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Cloning and Characterization of an Antifungal Class III Chitinase from Suspension-Cultured Bamboo (*Bambusa oldhamii*) Cells

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A class III chitinase cDNA (*BoChi3-1*) was cloned using a cDNA library from suspension-cultured bamboo (*Bambusa oldhamii*) cells and then transformed into yeast (*Pichia pastoris* X-33) for expression. Two recombinant chitinases with molecular masses of 28.3 and 35.7 kDa, respectively, were purified from the yeast's culture broth to electrophoretic homogeneity using sequential ammonium sulfate fractionation, Phenyl-Sepharose hydrophobic interaction chromatography, and Con A-Sepharose chromatography steps. N-Terminal sequencing and immunoblotting revealed that both recombinant chitinases were encoded by *BoChi3-1*, whereas SDS-PAGE and glycoprotein staining showed that the 35.7 kDa isoform (35.7 kDa BoCHI3-1) was glycosylated and the 28.3 kDa isoform (28.3 kDa BoCHI3-1) was not. For hydrolysis of ethylene glycol chitin (EGC), the optimal pH values were 3 and 4 for 35.7 and 28.3 kDa BoCHI3-1, respectively; the optimal temperatures were 80 and 70 °C, and the K_m values were 1.35 and 0.65 mg/mL. The purified 35.7 kDa BoCHI3-1 hydrolyzed EGC more efficiently than the 28.3 kDa isoform, as compared with their specific activity and activation energy. Both recombinant BoCHI3-1 isoforms showed antifungal activity against *Scolecobasidium longiphorum* and displayed remarkable thermal (up to 70 °C) and storage (up to a year at 4 °C) stabilities.

KEYWORDS: ***Bambusa oldhamii*; characterization; purification; recombinant class III chitinase; suspension-cultured cells**

INTRODUCTION

Chitin is an abundant *N*-acetylglucosamine (GlcNAc) polymer found in the cell walls of fungi and green algae and in the exoskeleton of many crustaceans and insects (*1*). Although higher plants do not endogenously synthesize chitin, they do express chitinases (EC 3.2.1.14) to hydrolyze it, and the results of several studies suggest that chitinases in some way help protect plants from pathogens (*2*−*6*) and from the cold (*7, 8*). In addition, many chitinases appear to be developmentally regulated, with specific isoforms appearing in certain organs only during certain stages of a plant's life. Although an endogenous function for chitinase in plants has not yet been demonstrated, specific isoforms may play roles in embryonic development (*8*), pollination, and sexual reproduction (*9*).

Plant chitinases have been divided into several groups on the basis of the structural features of their protein sequences (*10*−*12*). Class I chitinases have an N-terminal chitin binding domain (CBD) and a catalytic domain linked by a flexible hinge region. Class II chitinases show sequence similarity to class I chitinases, but lack the CBD and hinge region. Class III chitinases are structurally unrelated to other types of plant chitinase, but have a distant sequence similarity to bacterial and fungal chitinases. Class IV chitinases show sequence similarity to class I chitinases, but are smaller due to four deletions. Classes V, VI, and VII include only one or two examples at the present time (*13*−*16*). Within the glycosyl hydrolase classification system, chitinases are grouped into two families (*17*). All fungal, animal, and bacterial chitinases, as well as class III and VI plant chitinases, are classified into family 18, whereas classes I, II, IV, and V are classified into family 19. Among plant chitinases, the class III enzymes are of particular interest because of their remarkable thermostability (*18*).

Most plant chitinases are purified from the roots, stems, leaves, fruits, or seeds of plants (*19*), but some have also been

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isolated from suspension cultures of cells isolated from barley (*20*), carrot (*8*), or rice (*21*). In plant cell cultures, chitinase expression has been shown to be regulated by elicitors as well as plant hormones (*22, 23*), and we recently found that a thermostable chitinase with a molecular mass of 28 kDa was induced in suspensions of bamboo cells cultured in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D). Here we report on the cloning and expression of a class III chitinase cDNA (*BoChi3-1*) using a cDNA library from suspension-cultured bamboo cells. In addition, we describe the purification and characterization of two recombinant class III chitinase isoforms with molecular masses of 28.3 and 35.7 kDa, respectively.

MATERIALS AND METHODS

cDNA Library Construction. Suspension-cultured bamboo (*Bambusa oldhamii*) cells were maintained in Murashige−Skoog (MS) liquid medium (*24*) supplemented with 3% sucrose and 3 ppm of 2,4-D. The cultures were incubated at 25 °C in the dark with rotation at 125 rpm. Total RNA was extracted from 21-day-old suspension-cultured bamboo cells using TRIZOL reagent (Gibco BRL, Gaithersburg, MD) according to the manufacturer's instructions. Poly(A)$^+$ RNA was prepared using a PolyATtract mRNA isolation system (Promega, Madison, WI). A cDNA library was constructed using a SuperScript Choice System (Invitrogen, Carlsbad, CA) and a Lambda ZAP II RI Library Construction Kit (Stratagene, La Jolla, CA).

Cloning of Chitinase. Oligonucleotide primers [5′-GTCAAGGTCATCCTCTCCATCGGCGG-3′ (sense) and 5′-CCACAGCATGATGCCGCCGTA-3′ (antisense)] were designed on the basis of the regions conserved among rice class III chitinases, after which DNA amplification was carried out using these primers with the cDNA library as a template. The PCR product with the expected size (0.5 kb) was then subcloned into plasmid pGEM-T (Promega), and its identity as part of a chitinase cDNA was confirmed by sequencing. After the 0.5 kb chitinase cDNA fragment had been labeled with α-^{32}P-dCTP using the Random Priming Labeling System (Amersham Pharmacia Biotech, Amersham, U.K.), the labeled fragment was used as a probe to screen the cDNA library from suspension-cultured bamboo cells. Positive plaques were isolated, subjected to in vivo excision, and amplified as phagemids for further analysis.

