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Gene Cloning and Characterization of a Novel Recombinant Antifungal Chitinase from Papaya (*Carica papaya*)

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A chitinase cDNA clone (CpCHI, 1002 bp) was isolated from papaya fruit, which encoded a 275 amino acid protein containing a 28 amino acid signal peptide in the N-terminal end. The predicted molecular mass of the mature protein was 26.2 kDa, and its p*I* value was 6.32. On the basis of its amino acid sequence homology with other plant chitinases, it was classified as a class IV chitinase. An active recombinant CpCHI enzyme was overexpressed in *Escherichia coli*. The purified recombinant papaya chitinase showed an optimal reaction temperature at 30 °C and a broad optimal pH ranging from 5.0 to 9.0. The recombinant enzyme was quite stable, retaining >64% activity for 3 weeks at 30 °C. The spore germination of *Alternaria brassicicola* could be completely inhibited by a 76 nM level of recombinant CpCHI. Recombinant CpCHI also showed antibacterial activity in which 50% of *E. coli* was inhibited by a 2.5 μM concentration of the enzyme.

KEYWORDS: Chitinase; papaya; antifungal activity; recombinant expression

INTRODUCTION

Chitin, a linear β-1,4-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), functions as a structural polysaccharide and is widely distributed in fungal cell walls, the exoskeleton of arthropods, and the outer shell of crustaceans. Chitinases (EC 3.2.1.14), catalyzing the hydrolysis of chitin, occur in viruses, bacteria, fungi, insects, plants, and animals (*1*). Chitinolytic activity can be involved in the nutrition process, morphogenesis, and old cuticle degradation (*2*). In higher plants without endogenous substrates, chitinases are thought to be involved in the defense system against pathogen attack as a pathogenesis-related (PR) protein (*3*). Among PR proteins, chitinases and β-glucanases hydrolyze chitin and β-1,3-glucan, which are the major components in the cell wall of many phytopathogeneic fungi (*4*). Chitinases, like β-glucanases, contribute to plant defenses due to their ability to degrade the cell wall and inhibit the growth of fungal pathogens. In the early stage of pathogenesis, apoplastic chitinases release the elicitor molecule that activates the defense mechanisms of plants (*3*). Then the synthesis and secretion of apoplastic chitinases occur to enhance the signaling of infection, whereas the vacuolar chitinase degrades the fungal cell wall to inhibit pathogen growth (*3*). Chitinases have been demonstrated to be useful in agriculture for controlling plant pathogen (*5*).

On the basis of the amino acid sequence similarity of the catalytic domains, chitinolytic enzymes have been grouped into families 18 and 19 of glycosyl hydrolases (*6*). Family 18 chitinases, using the substrate-assisted catalysis mechanism, lead the retention of conformation at the anomeric carbon of the product (*7*); meanwhile, family 19 members, using the acid catalysis mechanism, invert the product into anomeric configuration (*8*). At present, the chitinases of both families are present in plants (*9*). Chitinases can be divided into three groups on the basis of their activities: exochitinases, endochitinases, and *N*-acetylglucosaminidases (*10*−*12*). Endochitinases cleave β-1,4-glycoside linkages randomly within the chain, generating a low molecular mass of GlcNac, such as chitiotetraose, chitotriose, and diacetylchitobiose (*10*). In comparison, exochitinases specifically cleave off diacetylchitobiose from the nonreducing end of the chitin chain (*11*), whereas *N*-acetylglucosaminidases cleave the oligomeric products of endochitinase and chitibiosidase, generating monomers of GlcNac (*12*, *13*). Some plant endochitinases also show lysozyme activity, which hydrolyzes the β-1,4-linkages between *N*-acetylmuramic acid and GlcNAc residues in peptidoglucan (*3*, *14*−*17*).

Pathogens are the most important factors that affect the yield and quality of papaya, an important tropical and subtropical economic crop due to its edible fruit and the rich papain in its latex. Class I and II chitinases have been found in the papaya's

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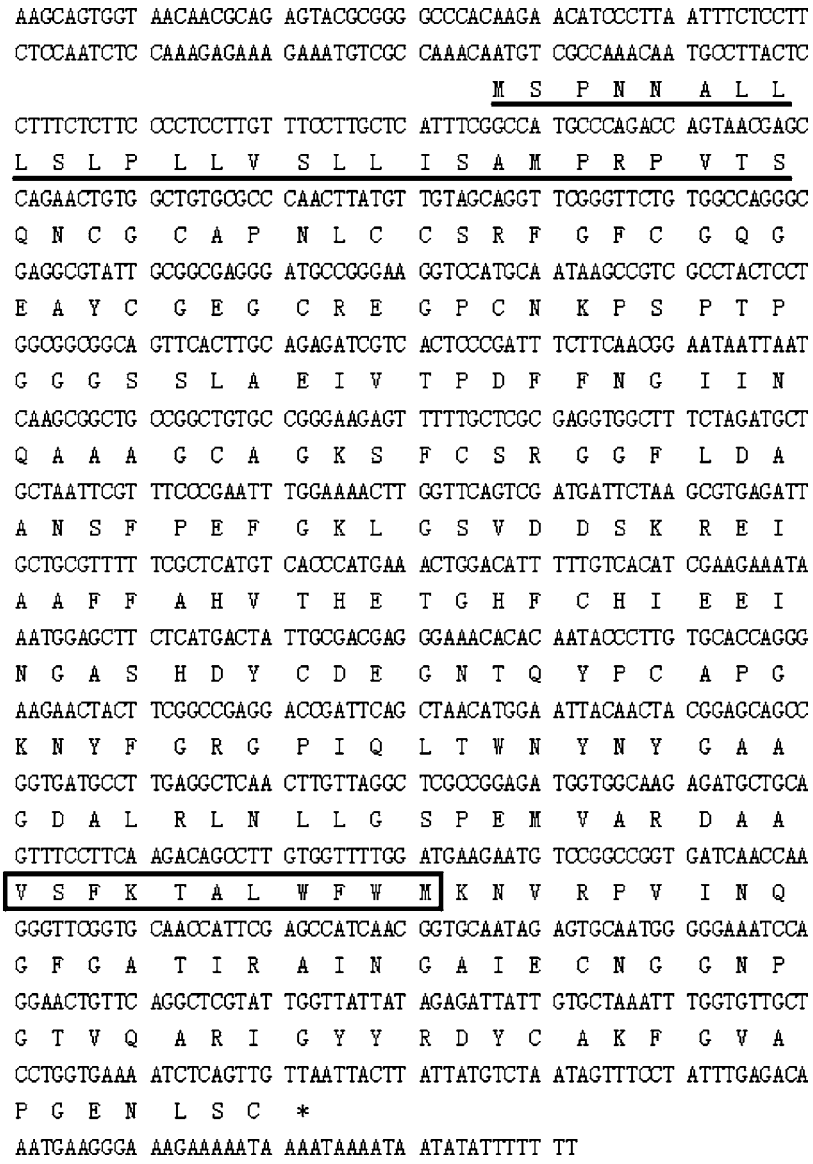

