Chapter 7
Bacterial Chitinase System as a Model of Chitin Biodegradation

Takafumi Itoh and Hisashi Kimoto

Abstract Chitin, a structural polysaccharide of β-1,4-linked *N*-acetyl-D-glucosamine residues, is the second most abundant natural biopolymer after cellulose. The metabolism of chitin affects the global carbon and nitrogen cycles, which are maintained by marine and soil-dwelling bacteria. The degradation products of chitin metabolism serve as important nutrient sources for the chitinolytic bacteria. Chitinolytic bacteria have elaborate enzymatic systems for the degradation of the recalcitrant chitin biopolymer. This chapter introduces chitin degradation and utilization systems of the chitinolytic bacteria. These bacteria secrete many chitin-degrading enzymes, including processive chitinases, endo-acting non-processive chitinases, lytic polysaccharide monooxygenases, and *N*-acetyl-hexosaminidases. Bacterial chitinases play a fundamental role in the degradation of chitin. Enzymatic properties, catalytic mechanisms, and three-dimensional structures of chitinases have been extensively studied by many scientists. These enzymes can be exploited to produce a range of chitin-derived products, e.g., biocontrol agents against many plant pathogenic fungi and insects. We introduce bacterial chitinases in terms of their reaction modes and structural features.

Keywords Bacterial chitinase · Bacterial chitinolysis · Endo-acting non-processive chitinase · Processive chitinase

7.1 Introduction

Chitin, a linear homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues, is widespread in nature and serves as a structural component in the cell walls of fungi, and the exoskeletons of insects, arthropods, sponges, and squid pens

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Q. Yang and T. Fukamizo (eds.), *Targeting Chitin-containing Organisms*, Advances in Experimental Medicine and Biology 1142,
https://doi.org/10.1007/978-981-13-7318-3_7

(Gooday 1990a, b). Chitin is classified by its crystalline forms, such as α-, β-, and γ-chitin, differing in the orientation of the microfibrils. To form complex structures in the cell walls, the crystalline chitin often associates with some proteins, or with other polysaccharides, such as glucans and mannans (Attwood and Zola 1967; Austin et al. 1981 Schaefer et al. 1987; Merzendorfer and Zimoch 2003). In accordance with the abundance and ubiquity of chitin, chitin-degrading enzymes (or chitin-related proteins) have ubiquitous distribution across bacteria, fungi (Gooday 1990a), archaea (Andronopoulou and Vorgias 2004), algae (Shirota et al. 2008; Kitaoku et al. 2017), plants (Grover 2012), and animals (Gooday 1990a). Although annual chitin production in natural ecosystems is estimated to be 10^{10}–10^{12} tons, most of the chitin is recycled by bacteria and fungi as their carbon and nitrogen sources (Tharanathan and Kittur 2003).