DNA Sequencing and Sequence Analysis. Both DNA strands were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit with an ABI 3730 XL DNA Analyzer (Applied Biosystems, Foster City, CA). Amino acid sequence analysis and alignment was carried out using Vector NTI Suite 9 Sequence Analysis Software (Invitrogen). Signal peptide cleavage sites were predicted using SignalP (*25*), and the N-linked glycosylation site was identified using the NetNGlyc 1.0 server. Protein structure homology−modeling was performed using the SWISS-MODEL online server (*26*). High-quality molecular graphics and predicted secondary structures were displayed using the molecular visualization system PyMOL 1.0 program.

Purification of Recombinant BoCHI3-1 Overexpressed in *Pichia pastoris* X-33. The coding region of *BoChi3-1*, without the putative BoCHI3-1 signal sequence, was amplified by PCR using *BoChi3-1* cDNA as a template with a pair of primers (5′-ATGGAATTCGCCGGCAACATCGCCGTGTAC-3′ and 5′-AGCTTCTAGA GCTCAGACGCTGTCCTTCAC-3′) containing *Eco*RI and *Xho*I sites, respectively. The amplified product was then ligated into pGEM-T plasmid to form pGEM- *BoChi3-1*, after which the *BoChi3-1* cDNA fragment was excised using *Eco*RI and *Xho*I and ligated into pPICZαA plasmid (Invitrogen) previously digested with the same enzymes. The resultant pPICZαA−*BoChi3-1* plasmid was digested with *Pme*I and then transformed into yeast (*P. pastoris* X-33; Invitrogen) by electroporation.

Growth of the transformants and induction of expression using methanol were carried out according to the manufacturer's instructions. After 72 h of induction, the culture was centrifuged for 10 min at 9600*g*, and the resultant pellet was discarded. The proteins in the supernatant fluid were then precipitated using 100% saturated ammonium sulfate, collected by centrifugation, dissolved in PB-7.4 (50 mM sodium phosphate, pH 7.4) containing 0.8 M $(NH_4)_2SO_4$, and applied to a Phenyl-Sepharose 6 Fast Flow column (2.6 × 9 cm) pre-equilibrated with PB-7.4 containing 0.8 M $(NH_4)_2SO_4$. The column was then washed with the same buffer to elute the unbound chitinase (peak I), after which the bound chitinase was eluted stepwise using PB-7.4 containing 0.6 or 0.4 M $(NH_4)_2SO_4$ (peaks II and III) or PB-7.4 without $(NH_4)_2SO_4$ (peak IV). The peak III fractions, which contained chitinase activity, were pooled and concentrated by ultrafiltration (Centricon YM-10). The concentrated enzyme solution was applied to a Con A-Sepharose column (1.6 × 5 cm) pre-equilibrated with PB-7.4 containing 0.5 M NaCl and then washed with the same buffer to elute the unbound chitinase. Chitinase bound to the column was then eluted stepwise with 0.1, 0.3, and 0.5 M methyl α-D-mannopyranoside in PB-7.4 containing 0.5 M NaCl. The flow-through unbound fractions and eluted bound fractions containing chitinase activity were separately pooled and concentrated as described above.

Measurement of Chitinase Activity and Michaelis Constant Determination. The reaction mixture contained 100 μL of 0.2% ethylene glycol chitin (EGC), 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. The reaction was carried out at 45 °C for 30 min. The reducing sugar produced was measured colorimetrically as a decrease in the absorbance of 405 nm using ferri-ferrocyanide reagent and the method of Imoto and Yagishita (*27*). One unit of enzyme activity (EGC hydrolysis) was defined as the amount of enzyme releasing 1 μmol of *N*-acetylglucosamine per minute under assay conditions. The Michaelis constant (K_m) for BoCHI3-1-catalyzed EGC hydrolysis was determined at substrate concentrations ranging from 0.05 to 2.5 mg/mL. The K_m was calculated from double-reciprocal plots according to the method of Lineweaver and Burk (*28*).

Determination of Optimal pH, Optimal Temperature, Thermostability, and Substrate Specificity. The optimal pH for the purified BoCHI3-1 was assayed in universal buffer (Britton and Robinson type) (*29*) at pH values ranging from 2 to 10 using EGC as the substrate (for 30 min at 45 °C). The optimal temperature for EGC hydrolysis was assayed in 0.1 M sodium acetate buffer at temperatures ranging from 20 to 90 °C (for 30 min at pH 4.0). To assess thermostability, enzyme solutions (pH 4.0) were immersed in a thermostatic water bath set to temperatures ranging from 20 to 90 °C. After 30 min, the remaining activities were measured as described above. Substrate specificity was determined by incubating the enzyme with various chitin and chitosan polymers. Hydrolysis of chitinous substrates, including chitin, colloidal chitin, CM-chitin, EGC, chitosan, CM-chitosan, and glycol chitosan, was carried out for 30 min at 45 °C in 0.1 M sodium acetate buffer (pH 4.0) containing 0.1% substrate. The reducing sugar produced was measured as described above.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot. Proteins were mixed with sample buffer containing 125 mM Tris (pH 6.8), 2 mM EDTA·2Na, 2% SDS, 10% glycerol, and 5% (v/v) β-mercaptoethanol, boiled for 5 min, and then subjected to SDS-PAGE using 12.5% acrylamide gel. Following the electrophoresis, the gel was stained with Coomassie blue R-250. For Western blotting, duplicate gels were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P transfer membrane purchased from Millipore), after which the membranes were immersed in urea−PBST (130 mM NaCl, 0.01 M NaH_2PO_4, 0.05% Tween 20, 6 M urea, pH 7.4) for 1 h and then washed with PBST three times for a total of 30 min. The membranes were then incubated with gelatin−NET (0.25% gelatin, 0.15 M NaCl, 0.05% Tween 20, 5 mM EDTA·2Na, 50 mM Tris, pH 8.0) containing a 1:5000 dilution of chicken anti-(jelly fig chitinase) polyclonal antibody (*30*) for 1 h at room temperature, washed with PBST three times for a total of 30 min, and incubated with gelatin−NET containing a 1:10000 dilution of horseradish peroxidase-conjugated goat anti-chicken IgG for 1 h at room temperature. After another three washings with PBST for a total of 30 min, the proteins were visualized using 3,3-diaminobenzidine/$NiCl_2$ substrate and enhanced chemiluminescence.