Figure 1. Nucleotide and deduced amino acid sequence of a papaya chitinase (CpCHI) gene. The numbers on the left refer to the nucleotide and amino acid residues. The underlined regions indicate the putative signal sequence predicted by CBS Prediction Servers (http://www.cbs.dtu.dk/services/) SignalP-NN prediction. The boxed region shows the highly conserved motif VSFKTALWFWM, which is located at 209−219 aa.

latex (*17*, *18*). Moreover, there were no reports on the gene cloning, sequencing, and biochemical characterization of the papaya chitinase. In this study, we cloned a cDNA encoding class IV chitinase from a papaya fruit by PCR-based subtractive hybridization. The biochemical analyses showed that recombinant CpCHI has a wide optimal pH value, long-term stability, and strong antifungal activity. These results suggest that recombinant CpCHI is potentially useful as a biocontrol agent that can protect plants against agriculture pathogens.

MATERIALS AND METHODS

Plants and Materials. Papaya (*Carica papaya* L. cv. Tainong 2) fruits were harvested from a local orchard at different development stages and then were immediately frozen and stored at −80 °C until their use. *Alternaria brassicicola* was provided by Dr. Jenn-Wen Hung of the Department of Plant Pathology of the National Chung-Hsing University. Glycol chitin was prepared through the method of Yamada and Imoto with reacetylation using acetic anhydride (*19*). All enzymes were purchased from Promega (Madison, WI). Chemicals were purchased from Merck (Darmstadt, Germany), whereas oligonucleotide primers were purchased from DNAFax (Taipei, Taiwan).

mRNA Preparation. The total RNA was extracted from papaya samples according to the Pine Tree method (*20*). The poly(A)+ RNA was isolated from the total RNA with an Oligotex mRNA Kit (Qiagen Inc., Valencia, CA). The cDNA was synthesized and used for cloning the differentially expressed cDNA, in which a PCR-based subtractive hybridization kit was used (Clontech Lab., Mountain View, CA).

PCR-Based Subtractive Hybridization and CapFinder PCR. Papaya cDNAs derived from both ripe and immature fruits were used in subtractive hybridization (Clontech, PCR-Select cDNA subtraction kit) according to the procedure developed by Wang and Brown (*21*). The cDNAs from the immature fruit served as the driver, and those from the ripe fruits served as the tester. Subtractive hybridization was intended to enrich cDNAs that are up-regulated in ripe papaya fruit. The CapFinder PCR method (Clontech) was used to isolate the 5′ and 3′ ends of chitinase cDNA, according to the directions provided by the manufacturer. The Cp-chi-R1 primer (5′ CGAATTAGCAGCATCTAGAAAGCCACCTCG 3′, located at 400−429) was synthesized according to the sequence of the partial length of papaya chitinase derived from subtractive hybridization. The 5′-end of the chitinase gene was cloned following the CapFinder PCR technique, using papaya cDNA from ripe fruit as template and the two primers Cp-chi-R1 and Cap-G (5′ AAGCAGTGGTAACAACG CAGAGTACGCGGG). Cp-chi-1 primer (5′ ATGCAGAACTGTGGCTGTGCG) and X primer

