

# Characterization of recombinant chitinase-like proteins of *Drosophila melanogaster* and *Tribolium castaneum*

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## Abstract

Insect chitinase (CHT) family proteins are encoded by as many as 16 genes depending upon the species of interest. We have classified these proteins in three species into five different groups based on amino acid sequence similarities (Zhu et al., companion paper). The functions of most of the individual proteins of this family during growth and development are largely unknown. To help determine their enzymatic properties and physiological roles, we expressed representative members belonging to this protein family from *Drosophila melanogaster* (Dm) and *Tribolium castaneum* (Tc), and characterized their kinetic and carbohydrate-binding properties. Seven proteins, including DmCHT 4, 5, 9 and DmDS47 from *Drosophila*, and TcCHT5, TcIDGF2 and TcIDGF4 from *Tribolium*, belonging to groups I, IV or V of the chitinase-like family were expressed in a baculovirus-insect cell line expression system, purified and characterized. Their enzymatic and chitin-binding properties were compared to those of the well-characterized chitinase, MsCHT535, from *Manduca sexta* (Ms). All of these proteins, except those belonging to group V that are related to imaginal disc growth factors (IDGFs), exhibited chitinolytic activity against the long polymeric substrate, CM-Chitin-RBV, and/or the short oligomeric substrate, MU-(GlcNAc)<sub>3</sub>. TcCHT5, DmCHT5 and MsCHT535, which are members of group I chitinases, cleaved both polymeric and oligomeric substrates. Their enzymatic properties, including pH optima, kinetic parameters, and susceptibility to substrate inhibition by chitooligosaccharides, were similar. Two group IV chitinases, DmCHT4 and DmCHT9, also were characterized. DmCHT4 had one optimum pH of 6 towards the polymeric substrate and no detectable chitinolytic activity towards an oligosaccharide substrate. DmCHT9 had high activity from pH 4 to 8 towards the polymeric substrate and exhibited low activity towards the oligosaccharide substrate. The group V proteins, TcIDGF2 and TcIDGF4, contain all of the catalytically critical residues within conserved region II of family 18 chitinases but neither exhibited chitinolytic activity. Another group V protein, DmDS47, which lacks the critical glutamate residue in region II and the C-terminal CBD, also exhibited no chitinolytic activity. However, all three of the group V proteins bound to chitin tightly. A comparison of the amino acid sequences and homology model structures of group V proteins with enzymatically active members of the chitinase family indicated that the presence of additional loops of amino acids within the ( $\beta\alpha$ )<sub>8</sub>-barrel structure of these proteins interferes with productive substrate binding and/or catalysis.

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**Keywords:** Insect; *Drosophila melanogaster*; *Tribolium castaneum*; Gene family; Enzymes; Properties; Chitin-binding; Imaginal disc growth factor

## 1. Introduction

Genes encoding chitinase-like proteins in dipteran and coleopteran insects constitute a large gene family of from 13 to 16 members depending upon the species (Kramer and Muthukrishnan, 2005). To date, a large number of putative chitinase-family genes have been identified from the completed databases of the genomes of three species,

**Abbreviations:** CBD, chitin binding domain; CHT, chitinase; GlcNAc, N-acetylglucosamine; NAG,  $\beta$ -N-acetylglucosaminidase; Tc, *Tribolium castaneum*; IDGF, imaginal disc growth factor

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*Tribolium*, *Drosophila* and *Anopheles*. Even though the genomes of lepidopteran and hemipteran insects have not been completely analyzed, available data indicate that even in these orders, chitinase-like proteins also are encoded by multiple genes. Phylogenetic analyses of 62 characterized insect chitinase-like proteins resulted in a tree made up of five groups of related proteins (Zhu et al., companion paper). Group I contains the prototypical and well-studied molting-associated chitinases from dipteran, lepidopteran and coleopteran insects. Group I enzymes are produced primarily by the epidermis and consist of one catalytic domain and one chitin-binding domain (CBD) together with an interconnecting serine/threonine (S/T)-rich linker domain. Their apparent function is to facilitate insect molting by degrading chitin in the exoskeleton. Chitinases with four or five catalytic domains and CBDs make up group II and group III enzymes have two catalytic domains and a CBD. Group IV includes both fat body- and/or gut-specific chitinases. Group V consists of the imaginal disc growth factors (IDGFs) that are chitinase-like in amino acid sequence but are devoid of catalytic activity and CBDs. Little is known about the functions of most of these groups of chitinase-like proteins or their properties with the exception of the group I enzymes and one group IV enzyme (Genta et al., 2006). We have compared some of the biochemical and kinetic properties of representative members of chitinase-like proteins from different groups found in two different species. One or two genes from each group were selected and cDNAs were either purchased or cloned by RT-PCR. Utilizing a baculovirus–insect cell line expression system, we successfully expressed and purified several chitinase-like proteins belonging to three of these groups and compared their properties. Group I chitinases efficiently cleaved both polymeric and oligomeric substrates. Group IV enzymes, however, preferred the soluble

polymeric substrate. Although the *Drosophila* IDGF, DmDS47, and the two *Tribolium* IDGFs, TcIDGF2 and TcIDGF4, belonging to group V, were devoid of chitinolytic activity, they acted as carbohydrate-binding proteins and bound very tightly to an insoluble ligand, colloidal chitin. Attempts to express chitinase genes encoding proteins belonging to groups II and III with multiple catalytic domains were unsuccessful.

## 2. Materials and methods

### 2.1. Construction of recombinant baculoviruses containing the coding regions of chitinase-like genes from *Drosophila* and *Tribolium*

Full-length cDNAs corresponding to *TcCHT5*, *TcIDGF2* and *TcIDGF4* were amplified from a larval cDNA preparation as described in Zhu et al. (accompanying manuscript). The full-length cDNA clones corresponding to *DmCHTs 4, 5, 7, 9, 10* and *DmDS47* were purchased from the *Drosophila* Genomics Resource Center at Indiana University (<http://dgrc.cgb.indiana.edu/>). These cDNAs were used as templates along with pairs of forward and reverse primers that were designed based on the sequence of each gene targeted for expression to amplify the protein coding sequences (Table 1). The primers contained appropriate restriction enzyme recognition sites to facilitate directional cloning of the coding sequence fragments into the pVL1393 (BD Pharmingen, San Diego, CA) expression vector behind the polyhedrin promoter. The reverse primers used for amplification of *DmCHT4, 5, 7, 9, 10, DmDS47, TcIDGF2* and *TcIDGF4* also contained “6-His tag” coding sequences immediately before the termination codon.

