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Chitinases from *Serratia marcescens*

May Bente Brurberg [1], Bjørnar Synstad [2], Sonja Sletner Klemsdal [1], Daan M.F. van Aalten [3] Leif Sundheim [1] and Vincent G.H. Eijsink [2]

[1] The Norwegian Crop Research Institute, Ås Norway, [2] Department of Chemistry and Biotechnology, Agricultural University of Norway, Ås Norway, [3] Department of Biochemistry, University of Dundee, Dundee, Scotland.

Abstract

Serratia marcescens is one of the most effective bacteria for degradation of chitin, a 1,4-β-linked polymer of N-acetyl-β-D-glucosamine (GlcNAc). Chitinolysis by *S. marcescens* involves at least four enzymes and a chitin-binding protein. Studies of the enzymology and the structures of the enzymes provide insight in how a natural set of chitinolytic enzymes may be built up. *S. marcescens* chitinases may find applications as biocontrol agents against fungi and insects.

Introduction

Chitin, a 1,4-β-linked polymer of N-acetyl-β-D-glucosamine (GlcNAc), is the second most abundant polymer in nature, after cellulose. It is a major structural component of the exoskeleton of insects and crustaceans and it occurs in the cell walls of a variety of fungi. In accordance with the abundance of chitin, chitin-degrading enzymes are found in a variety of organisms, varying from prokaryotes to man (reviewed in 1 - 7). In chitin-containing organisms, chitinases play an important role in normal life cycle functions such as morphogenesis and cell division, whereas plants produce chitinases as part of their defence against fungal pathogens. Many bacteria and fungi contain chitinolytic enzymes to convert chitin into compounds that can serve as energy source.

Chitinases belong to families 18 and 19 of glycosyl hydrolases (8). Family 18 contains enzymes from a variety of prokaryotic and eukaryotic organisms, whereas family 19 chitinases only have been found in higher plants and in the Gram-positive bacterium *Streptomyces* (1, 9). The two families contain both endochitinases, cleaving randomly in the chitin chain, and exochitinases. The latter cleave off chitobiose $(GlcNAc)_2$ (”chitobiosidase”; exo-N,N’-diacetylchitobiohydrolase) or chitotriose $(GlcNAc)_3$ (”chitotriosidase”; exo-*N*,*N*’,*N*’’-triacetylchitotriohydrolase) from the reducing or the non-reducing end of the chitin chain (10 - 15). In addition to endo- and exochitinases, chitin-degrading organisms contain chitobiases (*N*-acetyl-β-glucosaminidases), a third class of chitinolytic enzymes that convert GlcNAc dimers into monomers (16 – 19).

Chitinases are of great biotechnological interest. Firstly, these enzymes may be used to convert chitin-containing biomass into useful (depolymerised) components. Secondly, chitinases may be exploited for the control of fungal and insect pathogens of plants (6, 20 –

Table 1. Chitinases and CBP from *S. marcescens* [1].

SDS-PAGE band (kDa) [2]	Gene (protein name)	Localization in *S. marcescens*[3]	N-terminal signal peptide	Reference
57 - 58	*chiA* (ChiA)	extracellular	yes	27 - 29, 32
52 - 54	*chiB* (ChiB)	periplasm / extracellular	no	27 – 29, 31, 33, 34
48 - 52	*chiC* (ChiC1)	extracellular	no	14, 27, 28, 35
35 - 36	*chiC* (ChiC2)	extracellular	no	14, 27, 28, 35
95	*chb*	periplasm	yes	17, 18
21 - 22	*cbp* (CBP21)	extracellular	yes	27, 28, 34

1) See (26) for an early study on chtinolytic proteins produced by *S. marcescens.*
2) The ranges indicate variation in the apparent molecular masses that have appeared in the literature.
3) The literature is somewhat inconclusive concerning the localization of the various chitinases; see text for details.

23). Thirdly, chitinase inhibitors potentially nhibit growth of chitin-containing (plant-) pathogens and plague insects that need chitinases for normal development (24).

The Gram-negative bacterium *Serratia marcescens* secretes a variety of extracellular enzymes including chitinases (25). *S. marcescens* is one of the most effective bacteria for degradation of chitin (10). When this bacterium is cultivated in the presence of chitin, a variety of chitinolytic enzymes and chitin-binding proteins can be detected (26 – 28). The precise number of different enzymes is somewhat difficult to determine on the basis of biochemical studies only, because some of the enzymes occur in multiple forms, e.g. on an SDS-PAGE gel. The latter is because the chitinases have a multi-domain structure (see below), which makes them sensitive for partial proteolytic degradation. Thorough studies by a number of groups clearly show that *S. marcescens* produces at least three chitinases (ChiA, ChiB, ChiC), a chitobiase and a putative chitin-binding protein (CBP21) (13, 18, 26 – 35). It is conceivable, but not certain, that these five proteins represent the complete chitinolytic machinery of the bacterium. The chitinolytic machinery of *S. marcescens* is of great interest because it is one of the best characterized chitinolytic machineries known to date. Recently determined crystal structures of ChiA (36), ChiB (15) and the chitobiase (19) provide detailed insight in how a natural set of chitinolytic enzymes may be built up.

In this review we will provide an overview of current knowledge concerning the genes and proteins that make up the chitinolytic machinery of *S. marcescens*, with focus on the chitinases ChiA, ChiB and ChiC. In addition, several interesting and partly unresolved aspects of this machinery will be discussed.