Gel Activity Staining of Chitinase. Activity staining was carried out using SDS-PAGE with 12.5% polyacrylamide gel containing 0.25% EGC as the substrate. The sample buffer was prepared as described above, except without the 5% (v/v) β-mercaptoethanol. Following SDS-PAGE, renaturation of the enzyme activity was performed in 50 mM

sodium acetate buffer (pH 4.0) containing 1% (v/v) Triton X-100 at 37 °C with gentle shaking for 2 h. Chitinase activity was then detected as a dark (nonfluorescent) band against a UV fluorescent background of intact EGC stained with Calcofluor White M2R (Sigma) (*31*).

N-Terminal Amino Acid Sequencing and Periodic Acid/Schiff (PAS) Staining. Purified 28.3 and 35.7 kDa recombinant chitinases were subjected to SDS-PAGE with 12.5% acrylamide gel as described above. Thereafter, the two protein bands were electroblotted onto a PVDF membrane and then separately excised for N-terminal amino acid sequencing (ABI 491A protein sequencer, Perkin-Elmer). For glycoprotein staining, the PVDF membrane was gently washed in deionized H_2O for 5 min on a shaking agitator and then treated with 1% (w/v) periodic acid in 3% acetic acid (v/v) for 15 min. Then after two washes with deionized H_2O for 10 min each, the membrane was immersed in Schiff reagent and incubated in the dark for 15 min, after which it was destained in 0.5% (w/v) sodium metabisulfite for 5 min. Glycoproteins appear as a pink band on the membrane.

Thin-Layer Chromatography (TLC) of Hydrolysis Products. Seventy-five microliters of diluted enzyme was incubated with 25 μL of 0.1 M sodium acetate buffer (pH 4.0) and 100 μL of 5 mM *N*-acetylchitooligosaccharides ($GlcNAc_n$, $n = 1-6$) in a thermostatic water bath for 48 h at 50 °C. The enzymatic products were then subjected to TLC on a silica gel plate 60 F_{254} in a solvent system composed of *n*-propanol, water, and ammonia−water (70:30:1 v/v). The TLC plates were developed by first dipping them in acetone saturated with silver nitrate and then sprinkling them with ethanol containing 0.5 M NaOH.

Detection of Antifungal Activity. To determine whether the purified BoCHI3-1 could exert its antifungal activity on *Scolecobasidium longiphorum*, an important aerobic pathogen associated with bamboo rotten culm in Taiwan, we assessed the inhibitory effect of BoCHI3-1 on spore germination. The fungus was first grown for 2 weeks at 25 °C in a corn meal agar (DIFCO, Detroit, MI) plate containing 1% yeast extract. Once the fungus showed substantial sporulation, the spores were suspended in 1% (v/v) Tween 20 at a concentration of 10^6 spores mL^{-1}. One hundred microliters of this fungal spore stock was then seeded onto another corn meal agar plate, a sterile filter paper (8.5 mm diameter) was laid on the center of the agar surface, and 10 μg of recombinant chitinase or deionized H_2O was applied to the disk. The plates were then incubated for 7 days at 25 °C. The inhibition of spore germination was assessed with the naked eye and photographed.

RESULTS

Nucleotide and Amino Acid Analyses of Cloned Chitinase from Suspension-Cultured Bamboo Cells. By screening a cDNA library from suspension-cultured bamboo cells using a chitinase probe containing a region conserved among rice class III chitinases, we obtained a 1204 bp cDNA (designated *BoChi3-1*, accession no. EU047798) containing a 5′-UTR, a 900 bp open reading frame, a 3′-UTR, and a poly(A) tail (**Figure 1**). The amino acid sequence deduced from *BoChi3-1* showed little similarity to the class I and II chitinases from rice, but showed 66.8, 72.2, 70.6, 67.6, and 68.6% similarity to class III chitinases from white lupine (accession no. CAA76203), swamp oak (accession no. ABL74451), rice (accession no. BAA22266), jelly fig (accession no. AAQ07267). and rubber tree (accession no. 1HVQ), respectively. In addition, two motifs conserved in class III chitinases, KVLLSLGGG and LDGIDFDIE (*32*), as well as an N-terminal signal peptide sequence like that found in other plant class III chitinases, were present. On the basis of the sequence similarity and conserved regions, we conclude that the cDNA from suspension-cultured bamboo cells encodes a class III chitinase.

3D Structure of BoCHI3-1 by Homology Modeling. The predicted secondary structures of BoCHI3-1 are indicated on the top of the sequence (**Figure 2A**). The 3D structure of BoCHI3-1 was simulated by homology modeling using the crystal structure of the rubber tree chitinase as a template. The

