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Conservation of structural elements and catalytic mechanism in the chitinolytic enzymes from *Serratia marcescens*

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Introduction

Chitin degrading enzymes are ubiquitous and are found in prokaryotic and eukaryotic organisms as well as in archaea (Perrakis *et al.*, 1993; Tanaka *et al.*, 1999; Brumberg *et al.*, 2001). *Serratia marcescens* has been used as a model system for the utilization of chitin as a carbon source. In the presence of chitin, *S. marcescens* expresses chitinase A, chitinase B, chitinase C and chitobiase that degrade chitin to *N*-acetylglucosamine (NAG) (Warren, 1996). In addition these cells also express a chitin binding protein (CBP21) with unknown function (Folders *et al.*, 2000).

The structures of *S. marcescens* chitinolytic enzymes, chitinase A, chitinase B and chitobiase were solved and the catalytic domains were found to possess an α/β TIM barrel structure (Perrakis *et al.*, 1994; Tews *et al.*, 1996; van Aalten *et al.*, 2000). In contrast, very little is known about the function and structure of chitinase C. Chitinase A, B and C were assigned to glycosyl hydrolase protein family 18, while chitobiase belongs to protein family 20 (Henrissat, 1991). In these enzymes the active site is

located at the carboxy-terminal end of the β-strands of the α/β-barrel suggesting that these proteins, that greatly differ in their primary sequences, all have a common origin.

Information on the co-crystal structure of chitinase-substrate complexes is scant. Earlier attempts to obtain the co-crystal structures of oligoNAG-chitinase complexes in which the non-cleaved substrate occupies the catalytic site proved unsuccessful (Perrakis *et al.*, 1994; Terwisscha-van-Scheltinga *et al.*, 1996). Creation of mutations of the catalytic residues of *S. marcescens* chitinase A and chitobiase allowed us to obtain co-crystals with the native substrate (Prag *et al.*, 2000). Our investigations led us to suggest that *S. marcescens* enzymes participating in chitin metabolism are all employing a similar catalytic mechanism.

Results and Discussion

Sequence conservation at the active site

Multiple alignments of glycosyl hydrolases protein families 18 and 20, show the conservation of residues in the β4-loop #4; DXXDXDXE in family 18 and HXGGDE in family 20. Both signatures include Glu315 of chitinase A and Glu540 of chitobiase that act as the proton donor (Henrissat, 1991; Perrakis *et al.*, 1994; Tews *et al.*, 1996), (Figure 1).

Structural conservation at the active site

We have mutated the conserved Asp and Glu residues mentioned above of both chitinase A and chitobiase and found that both residues are essential for the catalytic activity. Moreover these mutants allowed us, to determine the crystal structures of chitinase A and chitobiase complexed with their native substrate at the active site (Prag *et al.*, 2000; Papanikolau, Y.; G. P.; Tavlas. G.; C. E. V.; A. B. O. and Petratos K., in preparation). The structure of the catalytic residues Asp and Glu are similar in both enzymes (Figure 2). However, only in chitinase A, Asp313 appears, in the wild-type, in two alternative conformations Papanikolau, Y.; G. P.; Tavlas. G.; C. E. V.; A. B. O. and Petratos K., in preparation). Comparison of the structures of the enzyme-substrate complexes show high similarity in the position of the -1 to +1 sugars. It was also observed that in both enzyme-substrate complexes, the planes of the sugars at –1 and +1 are tilted around the scissile bond in a similar manner. In both enzymes a critical chair to boat sugar conformational change is involved in the bending and rotation of the substrate upon binding (Figure 2). These energetically non-favored

structures may favor the hydrolysis reaction. Furthermore, the distance from the proton donor and the glycosidic oxygen is conserved. These findings suggest that chitinase A, chitinase B and chitobiase possess similar catalytic sites.

Family 20

CHB_SERMA	529	QPIKTWHFGGDEAK	542
HEX_STRPL	303	TPGRYLHIGGDEAH	316
HEXA_HUMAN	312	FPDFYLHLGGDEVD	325
HEXB_HUMAN	344	FPDQFIHLGGDEVE	357
HEX_VIBVU	508	QPLTDYHIGADETA	521
CHB_VIBHA	526	APLTTWHFGGDEAK	539
HEXB_MOUSE	323	FPDQFIHLGGDEVE	336
HEXA_MOUSE	312	FPDFYLHLGGDEVD	325
HEXB_ALTSO	520	VPLNTYHIGADETA	533
HEXB_FELCA	286	FPDHFVHLGGDEVE	299
HEX1_CANAL	313	FIDDVFHVGNDELQ	326
HEX1_ENTHI	290	FGTDYVHVGGDEVW	303
HEXA_DICDI	297	FIDNYFHTGGDELV	310
HEXC_BOMMO	359	ESTDMFHMGGDEVS	372
HEXA_PORGI	324	FPGTYFHIGGDECP	337
STRH_STRPN	349	KKTEIFNIGLDEYA	362
STRH_STRPN	793	GKTKIFNFGTDEYA	806

