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รายงานการวิจัย

การแสดงลักษณะเฉพาะของไกลโคซิเดสจากพืชตระกูลถั่วป่า

[Characterization of Glycosidases from Forest Legumes]

ได้รับทุนอุดหนุนการวิจัยจาก
มหาวิทยาลัยเทคโนโลยีสุรนารี

ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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[Characterization of Glycosidases from Forest Legumes]

คณะผู้วิจัย

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ผลงานวิจัยเป็นความรับผิดชอบของหัวหน้าโครงการวิจัยแต่เพียงผู้เดียว

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บทคัดย่อภาษาไทย

โครงการวิจัยนี้เป็นการศึกษาโครงสร้างปฐมภูมิ บทบาทหน้าที่และการนำไปประยุกต์ใช้ของเอนไซม์ในกลุ่มไกลโคซิลไฮโดรเลสจากไม้ยืนต้นตระกูลถั่วที่พบในป่าของประเทศไทย เนื่องจากข้อจำกัดของงบประมาณจึงได้ศึกษา 2 เอนไซม์ คือ เอนไซม์ไคติเนสจากกระถินบ้านและเบต้ากลูโคสิเดสจากฉนวน เอนไซม์ทั้งสองถูกแยกจากเมล็ดและต้นอ่อนและได้ทำการโคลน cDNA จากการสร้าง recombinant ไคติเนสใน *E.coli* พบว่าเอนไซม์นี้มีกิจกรรมคล้ายคลึงกับเอนไซม์ที่แยกได้จากพืชด้วยวิธี chitin-affinity adsorption เอนไซม์ทั้งจากพืชและ recombinant *E.coli* สามารถย่อยสลาย ซับสเตรตจำพวกไคตินได้ดี โดยสามารถย่อยสลาย colloidal chitin ได้ดีที่สุด ผลลัพท์จากการย่อยสลายส่วนใหญ่จะเป็น N, N'-diacetylchitobiose และ N, N', N"-triacetyl chitotriose ในระยะต้นและในระยะเวลาที่นานขึ้นจะได้ N-acetylglucosamine และ chitiobiose และยังพบว่า recombinant ไคติเนสสามารถยับยั้งการเจริญเติบโตของเชื้อรา 13 ชนิด ได้อย่างมีนัยสำคัญ ซึ่งอาจจะชี้ให้เห็นถึงศักยภาพการนำเอาไคติเนสนี้ไปประยุกต์ใช้ในการเกษตรกรรม

เบต้ากลูโคสิเดสจากเมล็ดฉนวนสามารถย่อยสลาย *p*-nitrophenol glycoside ได้ดีใกล้เคียงกับเบต้ากลูโคสิเดสจากเมล็ดพยุง แต่ความสามารถในการย่อยสลาย isoflavanoind และ steriod β-glucosides นั้นแตกต่างกัน เบต้ากลูโคสิเดสจากฉนวนสามารถย่อยสลาย isofavanoid glycosides 2 ชนิด ที่แยกจากเมล็ดฉนวนได้แต่ เอนไซม์จากพยุงไม่สามารถย่อยสลายซับสเตรตทั้ง 2 ตัวนี้ได้ ทั้งๆ ที่ลำดับสายกรดอะมิโนของเอนไซม์จากฉนวนนั้นมีความเหมือนกับเอนไซม์จากพยุงถึง 85% เนื่องจากcDNA ของเอนไซม์จากฉนวนไม่สามารถแสดงออกใน *E.coli หรือ Pichia pastoris* ได้ จึงไม่สามารถทำการเปรียบเทียบ recombinant เอนไซม์ได้ อย่างไรก็ตามลำดับกรดอะมิโนเกือบทุกตัวที่มีความสำคัญสำหรับการเกาะกับสับสเตรตและการย่อยสลายของเอนไซม์จากฉนวนและพยุงไม่มีความแตกต่างกัน ดังนั้น พื้นฐานของความแตกต่างกันในการย่อยสับสเตรตจะต้องทำการศึกษาต่อไป

จึงสรุปได้ว่า ไคติเนสจากกระถินบ้าน อาจนำไปใช้ประโยชน์ในการเกษตรโดยนำไปใช้ยับยั้งการเจริญเติบโตของราที่เข้าทำลายพืช และการศึกษาเอนไซม์เบต้ากลูโคซิเดสเป็นพื้นฐานที่จะพัฒนางานเกี่ยวกับความสามารถในการจับกับสับสเตรต ซึ่งสามารถนำไปประยุกต์ใช้ในการออกแบบเอนไซม์ให้เหมาะสมต่อไป

Abstract

This project set out to investigate the primary structures, functions and possible application of glycosidase enzymes from legumous trees found in Thai forests. In order to appropriately utilize the resources available, two enzymes were studied, chitinase from *Leucaena leucocephala* de Wit and beta-glucosidase from *Dalbergia nigrescens* Kurz. Both these enzymes were purified from seeds or young plants and their cDNAs cloned. The chitinase was expressed from the cDNA in *Escherichia coli* and the recombinant protein was found to have activity similar to that of protein purified from the plant by chitin-affinity adsorption. Both chitinases could hydrolyze chitinous substrates with the highest activity toward colloidal chitin. The products of both natural purified chitinase and recombinant chitinase hydrolysis were predominantly N,N'-diacetyl chitobiose and N,N',N''-triacetyl chitotriose at short time points and chitiobiose and N-acetylglucosamine at longer time periods. The recombinant protein was also found to significantly inhibit the growth of 13 strains of fugus, suggesting its potential use in agricultural applications. The *D. nigrescens* β-glucosidase was found to hydrolyze *p*-nitrophenol glycosides with similar preference to *D. cochinchinensis* β-glucosidase, but to have different specificity toward natural isoflavanoid and steriod β-glucosides. In particular, *D. nigrescens* β-glucosidase could hydrolyze two apparent isoflavanoid glycosides isolated from *D. nigrescens* seeds, while the *D. cochinchinensis* enzyme could not hydrolyze them well. Despite this, the protein deduced from the *D. nigrescens* cDNA was 85% identical to the *D. cochinchinensis* β-glucosidase and matched peptide sequences from the purified protein, though active protein could not be expressed by recombinant expression from the *D. nigrescens* cDNA in either *E. coli* or *Pichia pastoris*. Sequence analysis confirmed that almost all amino acid residues previously identified as being important for substrate binding and hydrolysis were identical between the *D. cochinchinensis* and *D. nigrescens* β-glucosidases, so the basis for their different specificities remains to be studied further. Thus, the *L. leucaena* chitinase provides a useful enzyme to be studied for agricultural application in preventing fungal disease, while the β-glucosidase work provides a basis for work on determinants of substrate specificity, which may be used to engineer enzymes for specific uses in the future.

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List of Abbreviations and Symbols

A	Absorbance
Amp	Ampicillin
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine Serum Albumin
°C	Degrees Celsius
cDNA	Complementary deoxynucleic acid
CTP	Cytosine triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	dATP, dCTP, dGTP and dTTP
EDTA	Ethylene diamine tetraacetic ccid
g	Gravitational acceleration
GlcNAc	N-acetyl glucosamine
GTP	Guanidine triphosphate
(m, n) g	(Milli, Nano) gram
h	Hour
HPLC	High performance liquid chromaography
IPTG	Isopropyl-β-D-thiogalactopyranoside
kDa	Kilo Dalton
min	Minute
(m, μ, n) M	(Milli, micro, nano) Molar
(m, μ) L	(Milli, micro) Litter
(μ, n, pmol) mol	(Micro, nano, pico) Mole

List of Abbrevations (continued)

mRNA	Messenger ribonucleic acid
Mw	Molecular weight
4MUGlc	4-Methylumbellriferyl-β-D-glucoside
OD	Optical density
PAGE	Poly acrylamide gel electrophoresis
PCR	Polymerase chain reaction
pI	Isoelectric point
*p*NP	*para*-Nitrophenol
*p*NPFuc	*para*-Nitrophenol-β-D-fucoside
*p*NPGlc	*para*-Nitrophenol-β-D-glucoside
RNA	Ribonucleic acid
RNase	Ribonuclease
SDS	Sodium dodecyl sulfate
s	Second
SSC	Saline sodium citrate
TEMED	Tetramethylenediamine
Tris	Tris-(hydroxymethyl)-aminoethane
TTP	Thymidine triphosphate
UV	Ultraviolet
U	Unit, μmol/min
v/v	Volume/volume
w/v	Weight/volume

บทที่ 1 บทนำ

1.1. Importance and Background of Reseach Problem (ความสำคัญและที่มาของปัญหาการวิจัย)

Background and literature survey

Legumes are plants that form symbiotic relationships with nitrogen fixing rhizobium bacteria. Because they produce more fixed nitrogen (in the form of ammonia) than is necessary for plant growth, they contribute to the nitrogen content of the soil. For this reason, legumes, such as beans, peas, and alfalfa are extensively used in agriculture for enriching the soil. Legumes are also integral parts of the forests of Thailand and include many native tree species, such as *Leucaena*, *Dalbergia*, and *Cassia* species, as well as smaller plants. These legumes are presumed to play an important role in forest soil fertility, and also contain many interesting natural products of potential use as pesticides and pharmaceuticals (Svasti et al. 1999).

Some natural products found in legumes, have been found to be glycosides. That is, they include one or more sugar residues as a part of their structure. These natural glycosides often have properties of medicinal interest in addition to their roles in the plant (Yoshikawa et al. 1998). Plants that contain such glycosides often contain enzymes which remove the sugars: glycosidases (King and Davies, 1995; Rubinelli, Hu, and Ma, 1998; Smith, Starrett, and Gross, 1998). Glycosidases that have been found in Thai legumes include: α-D-mannosidases, α-D-N-acetylglucosidases, α-D-Galactosidases, β-D-Fucosidases, β-D-Glucosidases (Surarit et. al., 1995) and chitinases (Poonsook Sriyotha, personal communication). These glycosidases fall into several families of evolutionarily related proteins (Henrisatt, 1991; Henrisatt and Bairoch 1993, 1996). Many of these enzymes function in defense against herbivores and parasites in other plants (Falk and Rask, 1995). Many plant β-glucosidases, for instance, hydrolyze glycosides to produce toxic substances upon disruption of their tissue integrity, such as linamarinase in cassava, which produces a cyanogenic product (Dunn, Hughes, and Sharif, 1994). Similar β-glucosidases play the same role in other plants (Cicek and Esen, 1998). Chitinases have been found to confer fungal resistance, suggesting they slow fungal growth by hydrolyzing the chitin in their cell walls (Collinge et al. 1993). Chitinases have also been shown to have a potential role in modulating legume-Rhizobium interactions. Soybean chitinase can inactivate NOD factor, a glycoside which is produced by Rhizobium to cause the legumes to make nodules in which the Rhizobium live (Collinge et al. 1993).

Using the evolutionary relationship between these enzymes, we previously cloned partial cDNA clones for α-mannosidase, β-galactosidase, β-glucosidase, and full-length cDNA for chitinase from Leucaena (Ketudat-Cairns et al, 1999, Kaomek et al. , 1999). In related work with the Biochemistry Laboratory of Chulabhorn Research Institute, we have previously cloned a novel rotenoid β-glucosidase from *Dalbergia cochinchinensis* Pierre, which had a potentially useful substrate (Svasti et al., 1999; Ketudat-Cairns et al., 2000). In order to further this work, here we completed the cloning and purification of chitinase from leucana, expressed recombinant protein in *E. coli* and tested the purified protein for antifungal activity. In addition, we have purified and cloned a new β-glucosidase from *Dalbergia nigrescens* Kurz, characterized its activity toward available substrates, and identified two natural glycosides from the same plant that appear to be natural substrates.

1.2. Research Objectives (วัตถุประสงค์ของการวิจัย)

2.1.2.1. Completion of the cloning of the chitinases from *Leucaena glauca* Benth., and β-glucosidases from *Dalbergia nigrescens* Kurz.

2.1.2.2. Comparison of the sequences of these proteins with those of known related enzymes sequences.

2.1.2.3. Purification and/or expression of the proteins.

2.1.2.4. Characterization of the activities and substrates for the enzymes and comparison to related enzymes.

1.3. Scope of Research (ขอบเขตของการวิจัย)

Due to the limitation on funding, the project concentrated on a few enzymes, chitinases from *Leucaena leucocephala* Benth and β-glucosidases from *Dalbergia nigrescens* Kurz. The enzymes from both plants were extracted, purified and characterized, and full-length cDNA were obtained for both genes. One chitinase from *L. leucocephala* Benth could be expressed from the cDNA in *E. coli*, and the protein from the plant and recombinant production were tested for anti-fungal activity. The β-glucosidase from *D. nigrescens* was also used to identify natural substrates from the same plant.

1.4. Short Description of Methods (ข้อตกลงเบื้องต้น)

This project involved the purification, cloning and characterization of chitinase from *Leucaena leucocephala* de Wit and β-glucosidase from *Dalbergia nigrescens* Kurz. The purification of both enzymes was done by standard protein purification methods of precipitation, adsorption and chromatography. Chitinase was purified from *L. leucephala* seeds by extraction, ammonium sulfate precipitation, and affinity adsorption and elution from colloidal chitin. Beta-glucosidase was purified from *D. nigrescens* seeds by extraction, ammonium sulfate precipitation, DEAE ion exchange chromatography and gel filtration chromatography on S300 resin. Both proteins were deemed pure by evidence of a single band on SDS-PAGE. Chitinase cDNA were cloned using degenerate primers based on conserved glycosyl hydrolase family 19 protein sequences to amplify a fragment by reverse-transcription and polymerase chain reaction (RT-PCR) from shoot RNA, followed by using these to screen a cDNA library and finally amplification of the 5' end by rapid amplification of cDNA ends (5' RACE). The full-length cDNA was then amplified and used to express protein as a thioredoxin fusion protein in *E. coli*, using the pET32a plasmid and the protein was characterized for hydrolysis of chitinase substrates and inhibition of fungal growth in a plate disk assay. The β-glucosidase cDNA was cloned by 5' and 3' RACE using primers from the closely related *D. cochinchinensis* β-glucosidase cDNA,

followed by full-length coding region cDNA amplification with primers from the derived sequences. Attempts to express protein from the cDNA were made in *E. coli* and *Pichia pastoris* systems, but no active enzyme could be achieved. The purified protein was used to identify two glycosides from *D. nigrescens* seeds that appear to be natural substrates. These were purified by extraction, LH20 gel filtration chromatography and preparative thin layer chromatography (TLC).