Proteins related to chitin degradation

Biochemical analyses of proteins produced by chitin-induced *S. marcescens* have revealed a number of relevant compounds, identifiable by their apparent molecular mass on an SDS-PAGE gel (Table 1). Subsequent genetic

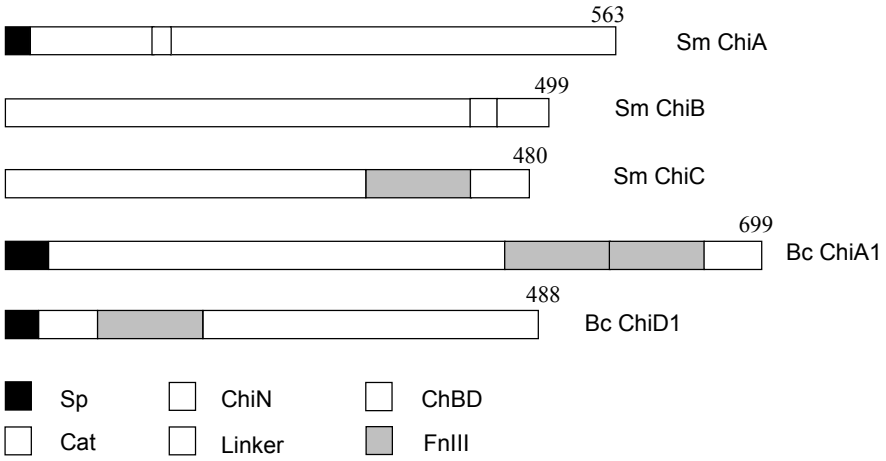


Figure 1. Modular structure of chitinases. In addition to the three chitinases from *S. marcescens* (Sm), two chitinases from *Bacillus circulans* (Bc; 50, 109) are shown. Sp, signal peptide; FnIII, fibronectin type III like domain; ChiN, domain with FnIII-like fold but with no detectable sequence homology to the indicated FnIII domains; see (60) for a discussion); Cat, catalytic domain; ChBD, chitin-binding domain. There exists structural information for complete Sm-ChiA (36), complete Sm-ChiB (15) and for the individual ChBD and Cat of Bc-ChiA1 (56, 61). See text for further details.

analyses have revealed further details (Table 1).

ChiA is produced as a 563-residue precursor, which is secreted from the cells with concomitant cleavage of an N-terminal signal peptide. The resulting enzyme has 540 residues and a calculated molecular mass of 58.5 kDa (32, 36). Apart from deletion of the N-terminal methionine residue, the primary product of the *chiB* gene does not seem to be processed, despite the fact that ChiB is exported in *S. marcescens* (see below). Mature ChiB contains 498 residues and its calculated molecular mass is 55.4 kDa (15, 32). The *chiC* gene gives rise to various variants of ChiC in *S. marcescens* and two variants in recombinant *E. coli.* Recombinant *E. coli* produces a full length variant (ChiC1) consisting of 479 residues and with a calculated molecular mass of 51.6 kDa. The other variant (ChiC2) is a C-terminally truncated variant of ChiC1 that consists of approximately 325 residues (14, 35; these two reports present different conclusions concerning the precise length of ChiC2). In *S. marcescens,* ChiC is exported without concomitant cleavage of an N-terminal signal peptide. Instead, unspecific N-terminal processing of the protein seems to occur which results in production of several slightly different ChiC species lacking 8 – 12 of the N-terminal residues (14, 35).

Analyses of the primary sequences of ChiA, ChiB, and ChiC1, as well as comparisons of available three-dimensional structures (see below) show that these enzymes have a modular structure, something which is very common for enzymes degrading insoluble biopolymers such as chitin and cellulose (Fig. 1; 13, 14, 37 – 41). All three *Serratia* chitinases contain a catalytic domain with the characteristics of a family 18 glycosyl hydrolase (e.g. the SXGG and DXXDXDXE sequence motifs; Fig. 2). In addition, each of the enzymes contains domains that are putatively involved in substrate binding (Fig. 1; see below). The sequence alignment of the