Table 1  
Primers used to amplify the coding region fragments of *Tribolium* chitinase-like proteins

Gene	Primers	Primer sequence (5'–3')
<i>TcCHT5</i>	TcCht5F6	<u>CGCGGATCC</u> ATGAAGCTGCTCCTAGCTGTAGC
	TcCht5R12	CCGGAATTC <b>T</b> TATTATTGTTCAATCTTTTTGCATTTTC
<i>TcIDGF2</i>	TcIDGF2F1	TGCTCTAGAAATGGGCTCCTTCAAGCCACTTC
	TcIDGF2R1	GAAGATCT <b>T</b> TAATGATGATGATGATGATGCAAAATGCGTTTTAGCGGTTG
<i>TcIDGF4</i>	TcIDGF4F1	TGCTCTAGAAATGAAAATTCTTATTTTTGCCT
	TcIDGF4R1	GAAGATCT <b>T</b> TAATGATGATGATGATGATGCAATCCAAGCTTAGCGGCACGC
<i>DmCHT4</i>	DmCht4F1	TCCCCCGGGATGAAGTTAGTATCGATTTGGGCATTG
	DmCht4R1	CCGGAATTC <b>T</b> TAATGATGATGATGATGATGCGGCCAGTCGCAATTTAAGGTAATG
<i>DmCHT5</i>	DmCht5F1	GCTCTAGAAATGGTTCGATATATTCGCTCCTAC
	DmCht5R1	GAAGATCT <b>T</b> TAATGATGATGATGATGATGATGCGGCCCTCGTTGCCCTTCTCC
<i>DmCHT7</i>	DmCht7F1	TCCCCCGGGATGTTTTCCACCGCGGTTGCTGCG
	DmCht7R1	CCGGAATTC <b>T</b> TAATGATGATGATGATGATGATGGGGCATCGATTCCATAATATTA
<i>DmCHT9</i>	DmCht9F1	TCCCCCGGGATGGGAAAGTTATTGGCACTACTC
	DmCht9R1	CCGGAATTC <b>T</b> TAATGATGATGATGATGATGATGCCAACGCAGCTTCTGTGTATG
<i>DmCHT10</i>	DmCht10F1	TCCCCCGGGATGCATCCGTATGTCCCAAGTGTG
	DmCht10R1	ATAAGAAATGCGGCCGCTTAATGATGATGATGATGAATGCAGTCCTTAGCCTTTGTCCAG
<i>DmDS47</i>	DmDS47F1	TCCCCCGGGATGATCATTAAAGCCCTAGCGATC
	DmDS47R1	CCGGAATTC <b>T</b> TAATGATGATGATGATGATGATGGTTGGTCAGACGATACTTAATGGC

Restriction enzyme sites are underlined. Translation start points and termination codons are in bold.

PCR fragments containing the coding sequences for chitinase-like proteins as well as pVL1393 plasmid DNA were digested with the desired restriction enzymes. Digestions were carried out at 37 °C for 2 h. Digestion products were subjected to electrophoresis in a low melting agarose gel and DNA was recovered and purified using Quantum Prep Freeze 'N Squeeze DNA gel extraction spin columns (Bio-Rad, Hercules, CA). After column purification of the digested DNA fragments, ligation was carried out for 15 min in a 10 µl reaction mixture that contained 100 ng pVL1393 DNA, 10 ng insert DNA, and 1 µl (400 units) T4 DNA ligase (New England Biolabs, Beverly, MA). After incubation for 15 min, the ligated products were used directly for transformation of *E. coli* JM109. After screening of the clones, recombinant pVL1393 plasmid DNA was prepared from the positive clones containing the desired inserts and subjected to DNA sequencing to verify correctness of the expression constructs.

Recombinant baculoviruses for expression of the chitinase-like genes were constructed using BaculoGold<sup>®</sup> DNA and 1 µg of recombinant baculovirus transfer vector pVL1393 containing the ORF for the desired chitinase-like protein as described previously (Gopalakrishnan et al., 1995) and amplified to obtain a high titer virus. An aliquot of this viral stock was used to prepare baculovirus genomic DNA according to the method of O'Reilly et al. (1994). The purified viral genomic DNA was used as a PCR template along with appropriate forward and reverse primers and PCR products were subjected to DNA sequencing at the Kansas State University Sequencing and Genotyping Facility (Manhattan, KS).

## 2.2. Purification of recombinant proteins expressed in Hi-5 cells

Hi-5 cells (*Trichoplusia ni* cell line) were used to express the desired proteins. Cells were seeded in 225 cm<sup>2</sup> flasks at 50–70% confluence. After the cells had attached to the flasks, high titer recombinant baculoviruses (10<sup>8</sup> pfu/ml) were added. The cells were incubated for 3 d at 27 °C. The medium was collected by centrifugation at 5000g for 10 min. SDS-PAGE followed by Coomassie Brilliant Blue staining was used to estimate the expression level of the proteins in the medium into which the expressed protein was secreted.

The supernatant was dialyzed against 20 mM sodium phosphate buffer (pH 8) overnight at 4 °C. To purify the recombinant proteins, DmCHT4, 5, 7, 9, 10, DS47, TcIDGF2 and TcIDGF4, all containing a six His tag [(His)<sub>6</sub>] at the C-terminal of the protein, Ni-NTA agarose chromatography was used. The dialyzed medium was mixed with Ni-NTA agarose at 4 °C for 1 h. Then the mixture of resin and medium was loaded onto a small column and washed with 50 ml washing buffer that contained 50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl and 20 mM imidazole at pH 8. The bound proteins were eluted with a 20 to 250 mM imidazole gradient. The fractions were

checked for protein content by SDS-PAGE and assayed for chitinolytic activity. The fractions containing recombinant proteins and free of other contaminants were pooled and concentrated using a Centrprep<sup>®</sup> ultrafilter (Amicon, Bedford, MA) with the desired mass cut-off consistent with the molecular weight of the protein being purified.

TcCHT5 was purified using DEAE-Sepharose column chromatography. After dialysis against 20 mM sodium phosphate buffer (pH 8.0), the medium was loaded onto an equilibrated DEAE-Sepharose (Sigma, St. Louis, MO) column (1.5 × 20 cm). The column was washed with the dialysis buffer followed by a 140 ml NaCl gradient ranging from 0 to 0.35 M (2 ml/min). The absorbance of every other 2 ml fraction was measured at 280 nm. The same fractions were also checked for protein content by SDS-PAGE and western blotting, and finally assayed for chitinolytic activity. The fractions containing the recombinant proteins and free of contaminants were pooled and concentrated using a Centrprep<sup>®</sup> ultrafilter with a 30 kDa mass cut-off (Amicon, Bedford, MA).

## 2.3. N-terminal amino acid sequencing

Proteins were separated using SDS-PAGE and then transferred onto a PVDF membrane. Coomassie Brilliant Blue R-250 was used to stain the proteins on the membrane. The apparent molecular weights of purified proteins were estimated by a comparison of their mobilities to those of standard size marker proteins. The desired recombinant protein bands were excised and subjected to N-terminal sequence analysis by automated Edman degradation using an Applied Biosystems Sequencer at the Kansas State University Biotechnology Core Facility (Manhattan, KS).

## 2.4. Immunoblot analysis

Purified protein samples were separated by 12% SDS-PAGE. After electrophoresis, the proteins in the gel were transferred to a PVDF membrane using the Semi-Dry Transfer System (Bio-Rad, Hercules, CA) and subjected to western blot analysis using the polyclonal antibody, anti-MsCHT386 (Arakane et al., 2003), together with the horseradish peroxidase-conjugated secondary antibody detection system (Bio-Rad, Hercules, CA).

