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Properties of catalytic, linker and chitin-binding domains of insect chitinase

Yasuyuki Arakane [a], Qingsong Zhu [a], Masahiro Matsumiya [b], Subbaratnam Muthukrishnan [a], Karl J. Kramer [c,*]

[a] *Department of Biochemistry, Kansas State University, Manhattan, KS 66506, USA*
[b] *College of Bioresource Sciences, Nihon University, Fujisawa, Kanagawa 252-8510, Japan*
[c] *Grain Marketing and Production Research Center, ARS-USDA, 1515 College Avenue, Manhattan, KS 66502, USA*

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Abstract

Manduca sexta (tobacco hornworm) chitinase is a glycoprotein that consists of an N-terminal catalytic domain, a Ser/Thr-rich linker region, and a C-terminal chitin-binding domain. To delineate the properties of these domains, we have generated truncated forms of chitinase, which were expressed in insect cells using baculovirus vectors. Three additional recombinant proteins composed of the catalytic domain fused with one or two insect or plant chitin-binding domains (CBDs) were also generated and characterized. The catalytic and chitin-binding activities are independent of each other because each activity is functional separately. When attached to the catalytic domain, the CBD enhanced activity toward the insoluble polymer but not the soluble chitin oligosaccharide primarily through an effect on the K_m for the former substrate. The linker region, which connects the two domains, facilitates secretion from the cell and helps to stabilize the enzyme in the presence of gut proteolytic enzymes. The linker region is extensively modified by *O*-glycosylation and the catalytic domain is moderately *N*-glycosylated. Immunological studies indicated that the linker region, along with elements of the CBD, is a major immunogenic epitope. The results support the hypothesis that the domain structure of insect chitinase evolved for efficient degradation of the insoluble polysaccharide to soluble oligosaccharides during the molting process.
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1. Introduction

Chitinolytic enzymes are now being used for biotechnological applications in agriculture and health care (Patil et al., 2000). Chitinases belonging to family 18 glycosylhydrolases (Coutinho and Henrissat, 1999) have been isolated from a wide variety of sources including bacteria, yeasts and other fungi, nematodes, arthropods and vertebrates such as humans, mice and chickens (Nagono et al., 2002; Suzuki et al., 2002). They are among a group of proteins that insects use to digest the structural polysaccharide chitin in their exoskeletons and gut linings during the molting process (Kramer et al., 1985; Kramer and Koga, 1986; Kramer and Muthukrish-

* Corresponding author. Tel.: +1-785-776-2711; fax: +1-785-537-5584.
E-mail address: kramer@gmprc.ksu.edu (K.J. Kramer).

Abbreviations: CBD, chitin-binding domain; GlcNAc; 2-acetamido-2-deoxyglucopyranoside; GalNAc, 2-acetamido-2-deoxygalactopyranoside; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; DEAE, diethylaminoethyl; Tris, tris(hydroxylmethyl)aminomethane; PCR, polymerase chain reaction; Chi535, full-length enzyme; Chi376, Chi386, Chi407 and Chi477: proteins consisting of amino acids 1–376, 1–386, 1–407 and 1–477, respectively; ChiLH, C-terminally His-tagged protein consisting of amino acids 377–535; ChiCH, C-terminally His-tagged protein consisting of amino acids 478–535; ChiLCH, C-terminally His-tagged protein consisting of amino acids 377–477; ChiMCBD, protein consisting of amino acids 1–386 fused with amino acids 478–535; Chi$(MCBD)_2$, protein consisting of amino acids 1–386 fused with two tandem repeats of amino acids 478–535; ChiRCBD, protein consisting of amino acids 1–386 fused with the rice chitinase CBD. The numbering refers to positions of the amino acids in the mature enzyme

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nan, 1997; Fukamizo, 2000). In plants, these enzymes are generally believed to serve protective functions, although the exact mechanism of such protection is unclear (Kramer et al., 1997; Ding et al., 1998; Gooday, 1999). We are interested in using a family 18 insect chitinase as a host plant resistance factor in transgenic plants and in improving the catalytic efficiency and stability of this enzyme so that its pesticidal activity would be enhanced. The enzyme of interest is the molting fluid chitinase from *Manduca sexta* (tobacco hornworm, GenBank accession number, AAC04924), which is a 535-amino acid-long glycoprotein (Chi535) (Koga et al., 1983a; Kramer et al., 1993; Choi et al., 1997; Kramer and Muthukrishnan, 1997).

Besides the cDNA of a chitinase from the tobacco hornworm (Kramer et al., 1993), other insect chitinase cDNAs have been cloned from the silkworm (Kim et al., 1998), the fall webworm (Kim et al., 1998), the common cutworm (Shinoda et al., 2001), the yellow mealworm (Royer et al., 2002), and the spruce budworm (Zheng et al., 2002). One of the structural features observed in many of these insect chitinases is a multidomain architecture that includes a signal peptide, one or more catalytic domains, cysteine-rich chitin-binding domains (CBD), fibronectin-like domains, and serine/threonine (S/T)-rich linker domains that are generally glycosylated (Tellam, 1996; Henrissat, 1999; Suzuki et al., 1999). In prior structure-function studies of tobacco hornworm chitinase, we investigated the properties of recombinant enzymes with substitutions of specific amino acids (aspartic acids 142 and 144, tryptophan 145 and glutamic acid 146) in the active site and also some C-terminal truncated derivatives to help identify residues and domains required for catalysis (Huang et al., 2000; Zhu et al., 2001; Lu et al., 2002; Zhang et al., 2002). The mature hornworm enzyme also has a modular structure, an N-terminal catalytic domain of about 376 amino acids and a C-terminal insoluble substrate (chitin)-binding domain (CBD) of approximately 58 amino acids, which are connected by an interdomain Ser/Thr-rich *O*-glycosylated linker of approximately 100 amino acid residues in length (Fig. 1). A similar domain structure also occurs in other insect chitinases, including those of the silkworm, *Bombyx mori* (Kim et al., 1998; Mikitani et al., 2000; Abdel-Banat and Koga, 2001), fall webworm, *Hyphantria cunea* (Kim et al., 1998), common cutworm, *Spodoptera litura* (Shinoda et al., 2001), and the spruce budworm, *Choristoneura fumiferana* (Zheng et al., 2002).

The interaction of insect chitinases with insoluble chitin in the exoskeleton and peritrophic matrix is believed to be a dynamic process that involves adsorption via the CBD, hydrolysis, desorption, and positioning of the catalytic domain on the surface of the substrate. This degradative process apparently requires a coordinated action of both domains by a mechanism that is not well understood. In addition to the catalytic events, the mechanism of binding of the enzyme onto the heterogeneous surface of native chitin is poorly characterized. In this study, we investigated some of the properties of recombinant forms of these domains and the linker region, and have also characterized three other recombinant proteins composed of the catalytic domain fused with one or two CBDs in order to better understand the contributions of the individual domains to the catalytic and substrate-binding processes.

2. Materials and methods

2.1. Construction of recombinant baculoviruses containing truncated, extended and individual domain forms of the M. sexta chitinase gene

Every expression construct was designed to have a signal peptide at the N-terminus. Their signal peptides allowed the expressed proteins to be secreted into the medium except for those with a deletion of the Ser/Thr-rich linker domain. Primers were synthesized at the Biotech Core Facility, Kansas State University. Primers used for the amplification of specific domain(s) are shown in Table 1. All DNA fragments with the exception of CBD_2 were amplified by PCR using *M. sexta* chitinase cDNA clone no. 10 as template. PCR reactions were conducted in a final volume of 50 μl containing 10 ng plasmid template, 0.4 μM of the primers, 0.2 mM dNTPs, 1× pfu buffer (20 mM Tris–HCl, pH 8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100 and 0.01% BSA), and 2.5 units of pfu polymerase using the PCR Gene Mate instrument (ISC Bio Express) as follows: denaturation at 94°C for 1 min, annealing at 60°C for 1 min and polymerization at 72°C for 1.5 min and 25 cycles. The PCR amplified fragments were purified from a low melting agarose gel after separation by electrophoresis, digested with *Eco*RI and *Pst*I, and ligated to similarly digested pVL1393 DNA. The desired combination of DNA fragments and the linearized pVL1393 vector DNA were ligated under standard conditions. The ligation mixtures were used to transform competent cells of *E. coli* JM 109 and recombinant clones were identified by standard methods. Table 1 shows the different constructs utilized for expression in insect cells of truncated and extended forms of *M. sexta* chitinase.

Recombinant baculoviruses were obtained by co-transfection of Sf21 cells with the appropriate transfer plasmid DNAs (pVL1393 constructs described above) and BaculoGold™ DNA from Pharmingen (San Diego, CA) (Gopalakrishnan et al., 1995). BaculoGold DNA is a modified baculoviral DNA (AcMNPV) with a lethal deletion. Recombination of the transfer plasmid pVL1393 DNA with BaculoGold DNA can rescue the

Table 1
Primers used for the amplification of DNAs for specific domains of insect chitinase

Construct/ fragment	Domains amplified	Forward primer (5′–3′)[a]	Reverse primer (5′–3′)[a]
Chi386	LP/CAT+10	TCTGAATTCAAG**ATG**CGAC	TCTCTGCAGA**TTA**TGTATGAGGAGGCG
Chi396	LP/CAT+20	TCTGAATTCAAG**ATG**CGAC	TCTCTGCAG**TTA**GGCCC ATTCAGGAG
MCBD	*M. sexta* CBD	TCTAAGCTTATCTGCAACTCAGACCAA	TCTCTGCAG**TTA**GGGTTGTTGACATTC
$M(CBD)_2$	*M. sexta* CBD	TCTATGCATATCTGCAACTCAGACC	TCTCTGCAG**TTA**GGGTTGTTGACATTC
RCBD	Rice CBD	TCTAAGCTTGAGCAGTGCGGCAGC	TCTATGCAT**TTA**GGGCGGGGTC
LH	LP/His_6	TCTAAGCTTAGCTCTTACACAAGTGCCG	TCTCTGCAG**TTA**ATGATGATGATG ATGATGTTCGCTACCATCGAC
CH	CBD/His_6	TCTAAGCTTATCTGCAAC TCAGAC CAA	TCTCTGCAG **TTA**ATGATGATGATG ATGATGGGGTTGTTGACATTC
LCH	Linker/MCBD/His_6	TCTAAGCTTATCTGCAAC TCAGAC CAA	TCTCTGCAG**TTA**ATGATGATGATG ATGATGGGGTTGTTGACATTC

[a] Restriction enzyme sites are underlined. The translation start codon (**ATG**) and the complement of the translation stop codon (**TTA**) are noted in bold.

lethal deletion as a result of integration of the transfer plasmid segments into AcMNPV DNA. Recombinant viruses were amplified 3–4 rounds in Sf 21 cells to obtain high titer viruses (about 1×10^8 pfu/ml). The plaque assay method was used to check the virus titer.