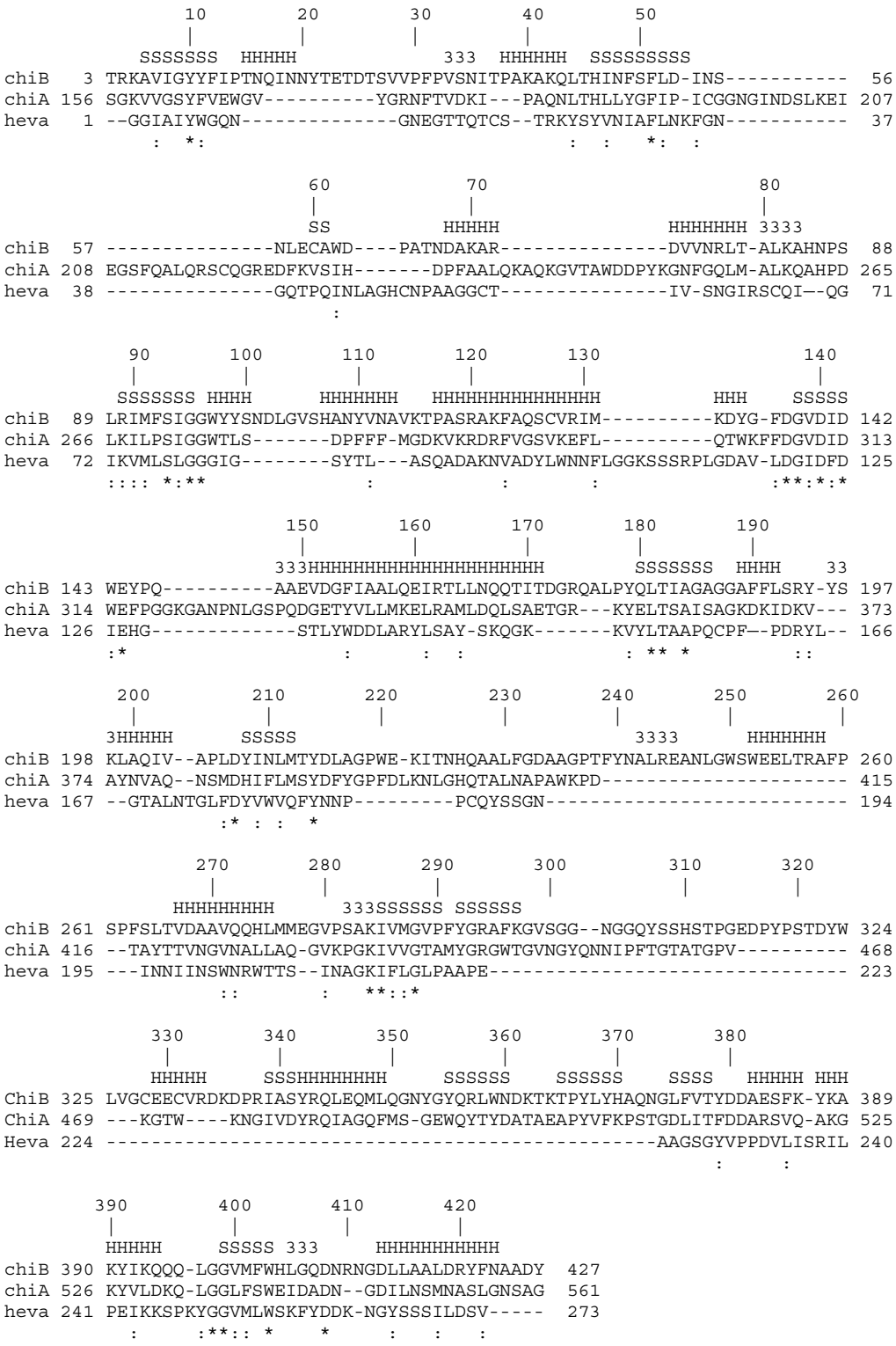

10 20 30 40 50
SSSSSSS HHHHH 333 HHHHHH SSSSSSSSS
chiB 3 TRKAVIGYYFIPTNQINNYTETDTSVVPFPVSNITPAKAKQLTHINFSFLD-INS---------- 56
chiA 156 SGKVVGSYFVEWGV----------YGRNFTVDKI---PAQNLTHLLYGFIP-ICGGNGINDSLKEI 207
heva 1 --GGIAIYWGQN--------------GNEGTTQTCS--TRKYSYVNIAFLNKFGN---------- 37
60 70 80
SS HHHHH HHHHHHH 3333
chiB 57 ---------------NLECAWD----PATNDAKAR---------------DVVNRLT-ALKAHNPS 88
chiA 208 EGSFQALQRSCQGREDFKVSIH-------DPFAALQKAQKGVTAWDDPYKGNFGQLM-ALKQAHPD 265
heva 38 ---------------GQTPQINLAGHCNPAAGGCT---------------IV-SNGIRSCQI--QG 71
90 100 110 120 130 140
SSSSSSS HHHH HHHHHHH HHHHHHHHHHHHHHH HHH SSSSS
chiB 89 LRIMFSIGGWYYSNDLGVSHANYVNAVKTPASRAKFAQSCVRIM----------KDYG-FDGVDID 142
chiA 266 LKILPSIGGWTLS-------DPFFF-MGDKVKRDRFVGSVKEFL----------QTWKFFDGVDID 313
heva 72 IKVMLSLGGGIG--------SYTL---ASQADAKNVADYLWNNFLGGKSSSRPLGDAV-LDGIDFD 125
150 160 170 180 190
333HHHHHHHHHHHHHHHHHHHHH SSSSSSS HHHH 33
chiB 143 WEYPQ----------AAEVDGFIAALQEIRTLLNQQTITDGRQALPYQLTIAGAGGAFFLSRY-YS 197
chiA 314 WEFPGGKGANPNLGSPQDGETYVLLMKELRAMLDQLSAETGR---KYELTSAISAGKDKIDKV--- 373
heva 126 IEHG-------------STLYWDDLARYLSAY-SKQGK-------KVYLTAAPQCPF--PDRYL-- 166
200 210 220 230 240 250 260
3HHHHH SSSSS 3333 HHHHHHH
chiB 198 KLAQIV--APLDYINLMTYDLAGPWE-KITNHQAALFGDAAGPTFYNALREANLGWSWEELTRAFP 260
chiA 374 AYNVAQ--NSMDHIFLMSYDFYGPFDLKNLGHQTALNAPAWKPD---------------------- 415
heva 167 --GTALNTGLFDYVWVQFYNNP---------PCQYSSGN--------------------------- 194
270 280 290 300 310 320
HHHHHHHHH 333SSSSSS SSSSSS
chiB 261 SPFSLTVDAAVQQHLMMEGVPSAKIVMGVPFYGRAFKGVSGG--NGGQYSSHSTPGEDPYPSTDYW 324
chiA 416 --TAYTTVNGVNALLAQ-GVKPGKIVVGTAMYGRGWTGVNGYQNNIPFTGTATGPV---------- 468
heva 195 ---INNIINSWNRWTTS--INAGKIFLGLPAAPE------------------------------- 223
330 340 350 360 370 380
HHHHH SSSHHHHHHHH SSSSSS SSSSSS SSSS HHHHH HHH
ChiB 325 LVGCEECVRDKDPRIASYRQLEQMLQGNYGYQRLWNDKTKTPYLYHAQNGLFVTYDDAESFK-YKA 389
ChiA 469 ---KGTW----KNGIVDYRQIAGQFMS-GEWQYTYDATAEAPYVFKPSTGDLITFDDARSVQ-AKG 525
Heva 224 --AAGSGYVPPDVLISRIL 240
390 400 410 420
HHHHH SSSSS 333 HHHHHHHHHHH
chiB 390 KYIKQQQ-LGGVMFWHLGQDNRNGDLLAALDRYFNAADY 427
chiA 526 KYVLDKQ-LGGLFSWEIDADN--GDILNSMNASLGNSAG 561
heva 241 PEIKKSPKYGGVMLWSKFYDDK-NGYSSSILDSV----- 273

Figure 2: Sequence alignment of catalytic domains of family 18 chitinases. ChiA, ChiB and hevamine, an endochitinase from *Hevea brasiliensis*, were aligned on the basis of a superposition of their crystal structures (15, 36, 55). Secondary structure is indicated in the top-line (H, α-helix; S, β-strand; 3, 3_{10}-helix). Fully conserved residues are marked by an asterisk. Characteristic sequence motifs include SXGG (residues 93-97) and DXXDXDXE (residues 137-144). See Figure 1 for details about domain structure. Hevamine contains 273 residues in total and has only one domain.

catalytic domains (Fig. 2) is characterized by the occurrence of relatively large numbers of long insertions and deletions. Several of these relate to variation in the structure and position of the extra domains, as illustrated in e.g. (15).

