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Homology modeling of the insect chitinase catalytic domain–oligosaccharide complex and the role of a putative active site tryptophan in catalysis

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Abstract

Knowledge-based protein modeling and substrate docking experiments as well as structural and sequence comparisons were performed to identify potential active-site residues in chitinase, a molting enzyme from the tobacco hornworm, *Manduca sexta*. We report here the identification of an active-site amino acid residue, W145. Several mutated forms of the gene encoding this protein were generated by site-directed mutagenesis, expressed in a baculovirus-insect cell-line system, and the corresponding mutant proteins were purified and characterized for their catalytic and substrate-binding properties. W145, which is present in the presumptive catalytic site, was selected for mutation to phenylalanine (F) and glycine (G), and the resulting mutant enzymes were characterized to evaluate the mechanistic role of this residue. The wild-type and W145F mutant proteins exhibited similar hydrolytic activities towards a tri-GlcNAc oligosaccharide substrate, but the former was approximately twofold more active towards a polymeric chitin-modified substrate. The W145G mutant protein was inactive towards both substrates, although it still retained its ability to bind chitin. Therefore, W145 is required for optimal catalytic activity but is not essential for binding to chitin. Measurement of kinetic constants of the wild-type and mutant proteins suggests that W145 increases the affinity of the enzyme for the polymeric substrate and also extends the alkaline pH range in which the enzyme is active. Published by Elsevier Science Ltd.

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1. Introduction

In several carbohydrases and carbohydrate-binding proteins, tryptophan residues are involved in substrate and/or ligand binding interactions because of their ability to form nonpolar or hydrogen bonds with pyranose rings of sugars (Vyas, 1991; Zeltins and Schrepf, 1997; Maenaka et al., 1998a). One of these types of proteins is the enzyme, chitinase, an insect-molting enzyme from the tobacco hornworm, *Manduca sexta* (Kramer and Muthukrishnan, 1997), with potential for development as a biopesticide in transgenic plants and entomopathogens

(Kramer et al., 1997; Ding et al., 1998). It digests the biopolymer chitin, which is found in the exoskeleton and gut lining of insects. The first cDNA encoding an insect chitinase was isolated from the tobacco hornworm by Kramer et al. (1993). This enzyme is classified as a distorted beta/alpha-barrel protein and consists mainly of α -helices and parallel β -sheets similar to proteins in the glycosyltransferase superfamily and in family 18 of hydrolytic enzymes, the members of which are noted for two conserved regions of amino acids that contain putative active-site residues including tryptophans (Kramer and Muthukrishnan, 1997).

To help determine what residues play critical roles in catalysis and/or substrate binding, we generated a homology model of the insect chitinase catalytic domain–oligosaccharide substrate complex based on the X-ray crystallographic structure of a homologous chitinase

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from *Serratia marcescens* (Kramer and Muthukrishnan, 1997); employed site-directed mutagenesis of one of the putative active-site residues, W145; and conducted a structure–function analysis of the wild type and mutant proteins. Residue W145 is located in the cleft where the N-acetylchitooligosaccharide binds to the enzyme. It occurs in the second conserved region, and sequence alignment analyses revealed that tryptophans equivalent to W145 are highly conserved in many other chitinases from other animal, microbial, and plant sources (Kramer and Muthukrishnan, 1997). In *Manduca* chitinase, this residue is “sandwiched” between two acidic amino acids, D144 and E146, which also are highly conserved and are postulated to participate in catalysis. Previous studies of some other carbohydrases indicated that mutations of tryptophans primarily affect substrate binding (Yamagami and Funatsu, 1997; Maenaka et al., 1998a,b; Koivula et al., 1998). To determine whether W145 plays a similar role in *M. sexta* chitinase, we examined the functional and structural properties of mutant forms of the protein in which this tryptophan had been replaced with either phenylalanine or glycine. Specifically, we were interested in determining how the mutations would impact the enzymatic parameters, k_{cat} and K_m as well as the pH dependence of enzymatic activity.

2. Materials and methods

2.1. Protein modeling and docking

To build a model structure for insect chitinase, automated protein homology-based molecular modeling software was used (<http://www.expasy.hcuge.ch/sprot/sprot-top.html>, <http://www.ncbi.nlm.nih.gov/BLAST/> and <http://www.expasy.ch/swissmod/SWISS-MODEL.html>; Peitsch, 1996; Kramer and Muthukrishnan, 1997). *S. marcescens* chitinase A, a similar protein of known three-dimensional structure, was used as the template to generate a model structure for insect chitinase (Greer, 1991; Kramer and Muthukrishnan, 1997). Using the three-dimensional coordinates for the atoms of the model, a molecular visualization program, RASMOL, was utilized to visualize the molecular model (<http://www.glxowellcome.co.uk/software/rasmol/>).

Protein docking of the chitinase model with the β -1,4-linked hexasaccharide of 2-acetamido-2-deoxyglucopyranoside (GlcNAc) as the substrate was conducted. The three-dimensional coordinates of hexa-GlcNAc were obtained by using QUANTA/CHARMm, a molecular modeling software program, which included a global energy minimization operation without conformational searching. The protein-docking program used was Global Range Molecular Matching (GRAMM; Vakser and Nikiforovich, 1995;

<http://reco3.musc.edu/gramm/index.html>), with the substrate and protein held rigid. Exhaustive six-dimensional searching was performed through relative translations and rotations of the two molecules, and an empirical approach was employed to smooth the intermolecular energy function by changing the range of the atom–atom potentials. Coordinates for the predicted complexes with different energies ranging from -34.7 to -30.5 for the first 10 matches thus were obtained. The model of the complex calculated to have the lowest free energy then was visualized using RASMOL.

2.2. Site-directed mutagenesis

The protocol and the reagents provided by the unique site elimination (U.S.E.) mutagenesis kit (Pharmacia Biotech) were used to conduct the site-directed mutagenesis on a recombinant pBSKS (+) plasmid, which contained the 1.8 kb *Eco* RI fragment devoid of a *Pvu* I site encoding the full-length *Manduca* chitinase (Kramer et al., 1993). One target mutagenic primer and two selection primers were employed for each desired mutation.

To replace the tryptophan at position 145 with phenylalanine or glycine, the target mutagenic primers, 5'CTA GAC CTT GAT TTC GAG TAC CCA GG 3' and 5'CTA GAC CTT GAT GGG GAG TAC CCA GG 3', respectively, were used. In the primers, the underlined codons for phenylalanine and glycine replaced the original TGG codon for tryptophan 145 in the chitinase gene. Designed to introduce a mutation into each of the two *Pvu* I sites at positions 500 and 2416 in the pBS KS(+) vector, the selection primers had the nucleotide sequences 5' GTT GGG AAG GGC AAT TGG TGC GGG CCT C 3' and 5' CTT CGG TCC TCC AAT TGT TGT CAG AAG 3', respectively. The recognition sequence CGATCG of *Pvu* I site was changed to the underlined sequence in both of the selection primers.