1.5. Benefits and output from this research project (ประโยชน์ที่ได้รับจากการวิจัย)

From the work on chitinase, we were able to purify chitinase from *Leucaena leucocephala* Benth, clone two chitinase cDNA from the same plant and express the protein for one of them in *E. coli*. This protein proved to have antifungal properties, which suggested we may be able to use it as an antifungal agent in the future. From this work one student was able to complete a Ph.D. thesis and one paper was published in an international journal (Kaomek *et al.*, 2003).

From the work on β-glucosidases from *Dalbergia* sp., we were able to purify and clone a β-glucosidase from *Dalbergia nigrescens* Kurz. In addition, two natural substrate glycosides were identified, though the structures of these compounds are yet to be resolved. This has provided the basis for one student's Ph.D. thesis and two manuscripts are currently in preparation for publication.

2. Materials and Methods

2.1 Materials.

Leucaena leucocephala Benth seeds were collected from trees at Suranaree University of Technology, Nakhon Ratchasima, Thailand. The seedlings were germinated and grown in soil in a growth chamber at 25°C with a 15 h/day light period for 1 week and then were transferred to the greenhouse for 3 weeks. *Dalbergia nigrescens* Kurz seeds were also collected at Suranaree University of Technology. Dry, mature seeds were used for enzyme extraction, while RNA was extracted from immature seeds that were near their final size, but still green in color. In some cases, RNA was extracted after germinating the *D. nigrescens* seeds overnight in water and approximately 3 days on moist tissue paper.

Degenerate oligonucleotides and SuperScript reverse transcriptase II, RNase H, and Trizol reagent were from GIBCO-BRL (Life Technologies, Grand Island, NY, USA). Non-degenerate oligonucleotides were ordered from Geneset/Proligo Oligos Ltd. (Singapore). Chitin from crab shells, purified chitin, purified chitosan and commercial glycoside substrates were products from Sigma, Fine Chemicals (St Louis, MO, USA). Restriction enzymes, deoxyribonucleotides, 5-Bromo-4chloro-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 from Promega (Madison, WI, USA). pT7blue, pET-23d, pET-32a and pET40a plasmids, BL21 (DE3), and Origami (DE3) *E. coli* were from Novagen (Madison, WI, USA). pUC19 plasmid, phage λgt11, and the 5′ RACE kit were from Takara (Toyoko, Japan). The cDNA synthesis kit was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The pPICZα, pPIC9K and pTOPO-Blunt plasmids were obtained from Invitrogen, as was Superscript II reverse transcriptase. Twelve strains of fungi were from Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand and Plant Pathology and Microbiology Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand.

2.2 Enzyme assays.

Chitinase activity measurements were performed using a colorimetric assay with colloidal chitin as substrate (Boller and Mauch, 1988). Assays for β-glucosidase, β-fucosidase and other glycosidase activities were tested by hydrolysis of p-nitrophenol (pNP) glycosides in 0.1 M sodium acetate, pH 5.0 for 10 min, as described by Surarit et al (1995).

To test hydrolysis of natural glucosides, enzyme was incubated with the substrate for 10 min, as above, and the glucose released was quantitated with a PGO glucose oxidase assay (Sigma Fine Chemicals).

2.3. Detection in plant and purification of chitinase from *L. leucocephala* plants.

L. leucocephala (40g) 4 week-old plants were ground in liquid nitrogen and dissolved in 80 ml of 100 mM sodium acetate buffer (pH 5.0) containing 1 mM phenylmethyl-sulfonylfluoride (PMSF) and 5% polyvinylpoly-pyrolidone. The extract was centrifuged at 13,800 g for 30 min at 4°C and the supernatant was analyzed for chitinase activity by colloidal chitin hydrolysis and protein content by Lowry assay (Lowry et al., 1951). The crude extract was precipitated by 70% ammonium sulfate, stirred at 4°C overnight and centrifuged at 13,800 g for 45 min at 4°C. The pellet was dissolved in 5 ml of 10 mM sodium acetate buffer pH 5.0, the pH was adjusted to 8.4 with sodium hydroxide and the solution dialyzed against two changes of 20 mM sodium bicarbonate pH 8.4 overnight. Approximately 3.5 g of regenerated chitin which had been preequilibated in 5 ml of 20 mM sodium bicarbonate pH 8 were suspended in the enzyme solution. The chitin was washed with 160 ml of 20 mM sodium bicarbonate pH 8.4 and centrifuged at 27,000 for 45 min at 4°C. The pellet was washed with 160 ml of 20 mM sodium acetate buffer pH 5.5 and centrifuged at 27,000 g for 45 min at 4°C. The pellet was eluted with 80 ml of 20 mM acetic acid pH 3.0 and the centrifugation repeated. The chitinase solution was concentrated with YM-10 centricon membranes (Millipore, Bedford, MA USA) to 5 ml and dialyzed against two changes of 10 mM sodium acetate pH 5.0. Isoelectric focusing of the extract and purified fractions was done on a Pharmacia pre made pI 3.5-9.0 ampholyte gel, followed by protein staining with Coomassie brilliant blue 250 to identify chitinase components. The extracts were also subjected to SDS-PAGE with Coomassie brilliant blue staining according to standard method (Laemli, 1970).

2.4 Cloning of chitinase.

Total RNA was extracted from four-week-old-shoot and root by the CTAB procedures of Clendennen and May, (1997). Oligonuclotide primers for DNA amplification were designed from conserved amino acid sequences identified in family 19 plant chitinases from the Swiss-Prot database (http://www.expasy.org/). Single-stranded cDNAs were synthesized by reverse transcription, using poly T_{17} primer and Superscript II reverse transcriptase (Gibco BRL) and amplified in PCR reactions containing 1/10 RT reaction of template, 1-2 μM outer primer set (forward 5′-GARRTIGCIGCITTYYTIGSICARAC-3′ and reverse 5′-CCRWAICCIGGIASRICYIYYSNGC-3′) and inner primer set (forward 5′-

CTGGATCCAYGARACIACIGGIGGNTGG-3′ and reverse 5′-AACTCGAGSIGTCATCCARAACCAIAWNGC-3′), 2mM dNTP, 1.5 mM $MgCl_2$ and 1 U/50μL *Taq* polymerase. Nested PCR utilized 30 cycles of 94°C 30s, 50°C 1 min, and 72°C extension 1 min. The specific products were purified by polyacrylamide gel electrophoresis and cloned into the pGEM-T vector according to the manufacturer's instructions (Promega). The fragment was sequenced by automated DNA sequencing and the sequence used to design oligonucleotide primers for 3′ rapid amplification of cDNA ends (3' RACE). 3′ RACE was carried out as described by Frohman and Martin (1989). The single-stranded cDNAs was synthesized by reverse transcription with QT primer (5′-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTT-3′) and used as template for PCR amplification with KB-1f (5′-TCCTTACGCCTGGGGTTACTGC-3′) and KB-1r primer (5′-CAGTGAGCAGAGTGACGAGGACTC-3′). RACE reactions were similar to nested PCR but the annealing was done at 58°C 10s for 3′ RACE and 60°C 30s for 5′ RACE. The 5′ end of leucaena chitinase cDNA was determined with using a 5′ Full RACE Core Set kit according to the manufacturer's instructions (TAKARA, Tokyo, Japan). The single-stranded cDNA was synthesized from the KB-2r primer (5′-GCACATGGATATTGGGAGCTGGG-3′) and used as template for amplification with the KB-1f (5′-TCCTTACGCCTGGGGTTACT-3′) and KB-3r (5′-CTCCCTCTTGCGCGTGGC-3′); then KB-1f and KB-4r (5′-GTCGTTGCGGTGTTGAGCATC-3′) primers.

2.5 Northern blot analysis.

Total RNA (10 μg) from shoots and roots was denatured and separated on 1.2% agarose formaldehyde gels, transferred to nylon membrane and cross-linked with UV light by standard methods (Maniatis et al., 1982). The blotted RNA was hybridized at 60°C to the random primer method generated ^{32}P-labelled 3' RACE product and washed in 2X SSC, 1% SDS at 60°C prior to autoradiography on Kodak X-ray film.

2.6 Construction and screening of the *Leucaena leucocephala* seedling cDNA library.

Poly(A)$^+$ mRNA was prepared from total RNA using the polyATtract mRNA isolation system IV. The cDNA was prepared from 5 μg of poly(A)$^+$ mRNA with a cDNA synthesis kit (Amershan Pharmacia Biotech) and λgt11 cDNA library constructed as described by Gulber and Hoffman (1983). Recombinant phage were screened on nitrocellulose filters with two ^{32}P-labelled fragments, 242 bp and 331 bp, derived from the *Bam*H I and *Xba* I digestion of the 3′ RACE product. Hybridization of the probe (2 ng/ml) to the membranes was done at 60°C for 16 h in 0.5 SSPE, 0.01% SDS, 0.1X Denhardt's

solution. The membrane was washed twice with 2X SSC, 1% SDS at 60°C. Each positive plaque was subjected three rounds of purification. The positive λgt11 recombinant phage were purified, their inserts excised with *Eco*R I and subcloned into the *Eco*R I site of the pUC19 vector and automated DNA sequencing was completed in both orientations.

2.7 Expression of chitinase.

For expression in *E.coli*, pET-23d and pET-32a were used. To obtain a cDNA fragment without prepeptide, single-stranded cDNAs were synthesized by reverse transcription, using poly T primer and Superscript II reverse transcriptase and amplified in PCR reactions containing 1/10 RT reaction of template, 1-2 μM of a 5′ *Nco* I linker-primer (5′ CCCATGGAGCAATGCGGCAGA-3′) and a 3′ *Xho* I linker-primer, Kbex-R2 (5′ CCTCGAGGACGTCGATGA-3′) for expression with pET23d or Kbex-R1 (5'-CCTCGAGTTAGAGGTCGA-3') for expression in pET32a, 2 mM dNTP, 1.5 mM $MgCl_2$ and 1 U *Pfu* DNA polymerase were amplified using 35 cycles of 95°C 30s, 50°C 1 min, and 72°C extension 2 min. The specific product was purified and cloned into 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 transformed into *E. coli* strains BL21(DE3) for pET23d and Origami(DE3) for pET32a and induced with 0.1 and 0.3 mM IPTG, respectively. After induction, growth was continued for 12 h at 15°C. Cells were harvested by centrifugation at 5,000g for 10 min at 4° C and stored at –70°C overnight. The cells were resuspended in 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM immidazole, pH 8.0, 100 μg/ml lysozyme, 1% triton X-100, 1mM PMSF and lysed by sonication on ice (Anderson et al., 1997). Soluble and insoluble proteins were separated by centrifugation at 14,000 g for 30 min. Protein concentrations were determined and SDS-PAGE was performed using 12 and 15% polyacrylamide gels after boiling of samples in 1% β-mercaptoethanol and gels were stained with Coomassie Brilliant Blue.

2.8 Purification and cleavage of recombinant chitinase protein.

Four milliliters of soluble recombinant protein was added in 1 milliliter of Ni-NTA superflow (QIAGEN corp, Hilden, Germany) column equilibrated with 10 volume of lysis buffer (500 mM, sodium chloride 50 mM sodium dihydrogen 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). After that, the recombinant protein was eluted with 5 volumes of elution buffer (250 mM immidazole in lysis buffer pH 8.0). The fractions with chitinase activity were pooled and concentrated with 10 kDa cutoff ultrafiltration membrane. The concentrated enzyme solution was loaded onto a G-100 gel filtration column and was washed with 20 mM

Tris-HCl pH 7.5. The fractions with chitinase activity were pooled and concentrated as before. Three hundred micrograms of recombinant protein were cleaved with 2 units of enterokinase in 30 μl of 200 mM Tris-HCl, 500 mM sodium chloride, 20 mM calcium chloride overnight at 23°C. The reaction was added to 100 μl of Ni-NTA superflow and incubated at 4°C for 30 min. The reaction was centrifuged at 4°C for 10 second at 15,000 g and the supernatant collected. Fractions were evaluated by SDS PAGE and by hydrolysis of colloidal chitin.

2.9 Characterization of chitinase enzyme activity.

Enzyme assays for endochitnase and exochitinase used the chromogenic substrates, *p*-nitrophenyl-*β*-D-*N*-acetylglucosaminidee, *p*-nitrophenyl-*β*-D-*N,N'*-diacetylchitobiose, *p*-nitrophenyl-*β*-D-*N,N'*, *N''*-triacetylchitotriose. They were dissolved at 10 mM in 100 mM sodium acetate buffer pH 5.0 purified chitinase (100 ml) were added and incubated at 50°C (Roberts and Selitrennikoff, 1988). Reactions were terminated by the addition of 2 ml 2 M sodium carbonate and absorbance measured at 400 nm.

To determined the pH and temperature optimum, the purified chitinase was assayed in 0.1 M acetate buffer pH 3.5-6.0, 0.1 M phosphate buffer pH 5.5-7.5, and in 0.1 M Tris-HCl pH 7.0-9.0. It was then assayed at temperatures varying from 25°C to 70°C at in 0.1 M acetate buffer pH 5.0.

To determined 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, *p*-nitrophenyl-*β*-D-*N,N'*-diacetylchitobiose, and *p*-nitrophenyl-*β*-D-*N,N'*, *N''*-triacetylchitotriose as substrate at 40°C for 10 min. Activity was measured by release of chitooligosaccharides and pNP as previously described.

The products of the chitinase reactions after the incubation with colloidal chitin at 40°C for 0, 15, 30 min, 1, 2, 4, 8, 16, and 24 h were analyzed on a Carbosep CHO-411 HPLC column (Transgenomic, Omaha, NE USA) using water as the mobile phase, at the flow rate 0.4 ml/min, 70°C, with detection by absorbance at 210 nm.

Kinetic parameters were determined by Lineweaver-Burk representation of data obtained under the assay conditions previously described using 0.2-6.5 mg/ml colloidal chitin (Pegg, 1988) or 0.25-5mM pNP-$(GlcNAc)_3$ (Hollis et al., 1997).