A fourth enzyme involved in chitin degradation is the 95 kDa chitobiase, which belongs to family 20 of glycosyl hydrolases (17 - 19). This enzyme is produced as an 885-residue precursor that is exported with concomitant removal of a typical 27-residue N-terminal signal peptide. The chitobiase consists of four domains, several of which have unknown functions. The catalytic domain has the same overall fold as the family 18 chitinases [$(\beta\alpha)_8$; TIM-barrel; see below], but the active site architecture is different.

Upon induction with chitin, *S. marcescens* produces at least one more protein which has no enzymatic activity but which binds to chitin (26, 27). This chitin-binding protein with an apparent molecular mass of 21 kDa (CBP21) is produced as a 197 residue precursor, which is exported with concomitant cleavage of a typical 27-residue N-terminal leader peptide. The mature protein consists of 170 residues and has a calculated mass of 18.8 kDa (28). The protein shares 45 % sequence identity with a chitin-binding protein from *Streptomyces olivaceoviridis* (42) and its general characteristics (e.g. the presence of aromatic amino acids) resemble that of known cellulose-binding domains from cellulases.

Export and localization

The export and localization of the chitinolytic machinery of *S. marcescens* has a number of interesting features. ChiA and CPB21 contain N-terminal signal peptides that are cleaved off during secretion of the proteins to the culture medium (27 – 29, 32). N-terminal signal peptides are signatures of *sec*-dependent protein export to the periplasm. The signal peptide is cleaved of by a periplasmic signal peptidase when the exported protein reaches the periplasm (43, 44). How ChiA and CHB21 are transported across the outer membrane is unknown. The chitobiase also contains an N-terminal signal peptide that is cleaved off during export. Current data indicate that its natural location is in the periplasm (17, 18).

ChiB and ChiC are both found in the periplasm and/or culture medium of *S. marcescens* but these two proteins do not contain typical N-terminal signal peptides (14, 27, 33). There are no indications of any proteolytic processing of ChiB (33), whereas proteolytic processing of ChiC does not seem to be linked to export but rather to some kind of unspecific process (see above). In *E. coli*, both enzymes undergo no N-terminal proteolytic processing and they are mainly found in the cytoplasmic fraction (14, 33). Thus, it seems that *S. marcescens* contains a type of export machinery that is absent in *E. coli.*

S. marcescens is known to possess mechanisms for *sec*-independent secretion of proteins. For example, secretion of the HasA and PrtSM proteases and of the lipase A involves ABC-transporter-like proteins (45 - 48). However, sequence features that are (putatively) linked to this type of export mechanism have so far not been detected in ChiB and ChiC (14, 33), meaning that the export mechanism for these two chitinases remains unknown. Export of proteins without any recognisable signals has

also been reported for *Sphingomonas paucimobilis* (49).

Although it is clear that ChiB and ChiC are exported from the cells, the precise localization of these enzymes is somewhat uncertain. Immunocytochemical studies showed that ChiB is located in the periplasm of *S. marcescens* BJL200, and cell fractionation studies confirmed that ChiB was found mainly in the periplasmic fraction (33). However, other groups have detected significant amounts of ChiB in the culture medium, leading to the conclusion that ChiB primarily is secreted to the surroundings of the cell (27, 29). It is conceivable, that differences in culture conditions and / or artefacts produced by cell fractionation methods (33) account for these different observations. ChiC is considered to be an extracellular enzyme (26, 27), but the localization of this enzyme has been studied in less detail than the localization of ChiB. Clearly, studies that are more rigorous are needed to determine the natural localization of ChiB and ChiC, and to identify (differences in) the mechanism by which these two enzymes are exported.

In summary, it seems that the chitinolytic machinery of *S. marcescens* involves at least two different export pathways that ensure translocation of the chitinolytic enzymes to the periplasm and the surrounding medium. Most known chitinases from other bacteria are secreted enzymes that carry standard N-terminal signal peptides (e.g. 38, 39, 50 - 54).

Structures of chia and chib

Publication of the crystal structures of the one-domain family 18 chitinase hevamine (55) and of the multi-domain *S. marcescens* ChiA (36) were landmarks in research on family 18 chitinases. These structures showed that the catalytic domains of family 18 chitinases have a TIM-barrel fold and they revealed aspects of the catalytic centers. The structure of ChiA is unique because of its completeness. There are very few examples of complete structures of multi-domain enzymes that act on insoluble substrates such as chitin and cellulose. This is presumably caused by the fact that these enzymes have a tendency to undergo proteolytic cleavage and by a great degree of flexibility between the individual domains, both of which may prevent crystallization. Since 1994, three more structures of family 18 chitinases have been published: the catalytic domain of chitinase A1 from *B. circulans* (56), the complete structure of a one-domain chitinase from the fungus *Coccidioides immitis* (57), and the complete structure of the two-domain ChiB from *S. marcescens* (15).

The structures of ChiA and ChiB are displayed in Figure 3 and provide a fascinating insight in how a natural chitinolytic machinery may be built up. Both enzymes contain a deep substrate-binding groove, which is extended by an aromatic surface on the putative chitin-binding domains (see also Fig. 1). Interestingly, these domains are located on opposite sides of the catalytic center and, thus, ChiA and ChiB are likely to have different orientations when bound to polymeric chitin chains. This and other aspects of the two structures are discussed in the next section.