Bacterial responses to chitin include chemotaxis and chemotropism, when the bacteria either migrate toward the chitin source or grow toward it; adhesion to chitin; secretion of extracellular chitin-degrading enzymes; and uptake of chitin oligosaccharides (Keyhani and Roseman 1999; Li and Roseman 2004; Meibom et al. 2005). Bacterial chitinolysis, the main step in chitin utilization, typically consists of three enzymatic steps, namely breaking down of crystalline chitin, hydrolyzing chitin chains into dimers, and dividing the dimers into monomers. In the extracellular environments, the first two steps are usually catalyzed by three types of enzymes, lytic polysaccharide monooxygenases (LPMO), endo-acting non-processive chitinases, and processive chitinases. LPMOs, classified as auxiliary activity (AA) families 10 and 11 in the CAZy database (Lombard et al. 2014), cleave glycosidic bonds on the surface of crystalline chitin by an oxidative reaction and introduce oxidized ends to promote further degradation by other secreted chitinases. Chitinases are typically grouped into glycoside hydrolase families 18 and 19 (GH18 and GH19). GH19 chitinases are found mostly in plants, nematodes, and some members of Streptomycetaceae (Ohno et al. 1996; Shimosaka et al. 2001; Lacombe-Harvey et al. 2018). The amino acid sequences of the two GH families share little or no similarities and their catalytic mechanisms are entirely different. It has been hypothesized that GH18 and GH19 chitinases have evolved separately (Fukamizo 2000). These chitinases are often regulated and induced by *N*-acetylglucosamine (GlcNAc monomer), a product of chitin hydrolysis (Bassler et al. 1991), or chitin oligomers $(GlcNAc)_n$, where n = 2 to 6 (Uchiyama et al. 2003). GlcNAc has also been reported to act as a suppressor of chitinase expression in some strains of *Streptomyces* (Miyashita et al. 2000) and *Paenibacillus* (Itoh et al. 2013). After the extracellular hydrolysis of chitin polysaccharides into monomers and oligomers, the products are imported into the periplasm or cytoplasm of the bacteria by efficient uptake systems. In some bacterial strains, phosphoenolpyruvate-dependent phosphotransferase systems (PTS) and ATP-binding cassette (ABC) transporters are responsible for the respective uptakes of GlcNAc monomers and oligomers (Berg et al. 2007; Colson *et al*. 2008; Świątek et al. 2012a). In the cytoplasm or the periplasmic space, β-*N*-acetyl-hexosaminidases, usually classified as GH3 or GH20, cleave the imported chitin oligomers into GlcNAc (Ito et al. 2013; Macdonald et al. 2015).

Chitinolytic bacteria, such as *Serratia marcescens* (Vaaje-Kolstad et al. 2013), *Bacillus circulans* WL-12 (Watanabe et al. 1990), *Streptomyces coelicolor* A3(2) (Saito et al. 2007), *Vibrio cholerae* (Li and Roseman 2004), and *Paenibacillus sp.* (Itoh et al. 2013; Kusaoke et al. 2017), produce many chitinases for efficient degradation of chitin. Nucleotide sequences of these chitinases are often almost completely conserved. This genetic conservation could be explained by the multiple gene duplication in a single cell. However, there are some bacteria that possess gene clusters of chitinases with unique sequences, thought to be acquired from other organisms via lateral gene transfer (Hunt et al. 2008).

7.2 Chitin Biodegradation System of *Serratia marcescens*

Serratia marcescens is one of the most efficient bacteria for the degradation of chitin (Monreal and Reese 1969) and has been extensively studied as the model for bacterial chitinolysis. *Serratia* is a genus of rod-shaped Gram-negative bacteria in the family Enterobacteriaceae. Production of chitin-degrading enzymes in *S. marcescens* can be induced by the presence of chitin in the culture medium (Monreal and Reese 1969). *S. marcescens* strain QMB1466 produces five different chitinolytic enzymes (Fuchs et al. 1986). These enzymes are named as ChiA, ChiB, ChiC1, ChiC2, and CBP21; ChiA and ChiB, two processive chitinases, produce disaccharides from chitin chain by sliding along the chain in opposite directions, i.e., ChiA from the reducing end and ChiB from the nonreducing end (Horn et al. 2006); ChiC1 and ChiC2, endo-acting non-processive chitinases, hydrolyze the chitin polymers randomly; and CBP21 is an LPMO that introduces chain breaks by oxidative cleavages (Vaaje-Kolstad et al. 2013) (Fig. 7.1). ChiC2 results from a posttranslational cleavage of ChiC1; the hydrolytic activity of ChiC2 is lower than that of ChiC1 on crystalline chitin (Suzuki et al. 1999). The biological function of