## 2.5. Enzyme activity

Purified enzymes were mixed with the polymeric substrate, carboxymethyl Remazol Brilliant Violet-Chitin (CM-Chitin-RBV, Loewe Biochemica, Munich, Germany) and incubated for 2 h. The reaction rate was linear during this period. The reaction was terminated by the addition of 0.1 ml of 2 N HCl, after which the mixture was placed on ice for 15 min and then centrifuged at 12 000 g for 5 min. The absorbance of the supernatant was measured at 550 nm. A zero time control was used to determine

background fluorescence, which was subtracted from the experimental values of the reaction samples. One unit of enzyme activity was defined as the amount of enzyme that resulted in an  $\Delta A_{550}$  of 1.0/h.

The enzyme reaction using the oligosaccharide substrate, 4-methylumbelliferyl  $\beta$ -*N*, *N'*, *N''*-triacetylchitotrioside [4MU-(GlcNAc)<sub>3</sub>, Sigma, St. Louis, MO] in a total volume of 50  $\mu$ l was stopped by addition of a 12.5  $\mu$ l of 2 N HCL. The reaction mixture was diluted 400 times with 0.15 M glycine–NaOH, pH 10.5. A 2 ml portion of the mixture was used to determine the released methylumbelliferone (MU) by fluorescence spectrophotometry (DyNA Quant 200 Fluorometer, Hoefer) (Hollis et al., 1997) using an excitation wavelength of 365 nm and an emission wavelength of 460 nm. A standard curve for free 4MU was used to determine the amount of the products formed. One unit of enzyme activity was defined as the amount needed to release 1 nmole of 4 MU/min.

### 2.6. Activity–pH profile

Chitinase activity as a function of pH was determined using the polymeric substrate, CM-Chitin-RBV, and the oligosaccharide substrate, 4MU-(GlcNAc)<sub>3</sub> as substrates. For the polymeric substrate, 0.5  $\mu$ g protein was diluted to 10  $\mu$ l with water and incubated with 100  $\mu$ l of CM-Chitin-RBV (2 mg/ml) and 290  $\mu$ l of 0.2 M universal buffer (Britton and Robinson, 1931), pH 3–12, at 37 °C for 2 h. To stop the reaction, 100  $\mu$ l of 2 N HCl was added.

For the oligosaccharide substrate, 2  $\mu$ l of enzyme preparation (0.1  $\mu$ g protein) was mixed with 16  $\mu$ l 4MU-(GlcNAc)<sub>3</sub> (0.2 mM) and 32  $\mu$ l universal buffer (0.2 M), pH 3–12. The reactions were incubated at 37 °C for 15 min. To stop the reactions, 12.5  $\mu$ l of 2 N HCl was added.

### 2.7. Enzyme kinetics assays

Kinetic parameters were determined by using the polymeric substrate, CM-Chitin-RBV, or the oligosaccharide substrate, 4MU-(GlcNAc)<sub>3</sub>. In the CM-Chitin-RBV assay, 100  $\mu$ l of enzyme (0.5  $\mu$ g), 200  $\mu$ l 0.1 M Tris-HCl (pH 9) and 100  $\mu$ l of CM-Chitin-RBV (concentration ranging from 0.1 to 1 mg/ml) were mixed together and the reaction was carried out at 37 °C for 1 h. In the assays with 4MU-(GlcNAc)<sub>3</sub> as the substrate, the reaction was carried out at 37 °C for 10 min by mixing 2  $\mu$ l (0.1  $\mu$ g) protein and 48  $\mu$ l of 4 MU-(GlcNAc)<sub>3</sub> (ranging from 20 to 200  $\mu$ M), which was dissolved in 0.1 M sodium phosphate, pH 6. The kinetic parameters,  $K_m$  and  $k_{cat}$ , were calculated by nonlinear least-square fitting using the double reciprocal plot method.

### 2.8. Colloidal chitin-binding properties

Crab-shell chitin (Sigma, St. Louis, MO) was used to prepare colloidal chitin as described by Shimahara and Takiguchi (1988) and suspended at a final concentration of

1  $\mu$ g/ $\mu$ l. The chitin-binding assay was done as described by Arakane et al. (2003). The chitin-binding assay was initiated by mixing 50  $\mu$ l of the chitin suspension (10 mM phosphate, pH 8) with 1  $\mu$ g of protein in an Eppendorf tube in a total volume of 100  $\mu$ l. Then 50  $\mu$ l of the protein–chitin mixture was removed and stored as the starting fraction. This mixture was incubated with end-over-end rotation for 1 h at room temperature and then centrifuged for 10 min. The supernatant was removed and saved as the unbound fraction. Then the pellet was washed one time with 50  $\mu$ l of 10 mM sodium phosphate buffer (pH 8), followed by centrifugation. The supernatants were saved as the wash fractions. The pellet was resuspended and washed with 10 mM sodium phosphate buffer containing 1 M NaCl (pH 8), and then with 0.1 M acetic acid. Finally, 50  $\mu$ l of 1 X SDS-PAGE sample buffer was added to the pellet and the mixture boiled for 5 min. The supernatant was collected as the bound fraction. All samples were subsequently analyzed by SDS-PAGE (12% gel) followed by staining with Coomassie Brilliant Blue.

### 2.9. Homology modeling of *Tribolium putative chitinase-like proteins*

The SWISS-MODEL program (<http://swissmodel.expasy.org//SWISS-MODEL.html>) was used to generate homology models of the catalytic domains of *Tribolium putative chitinase-like proteins*. TcCHT5 homology modeling was performed using human chitotriosidase (PDB entry code 1lq0A) (Fusetti et al., 2002) as the template. *Drosophila* IDGF2 (PDB entry code 1jndA) (Varela et al., 2002) was used as the template for homology modeling of TcIDGF2.

## 3. Results

### 3.1. Expression of TcCHT5, TcIDGF2, TcIDGF4, DmCHT 4, 5, 7, 9, 10, and DmDS47 in Hi-5 cells

We attempted to express at least one representative member of each of the five groups of chitinase-like family of proteins from *Tribolium* and/or *Drosophila* in a baculovirus–insect cell expression system. These five groups contain members that differ in the number of catalytic domains and/or CBDs (Zhu et al., companion paper; Fig. 1). Each construct for expression of a chitinase-like protein contained its own signal peptide-coding region and was under the control of the polyhedrin promoter. Therefore, the recombinant proteins were predicted to be expressed late in the infection process and to be secreted into the medium. TcCHT5, TcIDGF2, TcIDGF4, DmCHT 4, 5, 9 and DmDS47 proteins of the expected sizes were detected in the medium by immunoblotting and/or as a prominent band by Coomassie blue staining in SDS-PAGE gels, which were not present in control media. A protein of the expected size of DmCHT7 was found in the cell pellet fraction but not in the medium (data not



