

**Cloning, Expression, and Characterization of an Antifungal Chitinase
from *Leucaena leucocephala* de Wit**

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Chitinase cDNAs from *Leucaena leucocephala* seedlings were cloned by PCR amplification with degenerate primers based on conserved class I chitinase sequences and cDNA library screening. Two closely related chitinase cDNAs were sequenced and inferred to encode precursor proteins of 323 (KB1) and 326 (KB2) amino acids. Expression of the KB2 chitinase from a pET32a plasmid in Origami (DE3) *Escherichia coli* produced high chitinase activity in the cell lysate. The recombinant thioredoxin fusion protein was purified and cleaved to yield a 32-kDa chitinase. The recombinant chitinase hydrolyzed colloidal chitin with endochitinase-type activity. It also inhibited growth of 13 of the 14 fungal strains tested.

Key words: chitinases; *Leucaena leucocephala* de Wit; cloning; recombinant expression

Chitinases (EC 3.2.1.14) are enzymes that catalyze the hydrolysis of the β -1,4-*N*-acetyl-D-glucosaminidic linkages of the polysaccharide chitin.^{1,2} Chitin is present in the cell walls of many fungi and some algae and in the exoskeletons of arthropods such as insects and crustaceans. Chitinases are made by these organisms, which need them for growth and molting,³ and also by bacteria, which degrade chitin for use as a carbon and energy source,¹ and by higher plants and animals, which produce chitinases to defend themselves against pathogenic fungi, among other things.^{4–7}

The functions of plant chitinases are of both scientific and economic interest and have been the subject of many studies.² Most plant chitinases are endo-type chitinases, which randomly hydrolyze the

internal β -1,4-*N*-acetyl-D-glucosaminidic linkages of chitin, producing chitoooligosaccharides. It has been demonstrated that transgenic plants that overexpress chitinases have increased resistance to fungal attack,^{8,9} supporting the suggested role for plant chitinases in defense against fungi. Indeed, many chitinases have been shown to be upregulated in response to fungal elicitors, ethylene, and other stresses.^{4,5,10–12} However, chitinases may have other roles, such as regulation of legume response to rhizobial nod factors, which contain chitoooligosaccharides,¹³ and regulation of normal plant development, perhaps by hydrolysis of similar oligosaccharide substrates.^{14,15} Thus, though anti-fungal activity is an important role of chitinases, it is not their only function, which may be why plants produce many chitinases with different regulatory patterns.^{2,16}

Plant chitinases are monomeric proteins between 22 and 40 kDa in size. On the basis of their amino acid sequences, chitinases have been divided into six classes,^{2,17,18} which fall into two unrelated glycosyl hydrolases families, 18 and 19.¹⁹ Family 18 chitinases, which have also been classified as classes III, V, and VI, have been found in a wide range of species, including bacteria, fungi, plants, insects, mammals, and viruses.^{1,2,7,20} Family 19 chitinases have been found in plants, *Streptomyces* and certain other bacteria, and include classes I, II, and IV.^{2,19,21,22} Class I chitinases have molecular masses of approximately 32 kDa in size, and contain an N-terminal cysteine-rich, chitin-binding domain, a Gly or Pro rich linker region, and a C-terminal catalytic domain. Both the N-terminal and catalytic domains contain several disulfide bonds, which help to stabi-

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Abbreviations: CTAB, cetyltrimethylammonium bromide; DMAB, *p*-dimethylamino-benzaldehyde; GlcNAc, *N*-acetylglucosamine; GlcNAc₂, β -D-*N,N'*-diacetylchitobiose; GlcNAc₃, β -D-*N,N',N''*-triacetylchitotriose; IPTG, isopropyl- β -D-thiogalactopyranoside; PMSF, phenylmethyl-sulfonylfluoride; pNP, *p*-nitrophenol; PVPP, polyvinylpolypyrrolidone; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SSC, saline sodium citrate buffer; SSPE, saline sodium phosphate buffer with EDTA

lize the protein.^{23,24} Class II chitinases have catalytic domains closely related to those of class I chitinases, but lack the cysteine-rich domain and linker, so they are smaller (approx. 24 kDa). Class IV chitinases resemble class I chitinases in structure, but have 4 small deletions that make them somewhat smaller. Family 19 chitinases are of interest due to their role in fungal resistance, especially class I chitinases.

Researchers have primarily focussed on chitinases from crops and ornamental plants,^{2,11,17,18,25} though a few weeds have been studied.^{6,26} In an effort to expand the biodiversity being sampled, Sriyotha and colleagues (unpublished) screened various tropical plants for chitinase activity. In that preliminary study, *Leucaena leucocephala* de Wit, a leguminous tree used in reforestation in South Asia and in herbal medicine, showed high chitinase activity relative to other tropical plants assayed. In this study, the cloning, recombinant expression, and characterization of a class I chitinase from *Leucaena leucocephala* de Wit was undertaken. Production of the chitinase in redox-deficient *E. coli* that allow formation of disulfide bonds in the cytoplasm gave protein with high catalytic and antifungal activities.