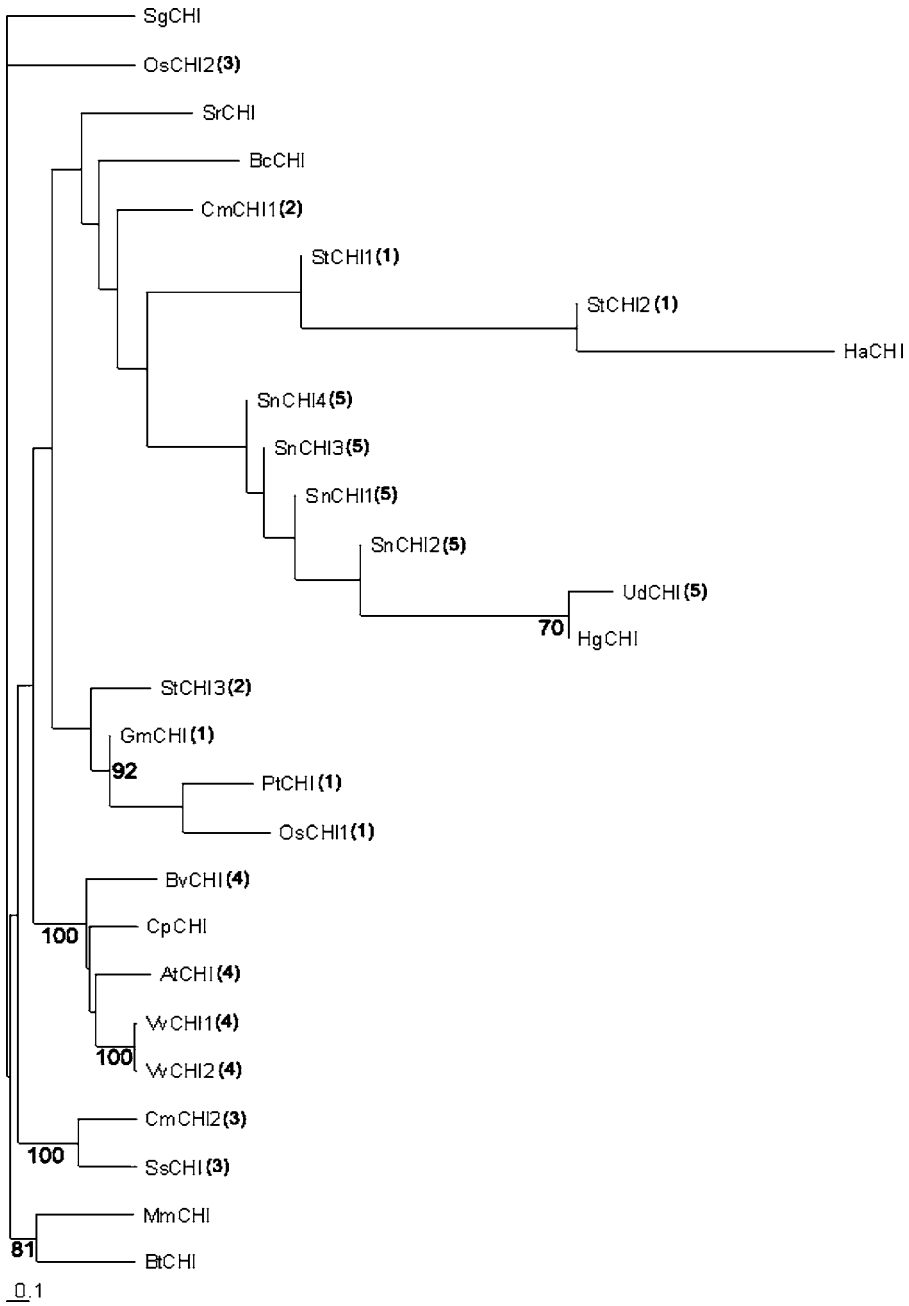

Figure 2. Cluster analysis was done using the PROTDIST and NEIGHBOR programs, and statistical evolution used Bootstrap analysis with 100 data sets. The number shown on the root indicates the bootstrap value, the percentage of replicates supporting that group. The number shown in parentheses indicates the classification of plant chitinase. The accession numbers of chitinase genes used in polygenetic relationship analysis are as follows: StCHI2, *Solanum tuberosum*, AF043248; StCHI1, *S. tuberosum*, AF043247; PtCHI, *Psophocarpus tetragonolobus*, AB048531; GmCHI, *Glycine max*, AF202731; StCHI, *S. tuberosum*, U49969; CmCHI, *Cucumis melo*, AF241538; OsCHI, *Oryza sativa*, AB012855; SnCHI1, *Sambucus nigra*, AF074387; SnCHI3, *S. nigra*, AF074385; SnCHI4, *S. nigra*, AF074386; SnCHI2, *S. nigra*, AF074388; UdCHI, *Urtica dioica*, AF059535; VvCHI1, *Vitis vinifera*, U97521; VvCHI2, *V. vinifera*, U97522; AtCHI, *Arabidopsis thaliana*, Y14590; BvCHI, *Beta vulgaris*, L25826; SgCHI, *Streptomyces griseobrunneus*, AY348315; SrCHI, *Streptomyces roseoflavus*, AY392156; BcCHI, *Bacillus cereus*, AF510723; BtCHI, *Bos taurus*, NP777124; HaCHI, *Helicoverpa armigera*, AAQ94193; MmCHI, *Mus musculus*, XP129391; HgCHI, *Heterodera glycines*, AF468679; OsCHI2, *O. sativa*, AF296279; SsCHI, *Sphenostylis stenocarpa*, AF137070; CmCHI2, *Cucurbita moschata*, AF082284.

(5′ AAGCAGTGGTAACAACGCAGAGTACT(30)N-1N) were used to amplify the 3′-end of papaya chitinase cDNA. One 0.4 kb fragment and one 0.9 kb fragment, obtaining the 5′- and 3′-ends of chitinase, respectively, were amplified by using the PCR technique. The PCR amplified fragment was subcloned into the pGEM-T easy vector (Promega) using *Escherichia coli* DH5α as host. The nucleotide sequence was determined in autosequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kits, Perkin-Elmer Co., Fremont, CA) with an ABI PRIZM 377 DNA sequencer.

Subcloning and Sequence Analysis of *Cp-chi* cDNA. The Cp-chi-1 and Cp-chi-2 primers (5′ ACAACTGAGATTTTCACCAGGAG) were synthesized to amplify the coding region of papaya chitinase. One 0.81 kb fragment was amplified using papaya cDNA from ripe fruit as template by the PCR technique and then was subcloned into the pGEM-T easy vector (Promega) using *E. coli* DH5α as host for further sequence analysis by autosequencing.

Recombinant DNA Preparation and Transformation. For subcloning into the expression vector pET-20b(+), full-length chitinase

cDNA was used as template. Two primers, Cp-chi-N (5′ GGAATTC-CATATGCAGAACTGTGGCTGTGCG) and Cp-chi-C (5' CCGCTC-GAGACAACTGAGATTTTCACCAGGAG) containing *Nde*I and *Xho*I restriction enzyme sites (underlined), respectively, were used to amplify the papaya chitinase cDNA and encode the mature protein. A 0.75 kb DNA fragment amplified by the PCR technique was digested with *Nde*I and *Xho* I and ligated to pET-20b(+) (pretreated with *Nde*I and *Xho* I) from Novagen (Darmstadt, Germany). The recombinant plasmid was transformed into *E. coli* Tunner (DE3) for protein expression.

Culture and Enzyme Extraction. The transformed *E. coli* cells were grown at 37 °C in a Luria−Bertani (LB) medium containing 100 μg mL^{-1} ampicillin until A_{600} reached 0.6. The culture was incubated at 37 °C for another 5 h at 150 rpm, after which 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) was added. The bacterial cell and the insoluble cell debris for inclusion body purification were washed with the BugBuster reagent (Novagen) and were harvested by centrifugation as described in the manual. The final pellet of inclusion bodies was resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and 6 M urea). The CpCHI protein, which contains 6× His tag at the N terminus, was purified with Ni-NTA agarose (Qiagen QIAexpress). Briefly, the supernatant was applied to the Ni-NTA resin column. After washing with a buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and 6 M urea), CpCHI was recovered by an elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and 6 M urea). Finally, the purified CpCHI was dialyzed in a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) for further activity assay.

Gel Activity Stain of Chitinase. For the gel activity staining, 20 μL of recombinant CpCHI was mixed with gel loading buffer without β-mercaptoethanol and was electrophoresed in 12% native polyacrylamide gel containing 0.1% glycol−chitin, according to the method of Trudel and Asselin (*22*). After electrophoresis, the gel was washed with 100 mM sodium acetate, pH 5.0, containing 1% Triton X-100 and 1% skimmed milk at 37 °C for 1−2 h and then was stained with 0.01% Calcoflour white M2R (Sigma) in 0.5 M Tris-HCl, pH 8.9, for 5−10 min. It was then exposed under UV light.