2.10 Antifungal activity characterization.

A disc of each fungus from fourteen strains of fungi, removed from an actively growing fungal culture, was placed in the center of a fresh potato dextrose agar medium. After incubation at 30°C for 3-4 days, 0.5 mm sterile paper discs containing 0.5 μg, 1 μg, and 2 μg

recombinant *L. leucocephala* chitinase and the control containing crude protein (including thioredoxin from pET32a plasmid without insert) without chitinase were placed around the perimeter of the fungal culture. Extension results in outward growth from the central disc. Inhibition of hyphal extension was detected as a crescent shaped zone of inhibition around the periferal discs as the fungus grew out from the central disc (Roberts and Selitrennikoff, 1988).

2.11 β-Glucosidase enzyme purification

Seeds were surface sterilized with 0.1% hypochlorite for 10 minutes, then washed with distilled water and soaked overnight. After this, all procedures were done at 4°C. Seeds were homogenized with 2 volumes of buffer 1 (0.025 M Tris-HCl, pH 8.0, 70% $(NH_4)_2SO_4$, 10 mM ascorbic acid, 0.025 M β-mercaptoethanol, 1 mM PMSF), then filtered with cheese cloth, and the solids were extracted 2 more times. Then, the solid was extracted with 4 volumes buffer 2 (0.2 M NaCl, 10 mM ascorbic acid, 0.025 M β-mercaptoethanol, 1 mM PMSF, 0.025 M Tris-HCl, pH 8.0), centrifuged at 12,000 rpm for 15 minutes at 4°C, and PVPP was added to the supernatant up to 2% and stirred at 4°C for 1 hr. The homogenate was centrifuged at 15,000 rpm for 20 minutes at 4°C, and the pellet was discarded. Then, activated Dowex 2X8 resin was added to the supernatant to 25% (w/v), mixed by stirring and removed by centrifugation at 15,000 rpm for 20 minutes at 4°C. The protein was fractionated from the supernatant by 35-75% $(NH_4)_2SO_4$ and the protein pellet was resuspended with the 25 mM Tris-HCl, pH 7.0. The resuspended protein was dialyzed in 12-14K molecular weight cut off dialysis tubing (Spectrum, USA) against the 500 ml of 25 mM Tris-HCl, pH 7.0. The $(NH_4)_2SO_4$ fractionated protein was separated on a DEAE-sepharose anion exchange column (1.25 cm × 23.5 cm, 115 cm^3) with a linear gradient of 0 to 1 M sodium chloride (NaCl) in 25 mM Tris-HCl, pH 7.0, at a flow rate of 0.65 ml/min. Fractions containing β-glucosidase and β-fucosidase activity were pooled, concentrated by ultrafiltration (YM-50, Amicron) and fractionated by chromatography in a Sephacryl S-300 gel filtration column (1.5 cm × 25 cm, 150 cm^3) equilibrated with 50 mM Tris-HCl pH 7.0, 0.3 M NaCl at a flow rate 0.8 ml/min. Fractions that had activity and appeared more than 90% pure by SDS were pooled and concentrated by ultrafiltration (YM-50, Amicron).

2.12 Determination of β-glucosidase and β-fucosidase activities

The reactions were performed in 0.1 M sodium acetate buffer, pH 5.0 containing *p*-nitrophenyl-β-D-glycoside. The reaction was incubated at 30°C for 10 minutes and stopped with two volumes 2 M sodium carbonate. The released *p*-nitrophenol (*p*NP) was determined by measuring absorbance at 400 nm (Montreuil et al., 1986). Hydrolysis of commercially

available natural glucosides was measured by glucose release, which was quantified with a PGO glucose oxidase assay (Sigma Fine Chemicals, St Louis, MO, USA and Babcock and Esen., 1994). Kinetic studies were analyzed by linear regression and non-linear regression with the Enzfitter computer program (Elsevier Biosoft, Cambridge, UK).

2.13 *β*-Glucosidase protein analysis

Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as standard, while column effluents were screened for protein by measurement of A_{280}. Denaturing or non-denaturing gel electrophoresis was run according to the general method of Laemmli (1970). SDS-denaturing polyacrylamide gel electrophoresis was performed on 10% polyacrylamide separating gels with Bio-RAD Low-range protein markers (Bio-RAD, Corp., Hercules, CA, USA), and stained with Coomassie Brilliant Blue R250. Non-denaturing polyacrylamide gel electrophoresis (activity gels), was performed with 5% polyacrylamide stacking gels and 7% polyacrylamide separating gels in *Laemmli* buffer without SDS. The gel was stained separately for β-glucosidase, β-fucosidase and β-galactosidase activity using 1 mM 4-methylumbelliferyl-β-glycosides. The fluorogenic bands of activity were detected using a Fluor-S™ MultiImager (Bio-RAD). The native molecular weight of protein was estimated by using Sephacryl S-300 (Amersham Phamacia) gel filtration chromatography. The Sephacryl S-300 column (1.5 cm × 25cm, 150 cm^3) was run in 50 mM Tris-HCl, pH 7.0 containing 0.3 M NaCl and calibrated with β-amylase (200 kDa), alcohol dehydrogenase (150 kDal), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa) as standards.

The N-terminus of the purified protein was sequenced with an ABI 471A Peptide Sequencers (Applied BioSystems Inc., CA, USA). The protein was digested with trypsin (Promega), and peptides were isolated by Vydac-C4 (4.6 mm × 250 mm (5 μm)) reverse phase column (Grace Vydac, Columbia, MD), dried onto a glass fiber filter and sequenced by the same methods.

2.14 β-Glucosidase pH and temperature optimum

To determine the pH optimum for enzyme activity, the reactions were performed in different 0.1 M buffers from pH 3-8.5 (citrate, pH 3-4; NaOAc, pH 4.5-5.5; potassium phosphate, pH 6-8.5), at 0.5 pH unit intervals. The activity at various pH values was measured by mixing the enzyme solution with 1 mM final concentration of *p*NP-β-D-glucoside or *p*NP-β-D-fucoside. The temperature optimum was determined by incubating the enzyme with 1 mM final concentration of *p*NP-β-D-glucoside or *p*NP-β-D-fucoside in 0.1 M

NaOAc, pH 5.0 and incubating at temperatures ranging of 35-85°C, at 5°C intervals for 10 min.

2.15 Effects of various substances on the activity of the β-glucosidase

The effects of some substances toward activity were determined by pre-incubating the purified enzyme with the substance for 10 min at 30°C in 0.1 M NaOAc, pH 5.0, then adding 1 mM *p*NP-β-D-fucoside or 2 mM *p*NP-β-D-glucoside and incubating for 10 min at 30°C.

2.16 HPLC and TLC analysis of hydrolysis products

The reaction products after enzymatic hydrolysis of natural substrates were separated and quantified with an Eclipse XDB-C18 (4.6 mm × 250 mm (5 μm)) reverse phase column on an HP-Series 1100 HPLC (Agilent Corp, Palo Alto, CA, USA) with a linear gradient of 0-100% methanol in 0.1% TFA/water.

TLC of hydrolyzed products was performed on analytical silica gel 60 F_{254} aluminum (Merck, Darmstadt, Germany) with $CHCl_3/MeOH/H_2O$ (15:3:1) as solvent.

2.17 β-Glucosidase cDNA cloning and sequencing

Total RNA was isolated from 2-3 day old *D. nigrescens* seedlings using Trizol Reagent (GIBCO-BRL, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The extracted total RNA was used as the template to synthesize the first strand cDNA with Q_T primer (5′-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GCT T_{16}-3′) and SuperScript II Reverse Transcriptase (GIBCO-BRL, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Amplification of the 3′ end of the *Dnbglu2* cDNA was achieved by 3′ RACE PCR using the PTDnF1 (5′-GGTGGCTTCTTAGATCGTAG -3′) and Q_T primers (Frohman, 1993). Amplification of the 5′ end of the *Dnbglu2* cDNA was carried out by 5′ RACE. Synthesis of the first strand cDNA was as described above, but it was primed with the PTDnR1 primer (5′-GCCATTTGTGGTGAAGACTTG-3′). After synthesis, the RNA was digested with RNaseH. The first strand cDNA was then precipitated with 2 volumes of absolute ethanol and 0.1 volume of 3 M NaOAc, pH 5.2. The product was tailed with dATP using terminal deoxyribonucleotide-transferase (TdT) (Promega, Madison, WI). After that, the product was amplified with Q_T and PTDnR1 primers. A second amplification was performed using PTDnR2 (5′-CGAAAATCATTTACAACCCTAC-3′) and Q_o (5′-CCA GTG AGC AGA GTG ACG-3′) primers. The PCR products were gel purified with a QIAQuick gel purification kit (QIAGEN), ligated into pGEM-T Easy vector (Promega, Madison, WI, U.S.A.) and transformed into *E. coli* DH5α, according to the suppliers' instructions.

Automated sequencing was done with T7 promoter and SP6 primers and an ABI Big Dye Dye-terminator sequencing kit (Applied BioSystems Inc., CA, USA) on an ABI PRISM 310 Genetic Analyzer. The sequences of the 3′ and 5′ RACE product were used to design the 3′ terminus (5′-AAATGTACCAAAGCCACAAAC-3′) and 5′ terminus (5′-TCCTTTCTTTCATCTCATGATTG-3′) primers (Dn2_3′UTRr1 and Dn2_5′UTRf1, respectively), which were used to amplify the full length cDNA with expected size, 1719 bp. The SignalP program (http://www.cbs.dtu.dk/services/SignalP/, Bendtsen et al., 2004) predicted the protein signal sequence and the mature protein MW and pI were predicted by the protein prediction program at expasy (http://www.expasy.org).

2.18 β-Glucosidase protein expression in *Pichia pastoris* and *E. coli*

The cDNA encoding the predicted mature protein of Dnbglu2 was amplified using the Dn2NTERMPstIf (5′-GGCTGCAGCTATTGCCTTTCCAAAAGAAGTC-3′) and Dn2CTERMXbaIr (5′-GGTCTAGATCAAAATGCTTGAATGGCCCACTTG-3′) primers with *Pfu* polymerase to introduced a *Pst* I site at the 5′ end and *Xba* I site at the 3′ end of the coding region, and sequenced to confirm the sequence. The cDNA was digested and cloned into the pPICZαB-Thrombin expression plasmid vector at the *Pst* I and *Xba* I sites. The plasmid was transformed into *Pichia pastoris* strain GS115 (Invitrogen, Carlsbad, CA, USA) according to the procedure recommended by Invitrogen. Transformants were grown on YPD (1% yeast extract, 2% peptone and 2% dextrose) agar plates containing 100 μg/ml zeocin. For expression, the selected clones were inoculated in BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4×10^{-5} g/mL biotin, and 1% glycerol) and grown at 30°C, 250 rpm until the OD_{600} reached 2-3, then the cell pellet was collected by centrifugation at 3,000×g at room temperature for 5 min and resuspended in one-fifth volume of BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate buffer, pH 6.0, 1.34% YNB, 4×10^{-5} g/mL biotin, and 1% MeOH) to induce protein expression and methanol was added to 1% each day. Media and cell extract fractions were assayed using *p*NP-β-D-glucoside, *p*NP-β-D-fucoside and natural substrates.

2.19 Characterization of natural β-glycosidase substrates from *D. nigrescens* Kurz

The *Dalbergia nigrescens* seed was collected from Suranaree University of Technology, Nakhon Ratchasima, Thailand, in January 2003. Dried seeds were ground, seed coats removed and the remainder ground to a powder. *D. nigrescens* β-glucosidase was isolated by extracting the seed powder with buffer A (0.025 M Tris-HCl buffer pH 8.0 containing 70% $(NH_4)_2SO_4$, 10 mM ascorbic acid, 0.025 M β-mercapto-ethanol, 1 mM PMSF) and buffer B (0.025 M Tris-HCl buffer pH 8.0 containing 0.2 M NaCl, 10 mM

ascorbic acid, 0.025 M β-mercaptoethanol, 1 mM PMSF). The crude protein was precipitated with 35-75% $(NH_4)_2SO_4$ and purified by using DEAE-sepharose anion exchange column chromatography and S-200 gel filtration, with detection by *p*-nitrophenol (pNP) release from pNP-glucose, as previously described (Srisomsap et al., 1996).

The powder was extracted with 3 volumes of absolute methanol by stirring overnight at room temperature. The extract was filtered and partitioned with 1 volume of hexane. The methanol fraction was dried by rotary evaporator and the residue dissolved with water. The water fraction was extracted with 2.5 volumes of ethyl acetate and then dried by speed vacuum. The dried residue was dissolved with methanol and separated by chromatography on a 1.2 × 85 cm Sephadex LH-20 gel filtration column (Amersham Biosciences, Uppsala, Sweden). The compounds were eluted with methanol and the fractions containing β-glucosidase substrates were pooled. The β-glucosidase substrates were identified by incubating aliquots of (10% by volume) fractions with and without β-glucosidase in 0.1 M sodium acetate 10 min at 30°C, then spotting onto analytical silica gel 60 F_{254} aluminum TLC sheets (Merck, Darmstadt, Germany) and developed. Compounds 1 and 2 were purified using the preparative silica gel 60 F_{254} thin-layer chromatography by developing twice with solvent A, ethyl acetate/methanol/H_2O/acetic acid 15:2:1:2 (by vol.). The compound 2 glycoside was digested with *D. nigrescens* β-glucosidase to get aglycone by incubating with 0.1 M sodium acetate buffer pH 5.0 at 30°C, overnight. The aglycone of compound 2 was purified by preparative silica gel 60 F_{254} TLC using solvent B, ethyl acetate/methanol/H_2O 5:2:1 (by vol.).

The compound 1 and 2 substrates were hydrolyzed with *D. nigrescens* β-glucosidase to form 1a and 2a, respectively. The reaction was performed with 0.1 M sodium acetate buffer pH 5.0 at 30 ^{O}C, for 10 minutes. The reactions were analyzed by TLC on silica gel 60 F_{254} aluminum sheets, developing twice with solvent A. The chromatograms were visualized under a UV-box at a wavelength of 254 nm. The aglycones produced by digestion of compound 1 and 2 had changed mobility on TLC. Initial structure analysis was done by nuclear magnetic resonance (NMR) with ^{1}H-NMR and ^{13}C-NMR, on a 300 MHz Varian Unity Inova spectrometer.