Materials and Methods

Plant materials and fungi. *Leucaena leucocephala* seeds were collected from trees at Suranaree University of Technology, Nakhon Ratchasima, Thailand. The seedlings were germinated and grown in clean soil at 25°C with a 15-h/day light period for 1 week. They were then transferred to a greenhouse for 3 weeks before RNA isolation. Fungal strains were obtained from the Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand and the Plant Pathology and Microbiology Divisions of the Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

Enzymes and reagents. Degenerate oligonucleotides, SuperScript reverse transcriptase II, RNase H, and Trizol reagent were from GIBCO-BRL (Invitrogen Corp., Carlsbad, CA). Non-degenerate oligonucleotides were ordered from Geneset Oligos Ltd. (Singapore). Chitin from crab shells, purified chitin, purified chitosan, chitoooligosaccharides, and *p*-nitrophenyl (pNP) glycosides were products of Sigma Fine Chemicals (St. Louis, MO). Restriction enzymes, deoxyribonucleotides, 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-Gal), pGEM-T plasmid, Taq polymerase, *Pfu* DNA polymerase, T4 DNA ligase, and Poly Tract mRNA isolation system IV were products of Promega Corp. (Madison, WI). pT7blue, pET23d, and pET32a plasmids, and BL21 (DE3), and Origami (DE3) *E. coli* were from Novagen (Madison, WI). pUC19 plasmid, phage

λ gt11, and the 5' RACE kit were from Takara (Takara Shuzo Corp., Tokyo, Japan). The cDNA synthesis kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Assessment of protein and chitinase activity. Throughout the study, protein concentrations were measured by the Lowry assay²⁷ and chitinase activity by colloidal chitin hydrolysis with *p*-dimethylaminobenzaldehyde (DMAB) detection of products.²⁸ Isoelectric focusing was done on precast PhastGel IEF—3.5–9.0 gels with a PhastSystem electrophoresis set (Amersham Pharmacia Biotech), followed by protein staining with PhastGel Blue R (Coomassie Blue R-350), according to the manufacturer's instructions. The apparent pI was determined by comparison to a calibration curve of Bio-Rad pI 4.45–9.60 IEF standards (Bio-Rad Corp., Hercules, CA, USA). SDS-PAGE was done on 12% and 15% acrylamide gels, after samples were boiled 5 min in 1% β -mercaptoethanol, and proteins were detected with Coomassie brilliant blue staining, according to standard methods.²⁹

Cloning of chitinase. Oligonucleotide primers for DNA amplification were designed from conserved amino acid sequences identified by multiple sequence alignment³⁰ of family 19 plant chitinases retrieved from the Swiss-Prot database. Total RNA was extracted from four-week-old shoots and roots by the CTAB procedure of Clendennen and May.³¹ Single-stranded cDNAs were synthesized by reverse transcription (RT), with poly T₁₇ primer and Superscript II reverse transcriptase (GIBCO-BRL), by the manufacturer's instructions. One tenth of the RT reaction was used as template for PCR amplification with 1 μ M Chit19E191f (GARRTIGCIGCITTTY-TIGSICARAC, in IUPAC code for degeneracies and I for inosine) and 2 μ M Chit19A326r (CCRWAIC-CIGGIASRICYIYYNSNGC) primers, 2 mM dNTP, 1.5 mM MgCl₂, and 1 U per 50 μ L Taq polymerase. Polymerase chain reaction (PCR) utilized melting at 94°C 5 min, then 30 cycles of 94°C 30 s, 50°C 1 min, and 72°C extension 1 min, with extension at 72°C 5 min after the last cycle. One microliter of the first strand product was reamplified under the same conditions with the inner primer set (Chit19H201f, CTGGATCCAYGARACIACIGGIGGNTGG, and Chit19P301r, AACTCGAGSIGTCATCCARAAC-CAIAWNGC). The specific products were purified by polyacrylamide gel electrophoresis and cloned into the pGEM-T vector according to the manufacturer's instructions (Promega). The cloned fragment was sequenced by automated DNA sequencing, and the sequence was used to design oligonucleotide primers for 3' RACE. 3' RACE was done as described by Frohman and Martin,³² with the Q_T primer (CCAGTGAGCAGAGTGACGAGGAC-

TCGAGCTCAAGCTTTTTTTTTTTTTTTTTTTT) to prime reverse transcription and the KB-1f (TCCTTACGCCTGGGGTTACTGC) and KB-1r (CAGTGAGCAGAGTGACGAGGACTC) primers for PCR amplification. RACE reactions were similar to nested PCR, but the annealing was done at 58°C 10 s for 3' RACE and 60°C 30 s for 5' RACE. The 5' end of leucaena chitinase cDNA was identified with a 5' Full RACE Core Set kit according to the manufacturer's instructions (Takara). The single-stranded cDNA was synthesized from the KB-2r primer (GCACATGGATATTGGGAGCTGGG) and used as template for inverse PCR amplification with the KB-1f and KB-3r (CTCCCTCTTGCGCGTGGC) primers. The product was then reamplified with the KB-1f and KB-4r (GTCGTTGCGGTGTTGAG-CATC) primers.

Construction and screening of the cDNA library. Poly(A)⁺ mRNA was prepared from total RNA with the polyATtract mRNA isolation system IV (Promega). The cDNA was prepared from 5 µg of poly(A)⁺ mRNA with a cDNA synthesis kit (Amersham Pharmacia Biotech) and a λgt11 cDNA library was constructed as described by Gulber and Hoffman.³³ Recombinant phage were screened on nitrocellulose filters with two ³²P-labelled fragments, 242 bp and 331 bp, derived from the *Bam*HI and *Xba*I digestion of the 3' RACE product. The probes (2 ng/ml) were hybridized to the membranes at 60°C for 16 h in 0.5 SSPE, 0.01% SDS, 0.1 × Denhardt's solution. The membrane was washed twice with 2 × SSC containing 1% SDS at 60°C. In total, approximately 3.6 × 10⁵ plaques were screened. Ten clones were isolated using the 242-bp fragment (3' region of 3' RACE product) and five with the 331-bp fragment (5' region of the 3' RACE product). Each positive plaque was purified three times. The recombinant λgt11 phage DNA was extracted, and inserts were excised with *Eco*RI and subcloned into the *Eco*RI site of the pUC19 vector. Automated DNA sequencing of the clones was completed in both orientations.

