Structural Basis of Chitin Hydrolysis in Bacteria

Constantinos. E. Vorgias

University of Athens, Dept. of Biochemistry-Molecular Biology,
Biochemistry Laboratory, Panepistimiopolis, Kouponia,
157 01 Athens, Hellas,
Tel. +30-1-7284514/7257572, Fax +30-1-7257572,
email cvorgias@biology.db.uoa.gr

Introduction

Chitin, the homopolymer of *N*-acetyl-D-glucosamine is not only the major constituent of the fungal cell wall and the arthropod exosceleton but also an important nutrient source of carbon and nitrogen for bacteria. Based on the currently available biochemical, genetical and structural information we have started to understand the interaction of bacteria chitin degrading enzymes with their insoluble substrate as well as the mode of their enzymatic action. Recent protein structure data have shed light on the enzymatic mechanism of the action of bacterial chitin hydrolyzing enzymes Chitinase A, Chitobiase and Chitosanase at the molecular level. The currently available information is going to be summarized in this paper

Chitin degradation in bacteria

The last five years the number of chitin hydrolyzing - modifying proteins - genes has been dramatically increased. However, the number of published bacteria chitin hydrolyzing enzymes are by far lower compared to plant and fungal chitin hydrolyzing/modifying enzymes.

Chitin degradation by bacteria has two alternative routes as shown in the scheme adopted from Muzzarelli's article (1993). From the enzymes presented in the scheme of Fig. 1, several enzymes have been isolated and for three of them we have extensive biochemical, genetical and structural analysis i.e Chitinase A, Chitobiase and Chitosanase. Although chitin deacetylase has been isolated and characterized from various organisms and the

modification pattern of chitin to chitosan is also well studied, there is not information about the structure of this protein and its enzymatic mechanism.

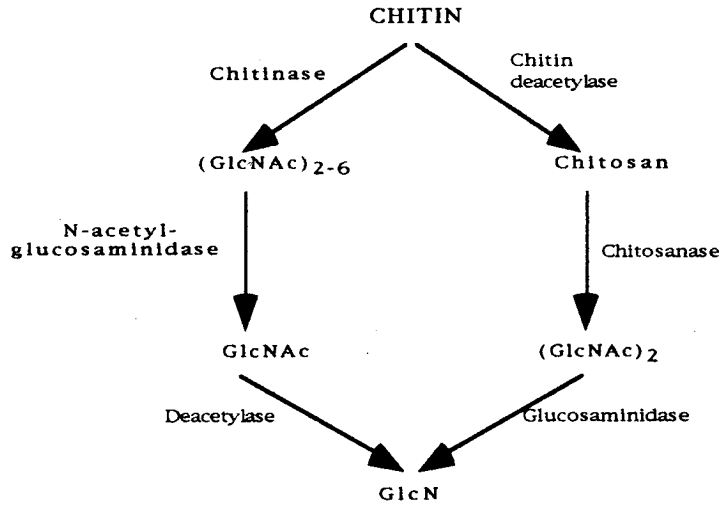

Figure 1

Alternative routes in the enzymatic degradation of chitin.

Primary structure of bacteria chitin degrading

Computer search in the current available data bases, results in a substantial number of new chitin hydrolyzing/modifying genes.

This paper focused on the bacteria genes and presents them in the following tables.

Table I presents the bacteria chitobiases, while table II summarizes bacteria chitosanases and table III bacteria chitinases

Organism	AC	Reference
Vibrio Harveyi	P13670	Soto-Gil, R.W. and Zyskind, J.W., PNAS 90, 6751 (1993)
Alteromonas sp.	I39596	Tsujibo, H., Fujimoto K., Tanno, H., Miyamoto K., Imada, C., Okami, Y., Inamori, Y. Gene 146, 111-115, (1994)
Vibrio parahaemolyticus	PH0889	Zhu B.C.R., Lo J.Y., Li Y.T., Li S.C., Jaynes J.M., Gildemeister O.S., Laine R.A., Ou, C.Y. J. Biochem. 112, 163-167 (1992)
Serratia marcescens	L43594	Tews, I., Vincentelli, R., Vorgias C.E. Gene 170, 63-67 (1996)