A mixture of 0.025 pmol plasmid template and 1.25 pmol of each phosphorylated primer was used in the site-directed mutagenesis according to the manufacturer's instructions. After completion of DNA synthesis and ligation, the reaction mixture first was treated with 10 units of *Pvu* I restriction enzyme and then used to transform competent cells of a repair-defective strain of *E. coli* (NM 522 *mutS*). Plasmid DNA was isolated and purified from the overnight bacterial culture representing a pool of presumptive mutants and then subjected to a second round of digestion with *Pvu* I to increase the proportion of mutant plasmids. An aliquot of the digested mixture containing about 27 ng of plasmid was used to transform 100 μ l *E. coli* JM109 cells. Plasmid DNAs were isolated from individual transformed colonies. The plasmids that were shown to be undigested by *Pvu* I, i.e. those containing mutated *Pvu* I restriction sites, were subjected to DNA sequencing.

2.3. DNA sequencing

The chain-termination DNA sequencing reaction was conducted using a sequencing kit from United States Biochemical, which employed Sequenase Version 2.0 T7 DNA polymerase. The forward primer had the nucleotide sequence 5' GCA CAG AAG AGC ACC CG 3', which was 67 bases away from the target mutation site. α -[³⁵S]dATP was employed as the label, and 1 pmol plasmid and 10 pmol primer were used for the reaction. DNA sequencing gel electrophoresis was performed in 1× TBE buffer at 45 W by using a 6% acrylamide/bis-acrylamide gel containing 8 M urea. The gel was then treated with 5% acetic acid and 15% methanol, dried at 80°C and exposed to an X-ray film.

2.4. Expression of mutant forms of chitinase in baculovirus-infected insect cells

The 1.8 kb *EcoR* I fragments isolated from plasmids, which were confirmed by DNA sequencing analysis to have the desired mutations at W145, were inserted into the baculovirus transfer plasmid pVL1393 behind the polyhedrin promoter as described by Gopalakrishnan et al. (1995). Recombinant baculoviruses with the mutant forms of the chitinase gene were obtained using BaculoGold™ Linearized Baculovirus DNA from Pharmingen (Gopalakrishnan et al., 1995). Plaques that expressed the insect chitinase proteins were identified by western blot analysis of culture medium and amplified to obtain viral stocks. Expression using SF21 insect cells in SF900 II medium was performed in monolayers in 75 cm² T-flasks. Culture media were collected 72 h after inoculation and stored at –20°C. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie Blue staining and western blot analyses were used to monitor the production of mutant forms of chitinase (Gopalakrishnan et al., 1995).

2.5. Purification of chitinase and N-terminal sequencing

Culture media containing expressed proteins first were desalted and solvent-exchanged by either dialysis or ultrafiltration. Dialysis was carried out using 30 K cutoff membrane tubing at 4°C against 20 mM phosphate buffer, pH 6.5. For ultrafiltration, centrifugation was carried out using Micron Centriprep-30 concentrators, and the retentates were reconstituted in 20 mM phosphate buffer pH 6.5.

After desalting and solvent-exchange, samples then were purified by DEAE Sepharose ion-exchange chromatography at 4°C. A 20-cm long, 2.5-cm i.d. Millipore Kontes Flex-column was used. The column was equilibrated with 20 mM phosphate buffer, pH 6.5, and the sample was loaded in the same buffer. A gradient of 0

to 0.5 M NaCl in the same buffer (0.75 ml/min) then was used to elute the proteins. Elution then was continued with the buffer containing 0.5 M NaCl. Samples of 1.5 ml were collected.

The absorbance at 280 nm of column effluents was monitored, and selected fractions then were examined by Coomassie Blue R250 staining after SDS-PAGE. Fractions containing homogeneous 85 kDa protein were pooled, desalted, and concentrated by ultrafiltration. Protein was subjected to automated Edman degradation using an Applied Biosystems Sequencer at the Biotechnology Microchemical Core Facility, Kansas State University. The Coomassie Protein Assay Reagent (Pierce) was used for protein quantitation with BSA as the standard.

2.6. Chitinase assay using CM–chitin–RBV as substrate

Colorimetric assays of chitinase activity was performed in triplicate using the purple dye-labeled biopolymeric substrate, CM–chitin–RBV (Loewe Biochemica GmbH, Sauerlach, Germany). For each assay, 0.1 ml of aqueous CM–chitin–RBV (2 mg/ml) was mixed with 0.2 ml of phosphate buffer at pH 7.5 and 0.1 ml of a solution containing 0.5 µg chitinase (Gopalakrishnan et al., 1995). For the blank, 0.1 ml CM–chitin–RBV was mixed with 0.2 ml of buffer and 0.1 ml of water. For kinetic analyses, the reaction time was 15 min. For the pH-rate profile, after incubation at 37°C for 2 h, the reactions were stopped by adding 0.1 ml of 2 M HCl, after which the reaction mixtures were cooled on ice for 10 min. After centrifugation for 5 min to remove nondegraded substrate, the absorbance of the supernatant at 550 nm was measured. The stabilities of the enzymes during a 5 h incubation period were similar over the entire pH range, pH 4–11.

2.7. Chitinase assay using 4-Mu-(NAG)₃ as substrate

The assays were conducted according to Hollis et al. (1997) with some modifications. 4-Methylumbelliferyl-N, N',N''-triacetylchitotrioside [(GlcNAc)₃-UMB] from Sigma was used as the substrate. For determination of pH optimum, the assays were conducted in a final volume of 50 µl with the following: 100 µM substrate, 1.5 µl enzyme (0.1 µg protein) and 12.5 µl 0.2 M universal buffer (Frugoni, 1957), pH ranging from 4.2 to 10.8. Assays for determination of kinetic constants were identical, except that they were carried out at pH 6.5 or 8.0 with W145F or W145 enzymes, respectively, and substrate concentrations ranged from 6 to 800 µM. All reactions were carried out in triplicate at 37°C for 10 min and stopped by adding 12.5 µl 2 N HCl. The reaction mixture was diluted to a final volume of 2.0 ml with 0.15 M glycine–NaOH buffer (pH 10.5). and the free

methylumbelliferone released by enzymatic hydrolysis was determined by fluorescence spectrophotometry. A model F-4010 Fluorescence Spectrophotometer (Hitachi Ltd, Tokyo, Japan) was used to measure product formed using an excitation wavelength of 360 nm and an emission wavelength of 450 nm.