Sequence analysis. Sequences were analyzed by the BLASTx facility at the National Center for Biotechnological Information (NCBI, USA) to find related protein sequences.³⁴ The protein sequences were translated and aligned with the GENETYNX program (Software Development, Shibuya-ku, Tokyo, Japan), signal peptide cleavage sites were predicted by Signal P,³⁵ and the molecular weights and isoelectric points were calculated by the ProtParam tool facility of the Expasy website (www.expasy.org).

Expression of chitinase. For expression in *E. coli*, pET23d and pET32a vectors were used. To obtain a cDNA fragment without the prepeptide for expres-

sion from pET23d with a C-terminal His₆ tag, single-stranded cDNAs were synthesized by reverse transcription and PCR as described above. PCR was done with 1–2 µM 5' *Nco*I-linker primer (KBex-F2: CCCATGGAGCAATGCGGCAGA) and 3' *Xho*I-linker primer (KBex-R2: CCTCGAGGACGTCGATGA), and 1 U *Pfu* DNA polymerase (in place of *Taq* polymerase). For expression from pET23d without a His₆-tag and from pET32a, cDNA were amplified in the same manner, except the KBex-R2 primer was replaced with KBex-R1 (CCTCGAGTTAGAGGTCGA). Reactions were amplified with 35 cycles of 95°C 30 s, 50°C 1 min, and 72°C 2 min. The specific products were purified and cloned in pUC19. After confirmation of the DNA sequence, the DNA fragment was excised by digestion with *Nco*I and *Xho*I, and ligated into the expression vectors pET23d and pET32a. The resulting plasmids were used to transform *E. coli* strains BL21 (DE3) for pET23d and Origami (DE3) for pET32a. Cells were grown to the appropriate density at 37°C and induced with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, or 1 mM IPTG, at 15°, 20°, 30°, or 37°C for various times to find optimum expression conditions. Optimal induction of protein expression was done with 0.3 mM and 0.1 mM IPTG for pET23d and pET32a, respectively, and the culture was incubated for 12 h at 15°C. Cells were harvested by centrifugation at 5,000 × *g* for 10 min at 4°C and stored at –70°C overnight. The cells were suspended in sonication buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 100 µg/ml lysozyme, 1% triton X-100, 1 mM PMSF, pH 8.0) and lysed by sonication on ice.³⁶ Soluble and insoluble proteins were separated by centrifugation at 14,000 × *g* (4°C) for 30 min. Protein concentrations were assayed, and SDS-PAGE was done, as described earlier.

Purification and cleavage of recombinant protein. Four milliliters of soluble recombinant protein was added to a one-milliliter Ni-NTA superflow resin (QIAGEN Corp., Hilden, Germany) column equilibrated with 10 volumes of lysis buffer (500 mM sodium chloride, 50 mM sodium phosphate, pH 8.0). The column was washed with 20 volumes of lysis buffer and wash buffer (10 mM imidazole in lysis buffer, pH 8.0), and the recombinant protein was eluted with 5 volumes of elution buffer (250 mM imidazole in lysis buffer, pH 8.0). The elution fractions with chitinase activity were pooled and concentrated 4-fold with a 10-kDa cutoff YM-10 Centricon ultrafiltration membrane. The concentrated enzyme solution was put on a G-100 gel filtration column and was eluted with 20 mM Tris-HCl, pH 7.5. The fractions containing chitinase activity were pooled and concentrated as before. Three hundred micrograms of recombinant protein was cleaved with 2 units of enterokinase (New England Biolabs, Beverly, MA) in

30 μ l of 200 mM Tris-HCl, 500 mM sodium chloride, 20 mM calcium chloride overnight at 23°C. The reaction mixture was added to 100 μ l of Ni-NTA superflow resin and incubated at 4°C for 30 min. The mixture was centrifuged at 4°C for 10 s at 15,000 \times g and the supernatant collected. Fractions were evaluated by SDS PAGE and by hydrolysis of colloidal chitin.

Characterization of enzyme activity. To identify the pH optimum, hydrolysis of colloidal chitin by the purified chitinase was assayed in 0.1 M acetate buffer at pH 3.5–6.0, 0.1 M phosphate buffer at pH 5.5–7.5, and 0.1 M Tris-HCl at pH 7.0–9.0. To identify the temperature optimum, the chitinase was assayed at temperatures from 25°C to 70°C in 0.1 M acetate buffer, pH 5.0.