Table I

Compilation of known bacteria chitobiases

Organism	AC	Reference
Bacillus Circulans	P33673	Ando A., Noguchi K., Yanagi M., Shinoyama H., Kagawa Y., Hirata H., Yabuki M., Fujii T. J. Gen. Appl. Microbiol. 38, 135-144 (1992)
Nocardioides sp. (Strain N106).	P48846	Masson J.Y., Boucher I., Neugebauer W.A., Ramotkar D.,Brzezinski R. (unpublished)
Streptomyces sp. (Strain N174).	P33665	Boucher I., Dupuy A., Vidal P., Neugebauer W.A., Brzezinski R.; Appl. Microbiol. Biotechnol. 38, 188-193 (1992)
Bacillus subtilis	BSU93875	Sorokin A., Bolotin A., Purnelle B., Hilbert H., Lauber J., Duesterhoeft A., Ehrlich S.D. (unpublished)

Table II

Compilation of known bacteria chitosanases

Organism	AC	Reference
Aeromonas caviae	U09139	J. Bacteriol. 177:4187-4189(1995).
Amycolatopsis methanolica	U31277	J. Bacteriol. 178:149-155(1996).
Alteromonas sp.	D13762	J. Bacteriol. 175:176-181(1993).
Aeromonas sp.	D63139-42	Biochim. Biophys. Acta 1305:44-48(1996).
Aeromonas sp.	D31818	J. Ferment. Bioeng. 78:205-211(1994).
Bacillus circulans	M57601-J05599; D10594; D90534;	J. Biol. Chem. 265:15659-15665(1990).
Bacillus circulans	D89568;	J. Ferment. Bioeng. 80:454-461(1995).
Bacillus licheniformis	U71214;	Unpublished
Clostridium thermocellum	Z68924	Unpublished
Ewingella americana	X90562;	Unpublished
Enterobacter agglomerans	U59304	Appl. Environ. Microbiol. 63:834-839(1997).
Janthinobacterium lividum	U07025	FEMS Microbiol. Lett. 131:279-288(1995).
Kurthia zopfii	D63702	Unpublished
Serratia marcescens	X03657	EMBO J. 5:467-473(1986).
Serratia marcescens	Z36294	FEMS Microbiol. Lett. 124:399-404(1994).
Serratia marcescens	Z36295	Microbiology 141:123-131(1995).
Streptomyces lividans	D13775	Biosci. Biotechnol. Biochem. 57:1691-1698(1993).
Streptomyces lividans	D12647	J. Gen. Microbiol. 139:677-686(1993).
Serratia marcescens	X15208	Nucleic Acids Res. 17:5395-5395(1989).
Serratia marcescens	L01455	Unpublished
Serratia marcescens	L38484	Unpublished.
Serratia marcescens	L41660	Unpublished
Streptomyces olivaceoviridis	X71080	Eur. J. Biochem. 214:659-669(1993).
Streptomyces plicatus	M18397	J. Biol. Chem. 263:443-447(1988).
Streptomyces plicatus	M82804	Proc. Natl. Acad. Sci. U.S.A. 89:1885-1889(1992).
Streptomyces thermoviolaceus	D14536	Gene 134:113-117(1993).
Vibrio harveyi	U81496	Unpublished

Table III

Compilation of known bacteria chitinases

3D-structure of bacteria chitin degrading enzymes

1. Chitinases A from *Serratia marcescens*

The X-ray structure of Chitinase A from the chitinolytic bacterium *Serratia marcescens* has been solved by multiple isomorphous replacement (MIR) and refined at 2.3 Å resolution resulting in a crystallographic R-factor of 16.2% (1,2). The structure of Chitinase A consists of three domains (Fig. 2). The amino-terminal domain (aa 24-137) which consists only of β-strands, connects through a hinge region (residues 138-158) to the main $(\alpha\beta)_8$-barrel domain (residues 159-442) and (517-563). The third domain has an α+β fold and is formed by an insertion in the barrel motif (residues 443-516).