Spectrophotometer Assay of Chitinase Activity. Chitinase activity was determined by the reducing end group *N*-acetaminoglucose produced from glycol chitin. The reducing end group produced was measured colorimetrically at 420 nm with 200 μL of potassium ferricyanide [$K_3Fe(CN)_6$] reagent according to Boller's method (*23*). In a typical reaction, 0.3 μg of purified enzyme and 2% (w/v) glycolchitin were incubated in 100 μL of 100 mM phosphate buffer (pH 6.0) at 30 °C for 1 h. Reduced sugar levels were determined relative to *N*-acetyl-β-D-glucosamine standards of 0−150 μg mL^{-1}. One unit of chitinase activity is defined as the amount of enzyme producing 1 μmol of reducing sugar per minute at 30 °C. Substrate and enzyme blanks were also prepared in which the enzyme or substrate was incubated with a buffer. All measured values of absorbency were corrected for the appropriate blank absorbencies.

To determine the optimal pH of the chitiolytic activity, the purified CpCHI was investigated within different pH levels (3−10) at 30 °C for 1 h using glycol chitin (2%) as the substrate. Acetate buffer (100 mM) was used for pH 3−5, phosphate buffer (100 mM) for pH 6−7, Tris-HCl buffer (100 mM) for pH 8, and 100 mM glycine−NaOH buffer for pH 9 and 10. The optimal temperature was assayed by incubating the purified chitinase in 100 mM phosphate buffer (pH 6.0) at different temperatures (10−90 °C) for 1 h using 2% glycol chitin as the substrate.

To determine the pH stability, chitinase was preincubated with different buffers as described above, without the substrate at 30 °C for 3 h. Then 2% glycol chitin was added as substrate for residual activity assay. The temperature stability was assayed by preincubating the purified chitinase in 100 mM phosphate buffer (pH 6.0) at different temperatures (10−90 °C) for 1, 2, 3, 7, 14, 21, or 28 days at 30, 37, or 50 °C without the substrate, and then 2% glycol chitin was added as substrate for residual activity assay.

To assay the effect of metals and other factors on chitinase activity, the enzyme (0.3 μg as protein) was incubated with a 10 mM concentration of different metals and inhibitors and 2% (w/v) glycol chitin in 100 μL of 100 mM phosphate buffer (pH 6.0) at 30 °C for 1 h. The highest activity was calculated as relative activity (RA) 100%.

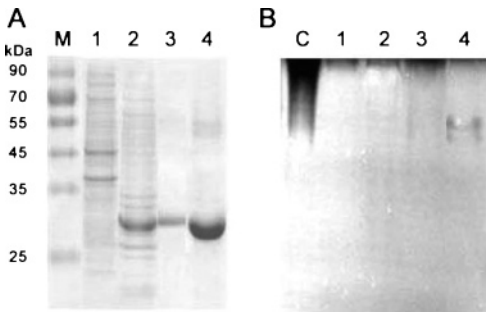

Figure 3. Coomassie blue stained and active staining of recombinant CpCHI. Each protein (different purified fraction by affinity chromatography using Ni-NTA resin) was subjected to SDS-PAGE or native-PAGE with 20 μL of protein sample loaded into each well: (**A**) Coomassie Brilliant Blue stained gel (12% SDS-PAGE); (**B**) chitinase activity staining (12% native-PAGE with 0.1% glycol chitin; the assay was performed as described under Materials and Methods). M, protein marker; the numbers indicate the molecular weight on the left; C, crude extract of *Bacillus circulans* WL-12 chitinase as positive control; 1, supernatant of cell extract; 2, pellet of cell extract; 3, eluting fraction of purified protein by Ni-NTA; 4, dialysis sample of 3.

HPLC Analysis of Chitin Hydrolysis by CpCHI. Chitin hydrolysis was carried out in a 100 mM phosphate buffer, pH 6, at 30 °C with shaking. The concentrations of chitinase and glycol chitin suspension were 5 μg mL^{-1} and 1%, respectively. The reactions were quenched with 10% (v/v) acetic acid. Following a centrifugation at 5 °C, the supernatant containing chitooligosaccharide products formed after 5 min was immediately injected into a 4.6 mm i.d. × 250 mm length Asahipak NH2P-50 4E column. The column was connected to an Agilent Technologies 1100 series HPLC system under the control of a Thermo Finnigan LCQ DECA electrospray mass spectrometer. The mass-to-charge ratios (*m*/*z*) of the expected oligosaccharides were selected as follows: Glc-NAc, 221.9; $(GlcNAc)_2$, 425.5; $(GlcNAc)_3$, 627.6; $(GlcNAc)_4$, 830.8; $(GlcNAc)_5$, 1034.0; $(GlcNAc)_6$, 1237.2; and $(GlcNAc)_7$, 1440.0.

Antifungal Activity Assay. *Alternaria brassicicola*, isolated from a cabbage, causes black spot disease in a wide range of *Brassica* crops. The fungus was cultured in potato dextrose agar (Difco) for 2 weeks. After sporulation, the spores of the pathogen were suspended in water using a concentration of 10^4 spores mL^{-1}. The fungal spore stock (approximately 500 spores) was cultured in water or TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) as control, with or without the purified CpCHI (38 or 76 nM) at 24 °C in total darkness for 12 h. The inhibition of spore germination was examined under a light microscope at 200× amplification (*24*), and the percentage of germination was calculated from 10 different observations. All experiments were conducted three times.

Antibacterial Activity Assay. *E. coli* ED2566 and *E. coli* AD494 were used for the inhibitory activity assay. The *E. coli* strain was grown at 37 °C in an LB medium with shaking at 225 rpm overnight. The next day, a 100 μL overnight culture was added to 10 mL of fresh LB medium with CpCHI in 1, 1.5, 2, 2.5, or 3 μM final concentration for further culture at 37 °C with shaking. A 300 μL TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) was added to 10 mL of LB medium as control. Growth was monitored in terms of changes in absorbance at 600 nm.