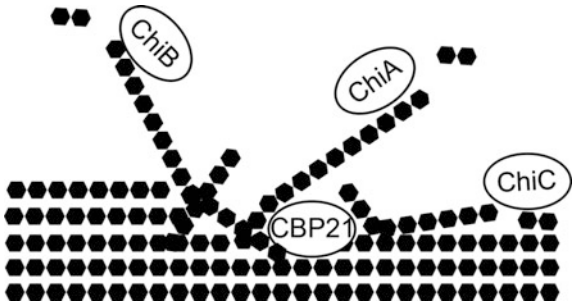

Fig. 7.1 Chitinolytic enzymes of *Serratia marcescens*. The catalytic domains of *S. marcescens* chitinases (ChiA, ChiB, and ChiC) belong to GH18. ChiA and ChiB are processive chitinases that bind closely to the ends of the detached chitin chain and release $(GlcNAc)_2$. ChiC works as an endo-acting non-processive chitinase. CBP21 is an AA10 LPMO and breaks chitin chain by oxidative cleavages

the posttranslational cleavage of ChiC1 remains unclear. These chitin-degrading enzymes work synergistically on chitin degradation (Suzuki et al. 2002). In the cytoplasm or the periplasmic space, *N*-acetyl-hexosaminidase (chitobiase) hydrolyzes oligosaccharides to GlcNAc. According to the CAZy database, in *S. marcescens*, all chitinases (ChiA, B, C1, and C2) belong to GH18, chitobiases belong to GH20, and CBP21 belongs to AA10.

7.3 Chitin Biodegradation System of *Bacillus circulans* WL-12

Bacillus circulans WL-12, a Gram-positive and rod-shaped cell, has been identified as a chitinolytic bacterium that degrades chitin in the cell walls of yeasts and fungi and is known to secrete multiple polysaccharide-degrading enzymes into the culture media (Rombouts and Phaff 1976; Watanabe et al. 1990). When grown in the presence of chitin, over 10 chitinases have been detected in the supernatant of the bacterial culture (Watanabe et al. 1990; Alam et al. 1996). These chitinases are derived from three genes, *chiA*, *chiC*, and *chiD*. Their gene products, ChiA, ChiC, and ChiD, are multi-modular chitinases and their posttranslational cleavages produce multiple chitinases in the culture supernatant. ChiA1, one of the cleavage products, the highest colloidal chitinase activity among others; it yields predominantly a dimer $(GlcNAc)_2$ by binding to insoluble chitin. The structure of ChiA1 contains an N-terminal GH18 catalytic domain, two fibronectin type III (FnIII) domains, and a C-terminal carbohydrate-binding module belonging to CBM12. The catalytic domain of ChiA1 has a deep substrate-binding cleft. On the cleft surface, aromatic residues are linearly located and are important for crystalline chitin hydrolysis (Watanabe et al. 2003).

7.4 Chitin Biodegradation System of *Streptomyces species*

Bacteria in the genus *Streptomyces* belong to a group of high GC content, Gram-positive bacteria (Actinomycetes) that have an extensive chitinolytic system supported by multiple chitinases (Berger and Reynolds 1958; Saito et al. 1999; Schrempf 2001; Kawase et al. 2006; Saito et al. 2007). In the *S. coelicolor* A3(2) genome, 13 chitinases have been found to date; 11 of these have been classified in the GH18 family of chitinases: subfamily A (Chi18aC, Chi18aD, Chi18aE, and Chi18aJ), subfamily B (Chi18bA, Chi18bB, and Chi18bI), and subfamily C (Chi18cH, Chi18cK, Chi18cL, and Chi18 cM), and two in the GH19 family of chitinases (Chi19F and Chi19G) (Kawase et al. 2006). Besides chitinases, *S. coelicolor* A3(2) secretes chitosan-related enzymes, such as GH46 chitobiase (Ghinet et al. 2010), GH20 chitobiase (Saito et al. 2013), and CE4 chitin deacetylase (Świątek et al. 2012b). The metabolism of chitin oligosaccharides in