Viral DNA was prepared from the high titer virus by phenol/chloroform extraction. PCR was used to amplify the construct using primers that were designed to amplify the entire chitinase-coding region. Fragments derived from the recombinant viral DNA had the same size as those obtained from the corresponding vector plasmids. Each viral PCR product was purified from a low melting agarose gel and analyzed by DNA sequencing using appropriate forward and reverse primers. The sequencing results confirmed that all fragments were ligated correctly as designed and encoded the desired protein (data not shown).

2.2. Expression of wild-type, truncated and extended forms in baculovirus-insect cell line gene expression system

Baculovirus-mediated recombinant chitinase gene expression was done by following the method of Zhu et al. (2001) using Hi-5 insect cells cultured in EX-CELL 405 serum-free medium containing L-glutamine (JRH Bioscience, Lenexa, KS) in 225 cm^2 flasks. Culture media were collected 3 d after incubation with recombinant viruses and clarified by centrifugation at 10,000 *g* for 10 min at 4°C. Each construct containing the signal peptide was predicted to result in secretion of the corresponding protein into the medium. Monolayers of Hi-5 cells were used as host cells to express the proteins encoded in the recombinant baculoviruses as described in Section 2. Previous results showed that the Hi-5 cell line had a higher level of expression than other cell lines (Gopalakrishnan et al., 1995). Another advantage was that the Hi-5 cell line could be cultured in serum-free medium, which facilitates protein purification. All of the proteins were secreted into the medium by baculovirus-infected Hi-5 cells except for ChiLH, which was retained inside the Hi-5 cells for unknown reasons.

2.3. Purification of chitinases

The supernatants collected by centrifugation of culture media were dialyzed against 20 mM sodium phosphate buffer, pH 8 for wild-type and truncated forms or against 20 mM Tris–HCl buffer, pH 9 for the extended forms. The dialyzed samples were subjected to anion-exchange chromatography on a DEAE-Sepharose column (2×7 cm, Pharmacia), which was previously equilibrated with the same buffer used for dialysis. The proteins were eluted using a linear gradient of NaCl from 0 to 0.4 M in the same buffer at a flow rate of 0.8 ml/min. Fractions of 1.8 ml were collected and analyzed by SDS-PAGE. Fractions containing the protein of interest were pooled and dialyzed against 10 mM sodium phosphate buffer, pH 8, and then subjected to chromatography on a hydroxylapatite column (1×8 cm, Bio-Rad) equilibrated with 10 mM sodium phosphate buffer, pH 8. Protein was eluted with a linear gradient of sodium phosphate buffer, pH 8, from 10 to 300 mM, after washing the column with 10 mM sodium phosphate buffer, pH 8. Fractions containing the protein of interest were pooled, desalted and concentrated by ultrafiltration.

The culture media supernatants containing either ChiLCH or ChiCH proteins were passed through a Ni-NTA agarose column and the bound proteins with C-terminal His-tags were eluted with an imidazole gradient. ChiLCH was eluted with a gradient of 10–50 mM imidazole, as a rather heterogeneous mixture of proteins with apparent molecular weights ranging from 21 to 46 kDa. ChiCH was eluted with an imidazole gradient of

50–250 mM. ChiCH was homogeneous and had an apparent size of 13 kDa.

ChiLH was not secreted into the medium. Therefore, the Hi-5 cell pellet containing the ChiLH protein was collected 72 h after virus infection and used as the starting material for purification of this protein. The cells were lysed by sonication for 2 mm at 40 W and 20 kHz. The lysate was centrifuged at 10,000 *g* for 10 mm at 4°C. The supernatant that contained the ChiLH protein was passed through a Ni-NTA agarose column. ChiLH was eluted by buffer containing 250 mM imidazole and had an apparent molecular weight of 20 kDa.

2.4. Protein determination

Absorbance at 280 nm was measured to monitor proteins during chromatographic separations. Protein concentration was measured using the bicinchoninic acid assay reagent (Pierce, Rockford, IL) using BSA as the standard protein.

2.5. Molecular weight and N-terminal sequence determinations

Protein samples obtained from hydroxylapatite column chromatography were used for molecular mass determination by laser desorption mass spectrometry and for N-terminal sequence analysis at the Biotechnology Core Facility, Kansas State University, Manhattan, KS. The proteins were resolved using SDS-PAGE and transferred onto a PVDF membrane. Coomassie Brilliant Blue R-250 staining was used to locate the protein bands, which were cut out from the membrane and subjected to N-terminal sequence analysis by automated Edman degradation using an Applied Biosystem Sequencer.

2.6. Carbohydrate analysis

Glycosyl composition analysis was performed at the Complex Carbohydrate Research Center, University of Georgia, Athens, GA, by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the samples by acidic methanolysis. Methyl glycosides were first prepared from dried samples by methanolysis in 1 M HCl in methanol at 80°C for 18–22 h, followed by re-*N*-acetylation with acetic anhydride in pyridine/methanol for detection of amino sugars. The samples were then per-*O*-trimethylsilylated by treatment with Tri-Sil reagent (Pierce Chem., Rockford, IL) at 80 °C for 0.5 h (York et al., 1985). GC/MS analysis of the TMS methyl glycosides was performed on an HP 5890 GC equipped with a Supelco EB 1 fused silica capillary column interfaced to an HP 5970 MSD detector.

Glycosidases were also used for enzymatic deglycosylation to digest carbohydrate side chains of the polypeptide backbone of the recombinant glycoproteins using the Glycopro deglycosylation kit from Prozyme (San Leandro, CA). PNGase F (Glycopro GE41 PNGase) was used to remove intact N-linked oligosaccharides, whereas a mixture of *O*-glycosidases was used to remove O-linked sugars (Tarentino and Plummer, 1994). Because there was no single enzyme available for removing the intact O-linked oligosaccharides, a mixture of exoglycosidases including sialidase A™, β(1-4) galactosidase, *N*-acetylglucosaminidase and endo-*O*-glycosidase (ProZyme, Inc., San Leandro, CA) was used to remove both simple and complex O-linked carbohydrates. First, β(1-4) galactosidase, glucosaminidase, sialidase A™ were used to remove side chain sugars until the Gal β(1-3)GalNAc core remained attached to the serine or threonine side chain. β(1-4) Galactosidase released any β(l-4) linked, non-reducing terminal galactose residues from complex carbohydrates and glycoproteins. Glucosaminidase cleaved any non-reducing terminal β-linked *N*-acetylglucosamine residues. Sialidase A™ removed any *N*-acetylneuraminic acid residues. Secondly, endo-*O*-glycosidase removed any core Gal β(1-3)GalNAc residues from the serine or threonine residues.

2.7. Immunoblotting

Immunoblotting was done by the method of Koga et al. (1992). After electrophoresis, the proteins in the gel were transblotted to a PVDF membrane (Millipore Co., Bedford, MA) using a semi-dry blotting apparatus (Bio-Rad) at 2.5 mA/cm^2 for 1 h in Tris–glycine–methanol buffer, pH 7.5. Two separate rabbit anti-sera that were raised against either purified Chi535 (wild-type) or the truncated form, Chi386, were used as the primary antibodies.

2.8. Kinetic analysis of truncated and extended forms

Previously, we had utilized pH 6 for kinetic analyses when using CM-Chitin-RBV as the substrate (Zhu et al., 2001; Zhang et al., 2002). However, because that pH was not intermediate to the pH of the locations, where insect chitinase is physiologically functional, i.e. the molting fluid (pH ~7) and midgut lumen (pH ~10), we changed the pH of the CM-Chitin-RBV and colloidal chitin assays to pH 9. Also, for the trisaccharide substrate, previously, a three parameter substrate inhibition model was used to calculate the kinetic parameters over a substrate concentration range of 0–50 μM. However, for this study, we used the Lineweaver–Burk model instead and a substrate concentration range of 20–200 μM.

2.8.1. CM-Chitin-RBV as the substrate

Kinetic experiments were done on the enzymatic hydrolysis of CM-Chitin-RBV (Loewe Biochemica GmbH, Sauerlach, Germany) in 50 mM Tris–HCl, pH 9. One-tenth milliliter of a reaction mixture consisting of substrate (0.1–1.0 mg/ml) and 0.5 μg of purified enzyme protein was incubated at 37°C for 1 h, and the reaction was stopped by adding 0.1 ml of 2 N HCl. The mixture was cooled on ice for 15 min and then centrifuged at 12,000 rpm for 5 min. The supernatant was collected and absorbance at 550 nm was measured.