To characterize the substrate specificity, purified chitinase was incubated separately with colloidal chitin, purified chitin, purified chitosan, glycol chitin, swollen chitin, *p*-nitrophenyl- β -D-*N*-acetylglucosaminide (pNP-GlcNAc), *p*-nitrophenyl- β -D-*N,N'*-diacetylchitobioside (pNP-GlcNAc₂), and *p*-nitrophenyl- β -D-*N,N',N''*-triacetylchitotrioside (pNP-GlcNAc₃) as substrates at 40°C for 10 min. Activity was measured by the release of chitoooligosaccharides and pNP, as previously described.^{28,37)}

Test of antifungal activity. The antifungal activity of recombinant chitinase fusion protein was assessed by the hyphal extension inhibition assay.³⁸⁾ Briefly, a disc of each of 14 strains of fungi, each removed from an actively growing fungal culture, was placed in the center of a fresh potato dextrose agar plate. After incubation at 30°C for 3–4 days, 5-mm sterile paper discs containing 0.5 μ g, 1 μ g, or 2 μ g recombinant *L. leucocephala* chitinase and a control disc were placed 5 mm from the perimeter of the fungal culture. The control contained 8 μ g of crude protein extract of induced Origami *E. coli* containing pET32a without a chitinase insert. Inhibition of hyphal extension was detected as a crescent shaped zone of inhibition around the peripheral discs as the fungus grew out from the central disc.

Results and Discussion

Isolation and sequencing of Leucaena leucocephala chitinase cDNAs

Preliminary studies showed that *L. leucocephala* de Wit seedlings contained high levels of chitinase activity, some of which bound tightly to colloidal chitin. Since class I chitinases contain chitin-binding domains and are of interest for their antifungal properties, an attempt was made to clone a class I chitinase from *L. leucocephala* seedlings. Oligonucleotide primers for nested PCR were designed from conserved amino acid sequences iden-

tified by multiple sequence alignment of glycosyl hydrolase family 19 plant chitinase sequences. These conserved sequences in the catalytic domain were used to produce two forward primers (chit19E191f, chit19H210f) and two reverse primers (chit19P301r, chit19A326r). A specific product of approximately 300 bp was amplified by RT-PCR, gel-purified, cloned, and sequenced. The resulting sequence was used to design oligonucleotide primers (see Fig. 1) for rapid amplification of the 3' cDNA end (3' RACE). A specific product of approximately 650 bp was purified by polyacrylamide gel electrophoresis, cloned, and sequenced.

Two separate ³²P-labeled cDNA fragments (242 and 331 bp) derived from the 3' RACE product were used as probes to screen a *Leucaena leucocephala* young shoot cDNA library in λ gt11. In total, 15 positive clones were isolated and their sequences were analyzed. The insert sizes of these clones ranged between 500–850 bp, but none contained the initiation codon (ATG). Therefore, 5' RACE was used to isolate eight independent clones, each with a length of 180 bp, which were found to include the initiation codon and 5' untranslated region. Combining the sequences of the 5' RACE and cDNA library clones showed that the total length of the leucaena chitinase cDNA was approximately 1,100 bp. This message size was confirmed by a northern blot of whole mRNA extracted from seedlings (data not shown).

L. leucocephala chitinase cDNA sequence analysis

Two *L. leucocephala* chitinase cDNA sequences were found by combining 5' RACE and cDNA library clone sequences (4/3.4 and 5/3.1, Genbank accession numbers: AF513017 and AF523071, respectively, Fig. 1). The two deduced amino acid sequences contained 323 (KB1 from clone 5/3.1) and 326 amino acids (KB2 from 4/3.4) and were 95% identical to each other and very similar to known plant class I chitinases, with 74% amino acid identity to kidney bean chitinase (Genbank accession number: S43926), as shown in Fig. 2. Based on comparison of the deduced full-length amino acid sequences with known class I chitinases, the leucaena cDNA clones appeared to encode class I chitinases with the following features: 1) a hydrophobic signal peptide of 23 and 24 amino acids; 2) a Cys-rich domain of approximately 40 amino acids with 8 cysteines; 3) a Gly- and Pro-rich hinge domain of 7–13 amino acids; and 4) a catalytic domain. The predicted mature chitinases encoded by the 5/3.1 and 4/3.4 cDNAs have calculated molecular masses of 31.9 and 32.2 kDa and the estimated pI are 7.2 and 7.5, respectively. These molecular masses and pIs are similar to those of basic class I chitinases, such as those from bean¹¹⁾ and tobacco.¹⁶⁾

The sequences of barley class II chitinase²⁴⁾ and hevein,²³⁾ which have known 3 dimensional structures