2.8. Glycol-chitin overlay assay

This assay was based on the procedure of Trudel and Asselin (1989) with some modifications. After electrophoresis of the enzyme preparation in a native 7.5% polyacrylamide minigel at pH 8.8, the resolving gel was soaked in 50 ml of 150 mM phosphate buffer at pH 6 or pH 7 for 5 min. A 7.5% polyacrylamide gel containing 0.2% glycol chitin was overlaid on the separating gel and incubated at 37°C for 2.5 h. Bands of chitinase activity were detected by staining with a fluorescent brightener, calcofluor white M2R (Sigma), viewed under UV light, and photographed.

2.9. Chitin-binding assay

The binding assay was conducted using a modification of the method of Venegas et al. (1996). First, 0.05 g finely ground chitin (crab shell, Sigma) was prepared by washing in 1 ml solution containing 1% SDS and 1% β -mercaptoethanol and boiling for 5 min. Then, 15 ml of water was added, and the suspension was filtered through a 0.45 μ m filter. The retentate was resuspended in 1 ml PBS buffer containing 0.2% NaN_3 . An aliquot of 400 μ l of the above suspension was centrifuged, and the chitin pellet was washed with 20 mM sodium phosphate, pH 6.5. The centrifugation and washing were repeated twice, and the chitin was resuspended in 400 μ l 20 mM sodium phosphate buffer, pH 6.5. An aliquot of 100 μ l chitin suspension was centrifuged and the pellet mixed with a 200 μ l chitinase sample in the same buffer, and the mixture was incubated at room temperature for 1 h with end-to-end shaking and then centrifuged for 15 min. The supernatant (approx. 150 μ l) was removed as the unbound fraction. The pellet was washed in 75 μ l buffer and centrifuged for 15 min, which was repeated once, and the supernatants were pooled as the wash fraction. The final pellet was resuspended in 120 μ l buffer, followed by mixing with 30 μ l 5X SDS gel-loading buffer and boiling. After centrifugation, the supernatant was removed as the bound fraction. The unbound fraction and wash fraction also were mixed with the same amount of loading buffer and boiled. An aliquot of 50 μ l of each fraction was concentrated using an evapoconcentrator and examined for the presence of the chitinase by SDS-PAGE followed by staining with Coomassie Brilliant Blue dye.

3. Results

3.1. Modeling and docking

To elucidate some of the structural characteristics of insect chitinase, several computer-modeling experiments were performed. First, a model structure for the *Manduca* chitinase was predicted computationally from the amino acid sequence deduced from its cDNA clone by using SWISS-MODEL software, an automated knowledge-based protein modeling server on the Internet. The server used *Serratia* chitinase A, whose three-dimensional crystal structure is known, as the template for the modeling. The overall sequence comparison between these two chitinases showed an 81% similarity in amino acid sequence for residues 80–387 of *Manduca* chitinase and residues 257–544 of *Serratia* chitinase A (Kramer and Muthukrishnan, 1997). The model structure of the central portion of the *Manduca* chitinase (positions 80–387, which represent the catalytic domain and include the two highly conserved regions found in all family 18 chitinases), thus was generated and had a cleft similar to that of *Serratia* chitinase. This structure revealed that the two conserved regions occur as two parallel β -strands (green ribbons), which make up a portion of the cleft in the molecule [Fig. 1(A)]. This cleft was predicted to be the substrate-binding site, and mutations of residues in this cleft likely would affect substrate binding and/or hydrolysis.

To understand how a substrate might interact with the chitinase, high-resolution protein–ligand docking was conducted using GRAMM molecular docking software and hexa-GlcNAc as the substrate [Fig. 1(A) and (B)]. In the predicted chitinase-substrate complex of lowest energy, the bond that apparently is cleaved is positioned just inside the cleft of the chitinase and very close to the second conserved region containing our target mutation site, W145 (purple residue located in the green ribbon just above the left-hand side of the oligosaccharide in Fig. 1(A) and two other putative catalytic residues, D144 and E146, on opposite sides of W145. Comparison of amino acid sequences conserved in region II of family 18 chitinases and related proteins revealed that a trypto-

Fig. 1. (a) Model structure of the lowest energy complex of the catalytic domain of *Manduca sexta* chitinase and the oligomeric substrate hexa-GlcNAc. The enzyme ribbon structure was obtained from SWISS-MODEL software using *Serratia* chitinase A as a template. The alpha-helical segments are shown in red, the beta-sheet structures in yellow, and the turn segments in blue. The two conserved regions are shown in green, and the target mutation site, tryptophan at position 145, is colored purple. A cleft is visible on the lower right, close to the conserved regions, which is probably the active site. The model of the complex was obtained using the GRAMM molecular docking software program (Vakser and Nikiforovich, 1995). The oligosaccharide is shown as a space-filling molecule in orange. (b) Side view of model shown in (a).

(a)



(b)



phan is present in the position equivalent to W145 in most of those proteins (Fig. 2). Therefore, W145 was selected as the target residue for mutation.

3.2. Site-directed mutagenesis and expression of mutant forms of chitinase

Site-directed mutagenesis of the chitinase gene was performed as described in Section 2 to introduce desired

mutations that resulted in substitutions of tryptophan 145 with either phenylalanine (W145F) or glycine (W145G). DNA sequencing was carried out to confirm that the mutants indeed contained the desired mutations. The mutant forms of the gene were used to express the corresponding mutated chitinase proteins in the baculovirus-insect cell line expression system (Gopalakrishnan et al., 1995). The expression levels were established to be 10–20 µg/ml.

3.3. Chitinase purification

Wild type and mutant forms (W145F and W145G) of chitinases were purified from the culture media as outlined in Section 2. A typical elution profile of the W145F chitinase after DEAE-Sepharose chromatography is shown in Fig. 3. The results of Coomassie Blue staining analyses of W145G chitinase fractions eluting between 200 and 215 ml and the original sample prior to the DEAE chromatography are shown in Fig. 4. They indicated that the wild-type chitinase and the mutant proteins could be purified to approximately 95% homogeneity by a single chromatographic step. N-Terminal amino acid sequencing revealed the same sequence, DSRAIV, for all three of the enzymes. The minor proteins present in the chitinase preparations did not exhibit chitinolytic activity or chitin-binding activity (see below).

3.4. Activity towards polymeric and oligomeric substrates

The properties of *M. sexta* chitinase and the two mutant proteins are shown in Table 1. Kinetic constants of the enzymes, assuming the Michaelis–Menten model, were determined with two substrates, the polymeric CM–chitin–RBV and the oligomeric MU-(GlcNAc)₃. W145G exhibited no activity towards either of the substrates. In the case of the polymeric substrate, the k_{cat}/K_m of W145F was approximately half that of the wild-type enzyme, whereas the values for the oligosaccharide substrate were about the same. Replacement of W145 by F increased only the K_m for the polymeric substrate, but both k_{cat} and K_m for the oligomeric substrate.