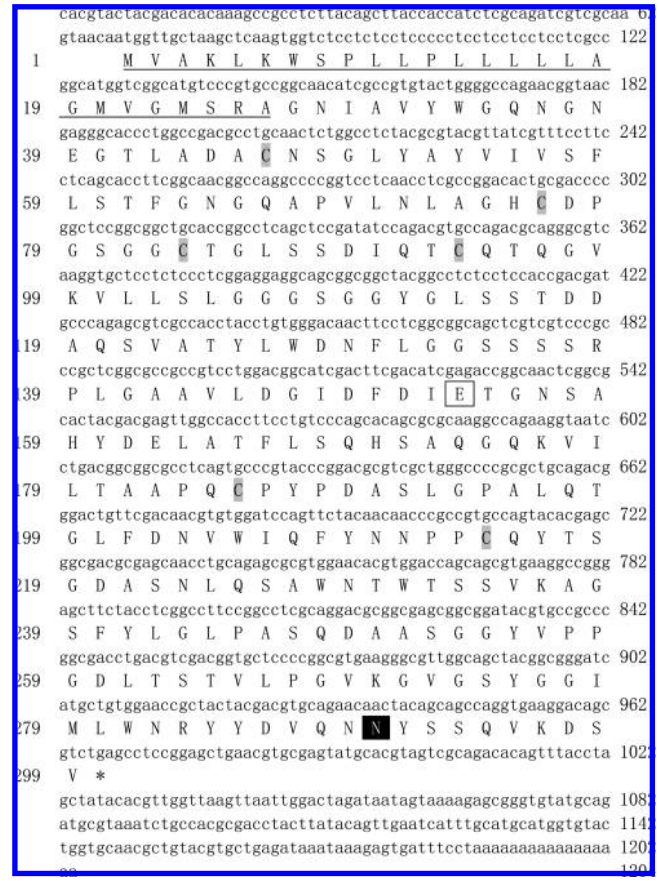

Figure 1. Nucleotide (GenBank accession no. EU047798) and deduced amino acid sequences of a class III chitinase (BoCHI3-1). The nucleotide sequence is numbered on the right. The deduced amino acids are shown in one-letter code below the corresponding codons and are numbered on the left. The potential asparagine-linked N-glycosylation site is shown in white on a black background. The catalytic glutamate residue in the chitinolytic active site is boxed. The six conserved cysteine residues that presumably form three intramolecular disulfide bonds are indicated by gray shading. The stop codon is indicated with an asterisk. The open reading frame (ORF) of class III chitinase from suspension-cultured bamboo (*Bambusa oldhamii*) cells encodes 299 amino acids (900 bp) with a putative N-terminal signal peptide of 26 amino acids (underlined).

eight β-strands, which were mainly composed of nonpolar amino acids, exhibited substantial hydrophobicity and were embedded in the interior of the molecule. Conversely, the eight α-helices, which were mainly composed of polar amino acids, exhibited substantial hydrophilicity and were situated on the exterior (**Figure 2B**).

Enzyme Purification. We initially optimized the incubation of *P. pastoris* X-33 for BoCHI3-1 expression. As shown in **Figure 3**, the extracellular chitinase activity expressed in the recombinant strain peaked on day 3. Note that a low level of chitinase activity was detected in the transformed strain harboring only empty pPICZαA, suggesting that *P. pastoris* X-33 expresses a small of amount of endogenous chitinase. On the basis of these results, culture filtrates from the recombinant strain harvested on day 3 were used for recombinant chitinase purification.

The purification protocol entailed ammonium sulfate precipitation, hydrophobic interaction chromatography, and Con A-Sepharose affinity chromatography. The chitinase precipitated in the saturated ammonium sulfate step was initially fractionated by hydrophobic interaction chromatography on a Phenyl-