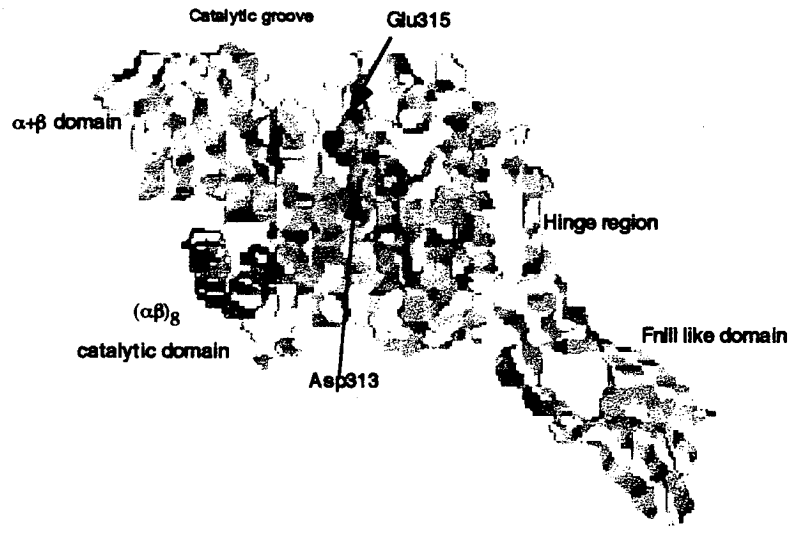

Figure 2

Ribbon diagram illustrating the structure of Chitinase A, its three domains and the groove of the active site where the catalytic amino acid Glu315 is located.

2. Chitobiase from *Serratia marcescens*

The 3D structure of chitobiase was solved by MIR and refined to a resolution of 1.9 Å. Chitobiase has an eight stranded $(\alpha\beta)_8$-barrel structure (domain III, see fig. 3) with three additional domains. The N-terminal domain I, 154 aa, comprises two β-sheets. Domain II, 122 aa, has α+β topology. Domain III, 483 aa, an $(\alpha\beta)_8$-barrel motif, is the core of the structure, around which the other domains are organised. The active site is on the C-terminal end of the barrel from where long loops interacting with domain I. The eight β-strands are surrounded by seven α-helices (3, 4).

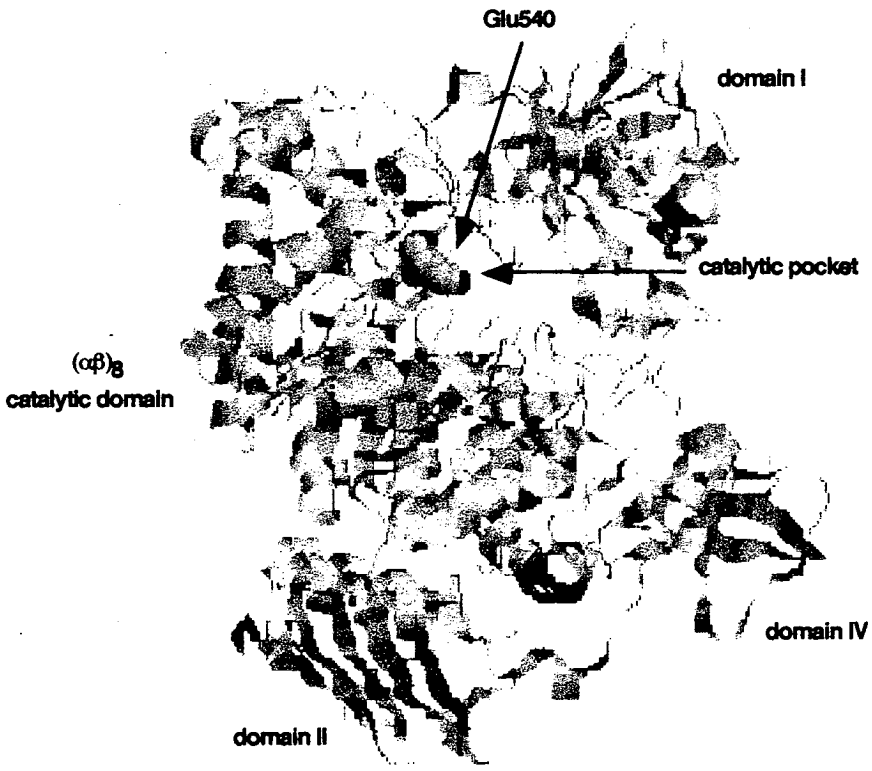

Figure 3.
Schematic diagram illustrating the structure of chitobiase and its domains. The amico acid residue responsible for catalysis Glu 540, is in space filling.

Several deviations from the classical $(\alpha\beta)_8$-barrel motif have been identified. Domain IV, as domain I, comprises two β-sheets, but

has only 67 aa. Overall dimensions of the protein are roughly 90 x 80 x 60 Å.

3. Chitosanase from *Streptomyces* N174

The 2.4 A X-ray crystal structure of chitosanase, a protein with chitosan endo-hydrolase activity isolated from *Streptomyces* N174, was previously published (5). The structure was solved using phases acquired by SIRAS from a two-site methyl mercury derivative combined with solvent flattening and non-crystallographic two-fold symmetry averaging, and refined to an R-factor of 18.5%. The mostly α-helical fold reveals a structural core (fig. 4) shared with several classes of lysozyme and barley endochitinase, in spite of a lack of shared sequence.