Family 18

*	**CHIA_SERMA**	307	FDGVDIDWEFP	317
*	**EBAG_STRPL**	124	LDGVDFDDEYA	134
*	**EBA1_FLAME**	120	LDGVFFDDEYS	130
*	**CHLY_HEVBR**	119	LDGIDFDIEHG	129
*	**CHIB_SERMA**	136	FDGVDIDWEYP	146
	CHIC_SERMA	133	FDGLDIDLEQA	143
	CHID_BACCI	295	FNGLDIDLEGS	305
	CHIX_STROI	376	LDGLDIDFEGH	386
	CHI1_APHAL	163	FDGIDIDWEYP	173
	CHI1_BACCI	196	FDGVDLDWEYP	206
	CHI1_CANAL	139	VDGFDFDIENK	149
	CHI1_COCIM	163	FDGIDIDWEYP	173
	CHI1_RHIOL	158	IDGVDLDIEGG	168
	CHI2_COCIM	167	VDGFDFDIEKG	177
	CHI3_CANAL	149	LDGFDFDIENN	159
	CHI4_TRIHA	163	FDGIDIDWEYP	173
	CHIA_ALTSO	305	YDGVDIDWEFP	315
	CHIB_TOBAC	143	LDGIDFDIELG	153
	CHIT_CAEEL	171	FDGIDIDWEYP	181
	DIAC_HUMAN	135	MDGINIDIEQE	145
	CHIT_YEAST	149	VDGFDFDIENN	159
**	C3L1_HUMAN	131	FDGLDLAWLYP	142
**	OGP_HUMAN	133	FDGLDLFFLYP	143
**	**NARBONIN**	124	IDGIDIHYEHI	134
**	**CONB_CANEN**	123	LDGIHFDIQKP	133

Figure 1. Sequence alignment of protein families 18 and 20 showing the conserved signatures. * Marks proteins whose structure was solved; ** proteins with no chitinase activity. Catalytic glutamic residues are shown in red and the conserved aspartic residues in blue.

The sequences of proteins belonging to glycosyl hydrolase families 18 and 20 are too different to be aligned on the basis of their primary

sequences only. However, the structure of the catalytic α/β-barrel domain is highly conserved. We found that the structure of residues in the conserved motives (DE of chitobiase and DWE of chitinase A) together with the structure of the -1/+1 diNAG is sufficient to obtain structural alignment of chitobiase with chitinase A. Chitinase B could be aligned with chitinase A as these proteins are very similar (Figure 3). This analysis revealed, for example, a conserved tyrosine residue (Tyr669 of chitobiase and Tyr390 of chitinase A). Mutations Tyr669Asp and Tyr390Phe showed reduced activity.

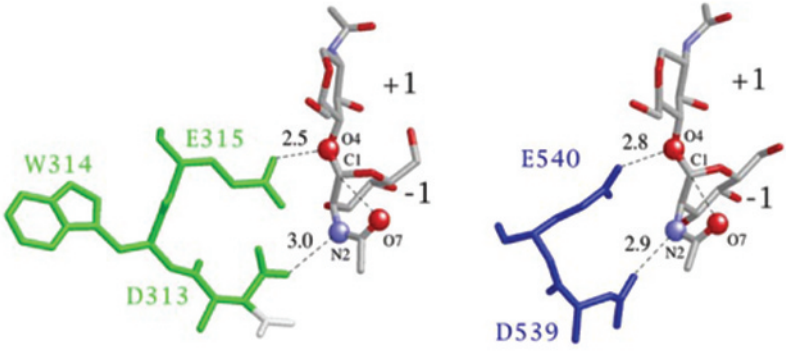

Figure 2. Conservation of chitinase A and chitobiase catalytic sites. The catalytic residues of chitinase A (left) and chitobiase (right) are shown and the overlap of the two enzymes (right). The -1 and +1 sugars are shown. Distances are given in Å. The complex of wild-type chitinase A with the substrate was modeled based on mutant complexes.