3.5. pH optima for hydrolysis of oligosaccharide and polymeric substrates

Activities vs pH profiles were determined for the three forms of insect chitinase over the pH range 4–11 as described in Section 2. The profiles for the 4-MU-(GlcNAc)₃ oligomeric substrate were similar for the wild type and W145F, with a broad pH optimum between pH 5 and 7 [Fig. 5(A)]. W145G was inactive over the entire pH range. On the other hand, the curves obtained using the polymeric substrate, CM–chitin–RBV, were substantially different for W145 and W145F [Fig. 5(B)]. The

A. Proteins with chitinolytic activity:

Species	Common Name	Amino Acid Sequence	Accession Number
<i>Manduca sexta</i>	Tobacco hornworm	FDGLDLLWEYP ^{^*}	A56596
<i>Bombyx mori</i>	Silkworm	FDGLDLLWEYP	U86876
<i>Hyphantria cunea</i>	Fall webworm	FDGLDLLWEYP	U86877
<i>Aedes aegypti 1</i>	Mosquito	FDGFDLLWEYP	AF026491
<i>Anopheles gambiae</i>	Mosquito	FDGIDLWEYP	AF008575
<i>Chelonus sp.</i>	Braconid Wasp	FDGFDDWEYP	A53918
<i>Chironomus tentans</i>	Midge	FDGLDLLWEYP	Y13233
<i>Aedes aegypti 2</i>	Mosquito	FDGLDLLWEYP	AF026492
<i>Penaeus japonicus</i>	Prawn	FDGLDLLWEYP	AB008027
<i>Brugia malayi</i>	Nematode	FDGFDLLWEYP	A38221
<i>B. pahangi</i>	Nematode	FDGFDLLWEYP	U59690
<i>Caenorhabditis elegans</i>	Nematode	FDGIDIDWEYP	U42835
<i>Aphanocladium album</i>	Fungus	FDGIDIDWEYP	P32470
<i>Trichoderma hamatum</i>	Fungus	FDGIDVDWEYP	Z71415
<i>Serratia marcescens</i>	Bacterium	FDGVDIDWEFP	AB15996
<i>Bacillus circulans</i>	Bacterium	FNGLDIDLEGS	D10594
<i>Homo sapiens 1</i>	Human	FDGLDLLWEYP	U29615
<i>Nicotiana tabacum</i>	Tobacco	FHGLDLLWEYP	X77111
<i>N. tabacum</i>	Tobacco	LDGIDFDIELG	P29061
<i>Hevea brasiliensis</i>	Rubber heveamine	LDGIDFDIEHG	P23473

B. Proteins without chitinolytic activity:

Species	Common Name	Amino Acid Sequence	Accession Number
<i>Drosophila melanogaster</i>	Fruitfly	FDGLDLGWQFP ^{^*}	AF011373
<i>D. melanogaster</i>	Fruitfly	FDGLDVAWQFP	U13825
<i>Homo sapiens 2</i>	Human	FDGLDVSWIYP	U49835
<i>H. sapiens GP39</i>	Human	FDGLDLAWLYP	P36222
<i>Mus musculus</i>	Mouse yml	FDGLNLDWQYP	M94584
<i>M. musculus BRP</i>	Mouse brp39	FDGLDLAWLYP	S61550
<i>Bos taurus</i>	Bovine oviductin	FDGLDLAWLYP	AF011373
<i>D. melanogaster</i>	Fruitfly	FDGLDLAYQFP	AF102237
<i>Vicia narbonensis</i>	Bean norbonin	IDGIDIHYEHI	494398
<i>D. melanogaster</i>	Fruitfly	FDGLDLAFQLP	AF102236
<i>Ovis aries</i>	Ovine	FDGLDLFFLYP	U17966
<i>D. melanogaster</i>	Fruitfly	FDGLDLALQLP	AF1002238
<i>Canavalia ensiformis</i>	Plant Con B	LDGIHFDIQKP	1421021

Fig. 2. Amino acid sequence comparison of conserved region II of glycosyl hydrolase family 18 chitinases and related proteins. Residues equivalent to W145 and E146 in *M. sexta* chitinase are denoted by [^] and *, respectively.

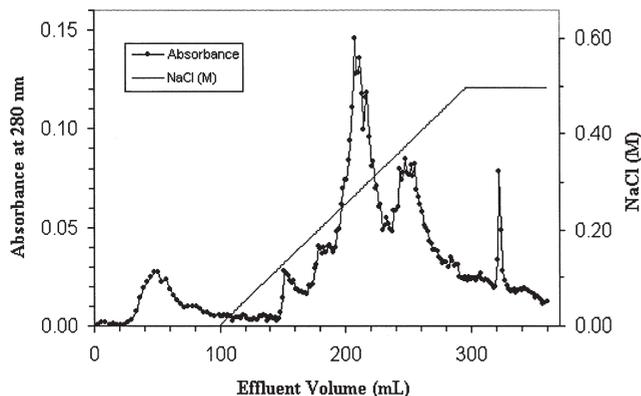


Fig. 3. DEAE-cellulose chromatography effluent profile of a sample containing W145F chitinase expressed by recombinant baculovirus-infected Sf21 cells. About 25 ml of the crude media after dialysis was loaded. The elution was carried out by an increasing salt gradient from 0 to 0.5 M NaCl. Fractions from 200 to 215 ml contained the chitinases.

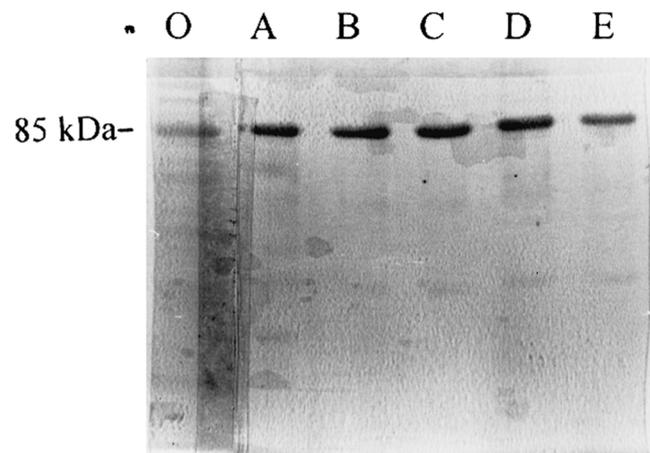


Fig. 4. Coomassie Blue-stained SDS-PAGE of W145G chitinase fractions from DEAE chromatography. The major bands at 85 kDa were the chitinase with W145G mutation. Fractions A, B, C, D, and E correspond to 204, 205.5, 210, 213, and 214.5 ml of the eluent. The original medium (O) prior to column chromatography also was analyzed for comparison.