Figure 1 displays three types of domains that presumably are involved in interactions with the substrate, ChiN, FnIII and ChBD. There is ample experimental evidence that the domains marked ChBD are true chitin-binding domains (58, 59). The information for the structurally similar (but not detectably homologous) domains marked FnIII and ChiN is less clear. These domains are highly likely to interact with the substrate but they may not be involved in the initial binding phase. It has been proposed that these domains interact somewhat loosely with the substrate and that they help directing

Table 2. Degradation of natural and synthetic substrates by ChiA and ChiB (pH 6.1, 37 °C).

	End products[1]		Other products in partial digests[2]	
Substrate	ChiA	ChiB	ChiA	ChiB
$(GlcNAc)_2$	2	2	-	-
$(GlcNAc)_3$	2 + 1	2 + 1	-	-
$(GlcNAc)_4$	2	2	-	-
$(GlcNAc)_5$	2 + 1	2 + 1	3	3
$(GlcNAc)_6$	2	2 + 1	4	3 + 4
chitin	2 + 1	2 + 1	-[3]	-[3]
4MU-GlcNAc	4MU-1	4MU-1	-	-
4MU-$(GlcNAc)_2$	4MU + 2	4MU + 2	-	-
4MU-$(GlcNAc)_3$	4MU + 1 + 2[4]	4MU-1 + 2[5]	3[6]	-

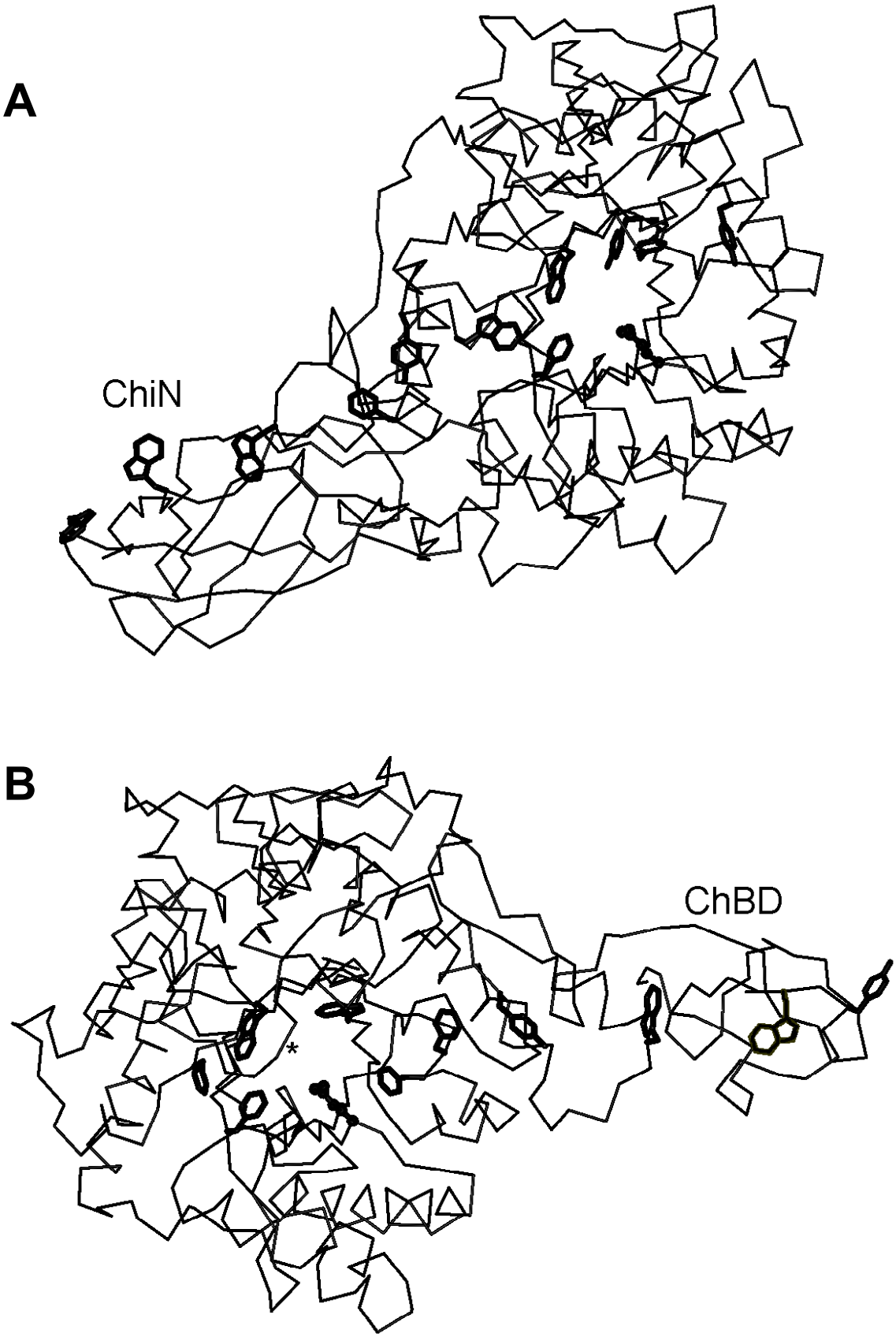
[1] Products are indicated by the number of GlcNAc moieties. Products were analyzed by TLC [chitin and $(GlcNAc)_n$ substrates] or by measuring 4-MU fluorescence [4MU-$(GlcNAc)_n$ substrates) (13).
[2] Additional products detected by TLC in partial digests of chitin and $(GlcNAc)_n$.
[3] Intermediate products were not detectable by TLC when applying samples that gave reasonable spots at $(GlcNAc)_2$ and GlcNAc. Apparently intermediate products do hardly accumulate.
[4] > 98% of the substrate was converted as indicated; it is probable that trace amounts of 4MU-GlcNAc were also present. Under standard assay conditions 4MU was produced at a speed similar to that in the reaction with 4MU-$(GlcNAc)_2$.
[5] Detected by measuring the disappearance of the substrate (by adding excess purified ChiA).
[6] No attempts were made to detect this intermediate. Since ChiA does not cleave 4MU-GlcNAc and $(GlcNAc)_2$, it *must* have been present.

the substrate to and through the active site cleft (58, 60, 61). The structure of ChiA (Fig. 3) strongly suggests that the ChiN domain functions by elongating the substrate-binding cleft (see also www.rcsb.org/pdb/, entry 1EHN). More experimental work is needed to unravel the precise roles of these domains.