4/3.4N	acatcaagtccaagagATG	GATAACATGAACAAGATGCGGGTCTCTTGTGTGTGCTGTACAGCTTCATGGTGGGAGGCTTAGCGGAGCAATGC	81
5/3.1N		T --- C A	78
4/3.1A		M D N M N K M R V L L C V L L Y S F M V G G L A E Q C	27
5/3.1A		L - L	26
4/3.4N	GGCAGACAGCAGGGGGTGCCTTGTGCCCGCCGCTCTGCTGCAGCCAAATTCGGTGGTGGTTCCTCAACGATTACTGCGGCCCGGTTGC		177
5/3.1N	T T	G T A A T	174
4/3.1A	G R Q A G G A L C P G R L C C S Q F G W C G S T N D Y C G P G C		59
5/3.1A		G N	58
4/3.4N	CAGAGCCAGTGTGGCGAAGTGGCCAGCCCGCCCTCCCTCCGGTGGCCCTCACCGGCATCATCTCCAGGGACACCTTCAACAGATGCTCAAG		273
5/3.1N	A C C	G A T	264
4/3.1A	Q S Q C G G S G P P A P P S G G L T G I I S R D T F N Q M L K		91
5/3.1A	S	A S	88
4/3.4N	CACCGCAACGACCCCGCTGCCCGCCAAATGGTTTCTACACTACAGCCCTTCAATTCGGCCCAAGTCTTCCCGCCTCCGGCAGCACCGGC		369
5/3.1N		T T A G T A T	360
4/3.1A	H R N D A A C P A N G F Y T Y D A F I L A A K S F P A F G S T G		123
5/3.1A		Q N Y	120
4/3.4N	GATGATGCCACGCGCAAGAGGGAGTTCGACGCTTCTCCGCGCAACTTCACAGAGACCACCGCGGTGGCCAGCGCTCCCGACGGTCTCTAC		465
5/3.1N	CC	G C A	456
4/3.1A	D D A T R K R E V A A F L G Q T S H E T T G G W P S A P D G P Y		155
5/3.1A	A		152
4/3.4N	GCCTGGGTTACTGCTTCAACAGGAACCGAACCACCAAGCGCTTACTGTCAACCCAGCTCCCAATATCCATGGCCTCCAGGCAAGCAACTAT		561
5/3.1N		C	552
4/3.1A	A W G Y C F K Q E R N P P S A Y C Q P S S Q Y P C A P G K Q Y Y		187
5/3.1A			184
4/3.4N	GGCCCGGACCCATGCAACTGCTCTGGAACACTACACTACGACAGTGGGAGAGCCATAGGAGCGGACTTGTCTCAACAGCCCCACCTGGTGGCT		657
5/3.1N		C A C	648
4/3.1A	G R G P M Q L S W N Y N Y G Q C G R A I G A D L L N S P D L V A		219
5/3.1A		N	216
4/3.4N	AGCGATGCTGTATATCCTTCAAGACGGCACTATGGTTCTGGATGACAGCGCAGTCCGCGAAGCCATCGTCCACAGCGTCAACAGGGAGATGG		753
5/3.1N		T G C T	744
4/3.1A	S D A V I S F K T A L W F W M T A Q S P K P S C H D V I T G R W		251
5/3.1A			248
4/3.4N	ACACCCTCCGGCGCTGACACGGCGGGTAGACTTCCAGGGTACGGAACGACACGAAACATCATCAACGGAGGGCTGGAGTGTGGGAGAGGGCAG		849
5/3.1N	C C A C C C	A C C A	840
4/3.1A	T P S G A D T A A G R L P G Y G T T T N I I N G G L E C G R G Q		283
5/3.1A			280
4/3.4N	GACCGGAGGGTTCGGATTCGATTCTTCAAGATACTGCGACATTCCTGCTGGTATGGCTCCAACCTCGACTGCTATTCCAGAGG		945
5/3.1N	C T	T G	936
4/3.1A	D A R V A D R I G F F K R Y C D I L R V G Y G S N L D C Y S Q R		315
5/3.1A	P	G	312
4/3.4N	CCATTCCGGCTCTTCCGCTCATCGAGCTCCTTAAATCTCATCTCACTAGTCTCATATGTGTGCTCTGCCCTACTCTTATTCATATAATTCATCT		1041
5/3.1N	A		1032
4/3.1A	P F G S S S L I D V L *		326
5/3.1A			323
4/3.4N	TCTTCCACAAAAA		1075
5/3.1N			1066

Fig. 1. Nucleotide Sequences and Deduced Amino Acid Sequences of Leucaena Chitinase cDNA 4/3.1 and 5/3.4.

Nucleotide sequences are designated by N and protein sequences by A. The dotted line under the protein sequence indicates the cysteine-rich domain. The putative polyadenylation signal of the leucaena chitinase 4/3.4 cDNA at nucleotides 1029-1035 is shown in a gray box, and the start codon and stop codon are marked with open boxes. The underlined and doubly underlined amino acid residues denote the conserved amino acid sequence regions used to design the nested PCR primers. The nucleotide sequences used for the 3' RACE and 5' RACE primers are underlined.

and are homologous to the catalytic domain and chitin-binding (cysteine-rich) domain, respectively, are aligned with the *L. leucocephala* chitinase sequences in Fig. 2. Most residues involved in carbohydrate binding and catalysis are identical. The putative chitin-binding domain is 70% identical to hevein and all 8 cysteines which form disulfide bonds in hevein are conserved in the leucaena chitinase sequences. The catalytic general acid of barley chitinase, Glu67, corresponds to Glu142 in leucaena chitinase 4-3.4 precursor (KB2), while the catalytic base, Glu89 in barley, corresponds to Glu164 in KB2. Brameld and Goddard³⁹ predicted that barley chitinase residues H66, E67, T69, W103, N124, Q162, K165, N199, and R215 form hydrogen bonds from their side chains to a chitohexose substrate. In leucaena chitinase KB2, all the corresponding residues are identical to barley, except for Tyr154, which corresponds to Trp103 in barley. This conservative amino acid change will like-

ly maintain hydrogen bonding with the phenolic OH replacing the indole NH. Garcia-Casado *et al.*⁴⁰ showed that mutagenesis of chestnut class I chitinase at residues E124, E146, Q173, T175, and N254 resulted in decreased chitinase activity. Again, all these positions are identical between *L. leucocephala* and barley chitinases, and chestnut chitinase is only different in the conservative replacement of Ser with Thr at T175. So, the chitin-binding and catalytic domains of KB2 appear to contain the functional groups needed for binding chitin and hydrolyzing it.