Table 1
Kinetic parameters of *M. sexta* chitinase using CM-Chitin-RBV (A) and MU-(GlcNAc)₃ (B) as substrates

Parameter		W145	W145F
A	k_{cat} ($\text{s}^{-1} \times 10^{-2}$)	1.30	0.90
	K_{m} (mg ml^{-1})	0.13	0.16
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{ml mg}^{-1} \text{s}^{-1}$)	0.10	0.05
B	k_{cat} ($\text{s}^{-1} \times 10^{-4}$)	2.60	6.20
	K_{m} (μM)	510.80	1163.30
	$k_{\text{cat}}/K_{\text{m}}$ ($\text{M}^{-1}\text{s}^{-1}$)	0.51	0.53

pH optimum curve for the W145F enzyme using the polymeric substrate was similar to that obtained with the oligosaccharide substrate, with a broad pH optimum between pH 5 and 7. The wild-type enzyme had a much higher activity than W145F with the polymeric substrate at pH values above 6 and an optimum around pH 8.5. Even at pH 9 and 10, the wild-type enzyme had substantial activity, whereas the W145F enzyme had little or no detectable activity. The W145G enzyme was inactive towards the polymeric substrate at all pH values tested. Another polymeric substrate, glycol chitin, also was tested as a substrate at pH 6 and 7 using an activity gel overlay assay (Gopalakrishnan et al., 1995). The wild-type and W145F mutant proteins also hydrolyzed glycol chitin, but W145G did not [Fig. 5(C)]. Whereas the activities of the W145 and W145F were similar at pH 6, the former enzyme was more active toward glycol chitin at $\text{pH} \geq 7$ and had a more alkaline pH optimum, results consistent with data obtained using another polymeric substrate, CM-chitin-RBV [Fig. 5(B)].

3.6. Chitin binding

The abilities of the mutant forms of chitinase to bind to the insoluble chitin substrate were studied using a method modified from Venegas et al. (1996). The results shown in Fig. 6 indicated that the wild type and mutant chitinases bound to the chitin matrix, whereas neither the unbound fractions nor the wash fractions contained any protein. Under the experimental conditions utilized, the mutant forms of chitinase apparently bound to chitin as well as the wild-type chitinase. Whereas both of the mutant forms bound to chitin, only the phenylalanine mutant had catalytic activity and degraded the polysaccharide and oligosaccharide substrates. The glycine mutant did not digest the chitin substrates. Together with the enzyme activity results, these binding data demonstrate that W145 is not essential for chitin binding but does influence the catalytic activity of chitinase.

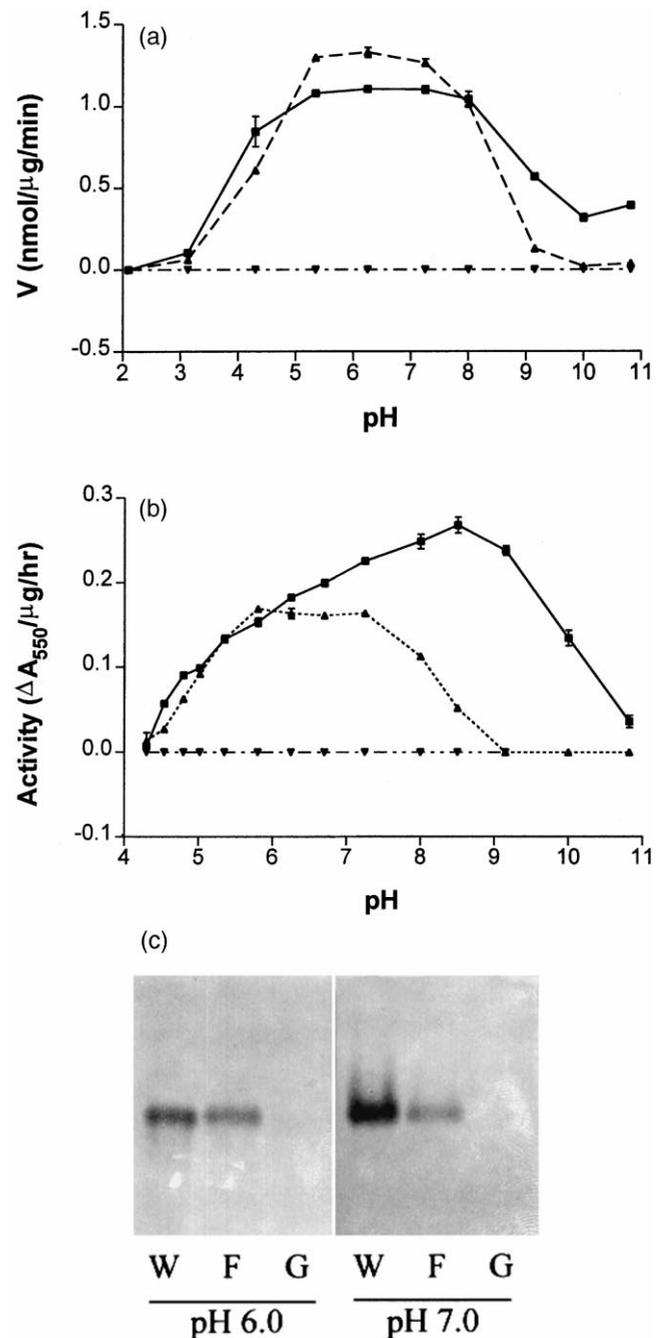


Fig. 5. Activity versus pH profiles of wild-type and mutant forms of *M. sexta* chitinase. (a) 4-MU-(GlcNAc)₃ as the substrate. The activities of wild-type and mutant proteins (0.02 mg/ml) were measured with the oligosaccharide substrate (100 μM) in 0.2 M universal buffer at 37°C for 10 minutes. (b) CM-chitin-RBV as the substrate. The activities of wild-type and mutant chitinases (0.00125 mg/ml) were measured with the polymeric substrate (0.5 mg/ml) in 0.2 M universal buffer at 37°C for 2 h. Wild-type, ■; W145F, ▲; and W145G, ▼. (c) Glycol chitin overlay assay of wild-type and mutant forms of *M. sexta* chitinase.

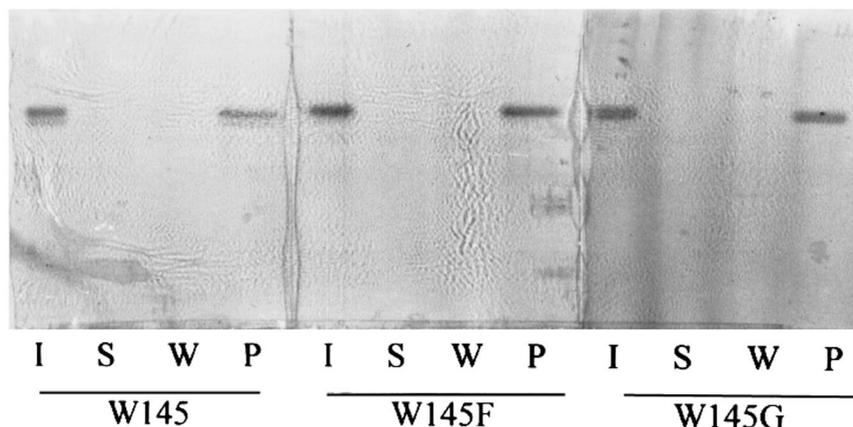


Fig. 6. Chitin-binding assay of wild-type and mutant forms of *M. sexta* chitinase. I, initial fraction; S, supernatant; W, wash fraction and P, pellet fraction. Proteins (2.25 μ g) were incubated with the chitin suspension (5 mg/0.1 ml) at room temperature for 1 h as described in Section 2.