Enzymatic activities of chia, chib and chic

In many studies, enzymatic analyses with artificial chito-oligosaccharides of varying lengths [e.g. 4-methylumbelliferyl-$(GlcNAc)_n$] have been used to draw conclusions about the character of newly purified chitinases. For example, it is often assumed that enzymes cleaving the 4-methylumbelliferyl group from 4-methylumbelliferyl-$(GlcNAc)_3$ have an endo-character whereas enzymes cleaving the same group from 4-methylumbelliferyl-$(GlcNAc)_2$ have an exo-character. It is now clear that studies with artificial substrates should not be used for determination of substrate preferences (discussed and illustrated in 13). For example, ChiA converts $(GlcNAc)_4$ exclusively to $(GlcNAc)_2$, whereas it converts the $(GlcNAc)_4$ analogue 4-methylumbelliferyl-$(GlcNAc)_3$ almost exclusively to 4-methylumbelliferyl, $(GlcNAc)_2$ and GlcNAc (Table 2). Unfortunately, analysis of substrate and product specificities with natural substrates is not straightforward. The main problem is that catalytic activity is strongly dependent on the type of substrate. Thus, shorter intermediate products may never accumulate in significant amounts because they are much more rapidly degraded than longer substrates.

Careful analysis of catalytic activity towards a number of substrates combined with structural studies (see above) may provide reasonably


A
ChiN
B
ChBD
*

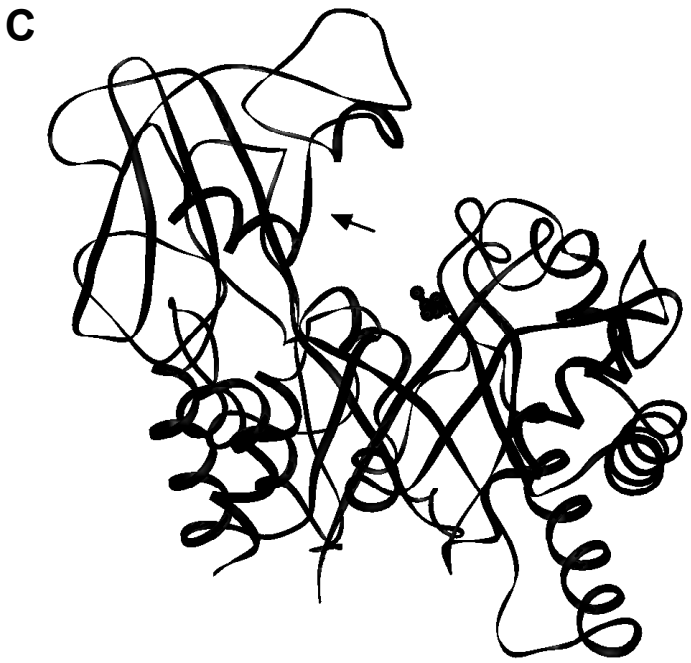


Figure 3. Crystal structures of ChiA and ChiB. Panels A, B: C-alpha trace of ChiA (A) and ChiB (B). The view is through the core of the catalytic TIM-barrel from the side of the catalytic acid (Glu315 and Glu144 in ChiA and ChiB, respectively; shown as ball-stick models). Side chains of aromatic residues presumably involved in substrate binding are drawn in bold sticks. Domains putatively involved in chitin-binding are indicated by their abbreviations (see text and Fig. 1). The asterisk in panel B indicates a loop (residues 315-327; Fig. 2) that covers the substrate-binding groove of ChiB and thus gives this enzyme more of a "tunnel"-character. Panel C: ChiB viewed along the substrate-binding groove. The arrow indicates the $\alpha + \beta$ domain making up one of the walls of the groove. The catalytic glutamate (Glu144) is shown as ball-stick model.

reliable clues as to the substrate and product specificities of chitinases. Results from an enzymatic analysis of ChiA and ChiB are presented in Table 2 (13). The data in Table 2 as well as analysis and comparisons of the structures of ChiA and ChiB (Fig. 3) indicate that both chitinases have a strong exo-character leading to the production of $(GlcNAc)_2$ (ChiA) or $(GlcNAc)_2$ plus $(GlcNAc)_3$ [ChiB; $(GlcNAc)_3$ is subsequently converted into $(GlcNAc)_2$ and GlcNAc].

ChiB seems to be a true exochitinase since it's active site cleft is blocked in front of the –3 subsite (15; Fig. 3). The substrate-binding cleft of ChiB is relatively closed, giving this cleft much of the "tunnel"-character that is characteristic for many exo-acting glycosyl hydrolases (62). ChiB has clearly defined subsites ranging from –3 to +2. The +-side (corresponding to the reducing end of the sugar chain) of the substrate-binding groove is extended by the aromatic surface on the chitin-binding domain (Fig. 3). The distance from the catalytic residue to the most distant aromatic residue on the chitin-binding domain is 55 Å, which corresponds to the length of a chitin chain with 10 sugar units. Taken together, the available information shows that ChiB degrades chitin chains from their nonreducing end by a combined chitobiosidase and chitotriosidase activity.

