]Data were obtained from 5 g of lyophilized bamboo-shoot sheath powder.

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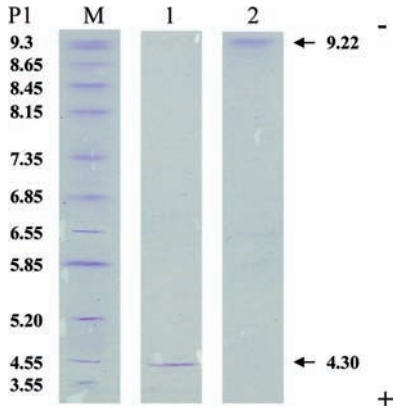

Figure 4. Isoelectric focusing electrophoresis of chitosanase isoforms A and B. Lane M contains p*I* marker proteins. Lanes 1 and 2 contain purified chitosanase isoforms A and B, respectively.

The optimum reaction temperatures were 70 and 60 °C for isoforms A and B, respectively (data not shown). To assess the stability of each isoform, we incubated each purified enzyme in a thermostated water bath at various temperatures between 30 and 80 °C. After incubation, the residual activity was measured under standard assay conditions. As shown in Figure 5,

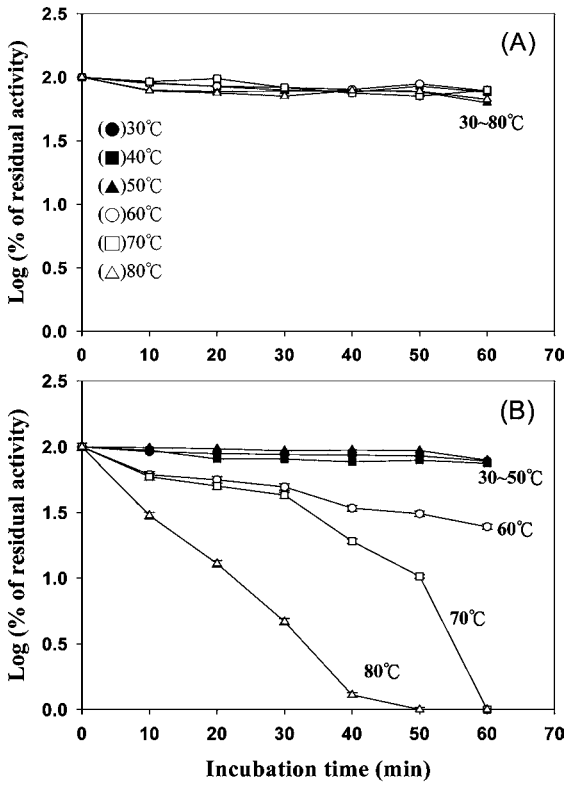

Figure 5. Thermal stability of chitosanase isoforms A (A) and B (B) from the sheaths of bamboo shoots. The log of the percent activity remaining is plotted against incubation time at various temperatures. Each point represents the mean value of duplicate measurements, and the range is indicated with a vertical bar.

isoform A was stable between 30 and 80 °C, whereas isoform B was stable between 30 and 50 °C. Isoform B experienced a relatively modest loss of activity between 60 and 70 °C but a rapid loss at 80 °C. According to eq 2, the half-lives of thermal inactivation for isoform B at 60 and 80 °C were 31.0 and 6.5 min, respectively. Similarly, chitosanase from a fig ficin preparation was fairly stable after 30 min at 40−70 °C, but its activity decreased significantly at temperatures >75 °C.[37] The thermal stabilities of chitosanases of *Streptomyces* N-174[40] and *Bacillus megaterium* P1[41] were improved by the addition of bovine serum albumin at 70 °C.

To determine the storage stability of the purified chitosanases, each isoform was stored at 4 °C for up to 56 days. Isoform A was rather stable during storage for 14 days but slowly became inactivated thereafter. The activity of isoform B did not perceptibly change during the first 7 days but slowly became inactivated thereafter (data not shown).

Effect of Metal Ions and EDTA. EDTA (5 mM) stimulated the activity of both isoforms A and B (Table 2). On the other hand, metal ions affected isoforms A and B very differently. For isoform A, the transition heavy metal ion Ag^+ (1 mM) significantly inhibited chitosanase activity, whereas the light alkali metal ions K^+ (5 mM) and Na^+ (5 mM) and transition ion Cu^{2+} (5 mM) significantly stimulated chitosanase activity ($p < 0.05$). The other ions tested did not alter the chitosanase activity of isoform A ($p > 0.05$). For isoform B, the transition heavy metal ions Ag^+ (1 mM) and Hg^{2+} (0.1, 0.25 mM) significantly inhibited chitosanase activity ($p < 0.05$), whereas the alkali earth metal ion Ca^{2+} (5 mM), the transition metal ion Zn^{2+} (5 mM), and the heavy metal ion Ag^+ (0.25 mM) significantly stimulated chitosanase activity ($p < 0.05$). The other ions tested did not significantly affect enzyme activity ($p > 0.05$). Most microbial chitosanases were reportedly inhibited by heavy metal ions.[10] The chitosanase from a fig ficin preparation was not inhibited or stimulated by any tested metal ions or EDTA.[37] However, our results indicate that the chitosanase of bamboo shoots was affected by several metal ions and EDTA.