2.8.2. MU-$(GlcNAc)_3$ as the substrate

Kinetic assays were conducted by the method of Zhu et al. (2001) with minor modifications. The assays were done using 4-methylumbelliferyl β-*N*, *N*′, *N*″-triacetylchitotrioside [MU-$(GlcNAc)_3$] (Sigma) as substrate in 0.1 M sodium phosphate buffer, pH 6. Fifty microliter of a reaction mixture consisting of substrate (0.02–0.2 mM) and 0.1 μg of protein were incubated at 37 °C for 10 min, and the reaction was stopped by adding 12.5 μl of 2 N HCl. The mixture was diluted 320-fold with 0.15 M glycine-NaOH buffer, pH 10.5. A 2-ml portion of the mixture was used to determine the free methylumbelliferone released by enzymatic hydrolysis. A DyNA Quant 200 fluorescence spectrophotometer (Pharmacia Biotech) was used to measure the product formed utilizing an excitation wavelength of 365 nm and an emission wavelength of 460 nm.

2.8.3. Colloidal chitin as the substrate

Colloidal chitin was prepared by the method of Shimahara and Takiguchi (1988) using crabshell chitin (Sigma). One-tenth milliliter of a reaction mixture consisting of colloidal chitin (1–5 mg/ml) and 0.4 μg of protein in 50 mM Tris–HCl, pH 9, was incubated at 37°C for 1 h. The reaction was stopped by adding 0.2 ml of ferri-ferrocyanide reagent and then the mixture was boiled for 15 min (Imoto and Yagishita, 1971). After centrifugation at 12,000 rpm for 5 min, the supernatant was collected and the reducing sugars were measured by the absorbance at 405 nm.

2.9. Chitin-binding assay

The chitin-binding assay was done using colloidal chitin as the affinity matrix. Previously, we had utilized native chitin instead of colloidal chitin as the ligand and pH 6.5 instead of pH 8 for the binding assay (Zhu et al., 2001; Zhang et al., 2002). However, because preparations of the latter were more reproducible than the former, and the latter pH was intermediate to the pH of the locations where chitinase is physiologically functional, i.e. the molting fluid (pH ~7) and midgut lumen (pH ~l0), we have modified the assay as follows: first, 0.5 mg of colloidal chitin was mixed with 1 μg of protein in 50 μl of 10 mM sodium phosphate buffer, pH 8. The mixture was incubated at room temperature for 1 h and then centrifuged for 3 min. The supernatant was collected as the fraction containing unbound protein. The pellet was washed after suspension in 50 μl of 10 mM sodium phosphate buffer, pH 8, and centrifuged. This second supernatant was denoted as wash fraction I. Then the pellet was washed after resuspension in 50 μl of 10 mM sodium phosphate buffer containing 1 M NaCl, pH 8, followed by another wash in 50 μl of 0.1 M acetic acid. Both of those supernatants were collected as wash fractions II and III. Finally, the pellet was resuspended in 50 μl of SDS-PAGE sample buffer and boiled for 10 min. After centrifugation, the supernatant was collected as the bound protein fraction. All fractions were analyzed by SDS-PAGE followed by protein staining with Coomassie Brilliant Blue R-250. The protein bands were quantified using densitometric analysis.

2.10. Stability of chitinases in presence of gut extract

2.10.1. Preparation of gut extract

Midguts were dissected from fifth instar larvae of *M. sexta* that were actively feeding and immediately frozen on dry ice. The tissue was homogenized in five volumes of 50 mM Tris–HCl, pH 9, using a mortar and pestle on ice. The homogenate was centrifuged at 12,000 rpm for 20 min and the supernatant was collected.

2.10.2. Resistance of recombinant proteins to gut proteases

To investigate the stability of proteins, 20 μl of a reaction mixture containing 1 μg of chitinase protein and gut extract (1 μg of total protein) in 50 mM Tris–HCl, pH 9, was incubated at 37°C for 0–60 min. The reaction was stopped by adding 7 μl of 4× SDS-PAGE sample buffer and immediately boiled for 5–15 mm. All samples were analyzed by SDS-PAGE followed by staining for proteins with Coomassie Brilliant Blue R-250. The protein bands were quantified using densitometric analysis.

2.11. Circular dichroism

The gross structures of wild-type, truncated and extended forms of *M. sexta* chitinase as well as the linker and CBDs were monitored by circular dichroism (CD). Proteins were diluted into 20 mM sodium phosphate buffer, pH 8. The CD spectra were measured using a Jasco J720 spectropolarimeter at 20 °C. After noise reduction and concentration adjustment, the ellipticity was converted to the molar ellipticity and plotted against the wavelength.

3. Results

3.1. Expression, secretion and purification of recombinant proteins

To investigate the functions of various domains present in *M. sexta* chitinase, we generated cDNA expression constructs encoding several recombinant truncated and extended forms of the protein in addition to the one coding for the full-length protein, Chi535 (Fig. 1). Five constructs containing the open reading frames for C-terminally truncated proteins with progressively shorter deletions of amino acids on the C-terminal side of residue 376, including Chi376, Chi386, Chi396, Chi407 and Chi477 (see list of abbreviations and Fig. 1 for the regions included in these proteins), were expressed in Hi-5 insect cells using the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) as the expression vector (Gopalakrishnan et al., 1995; Zhu et al., 2001). Also, three extended forms of the protein, in which the C-terminus of the catalytic domain encoded by construct Chi386 was fused to one or two putative chitin binding domains, MCBD (amino acid residues 478–535 of *M. sexta* chitinase (Kramer et al., 1993)) or a rice chitin binding domain, RCBD (amino acid residues 19–68 of a rice class I chitinase (Huang et al., 1991)), were generated. Three deletion forms of *M. sexta* chitinases devoid of the catalytic domain at the N-terminal end and with six histidines as a tag on the C-terminal end were produced as recombinant proteins.

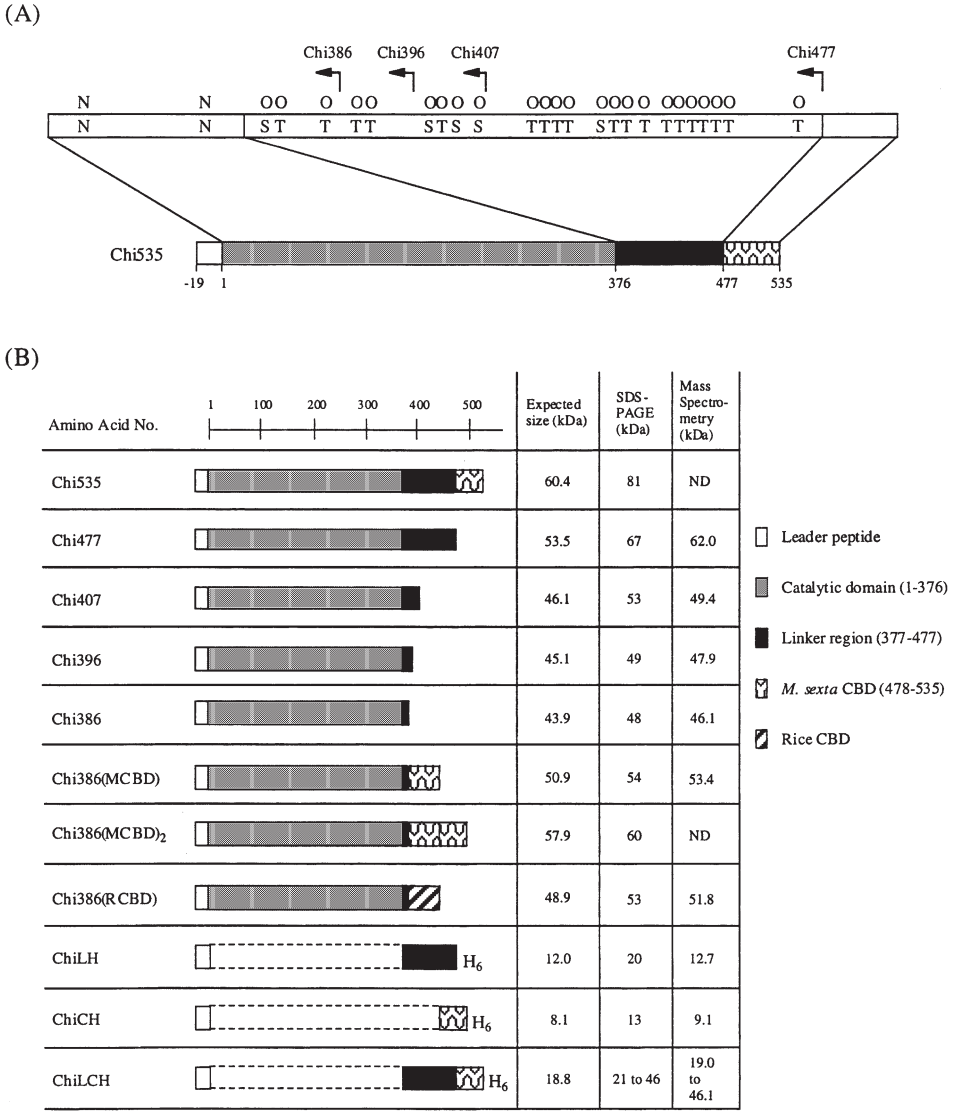

Fig. 1. Schematic diagram of recombinant full-length (Chi535), truncated (Chi386, Chi396, Chi407, Chi477, ChiLH, ChiCH and ChiLCH), and extended forms [(Chi386(MCBD), Chi386(MCBD)$_2$ and Chi386(RCBD)] of insect chitinase. (A) The full-length glycoprotein Chi535 with predicted locations of *O*- and N-linked residues (Hansen et al., 1997, 1998) denoted by the letters O and N. (B) The full-length, truncated and extended forms with masses determined from amino acid sequence, SDS-PAGE and mass spectrometry.