บทที่ 3 ผลการวิจัย

3 Results

3.1 *Leucaena leucocephala* de Wit chitinase

3.1.1 Purification of chitinase from *L. leucocephala* germinated seeds

L. leucocephala chitinase was purified from seeds geminated for 4 weeks. The leucaena chitinase was purified by 70% ammonium sulfate precipitation and affinity chromatography on a chitin. Most of the activity bound to chitin and was eluted with acetic acid (pH 3.0). The elution was dialyzed and concentrated. The homogenous protein was detected by SDS-PAGE at 32 kDa (Figure 1). Purification with chitin affinity chromatography 26.7 fold protein purification with a recovery of 15.9%. (Table 1).

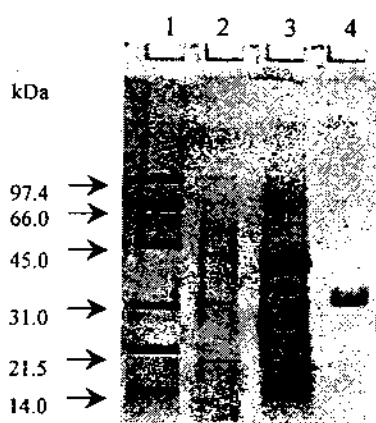

Figure 1 12 % SDS PAGE of purified chitinase from *L. leucocephala* germinated for 4 weeks. Lane 1: Protein mass makers (kDa), lane 2: Crude enzyme, lane 3: 70% Ammonium sulfate precipitate lane 4: Chitin affinity chromatography, eluted with acetic acid (pH 3.0)

Table 1 Purification of chitinase from *Leucaena leucocephala* de Wit

Step	Total activity (Units)	Total protein (mg)	Specific activity (Units/mg)	Recovery (%)	Purification (fold)
1. Crude extract	0.5640	202	0.0028	100.00	1
2. 70% $(NH_4)_2SO_4$	0.3675	20	0.0184	65.16	6.6
3. Chitin affinity chromatography	0.0898	1.2	0.0748	15.92	26.7

3.1.2 *Leucaena leucocephala* de Wit chitinase cloning, chitinase sequence and comparison to other chitinase

Oligonuclotide primers for Nested PCR were designed from amino acid sequences conserved in plant family 19 chitinases found in the NCBI Genbank nr databank. Multiple sequence alignment revealed highly conserved sequences in the catalytic domain, which were used to produce two forward primers (chit19E191f, chit19H210f) and two reverse primers (chit19P301r, chit19A326r). A specific band of about 300 bp was gel purified, cloned, and

sequenced. The initial clone sequence was used to design oligonucleotide primers for 3' rapid amplification of cDNA ends (RACE). The first strand cDNA was synthesized with the Q_T primer. Then, PCR amplification with the KB-1f forward and KB-1r reverse primers produced a specific band of about 650 bp, which was separated on a 5% polyacrylamide electrophoresis gel, eluted, cloned, and sequenced.

3.1.3 Northern blot analysis

Northern blot analysis of the total cellular RNA from seedling whole plants, shoots and roots (not shown) of *L. leucocephala* demonstrated that the chitinase gene is highly expressed in shoots of young plants. Figure 2B shows that the band of hybridization with 3′ RACE product gave a specific band with a size of about 1,100 bp compared with EtBr straining (Figure 2A) which was used to detect the λ Hind III marker and ribosomal RNA bands.

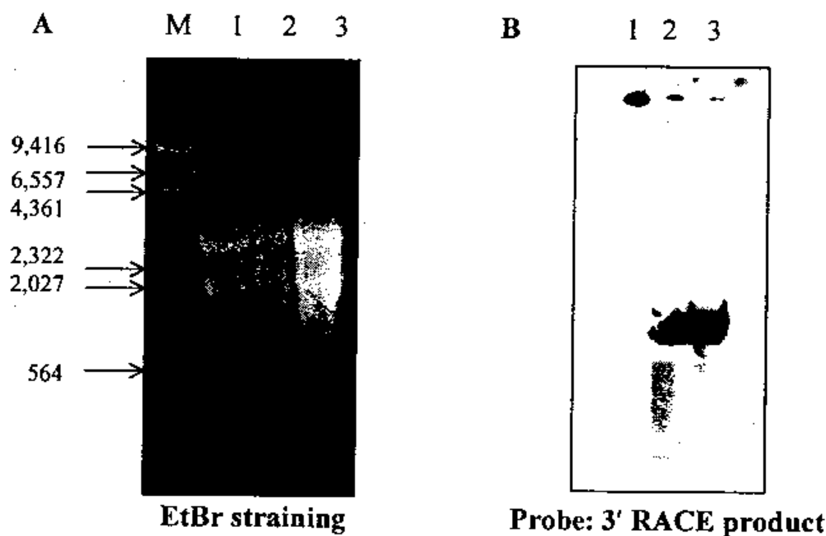

Figure 2 Leucaena chitinase RNA blot hybridization analysis. 10 μg portion of total RNA from rice (1), *Leucaena leucocephala* shoots (2), and whole seedlings (3) were separated on a 1.2% agarose gel after denaturation with glyoxal, transferred onto nylon membrane and hybridized to the 3′ RACE cDNA probe labeled with ^{32}P by random priming. The transcript size (kb), estimated by comparison with λHind III marker (M), was about 1.1 kb.

3.1.4 Isolation and Sequencing of a *Leucaena leucocephala* chitinase cDNA.

A *Leucaena leucocephala* λgt11 cDNA library was screened independently with two separate ^{32}P-labeled cDNA fragments (242 bp and 331 bp) derived from the 3'RACE product as probes. Approximately 3.6×10^5 plaques from a *Leucaena leucocephala* young shoot cDNA library in λgt11 were screened. In total, fifteen positive clones were isolated and the sequences of these clones were determined. Ten and five clones were isolated using the 242 bp fragment (3′ region of 3′ RACE product) and the 331 bp fragment (5′ region of 3′ RACE product), respectively, as probes. Insert sizes of these clones ranged between 500-850 bp. The longest insert contained a long open reading frame, a stop codon (TAA), but it did not

contain the 5′ noncoding region and the initiation codon (ATG). So, 5′ RACE was performed using leucaena chitinase-specific antisense primer prepared from the sequence data of the leucaena chitinase clone. Eight independent clones isolated by 5′ RACE contained 180 bp inserts. This included some of the 5′ noncoding region and the initiation codon ATG. These sequences fell into two closely related groups, with the 3' ends of the sequences matching two sets of cDNA library clones, which were joined to get the whole sequence. These sequences gave a total length of each leucaena chitinase cDNA of approximately 1,100 bp.

The nucleotide sequence and the deduced amino acid of the *L. leucocephala* chitinase cDNA fragment (designated leucaena chitinase) are shown in Figure 3. The two regions for the inner PCR primers were found to be conserved in many plant chitinases. This suggests the possibility that other chitinase genes from many different plants can be isolated by the same procedure.

Two chitinase cDNA from *L. leucocephala* were identified, 4-3.4 and 5-3.1. Based on the nucleotide and deduced amino acid sequence of the full-length open reading frames, the 4-3.4 and 5-3.1 cDNA apparently encode class I chitinases with the following features: (a) a hydrophobic signal peptide of 23 and 24 amino acids; (b) a Cys-rich domain of 40 amino acids with 7 cysteines; (c) a Pro- and Gly-rich hinge region of 13 and 11 amino acids; and (d) a catalytic domain. Based on the deduced amino acid sequences, the derived chitinases, 4-3.4 (326 amino acids) and 5-3.1 (323 amino acids) share 95% identity and they have 74% identity with kidney bean chitinase precursor. Computer calculation of molecular weight and isoelectric points showed that the predicted mature chitinases encoded by the 4-3.4 and 5-3.1 genes are 32.2 and 31.9 kDa, and pI values of 7.5 and 7.2, respectively (Table 2). These pI values would allow them to be classified as basic chitinases, since they are similar to the pI values of chitinase from bean (Brogile et al., 1986), potato (Gaynor, 1988), and tobacco (Shishi et al., 1987).

A comparison of the primary structures of leucaena chitinase with several other plant chitinases was done by multiple sequence alignment, as shown in Figure 4. As may be seen, the Cys-rich domain (putative chitin-binding domain) and catalytic domain are highly conserved with other plant type 1 (glycosyl hydrolase family 19) chitinases.

N1: Nucleotide of leucaena chitinase4/3.4 (1064 bp.)
A1: Amino acid of leucaena chitinase4/3.4 (326 A.A.)

N2: Nucleotide of leucaena chitinase5/3.1 (1075 bp.)
A2: Amino acid of leucaena chitinase5/3.1 (323 A.A.)

```
                    1
N1 acatcaagtccaagagATGGATAACATGAACAAGATGCGGGTGCTCTTGTGTGTGCTGCTGTACAGCTTCATGGTGGGAGGCTTAGCGGAGCAATGC   81
N2                                                      T       ---       C                 A          78
A1                  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
A2                                                      L        -        L                            26

N1 GGCAGACAGGCAGGGGGTGCCTTGTGCCCCGGCCGCCTCTGCTGCAGCCAATTCGGCTGGTGTGGTTCCACTAACGATTACTGCGGCCCCGGTTGC  177
N2    TT T                              G        T              T           A     A  T              174
A1  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
A2     C                              G                                         N                   58

N1 CAGAGCCAGTGTGGCGGAAGTGGCCCAGGCCCAGCCCCTCCCTCCGGTGGCCTCACCGGCATCATCTCCAGGGACACCTTCAACCAGATGCTCAAG  273
N2                 A     C  C  ------                         G  A                          T         264
A1  Q  S  Q  C  G  G  S  G  P  G  P  A  P  P  S  G  G  L  T  G  I  I  S  R  D  T  F  N  Q  M  L  K    91
A2        S           -  -                          A  S                             88

N1 CACCGCAACGACGCCGCCTGCCCGGCCAATGGTTTCTACACCTACGACGCCTTCATTCTGGCCGCCAAGTCTTTCCCCGCCTTCGGCAGCACCGGC  369
N2                                                  T  T     A     G     T  A     T              T   360
A1  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
A2                                                           Q        N  Y                          120

N1 GATGATGCCACGCGCAAGAGGGAGGTCGCAGCTTTCCTCGGGCAAACTTCACACGAGACCACCGGCGGTTGGCCCAGCGCTCCCGACGGTCCTTAC  465
N2   CC G              C                    A                                                       456
A1  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
A2     A                                                                                            152

N1 GCCTGGGGTTACTGCTTCAAACAGGAACGGAACCCACCAAGCGCTTACTGTCAACCCAGCTCCCAATATCCATGCGCTCCAGGCAAGCAATACTAT  561
N2                                                                       C                          552
A1  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
A2                                                                                                  184

N1 GGCCGCGGACCCATGCAACTGTCCTGGAACTACAACTACGGACAGTGCGGAAGAGCCATAGGAGCGGACTTGCTCAACAGCCCCGACCTGGTGGCT  657
N2                         C                                                        A              C 648
A1  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
A2                                                                                  N               216

N1 AGCGATGCTGTGATATCCTTCAAGACGGCACTATGGTTCTGGATGACAGCGCAGTCGCCGAAGCCATCGTGCCACGACGTCATCACGGGGAGATGG  753
N2                                   T  G        C                                             T      744
A1  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
A2                                                                                                  248

N1 ACACCCTCCGGCGCTGACACGGCGGCGGGTAGACTTCCAGGGTACGGAACGACCACGAACATCATCAACGGAGGGCTGGAGTGTGGGAGAGGGCAG  849
N2       C           C        A     C                    C     C                       A            840
A1  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
A2                                                                                                  280

N1 GACGCGAGGGTTGCGGATCGCATTGGATTCTTCAAGAGATACTGCGACATTCTGCGTGTCGGCTATGGCTCCAACCTCGACTGCTATTCCCAGAGG  945
N2       C                          T                       G                                       936
A1  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
A2       P                                                  G                                       312

N1 CCATTCGGCTCTTCCTCGCTCATCGACGTCCTCTAATCTCATCTCACTAGTCTCATATGTGTCGCTCTGCCCTACTCTTATTC[illegible]TCATCT  1041
N2                       A                                                                          1032
A1  P  F  G  S  S  S  L  I  D  V  L  *                                                               326
A2                                                                                                  323

N1 TCTTTCCACAAAAAAAAAAAAAAAAAAAAAAAA                                                                 1075
N2                                                                                                  1066
```