4. Discussion

4.1. Model structure for enzyme-substrate complex

Manduca sexta chitinase belongs to family 18 of the glycosyl hydrolase superfamily along with *S. marcescens* chitinase and hevine from rubber latex (Henrissat and Bairoch, 1993; Kramer and Muthukrishnan, 1997). The sequences of these two latter proteins are related most closely to *M. sexta* chitinase, and crystal structures of these latter enzymes are available (Perrakis et al., 1994; Van Scheltinga et al., 1994). Our model for the catalytic domain of the *M. sexta* chitinase was based upon the crystal structure of *S. marcescens* chitinase, although it lacks the N-terminal residues 1–79 and the C-terminal residues 388–554. Nonetheless, this model does provide a picture of the catalytic domain as well as its complex with an oligosaccharide substrate. The predicted structure is basically a partial $(\beta/\alpha)_8$ barrel structure that is similar to other members of the family 18 glycosyl hydrolases, although it contains only about four complete and several incomplete α -helices together with approximately eight stretches of β -sheets. Model structures of three other glycosyl hydrolase family proteins have been generated previously, and overall our insect chitinase model has similar features (Aronson et al., 1997). Although our model lacks a well-defined $(\beta/\alpha)_8$ barrel structure and is only an approximation of the true structure (Martin et al., 1997), these similarities provide confidence that our model is reliable enough for further study until experimental coordinates become available.

Two highly conserved regions of amino acids found in many family 18 glycosyl hydrolases are also present in *M. sexta* chitinase in separate β -sheets. These conserved regions are believed to be involved in substrate binding and/or catalysis (Watanabe et al., 1993). W145, the focus in this study, resides in one of these two β -sheets. Protein-substrate docking studies revealed that this tryptophan is very close to the probable cleavage

site of the bound substrate, $(\text{GlcNAc})_6$. The second conserved region of family 18 glycosyl hydrolases contains this tryptophan as well as several acidic residues, which are thought to be involved in catalysis either as proton donors, nucleophiles, or electrostatic stabilizers of the charged transition state intermediate (Brameld and Goddard, 1998). We employed site-directed mutagenesis to investigate the role(s) of this “sandwiched” tryptophan in the catalytic mechanism. We focused on tryptophan, because this residue has been suggested to have multiple functions in catalysis by glycosidases such as lysozymes and chitinases (Vyas, 1991). In many proteins, hydrophobic side chains like the indole group play important roles in stabilizing protein structure, but other functions include substrate hydrogen bonding interactions and altering the pK_a values of near-neighbor ionizable groups. The role of the two acidic residues that sandwich this tryptophan will be the subject of another study.

4.2. The role of W145 in *M. sexta* chitinase catalysis

Studies with mutants in which a glutamic acid in this conserved region was replaced with other amino acids have indicated that this residue is critically important for catalysis in many other family 18 glycosyl hydrolases. Presumably, it acts as a proton donor similar to the role of acidic residues in hen egg white lysozyme and *Bacillus circulans* chitinase (Watanabe et al., 1993; Maenaka et al., 1998b). The finding that many other members of the family 18 enzymes have a tryptophan in the adjacent position suggests a critical function for this residue as well. Results from studies of mutant forms of *M. sexta* chitinase, in which W145 was replaced by phenylalanine or glycine, provide some ideas about the role of this residue in catalysis. The finding that replacement of W145 with a glycine residue leads to a total loss of enzymatic activity suggests that the tryptophan is critical for either maintaining the structure of the catalytic site or for substrate binding and/or activity. However, this mutant still

retained the ability to bind chitin, indicating that hydrolysis cannot occur, perhaps because of a subtle alteration in the geometry of the catalytic site. Alternatively, the chitin binding observed in our study may represent an interaction between chitin and the enzyme at a site different from the catalytic cleft. Results of Yamagami and Funatsu (1997) support a two-binding-site model. Modification of several tryptophan residues of a rye seed chitinase with N-bromosuccinamide revealed that W23, a residue in the N-terminal chitin-binding domain that is highly exposed on the surface of the enzyme, contributed to chitin binding but not chitinase activity. Another tryptophan residue in rye chitinase, W131, which is buried partially in the catalytic cleft, was involved directly in catalysis.

Replacement of the W145 by a phenylalanine resulted in an enzyme with a lower k_{cat} and a higher K_{m} , when tested using the polymeric substrate, indicating that W145F is only partially effective in fulfilling the catalytic role played by tryptophan at this position. The vast majority of family 18 chitinases have a tryptophan equivalent to W145 in conserved region II of *M. sexta* chitinase (Fig. 2). In the chitinolytic protein hevamine from rubber latex, the corresponding tryptophan residue is replaced by an isoleucine (Van Scheltinga et al., 1994). These results suggest that the presence of either a bulky hydrophobic or an aromatic side chain at this position is essential for maintaining catalytic activity. Many proteins related to chitinase but with no demonstrable enzymatic activity, including some chitin-binding lectins, lack amino acids equivalent to E146 and/or W145 in conserved region II of *M. sexta* chitinase [Fig. 2(B)]. Tryptophans in other glycosidases or carbohydrate-binding proteins also are implicated in interactions that promote catalysis (Maenaka et al., 1998a; Koivula et al., 1998); cooperatively stabilize the proteins (Honda et al., 1999); or influence the mode of substrate/ligand binding (Asensio et al., 1995; Elgavish and Shannan, 1997; Maenaka et al., 1998b). We are currently constructing additional mutants with either tyrosine, isoleucine, or histidine at position 145 to learn more about the requirements for this residue as it relates to enzyme structure and function.