Table 2. Effect of Various Salts on the Activities of Chitosanase Isoforms A and B

		relative activity[a] (%)	
reagent	final concn (mM)	isoform A	isoform B
none		100	100
$AgNO_3$	0.25	98 ± 0.6	105 ± 0.5 *
$AgNO_3$	1	91 ± 0.8 *	86 ± 1.0 *
$HgCl_2$	0.1	100 ± 0.3	90 ± 1.7 *
$HgCl_2$	0.25	100 ± 0.4	77 ± 1.3 *
$CaCl_2$	5	100 ± 0.6	117 ± 0.6 *
$ZnCl_2$	5	100 ± 1.0	111 ± 0.5 *
$CuSO_4$	5	105 *	98 ± 3.3
$MgSO_4$	5	103 ± 0.2 *	104 ± 2.4
KCl	5	108 ± 0.9 *	106 ± 2.9
NaCl	5	111 ± 0.7 *	95 ± 4.0
EDTA[b]	5	107 ± 0.7 *	104 ± 0.1 *

[a]Data are presented as the mean ± standard deviation ($n = 3$). *, significant difference between the control and treated groups at $p < 0.05$. [b]Ethylenediaminetetraacetic acid disodium salt.

Effects of Chemical Modification Reagents. It is generally believed that an enzyme will be inhibited or inactivated if its amino acid side chains that are involved in catalytic activity are chemically modified. In this study, a number of reagents were used to chemically modify the amino acid side chains of chitosanase isoforms A and B (Table 3). Among the tested chemical modifiers, NBS, which usually acts on tryptophan and cysteine, and WRK, a selective modification reagent for aspartic and glutamic residues, significantly inhibited the activity of both isoforms ($p < 0.05$). EAM and PMSF, selective chemical modification reagents for lysine and

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Table 3. Effect of Various Chemical Modification Agents on the Activities of Chitosanase Isoforms A and B

		relative activity[a] (%)	
reagent	final concn (mM)	isoform A	isoform B
none		100	100
NBS	5	12 ± 1.8 *	0 *
NBS	0.5	48 ± 1.1 *	52 ± 1.5 *
NAI	2.5	96 ± 3.3	84 ± 4.2
*p*HMB	0.5	112 ± 0.6 *	102 ± 1.5
DEPC	2.5	111 ± 3.6	97 ± 2.4
DNFB	0.625	95 ± 2.1	84 ± 1.8
EAM	250	106 ± 4.2	56 ± 4.0 *
PMSF	2.5	113 ± 3.6	86 ± 1.3 *
WRK	50	40 ± 0.5 *	1 ± 1.6 *
CHD	1.25	104 ± 5.1	108 ± 3.3

[a]Data are presented as the mean ± standard deviation ($n = 3$). *, significant difference between the control and treated groups at $p < 0.05$.

serine residues, respectively, both significantly inhibited isoform B activity ($p < 0.05$). However, the sulfhydryl-modifying reagent, *p*HMB, significantly stimulated isoform A activity ($p < 0.05$). The other chemical modification reagents tested had no significant effect on the activity of either isoform ($p > 0.05$). Therefore, the indole group of tryptophan and the carboxyl group of aspartic acid and glutamic acid appear to be essential for the catalytic activities of both isoforms. The ε-amino group of lysine side chains and the hydroxyl group of serine are probably also essential for the catalytic activity of isoform B. Several microbial chitosanases were inhibited by *p*HMB and NBS, and so sulfhydryl and indole groups were essential for catalysis.[10] In addition, the chitosanase from a fig ficin preparation was completely inhibited by NBS.[37]

Effects of Substrate Concentration. The substrate–saturation curves for both isoforms A and B fit Michaelis–Menten kinetics (data not shown). The K_m values calculated from double-reciprocal plots for isoforms A and B were 0.539 and 0.183 mg/mL, respectively, whereas the V_{max} values for isoforms A and B were 0.262 and 0.092 μmol min^{-1} mg^{-1}, respectively. These results indicate that isoform B had lower apparent dissociation and decomposition rate constants for the enzyme–substrate complex than isoform A.