Expression and purification of recombinant chitinase

The leucaena chitinase cDNA (4/3.4) was cloned into the prokaryotic expression vector pET23d. The cloning strategy was designed such that a protein identical to a protein containing an additional N-terminal methionine residue and C-terminal His₆ tag

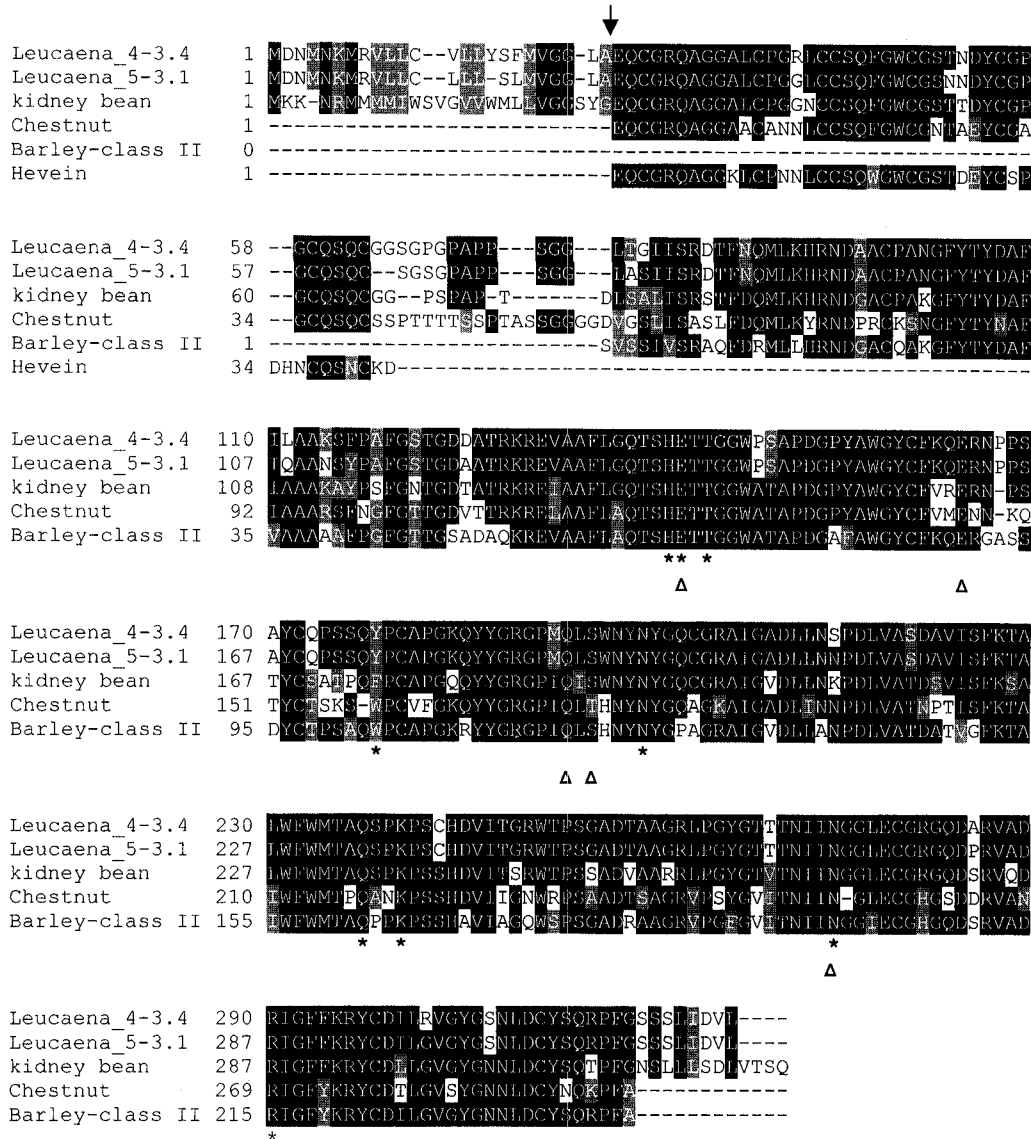


Fig. 2. Alignment of *L. leucocephala* Chitinase Clone Proteins with Related Proteins.

The leucaena chitinase clone 4/3.4 and 5/3.1 predicted protein sequences are aligned with class I chitinases from kidney bean (*Phaseolus vulgaris*, Genbank Ac: S43926) and chestnut,⁴⁰ class II chitinase from barley (Ac: P23951), and rubber hevein (Ac: AF287016). Positions identified by Brameld and Goddard³⁹ as forming hydrogen bonds with substrate in barley class II chitinase are marked with asterisks. Those residues at which mutations decreased the catalytic activity of chestnut chitinase⁴⁰ are marked by deltas (Δ). The arrow indicates the predicted signal sequence cleavage site. The alignment shading was done with the Boxshade program by K. Hoffman.

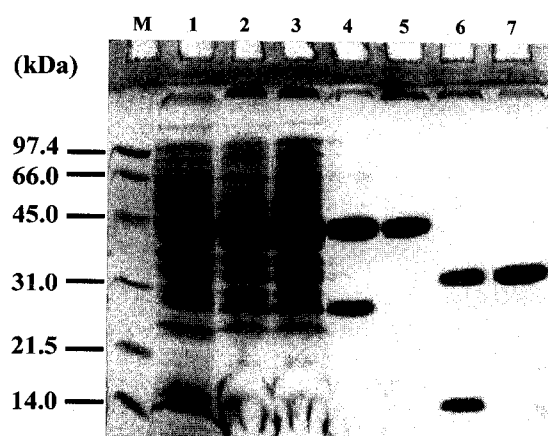
would be produced. Expression of active chitinase was optimized by varying the IPTG concentration, temperature, and time of induction. Optimal induction of expression at 15°C with 0.3 mM IPTG resulted in an approximately 32-kDa protein appearing in low amounts in both insoluble and soluble fractions of cell extracts. This expression provided 0.0146 unit chitinase activity per milliliter of culture (Table 1). Expression of protein without the His₆ tag did not improve the expression levels (not shown), so an alternative system was sought.