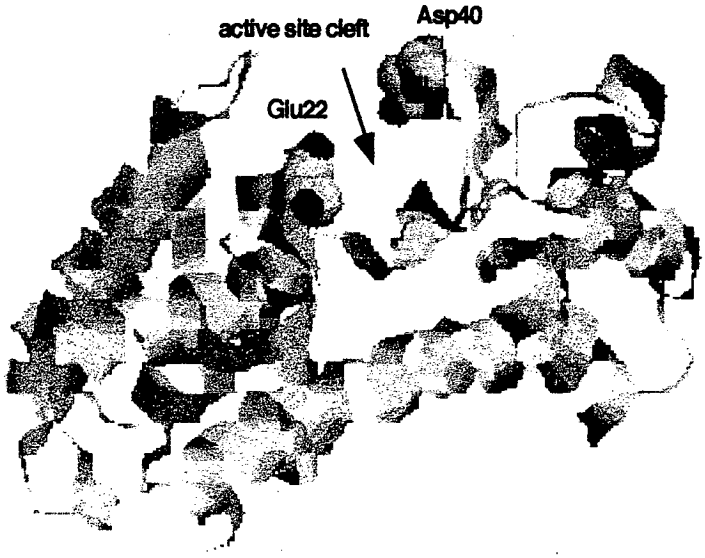

Figure 4.

Schematic diagram illustrating the structure of chitosanase. The amico acid residues responsible for catalysis, Glu22 and Asp40 are in space filling and located in both site of the active site cleft.

Based on the structural similarity with lysozyme and barley endochitinase, the authors postulate a putative active site, an acid/base mechanism of catalysis as well as the mode of substrate recognition. It appears that Glu22 acts as an acid and Asp40 serves as a general base to activate a water molecule for an SN_2 attack on the glycosidic bond. A series of amino-acid side chains and backbone carbonyl groups may bind the polycationic chitosan substrate in a deep electronegative binding cleft.

Current knowledge on the catalytic mechanism and stereochemistry of chitin hydrolysis

Enzymatic hydrolysis of the glycosidic bond takes place *via* general acid-base catalysis that requires two critical residues: a proton donor and a nucleophile base. This hydrolysis can give rise to either an overall retention or an inversion of anomeric configuration.

There are two variations of the classical acid-base catalysis mechanism which is common for glycosyl hydrolases.

1. The first variation involves a protonated acidic residue as proton (H^+) donor which is the -COOH group of a Glu residue and a second negatively charged amino acid residue which electrostatically stabilizes the positive charge at C_1 fromed while catalysis. The -COOH of the Glu donates a H^+ to the glycosidic oxygen causing it to be a better leaving group due to the polarisation of the scissile bond. The product leaves and the remaining sugar acquires a positive charge, called *carbonium ion* or *oxocarbonium* intermediate. The second negatively charged residue is thought to stabilise this oxocarbonium ion intermediate, or to act as a nucleophile to form a covalent intermediate (glycosyl-enzyme). Then the carbonium ion reacts with an activated H_2O (OH^-) from the equatorial side leading to *retention* of the anomeric configuration of the C1.
2. In the second variation, however, the stabilising base (negative charged residue) is too far from the C_1 atom to stabilize the positively charged carbonium ion intermediate. That residue facilitates the polarisation of a water molecule from the solution, which attacks directly from the free axial side, thus leading to *inversion* of the anomeric configuration.

The distance between the two catalytic residues is characteristic of the mode and stereochemistry of the reaction mechanism: 4.8 - 5.3 Å is typical for hydrolysis with retention of anomeric configuration (via a double displacement mechanism), and roughly 9 - 9.6 Å for inversion (single displacement), where an additional water is positioned between the anomeric carbon and the second protein carboxylate.