Catalytic mechanism

In general, glycosidases degrade carbohydrates by a general acid-base catalysis that involves two amino acid residues, a proton donor and nucleophile. Hydrolysis of the scissile bond results in either the retention or the inversion of the C1-carbon anomeric configuration. While the catalytic glutamate is highly conserved, no amino acid residue that could act as a nucleophile was identified in protein families 18 and 20. A different catalytic mechanism was therefore proposed. Based on the complex of hevamine (a family 18 plant chitinase) with allosamidin and on the complex of chitobiase with diNAG it was suggested that catalysis take

place via substrate-assisted mechanism (Figure 4), (van Scheltinga *et al.*, 1994; Tews *et al.*, 1996 and Prag *et al.*, 2000). In this proposed mechanism the glutamate residue acts as a proton donor, while the terminal oxygen (O7) of the acetamido group of the –1 NAG acts as a nucleophile. In order to act as a nucleophile the acetamido group has to rotate around the C2-N2 bond as was experimentally observed (Prag *et al.*, 2000). However, direct evidence for substrate-assisted catalysis is not yet available.

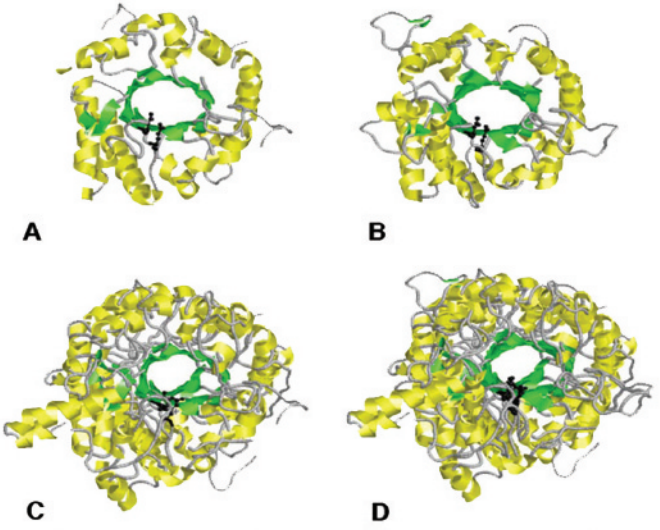

Figure 3. Structural comparison of the TIM-barrel domain of chitinase A (A), chitinase B (B), chitobiase (C) and superposition of all enzymes (D)

What is then the function of the essential aspartic residue in these two protein families? Structural analysis of alanine replacement mutations in chitobiase and chitinase A shed some light on this question. In chitobiase, the complex of Asp539Ala-diNAG the acetamido group is flipped by about 180^0 with respect to that found in the wild-type and in the Glu540Asp complexes (Prag *et al.*, 2000). In this position the acetamido group cannot participate in the substrate-assisted reaction. Thus we suggested that one function of Asp539 is to ensure placing the acetamido group in the conformation that favors the catalysis via the substrate assisted catalysis. In addition, Asp539 appears to be required for restraining the movement of Glu540. Asp539 may provide additional negative charge at the active site

and stabilize the partial positive charge of the acetamido group while forming the oxazoline ring.

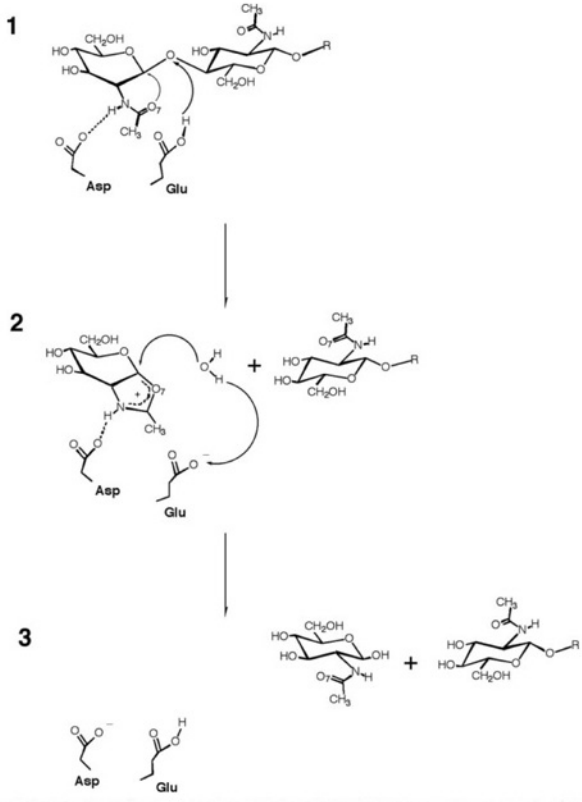

Figure 4. Model for the catalytic mechanism of chitinase A and chitobiase focusing on the catalytic Asp and Glu residues at the active site.