Computational Methods Used To Analyze the Sequence. After autosequencing, the amino acid sequence was predicted by using GCG software (Genetics Computer Group, Madison, WI). SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) predicts the presence and location of signal peptide cleavage sites in amino acid sequences (*25*). The amino acid sequences were compared with those deposited in the database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). For sequence phylogeny, a majority-rule consensus tree was built using the PROTDIST and NEIGHBOR programs, whereas the statistical

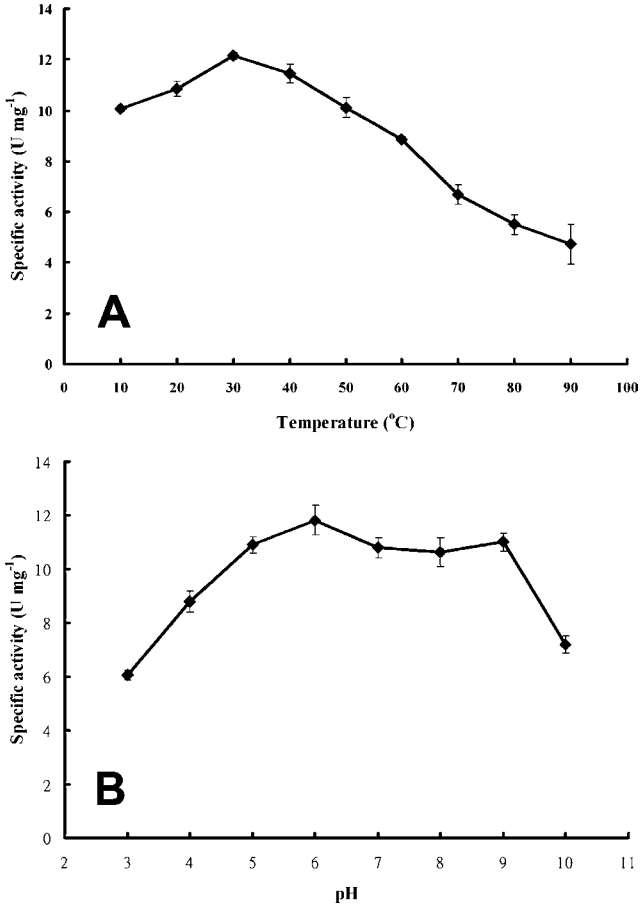

Figure 4. Effects of pH and temperature on the chitinase activity of CpCHI. (**A**) To measure the optimal temperature, the chitinase activity was carried out at various temperatures for 1 h at pH 6.0. (**B**) The following buffer systems were used: 100 mM acetate buffer (pH 3.0–5.0), 100 mM phosphate buffer (pH 6.0–7.0), 100 mM Tris-HCl buffer (pH 8.0), and 100 mM glycine–NaOH buffer (pH 9.0–10.0). The reaction mixture was incubated at 30 °C for 1 h. Chitinase activity was determined by spectrophtometric assay as described under Materials and Methods. All of the results were averaged from three independent experiments, and the standard deviations are shown.

evolution used Bootstrap analysis with 100 data sets. The species and accession numbers of the amino acid sequences used to construct the tree form of the NCBI database (http://www.ncbi.nlm.nih.gov/) are described in the caption of **Figure 2**.

RESULTS

Fruit ripening is a regulated development process that is affected by hormones and environmental factors. The ripe fruit changes significantly in terms of its firmness, pigmentation, flavor, nutrients, and increased susceptibility to pathogens. Many genes are involved in metabolism processes including pigment biosynthesis, cell wall degradation, carbohydrate metabolism, protein degradation, lipid metabolism, nucleic acid metabolism, ethylene biosynthesis, and pathogen defense. The identification of ripening-regulatory genes can be applied to the manipulation of fruit ripening, which affects the fruit's quality and shelf life. In our previous study, we isolated over 400 cDNAs up-regulated in ripe papaya fruit by subtractive hybridization (Chen et al., unpublished data). Among them, chitinase was thought to be involved in the defense system and was isolated for further cloning and characterization in this study.

Cloning, Sequencing, and Identification of Papaya *CpCHI* cDNA. Chitinase *CpCHI* cDNA was first isolated from the papaya fruit. Its full-length cDNA gene was cloned using subtractive hybridization and the CapFinder PCR technique. CpCHI was 1002 bp in length and contained an open reading frame of 825 bp (**Figure 1**). According to CBS Prediction Servers (http://www.cbs.dtu.dk/services/) SignalP-NN prediction, CpCHI contains a 28 amino acid signal peptide in the N-terminal end. Therefore, the predicated molecular mass of a mature CpCHI was 26.2 kDa, with an isoelectric point of 6.32.

As compared to the amino acid sequences deposited in the database by using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), CpCHI is most similar to *Arabidopsis thaliana* chitinase (Y14590), with 63% identity and 78% similarity.

A phylogenetic tree was constructed from the deduced amino acid sequences of CpCHI with chitinases from other species in the database using the PROTDIST and NEIGHBOR programs of the Phylip software. CpCHI belongs to the same subgroup with other class IV chitinases, that is, BvCHI, AtCHI, VvCHI1, and VvCHI2 (**Figure 2**).

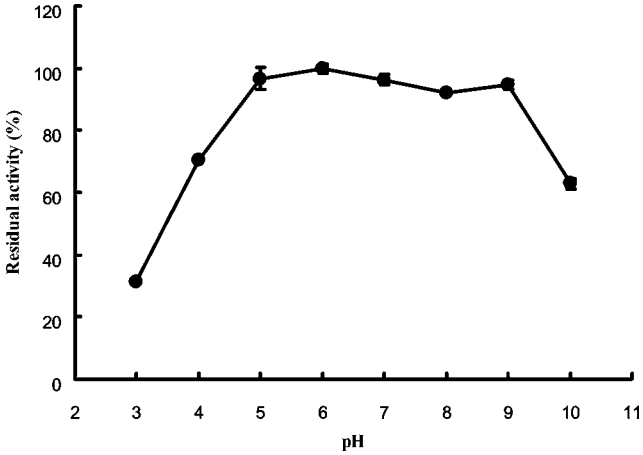

Figure 5. Effects of pH on the stability of CpCHI. The purified recombinant CpCHI was incubated at 30 °C from pH 3.0 to 10.0 (pH 3.0–5.0, 100 mM acetate buffer; pH 6.0–7.0, 100 mM phosphate buffer; pH 8.0, 100 mM Tris-HCl buffer; pH 9.0–10.0, 100 mM glycine–NaOH buffer) for 3 h, and its residual activity was assayed at a typical condition (30 °C for 1 h at pH 6.0). Chitinase activity was determined by spectrophtometric assay as described under Materials and Methods. The results were averaged from three independent experiments, and the standard deviations are shown.