Figure 3 Nucleotide sequence and predicted amino acid sequence of *Leucaena leucocephala* de Wit chitinase cDNA, 4/3.4 and 5/3.1. The dotted line shows the cysteine-rich domain. The putative polyadenylation signal of leucaena chitinase 4/3.4 and leucaena chitinase 5/3.1 at 1029-1035 and 1020-1026 is in a gray box, the start codon and stop codon of leucaena chitinase 4/3.4 and leucaena chitinase 5/3.1 at nucleotides 1-3 and 979-981, 970-972 respectively are marked with open boxes. The underlined and double underlined portions denote the conserved amino acid sequence regions which were used for designing the Nested PCR primers. The bold alphabet was used for designing the 3′ RACE PCR primer and the 5′ RACE primers are shown in the dotted boxes.

```
Leucaena Chitinase5-3     1:MDNMNKMRVLLC--LLL-SLMVGG-LAEQCGCQAGGALCPGGLCCSQFGWCGSNNDYCGP  56
Leucaena Chitinase4-3     1:MDNMNKMRVLLC--VLLYSFMVGG-LAEQCGRQAGGALCPGRLCCSQFGWCGSTNDYCGP  57
kidney bean CH5B          1:MKK-NRMMIMICSVGVVWMLLVGGSYGEQCGRQAGGALCPGGNCCSQFGWCGSTTDYCGK  59
cowpea                    1:IPK-NRMIWSV-AV-VVWTVLVGGSWGEQCGSQAGGALCPGGLCCSQFGWCGSTDDYCGK  57
M.sativa                  1:---M-LMKMRLALVTTVVLLIIGCSFAEQCGKQAGGALCPGGLCCSKFGWCGSTGEYCGD  56
M.truncatula              1:---M-MMRLALVVTTAVLLVIIGCSFADECGKQAGGALCPGGLCCSKFGWCGSTGDYCGD  56
pea endochitinase A2      1:------M-SKLRIPILLVLFIVSCCSAEQCGTQAGGALCPGGLCCSKFGWCGSTSEYCGD  53

Leucaena Chitinase5-3    57:GCQSQC--SGSGPAPPSGGLASIISRDTFNQMLKHRNDAACPANGFYTYDAFIQAANSYP 114
Leucaena Chitinase4-3    58:GCQSQCGGSGPGPAPPSGGLTGIISRDTFNQMLKHRNDAACPANGFYTYDAFILAAKSFP 117
kidney bean CH5B         60:DCQSQC-G-GPSPA-P-TDLSALISRSTFDQVLKHRNDGACPAKGFYTYDAFIAAAKAYP 115
cowpea                   58:GCQSQC-G-GQ-PA-P-SDLSALIP-GHFDQMLKHRNDGACPARGFYTYDAFIAGARAFP 111
M.sativa                 57:GCQSQCGGSS-G-G--GGDLGSLISRDTFNNMLKHRDDSGCQGKGLYTYDAFISAAKAFP 112
M.truncatula             57:GCQSQCSGSS-------GDLGSLISRDTFNNMLKHRDDSGCQGKRLYTYDAFISAAKAFP 109
pea endochitinase A2     54:GCQSQCSGSS---G--GGTLSSLISGDTFNNMLKHRNDNACQGKPFYTYDAFLSAAKAFP 108

Leucaena Chitinase5-3   115:AFGSTGDAATRKREVAAFLGQTSHETTGGWPSAPDGPYAWGYCFKQERNPPSAYCQPSSQ 174
Leucaena Chitinase4-3   118:AFGSTGDDATRKREVAAFLGQTSHETTGGWPSAPDGPYAWGYCFKQERNPPSAYCQPSSQ 177
kidney bean CH5B        116:SFGNTGDTATRKREIAAFLGQTSHETTGGWATAPDGPYAWGYCFVRERN-PSAYCSATPQ 174
cowpea                  112:SFGNTGDTATRKREIAAFLGQTSHETTGGWPSAPDGPYAWGYCFVREQN-PSAYCSPTPQ 170
M.sativa                113:NFANNGDTATKKREIAAFLGQTSHETTGGWATAPDGPYAWGYCFVREQNP-STYCQPSSE 171
M.truncatula            110:NFANNGDTATKKREIAAFLGQTSHETTGGWATAPDGPYAWGYCFVREQNPSSTYCQPSSE 169
pea endochitinase A2    109:NFANKGDTATKKREIAAFLGQTSHETTGGWPTAPDGPYAWGYCFLREQNP-STYCQASSE 167

Leucaena Chitinase5-3   175:YPCAPGKQYYGRGPMQLSWNYNYGQCGRAIGADLLNNPDLVASDAVISFKTALWFWMTAQ 234
Leucaena Chitinase4-3   178:YPCAPGKQYYGRGPMQLSWNYNYGQCGRAIGADLLNSPDLVASDAVISFKTALWFWMTAQ 237
kidney bean CH5B        175:FPCAPGQQYYGRGPIQISWNYNYGQCGRAIGVDLLNKPDLVATDSVISFKSALWFWMTAQ 234
cowpea                  171:FPCASGQQYYGRGPIQISWNYNYGQCGNAIGVDLINNPDLVATDPVVSFKSAIWFWMTPQ 230
M.sativa                172:FPCASGKQYYGRGPIQISWNYNYGQCGRAIGVDLLNNPDLVATDPVISFKTALWFWMTPQ 231
M.truncatula            170:FPCASGKQYYGRGPIQISWNYNYGQCGRAIGVDLLNNPDLVATDPVISFKTALWFWMTPQ 229
pea endochitinase A2    168:FPCASGKQYYGRGPIQISWNYNYGQCGRAIGVDLLNNPDLVATDPVISFKTALWFWMTPQ 227

Leucaena Chitinase5-3   235:SPKPSCHDVITGRWTPSGADTAAGRLPGYGTTTNIINGGLECGRGQDPRVADRIGFFKRY 294
Leucaena Chitinase4-3   238:SPKPSCHDVITGRWTPSGADTAAGRLPGYGTTTNIINGGLECGRGQDARVADRIGFFKRY 297
kidney bean CH5B        235:SPKPSSHDVITSRWTPSSADVAARRLPGYGTVTNIINGGLECGRGQDSRVQDRIGFFKRY 294
cowpea                  231:SPKPSSHDVITSQWTPSAADVAAGRLPGYGTVTNIINGGLECGRGQDSRVEDRIGFFKRY 290
M.sativa                232:SPKPSCHDVITGRWSPSSADRAAGRLSGYGTVTNIINGGLECGRGQDG-VQDRIGFYKRY 290
M.truncatula            230:SPKPSCHDVITGRWSPSSADRAAGRLPGYGTVTNIINGGLECGRGQDGRVQDRIGFYKRY 289
pea endochitinase A2    228:SPKPSCHDVITGGWTPSSADRAAGRLPGYGTVTNIINGGLECGRGQDSRVQDRIGFYKRY 287

Leucaena Chitinase5-3   295:CDILGVGYGSNLDCYSQRPFGSS--SLIDVL------                        323
Leucaena Chitinase4-3   298:CDILRVGYGSNLDCYSQRPFGSS--SLIDVL------                        326
kidney bean CH5B        295:CDLLGVGYGNNLDCYSQTPFGNS--LFLSDLVTSQ--                        327
cowpea                  291:CDLFGVGYGNNLDCYSQAPFGNS--L-L-NLHPIV--                        321
M.sativa                291:CDILGVGYGANLDCFSQRPFGSSLSLSSLFLNSIDT-                        326
M.truncatula            290:CDILGVGYGDNLDCFSQRPFGSSLSLSSLFLNSIDT-                        325
pea endochitinase A2    288:CDIFGIGYGDNLDCYSQRPFGSSLPLSSILLDTVAAA                        324
```

Figure 4: Alignment of *L. leucocephala* chitinase clones with Class I Chitinases. Legend: Clones from a *Leucaena leucocephala* cDNA library were combined with corresponding 5′ RACE clones to give the leucaena chitinase 5/3.1 and 4/3.4 sequences. Translations of these sequences are aligned with the closely related class I chitinase protein sequences. The sequences aligned include the class I chitinases from: G.hirsutum, *Gossypium hirsutum*; kidney bean, *Phaseolus vulgaris*; cow pea, *Vigna unguiculata*; M.sativa, *Medicago sativa*; M.trucatula, *Medicago truncatula*; and pea, *Pisum sativum*. Sequences were downloaded from the Genbank nr database and aligned with Clustal X.

Table 2 Predicted chitinase protein properties

Leucaena chitinase.	Precursor length (A.A.)	Precursor MW. (dalton)	Precursor pI	Mature length (A.A.)	Mature MW. (dalton)	Mature pI
Clone 5/3.1	323	34,388	7.57	300	31,870	7.15
Clone 4/3.4	326	34,908	7.87	302	32,207	7.53

3.1.5 Optimum of expression system

The leucaena chitinase gene was cloned into the prokaryotic expression vector pET23d. The cloning strategy was designed such that a protein identical to a predicted mature protein

containing an additional N-terminal methionine residue and linked to His_6 at the C-terminus would be produced, depending on processing events in the host. Induction of expression at 15°C resulted in an approximately 32 kDa protein. The expressed protein was present in both insoluble inclusion bodies and soluble form from pET23d. For pET32a, a protein was produced with contained an additional N-terminal thioredoxin (Trx) tag, an S tag, a His_6 tag and cleavage sites for thrombin and enterokinase. Induction of expression at 15°C resulted in an approximately 46 kDa recombinant protein. The activity of the enzyme produced was much higher for the pET32a system.

When the *Leucaena chitinase* gene was expressed in Origami (DE3) *E. coli* using the pET32a expression vector, the crude enzyme extract from this expression showed high activity, comparable to extracts of young *Leucaena chitinase* de Wit plants. 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.

3.1.6 Purification and cleavage of recombinant protein.

The recombinant fusion protein has about 46 kDa, which contained the thioredoxin tag protein of 14 kDa and 32 kDa of chitinase. After purification using a Ni-NTA column to bind the His_6-tag, elution with imidazole and a further G150 gel filtration chromatography purification, a single band of recombinant protein was seen on SDS-PAGE at 46 kDa, as seen in Figure 5. The N-terminal fusion tag, containing the Trx, S and His tags could be removed by digestion with enterokinase. Then, the fusion tag could be removed by passing it over the Ni-NTA IMAC resin again to bind the His tag in the fusion tag (Figure 5B, lane 6).

3.1.7 Chitinase enzyme activity

The isoelectric points of leucaena chitinase from germinated seeds and recombinant protein were 7.6 and 7.5. They had the same pH and temperature optimum at pH 4.5 and 55°C (Table 4). Hydrolysis of *p*-nitrophenyl-triacetylchitotriose by *L. leucocephala* chitinase is probably a refection of the endochitnase mechanism of action of this enzyme. The endochitinolysis splitting of triacetylchitotriose from this substrate liberates *p*-nitrophenol (*p*NP) as the product, but essentially no release of *p*NP was seen from *p*-nitrophenyl-*N,N'*-diacetyl-D-glucosaminide and *p*-nitrophenyl-*N*-acetyl-D-glucosamine. The K_m calculated for the recombinant protein with colloidal chitin, pNP-$(GlcNAc)_3$ and tetra-acetyl-chitotetraose were 14.60 mg dry weight chitin/ml, 48.78 μM, and 2.05 μM, respectively. It was not active on *p*NP-GlcNAc and *p*NP-$(GlcNAc)_2$ substrates. The V_{max} values for the hydrolysis of

colloidal chitin, *p*NP-$(GlcNAc)_3$ and tetra-acetyl-chitotetraose were 0.19 μmol/min, 0.77 μ mol/min, and 2.07 μmol/min, respectively.

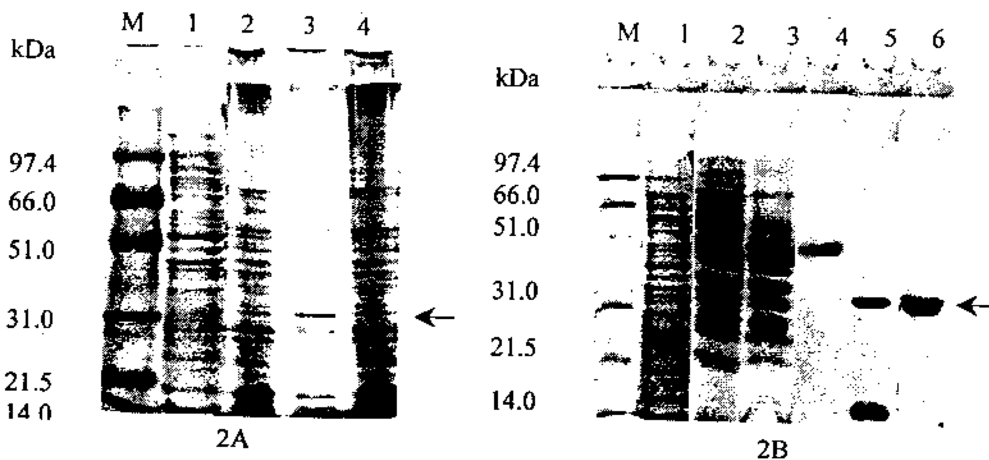

Figure 5 SDS-PAGE of chitinase protein expression. Figure 5A, 12 % SDS PAGE of the pET23d expression from *L. leucocephala* chitinase in *E.coli* BL21(DE3). BL21(DE3) cells containing the expressed plasmid were induced with 0.3 mM IPTG for 12 h at 15°C. M: molecular mass markers (kDa), 1: Control (Crude extract of *E. coli* containing pET-23d), 2: Crude *E. coli* extract of recombinant chitinase, 3: Insoluble material from pET23d-chit4/3.4 *E. coli* extract, 4: soluble material from pET23d-chit4/3.4 *E. coli* extract. Figure 5B, 12 % SDS PAGE of the pET32a expression from *L. leucocephala* chitinase in Origami (DE3) *E.coli.* Origami (DE3) cells containing the pET32a plasmid with or without insert were induced for 12 h at 15°C. M: molecular mass markers (kDa), 1: Control pET32a induced *E. coli* extract, 2: Soluble crude pET32a-chit4/3.4 *E. coli* extract, uninduced, 3: Soluble crude pET23d-chit4/3.4 *E. coli* extract induced with 0.1 mM IPTG, 4: Purified recombinant fusion protein, 5: Cleavage of recombinant protein with enterokinase 6: Purified, cleaved chitinase.

Table 3 Comparison of activity of leucaena chitnase from germinated seeds and expressed in *E. coli.*

Sample	Activity (Unit/ml)	Protein (mg/ml)	Specific activity
- Control	0.0015	2.579	0.0005
- Purification of germinated seeds	0.0073	0.350	0.0210
- Crude soluble protein pET-23d	0.0146	2.100	0.0069
- Crude soluble recombinant protein pET-32a	0.0605	2.416	0.0252
- Purified recombinant fusion protein	0.0645	1.470	0.0439
- Purified, cleaved recombinant chitinase	0.0597	1.047	0.0571

Table 4 Enzyme properties of purified chitinase from germinated seeds and recombinant protein

Chitinase	MW (kDa)	pI	pH	Tem (°C)	K_m			V_{max}		
					Colloidal chitin (mg/ml)	pNP-$(GlcNAc)_3$ (μM)	Chito-tetraose (μM)	Colloidal chitin (μmol/ min)	pNP-$(GlcNAc)_3$ (μmol/ min)	Chito-tetraose (μmol/ mni)
Germinated	32	7.6	4.5	55	-	-	-	-	-	-
Recombinant	32	7.5	4.5	55	14.60	48.78	2.05	0.19	0.77	2.07

3.1.8 Substrate specificity of leucaena chitinase

The purified chitinases from germinated seeds and recombinant protein were able to hydrolyze colloidal chitin, purified chitin, swollen chitin, glycol chitin, and chitosan, from fastest to slowest, respectively (Table 3.2.5).