Because the difficulty in forming active protein might be related to the large number of cysteine

residues in the chitinase, expression of the chitinase as a thioredoxin fusion from the pET32a vector in Origami (DE3) *E. coli* was tried. These bacteria are redox-deficient, so they allow disulfide bond formation in the cytoplasm, while the thioredoxin fusion tag can assist folding and catalyze disulfide bond formation under these conditions (pET manual, Novagen). In this system, a protein was produced which contained a thioredoxin (Trx)-Tag, a His₆-Tag, an S-Tag, and cleavage sites for thrombin and enterokinase at the N-terminus. Induction of expression at 15°C resulted in high chitinase activity in the cell extract (0.061 U/mL, Table 1) and an approxi-

Table 1. Comparison of Activities of Recombinant Leucaena Chitinases Produced in pET23d and pET32a *E. coli* Expression Systems Purified chitinase fusion protein and cleaved chitinase are from pET32a expressed in Origami *E. coli*.

Sample	Activity (unit/ml)	Protein (mg/ml)	Specific activity
Control	0.0015	2.58	0.0005
Crude soluble protein from pET23d	0.0146	2.10	0.0069
Crude soluble protein from pET32a	0.0605	2.42	0.0252
Purified recombinant fusion protein	0.0645	1.47	0.0439
Purified, cleaved recombinant chitinase	0.0597	1.05	0.0571

**Fig. 3.** SDS-PAGE of Chitinase Protein Expression.

12% SDS-PAGE of the *L. leucocephala* chitinase expression from pET32a in Origami (DE3) *E. coli*. Origami (DE3) cells containing the pET32a plasmid with or without insert were induced for 12 h at 15°C. The gel lanes are: M, molecular mass markers (kDa); 1, pET32a control (induced, no insert); 2, Soluble crude Origami *E. coli* pET32a/chitinase extract, uninduced; 3, Soluble crude *E. coli* pET32a/chitinase extract induced with 0.1 mM IPTG; 4, Recombinant thioredoxin-chitinase fusion protein purified on a Ni-NTA column; 5, Purified recombinant thioredoxin-chitinase fusion protein after Sephadex G-100 chromatography; 6, Cleavage of recombinant protein with enterokinase; and 7, Purified, cleaved chitinase after adsorption of the cleaved tag with Ni-NTA resin.

mately 46-kDa recombinant protein on SDS PAGE of soluble extract (Fig. 3). The amount of soluble recombinant protein was dependent on the IPTG concentration, time, and temperature, with optimal expression achieved on induction with 0.1 mM IPTG at 15°C for 12 h. After purification with a Ni-NTA column to bind the His₆-Tag and gel filtration to remove a 25 kDa contaminant band, a single band of recombinant protein was seen on SDS-PAGE at 46 kDa. Cleavage of this protein with enterokinase resulted in a 32-kDa chitinase band and a 14-kDa thioredoxin-His₆ tag protein. The thioredoxin-His₆ tag protein was completely removed by adsorbing it to Ni-NTA resin to leave chitinase in the supernatant. Hydrolysis of colloidal chitin with the purified fusion protein or the purified cleaved chitinase produced the same products in HPLC analysis (data not shown), so the pure fusion protein was used for further enzymatic characterization.

Table 2. Substrate Specificity of Chitinase Activity from *L. leucocephala*

Substrate	Enzyme activity (U/ml)	Relative activity (%)
Colloidal chitin	0.080	100
Purified chitin	0.040	50
Swollen chitin	0.025	31
Glycol chitin	0.018	23
Chitosan	0.015	19

Characterization of the chitinase

The isoelectric point of the recombinant (cleaved) chitinase was 7.5–7.6, similar to the 7.5 predicted from its sequence. It had a pH optimum of 4.5 and temperature optimum of 55°C. The recombinant chitinase hydrolyzed *p*-nitrophenyl-*N,N,N'*-triacetyl-*D*-chitotriose to liberate *p*-nitrophenol (pNP), but almost no pNP was released from pNP-*N,N'*-diacetyl-*D*-chitobioside or pNP-*N*-acetyl-*D*-glucosaminide. Efficient hydrolysis of pNP-*N,N,N'*-triacetyl-*D*-chitotriose by *L. leucocephala* chitinase and not pNP-*N,N'*-diacetyl-*D*-chitobioside is characteristic of endochitinases, whereas exochitinases (chitobiosidases) would be expected to release pNP from pNP-chitobioside, but not from pNP-chitotriose.³⁷⁾ The substrate specificity of the chitinase was also tested on various chitinous substrates (Table 2). The recombinant expression showed highest activity toward colloidal chitin, followed by purified chitin, swollen chitin, glycol chitin, and chitosan, respectively. This substrate preference is fairly typical of plant chitinases, though some have been reported to hydrolyze glycol chitin or other substrates better.³⁷⁾ The preference for hydrolysis of colloidal chitin over purified chitin or swollen chitin probably reflects a structure that is more accessible to the chitinase active site, while glycol chitin and chitosan are chemically different.