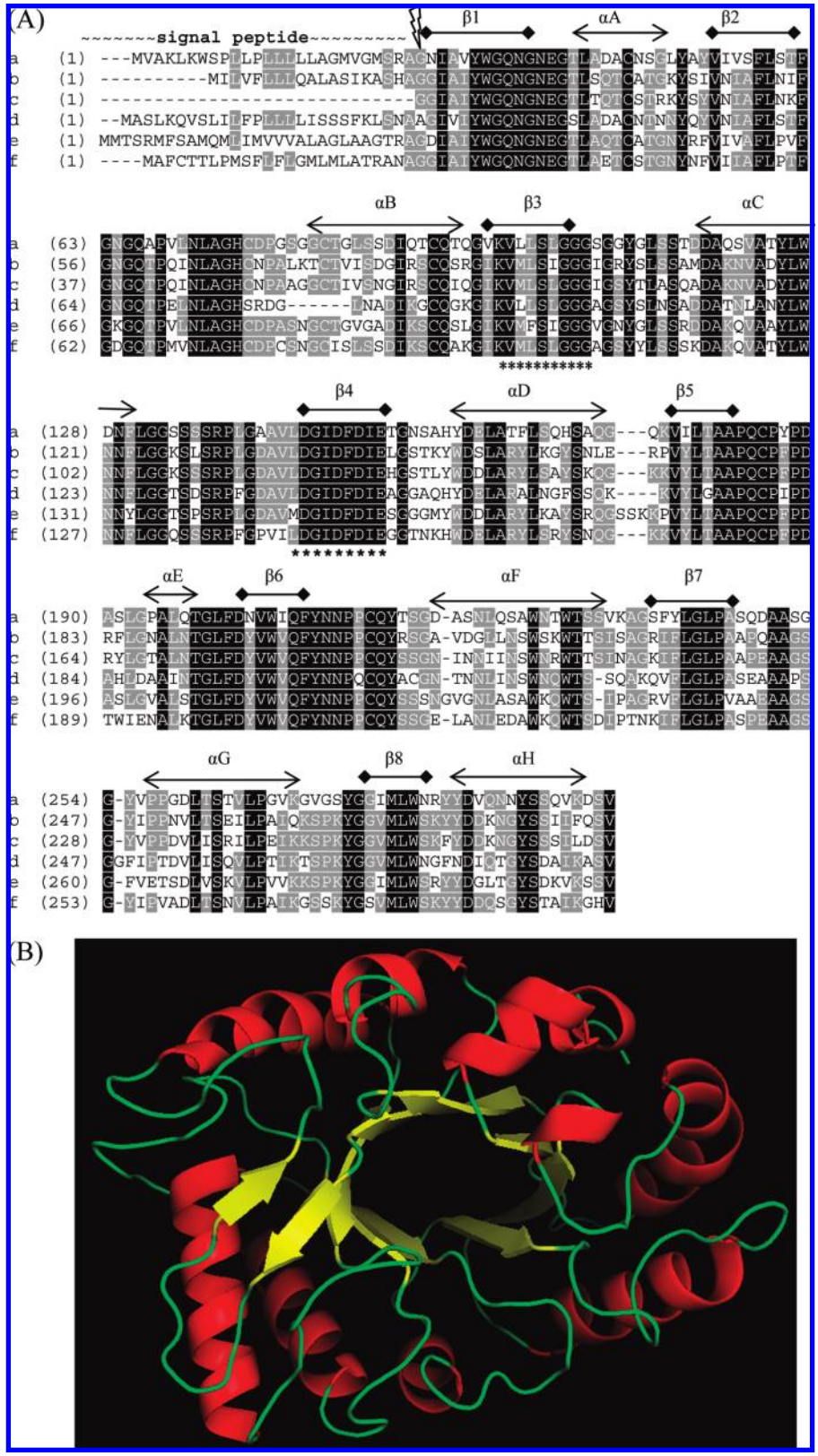

Figure 2. Alignment of the amino acid sequences of class III chitinases and the simulated 3D structure of BoCHI3-1. (**A**) The amino acid sequence of BoCHI3-1 (a) is aligned with those of class III chitinases from jelly fig (*Ficus awkeotsang* Makino) (b; accession no. AAQ07267), rubber tree latex (*Hevea brasiliensis*) (c; accession no. 1HVQ), white lupus (*Lupinus albus*) (d; accession no. CAA76203), rice [*Oryza sativa* (japonica cultivar group)] (e; accession no. BAA22266), and swamp oak (*Casuarina glauca*) (f; accession no. ABL74451). The number of the last amino acid residue in each line is indicated on the left for each species. The predicted secondary structural elements of BoCHI3-1 are shown above the sequence. Double-headed arrows indicate α-helices, and rods indicate β-strands. Residues identical across all species are highlighted by black shading. Weakly similar regions are indicated by gray shading. Gaps employed for optimal alignment of the sequences are indicated by broken lines. The lightning symbol indicates the cleavage site of the N-terminal signal peptide. Conserved regions found only in class III chitinases are marked with asterisks. (**B**) 3D structure of BoCHI3-1 was simulated by homology modeling using the crystal structure of the rubber tree chitinase as a template.

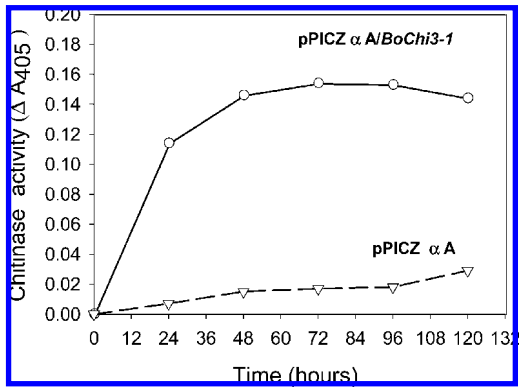

Figure 3. Chitinase activity isolated from recombinant strains harboring pPICZαA−*BoChi3-1* or pPICZαA. Expression was induced with 1% methanol at 30 °C for the indicated times. Ethylene glycol chitin was used as the substrate for chitinase activity assays: (○) pPICZαA−*BoChi3-1*; (▽) pPICZαA.

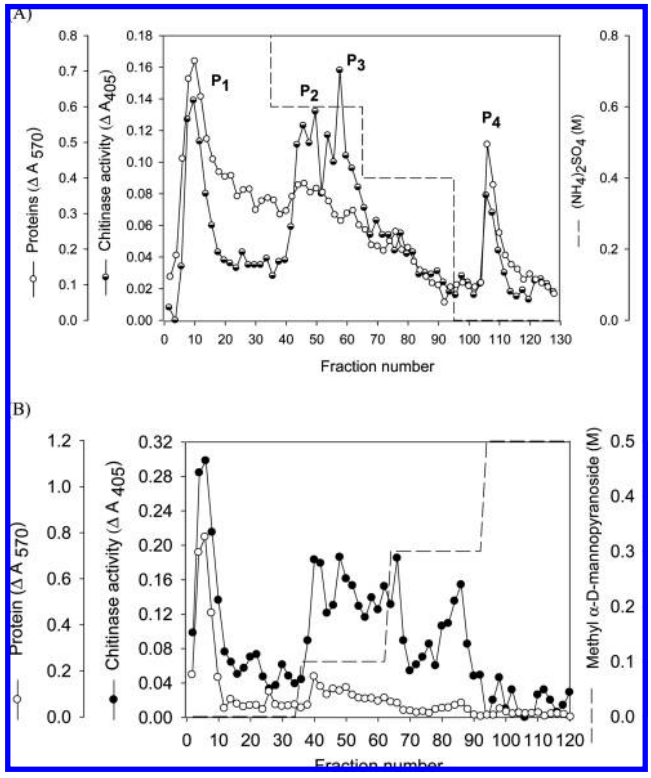

Figure 4. Hydrophobic and affinity chromatography of chitinase. (**A**) Hydrophobic chromatography of chitinase on a Phenyl-Sepharose 6 Fast Flow column. The column was equilibrated with 50 mM PB-7.4 (pH 7.4) containing 0.8 M $NH_4(SO_4)_2$. Proteins bound to the column were eluted with a stepwise gradient of ammonium sulfate (0.8−0 M) at a flow rate of 120 mL/h; 8 mL fractions were collected. (**B**) Con A-Sepharose affinity chromatography. The column was pre-equilibrated with 50 mM PB-7.4 containing 0.5 M NaCl. Proteins bound to the column were eluted stepwise with 0.1, 0.3, and 0.5 M methyl α-D-mannopyranoside in 50 mM PB-7.4 containing 0.5 M NaCl at a flow rate of 25 mL/h; 1.5 mL fractions were collected.