Table 5 Substrate specificity of chitinase activity from *L. leucocephala*

Substrate	Enzyme activity (U/ml)		Relative activity (%)	
	Germinated Seeds (KB1)	Recombinant Protein (KB4/3.4)	Germinated Seeds (KB1)	Recombinant Protein (KB4/3.4)
Colloidal chitin	0.063	0.080	100	100
Purified chitin	0.032	0.040	50.79	50.00
Swollen chitin	0.021	0.025	33.33	31.25
Glycol chitin	0.014	0.018	22.22	22.50
Chitosan	0.012	0.015	19.05	18.75

3.1.9 Reaction products of leucaena chitinase

The hydrolysis products generated by cleavage of colloidal chitin and various oligomers of GlcNAc by recombinant protein were determined. The main products were $(GlcNAc)_2$ and $(GlcNAc)_3$ at incubation time of 30 min as shown in Figure 6. After prolonged incubation times to 24 h, the main products were GlcNAc and $(GlcNAc)_2$. This result is similar to that seen with tobacco chitinase, another class I chitinase (9).

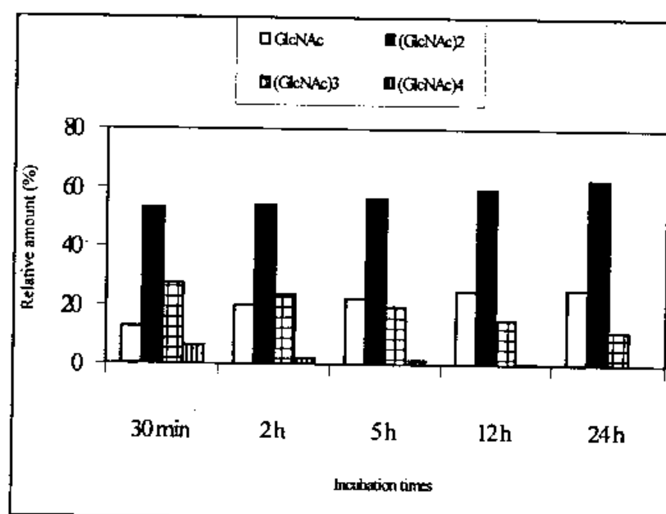

Figure 6 Analysis of hydrolysis products released from colloidal chitin by leucaena chitinase.

3.1.10 Antifungal activity of leucaena chitinase

Antifungal activities were tested on purified recombinant chitinase at concentration of 0.5, 1, 2 µg/well with 14 fungal strains. Purified recombinant chitinase inhibited *Collectotrichum* sp.1, sp.2., and *Pestalestiopsis* sp.1 at a concentration of 0.5 µg per well; *Anthanose collectotrichum, Fusarium* sp.1, *Fusarium monoliforme, Fusarium* oxysporum, *Pestalestiopsis* sp.2, sp.3, and sp4. at a

concentraion of 1 μg per well; and *Cercospora* sp., *Drechslera* sp., and *Sclerotium* sp. at a concentraion of 2 μg per well (Figure 7), whereas *Cladosporium* sp. seemed to not be inhibited at these levels.

Table 6 Inhibition of fungal growth by recombinant chitinase.

Fungal strains	Concentration of purified recombinant protein (μg/well 5mm disc)
- *Anthanose collectotrichum* (A)	1.0
- *Cercospora* sp. (B)	2.0
- *Cladosporium* sp. (-)	NI
- *Collectotrichum* sp.1 (C)	0.5
- *Collectotrichum* sp.2 (D)	0.5
- *Drechslera* sp. (E)	2.0
- *Fusarium* sp.1 (F)	1.0
- *Fusarium monoliforme*(G)	1.0
- *Fusarium* oxysporum (H)	1.0
- *Pestalestiopsis* sp.1 (I)	0.5
- *Pestalestiopsis* sp.2 (J)	1.0
- *Pestalestiopsis* sp.3 (K)	1.0
- *Pestalestiopsis* sp4. (L)	1.0
- *Sclerotium* sp. (-)	2.0

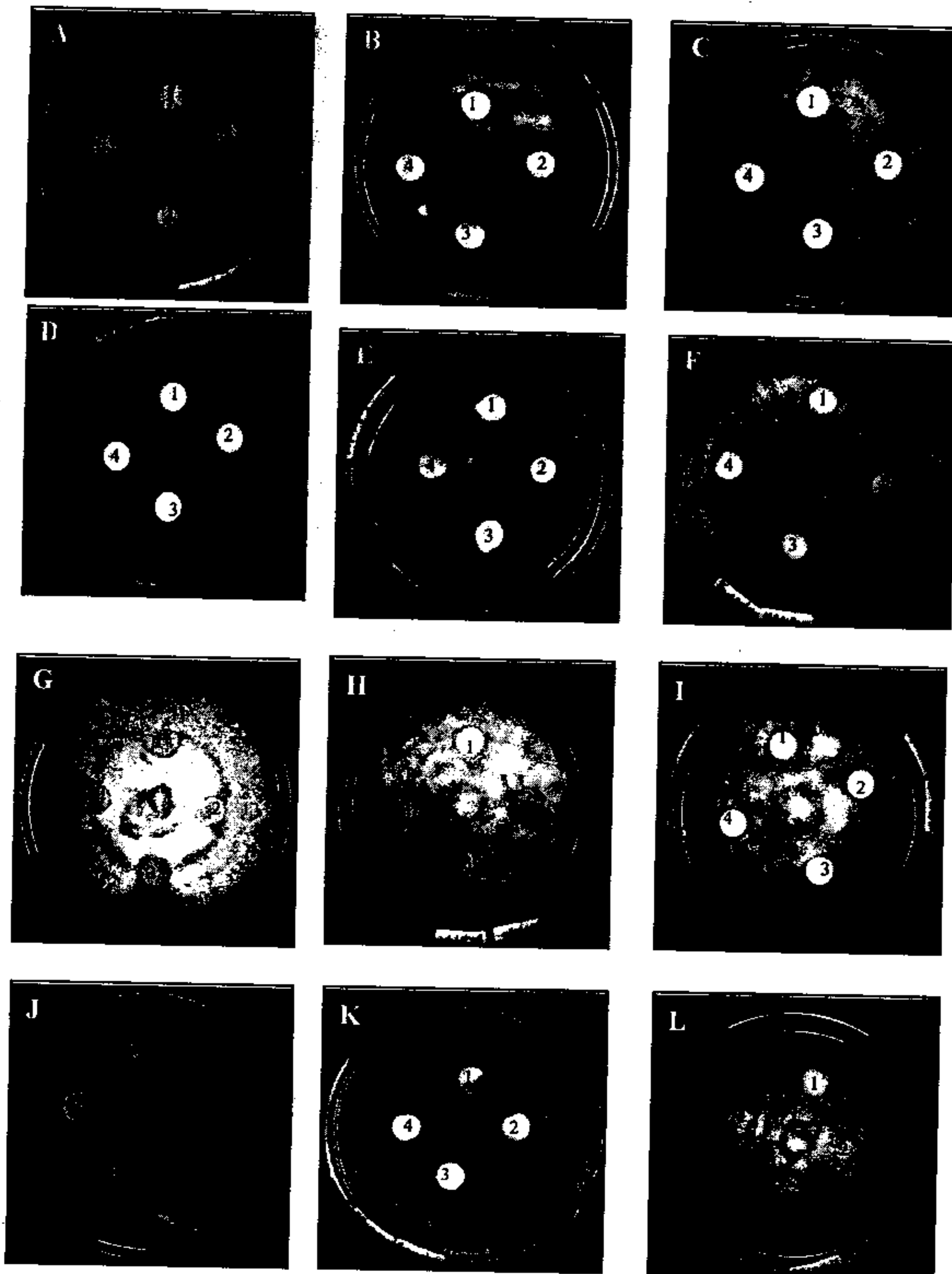

Figure 7 Antifungal activity of *L. leucocephala* chitinase against agricultural fungi isolates. Number 1, 2, 3, and 4 are control (pET32a), 0.5, 1, 2 μg per well of purified recombinant protein, respectively. The fungal strains are designated by the letters given in Table 6.

3.2 *Dalbergia nigrescens* β-glucosidase

3.2.1 Purification of β-glucosidase/β-fucosidase from *D. nigrescens* Kurz seed

The enzyme was extracted from seeds and purified by 35-75% ammonium sulfate fractionation, DEAE anion exchange chromatography and S-300 gel filtration chromatography, as described in the methods. The crude extract of *D. nigrescens* seeds contained hydrolytic activity toward both *p*NP-β-D-glucoside and *p*-NP-β-D-fucoside. Therefore, both activities were determined during the purification. The results of purification, which are summarized in Table 7, indicated the yield for both β-glucosidase and β-fucosidase were similar, but β-fucosidase total and specific activities were 2 times higher than those of β-glucosidase at each step. The enzyme was purified 33 and 49 fold over the crude extract with 2% and 3% yield for β-glucosidase and β-fucosidase, to give about 1 mg of enzyme per 50 g of seed.

Table 7 Purification of β-glucosidase/β-fucosidase from *D. nigrescens* seeds

Fraction	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification (fold)	Yield (%)
Crude extract		3040			
β-Glucosidase	6125		2.02	1.0	100
β-Fucosidase	9467		3.11	1.0	100
35-75% $(NH_4)_2SO_4$		1390			
β-Glucosidase	4220		3.04	1.5	69
β-fucosidase	8,675		6.26	2.0	92
DEAE		12.3			
β-Glucosidase	574		46.8	23	9.4
β-fucosidase	1008		82.2	26	10.6
S 300 gel filtration		1.8			
β-Glucosidase	119		66.6	33	1.9
β-fucosidase	271		152	49	2.9

3.2.2 Analytical gel electrophoresis and analytical gel filtration of β-glucosidase

The apparent mass on 10% SDS-polyacrylamide gel electrophoresis was 62.3 kDa (from a linear plot of the relative mobility of the protein against the logarithm of relative molecular mass) (Figure 8). Gel filtration of the native enzyme on Sephacryl S-300 gave a molecular weight of approximately 240 kDa, so the native enzyme appeared to consist of 4 subunits (data not show). Non-denaturing polyacrylamide gel electrophoresis of the purified enzyme followed by activity staining with 4-MU-β-D-glucoside, 4-MU-β-D-fucoside and 4-

MU-β-D-βgalactoside showed the hydrolytic activity of a single fluorogenic activity band for each substrate at the same position as the main protein band (Figure 9).

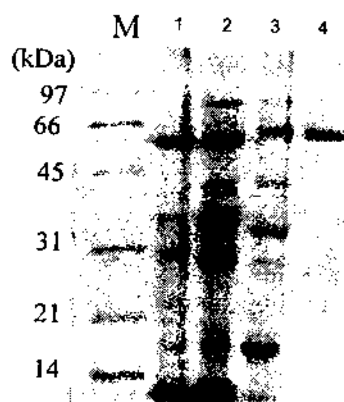

Figure 8 SDS-PAGE analysis of purified β-glucosidase/β-fucosidase enzyme from *D. nigrescens* seeds. Lane M, Low-range protein markers; 1, crude extract; 2, 35-75% $(NH_4)_2SO_4$ fraction; 3, DEAE pooled fractions; 4, S 300 gel filtration fraction.

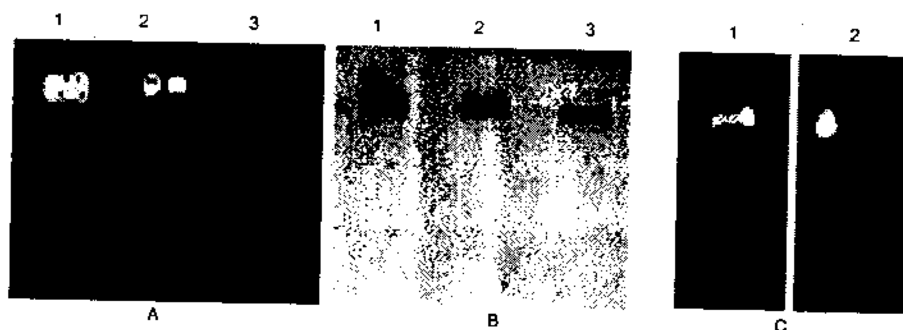

Figure 9 Activity staining of non-denaturing polyacrylamide gel electrophoresis of *D. nigrescens* β-glucosidase/β-fucosidase enzyme. A. Activity staining with 1 mM 4-MU-β-D-glucoside; lane 1, 4.8 μg; lane 2, 2.4 μg; lane 3, 1.2 μg of enzyme. B. Protein stained with Coomassie blue R-250; lane 1, 4.8 μg; lane 2, 2.4 μg; lane 3, 1.2 μg of enzyme. C. 10 μg of enzyme stained with 1 mM 4-MU-β-D-fucoside (1) and 1 mM 4-MU-β-D-galactoside (2).

3.2.3 Catalytic activity of purified β-glucosidase/β-fucosidase

The pH and temperature optima of the purified enzyme were pH 5-5.5 and 65°C, respectively, for both β-glucosidase and β-fucosidase activities (data not shown). Of the various *p*NP-glycosides tested as substrates at 5 mM (Table 8), the enzyme showed the

highest activity toward *p*NP-β-D-fucoside and *p*NP-β-D-glucoside, which was similar to *D. cochinchinensis* β-glucosidase. In contrast, *D. nigrescens* enzyme hydrolyzed *p*NP-β-D-xyloside and *p*NP-β-D-thioglucoside with relative activities 2 and 34 times higher than *D. cochinchinensis* β-glucosidase and *p*NP-β-D-galactoside with lower relative activity.

Table 8 Comparison of hydrolysis of synthetic substrates by *D. nigrescens* and *D. cochinchinensis* β-glucosidases. Enzyme reactions were performed with 5 mM glycosides in 0.1 M sodium acetate, pH 5.0 at 30°C for 10 min. Relative activities for *D. cochinchinensis* β-glucosidase are taken from Srisomsap et al. (1996).