Antifungal activity

The antifungal activity of purified recombinant chitinase was tested at concentrations of 0.5, 1, and 2 µg/disc on 14 fungal strains. Purified recombinant chitinase inhibited *Collectotrichum* sp. 1, sp. 2, and *Pestalotiopsis* sp. 1 at a concentration of 0.5 µg per disc; *Anthanose collectotrichum*, *Fusarium* sp. 1,

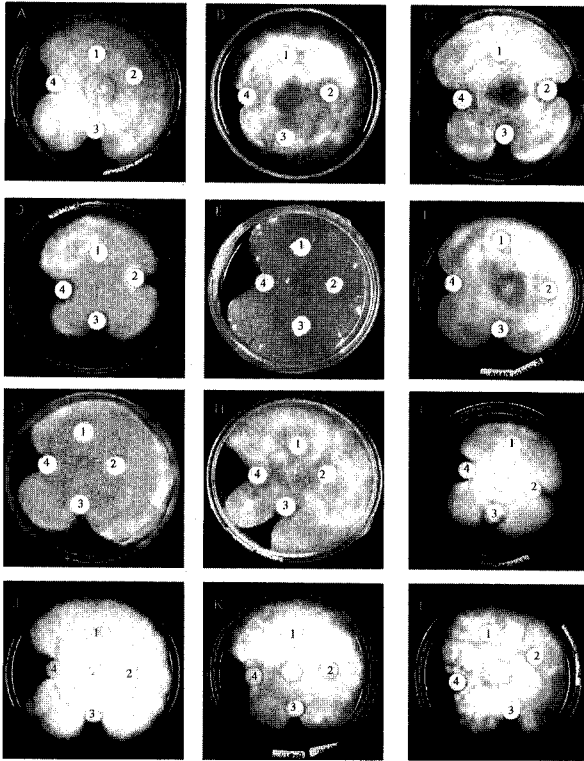


Fig. 4. Antifungal Activity of *L. leucocephala* Chitinase.

Disc numbers 1, 2, 3, and 4 are control (8 µg of crude protein extract of Origami (DE3) pET32a), and 0.5, 1, and 2 µg of purified recombinant protein per 5-mm disc, respectively. The fungal strains are: **A.** *Anthanose collectotrichum*, **B.** *Cercospora* sp., **C.** *Collectotrichum* sp., **D.** *Collectotrichum* sp. 2, **E.** *Drechslera* sp., **F.** *Fusarium* sp. 1, **G.** *Fusarium moniliforme*, **H.** *Fusarium oxysporum*, **I.** *Pestalestiopsis* sp. 1, **J.** *Pestalestiopsis* sp. 2, **K.** *Pestalestiopsis* sp. 3, and **L.** *Pestalestiopsis* sp. 4. Not shown are *Cladosporium* sp. (not inhibited) and *Sclerotium* sp. (inhibited at 2 µg).

Fusarium moniliforme, *Fusarium oxysporum*, *Pestalestiopsis* sp. 2, sp. 3, and sp. 4 at a concentration of 1 µg per disc; and *Cercospora* sp., *Drechslera* sp., and *Sclerotium* sp. at a concentration of 2 µg per disc (Fig. 4), whereas *Cladosporium* sp. was not inhibited at these levels. In comparison with other plant chitinases, purified recombinant *L. leucocephala* chitinase inhibited these fungi at similar or better levels. Chitinases from *Arabidopsis thaliana* and sorghum seed could inhibit *Fusarium moniliforme* and *Fusarium oxysporum* at concentrations of 8 µg and 5 µg per 5-mm disc, respectively, while leucaena chitinase inhibited at only 1 µg per disc.⁶ *L. leucocephala* chitinase also inhibited *Sclerotium* sp. at a concentration of 2.0 µg/disc, while *Arabidopsis thaliana* inhibited it at a concentration of 8 µg/disc.⁶ Though differences in strains used and experimental differences may account for some of these differences, *L. leucocephala* chitinase appears to be comparable to or better than other plant chitinases in fungal inhibition, and may be a good candidate for this application. As transgenic plants expressing

chitinases have been shown to have improved fungal resistance,^{2,7,8} it may be useful to express the *L. leucocephala* chitinase cDNA in crop plants.

The class I chitinase that was cloned from *Leucaena leucocephala* de Wit had properties similar to those of many plant chitinases that have been characterized in the past. It showed a strong preference for colloidal chitin over other chitinous substrates, and seemed to need substrates with at least 3 *N*-acetylglucosamine residues for efficient hydrolysis. The expression of the protein as a thioredoxin fusion protein in redox-deficient *E. coli*, greatly increased its solubility and activity. This suggests that this system is likely to work well for chitinases, which are rich in disulfide bonds, though we have not directly demonstrated proper disulfide bond formation. The high activity and temperature optimum suggests that the disulfide bonds in the catalytic domain, at least, are formed correctly. Garcia-Casado *et al.*⁴⁰ expressed chestnut class I chitinase in *E. coli* without cytoplasmic disulfide bond formation and tested which amino acids are responsible for catalysis and antifungal activity by site-directed mutagenesis. However, their catalytic and antifungal activity was rather low, as was the activity of chitinase expressed from pET23d in normal BL21 (DE3) *E. coli* in our experiments. Therefore, the pET32a/Origami *E. coli* expression system appears better for class I chitinase production than other systems that have been tried. The recombinant *L. leucocephala* chitinase produced shows good catalytic and antifungal properties, which will be useful for future applications.

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