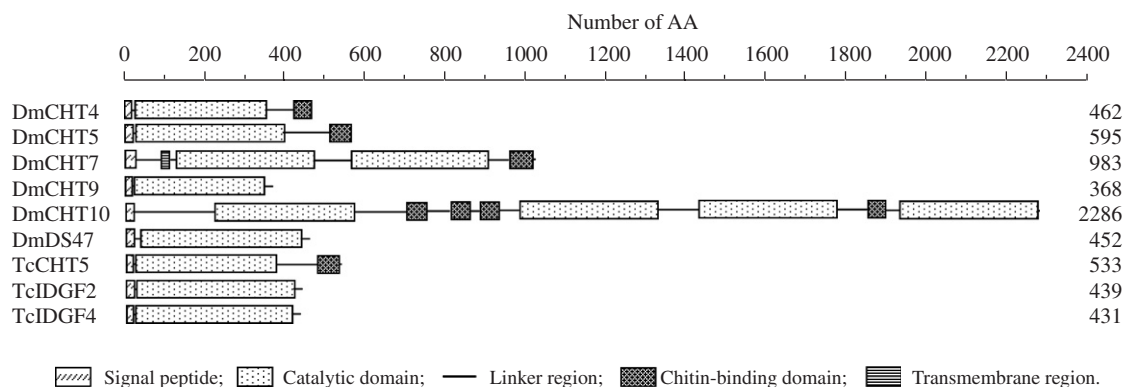


Fig. 1. The domain structures of insect chitinase-like proteins, DmCHT 4, 5, 7, 9, 10 and DmDS47 from *Drosophila* and TcCHT5, TcIDGF2 and TcIDGF4 from *Tribolium*.

shown), a result consistent with the prediction that this protein has a membrane-anchoring segment at the N-terminus (Zhu et al., companion paper). However, we have not yet been able to purify DmCHT7 to study its biochemical properties in detail. No protein corresponding to the expected size of DmCHT10 (~300 kDa) was detected in either the culture medium or cell lysate. The reason for the failure to detect expression of this chitinase with multiple catalytic domains is unknown. The expression levels of TcCHT5 and DmCHT5 were estimated to be approximately 10 mg protein/l of culture medium. The yields of TcIDGF2, TcIDGF4, DmCHT4, 9 and DmDS47 were five-fold lower at about 2 mg/l, but nonetheless all of the yields were sufficient for purification of these proteins by standard chromatographic methods.

### 3.2. Analysis of chitinase family proteins by SDS-PAGE and N-terminal sequencing

The electrophoretic mobilities of all of the eight purified proteins, TcCHT5, TcIDGF2, TcIDGF4, DmCHT4, 5, 9 and DmDS47, were determined by SDS-PAGE along with those of a set of standard protein size markers (Fig. 2A and C). The apparent molecular weights estimated from SDS-PAGE were 67, 53, 52, 51, 83, 40 and 51 kDa for TcCHT5, TcIDGF2, TcIDGF4, DmCHT4, 5, 9 and DmDS47, respectively. The theoretical molecular sizes of these proteins were 58, 47, 46, 48, 65, 39 and 48 kDa for TcCHT5, TcIDGF2, TcIDGF4, DmCHT4, 5, 9 and DmDS47, respectively. The experimentally determined values were larger than those predicted from their amino acid sequences after cleavage of the leader peptide. The most striking example of this anomalous migration is reflected in the case of DmCHT5. Its apparent molecular weight estimated by SDS-PAGE was 83 kDa, which is much larger than the size of 65 kDa predicted from its amino acid sequence. The slower than predicted migration of these recombinant proteins is probably due to glycosylation, as has been observed with other insect chitinases (Gopalakrishnan et al., 1995; Zheng et al., 2002; Arakane et al., 2003). For comparison, the *Tribolium* homolog

TcCHT5 apparently has a lower extent of glycosylation compared to DmCHT5.

The N-terminal amino acid sequences of all of the chitinase-like proteins purified from the culture media of Hi-5 cells were in agreement with the predicted sequences of the mature proteins, after cleavage of the leader peptide, indicating the occurrence of proper processing and secretion of these proteins by the insect cell line (data not shown).

### 3.3. Immunological cross-reactivity of chitinase-like family proteins

For immunological analysis of the chitinase-like family proteins, we used an antibody raised against MsCHT386, a truncated form of *M. sexta* chitinase expressed in the insect cell line, which consisted of the catalytic domain plus only 10 amino acids of the linker region (Arakane et al., 2003). As shown in Fig. 2B and D, anti-MsCHT386 antibody recognized DmCHT5 and TcCHT5, which belong to group I chitinases. This antibody, however, did not recognize DmCHT4 and DmCHT9 at 100 ng levels, both of which belong to group IV. DmCHT4 and DmCHT9 were detected by the antibody as very faint bands only when 400 ng of purified recombinant proteins were loaded onto the gel, indicating only a very weak cross-reactivity (data not shown). Anti-MsCHT386 antibody also did not cross-react with group V chitinase-like IDGFs, DmDS47 at a 400 ng level, or TcIDGF2 or TcIDGF4 even at a 1000 ng level. These results suggested that group I chitinase-like proteins belonging to dipteran and coleopteran lineages share antigenic determinants with a lepidopteran chitinase, but members of other groups of chitinase-like proteins even from the same species do not contain comparable determinants.

### 3.4. Colloidal chitin binding properties of chitinase-like family proteins

The presence within *Drosophila* and *Tribolium* chitinase-like proteins of sequences homologous to chitin-binding