In general terms, the substrate-binding groove in ChiA looks very much like that in ChiB: both enzymes have a deep cleft with "high

walls" (Fig. 3C). Nevertheless, there are several important differences between the two enzymes. ChiA has less of a tunnel character than ChiB because a loop making up the "roof" of the tunnel in ChiB is lacking (Fig. 2; sequence number 315-327; see also Fig. 3B). ChiA has six well-defined subsites (-4 - +2) in the groove and the groove is open at both sides. So, in contrast to ChiB, chitin chains can extend from the active site in both directions. Substrate binding is enhanced only on the non-reducing side of the active center by a putative chitin-binding domain that has an aromatic surface (Fig. 3A). The distance from the most remote aromatic residue to the catalytic center is in the order of 65 Å, corresponding to a chitin chain with 12 sugar units. The more open character of the groove in ChiA suggests that the enzyme could have some endo-activity and this is confirmed by the observation that ChiA can produce GlcNAc monomers out of chitin. Thus, ChiA seems to be a chitobiosidase with some endochitinase activity (13). Note that the exo-activity of ChiA results in degradation of the chitin chain from the reducing end (as opposed to non-reducing end for ChiB).

Low sequence identity as well as the presence of many insertions and deletions makes it very difficult to align the ChiC sequence to the sequences displayed in Figure 2 in a meaningful way. The sequence of ChiC (14) clearly shows, however, that the catalytic domain of this chitinase is smaller than that of ChiA and ChiB. The most prominent difference between ChiC and the other two *Serratia* chitinases is the lack of the so-called $\alpha+\beta$ domain in the former. This domain (residues 295 – 373 in Fig. 2) makes up one of the walls of the substrate-binding groove in ChiA and ChiB (Figure 3C). This suggests that the substrate-binding groove is much more open in ChiC, which is characteristic for endochitinases such as hevamine (Fig. 2; 55, 63).

Synergistic effects on the rate of degradation of colloidal chitin when combining ChiA and ChiB, have been observed (13). Adding ChiC to the mixture of enzymes also gives synergistic effects (14). These observations confirm the idea that the three chitinases have different roles in the degradation of chitin and they may be taken to confirm the suggested endo-character of ChiC.

Catalytic mechanism of ChiA, B, and C

Hydrolysis of glycosidic bonds normally follows one of two "classical" mechanisms: a double displacement mechanism leading to retention of the anomeric configuration, or a single displacement mechanism that leads to inversion of the anomeric configuration (62, 64). Catalysis by family 19 chitinases proceeds via a single displacement mechanism, thus leading to inversion of the anomeric configuration (65, 66).

Catalysis by family 18 chitinases such as ChiA, ChiB and ChiC, leads to retention of anomeric configuration (67), but the active sites of these enzymes lack the second acidic residue that is needed to stabilize the oxocarbonium intermediate that is formed in the classical double displacement mechanism. Work on the family 18 chitinases ChiA and hevamine, as well as studies of the family 20 chitobiase from *S. marcescens* have revealed a new mechanism in which initial protonation of the scissile glycosidic bond by the catalytic acid is followed by nucleophilic attack of the N-acetyl group of the sugar in the –1 subsite (19, 68 – 70). This substrate-assisted catalytic mechanism is displayed in Figure 4 and explains why family 18 chitinases have an absolute preference for substrates with an N-acetylglucosamine moiety in the –1 position (71).

The glutamate in the DXXDXDXE signature-

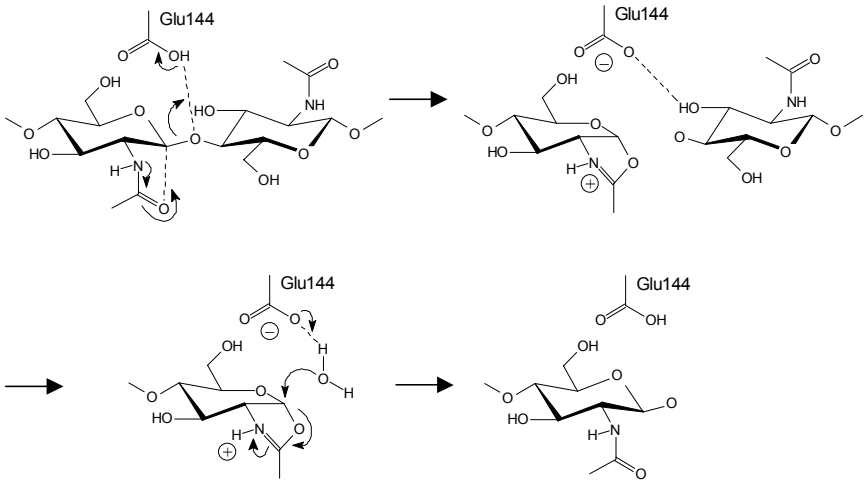


Figure 4. Catalytic mechanism of family 18 chitinases as proposed in (69). Glu144 is the catalytic glutamate residue as found in ChiB (the corresponding residue in ChiA is Glu315).

motif in family 18 chitinases acts as the catalytic acid (36, 55). Site-directed mutagenesis studies have not only confirmed the importance of this glutamate, but also shown that all aspartates in the DXXDXDXE motif contribute to catalysis (58, 72 - 74). The roles of these aspartates during catalysis are largely unknown and are a subject of current studies (74).

Genetic organisation and regulation

The genes of encoding chitinases A, B and C, have been cloned and sequenced from four different strains of *S. marcescens* (14, 27, 29, 31 – 34). Despite this molecular effort, little has been achieved in understanding the genetic regulation of the chitinase production in *S. marcescens*.

The organization of chitinase genes on the *S. marcescens* chromosome is not precisely known. Hybridisation studies have shown that the genes encoding ChiA and ChiB are not closely linked (M.B. Brurberg, unpublished observations). The genes encoding ChiB and CPB21 are linked (34), but the DNA sequence suggests that transcription of the two genes is not coupled (to our knowledge there are no transcriptional analyses in the literature). It is interesting to note that a recent study of eight chitinase genes in *Streptococcus coelicolor* showed that these genes are scattered on the chromosome (39).