Purification and Characterization of Recombinant CpCHI. To characterize the papaya chitinase, we subcloned the coding region of the predicted mature protein, CpCHI, into pET-20b(+). CpCHI was expressed in *E. coli* Tunner (DE3) with the induction of IPTG, but the protein formed the inclusion body. After dissolution in the binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9, and 6 M urea), it was subjected to an Ni-NTA His·Bind Resin (Qiagen QIAexpress) column for CpCHI purification. The purified CpCHI was estimated to be 26 kDa by SDS-PAGE as a major band (**Figure 3A**). After dialysis, the recombinant protein showed chitinase activity using the gel activity assay with glycol chitin as substrate (**Figure 3B**). Then its specific activity was determined as 12.1 units mg^{-1} by spectrophtometric assay. The optimal conditions for the enzyme activity of recombinant CpCHI were 30 °C and pH 6.0 (**Figure 4**), whereas the enzyme appears to have broad pH optima between pH 5.0 and 9.0 (**Figure 4B**). The purified enzyme lost 45 and 49% of its activity at 70 °C and pH 3.0, respectively. The determination of pH stability of recombinant CpCHI indicated that the enzyme was stable between pH 5.0 and 9.0 with at least 90% activity (**Figure 5**). For application

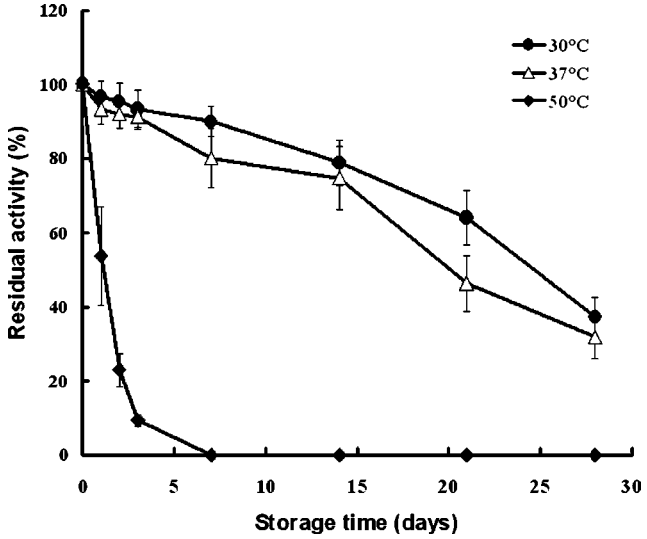

Figure 6. Effects of temperature on the stability of CpCHI. The purified recombinant CpCHI was incubated at various temperatures for different storage times, and its residual activity was assayed at a typical condition (30 °C for 1 h at pH 6.0). Chitinase activity was determined by spectrophtometric assay as described under Materials and Methods. The results were averaged from three independent experiments, and the standard deviations are shown.

Table 1. Effect of Various Reagents and Cations on the Chitinase Activity of CpCHI

	relative activity (%)		relative activity (%)
none	100	$ZnSO_4$	38.0 ± 3.6
EDTA	93.3 ± 3.1	$MnCl_2$	35.3 ± 3.1
KCl	92.3 ± 1.5	$HgCl_2$	0
$AgNO_3$	83.7 ± 2.5	$FeCl_3$	70.7 ± 3.1
$CuSO_4$	40.0 ± 7.2		

[a] To assay the effect of metals and EDTA on chitinase activity, 0.3 μg of recombinant CpCHI was preincubated with 10 mM EDTA, KCl, $FeCl_3$, $MnCl_2$, $ZnSO_4$, $AgNO_3$, $CuSO_4$, and $HgCl_2$ at 30 °C in 100 mM phosphate buffer, pH 6.0, with 2% (w/v) glycol chitin. After 30 min, the remaining chitinase activity was measured under the standard condition using the spectrophotometric assay as described under Materials and Methods. The results were averaged from three independent experiments, and the standard deviations are shown.

as a biocontrol agent, the stability of recombinant CpCHI during long-term storage was analyzed. The purified recombinant CpCHI was stable for long-term storage. It retained >60% of its activity for 3 weeks at 30 °C and >50% of its activity for 3 weeks at 37 °C (**Figure 6**).

To identify the hydrolyzed products of papaya chitinase, glycol chitin was incubated with chitinase in a 100 mM phosphate buffer, pH 6, at 30 °C with shaking for different reaction times. The results showed that within 0.5 and 1 h of reaction, the hydrolytic product $(GlcNAc)_3$ was accumulated (**Figure 7**). Moreover, the hydrolytic product was turned into $(GlcNAc)_3$, $(GlcNAc)_2$, and (GlcNAc) after a 2 h reaction (**Figure 7**). Finally, the hydrolytic products were $(GlcNAc)_2$ and (GlcNAc) (**Figure 7**). This suggests that CpCHI is a novel type of chitinase which has an initially exochitinase activity that yields $(GlcNAc)_3$, and then when the $(GlcNAc)_3$ is accumulated, the $(GlcNAc)_3$ is further cleaved into $(GlcNAc)_2$ and (GlcNAc). This is different from other exochitinases or endochitinases, which produced $(GlcNAc)_2$ specifically or a series of $(GlcNAc)_2$, $(GlcNAc)_3$, or $(GlcNAc)_4$, respectively (*21*).

EDTA and cations were tested with respect to a possible inhibitory or stimulating effect on chitinase activity (**Table 1**).