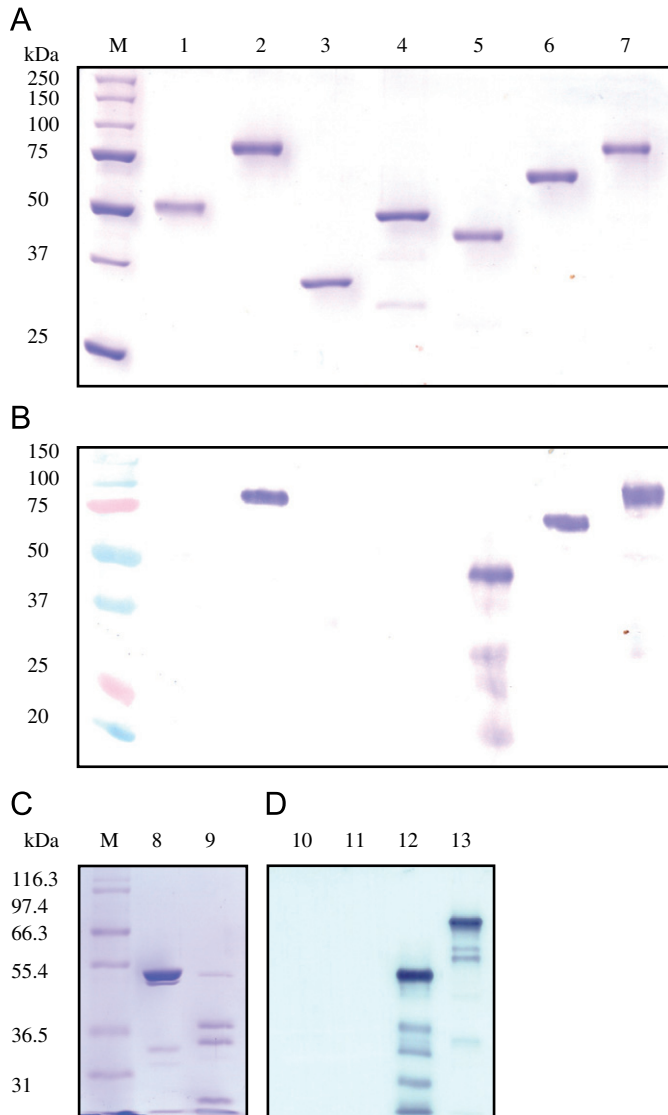


Fig. 2. SDS-PAGE analysis and immunoblotting of recombinant *Drosophila* and *Tribolium* chitinase-like proteins. Panels A and C: electrophoretic analysis of purified chitinases. One  $\mu\text{g}$  of the purified recombinant proteins were analyzed by SDS-PAGE followed by staining with Coomassie blue; panels B and D: immunoblots. A 100 ng (panel B) or 1  $\mu\text{g}$  (panel D) of purified recombinant proteins were subjected to SDS-PAGE run in parallel with the gels in panels A and C and probed with anti-MsCht386 antibody that was raised in a rabbit using purified recombinant *Manduca* Cht386 protein purified from insect cells (Arakane et al., 2003). Purified recombinant *Manduca* chitinase MsCht386 and MsCht535 were also analyzed as positive controls. Lanes: M, protein standard size markers; 1, DmCht4; 2, DmCht5; 3, DmCht9; 4, DmDs47; 5, and 12, MsCht386; 6, TcCht5; 7 and 13, MsCht535; 8 and 10, TcIDGF2; 9 and 11, TcIDGF4.

domains (CBDs) in other well-characterized proteins suggested that the CBDs may enhance the affinity of these proteins for their insoluble substrate, chitin. To evaluate the chitin-binding ability of the recombinant *Drosophila* and *Tribolium* chitinase-like proteins including the IDGFs, a chitin-binding assay was performed using colloidal chitin as described in Materials and Methods. Approximately 90% of both DmCht4 and DmCht5 proteins and 65%

of TcCht5 were bound to colloidal chitin in our standard assay that utilizes colloidal chitin (Fig. 3). These observations are consistent with the presence of a CBD-diagnostic sequence containing six conserved cysteines at the C-termini of DmCht4, DmCht5 and TcCht5. One of the chitinases, DmCht9, which contains only a catalytic domain and no CBD, did not bind to colloidal chitin. Only around 5% of bovine serum albumin (BSA), which was used as a non-specific protein control, bound to colloidal chitin. Although DmDs47 and TcIDGF2 were predicted to lack chitinase activity and have no CBD, nonetheless they also bound to colloidal chitin very tightly. Around 70% of these proteins bound to colloidal chitin, indicating a binding comparable to chitinases that contain a CBD.

### 3.5. Activity-pH profiles of insect chitinase-like proteins using a polymeric substrate

The activity-pH profiles of purified *Tribolium* and *Drosophila* chitinases were determined over the pH range of 3–12 in a borate-phosphate-acetate universal buffer using the soluble polysaccharide substrate, CM-Chitin-RBV (Fig. 4). Both group I chitinase-like proteins, TcCht5 and DmCht5, had two optima at approximately pH 6 and 9 as is typical for lepidopteran molting fluid chitinases (Kramer and Muthukrishnan, 2005). DmCht4 exhibited only one optimum around pH 6. DmCht9 had a very broad pH-activity profile. It had very high activity from pH 4 to 9. The group V proteins, TcIDGF2,

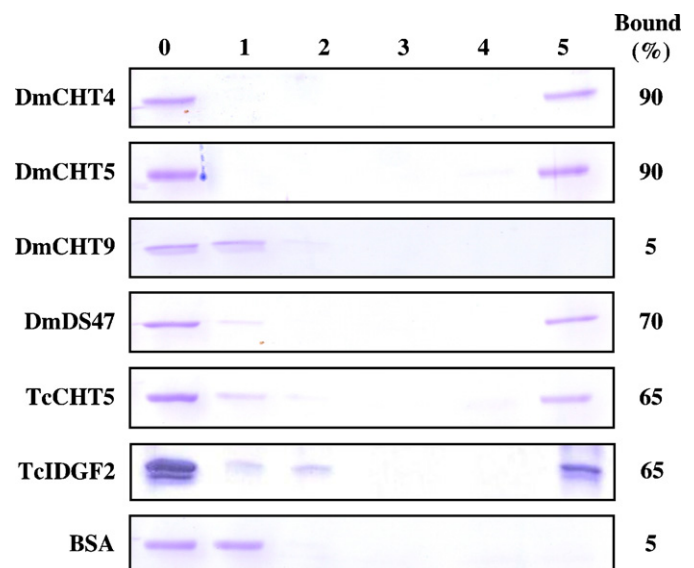


Fig. 3. Colloidal chitin-binding assay of purified recombinant *Drosophila* and *Tribolium* chitinase-like proteins. Lanes: 0, Protein extract loaded onto the colloidal chitin column; 1, flow-through fraction; 2, 10 mM sodium phosphate buffer, pH 8.0 wash; 3, 10 mM sodium phosphate buffer containing 1 M NaCl, pH 8.0 wash; 4, 0.1 M acetic acid eluate; 5, protein eluted by boiling for 5 min in 50  $\mu\text{l}$  1 $\times$  SDS-PAGE sample buffer. The samples were run on a 12% SDS-PAGE gel and stained with Coomassie blue R250.

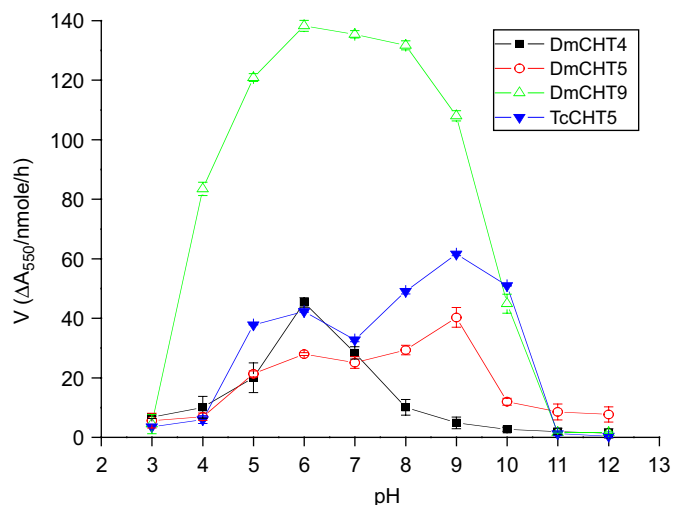


Fig. 4. Activity vs. pH profiles of purified *Tribolium* and *Drosophila* chitinase-like proteins using the polymeric substrate, CM-Chitin-RBV. Activities were measured with 0.5 mg/ml substrate and 0.1  $\mu$ g of enzyme in 0.2 M universal buffer at the indicated pH (total volume 0.4 ml) at 37 °C for 2 h. Values are expressed as absorbance changes at 550 nm per nmole of enzyme/h. DmCht4 (■), DmCht5 (○), DmCht9 (△) and TcCht5 (▼).