These forms were the following: ChiLH (consisting of amino acids 377–477 (the linker domain) followed by a C-terminal $(His)_6$ tag); ChiCH (consisting of amino acids 478–535 (the CBD) followed by a C-terminal $(His)_6$ tag); and ChiLCH (consisting of amino acids 377–535 (linker domain and the CBD) followed by a C-terminal $(His)_6$ tag). Finally, Chi80–535, a recombinant N-terminal truncated form missing the first 79 residues of the catalytic domain, was also purified from the culture medium but was found to be enzymatically inactive.

All of the recombinant proteins except for Chi376 and ChiLH were secreted into the medium by the AcMNPV-infected insect cells. These results agreed with those of Zhu et al. (2001), who also reported that Chi376 was not secreted and remained inside the cells even though it contained the 19 amino acid-long signal peptide in the preprocessed protein. When Chi376 was fused with the CBD from either *M. sexta* or rice chitinase, those constructs also remained inside the cells and were not secreted. However, addition of only 10 amino acids, consisting of residues 377–386 (SSYTVPPPHT), to the C-terminal of Chi376 did result in the secretion of recombinant proteins Chi386 and the domain-shuffled extended proteins, Chi386MCBD, $Chi386(MCBD)_2$ and Chi386RCBD, into the media. Other longer proteins with C-terminal CBDs, Chi407 and Chi477, which also contained residues 377–386, were likewise secreted from the cells into the media.

In terms of relative expression efficiency of the various proteins secreted by the insect cells, constructs encoding the full-length protein Chi535 and C-terminal truncated protein Chi477 exhibited the highest yields of approximately 20 mg protein/l of culture media. Yields of three other truncated proteins, Chi386, Chi396 and Chi407, were lower, ~10 mg/l, or only about 50% that of Chi535 and Chi477. The extended forms, Chi386MCBD, $Chi386(MCBD)_2$ and Chi386RCBD, were also secreted into the media but reached lower levels of <5 mg/l. The highest accumulations of secreted protein occurred when the linker region of the recombinant proteins remained intact. Apparently, the presence of CBDs (MCBD or RCBD) did not affect the secretory efficiency of these proteins, although their level of expression was slightly lower relative to Chi535 and Chi477. The yields of ChiLH, ChiCH and ChiLCH were rather low. The former protein was not secreted into the medium at all and had to be prepared from extracts of lysed cells. ChiCH and ChiLCH were secreted at concentrations ranging from 0.5 to 2 mg/l.

All of the secreted proteins were purified from culture media by chromatographic methods as described in Section 2. The purity of each protein was examined by SDS-PAGE. As shown in Fig. 2A, preparations of Chi535, Chi477, Chi407, Chi396, Chi386, Chi386MCBD, $Chi386(MCBD)_2$ and Chi386RCBD exhibited single protein bands and their apparent molecular masses were

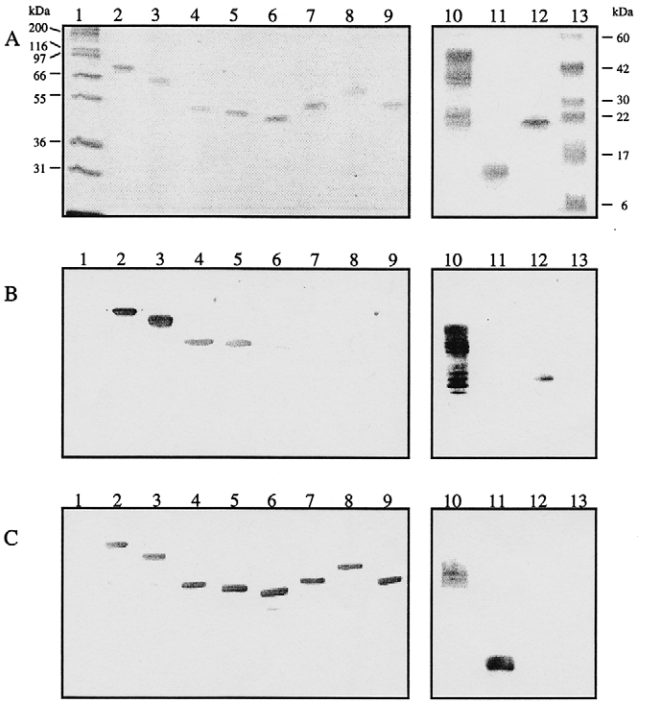

Fig. 2. SDS-PAGE analysis and immunoblotting of recombinant full-length, truncated and extended forms of insect chitinase. Proteins (2 μg) obtained by hydroxylapatite chromatography were subjected to electrophoresis on a 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250 (A). Immunoblotting was done using anti-Chi535 (B) and anti-Chi386 (C) antibodies. Lane 1, protein standard markers; lane 2, Chi535; lane 3, Chi477; lane 4, Chi407; lane 5, Chi396; lane 6, Chi386; lane 7, Chi386(MCBD); lane 8, $Chi386(MCBD)_2$; lane 9, Chi386(RCBD); lane 10, ChiLCH; lane 11, ChiCH; lane 12, ChiLH and lane 13, protein standard markers.

estimated to be 81, 67, 53, 49, 48, 54, 60 and 53 kDa, respectively (Fig. 1B). However, since the theoretical sizes of the truncated proteins based on their amino acid compositions were smaller in each case, 60.4, 53.5, 46.1, 45.1 and 43.9 kDa, respectively, and the masses determined by mass spectrometry were smaller than the apparent masses estimated from mobilities during SDS-PAGE, all of those proteins were probably post translationally glycosylated but to varying extents (Zhu et al., 2001; see Section 3.2). The apparent masses of the extended forms (lacking the linker region) were also larger than the predicted masses of 50.9, 57.9 and 48.9 kDa, respectively, for Chi386MCBD, $Chi386(MCBD)_2$ and Chi386RCB. Thus, those extended forms were apparently glycosylated as well but probably to a lower extent than the forms with the intact linker region. Thus, the relative differences between the observed and predicted masses ranged from about 2 to 30%. The degree of glycosylation apparently increased with the length of the truncated proteins. Mass spectrometry confirmed that the masses of all proteins were larger than those predicted from their amino acid sequences, indicating post-translational glycosylation.

The mobilities of ChiLH, ChiLCH and ChiCH as determined by SDS-PAGE were also greater than those predicted from their masses derived from the amino acid sequences. Laser desorption mass spectrometry was used to determine their molecular masses more accurately. Whereas the SDS-PAGE results indicated that the molecular weights of ChiCH and ChiLH are 13 and 20 kDa, respectively (Fig. 1 and Table 2), mass spectrometry indicated that these proteins had molecular weights of only 9.1 and 12.7 kDa, respectively. The masses predicted for ChiCH and ChiLH from their amino acid sequences (8.1 and 12.0 kDa, respectively) were close to the values determined by mass spectrometry. Unexpectedly, ChiLCH was quite heterogeneous (21–46 kDa) when examined by SDS-PAGE, and mass spectrometry revealed the presence in the preparation of a major protein with a size of 19 kDa. Its theoretical molecular weight based on amino acid sequence data was 18.8 kDa. Fig. 1 and Table 2 summarize the properties of all recombinant forms of these proteins.

The N-terminal sequence of both ChiLCH, a heterogeneous preparation consisting of a mixture of proteins ranging in size from 19 to 46 kDa, and ChiLH, which was a homogeneous preparation, was DKLSS. These data were in agreement with the predicated amino acid sequences of the mature forms of these proteins encoded by their constructs after cleavage of the leader peptide. There was no evidence of N-terminal sequence heterogeneity in the sequence of ChiLCH, even though it consisted of a mixture of proteins that apparently were heterogeneously glycosylated. The N-terminal sequence of ChiCH was DKLI, which was also exactly as predicted. Thus, the N-terminal sequencing results demonstrated that cleavage of the leader peptide of the precursors of these truncated forms had occurred in the insect cells as expected. Since these proteins were purified by affinity chromatography on Ni-NTA column, they all have an intact His_6 tag at the C-terminus. Therefore, it is probable that the heterogeneity observed in the ChiLCH preparation was due to a heterogeneous post-translational glycosylation.

3.2. Carbohydrate analysis

M. sexta chitinase is a glycoprotein, but the specific amino acid residues that are glycosylated are unknown (Gopalakrishnan et al., 1995; Zhu et al., 2001). When the amino acid sequence of insect chitinase was subjected to analysis by *O*-glycosylation site prediction software (Hansen et al., 1997, 1998), many residues in the linker region were predicted to be *O*-glycosylated, including 19 threonine residues: no. 380, 386, 390, 392, 401, 413–416, 422, 423, 426, 429–434 and 469, and five serine residues: no. 378, 400, 403, 406 and 421 (Fig. 1A). Only two of the threonines and three of the serines in the linker region, and all of the other threonines and serines outside of the linker region were not predicted to be glycosylated. On the other hand, when the chitinase sequence was subjected to analysis by *N*-glycosylation site prediction software (Gupta et al., 2003), out of the four asparagine-X-Ser/Thr residues present in this protein, only two (asparagines 66 and 285) were predicted to be *N*-glycosylated and those residues are outside of the linker region. Thus, most of the glycosylation in insect chitinase is predicted to occur in the linker region as *O*-glycosylated threonines and serines.