S. coelicolor A3(2) is well understood (Świątek et al. 2012a, b) (Fig. 7.2). The degradation monomer product, GlcNAc, is imported and phosphorylated to *N*-acetyl-D-glucosamine-6-phosphate (GlcNAc-6P) by the PTS through the cell membrane. In the PTS reactions, a phosphoryl group is transferred from phosphoenolpyruvate to phosphotransferase enzyme I (EI) and then the group is transferred from EI to histidine protein (HPr). The phosphoryl group of HPr is further transferred to the enzyme complex of enzyme IIA (EIIA), enzyme IIB (EIIB), and enzyme IIC (EIIC). GlcNAc, transported by enzyme IIC (EIIC), is phosphorylated to GlcNAc-6P by EIIA. Some ABC transporters, like NgcEFG-MsiK and DasABC-MsiK, can also transport $(GlcNAc)_2$ dimers. Transported $(GlcNAc)_2$ dimer is divided into GlcNAc monomers by β-*N*-acetyl-D-glucosaminidase (DasD) in the cytoplasm. An *N*-acetyl-D-glucosamine kinase (NagK) phosphorylates GlcNAc to GlcNAc-6P, which is then deacetylated to glucosamine-6-phosphate (GlcN-6P) by *N*-acetyl-D-glucosamine-6-phosphate deacetylase (NagA). Thereafter, glucosamine-6-phosphate deaminase (NagB) deaminates GlcN-6P to fructose-6-phosphate (Frc-6P) that would enter the glycolytic pathway.

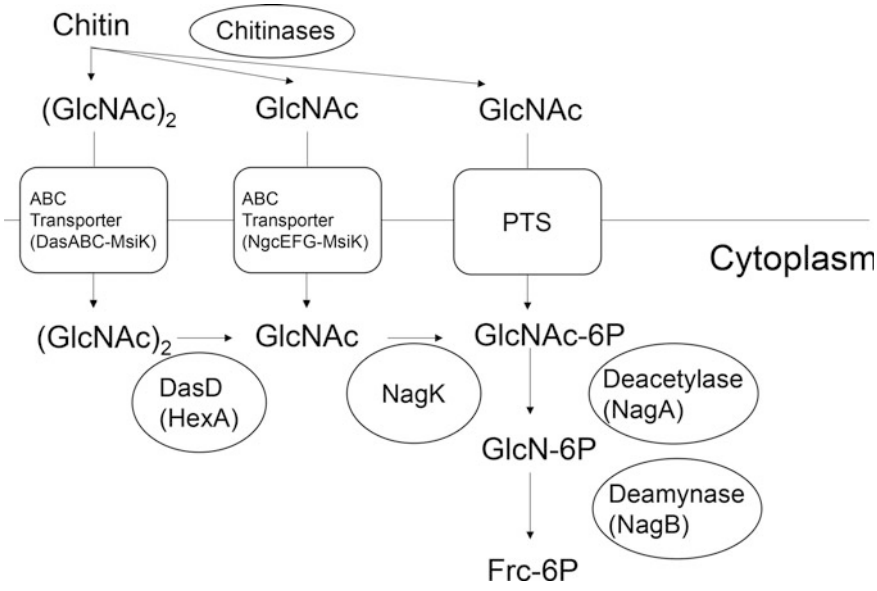

Fig. 7.2 Chitin uptake by *Streptomyces* species. Chitin is hydrolyzed into GlcNAc and $(GlcNAc)_2$ by GH18 and GH19 chitinases in the extracellular space. The degradation product, GlcNAc, is transported through PTS across the cell membrane and is phosphorylated to GlcNAc-6P by the PTS. An ABC transporter, NgcEFG-MsiK can import GlcNAc and $(GlcNAc)_2$. Another ABC transporter, DasABC-MsiK, can also import $(GlcNAc)_2$. Imported $(GlcNAc)_2$ is hydrolyzed into GlcNAc by chitobiase (DasD). GlcNAc is phosphorylated to GlcNAc-6P by GlcNAc kinase (NagK). GlcNAc-6P deacetylase (NagA) converts GlcNAc-6P to GlcN-6P, which is then deaminated to Frc-6P by GlcN-6P deaminase (NagB). Frc-6P would enter the glycolytic pathway