Sepharose 6B column, which separated the chitinase into four peaks: P1, P2, P3, and P4 (**Figure 4A**). Subsequent SDS-PAGE and gel activity staining revealed that the P2 fraction had only a single protein band (**Figure 5A**, lane 2), which contained chitinase activity (**Figure 5B**, lane 2). The molecular mass of the P2 chitinase was 37.5 kDa, as estimated by gel filtration on Sephacryl S-100. This purified enzyme was deemed to be a recombinant class III chitinase because it was recognized by a

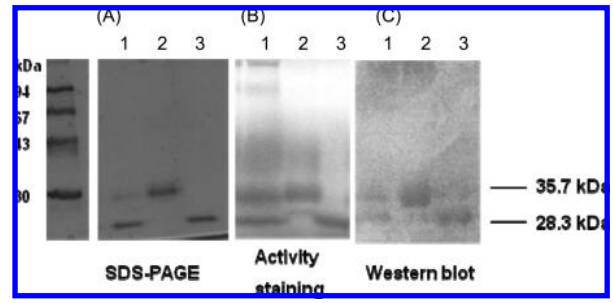

Figure 5. SDS-PAGE of recombinant chitinase after different purification steps: (**A**) Coomassie brilliant blue R-250 staining; (**B**) gel chitinase activity staining; (**C**) immunoblotting detected by an anti-(jelly fig chitinase) antibody. Lanes: 1, BoCHI3-1 purified from the P3 hydrophobic chromatography fraction; 2, BoCHI3-1 purified from the P2 hydrophobic chromatography fraction; 3, BoCHI3-1 purified from the Con A nonbinding fraction after Con A affinity chromatography. Labels on the right indicate the molecular masses of the two purified recombinant chitinases. Numbers on the left indicate the protein size markers (in kDa).

polyclonal antibody raised against the 30 kDa chitinase from jelly fig achene (*30*) (**Figure 5C**, lane 2) and hereafter will be referred to as 37.5 kDa BoCHI3-1.

The P3 fraction yielded one major and one minor band (**Figure 5A**, lane 1). Both bands showed chitinase activity (**Figure 5B**, lane 1) and were recognized by anti-(jelly fig chitinase) antibody (**Figure 5C**, lane 1). In addition, trace amounts of chitinase activity were detected in bands that had a molecular mass larger than 43 kDa and were not recognized by the anti-(jelly fig chitinase) antibody. This likely reflects the presence of endogenous chitinase expressed by the *P. pastoris* X-33 host. Further purification of the P3 fraction was carried out using Con A-Sepharose affinity chromatography, which also yielded a fraction with chitinase activity (**Figure 4B**, the flow-through unbound chitinase fraction). This fraction gave a single protein band (**Figure 5A**, lane 3), which showed chitinase activity (**Figure 5B**, lane 3) and was recognized by the anti-(jelly fig chitinase) antibody (**Figure 5C**, lane 3). Apparently, the other recombinant and endogenous chitinase isoforms were removed with this step. The molecular mass of the purified enzyme was 27.6 kDa, as estimated by gel filtration on Sephacryl S-100, which was close to that (28.3 kDa) calculated from the deduced amino acid sequences of *BoChi3-1*. Hereafter, this recombinant chitinase will be referred to as 28.3 kDa BoCHI3-1.

The purification results for 35.7 and 28.3 kDa BoCHI3-1 are summarized in **Table 1**. Through these steps, 35.7 kDa BoCHI3-1 was purified 6-fold with a yield of 10.3%, whereas 28.3 kDa BoCHI3-1 was purified 2.4-fold with a yield of 1.5%. For both isoforms, the amount of protein recovered by the final step was rather small due to the small amount of protein recovered after the ammonium sulfate precipitation step. Most likely, the recombinant chitinase was not completely precipitated at 100% saturated $(NH_4)_2SO_4$, or the enzyme was completely precipitated but, due to its hydrophobicity, was not completely dissolved in the small amount of solvent buffer used.

Glycosylation of the Recombinant BoCHI3-1. To determine whether or not 35.7 and 28.3 kDa BoCHI3-1 were glycosylated, both isoforms were resolved by SDS-PAGE, transferred to a PVDF membrane, and assayed for carbohydrate using PAS staining. Using the same amount of protein in each case (**Figure 6A**, lanes 4 and 5), 35.7 kDa BoCHI3-1 was positively stained (**Figure 6B**, lane 5), whereas 28.3 kDa BoCHI3-1 was not, although there was some contamination with trace amounts of 35.7 kDa BoCHI3-1 (**Figure 6B**, lane 4). Thus, 35.7 kDa

Table 1. Purification of Recombinant Chitinase BoCHI3-1 Overexpressed in *Pichia pastoris* X-33

procedure	total activity[b] (mU)[c]	total protein (mg)	specific activity (mU/mg)	purification (fold)	yield (%)
crude enzyme solution[a]	14540	306.3	47.5	1	100
$(NH_4)_2SO_4$ fractionation (0−100% saturation)	4400	69.5	63.3	1.3	30.3
Phenyl-Sepharose 6 Fast Flow					
P2 fraction (35.7 kDa)	1500	5.2	289	6.1	10.3
P3 fraction (28.3 kDa and other isoforms)	650	2.5	260	5.5	4.5
Con A-Sepharose					
Con A nonbinding fraction (28.3 kDa)	210	1.8	117	2.5	1.4

[a] Data were obtained from 1000 mL of culture medium on day 3. [b] One chitinase unit is defined as the amount of enzyme releasing 1 μmol of *N*-acetylglucosamine per minute under assay conditions. [c] One mU is 10^{-3} unit.