Chemical analysis of carbohydrates present in the full-length enzyme and the truncated proteins confirmed the prediction that all of these recombinant proteins were indeed glycosylated with mannose and/or GalNAc being the most abundant sugars. These analyses also revealed that the degree of glycosylation varied as longer stretches of amino acids were deleted from the C-terminal region (Table 2).The catalytic domain with the

Table 2
Carbohydrate compositions and masses of recombinant full-length and truncated forms of insect chitinase[a]

Carbohydrate	Chi535	Chi477	Chi407	Chi396	Chi386	ChiLH	ChiCH	ChiLCH
GalNAc	20 ± 14	16 ± 12	4 ± 3	1 ± 1	0	1	ND	8
Mannose	8 ± 4	8 ± 3	38 ± 36	14 ± 7	9 ± 3	1	5	4
GlcNAc	4 ± 2	3 ± 1	4 ± 3	3 ± 2	4 ± 1	0	2	1
Glucose	2 ± 0	3 ± 1	13 ± 10	4 ± 1	2 ± 1	1	1	0
Galactose	4 ± 3	4 ± 1	3 ± 2	2 ± 0	1 ± 0	0	ND	2
Total	38	34	62	24	16	3	8	15
Mass (kDa)								
aa	60	54	46	45	44	12	8	19
aa + carbohydrate	68	61	58	49	47	13	10	22
SDS	81	67	53	49	48	20	13	21–46
Mass spectrometry	ND	62.0	49.4	47.9	46.1	12.7	9.1	19–46

[a] Moles of sugar per mole of protein. Mean value ±SD (n = 3) for Chi535, Chi477 and Chi407. Mean value ±0.5 range (n = 2) for Chi396 and Chi386. ND=Not detected.

minimal length of linker (Chi386) contained GlcNAc, mannose and trace amounts of glucose and galactose. The proteins with an increasingly longer linker have progressive increases in GalNAc, while retaining nearly the same amount of GlcNAc, galactose and mannose as Chi386. Some preparations of Chi407 had unusually high amounts of mannose and glucose, which resulted in a large standard error in the carbohydrate composition for that protein. The reason for this large variation is unknown, but it may be related to improper or variable folding of the truncated protein in the absence of a full-length linker region. The data for the other truncated forms of chitinase are consistent with the notion that the catalytic domain has only one or two N-linked oligosaccharides rich in mannose and that the linker region is *O*-glycosylated (containing Gal and GalNAc) over much of its length. The difference in the sizes determined by mass spectrometry from those predicted only from amino acid sequences also increased as larger portions of the linker were added to the catalytic domain, which suggested that glycosylation occurred over the entire linker region. However, there was little or no carbohydrate in the linker or CBD when these recombinant proteins were expressed individually. Nonetheless, both of these domains migrated non-ideally during SDS-PAGE, especially ChiLH, which behaved like a protein twice as large as predicted (Table 2). Apparently, the linker does not associate with SDS in a complex with a charge:mass ratio comparable to those of the standard marker proteins.

We also examined the susceptibility of the recombinant proteins to various glycosidases as monitored by a comparison of their mobilities upon SDS-PAGE before and after enzyme treatment. Treatment of Chi535 with PNGase F overnight to remove N-linked glycosides resulted in a mobility shift corresponding to a size reduction of about 3 kDa (data not shown). If the removal of N-linked sugars was assumed to be complete, this result suggested that there was approximately 3 kDa of N-linked carbohydrates in this protein. Treatment with an *O*-glycosidase mixture (exo-*O*-glycosidases+endo-*O*-glycosidases) removed about 4 kDa of sugar from Chi535, a result suggesting that there was about 4 kDa of O-linked sugars in insect chitinase. A mixture of both *N*- and *O*-glycosidases removed approximately 6 kDa of carbohydrate. Overall, these data indicate that there was approximately 6 kDa of N- and O-linked oligosaccharides in the full-length protein Chi535.

Treatment of Chi386 with *N*-glycosidase, but not *O*-glycosidase, resulted in a mobility shift consistent with a reduction in size of about 2 kDa (data not shown). In contrast, *O*-glycosidase treatment, but not *N*-glycosidase treatment, resulted in a mobility shift equivalent to 3 kDa of the slowest moving band in the ChiLCH preparation. Several other bands in this preparation showed no alteration in mobility (size) after treatment with a mixture of *N*- and *O*-glycosidases (data not shown). ChiCH and ChiLH preparations showed no changes in mobility (size) after treatment with the mixture of *N*- and *O*-glycosidases. From all of the data described above including the carbohydrate content and glycosidase treatments, we conclude that the catalytic domain of *M. sexta* chitinase has one or two sites of *N*-glycosylation and that the linker domains in Chi535, Chi477, Chi407, Chi396, Chi386 and ChiLCH, but not ChiLH, have multiple sites of *O*-glycosylation (Fig. 1).

3.3. Immunoblot analysis

In immunological studies, we found that the anti-Chi535 antibody, our first polyclonal antibody that was raised against the full-length glycoprotein prepared from the molting fluid (Koga et al., 1983b), did not react well with the C-terminal-truncated proteins, Chi386 and Chi376, or ChiCH (Fig. 2B; Zhu et al., 2001). We attributed this observation to an inability of that polyclonal antibody, which was raised against the full-length native 81 kDa *M. sexta* chitinase, to bind to the catalytic domain (residues 1–376) or CBD (residues 478–535). Therefore, we raised a second polyclonal antibody to the smallest secreted enzymatic protein, Chi386, which contained the entire catalytic domain and lacked most of the C-terminal linker (except for the first 10 amino acids) and the entire CBD.

To investigate further the specificities of these two antibodies, immunoblot analysis was done using the antibodies elicited against either Chi535 or Chi386. As shown in Fig. 2B, anti-Chi535 antibody recognized Chi535 and Chi477 well, but the recognition of Chi407 and Chi396 was much weaker. Chi386, Chi386MCBD, Chi386$(MCBD)_2$ and Chi386RCBD were unrecognized or hardly at all, suggesting that the linker region (positions 386–477) and/or its associated glycosyl residues are important for recognition by the anti-Chi535 antibody. However, this antibody recognized ChiLH, which contains the entire linker domain and a C-terminal His-tag, only very weakly and did not recognize the CBD fragment. Interestingly, the Chi535-antibody cross-reacted strongly with ChiLCH, which contains both the linker region and the CBD and also is glycosylated. It appears that the anti-Chi535 antibody strongly recognizes epitopes that are composed of elements from both the linker and the CBDs, especially the *O*-glycosylated sites.

The linker region is a S/T-rich region with a high potential for *O*-glycosylation. The possibility that the anti-Chi535 antibody recognizes epitopes consisting of these sugars was tested by treating Chi535 and ChiLCH with a mixture of *N*- and *O*-glycosidases. We found no evidence of any reduction in immunological activity of these proteins even though we could demonstrate removal of most of the carbohydrate residues because

of changes in the mobility of the protein band(s) after glycosidase treatment, suggesting that the epitopes recognized by the antibody involve primarily amino acids and not sugars. However, we could not be certain that all of the sugars had been removed from the linker region by the glycosidase treatment. ChiLCH expressed from *E. coli* may help to clarify the immunological specificity or antibody recognition properties. Thus, the nature of the residues that interact with the anti-Chi535 antibody remains unresolved.

To detect protein forms that lacked this linker region, another antibody, anti-Chi386, had to be generated and utilized. This antibody recognized all of the deleted and extended forms of insect chitinase that contained the catalytic domain quite well (Fig. 2C). Anti-Chi386 detected Chi535 and Chi386 about equally well. Even though the Chi386 antigen did not contain the CBD region and only the first 10 amino acids of the linker region, western blotting analysis showed that anti-Chi386 recognized ChiCH well. Chi386 and ChiCH apparently share a common epitope that is perhaps localized in their carbohydrate binding sites. Anti-Chi386 also cross reacted with a set of larger molecular weight proteins present in the ChiLCH preparation but not with the lower molecular weight proteins that cross reacted with the Chi535 antibody.

3.4. Chitin binding

To compare the ability of the truncated and extended forms of insect chitinase to bind to the insoluble substrate chitin, a binding assay was conducted at pH 8 using colloidal chitin as the affinity matrix. The bound and unbound fractions were resolved by SDS-PAGE and stained for proteins. Chi535 was bound to colloidal chitin and the percentage bound was approximately 80% under our experimental conditions (Fig. 3). Non-specific adsorption of proteins to colloidal chitin was tested using bovine serum albumin as the test protein, which was bound to only about 5% with chitin. The binding abilities of the four C-terminal truncated forms were substantially lower than that of Chi535, with the binding of Chi477, Chi407, Chi396 and Chi386 occurring at only 15, 50, 33 and 37%, respectively. The reduced adsorption of these truncated forms (compared to the full-length enzyme) was not unexpected because the putative CBD (residues 478–535) was absent from those proteins. However, the higher percentage of binding observed with Chi407 compared to Chi477 suggested that the full-length linker region, perhaps as a result of the unusually high content of mannose and glucose, did influence ligand binding but in an unknown manner. The presence of amino acid residues 408–477 was detrimental to binding, perhaps because of steric interference. When the CBDs from insect and rice chitinases were fused with Chi386 (with a binding ability of 37%), the resulting products,

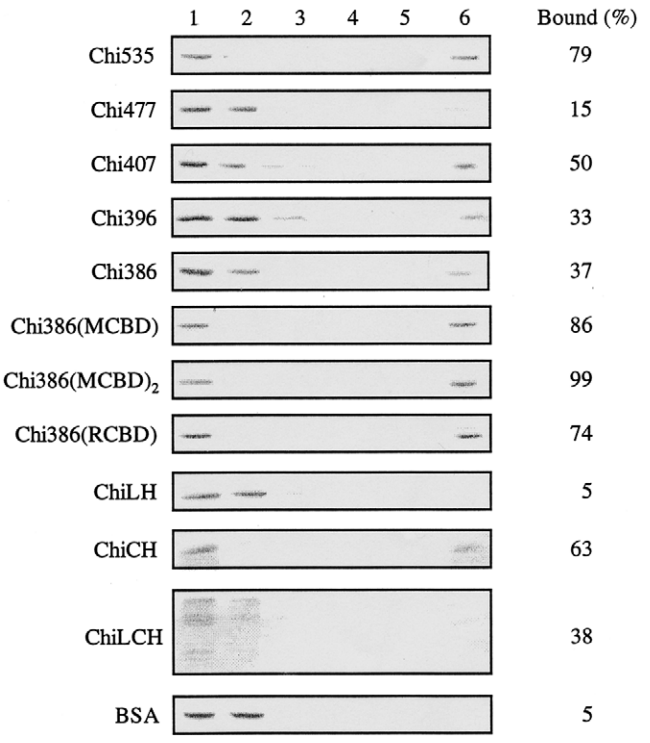

Fig. 3. Chitin-binding assay of recombinant full-length, truncated and extended forms of insect chitinase. Chitin binding assays were done as described in Section 2. The assay mixtures contained 1 μg of chitinase and 0.5 mg of colloidal chitin. All fractions were subjected to SDS-PAGE and proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, starting fraction; lane 2, unbound fraction; lane 3, wash fraction I (10 mM sodium phosphate, pH 8); lane 4, wash fraction II (10 mM sodium phosphate containing 1 M NaCl, pH 8); lane 5, wash fraction III (0.1 M acetic acid; and lane 6, bound fraction. Bound percentage = bound protein (lane 6)/starting protein (lane1). BSA = bovine serum albumin.