7.5 Chitin Biodegradation System of Marine Bacteria *Vibrio species*

Despite being a ubiquitous biopolymer, chitin is not known to accumulate in ocean sediments; marine chitinolytic bacteria, mainly from the family Vibrionaceae, contribute to the rapid recycling of chitin (Hirono et al. 1998). Chitinolytic and signal transduction systems of *Vibrio* species have been widely studied. The system constitutes five processes: finding chitin (chemotaxis); adhering to chitin via the bacterial cell surface; degrading chitin to oligosaccharides; transporting the degraded oligosaccharides to the cytoplasm by a sugar-specific porin (chitoporin) on the outer membrane and by a sugar-specific ABC transporter on the inner membrane; and converting the degradation products to Frc-6P in the cytosol for the glycolytic pathway (Keyhani and Roseman 1999; Li and Roseman 2004; Meibom et al. 2004; Hunt et al. 2008). Inducing chitinolytic enzymes of *Vibrio* species is intricately regulated by histidine kinases and two-component systems. In the resting state, a binding protein for chitin oligosaccharides (CBP) binds to the periplasmic domain of the membrane protein ChiS. When secreted chitinases degrade chitin to oligosaccharides in the extracellular space, the oligosaccharides are transported by the chitoporin and are bound by the CBP. In the binding state, the CBP/ChiS complex dissociates and transports the signal to express the chitinolytic genes (Fig. 7.3). The domain of ChiS in the cytoplasmic space is composed of three subdomains: the ATP-dependent His kinase/phosphatase (HK) domain; the Asp response regulator (RR) domain; and the histidine phosphotransfer (HP) domain.

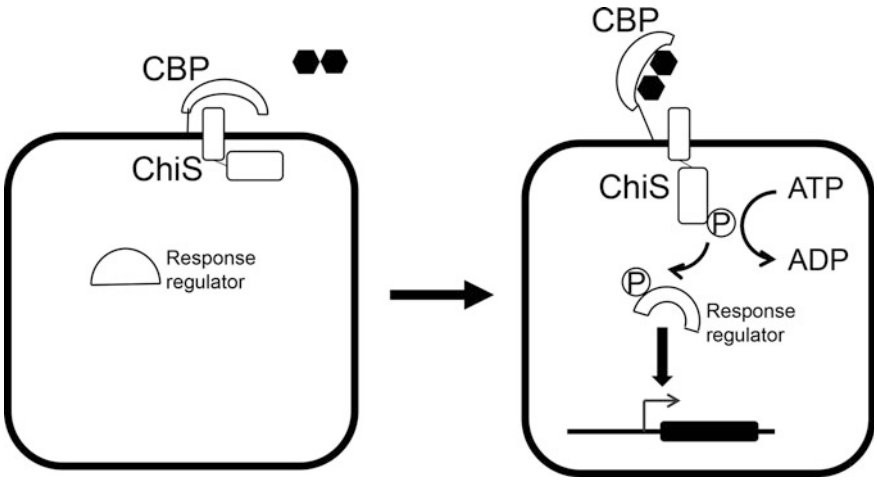

Fig. 7.3 Induction of chitinolytic enzymes of *Vibrio* species. The induction is regulated by a histidine kinase and a two-component system (ChiS). In the resting state, chitin-binding protein (CBP) binds to the periplasmic domain of the membrane protein ChiS. When the oligosaccharides bind to CBP, the CBP/ChiS complex dissociates and transports the signal for the expression of chitin-degrading enzymes

The phosphoryl group is transferred sequentially from ATP to HK, to RR, to HP, and finally to Asp on a response regulator that interacts with the genome to induce the expression of chitinolytic enzymes (Li and Roseman 2004).