Substrate	*D. nigrescens* % Relative activity	*D. cochinchinensis* %Relative activity
*p*NP-β-D-glucoside	100	100
*p*NP-β-D-fucoside	124	124
*p*NP-β-D-galactoside	3.97	8.95
*p*NP-β-D-xyloside	7.55	3.91
*p*NP-α-L-arabinoside	1.91	4.89
*p*NP-β-D-thioglucoside	0.68	0.02
*p*NP-β-L-arabinoside	0.47	-
*p*NP-β-D-mannoside	0.40	0.26

Release of glucose from commercially available natural glycosides and oligosaccharides was also tested (Table 9). The rates of hydrolysis were extremely low compared to the hydrolysis of *p*NP-β-D-glucoside, with (β1→3) laminaribiose releasing the most glucose and cellobiose and linamrin less. No hydrolysis of salicin, Dhurrin, DIMBOA glucoside, sophorose, cellotriose, laminaritriose, torvoside A or amygdalin could be observed. In addition, dalcochinin-8'-O-β-D-glucoside, the natural substrate of Thai rosewood β-glucosidase, was hydrolyzed dalcochinin-8'-O-β-D-glucoside as judged by TLC analysis (data not shown). However, two isoflavanoid glycosides could be isolated from *D. nigrescens* seeds appeared to be the best substrates (see below).

Table 9 Relative hydrolytic activity of purified *D. nigrescens* β-glucosidase/β-fucosidase toward natural substrates and oligosaccharides. Enzyme reactions were performed with 2 mM substrate in 0.1 M sodium acetate, pH 5.0 at 30°C for 10 min. ND indicates not detectable.

Substrate	% Relative activity
*p*NP-β-D-glucoside	100
Laminaribiose (β1→3)	1.01
Laminaritriose (β1→3)	ND
Cellobiose (β1→4)	0.33
Cellotriose (β1→4)	ND
Linamarin	0.37
Salicin	ND
Dhurrin	ND
DIMBOA glucoside	ND
Sophorose	ND
Amygdalin	ND

3.2.4 Purification of *D. nigrescens* natural glycoside substrates

In preliminary experiments, β-glucosidase from *D. cochinchinesis* was found to hydrolyzed a glycoside from *D. nigrescens* seed extract, but not as well as crude *D. nigrescens* β-glucosidase, as judged by the shift in mobility of a spot on TLC. Therefore, the *D. nigrescens* β-glucosidase was used to identify its substrates. The substrates were extracted from ground seed with methanol, back extracted with hexanol and ethylacetate, and fractionated by LH-20 gel filtration chromatography and preparative silica gel TLC to produce two pure compounds.

Compound S1 glycoside was obtained as a light yellow powder. From FTMS, this compound showed a molecular ion peak at m/z 659 $[M+Na]^+$ and future fragmentation of this peak gave a peak at m/z 365 $[M+Na]^+$. The 294 amu derived from mass subtraction indicated 2 sugar rings of five and six carbon which corresponded to the molecular formula $C_{29}O_{16}H_{32}$ for the glycoside. The UV spectrum showed the characteristic absorption of an isoflavone compound (λ^{MeOH}_{max} 260 and 315 nm). The formula $C_{18}H_{13}O_8$ is consistent with the mass of the aglycone.

Compound S2 glycoside was also obtained as a light yellow powder and had a molecular ion peak $[M+Na]^+$ at m/z 675 in MS, which corresponded to the molecular formula $C_{30}O_{16}H_{36}$. The future fragmentation of this peak gave a peak at m/z 381 $[M+Na]^+$. This indicated a hexaose and a pentaose sugar, as in the case of compound S1. The UV spectrum

showed the characteristic absorption of an isoflavone compound (λ^{MeOH}_{max} 260 and 315 nm). Analytical TLC of both substrates after digestion with enzyme or acid hydrolysis gave two sugar spots that migrated at the same R_f for both both substrates.

The K_m and k_{cat} values were determined by using *p*NP-β-D-glucoside, *p*NP-β-D-fucoside and 2 purified natural substrates, namely S1 and S2, from *D. nigrescens* seed (Table 3.2.10). The k_{cat} values of the enzyme were calculated per subunit assuming a MW of 61.85 kDa, as predicted from the predicted mature protein sequence. The data indicated that the best substrates for native and recombinant enzymes were the 2 natural glycosides, S1 and S2, isolated from *D. nigrescens* seed. The K_m of native and recombinant enzymes for S1 and S2 were similar, 0.5 and 0.7, respectively. Their K_m values were about 3 and 30 times larger than *p*NP-β-D-fucoside and *p*NP-β-D-glucoside, respectively. In addition, the enzyme showed higher k_{cat} for S1 and S2 than *p*NP-β-D-glucoside and *p*NP-β-D-fucoside, while *p*NP-β-D-fucoside was efficiently hydrolyzed than *p*NP-β-D-glucoside. This suggests that the S1 and S2 may be the natural substrates of this enzyme.

Table 10 The kinetic properties of purified native *D. nigrescens* β-glucosidase/β-fucosidase enzymes. Enzyme reactions were performed in 0.1 M sodium acetate buffer, pH 5, at 30°C for 10 min. The values of k_{cat} and k_{cat}/K_m of native enzyme were estimated assuming a subunit molecular weight of 61,858 Da.

Substrate	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
*p*NP-β-D-glucoside	14.7	10.4	876
*p*NP-β-D-fucoside	1.8	7.0	4,020
D. nigrescens S1 glycoside	0.5	465	9.9×10^5
D. nigrescens S2 glycoside	0.7	334	4.4×10^5

The effect of various compounds was tested on the hydrolysis of *p*NP-β-D-glucoside and *p*-NP-β-D-fucoside (Table 11). The most metal ions had little effect on either β-glucosidase or β-fucosidase and δ-gluconolactone was a somewhat weak inhibitor, though $HgCl_2$ and δ-gluconolactone are strong inhibitors of the closely related *D. cochinchinensis* β-glucosidase.

Table 11 Effect of metal ions and δ-gluconolactone on *D. nigrescens* β-glucosidase and β-fucosidase activities. Substances were preincubated with the enzyme for 10 min before assaying the hydrolysis of *p*NP-β-D-glucoside (2 mM) and *p*NP-β-D-fucoside (1 mM) in 0.1 M sodium acetate buffer, pH 5.0 at 30°C for 10 min.

Substrate	Final concentration	% β-D-glucosidase activity remaining	% β-D-fucosidase activity remaining
control	1 mM	100	100
$FeSO_4$	1 mM	114.8	108.6
$CaCl_2$	1 mM	115.2	109.4
EDTA	1 mM	117.6	115
$MnCl_2$	1 mM	113.3	114.1
$ZnSO_4$	1 mM	112.1	116.0
$HgCl_2$	1 mM	90.2	94.0
δ-gluconolactone	1 mM	66.1	55.2
	3 mM	55.0	39.9

3.2.5 *D. nigrescens* β-glucosidase protein sequence and recombinant expression

To find the protein sequence of the *D. nigrescens* β-glucosidase, cDNA was amplified from the RNA of immature seeds using primers from the *D. cochinchinensis* β-glucosidase to amplify fragments, followed by 3' and 5' RACE with specific primers to obtain full coding region cDNA. A unique cDNA was obtained. As expected, the amino acid sequences of the *D. nigrescens* β-glucosidase, Dnbglu1, indicated that it belonged to the glycosyl hydrolase family 1 and was most similar to dalcochinin 8′-O-β-glucoside β-glucosidase (JC7539) from the Thai rosewood, *D. cochinchinensis* Pierre with 85% amino acid sequence identity. The Dnbglu1 could not be expressed to produce active enzyme in *P. pastoris* as could *D. cochinchinensis* β-glucosidase (Ketudat Cairns et al., 2000) on several attempts with different constructs. To verify that the cloned cDNA corresponded to the purified β-glucosidase, the N-terminus and 4 tryptic peptides of the protein purified from seeds were sequenced. As shown in Figure 10, the N-terminus and the tryp1 peptide matched the cDNA exactly, tryp 2 matched, except for the absence of N at a likely glycosylation site, while the tryp 3 and 4 peptides matched Dnbglu1, but with some nonmatching residues and secondary signals, indicating there may be a mix of peptides in the fractions.

Figure 10 Protein sequence alignment of β-glucosidases. The alignment includes β-glucosidases from *D. nigrescens*; Dnbglu1, Dnbglu2, *D. cochinchinensis* (Thai rosewood), and cyanogenic β-glucosidase from white clover; 1CBG. N-terminal and tryptic peptide sequences obtained by Edman degradation of the purified protein are aligned above the full sequences with secondary signals in parentheses above the peptide. The sites indicated by Czjzek *et al* (2000) as interacting with the glycone (•) or aglycone (∇) of maize β-glucosidase are marked and the catalytic acid/base and nucleophile consensus sequences are underlined.

```
>N-terminal seq                                      ATITEV                     •
Dnbglu1_           -23 MHAMTFKAILLLGLLALVSTSASIAFAKEVRATITEVPPFNRNSFPSDFIFGTAASSYQY 37
Thai_rosewood      -23 MLAMTSKAILLLGLLALVSTSASIDFAKEVRETITEVPPFNRSCFPSDFIFGTASSSYQY 37
1CBG                 1 ---------------------------FKPLPISFDDFSDLNRSCFAPGFVFGTASSAFQY 34
ZM_Glu1              1 --------------------SARVGSQNGVQMLSPSEIPQ-RDWFPSDFTFGAATSAYQI 39

>peptide Tryp 1                                                         YMNLDAYR
Dnbglu1_            38 EG----EGRVPSIWDNFTHQYPEKIADGSNGDVAVDQFHHYKEDVAIMKYMNLDAYRLSI 93
Thai_rosewood       38 EG----EGRVPSIWDNFTHQYPEKIADRSNGDVAVDQFHRYKKDIAIMKDMNLDAYRMSI 93
1CBG                35 EGAAFEDGKGPSIWDTFTHKYPEKIKDRTNGDVAIDEYHRYKEDIGIMKDMNLDAYRFSI 94
ZM_Glu1             40 EGAWNEDGKGESNWDHFCHNHPERILDGSNSDIGANSYHMYKTDVRLLKEMGMDAYRFSI 99

>peptides Tryp 2 & 3             ASGGI-STGVD    LINETLANGI       •
Dnbglu1_            94 SWPRILPTGRASGGINSTGVDYYNRLINELLANDITPFVTIFHWDLPQALEDEYGGFLNH 153
Thai_rosewood       94 SWPRILPTGRVSGGINQTGVDYYNRLINESLANGITPFVTIFHWDLPQALEDEYGGFLNH 153
1CBG                95 SWPRVLPKGKLSGGVNREGINYYNNLINEVLANGMQPYVTLFHWDVPQALEDEYRGFLGR 154
ZM_Glu1            100 SWPRILPKGTKEGGINPDGIKYYRNLINLLLENGIEPYVTIFHWDVPQALEEKYGGFLDK 159

                                                      • Q    ∇     M∇
>peptide Tryp 4                                 HWITVNEPSIFTMNGYAYGIFAPGR
Dnbglu1_           154 ---TIVNDFRDYADLCFNLFGDRVKHWITVNEPSIFTMNGYAYGIFAPGRCSPSYNPTCT 210
Thai_rosewood      154 ---SVVNDFQDYADLCFQLFGDRVKHWITLNEPSIFTANGYAYGMFAPGRCSPSYNPTCT 210
1CBG               155 ---NIVDDFRDYAELCFKEFGDRVKHWITLNEPWGVSMNAYAYGTFAPGRCSDWLKLNCT 211
ZM_Glu1            160 SHKSIVEDYTYFAKVCFDNFGDKVKNWLTFNEPQTFTSFSYGTGVFAPGRCSPGLDCAYP 219

Dnbglu1_           211 GGDAGTEPDLVAHNLILSHAATVQVYKKKYQEHQNGIIGISLQIIWAVPLSNSTSDQKAA 270
Thai_rosewood      211 GGDAGTETYLVAHNLILSHAATVQVYKRKYQEHQKGTIGISLHVVWVIPLSNSTSDQNAT 270
1CBG               212 GGDSGREPYLAAHYQLLAHAAAARLYKTKYQASQNGIIGITLVSHWFEPASKEKADVDAA 271
ZM_Glu1            220 TGNSLVEPYTAGHNILLAHAEAVDLYNKHYKRDDTR-IGLAFDVMGRVPYGTSFLDKQAE 278

                                                                              ∇
Dnbglu1_           271 QRYLDFTGGWFLDPLTAGQYPESMQYLVGDRLPKFTTDEAKLVKGSFDFVGINYYTSSYL 330
Thai_rosewood      271 QRYLDFTCGWFMDPLTAGRYPDSMQYLVGDRLPKFTTDQAKLVKGSFDFIGLNYYTTNYA 330
1CBG               272 KRGLDFMLGWFMHPLTKGRYPESMRYLVRKRLPKFSTEESKELTGSFDFLGLNYYSSYYA 331
ZM_Glu1            279 ERSWDINLGWFLEPVVRGDYPFSMRSLARERLPFFKDEQKEKLAGSYNMLGLNYYTSRFS 338

                                                          ∇    ∇
Dnbglu1_           331 TSSDASTCCPPSYLTDSQVTFSSQR--NGVFIGPVTPSGWMCIYPKGLRDLLLYIKEKYN 388
Thai_rosewood      331 TKSDASTCCPPSYLTDPQVTLLQQR--NGVFIGPVTPSGWMCIYPKGLRDLLLYFKEKYN 388
1CBG               332 AKAPRIPNARPAIQTDSLINATFEH--NGKPLGPMAASSWLCIYPQGIRKLLLYVKNHYN 389
ZM_Glu1            339 KNIDISPNYSPVLNTDDAYASQEVNGPDGKPIGPPMGNPWIYMYPEGLKDLLMIMKNKYG 398

                              •                                                   •
Dnbglu1_           389 NPLVYITENGMDELD--DPSQSLEESLIDTYRIDSYYRHLFYVRSAIGSGANVKGFFAWS 446
Thai_rosewood      389 NPLVYITENGIDEKN--DASLSLEESLIDTYRIDSYYRHLFYVRYAIRSGANVKGFFAWS 446
1CBG               390 NPVIYITENGRNEFN--DPTLSLQESLLDTPRIDYYYRHLYYVLTAIGDGVNVKGYFAWS 447
ZM_Glu1            399 NPPIYITENGIGDVDTKETPLPMEAALNDYKRLDYIQRHIATLKESIDLGSNVQGYFAWS 458

                            ••∇∇     ∇
Dnbglu1_           447 LLDNFEWNEGFTSRFGLNFVNYT-TLTRYHKLSATWFKYFLARDQEIAKLDISAPKARWS 505
Thai_rosewood      447 LLDNFEWAEGYTSRFGLYFVNYT-TLNRYPKLSATWFKYFLARDQESAKLEILAPKARWS 505
1CBG               448 LFDNMEWDSGYTVRFGLVFVDFKNNLKRHPKLSAHWFKSFLKK----------------- 490
ZM_Glu1            459 LLDNFEWFAGFTERYGIVYVDRNNNCTRYMKESAKWLKEFNTAKKPSKKILTPA------ 512

Dnbglu1_           506 SSTMIKEEKRKPKWAIQAF 524
Thai_rosewood      506 LSTMIKEEKTKPKRGIEGF 524
```

Attempts were made to express the protein in *E. coli* as a fusion protein. The cDNA used for expression started with the codon for the amino acid corresponding to the first amino acid in Thai rosewood β-glucosidase. This was fused with N-terminal tags of thioredoxin in pET32a and a secreted disulfide isomerase C in pET40b or with just a methionine start codon in pET23d. In the case of pET32a, a large amount of protein production could be detected by SDS-PAGE, but it was insoluble and in active. In all cases, small amounts of β-glucosidase activity could be detected in the soluble fraction of the cells, but they were not clearly above the levels seen in control cells containing plasmids without inserts. However, the inclusion body (insoluble) protein may be useful for making antibodies. Attempts to express the protein in *Pichia pastoris* did not give activity and the protein in the media, as judged by SDS-PAGE could not be distinguished from media from control cells.