Biochemical experiments have clearly shown that Chitinase A and Chitobiase and several other bacterial chitin hydrolyzing enzymes are retaining enzymes (6,7) A structurally conserved Glu has been assigned to be the proton donor in acid-catalysis (i.e Glu315 in the Chitinase A, and Glu540 in Chitobiase). In the strucutre of Chitinase A and Chitobiase, no negatively charged residue can be unambiguously assigned to stabilise the carbonium ion intermediate. Therefore, the positive charge at C_1 is proposed to

be stabilised at its axial side through the O7 carbonyl oxygen of the *N*-acetyl group, which has a partial negative charge. In this way a water molecule can attack C1 from the equatorial side, resulting in retention of the anomeric configuration. This kind of stabilisation is in contrast to many other retaining glycosyl hydrolases, however it has been prosoped few decades ago by Koshland (7, 8, 9) and called *substrate assisted catalysis or anchimeric assistance of the N-acetyl group*. The current crystallographic and biochemical data provide strong evidence for this mechanism (10, 11, 12).

The role of FnIII domain on the interaction of chitinase with chitin

The 3D structure of Chitinase A has revealed that the N-terminus of the protein forms a domain which has an immunoglobulin-like fold. This domain is likely to facilitate the interaction of Chitinase A with its insoluble substrate.

Sequence searches performed using the BLASTP suite of programs has intentified this domain in three chitinases and three viral putative chitinase genes, as shown in fig. 5.

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            ..26.29..33...38.......    50.............66.69 .........
            ..*..*...*....*.........*.............*..*
Chia_Serma  AAPGKPTIAWGNTKFAIVEVDQAAT AYNNLVKVKN AADVSVSWNLWNGDAGTGPKIL      57
Chit_Aerca  ---A----GS-P--------N---S ---Q--T-HKDC-P---T----S--V-QTA-V-      58
Chit_Altso  ---ST--LD-QPQQYSF---NVDCLGS-KQ--KAKD VV-ISIK--A-S-SG-DNY-VY      58
Chit_Npvac  -L--T-V-D-ADRNY-L--INYE-- --E--IKPKE QV--Q----V-N--I-DIAYV-      57
Chit_Npvcf  -L--T-V-D-ADRNY-L-KINSD-- --E--IQRND HVS-Q----V-N.........      47
Chit_Npvbm  -I--T-V-D-AERNY-L-KINYE-- --E--IKLKE QV-...................      38
                  + +  +   *    *  +         *  +            *  *

Chia_Serma  LNGKEAWSG  PSTGSSGTANFKVNKGGRYQMQVALCNADGCTASDATEIVVADTDGSH      114
Chit_Aerca  -DV--V---  -AS AA-------T-----------------L--KK-LM--------      114
Chit_Altso  FDDLLVNQ-SL-AGTK--VVQ-PYT-S--H-LYLE--EGTV-AR-AGK---I-----A-      117
Chit_Npvac  FDEQQV-K-  DAE  -KR-TI--LVS-QFN-R-K--NE-G-SV--PVLV-------G-      112
Chit_Npvcf  ..........................................................
Chit_Npvbm  ..........................................................
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Figure 5.

Alignment of the sequences with the FnIII motif. Chia_Serma is for *Serratia marcescens* chitinase; Chit_Aerca is for *Aeromonas caviae*; Chit_Altso for *Alteromonas* sp. Chit_Npvac, Chit_Npvcf and Chit_Npvbm are for the putative chitinases from *Autographica californica*, *Bombyx mori* and *Choristoncura fumiferana* nuclear polyhedrosis viruses, respectively.

In two bacteria *Streptomyces* and one *Bacillus* sp. the same domain has also been identified and is located either either at the

end, the middle or the start of the molecule. Table IV summarizes the occurence of the FnIII domain in various bacteria chitinases.

Current available information does not support the direct involvement of the FnIII domain to the binding of chitinases to chitin (13,14). However, it is beleived that this domain is participating in the efficient degradation of chitin polymers by various bacteria chitinases.

Organism	Gene	Position
Serratia marcescens	CHIA_SERMA	Start of the molecule
Alteromonas sp.	CHIT_ALTO	Start of the molecule
Aeromonas caviae	U09139	Start of the molecule
Bacillus circulans	CHI1_BACCI	End of the molecule
Bacillus circulans	CHID_BACCI	Start of the molecule
Streptomyces lividans	CHIT_STRLI	Middle of the molecule
Streptomyces plicatus	CHIT_STRPL	Start of the molecule
Streptomyces olivaceoviridis	CHIX_STROI	Start of the molecule
Autographa caliphornica Nuclear Polyhedrosis Virus	CHIT_NPVAC	Start of the molecule
Bombyx mori Nuclear Polyhedrosis Virus	U12688	Start of the molecule
Choristoneura fumiferana Nuclear Polyhedrosis Virus	M97906	Start of the molecule

Table IV.

Occurence and location of FnIII-like domain in bacteria chitinases and viral putative chitinases.