TcIDGF4 and DmDS49, had no detectable activity over the pH range tested.

### 3.6. Activity–pH profiles of insect chitinases using an oligosaccharide substrate

The activity vs. pH profiles using the oligosaccharide, MU-(GlcNAc)<sub>3</sub>, as substrate were determined as outlined in Materials and Methods (Fig. 5). TcCht5 and DmCht5 had very broad pH profiles with relatively high activity from pH 4 to 8. These enzymes had a very high activity even at an acidic pH (pH 4) and only one optimum between pH 5 and 6. Compared to TcCht5 and DmCht5, DmCht9 had a lower specific activity and an optimum at pH 4. DmCht4, TcIDGF2, TcIDGF4 and DmDS49 exhibited no enzymatic activity towards the oligosaccharide substrate.

### 3.7. Kinetic parameters of chitinolytic enzymes

To obtain kinetic parameters such as  $K_m$  and  $k_{cat}$  of the purified recombinant chitinase-like proteins against long and short substrates, CM-Chitin-RBV and MU-(GlcNAc)<sub>3</sub> were used as model substrates. DmDS47, TcIDGF2 and TcIDGF4 exhibited no enzymatic activity when assayed with either of the substrates and, therefore, were not included in these studies. As shown in Tables 2 and 3, DmCht5 and MsCht535, which belong to group I chitinases have lower  $K_m$  values for the longer substrate (CM-chitin-RBV) than the enzymes belonging to group IV. The group I enzymes also have greater catalytic efficiencies as measured by the  $V_{max}/K_m$  parameter than the class IV enzymes. With the oligomeric substrate, MU-(GlcNAc)<sub>3</sub>,

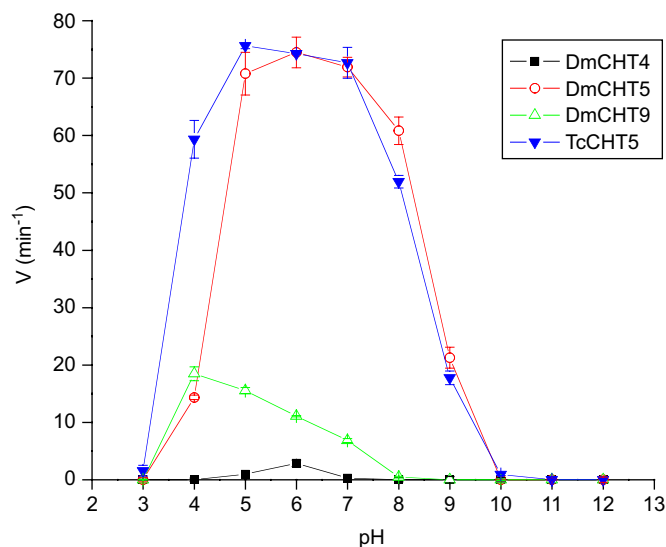


Fig. 5. Activity vs. pH profiles of purified *Tribolium* and *Drosophila* chitinase-like proteins using the oligosaccharide substrate, 4MU-(GlcNAc)<sub>3</sub>. Activities were measured at a substrate concentration of 64  $\mu$ M using reactions with 0.1  $\mu$ g enzyme in 0.2 M universal buffer (total volume 50  $\mu$ l) at 37 °C for 10 min. Velocities are expressed as units/min. One unit of enzyme activity was defined as the amount needed to release 1 nmole of 4MU per minute. DmCht4 (■), DmCht5 (○), DmCht9 (△) and TcCht5 (▼).

Table 2

Kinetic parameters of purified *Drosophila* and *Tribolium* chitinase-like proteins using CM-Chitin-RBV as the substrate

Enzyme	Group	$K_m$ (mg/ml)	$V_{max}$ ( $\Delta A_{550}/h/\mu\text{mol}$ )	$V_{max}/K_m^a$
DmCht4	IV	3.36	12.8	3.80
DmCht9	IV	7.14	27.7	3.88
DmDS47	V	N/A	N/A	N/A
DmCht5	I	0.86	4.7	5.52
TcCht5	I	1.41	7.3	5.17
MsCht535 <sup>b</sup>	I	1.83	14.3	7.80

N/A, no activity was detected.

<sup>a</sup>The  $V_{max}/K_m$  catalytic efficiency parameter rather than the  $k_{cat}/K_m$  parameter was calculated because the molar concentration of the CM-Chitin-RBV substrate is unknown.

<sup>b</sup>MsCht535 kinetic parameters were from Arakane et al. (2003).

DmCht4 has no detectable enzymatic activity. The group IV enzyme, DmCht9, had a  $K_m$  in the same range as the class I enzymes, DmCht5 and TcCht5, but the  $K_m$  is slightly lower than that of the related MsCht535. Once again, the  $V_{max}/K_m$  parameter was higher for the class I enzymes than that for the class IV enzyme, DmCht9. All four of the chitinases, TcCht5, DmCht5, DmCht9 and MsCht535, exhibited substrate inhibition at high concentrations of the oligomeric substrate, MU-(GlcNAc)<sub>3</sub>. On the other hand, no substrate inhibition was observed when the soluble polymeric substrate, CM-Chitin-RBV was tested (data not shown).

Table 3  
Kinetic parameters of purified *Drosophila* and *Tribolium* chitinase-like proteins using MU-(GlcNAc)<sub>3</sub> as the substrate

Enzyme	Group	$K_m$ (mM)	$k_{cat}$ (S <sup>-1</sup> )	$k_{cat}/K_m$ (mmol <sup>-1</sup> S <sup>-1</sup> )
DmCHT4	IV	N/A	N/A	N/A
DmCHT9	IV	0.27	0.76	2.8
DmDS47	V	N/A	N/A	N/A
DmCHT5	I	0.19	3.14	16.3
TcCHT5	I	0.43	5.26	12.2
MsCHT535 <sup>a</sup>	I	1.02	10.7	10.4

N/A, no activity was detected.

<sup>a</sup>MsCHT535 kinetic parameters was taken from Arakane et al. (2003).

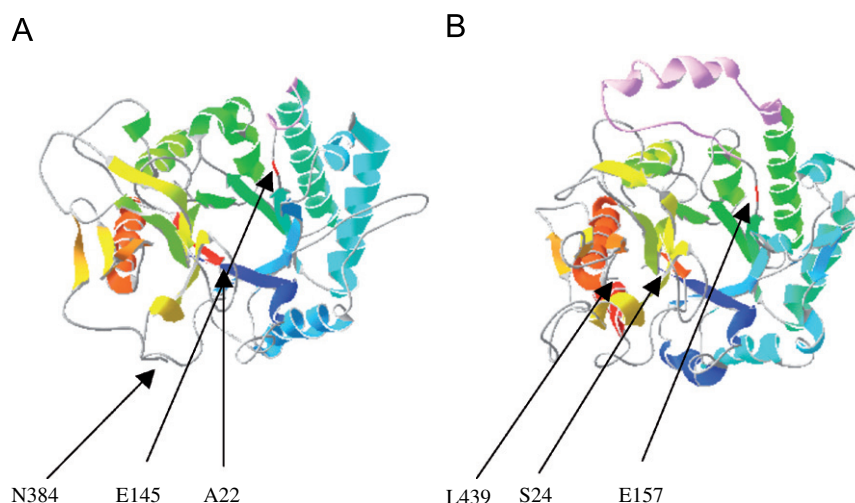


Fig. 6. Homology modeling of catalytic domains for *Tribolium* chitinase TcCHT5 and chitinase-like protein TcIDGF2. The SWISS-MODEL program was used to generate the models. The loop region between  $\beta$ -sheet 4 and  $\alpha$ -helix 4 is labeled in purple. A. TcCHT5 homology modeling was conducted using human chitotriosidase (PDB entry code 1lq0A) (Fusetti et al., 2002) as the template. B. TcIDGF2, *Drosophila* IDGF2 (PDB entry code 1jndA) (Varela et al., 2002) was used as the template. The glutamate residues in the conserved region II (E145 and E157) in the two proteins are indicated as are the beginnings (A22 and S24) and ends (N384 and L439) of the catalytic domains.