Substrate Specificity. As shown in Table 4, the purified chitosanases hydrolyzed chitosan and chitin polymers. Both

Table 4. Substrate Specificity of Chitosanase Isoforms A and B

	relative activity (%)	
substrate	isoform A	isoform B
soluble substrate		
commercial chitosan		
crab chitosan (1100 kDa)	100	100
crab chitosan (750 kDa)	81.3	117.4
crab chitosan (150 kDa)	47.0	67.8
crab chitosan (70 kDa)	38.8	51.2
water-soluble chitin		
glycol chitin	29.7	14.1
carboxymethyl chitin	41.8	17.2
insoluble substrate		
commercial chitin (crab) (Sigma, C364)	7.8	10.8

isoforms appeared to prefer chitosan polymer substrates, although they also showed activity toward chitin polymers. In addition, both isoforms showed greater activity toward high molecular weight chitosans (1100 and 750 kDa) than low molecular weight chitosans (150 and 70 kDa). These results imply that both isoforms are chitosan-preferred hydrolases with dual chitinase and chitosanase activities. The chitosanase isolated from a fig ficin preparation showed similar substrate specificity; although the fig-derived enzyme preferred chitin substrate, it also exhibited dual chitinase and chitosanase activities.[37]

Effect of Chitosan Deacetylation on Enzyme Activity. As shown in Figure 6, chitosan polymers with various degrees

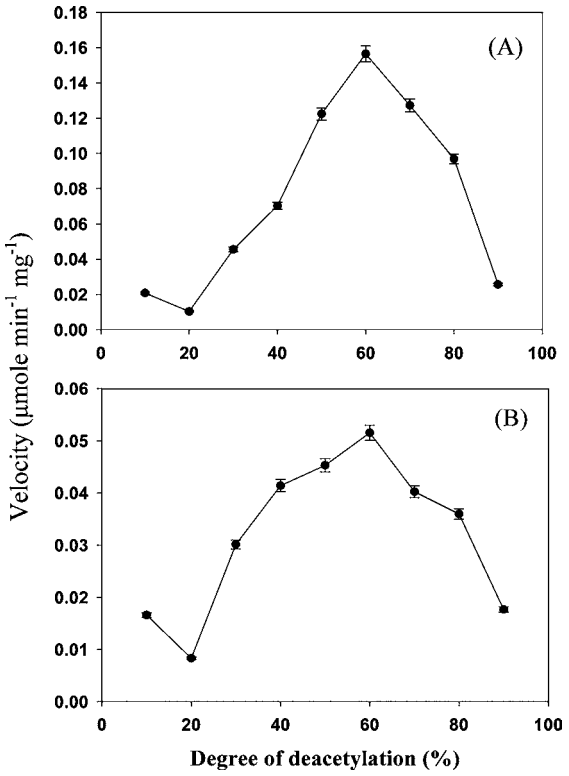

Figure 6. Effect of the degree of chitosan deacetylation on the activity of chitosanase isoforms A (A) and B (B). Each point represents the mean value of duplicate measurements, and the range is shown with a vertical bar.

of deacetylation (10–94%) were all susceptible to hydrolysis by both isoforms. Polymers with 50–70% deacetylation were most susceptible to hydrolysis by isoform A; polymers with 40–80% deacetylation were most susceptible to isoform B. These results indicate that the degree of deacetylation of chitosan affects the efficiency of chitosan hydrolysis catalyzed by chitosanase. The effects of chitosan deacetylation on chitosanase activity have been described for enzymes from a pineapple stem preparation,[39] a fig ficin preparation,[37] *Penicillium islandum*,[42] *Bacillus circulans* MH-K1,[43] *Bacillus* SP. PI-7S,[44] and *Bacillus subtilis* IMR-NK1.[45] Chitosan polymers exhibiting 20–90% deacetylation were all susceptible to the pineapple-derived chitosanase, with the most susceptible substrates being 60–80% deacetylated. On the other hand, 52–70% deacetylated chitosan was most susceptible to the fig chitosanase. The fungal (*P. islandium*) enzyme degraded 40–70% deacetylated chitosan most effectively, whereas the *Bacillus* chitosanases were most active toward highly deacetylated chitosan.

Products of Chitosan Hydrolysis. The products of the chitosan hydrolysis by the purified isoforms A and B were determined by gel filtration on a Superose 12 HR column, and