บทที่ 4 ข้อวิจารณ์

4.1 Discussion:

In this project, two enzymes from native Thai legumous trees were characterized, chitinase from *Leucaena leucocephala* de Wit and β-glucosidase from *Dalbergia nigrescens* Kurz. Both enzymes were purified from the plant and also had the genes cloned based on homology with other enzymes of the same type. For the chitinase, we were able to express the recombinant protein with good activity in *E. coli*, while the β-glucosidase could only be produced as an inactive protein in *E. coli*.

Two full-length coding sequences (CDS) of chitinase cDNA from *L. leucocephala* were determined. The two proteins were very similar with each other and other glycosyl hydrolase family 19 chitinases, especially class I chitinases from plants. They contained an N-terminal signal sequence that was predicted to target them to the secretory pathway, followed by a cysteine-rich domain, which is homologous to the lectin hevein from rubber tree latex. This domain would be expected to act as a carbohydrate-binding domain due to its similarity to the lectin, and has been termed the chitin-binding domain (Collinge et al., 1993). This domain was followed by a short Gly and Pro-rich region, thought to act as a flexible hinge between the carbohydrate-binding and catalytic domain, which follows it. The catalytic domain is also homologous to Class II plant chitinases, and Class IV chitinases are similar over the length of the protein to the Class I chitinases, like *L. leucocephala* 4/3.2 and 5/3.1, except for 4 small deletions, which make Class IV enzymes a bit shorter.

The expression of *L. leucocephala* chitinase as a thioredoxin fusion protein from the pET32a plasmid in Origami *E. coli* was quite successful. This system was chosen because the chitinase is expected to have several disulfide bonds, based on homology to other glycosyl hydrolase family 1 class I chitinases (Collinge et al., 1993). The Origami *E. coli* strain has mutations in genes controlling the redox level of the *E. coli* protoplasm, resulting in a more oxidizing environment in these cells, which may allow disulfide bond formation. In addition, the thioredoxin in the N-terminal thioredoxin/His6/myc-tag fusion of this construct, would be expected to help catalyze disulfide bond formation under these conditions.

The fact that the *L. leucocephala* chitinase could hydrolyze pNP-N, N′, N′′-triacetyl-D-chitotriose, but not pNP-N,N′-diacetyl-D-chitobiose or pNP-N-acetyl-D-glucosamine, suggests the enzyme acts as an endochitinase. It appears to need at least three N-acetyl-glucosamine residues at the nonreducing end to allow hydrolysis to release pNP

from these artificial substrates. However, hydrolysis of the polysaccharide, colloidal chitin, resulted in release of primarily N,N′-diacetyl chitobiose, suggesting that, either the enzyme can cut bonds with as few as one N-acetyl glucosamine on the reducing end, or that the cleavage of N-acetyl glucosamine from the chito oligosaccharides is different from release of pNP from the glycosides. When different chitinous polymers were tested, the enzyme showed the greatest ability to hydrolyze colloidal chitin and the least to chitosan. Since chitosan contains glucosamine residues rather than N-acetyl-glucosamine, this indicates the enzyme shows typical chitinase rather than chitosanase activity (Collinge et al., 1993).

The chitinase had the ability to inhibit the growth of several strains of fungi, which were originally isolated from agricultural sources. Thus, the chitinase has a potential to act as a fungal inhibitor for agricultural use. To that end, we have begun to collaborate to try to express the protein in rice and grapes and test whether it will convey fungal resistance to these plants. Though no chitinase has been shown to offer absolute protection from fungal pathogens, recombinant expression of plant chitinases in various crop plants have been shown to increase resistance to fungal pathogens. Transgenic tobacco and rape expressing a bean chitinase had increased resistance to *Rhizoctonia solani* (Broglie et al., 1991), and oilseed rape producing tomato chitinase showed a similar increased resistance to fungal disease (Grison et al., 1996). In rice, Nishizawa et al. (1999) showed the increased resistance to rice blast (*Magnaporthe grisea*) in recombinant Japonica rice overexpressing a rice chitinase. Therefore, the *L. leucocephala* chitinase may be useful in producing transgenic crops with fungal resistance in Thailand, so such technologies need not be imported from abroad.

The *Dalbergia nigrescens* β-glucosidase/β-fucosidase was found to have the ability to hydrolyze several substrates similar to the previously described *D. cochinchinensis* β-glucosidase, but to have distinct specificity toward other substrates compared to this closely related enzyme. Though *D. nigrescens* and *D. cochinchinensis* β-glucosidases had similar preference for the β-D-glucoside and β-D-fucoside among pNP-glycosides, its preference for other glycosides, like mannoside and galactoside were somewhat different. However, the major differences appeared to be in the aglycone specificity. Though the *D. nigrescens* β-glycosidase could hydrolyze the dalcochinin-8'-O-β-D-glucoside, the *D. cochinchinensis* natural substrate (Svasti et al., 1999), the *D. cochinchinensis* enzyme showed little ability to hydrolyze the S1 and S2 glycosides purified from *D. nigrescens* seeds. Also, the *D. nigrescens* enzyme could not hydrolyze torvosides A and H, which are glucosides found to be hydrolyzed by *D. cochinchinensis* β-glucosidase (Arthan et al., 2002). This suggests that the

aglycone structure is critical, as has been seen for some cyanogenic glucosidases, which display narrow specificities for aglycone moiety (Hösel and Conn, 1982). Some other glucosidases show broader specificities for aglycone moiety, such as maize, *Hevea* and almond β-glucosidase (Babcock and Esen, 1994). However, as seen for rice BGlu1, β-glucosidases that hydrolyze many substrates often show a much more rapid rate of hydrolysis of some substrates than others (Opassiri et al., 2003, 2004). *D. nigrescens* β-glucosidase can probably considered in the somewhat intermediate range of substrate specificity, but it is interesting that it displays some specificity different from the closely *related D. cochinchinensis* β-glucosidase.

In terms of inhibition, it was interesting that *D. nigrescens* showed little effect from metal ions and other inhibitors, including those that had been shown to strongly affect *D. cochinchinensis* β-glucosidase. One example is the rather low inhibition by δ-gluconolactone, which is a transitions-state-like compound that strongly inhibits many β-glucosidases. However, aldonolactones are unstable species, interacting with water and producing complex conformational depend on the temperature, pH and age. δ-gluconolactone in solution can transform into D-gluconic acid and D-glucono-1,4-lactone (Combes and Birch, 1988). The 1,5-lactone is the most effectively inhibitor but inhibition by the 1,4-lactone can explained in the term of the rapid interconversion in aqueous solution and the opening of the ring destroys the effectiveness of inhibition (Sanz-Aparicio, et al., 1998). This general explanation is not very acceptable, though, given that *D. cochinchinensis* was more strongly inhibited by δ-gluconolactone. So, it is likely due to the physical shape and properties of the *D. nigrescens* active site being slightly different, so it binds this compound less tightly. The *D. cochinchinensis* enzyme is also strongly inhibited by Hg, which did not have a significant affect on *D. nigrescens* β-glucosidase. Though Hg is often seen to bind strongly to sulfhydryls and, sometimes, incorporate into disulfide bonds, other cysteine modifying agents had little effect on *D. cochinchinensis* β-glucosidase activity, leading Srisomsap et al. (1996) to postulate that the Hg ion was somehow chelated in the active site. The fact that almost all Cys residues are conserved between the two enzymes, including all those near the active site, supports this argument and, again, suggest some small difference in the active site properties between *D. cochinchinensis* and *D. nigrescens* β-glucosidases.

Multiple sequence alignment placed E182 and E396 (bold in the underlined residues in Figure 3.2.10) in the positions of the catalytic acid/base and nucleophile, respectively. These residues are found in the TXNEP and ITENG motifs which are highly conserved in all

glycosyl hydrolase family 1 β-glucosidases, and the corresponding residues demonstrated to served as the general acid/base catalyst and nucleophile by site directed mutagenesis in *Agrobacterium feacalis* β-glucosidase (Withers et al.,1990; Withers et al.,1992 and Wang et al., 1995) and kinetic and mutational studies in cassava β-glucosidase (Kerrestessy et al., 1994, 2001). Compared with known three dimensional structure of cyanogenic β-glucosidase from white clover (1CBG), the conserved E182 and E396 were found close to the carboxy terminal ends of β-strands 4 and 7 of the $(\beta/\alpha)_8$ barrel, respectively. Czjzek et al. (2000) determined the x-ray crystal structure of maize β-glucosidase with its natural substrate, DIMBOAGlc, and found that the aglycone moiety of the substrate is sandwiched between W378 on one site and F198, F205 and F466 on the other. The W378 position is highly conserved among family 1 plant β-glucosidases, but the phenylalanine residues are not, so they were proposed to allow for substrate specificity. The residues equivalent to F198, F205, W378 and F466 are N189, F196, W369, and N/A454, respectively, in *Dalbergia nigrescens/D. cochinchinensis* β-glucosidases. Four addition residues, T334, M374, A467 and Y473 in maize β-glucosidase, may be involved in aglycone binding and the equivalent residues are T326, T364, E455 and F461 in the *Dalbergia* β-glucosidases. Because W369 (maize W378) is conserved among family 1 β-glucosidases, but all other residues in this group are not, their interaction with the substrate may differ and provide the basis of aglycone recognition and specificity in these β-glucosidases (Czjzek et al., 2000). So, the different amino acids at residue 454 in the *D. nigrescens* and *D. cochinchinensis* enzymes may cause the different substrate specificity between these enzymes, so Thai rosewood cannot hydrolyze the S1 and S2 natural substrates of *D. nigrescens* β-glucosidase, while *D. nigrescens* cannot hydrolyze the torvosides.

The glycone binding pocket of plant β-glucosidase contains the most highly conserved residues, as Czjzek et al (2000) noted. In maize β-glucosidase, these residues were Q38, H142, E191, E406, E464 and W465, equivalent to Q36, H136, E182, E396, E452 and W453 in the *Dalbergia* β-glucosidases. In addition, W445, which was highly conserved among family 1, was found to make stacking interaction with the plane defined by the ring-like gluconate chain in the *Bacillus polymyxa* β-glucosidase A/δ-gluconolactone crystal structure (Sanz-Aparicio et al., 1998), so it also seems to be involved in sugar binding. This interaction is typical for sugar binding sites of proteins, with hydrogen bonds between the sugar hydroxyls and polar side chains providing binding affinity and specificity, and aromatic residues oriented against the hydrophobic faces of sugar (Vyas, 1991). Since these sugar-

binding residues are all the same for the *Dalbergia* (and other) β-glucosidases, any differences in sugar-binding, which appear to be rather subtle, may be due to subtle differences in the positioning of these residues within the active site, or to other, as yet unnoted residues.

The two glycoside substrates from *D. nigrescens* appear to be isoflavanoids with a disaccharide attached to a hydroxyl, though the full structures are yet to be elucidated. The similar sugar specificity and completely conserved sugar binding sites of the *Dalbergia* β-glucosidases suggest that the difference in ability to hydrolyze these substrates is likely due to the aglycone moiety, but this is yet to be proven. The glycosides and aglycones will be further investigated for their bioactivity and complete structures, which are no doubt unique among substrates for which the enzyme has been characterized at the molecular level. Knowing these substrates structures will help us to learn how to engineer the enzymes better, so that they may use to hydrolyze or synthesize products of interest.

4.2. Conclusions and Comments

In this project, we cloned and sequenced chitinase from *Leucaena leucocephala* de Wit and β-glucosidase from *Dalbergia nigrescens* Kurz, purified these enzymes and characterized their activities. The chitinase was found to prefer colloidal chitin as a substrate and to have significant antifungal activity, which suggested it may be useful for agricultural applications. In fact, projects are currently underway to test its affect against fungal diseases in grapes and rice. The β-glucosidase showed a unique isoflavonoid specificities and catalytic properties that differed from the closely related β-glucosidase from *D. cochinchinensis* Pierre, which allows some amino acids to be noted for future investigation of structure-function relationships. This may allow engineering of β-glucosidases for applied functions, such as cleaving vitamin glycosides in feeds and aromatic glycosides in beverages, as well as other applications, in the future. So, this work has developed the background for application of these enzymes. In addition, it has provided at least part of the training for 3 Ph.D. students and a research assistant to help develop Thailand's technological workforce for biochemical education and biotechnology development.

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1. Principle investigator: Isolation and Characterization of Rice β-glucosidase cDNAs
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