7.6 Chitin Biodegradation Systems of *Paenibacillus* sp. str. IK-5 and FPU-7

Similar to the *Streptomyces* species, *Paenibacillus* sp. str. IK-5 produces a chitosanase (ChiE) and a GH19 chitinase (ChiD) in addition to two GH18 chitinases (ChiA and ChiB) and an AA10 LPMO (ChiC) (Kusaoke et al. 2017; Fig. 7.4). The two chitinases (ChiA and ChiB) contain one GH18 catalytic domain and two CBM5 chitin-binding modules. The AA10 LPMO (ChiC) also has a CBM5 module in addition to the catalytic domain. The chitinase ChiD has a GH19 catalytic domain. Chitosanase (ChiE) contains a GH8 catalytic domain at the N-terminus and two discoidin domains (CBM32) at the C-terminus. The CBM32 domains of ChiE can bind specifically and tightly to chitosan. These five enzymes (ChiA, ChiB, ChiC, ChiD, and ChiE) form a huge protein complex called a "chitinasome". When the culture medium contains only chitin, the complex consists of four enzymes (ChiA, ChiB, ChiC, and ChiD); when the culture medium contains both chitin and chitosan, the complex comprises all five enzymes. *Paenibacillus* sp. str. IK-5 cells also produce multi-modular chitobiase composed of the GH20 catalytic domain and S-layer homology domains (SLH) on their surface.

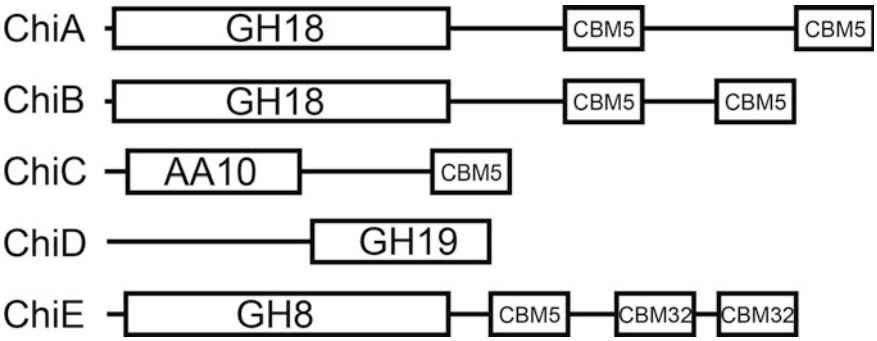

Fig. 7.4 Schematic representations of the *P.* str. IK-5 chitin and chitosan degrading enzymes. The enzymes (ChiA, ChiB, ChiC, ChiD, and ChiE) are produced by *P.* str. IK-5 and have signal peptides for secretion at the N-terminus. ChiA and ChiB are GH18 chitinases with catalytic domains at the N-terminus and two CBM 5 domains at the C-terminus. ChiC has an AA10 catalytic domain as LPMO and a CBM5 at the C-terminus. The catalytic domain of ChiD belongs to GH19. Chitosanase ChiE contains a GH8 catalytic domain at the N-terminus and two CBM32 at the C-terminus. These chitin and chitosan-related enzymes (ChiA, ChiB, ChiC, ChiD, and ChiE) are found as a huge protein complex (chitinasome). When the culture medium contains chitin and chitosan, ChiE is assembled into four enzyme complexes (ChiA, ChiB, ChiC, and ChiD)

Paenibacillus sp. str. FPU-7 (*P.* str. FPU-7), a bacterium with high chitinolytic activity, was recently isolated from soil using a medium containing solid chitin flakes (Itoh et al. 2013). Subject to carbon catabolite repression by GlcNAc, the bacterium constitutively secretes several chitinases into the culture medium. Genes for at least six chitinases (ChiA, ChiB, ChiC, ChiD, ChiE, and ChiF) are found in the genome, each containing a GH18 catalytic domain and one or more auxiliary domains, such as CBM5, CBM12, and FnIII. On a synthetic substrate, pNP-$(GlcNAc)_2$, ChiE shows high activity; ChiA, ChiD, and ChiF are less active; ChiB and ChiC are moderately active. All the chitinases, except ChiD, are highly active on insoluble colloidal chitin. Efficient degradation of chitin flakes by *P.* str. FPU-7 requires not only the chitinase secretions in the culture medium, but also the living cells, suggesting an involvement of cell-bound chitinase(s). Apart from the six chitinases extensively studied in its genome, chitinase ChiW is expressed on the cell surface of *P.* str. FPU-7 in the presence of $(GlcNAc)_2$. ChiW produces $(GlcNAc)_2$ as the final reaction product on the cell surface. The product $(GlcNAc)_2$ would then be transported into the cytosol by some transporters and induce the chitin-related gene expression such as ChiW.