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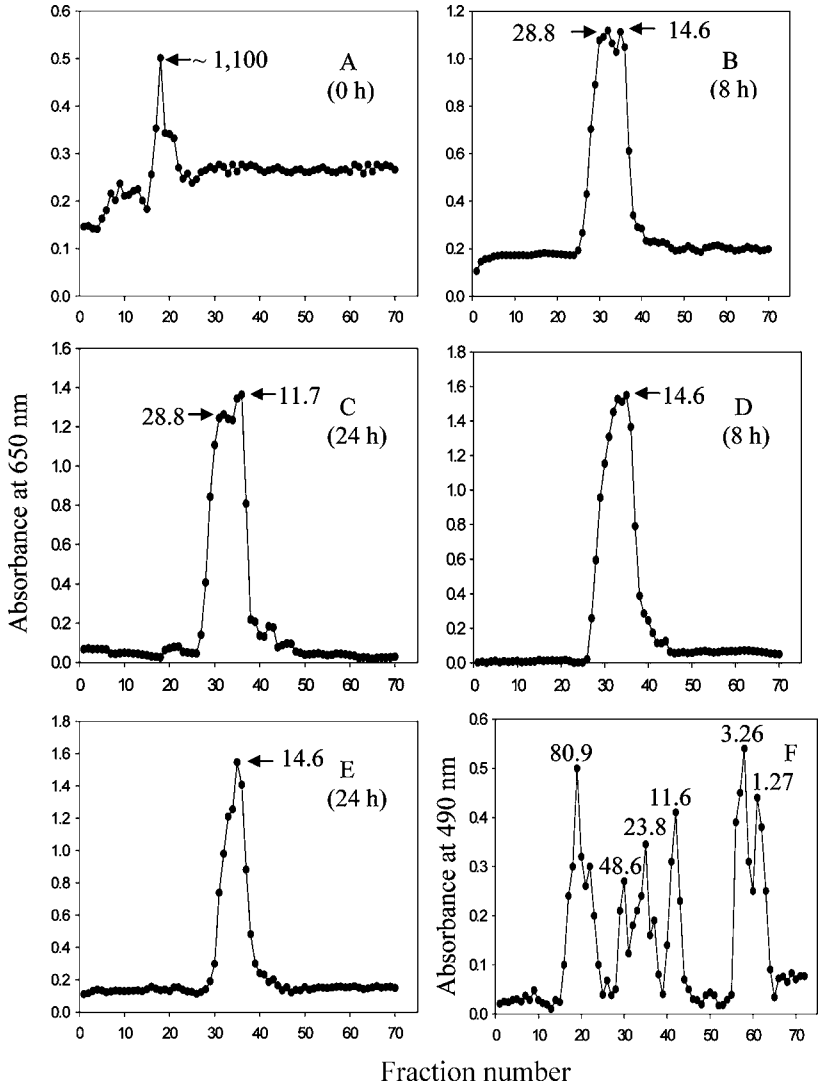

Figure 7. Gel filtration of chitosan hydrolysis products on a Superose 12 HR column: (A) unhydrolyzed high molecular weight chitosan (~1100 kDa); (B, C) products of chitosan hydrolysis catalyzed by chitosanase isoform A for 8 h (B) and 24 h (C); (D, E) products of chitosan hydrolysis catalyzed by chitosanase isoform B for 8 h (D) and 24 h (E); (F) standard molecular weight dextrans. Numbers on the peaks indicate the molecular size of chitosan hydrolysis products or molecular weight marker dextrans in kDa.

dextrans of various molecular weights were used for calibration. As shown in Figure 7, unhydrolyzed high molecular weight chitosan (1100 kDa) eluted from the column as a major peak at fraction 18 (Figure 7A). The calibration curve obtained from elution volumes of dextran standards is shown in Figure 7F. After 8 and 24 h of hydrolysis by isoform A, the hydrolysate was fractionated into two peaks with molecular masses ranging from 28.8 to 14.6 kDa (Figure 7B) and from 28.8 to 11.7 kDa (Figure 7C). A single major peak with a molecular mass of 14.6 kDa (Figure 7D,E) appeared in isoform B-catalyzed hydrolysates after both 8 and 24 h of incubation. On the basis of these results, we believe both isoforms are endosplitting enzymes and will be useful for the production of low molecular weight chitosan. Low molecular weight chitosan and chitosan oligomers have various biological activities; they can be used in the food and pharmaceutical fields[46,47] or in agriculture for the biocontrol of fungi and insects that are pathogenic to plants.

In summary, bamboo chitosanase isoforms A and B are chitosan-preferred hydrolases with dual chitosanase and chitinase activities. Both isoforms possess a wide chitosan substrate specificity and remain stable over a wide range of temperatures. The major products of chitosan hydrolysis catalyzed by both isoforms are low molecular weight polymers with molecular masses ranging from 28.8 to 11.7 kDa. Therefore, bamboo sheaths are a potential source of chitosanase for chitosan degradation. Biotechnological applications may include the preservation of fresh-cut bamboo shoots. In addition, these results provide information for exploring the roles of chitosanase in the defense system of postharvested bamboo shoots. However, further characterization including the identification of substrate cleavage position specificity, elucidation of the mechanism of substrate binding and catalysis, molecular sequencing, and identification of antifungal activity remains to be performed for both isoforms.

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