### 3.8. Homology modeling of two *Tribolium* putative chitinase-like proteins

Homology modeling showed that the catalytic domains of both TcCHT5 and TcIDGF2 probably have a  $(\beta\alpha)_8$  TIM barrel structure as expected (Fig. 6). It was determined that the extra amino acids found in TcIDGF2 between the  $\beta$ -4 and  $\alpha$ -4 components of the  $(\beta\alpha)_8$  TIM barrel formed an additional  $\alpha$ -helix (not present in TcCHT5), which is shown in purple in Fig. 6. The presence of this extra helix has been observed in the crystal structures of IDGF2 from *D. melanogaster* (Varela et al., 2002) and human chitotriosidase (Fusetti et al., 2002).

## 4. Discussion

We attempted to express at least one member of each group of chitinase-like proteins found in insects. Recombinant AcMNPVs containing the ORFs of the group I genes, *TcCht5* and *DmCht5*, the group II gene, *DmCht10*, the group III gene, *DmCht7*, the group IV genes, *DmCht4* and

*DmCht9*, and the group V genes, *TcIDGF2*, *TcIDGF4* and *DmDS47*, were constructed for expression in the baculovirus–insect cell system. Unfortunately, we could not detect expression of one of the most interesting and largest proteins, DmCHT10, which contains multiple catalytic domains, in either the medium or the cell lysate. DmCHT7 was predicted to have a transmembrane domain following the cleavable leader peptide. As expected, Coomassie blue staining after SDS-PAGE of the cell pellet after lysis of the cells infected with the AcMNPV containing this gene, and not the cell culture medium, revealed the presence of a new band with the size expected for this protein. On the other hand, recombinant *Tribolium* chitinase family proteins, TcCHT5, TcIDGF2 and TcIDGF4, and *Drosophila* chitinase-family proteins, DmCHT4, DmCHT5, DmCHT9 and DmDS47, which have predicted leader peptide sequences, were found to be secreted into the medium where they accumulated at relatively high levels, varying from 2 to 10 mg/l of medium depending on the gene being expressed. The ability to purify many of the proteins belonging to three different groups has allowed



a comparison of the physical, enzymatic and binding properties of these proteins, and also an assessment of the differences in their properties. The finding that the protein, DmCHT7, was present only in the cell pellet fraction in baculovirus infected insect cells suggested that this protein is membrane-bound and is not cytosolic or secreted.

#### 4.1. Differences in physical properties of insect chitinase-like proteins

The apparent molecular weights of these chitinase family proteins as determined by SDS-PAGE were larger than the theoretical masses based on their amino acid compositions. The differences in sizes ranged from 1 to 18 kDa. The difference was the largest for the group I chitinase-like proteins. For TcCHT5 and DmCHT5, the differences were 7 and 18 kDa, respectively, and this is likely to be due to glycosylation as has been shown to be the case for *M. sexta* chitinase, a group I chitinase which had a greater than 20 kDa difference between the experimentally determined and theoretical sizes (Arakane et al., 2003). The variable differences between the molecular weights determined by SDS-PAGE and the predicted masses of chitinase-like proteins from different groups also suggest that the group I enzymes containing S/T-rich linker regions are more extensively glycosylated compared to chitinases belonging to other groups that have very short or no S/T-rich linkers. In the cases of *M. sexta* and *C. fumiferana* chitinases, glycosylation appeared to be necessary for secretion of recombinant proteins (Gopalakrishnan et al., 1995; Zheng et al., 2002). Tunicamycin, a glycosylation inhibitor, inhibited both glycosylation and secretion of the two lepidopteran chitinases into the culture medium (Gopalakrishnan et al., 1995; Zheng et al., 2002).

#### 4.2. Immunological properties of chitinase-like proteins

An antiserum raised against MsCHT386, which contains only the catalytic domain, was used to test the cross-reactivity against chitinases of other groups. This antibody detected all proteins belonging to group I chitinase-family, including DmCHT5, TcCHT5, and MsCHT535, but it had very little immunological cross-reactivity with group IV chitinase-like proteins, DmCHT4 and DmCHT9, or with group V proteins, DmDS47, TcIDGF2 and TcIDGF4. Since the catalytic domains of chitinases of all groups including the IDGFs are expected to assume the  $(\beta\alpha)_8$  barrel core structure, the failure of the anti-CHT386 antibody to detect group IV and V chitinase-like proteins suggests that the antigenic determinants of the different classes of chitinases are determined by differences in the loop regions of amino acids that connect the  $\alpha$ -helices and the  $\beta$ -sheets, which are likely to be exposed to the solvent and elicit immune reactions (Varela et al., 2002; Fusetti et al., 2002).

#### 4.3. Biochemical properties of chitinase-like family proteins

Insect chitinases are family 18 glycosylhydrolases that catalyze the random hydrolysis of the  $\beta$ -(1,4)-glycosidic bonds in chitin (Henrissat and Davies, 1997; Coutinho and Henrissat, 1999). The insoluble polymeric chitin is digested, yielding soluble low molecular mass oligomers of GlcNAc, such as chitotetraose, chitotriose and chitobiose (Kramer and Koga, 1986; Samuels and Reynolds, 1993; Kramer and Muthukrishnan, 2005). Two substrates commonly used to determine chitinolytic activity are a polymeric soluble substrate, CM-chitin-RBV, and a small oligosaccharide substrate, 4MU-(GlcNAc)<sub>3</sub>. Use of these substrates provided us with some insight about the differences in chitinolytic activities of chitinase-like proteins belonging to different phylogenetic groups from the same insect species. The pH-activity profiles and kinetic constants of group I chitinases, TcCHT5 and DmCHT5 (Figs. 4 and 5), are similar to each other and to those of several previously characterized group I chitinases purified from *M. sexta* (Huang et al., 2000; Lu et al., 2002; Zhang et al., 2002), *C. fumiferana* (Zheng et al., 2002) and *L. oleracea* (Fitches et al., 2004). All of them exhibited two optima, one at around pH 6 and the other at pH 9 when CM-chitin-RBV was used as the substrate. Group I insect chitinases are expressed both in the epidermis and gut, and are active over a wide range of pH values. One of the remarkable properties of lepidopteran insects is the pH of the larval midgut lumen, which is highly alkaline (Waterhouse, 1949; Berenbaum, 1980; Dow, 1992). The pH-activity values of the larval lepidopteran midgut range from pH 10 to 12 (Dow, 1984, 1992; Gringorten et al., 1993). Similar high pH values also have been recorded in midguts of certain dipteran (Bignell and Anderson, 1980) and coleopteran species (Bayon, 1980). A microelectrode technique was used to measure a pH gradient in the gut of *A. aegypti*. The pH values were approximately 11 and 8 in the anterior and posterior midgut lumen, respectively (Boudko et al., 2001). Thus, group I chitinases show two optima at pH 6 and 9, which might be useful to ensure that their activity is substantial at the slightly acidic environment of the molting fluid and in the alkaline environment of the gut. Relatively broad pH profiles were also detected using 4MU-(GlcNAc)<sub>3</sub> as the substrate for both TcCHT5 and DmCHT5. These enzymes retained high activity from pH 4 to 8, which was also observed with tobacco hornworm and common cutworm chitinases, which belong to group I (Huang et al., 2000; Shinoda et al., 2001; Zhang et al., 2002).