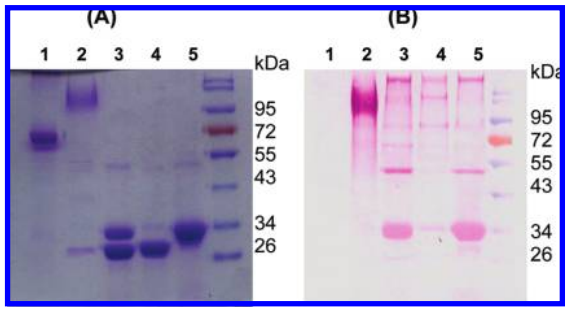

Figure 6. SDS-PAGE and PAS carbohydrate staining of recombinant chitinase overexpressed in *Pichia pastoris* X-33. Lanes: 1, bovine serum albumin (BSA); 2, invertase (a known glycoprotein); 3, BoCHI3-1purified from the P3 hydrophobic chromatography fraction; 4, BoCHI3-1 purified from the Con A nonbinding fraction after Con A affinity chromatography; Lane 5, BoCHI3-1 purified from the P2 hydrophobic chromatography fraction. Proteins were resolved in duplicate SDS-PAGE gels, which were subjected to Coomassie blue staining (**A**) or PAS carbohydrate staining (**B**). Numbers on the right indicate protein size markers (in kDa).

BoCHI3-1 appears to be a glycosylated protein, whereas 28.3 kDa BoCH3-1 appears not to be glycosylated. Notably, several carbohydrate-positive staining bands with molecular masses higher than 43 kDa were also observed on the PVDF membranes with the two recombinant enzymes. This positive staining likely reflects contamination of the concentrated purified enzyme samples by trace amounts of carbohydrate or glycoproteins, which was also observed when the concentrated P3 fraction from the hydrophobic chromatography step was analyzed (**Figure 6B**, lane 3).

Hydrolytic Products from Chitin Oligosaccharides. In addition to cleaving chitin polymer, both recombinant enzymes also cleaved chitin oligomers. As shown in **Figure 7**, 35.7 or 28.3 kDa BoCHI3-1 cleaved chitin trimer ($GlcNAc_3$) into the monomer (GlcNAc) and dimer ($GlcNAc_2$) and cleaved chitin tetramer to hexamer ($GlcNAc_{4-6}$) into a mixture of monomers, dimers, and trimers (GlcNAc, $GlcNAc_2$, and $GlcNAc_3$), suggesting that the two BoCHI3-1 isoforms act as endohydrolases.

Antifungal Activity of the Recombinant BoCHI3-1. *Scolecobasidium longiphorum* is an aerobic pathogen found in Taiwan that is associated with bamboo rotten culm. Germination of spores from this fungus was significantly inhibited by both recombinant chitinases (data not shown).

Enzyme Properties of Recombinant BoCHI3-1. As shown in **Table 2**, the optimal pH values for EGC hydrolysis by 35.7 and 28.3 kDa BoCHI3-1 were 3 and 4, respectively. Both enzymes were stable at pH 3, and the optimal temperature for maximum activity was 80 °C for 35.7 kDa BoCHI3-1 and 70 °C for 28.3 kDa BoCHI3-1. Examination of their activities at temperatures ranging from 20 to 90 °C revealed both BoCHI3-1 isoforms to be highly thermostable, as they retained nearly all of their activity (85−88%) after incubation at 70 °C for 30 min. In addition, the two purified enzymes could be stored in sodium phosphate buffer (50 mM, pH 7.4) at 4 °C for a year without a significant loss of activity. The isoelectric point for 28.3 kDa BoCHI3-1 predicted from its sequence was 4.12, suggesting that this enzyme is an acidic chitinase. For EGC hydrolysis, the K_m values were 1.35 and 0.65 mg/mL for 35.7 and 28.3 kDa BoCHI3-1, respectively, and their activation energies were 13.4 and 15.5 kJ/mol, respectively.

Substrate Specificity. As shown in **Table 3**, both recombinant chitinases hydrolyzed chitin and chitosan polymers. These two chitinases appear to prefer chitin polymer substrates, although they also showed some activity toward chitosan polymers. However, they showed much less activity toward insoluble chitin and colloidal chitin than toward soluble EGC.

DISCUSSION

Sequence analysis using the BLASTx facility revealed that the BoCHI3-1 sequence shows 68.6% similarity to the corresponding residues of rubber tree chitinase, suggesting that their 3D structures are similar. X-ray crystallographic analysis revealed the 3D structure of rubber tree chitinase to be a stable $(\beta\alpha)_8$ barrel fold with three intramolecular disulfide bonds (*33*). Consequently, a model of BoCHI3-1's 3D structure assembled through homology modeling (*26*) using the crystal structure of rubber tree chitinase as a template contained eight alternating α-helices and parallel β-strands displayed as a stable $(\beta\alpha)_8$ barrel fold, together with three intramolecular disulfide bonds formed by six conserved cysteine residues. The eight α-helices on the outer surface packed the eight parallel β-strands into the inner core of the simulated 3D structure of BoCHI3-1 (**Figure 2B**). The loop region linking the carboxyl end of the fourth β-strand to the amino end of the fifth α-helix contained the catalytic glutamic acid residue (data not shown), as in the simulated 3D structure of jelly fig chitinase (*30*). These results indicated that the 3D structure of BoCHI3-1 comprised a stable $(\beta\alpha)_8$ barrel structure accounting for its thermal stability. Analysis of the deduced amino acid sequence of BoCHI3-1 revealed that BoCHI3-1 contained one glycosylation site with a consensus sequence of Asn-Xaa-Ser/Thr, where Xaa can be any amino acid except proline, which was situated at residues 290−292 (**Figure 1**). Glycosylation almost resulted in a shift in molecular weight of BoCHI3-1 from 28.3 to 35.7 kDa (**Figure 6**). On the basis of the molecular masses of native and glycosylated