Production of chitinolytic enzymes in bacteria is normally induced by the presence of chitin in the culture medium (10). Since chitin is insoluble, the microorganisms are unable to utilize it unless it has been hydrolysed to soluble oligomers of *N*-acetyl-glucosamine. Several proposals have been made to explain how induction may occur in this situation. The most probable inducers of *S. marcescens* chitinolytic enzymes are soluble oligomers derived from chitin. Such soluble oligomers could be produced by the action of trace amounts of chitinolytic enzymes that are present because of constitutive low level expression of the chitinase genes. Alternatively, the signal for induction of synthesis of

chitinases could result from physical contact between the cell and the insoluble substrate. However, it has been shown that production of chitinolytic enzymes could indeed be induced by adding only $(GlcNAc)_2$ or $(GlcNAc)_{3-4}$ to the growth medium of *S. marcescens* (10, 27, 28). Whether all the chitinases in *S. marcescens* are induced with oligomers remains to be tested. In addition to being induced by chitin, the chitinases of *S. marcescens* are also induced by the SOS-response inducers mitomycin C and nalidixic acid (75, 76).

The production of chitinase by *S. marcescens* growing on chitin is inhibited by addition of glucose and GlcNAc (10, 27). A glucose effect has also been demonstrated for other bacteria (39, 77), indicating that catabolic repression is generally involved in regulating the chitinolytic system of bacteria.

Knowledge about the molecular mechanisms governing the regulation of chitinases in *S. marcescens* is limited. Studies in other microorganisms have provided some links as to how regulation may be achieved (reviewed in 7). In *Streptomyces plicatus* a 12-basepair direct repeat sequence overlapping with the putative RNA polymerase binding sites of two chitinase genes was shown to be involved in the induction by chitin and the repression of glucose (77, 78). The same direct repeat was found in the promoter regions of eight chitinase genes scattered on the *Streptomyces coelicolor* chromosome (39).

Quorum sensing control mediated by N-acyl lactone (AHL) signalling molecules has been established as a key feature of the regulation of exozyme production in many Gram-negative bacteria. In *Serratia liquefaciens*, a less studied chitinase producing (79, 80) relative of *S. marcescens*, control of protease production, biosurfactant production and swarming motility is governed by a quorum sensing mechanism (81 - 83). Another study, using *Serratia* sp. ATCC3906, showed that biosynthesis of carbapenem antibiotic and prodigiosin pigment is under quorum sensing control (84), which manifest the presence of a quorum sensing mechanism in the genus *Serratia*. Whether quorum sensing also controls chitinase production, in a manner similar to what has been observed in *Chromobacterium violaceum* (85) remains to be seen.

Biocontrol

One of the first biotechnological applications of chitinases concerned their use in biocontrol of plant pathogens. *S. marcescens* cultures, its chitinases, and its chitinase genes have shown potential as biocontrol agents in a variety of experimental set-ups. A highly chitinolytic strain of *S. marcescens* was found to suppress the growth of *Botrytis* spp. *in vitro* (86). In a greenhouse setting, *S. marcescens* has been shown to control *B. cinerea*, *Rhizoctonia solani*, and *Fusarium oxysporum* f. sp. *cyclaminis*, all pathogens of cyclamen (86). Similarly, *S. marcescens* controlled growth of *Sclerotinia minor*, the casual agent of basal drop disease, in lettuce grown in green house (87).

The *chiA* and *chiB* genes from *S. marcescens* have been transformed into other bacterial species like *Pseudomonas fluorescens* and *E. coli* in an attempt to improve their ability to control fungal plant pathogens (30, 88, 89) or to create new biocontrol agents (21, 90). Furthermore, chitinase ChiA partially purified after cloning into *E. coli*, was found to reduce disease caused by *Sclerotium rolfsii* in beans and *Rhizoctonia solani* in cotton (90). When ChiA was combined with *Bacillus thuringiensis* or with low concentrations of the *B. thuringiensis* delta-endotoxin, a synergistic toxic effect was seen on insect larvae (91, 92). A synergistic effect was also found when transgenic *P. fluorescens* expressing delta-

P. fluorescens carrying the *chiA* gene from *S. marcescens* (93).

Several transgenic plants overexpressing plant, fungal or bacterial chitinases have been described (e.g. 94 - 97). Transgenic tobacco plants expressing high levels of *S. marcescens* ChiA exhibited increased tolerance to *R. solani* as compared to untransformed control plants (98).

Perspectives

The past decade has yielded a large increase in knowledge of the enzymes and mechanisms involved in degradation of insoluble abundant polymers such as cellulose and chitin (e.g. 1, 40). This new knowledge brings us closer to a comprehensive understanding of how nature accomplishes the turnover of enormous amounts of these biopolymers. Many aspects of natural chitinolysis remain to be elucidated. For example: 1. What is the role of the CBPs? 2. Do individual chitinases interact and how? Can there be such a thing as a chitinosome (40); 3. How do chitinases interact with their substrate and how is this polymer displaced between individual catalytic cycles? 4. How is chitinase production regulated? In the case of *S. marcescens,* an additional interesting aspect concerns the localization and export mechanism of ChiB and ChiC.

As mentioned above, detailed knowledge of chitinases may have profound effects on several fields in biotechnology. The enzymes may be used directly as anti-fungal agents or they may be used as industrial biocatalysts. Chitinases and other enzymes related to chitin synthesis and modification may form an ”enzymatic toolbox” for converting an abundant raw material into bioactive oligosaccharides with a variety of appealing potential functions (e.g. 99 - 105). Finally, the increased knowledge of chitinases may be used to design inhibitors that may help to inhibit chitinase-dependent pathogens, including, for example *Plasmodium falciparum* (106, 107) and *Leishmania* (108).

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