7.7 Overview of Bacterial GH18 Chitinases

Chitinolytic bacteria secrete multiple chitinases as described above. Some bacterial chitinases can be active over a range of temperatures and pH (Meena et al. 2014; Rathore and Gupta 2015) and, thus, these enzymes are used in many industrial processes. Some of these enzymes also have antifungal and antibacterial properties and are used as biocontrol agents against many plant pathogenic fungi and insects, since chitin is a major constituent of fungal cell walls and insect cuticles (Bhattacharya et al. 2007; Rathore and Gupta 2015).

These bacterial chitinases are typically classified in the GH18 and GH19 families; as described above, bacterial chitinolysis is mainly carried out by GH18 chitinases. Based on their amino acid sequences, bacterial GH18 chitinases can be separated into three subfamilies, A, B, and C (Li and Greene 2010). Subfamily A chitinases have a small subdomain, chitin insertion domain (CID), in the TIM-barrel. CID is absent in the chitinases belonging to subfamilies B and C. Bacterial GH18 chitinases are usually multi-modular. For example, in *S. marcescens*, in addition to the catalytic domains, ChiA (SmChiA) contains an N-terminal FnIII domain; SmChiB contains a CBM5 at the C-terminus; SmChiC contains a FnIII domain and CBM12 at the C-terminus (Vaaje-Kolstad et al. 2013). Similarly, *B. circulans* WL-12 ChiA1 (BcChiA1) contains two FnIII and CBM12 domains at the C-terminus (Watanabe et al. 2003). Considering the enzymatic reaction modes, GH18 chitinases are classified into two types: processive chitinases and endo-acting non-processive chitinases. These chitin-degrading enzymes synergistically operate on the polymers. First, non-processive chitinases and LPMO break the glycosidic bond of surface-bound

crystalline chitin. Then, the newly produced terminuses of the detached chains from the solid surface become the sites for the attachment of processive chitinases.

Many crystal structures of bacterial GH18 chitinases are available on the Protein Data Bank. The typical catalytic domain of GH18 chitinase folds a TIM-barrel consisting of a substrate-binding cleft (Perrakis et al. 1994). GH18 chitinases hydrolyze chitin through a substrate-assisted mechanism that retains the stereochemistry of the anomeric carbon of the newly synthesized reducing end (Tews et al. 1997; Brameld and Goddard 1998; van Aalten et al. 2001) (Fig. 7.5). The catalytic residues are in a conserved DxDxE motif located on fourth β-strand of the core barrel. The catalysis is initiated with a distortion into a boat (or skewed boat) conformation of the GlcNAc residue at the -1 subsite. Substrate binding leads the rotation of a second aspartic acid residue of the DxDxE motif from the first aspartic acid to the catalytic glutamic acid of the DxDxE motif. The second aspartic acid residue then forms hydrogen bonds with the glutamic acid and the *N*-acetyl group of GlcNAc at the -1 subsite. The catalytic glutamic acid protonates the glycosidic bond followed by a nucleophilic attack on the anomeric carbon by the oxygen atom of the *N*-acetyl group. After the scission of the glycosidic bond, an oxazolinium ion intermediate is formed and subsequently hydrolyzed to complete the reaction. Other