Chi386MCBD and Chi386RCBD, exhibited a binding ability of 86 and 74%, respectively, which were comparable in strength to that of the full-length chitinase, Chi535. The absence of the linker region in those constructs did not negatively affect the extent of binding to chitin. Furthermore, when a second insect CBD domain was added, the resulting protein, Chi386$(MCBD)_2$, showed the highest binding capacity of all at 99%. Thus, addition of either an insect or a plant CBD to Chi386 resulted in increased adsorption to the insoluble substrate, colloidal chitin.

Regarding the ability of the linker (ChiLH) and CBDs (ChiCH) to bind to insoluble chitin, ChiCH had an affinity for insoluble chitin similar to Chi535. About 65% of ChiCH was bound to colloidal chitin (Fig. 3). The linker domain ChiLH did not bind to colloidal chitin. When the linker region was fused with the CBD (ChiLCH), that recombinant protein exhibited only about half of the binding ability of Chi535 and ChiCH. The linker region interfered with the chitin binding of ChiCH when the catalytic domain was not attached to the N-terminal of the linker region.

3.5. Circular dichroism

The gross structures of wild-type and several of the truncated and extended forms of *M. sexta* chitinase were analyzed using circular dichroism (CD). As shown in Fig. 4, the CD spectra are consistent with the full-length enzyme and the C-terminally truncated forms, Chi477, Chi407, Chi396 and Chi386, having both α-helices and β-sheets in agreement with the prediction that the catalytic domain of family 18 chitinases consists of a $(\beta\alpha)_8$ barrel structure (Terwisscha van Scheltinga et al., 1996). As the length of the truncated proteins decreased, the absorbance at approximately 220 nm slightly decreased, while that at about 207 nm increased, indicating a moderate change in the relative proportion of α-helix to β-sheet. On the other hand, ChiLH exhibited a CD spectrum indicative of little, if any, secondary structure. The spectrum of ChiCH showed predominance of β-strands. These results are consistent with the catalytic domain and CBD retaining most of their characteristic secondary structures both individually and when present in the full-length enzyme. The linker region, however, appeared to be devoid of any ordered structure, which did not significantly affect the structure of the other domains.

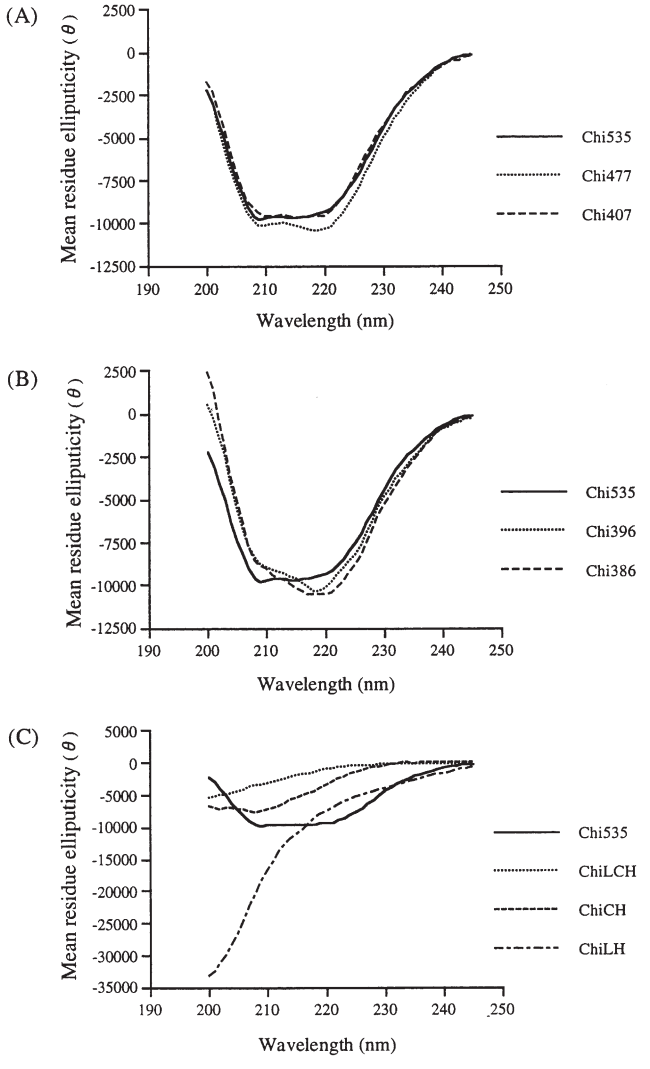

Fig. 4. (A) Circular dichroism spectra of recombinant full-length, truncated and extended forms of insect chitinase. CD spectra were measured in 20 mM phosphate buffer, pH 6.5 at room temperature in a 0.1 cm cuvette. (A) Chi535, Chi477 and Chi407. (B) Chi535, Chi396 and Chi386. (C) Chi535, ChiLCH, ChiCH and ChiLH. The concentrations of Chi535, Chi477, Chi407, Chi396, Chi386, ChiLCH, ChiCH and ChiLH were 18, 17, 16, 16, 16, 410, 112 and 140 μM, respectively.

3.6. Resistance to gut extract proteases

To investigate the influence of the domains on chitinase stability in its natural gut environment, each of the recombinant proteins was incubated with proteinases present in an extract prepared from midguts of feeding fifth instar *M. sexta* larvae. As shown in Fig. 5, Chi535 and Chi477 were more stable than the other truncated forms, with half-lives of >60 min. Chi407 had a half-life of approximately 50 min, whereas those of Chi386 and Chi396 were only about 15 min. These results suggested that the S/T-rich linker region and/or glycosylation might increase the stability of insect chitinase. With regard to extended forms, addition of either MCBD or RCBD increased stability only slightly. However, addition of a second MCBD increased the half-life of

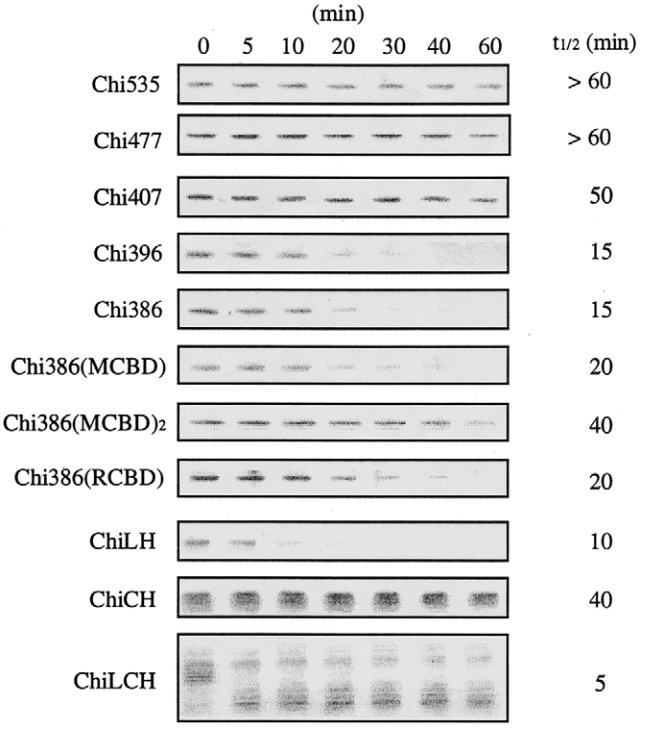

Fig. 5. Stability of recombinant full-length, truncated and extended forms of insect chitinase in the presence of gut extract. Enzyme stability assay was done by the method described in Section 2. Chitinases (1 μg) were incubated at 37 °C for 0–60 min in the presence of gut extract (1 μg of total protein). All fractions were subjected to SDS-PAGE and proteins were stained with Coomassie Brilliant Blue R-250.

the protein from 15 to 40 min. These results suggested that the S/T-rich linker region and/or glycosylation contributed to the stability of insect chitinase. It is possible that the CBDs improved stability by shielding some of the target sites from the gut proteinases, which helped to diminish protein degradation.

ChiCH was as stable in the presence of gut proteases as the full-length enzyme, but ChiLCH, in which the linker domain was attached to CBD, was degraded rapidly ($t_{1/2}$ = 5 min) from a protein of about 45 kDa to a mixture of products with sizes ranging from ~20 to 25 kDa. Those products were stable over the duration of the experiment. ChiLH also was unstable in the presence of gut proteases and was completely degraded by 10 min.