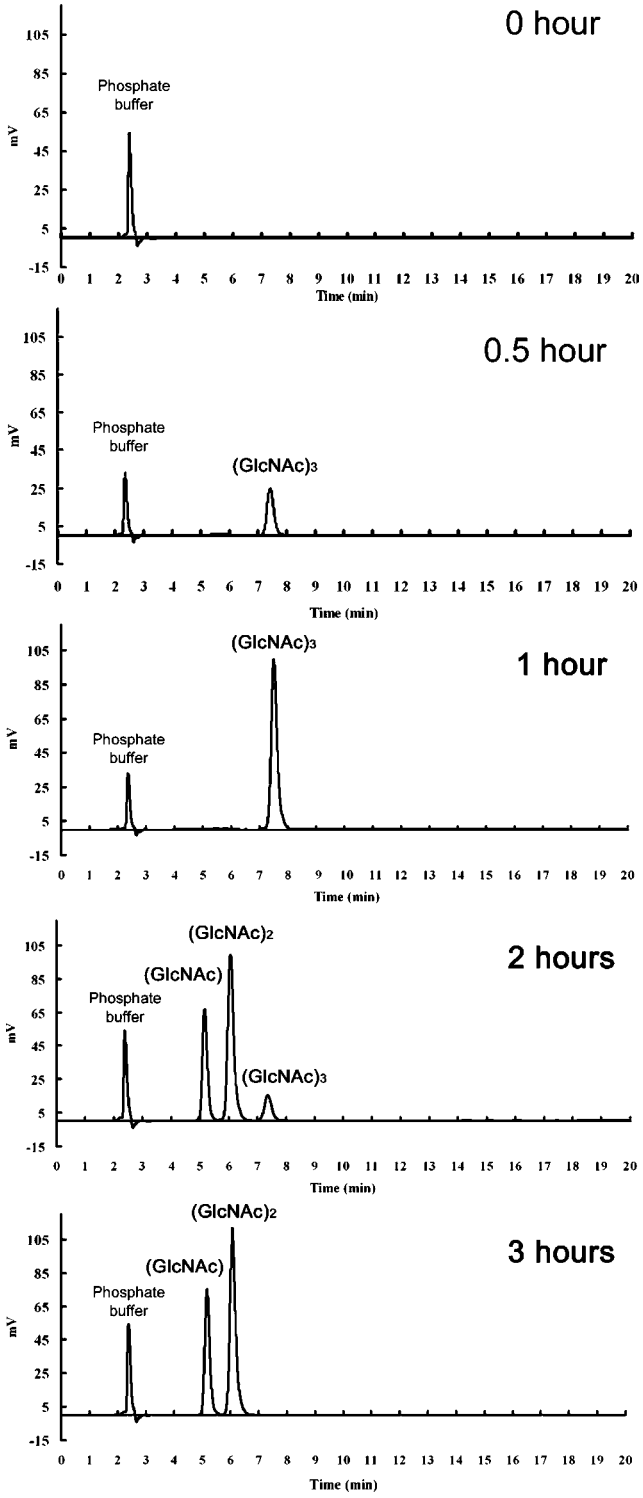

Figure 7. HPLC analysis of the hydrolytic products of glycol chitin by CpCHI at different reaction times. The enzyme reaction was conducted in a 100 mM phosphate buffer, pH 6.0, at 30 °C for 0, 0.5, 1, 2, or 3 h.

K^+, Ag^+, Fe^{3+}, and EDTA were found to slightly reduce CpCHI activity by 7.67, 16.33, 29.33, and 6.67% at the concentration of 10 mM, respectively. Some divalent metal ions reduced CpCHI activity by 64.7, 62.0, and 60% in the presence of 10 mM Mn^{2+}, Zn^{2+}, and Cu^{2+}. Ten millimolar Hg^{2+} was found to inhibit the chitiolytic activity of CpCHI completely.

Antifungal Activity of CpCHI. *Alternaria brassicicola*, which was isolated from cabbage, is considered to be a necrotrophic or death-causing plant pathogenic fungus and has been shown to secrete numerous toxic secondary metabolites

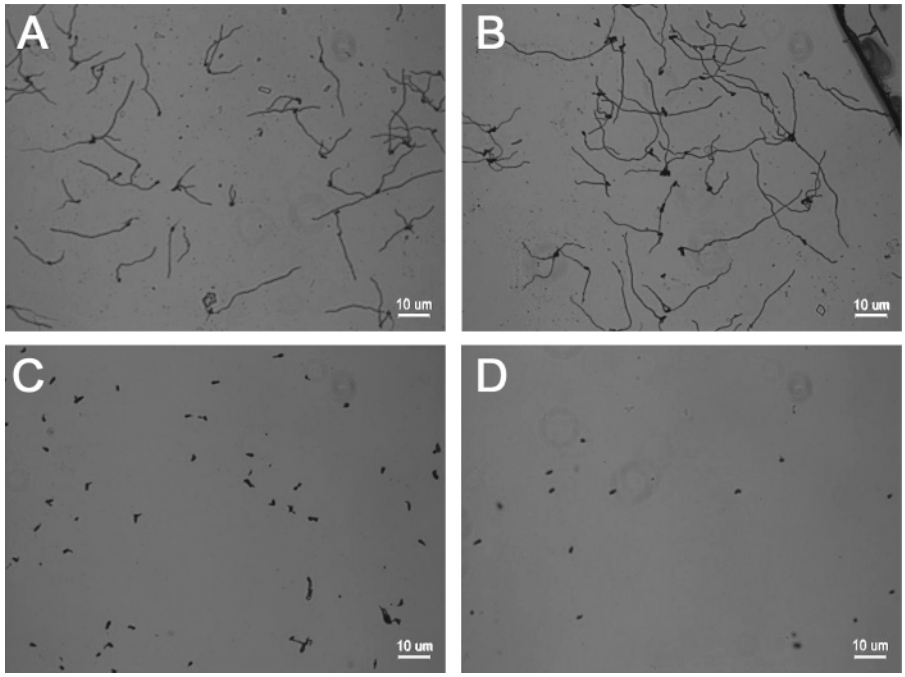

Figure 8. Spore germination inhibition assay. The inhibition of *Alternaria brassicicola* spore germination was observed under a light microscope at 200× amplification after the spores were cultured at 24 °C for 24 h in the following solutions: (**A**) H_2O; (**B**) TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA); (**C**) 38 nM CpCHI in TE buffer; (**D**) 76 nM CpCHI in TE buffer.

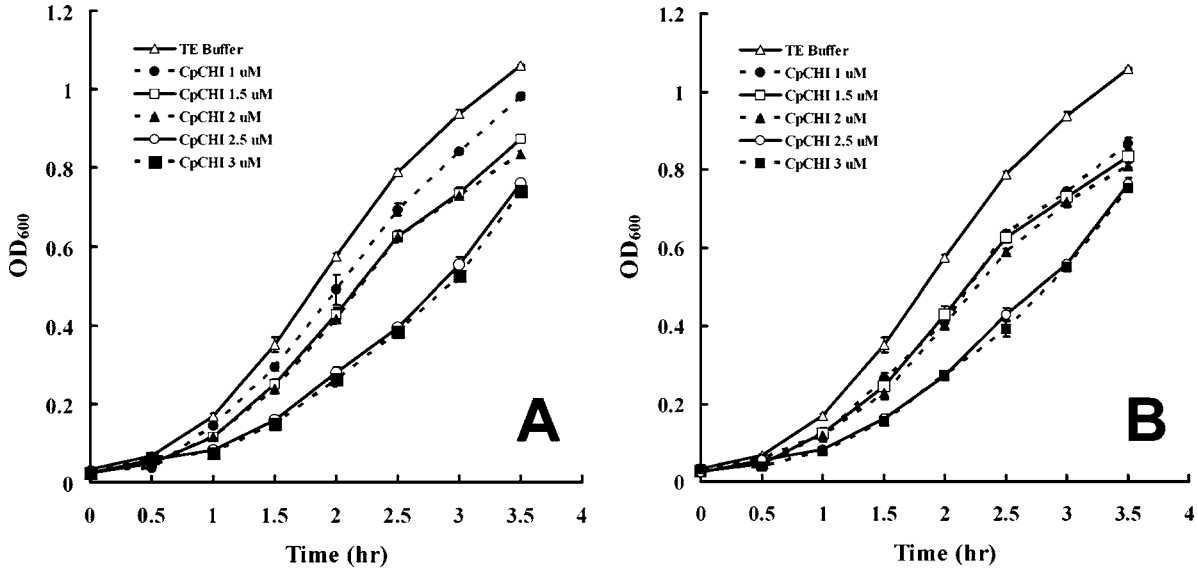

Figure 9. Antibacterial activity of recombinant CpCHI toward (**A**) *Escherichia coli* ED2566 and (**B**) *E. coli* AD494. The inhibition of bacterial growth was monitored from the changes in absorbance at 600 nm after the 100× diluted overnight culture of *E. coli* was incubated with CpCHI at different concentrations (1, 1.5, 2, 2.5, or 3 μM) at 37 °C. TE buffer was added as the control. The results were averaged from three independent experiments, and the standard deviations are shown.