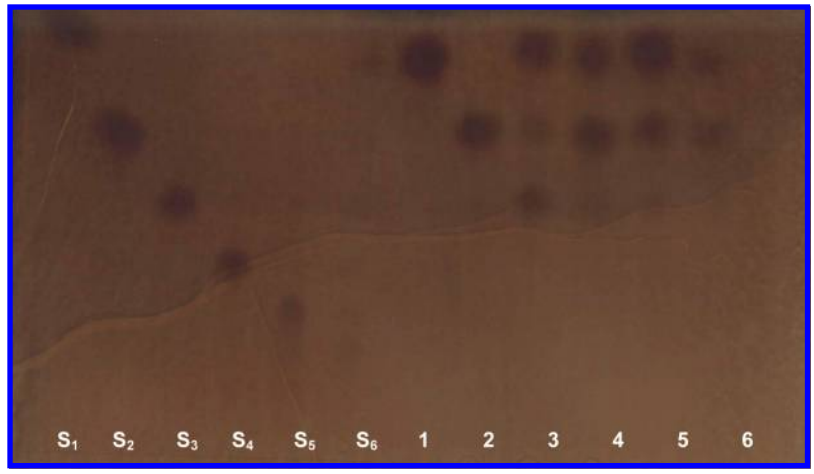

Figure 7. Thin-layer chromatogram showing hydrolysis of *N*-acetylglucosamine (GlcNAc) oligomers by 28.3 or 35.7 kDa BoCHI3-1. Lanes S_1-S_6 contain standard *N*-acetylglucosamine (GlcNAc) oligomers GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, and $(GlcNAc)_6$, respectively. Lanes 1−6 contain hydrolysis products of *N*-acetylglucosamine oligomers from monomer to hexamer, respectively.

Table 2. Biochemical Properties of 35.7 and 28.7 kDa Recombinant Chitinase BoCHI3-1

BoCHI3-1	K_m (mg/ mL)	optimal pH	optimal temperature (°C)	thermal stability (°C)	pH stability	activation energy (kJ/mol)	pI	action type	shortest chitin oligomer substrate
35.7 kDa	1.35	3	80	<70	3	13.4		endo	$GlcNAc_3$
28.3 kDa	0.65	4	70	<70	3	15.5	4.12	endo	$GlcNAc_3$

Table 3. Substrate Specificity of 35.7 and 28.7 kDa Recombinant Chitinase BoCHI3-1

	relative activity (%)	
substrate	35.7 kDa BoCHI3-1	28.3 kDa BoCHI3-1
soluble substrates		
ethylene glycol chitin (EGC)	100	100
chitosan (soluble at pH 3−4)	13.4	25.5
carboxymethyl chitin (CM-chitin)	8.93	9.96
carboxymethyl chitosan (CM-chitosan)	1.79	5.17
glycol chitosan	2.68	2.95
insoluble substrates		
colloidal chitin	12.1	9.96
chitin	5.8	8.12

recombinant chitinases, the degree of polymerization of the carbohydrate moiety in the glycosylated BoCHI3-1 was estimated to be about 40.

N- and O-linked oligosaccharides have been shown to facilitate the folding, subunit assembly, and secretion of some glycoproteins (*34*−*37*). In addition, a glycoprotein's oligosaccharides also reportedly have a significant effect on the protein's solubility, proteolytic degradation, thermal stability, and specific activity (*37*−*40*). Although different isoforms of chitinases have been reported, there is no report about the significance of differential glycosylation of plant chitinases. In our study, the presence of polysaccharide substantially enhanced the specific activity of glycosylated BoCHI3-1 (**Table 1**). Kinetic analysis revealed that glycosylation of BoCHI3-1 increased the K_m and decreased the enzyme activation energy (**Table 2**). This suggests that the polysaccharide chain on glycosylated BoCHI3-1 facilitates the conversion of the bound substrate to product, which would increase the K_m value of the enzyme by increasing the rate constant for the decomposition of enzyme−substrate complex to product (k_{cat}).

Some chitinases were known to possess extremely stable protein folding that could be rescued after denaturation in the presence of SDS and reducing agent, whereas some chitinases could be renatured only if denatured in the presence of SDS alone (*31*). We thus compared the effect of denaturation with SDS alone and SDS plus β-mercaptoethanol on renaturation of BoCHI3-1. Neither isoform of BoCHI3-1 could be efficiently renatured after SDS and β-mercaptoethanol denaturation (data not shown). However, both isoforms could be renatured after denaturation with SDS alone. These results indicated that the presence of disulfide bonds was essential for rescuing the enzyme's activity and were in agreement with the proposed formation of intramolecular disulfide linkage of BoCHI3-1's 3D structure.

Recently we isolated three class III chitinase cDNAs, *BoChi3-1*, *BoChi3-2*, and *BoChi3-3*, from suspension-cultured bamboo cells and found that two chitinases with molecular masses of ∼30 and ∼90 kDa were secreted into the medium during cultivation. The ∼90 kDa chitinase was purified and characterized as a chitin oligomer preferred endotype hydrolase. This native chitinase was different from both recombinant chitinases (35 and 28.3 kDa BoCHI3-1) expressed in *P. pastoris* X-33, as compared with their molecular mass and substrate specificity. Further cloning, characterization, and expression of the other two class III chitinase cDNAs (*BoChi3-2* and *BoChi3-3*) are in progress.

In sum, our findings indicate that both 35.7 and 28.3 kDa BoCHI3-1 are thermostable, endotype chitinases with chitosanase activity and antifungal activity. These two recombinant chitinases appear to represent a valuable source of enzyme for bioconversion of chitin polymer to oligomers and preparation of biocontrol agents, although further characterization of native and glycosylated recombinant BoCHI3-1 remains to be done.

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