3.7. Kinetic analyses

To compare the enzymatic behavior of these modified forms of insect chitinase, kinetic analyses were done at pH 9 using several substrates, including colloidal chitin as an insoluble polymeric substrate, CM-Chitin-RBV as a soluble polymeric substrate, and MU-$(GlcNAc)_3$ as a soluble oligomeric substrate. All of the recombinant proteins with the intact catalytic domain were active towards each of the substrates. With colloidal chitin as the substrate, it should be noted that the K_m values of all of the truncated forms were larger than that of Chi535, indicating that those enzymes had a diminished affinity for the insoluble large substrate presumably because they lacked the CBD (Table 3). The kinetic behavior of Chi407, however, was non-ideal such that the kinetic parameters could not be calculated because apparently the enzyme was highly susceptible to substrate and/or product inhibition (data not shown). The K_m values of the three extended forms were smaller than that of Chi386 and similar to that of Chi535, suggesting that all had a high substrate affinity. As expected, addition of CBDs increased the affinity of the catalytic domain of the insect chitinase for the insoluble substrate. These results were in good agreement with those obtained from chitin-binding analysis. It is likely that the binding of the insoluble substrate to the CBD increased the local concentration of the substrate in the neighborhood of the catalytic site. With respect to the oligosaccharide substrate, MU-$(GlcNAc)_3$, all of the enzymes were susceptible to substrate inhibition. Interestingly, Chi407 was particularly susceptible to inhibition by the small substrate (Table 4). On the other hand, with regard to the soluble polymeric substrate, CM-Chitin-RBV, there was no substrate inhibition behavior and it was possible to derive V_{max} and K_m values for all of the recombinant proteins (Table 5). The K_m values of the extended forms were the smallest for all of the substrates. However, the turnover numbers did not vary substantially and the most active protein was the full-length enzyme.

Table 3
Kinetic parameters of truncated and extended forms of insect chitinase using colloidal chitin as the substrate[a]

Enzyme	V_{max} (△A405/h/μM)	K_m (mg/ml)	V_{max}/K_m
Chi535	8.70	4.29	2.05
Chi477	8.40	9.96	0.89
Chi407[b]	–	–	–
Chi396	11.8	20.0	0.59
Chi386	13.5	29.0	0.47
Chi386(MCBD)	3.49	4.98	0.70
Chi386$(MCBD)_2$	4.88	4.67	1.04
Chi386(RCBD)	3.11	3.18	0.99

[a] Substrate concentration ranged from 1 to 5 mg/ml and enzyme concentration from 49 to 83 nM.
[b] No parameters were determined because of strong substrate/product inhibition.

4. Discussion

A multi-domain structural organization is often observed in polysaccharide-degrading enzymes, where one or more domains are responsible for hydrolysis and one or more domains are responsible for associating with the solid polysaccharide substrate. In addition, there are usually linker regions between the two types of domains, which also may be responsible, at least in part, for some functional properties of the enzymes. For example, a chitinase from the parasitic nematode, *Brugia malayi*, contains catalytic, linker and CBDs (Venegas et al., 1996). Insect chitinases possess such a structural organization as do other nematode, microbial, and plant chitinases and fungal cellulases. The most novel multidomain structure exhibited by an insect chitinase, which we are aware of, is that of the enzyme from the beetle, *Tenebrio molitor* (Royer et al., 2002). This protein is very large, with a calculated molecular mass of the deduced protein being 320 kDa. It contains five catalytic domains, five Ser/Thr-rich linker domains, four CBDs, and two mucin-like domains. The chitinases from the bacterium, *Serratia marcescens*, fall into three classes (with sizes ranging from 36 to 52 kDa), which are composed of different combinations of catalytic domains, fibronectin type-III-like domains, and N- or C-terminal CBDs (Suzuki et al., 1999). The *M. sexta* chitinase, however, is much smaller than the *Tenebrio* enzyme and less complex in domain structure with only a single N-terminal catalytic domain, a linker domain, and a C-terminal CBD. Classes I and IV plant chitinases contain an N-terminal CBD and a G/P-rich linker preceding the catalytic domain (Raikhel et al., 1993; Neuhaus, 1999), whereas the fungal cellulases possess a threonine/serine/proline-rich linker between the N-terminal catalytic domain and the C-terminal cellulose-binding domain (Srisodsuk et al., 1993). The *Manduca* chitinase linker region that is rich in T

Table 4
Kinetic parameters of truncated and extended forms of insect chitinase using MU-$(GlcNAc)_3$ as the substrate[a]

Enzyme	Substrate inhibition (mM)	k_{cat} (1/s)	K_m (mM)	k_{cat}/K_m (1/s/mM)
Chi535	>0.10	0.76	1.13	0.67
Chi477	>0.20	0.31	0.51	0.62
Chi407[b]	>0.03	–	–	–
Chi396	>0.10	0.27	0.64	0.42
Chi386	>0.10	0.37	0.93	0.40
Chi386(MCBD)	>0.10	0.14	0.29	0.50
$Chi386(MCBD)_2$	>0.05	0.17	0.31	0.54
Chi386(RCBD)	>0.05	0.22	0.48	0.46

[a] Substrate concentration ranged from 0.02 to 0.2 mM and enzyme concentration from 25 to 43 nM.
[b] No parameter were determined because of strong substrate inhibition.

Table 5
Kinetic parameters of truncated and extended forms of insect chitinase using CM-Chitin-RBV as the substrate[a]

Enzyme	V_{max} (△A550/h/μM)	K_m (mg/ml)	V_{max}/K_m
Chi535	18.2	1.84	9.89
Chi477	13.5	1.58	8.54
Chi407	3.39	0.63	5.41
Chi396	9.52	1.49	6.39
Chi386	6.54	1.05	6.23
Chi386(MCBD)	2.30	0.28	8.19
$Chi386(MCBD)_2$	2.54	0.35	7.28
Chi386(RCBD)	2.99	0.52	5.81

[a] Substrate concentration ranged from 0.1 to 1.0 mg/ml and enzyme concentration from 62 to 107 nM.

and S residues is also rich in P, D and E residues, which qualifies it as a PEST sequence according to Rogers et al. (1986). This composition suggests that the insect chitinase might be a rapidly degraded protein. Intracellular ubiquitin-conjugating enzymes recognize such a sequence so that proteasomes can digest the conjugated protein when it is localized intracellularly. However, insect chitinase is a secreted protein and, therefore, would be expected to be exposed to intracellular proteases or the ubiquitin-conjugating system for only a short period of time. Conversely, because Chi477 and Chi535, which contain the linker region, were more stable in the presence of midgut proteases than the other C-terminal truncated forms, the linker region apparently helps to stabilize the enzyme and protects protease-susceptible bonds in the catalytic domain from hydrolysis in the gut.

Recombinant enzymes lacking amino acid residues from position 377 to 386 accumulated intracellularly (e.g. Chi376 with or without CBDs, data not shown), whereas all of the forms that had these 10 amino acids were secreted into the media. We conclude, therefore, that the N-terminal portion of the linker region (residues 377–386) must be present, in addition to the 19 amino acid-long N-terminal leader peptide, for secretion to occur outside of the cells. That region may also need to be *O*-glycosylated because ChiLH, which contains residues 377–386, accumulates intracellularly and, based upon its size determined by mass spectrometry, ChiLH does not appear to be glycosylated. ChiCH, which does not contain residues 377–386, is secreted into the medium and has approximately the same size predicted from its amino acid sequence. Thus, ChiCH does not appear to be glycosylated (Table 2). On the other hand, during SDS-PAGE, ChiLCH behaved like a protein larger than predicted because it is glycosylated (15 mol of sugar). It has very little GlcNAc. Thus, the *M. sexta* chitinase linker region is probably *O*-glycosylated. However, both secretion from the cell and glycosylation of ChiLCH appear to be dependent upon the presence of the CBD because ChiLH is localized intracellularly and not glycosylated. The glycosylating enzyme system in the ER and/or Golgi may recognize sites for glycosylation, which are present only in molecules with both of those domains. This idea is further supported by the reaction of these domains to anti-Chi535, which recognizes ChiLCH but not ChiCH and recognizes ChiLH only very weakly. The critical residues for glycosylation, therefore, may involve residues between amino acids 376 and 386 (which includes two threonines) because Chi376 accumulated intracellularly, whereas Chi386 was secreted. Site-directed mutagenesis of these residues might help to answer the question about whether these residues are required for secretion.

The primary epitope recognized by the antibody elicited by the wild-type glycoprotein is the highly glycosylated Ser/Thr-rich linker region of *M. sexta* chitinase. Other highly immunogenic insect proteins that apparently are extensively *O*-glycosylated in threonine-rich domains similar to the linker region of *Manduca* chitinase are peritrophins-55 and -95 from the sheep blowfly, *Lucilia cuprina* (Tellam et al., 2000, 2003). The sera of sheep vaccinated with these peritrophins exhibited a strong immune response that inhibited the growth of blowfly larvae (Casu et al., 1997; Tellam et al., 2003).

M. sexta chitinase is probably *N*-glycosylated in the catalytic domain and *O*-glycosylated in the linker region. The insect cell line used here, TN-5B1-4 (Hi-5), which is used routinely for foreign glycoprotein production, synthesizes proteins with both N- and O-linked oligosaccharides (Davidson et al., 1990; Davis and Wood, 1995; Jarvis and Finn, 1995; Hsu et al., 1997). Various studies of the glycosylation patterns of endogenous and recombinant glycoproteins produced by insect cells have revealed a large variety of glycan structures (Marchal et al., 2001). When human fucosyltransferase III was produced as a recombinant protein in both insect and mammalian cells, *N*-glycosylation was required for its proper folding in vivo (Morais et al., 2002). Whether the same effect of *N*-glycosylation is true for insect chitinase remains to be determined. Previously, results of experiments investigating the effects of the *N*-glycosylation inhibitor, tunicamycin, on recombinant expression of insect chitinases in these cells indicated that the proteins were glycosylated prior to being secreted by the cells (Gopalakrishnan et al., 1995; Zheng et al., 2002). Direct chemical and enzymatic analyses have confirmed that *M. sexta* chitinase is both *N*- and *O*-glycosylated. Prolonged (overnight) deglycosylation of Chi535 with a mixture of *N*- and *O*-glycosidases resulted in a protein that appears to be smaller by about 6 kDa accounting for about 30 sugar residues per mole of protein. Because N-linked oligosaccharides in insects typically have 6–7 residues, two of which are GlcNAc (Paulson, 1989; Kubelka et al., 1995), our best estimate of the distribution of *N*-glycosylation involves a single or possibly two sites of *N*-glycosylation in the catalytic domain and *O*-glycosylation of between 10 and 20 serine or threonine residues in the linker region. *O*-Glycosylation may involve mainly addition of galactose and *N*-acetylgalactosamine. The structures of the N- and O-linked glycans may be comparable to those identified in other invertebrates (Marchal et al., 2001; Wilson, 2002).