PDB	Protein
1ctn	Chitinase a
1fnf	fibronectin
1bec	14.3.D t cell antigen receptor M
2hft	human tissue factor
8fab-B	Fab fragment from human immunoglobulin
1clc	endoglucanase

Table V.

Fold similarity of the FnIII-like domain from Chitinase A to various other domains or structures in the PDB.

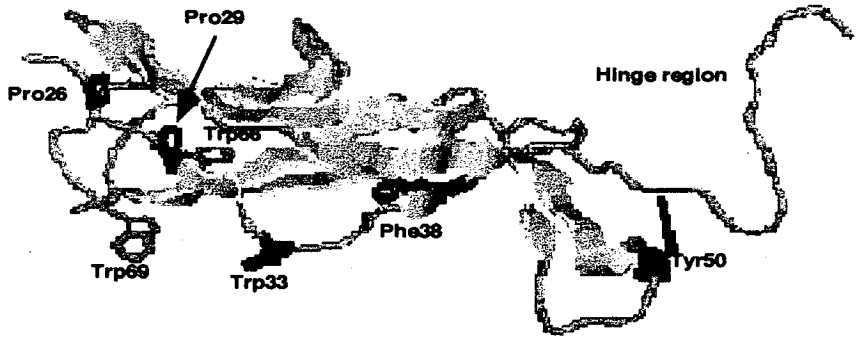

Figure 6.
Schematic diagram illustrating the structure of FnIII domain of *Serratia marcescens* Chitinase A. Higly conserved amino acid residues indicated on Fig. 5 are presented on the 3D-model of this domain, (aa numbering is according the 1ctn PDB file).

Structural data on carbohydrate binding: How would chitin substrate binds on the enzyme?

The modes of binding of a carbohydrate substrate on its hydrolase is difficult to be established directly by X-ray crystallography since the substrate is rapidly degraded. The first insight was provided by the X-ray structure of a$(NAG)_3$ (a strong competitive inhibitor of lysozyme) with lysozyme. The results of this study were used as the initial model for protein-carbohydarte interactions. The first protein-carbohydrate review appeared in the middle of '80s. Since then, the number of well refined proteins with bound carbohydrates is constantly increasing.

From the elucidation of the structure of Hevamine and Chitinase A with $(NAG)_3$ and $(NAG)_2$ oligomers, respectively and allosamidine, several subsites have been defined for the binding of the sugar substrate on the cleft (groove) around the catalytic site.

The (+2) subsite in Chitinase A is mainly defined by the stacking of the aromatic residue Phe396 against the hydrophobic face of the sugar. Two direct hydrogen bonds are present: (i) between the carboxylate oxygen of Asp391 and the N2 of the N-acetyl group of the sugar and (ii) between the Nz atom of Lys320

and O8 atom of the sugar. The Phe396 ring which is characteristic for the (+2) site in Chitinase A is absent in Hevamine.

The (+1) subsite in Chitinase A, is defined by the aromatic ring of Trp275 which is packed against the hydrophobic face of the sugar. No direct hydrogen bonds exist with this sugar and the affinity is modulated via 3 water molecules by mediating hydrogen bonds betwen the O3 and O6 sugar atoms and the protein.

The Trp275 ring is only present in Chitinase A. This supports the assumed higher affinity of this site in Chitinase A compared to Hevamine.

In Chitinase A and Hevamine complexed with allosamidin, an aromatic ring (Trp539) is packed against the hydrophobic face of the double ring of allosamidin, defining subsite (-1). This Trp residue is present in all three enzymes. The interactions of the oxazoline group in the Chitinase A-allosamidin complex are similar to the ones described for the complex with Hevamine.

The second sugar in subsite (-2) forms hydrogen bonds to the carboxyl oxygens of Glu540 and Glu473 with the O7 and N2 atoms of the *N*-acetyl group respectively. The O6 atom of the sugar hydrogen bonds with the main chain nitrogen of Thr276.

The (-3) subsite is defined in Chitinase A with Trp167, which also packs almost perfectly against the sugar ring as modelled in Hevamine, where that Trp is absent. The two direct hydrogen bonds in Hevamine are disrupted and presumably substituted by the hydrophobic interaction.

The sugar in the (-4) subsite of Chitinase A binds in a different manner than in Hevamine. While getting further from the active site it is expected that the mode of binding is not well conserved and this sugar ring could adopt a different position.

Further experiments are required to fully understand this complex protein-sugar interaction.

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