A comparison of the dependence of glycosidic activity of the wild-type and W145F mutant proteins on pH showed that the activity of the wild-type enzyme toward the polymeric substrate continued to increase until pH 8.5 and was substantial even at pH 10 or greater. This extension of catalytic activity into the alkaline pH range suggests that W145 in the wild-type enzyme influences the ionization of a group with a $\text{p}K_{\text{a}}$ of about pH 9.5. In the W145F enzyme, this group (assuming it is the same) has a lower $\text{p}K_{\text{a}}$ of about pH 8. A comparable shift in the optimum pH of lysozyme from pH 5 to pH 7 was reported in the W62H mutant of lysozyme (Kumagai et al., 1993; Maenaka et al., 1998b). Extending the activity

of the enzyme into the alkaline range is critical for the digestion of chitin by *M. sexta* in vivo, which occurs in the alkaline (pH > 10) midgut during the molting process. A chitinase with a phenylalanine instead of tryptophan at position 145 would not be enzymatically active in vivo under the alkaline conditions of the lepidopteran gut. Apparently, the tryptophan sandwiched between the two acidic residues in insect chitinase helps to maintain a relatively hydrophobic local environment, such that the $\text{p}K_{\text{a}}$ for one or both of those amino acids is elevated, which results in the hydrolytic activity being extended further into the alkaline range of pH.

Among the members of family 18 glycosyl hydrolases, only the *M. sexta* and *B. mori* chitinases have alkaline optimum pH values (Koga et al. 1983, 1997). Most of the other chitinases of plant, bacterial, and animal origins have optima of approximately pH 6 (Koga et al., 1997). Because this higher pH optimum is observed only with a polymeric substrate, binding of the polymer at all of the subsites of the catalytic cleft (A–F in the case of lysozyme) probably is necessary to bring about the alkaline range of activity. Possibly, W145 is a member of the hydrogen-bonding network utilized for binding of sugars at subsites apart from the bond-cleaving subsites (between subsites D and E in the case of lysozyme). The binding of an oligomeric substrate involves fewer subsites than binding of a polymeric substrate (presumably only three for MU-(GlcNAc)₃) and is expected to involve fewer hydrogen bonds. The polymeric substrate may have hydrogen bonding interactions with residues having $\text{p}K_{\text{a}}$ values in the alkaline range, which could be reflected in the broader pH range for enzyme activity. W145 could play a role in the formation of “cooperative” hydrogen bonds as observed in the case of lysozyme (Maenaka et al., 1998b). The measured kinetic constants are consistent with the above interpretation. For the polymeric substrate, the K_{m} and value of the wild-type enzyme is only slightly lower than that of the W145F protein, but for the oligomeric substrate, the K_{m} is much lower for the wild-type chitinase than for the W145F enzyme, suggesting a substantial contribution of this tryptophan in binding to the oligomeric substrate, presumably because fewer residues are involved in this binding compared to the polymeric substrate. However, the weaker affinity for the oligosaccharide substrate is compensated for by a greater k_{cat} of the W145F mutant compared to the wild-type enzyme. One possible explanation for this observation is that alternate nonproductive binding of oligosaccharide products occurs with the wild-type enzyme but not with the W145F mutant. This would be analogous to the fact that the W62H mutant of lysozyme hydrolyzes (GlcNAc)₃, whereas this oligosaccharide inhibits the wild-type enzyme by binding in a nonproductive mode (Maenaka et al., 1998b).

Thus, both tryptophan and phenylalanine have positive effects on the kinetic parameters; the former

improves substrate affinity, and the latter improves the turnover number. The k_{cat}/K_m values, which are the best measures of the catalytic prowess of enzymes, are very similar for the oligomeric substrate, but for the polymeric substrate, the k_{cat}/K_m value for the wild-type enzyme is twice that of the W145F mutant. Thus, W145 appears to be more important in the interaction with a polymeric substrate than with an oligosaccharide substrate. This is consistent with the notion that the major function of endochitinases is depolymerization of the insoluble polymeric substrate, and further breakdown of product oligosaccharides is a major function of the exochitinolytic N-acetylglucosaminidases (Fukamizo and Kramer, 1985).

The chitinase-substrate model was constructed to allow us to better test hypotheses about catalytic activity, substrate preference, stability, domain structure, and insecticidal activity. In the future, it will allow us to improve our ability to rationally design modified forms of protein with improved properties. Mutation of the W145 to glycine resulted in a protein devoid of enzymatic activity but with its carbohydrate-binding property retained. Thus, this mutant has become a lectin-like protein. A similar result was obtained when E122 or E144 of the catalytic domain of a tobacco class I chitinase was mutated to alanine (Iseli-Gamboni et al., 1998). Other carbohydrate-binding proteins, such as plant lectins and vicilins, also bind to chitin-containing structures in insects and exhibit anti-insect activity (Czapla and Lang, 1990; Yunes et al., 1998; Van Damme et al., 1998; Zhu-Salzman et al., 1998).