and proteins that cause cell death via induction of apoptosis in plants or by directly damaging cells. Spore germination was significantly inhibited (**Figure 8**) by 50 and 98%, respectively, after incubation with 38 and 76 nM recombinant CpCHI at 24 °C for 24 h.

Antibacterial Activity of CpCHI. Recombinant CpCHI could also suppress the growth of *E. coli* ED2566 and *E. coli* AD494. As shown in **Figure 9**, the IC_{50} values of *E. coli* ED2566 and *E. coli* AD494 were about 2.5 μM CpCHI.

DISCUSSION

Characterization of Purified Recombinant CpCHI. The present work clearly showed that recombinant CpCHI is a novel type of chitinase with distinct hydrolytic reaction and antifungal activity. On the basis of the amino acid sequence phylogeny analysis, CpCHI was classified as a class IV chitinase (**Figure 2**). The hydrolytic products of endochitinases were mixed with $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, and that of exochitinases is $(GlcNAc)_2$ (*26*). Unlike the reported chitinases, the final hydrolytic products of CpCHI were $(GlcNAc)_2$ and (GlcNAc). However, at a shorter reaction time, glycol chitin was specifically cleaved by CpCHI into $(GlcNAc)_3$. Therefore, we assume that CpCHI is a novel type of chitinase which has an initially exochitinase activity that yields $(GlcNAc)_3$ and then when $(GlcNAc)_3$ accumulates, $(GlcNAc)_3$ would further cleave into $(GlcNAc)_2$ and (GlcNAc). The novel cleavage patterns of

recombinant CpCHI might indicate that the structures of the active site or substrate binding motif are different from those of other types of chitinase. Thus, the relationship between hydrolytic mechanism and the three-dimensional structure of CpCHI requires further investigation.

CpCHI showed broad pH optima and good stability. Several chitinases have broad pH optima, such as pH 4.5−7.5 of chitinase from *Bacillus cereus* (*28*), pH 5.0−8.0 for *Aeromonas hydrophila* H-2330 (*29*), and pH 7.5−9.0 for *Bacillus* BG-11 (*30*). The pH optima for other plant chitinases reported were pH 5.4 for mung bean chitinase (*31*), pH 4.5 for *Leucaena* chitinase (*32*), and pH 4 for jelly fig chitinase (*24*). As compared to the reported chitinases, recombinant CpCHI has a much broader pH range, that is, pH 5.0−9.0, than the others. The optimum temperature of recombinant CpCHI was 30 °C, and it had 80% activity between 10 and 50 °C. It retained >60% activity at 30 °C for 3 weeks and >50% activity at 37 °C for 3 weeks. The thermostability of recombinant CpCHI was also significantly higher than that of the chitinases from bean (*23*), *Bacollus* BG-11 (*30*), and mung bean (*31*). On the basis of its broad pH optimum, thermostability, and insensitivity to some metal ions, recombinant CpCHI could thus be very useful in the agriculture, food, and medicine industries.

The yield of major food and economic crops is reduced to about 20% by pathogenic fungi. It is well-known that plant chitinases are part of the defense mechanisms against fungal pathogens (*33*). Their chitinolytic activities can be applied in biocontrol agents and crop protection by means of genetic engineering (*34*, *35*). In this study, we found that recombinant CpCHI could inhibit the spore germination of *A. brassicicola*. In comparison with CHIT 32, a chitinase from *Talaromyces flavus* inhibited the spore germination of *A. brassicicola* by 250 μg mL^{-1} (*36*), whereas recombinant CpCHI inhibited the spore germination of *A. brassicicola* by only 76 nM (about 2 μg mL^{-1}). Therefore, recombinant CpCHI appears to be much better than CHIT in fungal inhibition. Some transgenic plants expressing chitinases have been shown to have improved fungal resistance (*31*, *32*). We thus propose expressing papaya chitinase cDNA in crop plants to improve its antifugal activity or to produce recombinant CpCHI as a biofactory. In conclusion, recombinant CpCHI, with its long storage characteristic and significant inhibitory effect against the growth of fungus, may be used as a natural biofungicide to prolong the storage period of many fruits or economic crops.

Recombinant CpCHI suppressed the growth of *E. coli* ED2566 and *E. coli* AD494. As the results showed, IC_{50} was about 2.5 μM. Studies on the antibacterial and antifungal effects of chitinase are very few, with most papers addressing lysozyme activities in plant chitinases (*15*, *37*). Although there are reports on chitinolytic microorganisms that are antagonistic to fungal phytopathogens (*38*), the chitinases produced by these microorganisms are not known to have antibacterial effects. So far, only the recombinant CpCHI reported here and the CCRC31499 chitinase from *Monascus purpureus* (*39*) have shown antibacterial activity. As compared to the CCRC31499 chitinase from *M. purpureus* against bacteria with 80 μg of enzyme solution (*39*), the recombinant CpCHI inhibited *E. coli* with 2.5 μM (about 6.5 μg mL^{-1}). Therefore, it is very interesting to study the antibacterial mechanism of chitinases, which might provide further applications for this novel type of enzyme.

In conclusion, in this study, an active recombinant papaya chitinase was overexpressed in *E. coli*. The purified recombinant CpCHI showed a broad optimal pH ranging from 5.0 to 9.0 and long-term stability, retaining >64% activity for 3 weeks at 30 °C. The spore germination of *A. brassicicola* could be completely inhibited by 76 nM recombinant CpCHI. Moreover, recombinant CpCHI showed antibacterial activity in which 50% of *E. coli* was inhibited by a 2.5 μM concentration of the enzyme. In the future, the antifungal activity of CpCHI will be investigated using fungus from other plants, including fruit fungus, for the application of CpCHI as a biofungicide.

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