The analysis of a number of chitinase A-substrate complexes suggests that Asp313 play a similar role in ensuring the positioning of the acetamido group in the conformation that favors the catalysis via the substrate-assisted reaction. Furthermore, we identified in improved crystal structure of wild-type chitinase A that Asp313 is found in two alternative conformations, suggesting that the flexibility of Asp313 is an essential part of the catalytic reaction.

Evolutionary consideration

This study provides further information on the possible evolution of the pathway for chitin metabolism in which the catalytic mechanism and key catalytic residues are conserved. The large differences in amino acid sequence and the presence of additional domains led to the suggestion that proteins possessing an α/β TIM-barrel domain evolved by convergent evolution (Branden & Tooze, 1991; Perrakis *et al.*, 1996). Our analysis lead us to favor the hypothesis that the genes coding for protein families 18 and 20 diverged from a common ancestral gene coding for a TIM-barrel domain. These diverge to acquire different substrate specificity. Evolutionary forces, however, conserved loop #4 with its catalytic glutamate residue. Further tinkering led to the establishment of the signatures around the catalytic sites while conserving the Asp-Glu structure required for catalysis. Interestingly, family 19 chitinases, that do not possess a TIM-barrel structure, utilize an alternative, acid-base catalytic mechanism (Hart *et al.*, 1995). We anticipate that it will be possible to convert the specificity of families 18 and 20 enzymes by modifying their substrate binding properties without altering the catalytic residues (Altamirano *et al.*, 2000).

Several members of family 18, such as the plant seed storage proteins concanavalin B and narbonin, are known to be devoid of catalytic activity (Figure 1, Hennig *et al.*, 1995; Hennig *et al.*, 1992). The structure of the inactive catalytic domain of these proteins is similar to chitinase A and other chitinases. In both inactive proteins, the conserved motif is modified to DIQ in concanavalin B and HYE in narbonin (Terwisscha-van-Scheltinga *et al.*, 1996). Similarly, two human proteins, C3L1 and OGP39, were recently identified as inactive family 18 proteins. Our model for the function of Asp313 and Glu315 of chitinase A provides a molecular explanation for the lack of catalytic activity of these proteins.

Cooperative action in chitin degradation

The biochemical and structural information presented here provide a more complete picture to explain how *S. marcescens* employs its enzymes in chitin degrading. Chitin degradation is probably initiated by the action of endochitinases, probably chitinase A and chitinase C (Watanabe *et al.*, 1997), (Figure 5). Chitinase A also acts as an exochitinase, cleaving diNAG dimers from the reduced end. Chitinase B acts as an exochitinase

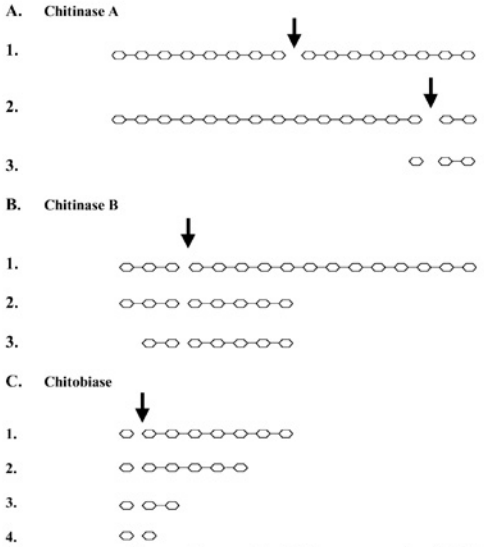

Figure 5. The cooperative action of chitinases and chitobiase in chitin degradation.

cleaving triNAG and diNAG from the non-reducing end of the chitin oligoNAG chains generated by the action of chitinase A and C (Brurberg *et al.*, 1996; van Aalten et al., 2000). The oligomers, triNAG and diNAG, are subsequently degraded to metabolizable NAG monomers by chitobiase. This analysis clearly explains the synergistic activity of these enzymes that has been reported previously (Brurberg *et al.*, 1996; Watanabe *et al.*, 1999).

Conclusion

Developments in solving the detailed structures of the chitinolytic enzyme-substrate complexes are expected to result in deeper understanding of the mechanism of action of these enzymes. The accumulated genetic, biochemical and structural data lead us to propose that both chitinase A and chitobiase share a similar catalytic mechanism. This conclusion is based on the following key findings:

- The catalytic domains assume a TIM-barrel structure in which a glutamate residue, located in loop # 4, acts as a proton donor.
- The spatial organization of the conserved Asp and Glu catalytic residues is conserved.

- The structure of the sugar residues where cleavage is to take place is highly conserved.

Furthermore, a model of how *S. marcescens* degrades chitin was developed.

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