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References

- Aronson, N.N., Blanchard, C.J., Madura, J.D., 1997. Homology modeling of glycosyl family 18 enzymes and proteins. *J. Chem. Inf. Comput. Sci.* 37, 999–1005.
- Asensio, J.L., Canada, F.I., Bruix, M., Rodriguez-Romero, A., Jimenez-Barbero, J., 1995. The interactions of hevein with N-acetylglucosamine-containing oligosaccharides. Solution structure of hevein complexed to chitobiose. *Eur. J. Biochem.* 230, 621–633.
- Brameld, K.A., Goddard, W.A., 1998. Substrate distortion to a boat conformation at subsite-1 is critical in the mechanism of family 18 chitinases. *J. Am. Chem. Soc.* 120, 3571–3580.
- Czapla, T.H., Lang, B.A., 1990. Effect of plant lectins on the larval development of European corn borer (Lepidoptera: Pyralidae) and southern corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 83, 2480–2485.
- Ding, X., Gopalakrishnan, B., Johnson, L.B., White, F.F., Wang, X., Morgan, T.D., Kramer, K.J., Muthukrishnan, S., 1998. Resistance to tobacco budworm feeding by transgenic plants expressing an insect chitinase gene. *Transgenic Res.* 7, 77–84.
- Elgavish, S., Shannan, B., 1997. Lectin-carbohydrate interactions: different folds, common recognition principles. *Trends Biochem. Sci.* 2, 462–467.
- Frugoni, J.A.C., 1957. Universal buffer of Britton and Robinson at constant ionic strength. *Gazz. Chim. Ital.* 87, 403–407.
- Fukamizo, T., Kramer, K.J., 1985. Mechanism of chitin hydrolysis by the binary chitinase system in insect moulting fluid. *Insect Biochem.* 15, 141–145.
- Greer, J., 1991. Comparative modeling of homologous proteins. *Meth. Enzymol.* 202, 239–252.
- Gopalakrishnan, B., Muthukrishnan, S., Kramer, K.J., 1995. Baculovirus-mediated expression of a *Manduca sexta* chitinase gene: properties of the recombinant protein. *Insect Biochem. Molec. Biol.* 25, 255–265.
- Henrissat, B., Bairoch, A., 1993. New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 293, 781–788.
- Hollis, T., Honda, Y., Fukamizo, T., Marcotte, E., Day, P.J., Robertus, J.D., 1997. Kinetic analysis of barley chitinase. *Arch. Biochem. Biophys.* 344, 335–342.
- Honda, Y., Fukamizo, T., Okajima, T., Goto, S., Boucher, I., Brzezinski, R., 1999. Thermal unfolding of chitosanase from *Streptomyces* sp. N174: role of tryptophan residues in the protein structure stabilization. *Biochim. Biophys. Acta* 1429, 365–376.
- Iseli-Gamboni, B., Boller, T., Neuhaus, J.M., 1998. Mutation of either of two essential glutamates converts the catalytic domain of tobacco class I chitinase into a chitin-binding lectin. *Plant Sci.* 134, 45–51.
- Koga, D., Jilka, J., Speirs, R.D., Kramer, K.J., 1983. Immunological relationships between β -N-acetylglucosaminidases from the tobacco hornworm, *Manduca sexta* (L.). *Insect Biochem.* 13, 407–410.
- Koga, D., Sasaki, Y., Uchiumi, Y., Hirai, N., Arakane, Y., Nagamatsu, Y., 1997. Purification and characterization of *Bombyx mori* chitinases. *Insect Biochem. Molec. Biol.* 27, 757–767.
- Koivula, A., Kinnari, T., Harjunpaa, V., Ruohonen, L., Teleman, A., Drakenberg, T., Rouvinen, J., Jones, A., Teeri, T.T., 1998. Tryptophan 272: an essential determinant of crystalline cellulose degradation by *Trichoderma reesei* cellobiohydrolase Cel6A. *FEBS Lett.* 429, 341–346.
- Kramer, K.J., Corpuz, L., Choi, H., Muthukrishnan, S., 1993. Sequence of a cDNA and expression of the genes encoding epidermal and gut chitinases of *Manduca sexta*. *Insect Biochem. Molec. Biol.* 23, 691–701.
- Kramer, K.J., Muthukrishnan, S., 1997. Insect chitinases: molecular biology and potential use as biopesticides. *Insect Biochem. Molec. Biol.* 27, 887–900.
- Kramer, K.J., Muthukrishnan, S., Johnson, L., White, F., 1997. Chitinases for insect control. In: Carozzi, N., Koziel, M. (Eds.) *Advances in Insect Control: The Role of Transgenic Plants*. Taylor & Francis, Bristol, PA, pp. 185–193.
- Kumagai, I., Maenaka, K., Sunada, F., Takeda, S., Miura, K., 1993. Effects of subsite alterations on substrate-binding mode in the active site of hen egg-white lysozyme. *Eur. J. Biochem.* 212, 151–156.
- Maenaka, K., Matsushima, M., Kawai, G., Kidera, A., Watanabe, K., Kuroki, R., Kumagai, I., 1998a. Structural and functional effect of Trp-62→Gly and Asp-101→Gly substitutions on substrate-binding modes of mutant hen egg-white lysozymes. *Biochem. J.* 333, 71–76.

- Maenaka, K., Matsushima, M., Kawai, G., Watanabe, K., Kuroki, R., Kumagai, I., 1998b. Structural analysis of a mutant hen egg-white lysozyme preferring a minor binding mode. *Biochim Biophys. Acta* 1384, 23–31.
- Martin, A.C., MacArthur, M.W., Thornton, J.M., 1997. Assessment of comparative modeling in CAPS2. *Proteins: Struct., Func., Gen. Suppl.* 1, 14–28.
- Peitsch, M.C., 1996. ProMod and Swiss-model: Internet based tools for automated comparative protein modeling. *Biochem. Soc. Trans.* 24, 274–279.
- Perrakis, A., Tews, I., Dauter, Z., Oppenheim, A.B., Chet, I., Wilson, K.S., Vorgias, C.E., 1994. Crystal structure of a bacterial chitinase at 2.3 Å resolution. *Structure* 2, 1169–1180.
- Trudel, J., Asselin, A., 1989. Detection of chitinase activity after polyacrylamide gel electrophoresis. *Anal. Biochem* 178, 362–366.
- Vakser, A., Nikiforovich, G.V., 1995. Protein docking in the absence of detailed molecular structures. In: Atassi, M.Z., Appella, E. (Eds.) *Methods in Protein Structure Analysis*. Plenum Press, New York, pp. 505–514.
- Van Damme, E.J.M., Peumans, W.J., Barre, A., Rougé, P., 1998. Plant lectins: a composite of several distinct families of structurally and evolutionary related proteins with diverse ecological roles. *Crit. Rev. Plant Sci.* 17, 575–692.
- Van Scheltinga, A.C.T., Kalk, K.H., Beintema, J., Dijkstra, B.W., 1994. Crystal structures of hevamine, a plant defence protein with chitinase and lysozyme activity, and its complex with an inhibitor. *Structure* 2, 1181–1189.
- Venegas, A., Goldstein, J.C., Beauregard, K., Oles, A., Abdulhayoglu, N., Fuhrman, J.A., 1996. Expression of recombinant microfilarial chitinase and analysis of domain function. *Molec. Biochem Parasitol.* 78, 149–159.
- Vyas, N.K., 1991. Atomic features of protein–carbohydrate interactions. *Curr. Opin. Struct. Biol.* 1, 732–740.
- Watanabe, T., Kobori, K., Miyashita, K., Fujii, T., Sakai, H., Uchida, M., Tanaka, H., 1993. Identification of glutamic acid 204 and aspartic acid 200 in chitinase A1 of *Bacillus circulans* WL-12 as essential residues for chitinase activity. *J. Biol. Chem.* 268, 18567–18572.
- Yamagami, T., Funatsu, G., 1997. Involvement of Trp 23 in the chitin binding and of Trp 131 in the chitinase activity of rye seed chitinase-A. *Biosci. Biotech. Biochem.* 61, 1819–1825.
- Yunes, A.N., Andrade, M.T., Sales, M.P., Morais, R.A., Fernandes, K.V.S., Games, V.M., Xavier-Filho, J., 1998. Legume seed vicilins (7S storage proteins) interfere with the development of the cowpea weevil (*Callosobruchus maculatus* (F)). *J. Sci. Food Agric.* 76, 111–116.
- Zeltins, A., Schrempf, H., 1997. Specific interaction of the *Streptomyces* chitin-binding protein CHB1 with α -chitin. The role of individual tryptophan residues. *Eur. J. Biochem* 246, 557–564.
- Zhu-Salzman, K., Shade, R.E., Koiwa, H., Salzman, R.A., Narasimhan, M., Bressan, R.A., Hasegawa, P.M., Murdock, L.L., 1998. Carbohydrate binding and resistance to proteolysis control insecticidal activity of *Griffonia simplicifolia* lectin II. *Proc. Natl. Acad. Sci.* 95, 15123–15128.