Several group IV insect chitinases have been identified from numerous insect species including *A. gambiae*, *Chelonas sp.*, *G. morsitans*, *L. longipalpis*, and *P. cochleariae* (Krishnan et al., 1994; Shen and Jacobs-Lorena, 1997; Girard and Jouanin, 1999; Yan et al., 2002; Ramalho-Ortigao and Traub-Cseko, 2003; Genta et al., 2006). The enzymatic properties of *G. morsitans*, *Chelonas* and *Tenebrio* chitinases have been reported using only the

oligosaccharide substrates, 4MU-(GlcNAc)<sub>3</sub> or pNp- $\beta$ -N,N',N''-triacetylchitotriose. The first two enzymes were not tested using a polymeric substrate. In our experiments, we assayed two group IV chitinase-like proteins, DmCHT4 and DmCHT9, using both polymeric and oligomeric substrates. DmCHT4, which has a catalytic domain and a CBD, exhibited only one optimum at pH 6 towards the polymeric substrate, whereas it had very little activity towards the oligosaccharide substrate. Another group IV enzyme, DmCHT9, which is closely related to DmCHT4 (64% sequence identity in the catalytic domain), lacks a CBD. This enzyme had a broad pH optimum and had relatively high activity in the pH range 4–8 towards the polymeric substrate, CM-Chitin-RBV. It had only a very low activity towards the oligosaccharide substrate, 4MU-(GlcNAc)<sub>3</sub>, with an acidic pH optimum. Therefore, it appears that the two group IV enzymes studied here prefer soluble polymeric substrates over oligomeric substrates and also differ in their enzymatic properties. Although DmCHT9 retained high activity towards the soluble polymeric substrate, it did not bind to insoluble colloidal chitin. However, the only other well-studied group IV enzyme from *T. molitor*, which lacks a CBD, also prefers the oligomeric substrate to the polymeric colloidal chitin substrate (Genta et al., 2006). It has been proposed that the CBD of insect chitinase facilitates hydrolysis of insoluble substrates but not the soluble ones (Arakane et al., 2003). In view of the preference of the *Tenebrio* gut chitinase for the oligomeric substrates, the idea that the presence of the CBD dictates the preference for insoluble polymeric substrate may have to be revisited. Group I enzymes, TcCHT5 and DmCHT5, and one group IV enzyme, DmCHT4, all of which contain a CBD in their C-terminal region, bound to colloidal chitin very tightly as expected. A comparison of the  $V_{\max}/K_m$  or  $k_{\text{cat}}/K_m$  parameters indicated that group I enzymes are more efficient than the group IV enzymes at hydrolyzing both of the substrates that we have tested.

Although the group V protein, DmDS47, exhibited no chitinolytic activity towards both polymeric and oligomeric substrates, it exhibited chitin-binding ability (Fig. 2). DmDS47 was first identified as a chitinase-like protein from the *Drosophila* Schneider cell line-2 (S2, Kirkpatrick et al., 1995). It was subsequently detected throughout the *Drosophila* life cycle, expressed mainly in fat body and hemocytes, and secreted into the hemolymph. As a result of its sequence homology to chitinases, DmDS47 was predicted to interact with glycan structures, but the function of DmDS47 is still unclear (Kirkpatrick et al., 1995). Kawamura et al. (1999) reported a family of polypeptide growth factors (IDGFs) from *Drosophila*, which are similar to chitinases in amino acid sequence. IDGFs are primarily expressed in yolk cells and fat body. The recombinant IDGFs cooperate with insulin and promote proliferation of wing imaginal disc cells (Kawamura et al., 1999). DmDS47 is highly similar to IDGFs in amino acid sequence. It is unknown whether DmDS47 can function

in proliferation, polarization, and/or motility of imaginal disc cells. Two IDGFs from *Tribolium* were also expressed and purified from the insect cell line expression system. Neither of the two proteins exhibited chitinolytic activity. These proteins were proteolytically cleaved to yield truncated products (Fig. 1) during their purification and storage, which has also been reported for *Drosophila* IDGF2 (Varela et al., 2002).

The domain structures of the insect chitinase-like proteins examined here are shown in Fig. 1. Some are enzymatically active, whereas others are not. Some of the enzymes are active towards both polymeric substrates, whereas others are not. In contrast to most of the enzymatically active chitinase-like proteins, IDGF chitinase-like proteins do not contain a CBD and do not have chitinase activity. All of them with the exception of TcIDGF2 and TcIDGF4 lack the catalytically critical glutamate residue in conserved region II which acts as the proton donor (Zhu et al., accompanying paper). The IDGF-like proteins probably are carbohydrate-binding proteins that bind to  $\beta$ (1-4)-linked GlcNAc-containing ligands in cell walls or membranes, which act as growth factors. The extracellular IDGFs may interact with membrane-bound glycoprotein receptors and modulate their signal transduction cascades. Alternatively, they may affect the adhesive properties of the target cells. Homology modeling indicates that both TcCHT5 and TcIDGF4 have the same  $(\beta\alpha)_8$  TIM barrel structure, but the latter has an additional  $\alpha$ -helix between the  $\beta$ -4 and  $\alpha$ -4 components of the barrel, which partially blocks the substrate-binding pocket. This structural feature might explain why TcIDGF2 and TcIDGF4 lack chitinase activity even though they do have the catalytically important glutamate residue in conserved region II.

In summary, this study compared some of the properties of several chitinase-like proteins from two insect species, *Drosophila* and *Tribolium*, in an attempt to link protein diversity with different biological functions. Among individual chitinase-like proteins, there are substantial differences in domain organization and physical and enzymatic properties including size, extent of post-translational modification (glycosylation), substrate preference, pH optimum and substrate inhibition as well as in their ability to bind to insoluble chitin. There also are differences in their cellular locations and developmental patterns of expression (Zhu et al., unpublished data). These differences are likely to be important for these proteins to carry out their diverse functions in various tissues and stages during insect development.

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