Glycosylation of the linker region may help to prevent proteolytic cleavage(s) at sites between the catalytic and CBDs. Such a functional role of glycosylated regions has been observed in some bacterial cellulases (Langsford et al., 1987). The full-length and near full-length *O*-glycosylated forms, Chi535 and Chi477, were the most stable proteins when incubated with the hornworm's midgut proteinases. The linker region connects the catalytic domain and the cysteine-rich CBD, both of which are predicted to have compact structures. Protein modeling studies using the crystal structures of other family 18 glycosylhydrolases as templates suggested that the catalytic domain of *M. sexta* chitinase has an eight-fold $(\beta\alpha)_8$-TIM barrel structure (Kramer and Muthukrishnan, 1997; Nagano et al., 2002). The CBD probably exhibits a multi-stranded β-sheet structure based on similarity to tachycitin (Suetake et al., 2000). We know of no structures computed or proposed for linker domains which may be rather flexible and potentially susceptible to proteolytic degradation unless they are protected by glycosylation. The CD spectrum of the linker domain is consistent with the lack of any secondary structure in this domain. It is conceivable that during the period of maximum chitinase activity, the enzyme is fully glycosylated. When required, a glycosidase(s) could be produced that would remove sugar residues, thus exposing those amino acid residues for proteolytic cleavage. Alternatively, proteolytic cleavage may be reduced because of glycosylation. Consistent with this notion is the finding that analysis of molting fluid from *M. sexta* and *Bombyx mori* revealed the presence of truncated forms of catalytically active chitinaes with sizes ranging from 50 to 60 kDa (Kramer and Koga, 1986; Koga et al., 1997; Abdel-Banat et al., 1999). We have detected similar truncated forms in our insect cell expression system, especially several days subsequent to infection with the recombinant baculovirus (Gopalakrishnan et al., 1995).

Peptides linking protein domains are very common in nature and many are believed to join domains rather passively without disturbing their function or affecting their susceptibility to cleavage by host proteases (Argos, 1990; Gilkes et al., 1991). Linker peptides with G, T or S residues are most common, perhaps because those residues are relatively small with G providing flexibility and T and S being uncharged but polar enough to interact with solvent or by their ability to hydrogen bond to water or the protein backbone to achieve conformational and energetic stability. The interdomain linker peptide of a fungal cellobiohydrolase apparently has a dual role in providing the necessary distance between the two functional domains and facilitating the dynamic adsorption process led by the cellulose-binding domain (Srisodsuk et al., 1993). Solution conformation studies of a fungal two-domain cellulase revelaed that its linker exhibited an extended conformation leading to maximum extension between the two domains and that heterogeneous glycosylation of the linker was likely a key factor defining its extended conformation (Receveur et al., 2002). Since the domain structure of *M. sexta* chitinase is similar to that of this fungal cellulase, these enzymes may have similar global structural characteristics. CD spectra of the proteins generated here were consistent with the hypothesis that the catalytic and CBDs possess substantial secondary structure, whereas the linker region does not.

The basic function of CBDs is thought to help localize the enzyme on the insoluble substrate to enhance the efficiency of degradation (Linder and Terri, 1997). In general, for many glycosyl hydrolases, the binding specificity of the carbohydrate binding module mirrors that of the catalytic module and these two domains are usually in relatively close association. The CBD of insect chitinase belongs to carbohydrate-binding module family 14, which consists of approximately 70 residues

(Coutinho and Henrissat, 1999). Of the chitin-binding modules, there are only three subfamilies identified so far and the *M. sexta* chitinase CBD is a member of Subfamily 1 (Henrissat, 1999). Such a carbohydrate-binding function has been demonstrated in several carbohydrolases and other carbohydrate-binding proteins. These modules are attached to catalytic domains of several chitinases and also to chitinase-like proteins devoid of enzyme activity. These CBDs can be either N- or C-terminal and may be present as a single copy or as multiple repeats. These domains are cysteine-rich and have several highly conserved aromatic residues (Shen and Jacobs-Lorena, 1999). The cysteine residues help to maintain protein folding by forming disulfide bridges and the aromatic residues probably interact with saccharides in the ligand-binding pocket. There are peritrophic matrix proteins, mucins, with affinity for chitin, which contain a similar six-cysteine peritrophin-A/mucin consensus sequence (Tellam et al., 1999; Morlais and Severson, 2001).

Insoluble substrate-binding domains apparently increase the enzyme concentration at the substrate surface and help to juxtaposition the catalytic domain so that hydrolysis occurs more readily on the insoluble substrates (Linder and Terri, 1997; Black et al., 1997). When fused with the catalytic domain of *M. sexta* chitinase, both insect and rice CBDs were similar in their ability to promote binding to and hydrolysis of chitin. The influence of extra substrate-binding domains has been examined previously using a fungal chitinase that was constructed to include plant and fungal carbohydrate-binding domains (Limón et al., 2001). The addition of those domains increased the substrate-binding capacity and specific activity of the enzyme toward high molecular mass insoluble substrates, such as ground chitin or chitin-rich fungal cell walls. Removal or addition of cellulose-binding domains can reduce or enhance, respectively, the ability of cellulases to degrade crystalline cellulose (Chhabra and Kelly, 2002). When a second cellulose-binding domain was fused to *Trichoderma reesei* cellulase, the resulting protein had a much higher affinity for cellulose than the protein with only a single binding domain (Linder et al., 1996). Likewise, the *M. sexta* chitinase catalytic domain fused with two CBDs associated with chitin more strongly than any of the single CBD-containing proteins described in this study. This domain apparently targets the secreted enzyme to its substrate.

Rye seed contains two chitinases, one with and the other lacking a CBD (Taira et al., 2002). In terms of fungicidal activity, the former class I enzyme inhibited fungal growth more effectively than the latter class II enzyme. Apparently, the CBD of the class I chitinase facilitates binding to the fungal hyphae, whereas the other enzyme cannot associate with this insoluble substrate as well. Similarly, removal of the N-terminal CBD from a tobacco class I chitinase resulted in increases in K_m and V_{max} of the enzyme (Iseli et al., 1993). The CBD of insect chitinase probably has the function of associating with insoluble chitin and helping to direct the chitin chain into the active site of the catalytic domain in a manner similar to the processive hydrolysis mechanism of *S. marcescens* chitinase A (ChiA, Uchiyama et al., 2001). The only difference is that in the case of insect chitinase, hydrolysis proceeds toward the reducing end instead of the non-reducing end (Kramer and Koga, 1986).

Catalytically, none of the modified forms of insect chitinase reported in this study was more efficient at substrate hydrolysis than the full-length enzyme. Chi535 was from 2- to 4-fold more active in hydrolyzing insoluble colloidal chitin than any of the other enzymes, but all of the recombinant forms were nearly comparable in turnover rate when the two soluble substrates, CM-chitin-RBV, which is a chitin derivative that is *O*-carboxymethylated, and MU-$(GlcNAc)_3$, an oligosaccharide substrate, were tested. Thus, a moderate increase in catalytic efficiency was observed when the catalytic domain fused with the CBD hydrolyzed the insoluble substrate. When the C-terminal CBD was deleted from a bacterial chitinase from *A. caviae*, this truncated chitinase was also active, but it liberated longer oligosaccharide products than did the full-length enzyme (Zhou et al., 2002). Thus, as was observed with other carbohydrolases such as xylanases (Gill et al., 1999), the CBD of insect chitinase facilitates hydrolysis of insoluble but not soluble substrates, and also influences the size of the oligosaccharide products generated. The linker region can also influence the functionality of the carbohydrate-binding domain. When a fungal cellulose-binding domain was fused with a fungal threonine/serine-rich linker peptide, the fusion protein adsorbed to both crystalline and amorphous cellulose. However, deletion of the linker peptide caused a decrease in cellulose adsorption and a higher sensitivity to protease digestion (Quentin et al., 2002).

The results of this study are consistent with the hypothesis that *M. sexta* chitinase consists of two domains connected by a linker region with the N-terminal domain harboring the catalytic activity and the C-terminal one being a CBD capable of specific recognition of the insoluble chitin polymer. These activities are independent of each other because each domain was functional separately when they were expressed as recombinant proteins. The linker region between these domains, which is highly glycosylated, not only connects them but also facilitates protein secretion from the cell and helps to stabilize the enzyme in the presence of proteolytic enzymes. Proteolysis of cuticle protein is probably necessary to expose chitin in the exoskeleton and PM so that chitinases can digest the polysaccharide. Insect chitinase may have evolved resistance to those

proteases by utilizing a linker region that is highly glycosylated. Also, the linker region is probably exposed sufficiently to the aqueous environment such that it is strongly immunogenic. The data generated here also support the hypothesis that the domain structure of insect chitinase was evolved for efficient degradation of the insoluble substrate to soluble β(1→4)-linked oligosaccharides of GlcNac during the molting process. The unique properties of the two domains and the linker region suggest that each could be used to manipulate interactions of proteins with chitin in chitin-containing organisms, such as pest insects and pathogenic fungi, which could lead to adverse effects on their life cycles.

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