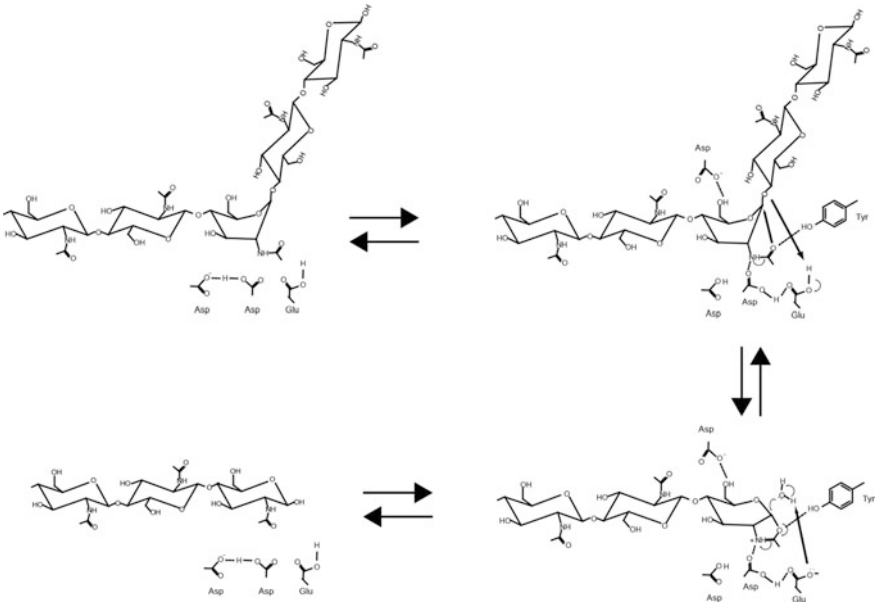

Fig. 7.5 Substrate-assisted catalytic mechanism of GH18 chitinase. The catalysis is initiated when the GlcNAc residue at the -1 subsite is distorted into a boat conformation. The catalytic glutamic acid protonates the glycosidic bond. This is followed by a nucleophilic attack on the anomeric carbon by the oxygen atom of *N*-acetyl group of the substrate GlcNAc at the -1 subsite. After the scission of the glycosidic bond, an oxazolinium ion intermediate is formed and is subsequently hydrolyzed

conserved residues, tyrosine, and aspartic acid (or asparagine), interact with GlcNAc residues at the subsite −1 and thus, serve as important residues for catalysis.

Chitooligosaccharides (CHOS) produced by the hydrolysis of chitin have potential biological applications in the food, medicine, and agriculture sectors. Depending on the length and pattern of acetylation, CHOS exhibit antimicrobial, antitumor, and immuno-enhancing properties (Mallakuntla et al. 2017). Since the chemical synthesis of CHOS involves nonspecific random hydrolysis, controlling the length of the product is very difficult. Besides hydrolytic activities, some GH18 chitinases exhibit transglycosylation (TG) activities. The enzymes can condensate other saccharide molecules without a water molecule as an acceptor, with the oxazolinium ion intermediate as a donor (Umemoto et al. 2015). TG activities, focusing on the enzymatic production of longer CHOS from shorter oligosaccharides, have received considerable attention. TG activities of GH18 are improved by mutagenesis. Mutations on specific sites can reduce the hydrolytic activities and optimize the subsite affinities and/or stability of the oxazolium ion intermediate. Mutations of aspartates in the DXDXE sequence motif affect the stability of the oxazolium ion intermediate and reduce hydrolytic activities. As a result, these mutant enzymes exhibit improved TG activities. Mutations of aromatic residues in the minus subsite (donor site), such as W167A in the −3 subsite of SmChiA; Y163A and Y390F in the -1 subsite of SmChiA; and Y28A and Y222A in the −1 subsite of *Serratia proteamaculans* ChiD (SpChiD), are known to enhance the TG activities of the enzymes. In contrast to the minus subsite (donor site), introducing aromatic side chains and increasing hydrophobicity at the +1 and +2 subsite surfaces, such as Y226W of SpChiD, also improve the TG activities (Madhuprakash et al. 2018).

7.8 Structure and Function of GH18 Processive Chitinases

The GH18 processive chitinases bind single polymer chains detached from the crystalline chitin in the long tunnels or deep clefts of the TIM-barrel, hydrolyze glycosidic bonds along the same chain, and release disaccharides $(GlcNAc)_2$, before dissociation from the chain (Vaaje-Kolstad et al. 2013). Processive chitinases often have a subdomain, CID, as described previously. The CID is composed of five or six antiparallel β-strands and a α-helix. The CID forms a wall alongside the substrate-binding cleft by inserting itself between the seventh and eighth β-strand of the core barrel. It has been suggested that the CID interacts with the substrate through four conserved amino acid residues (Li and Greene 2010). Besides the TIM domain and the CID, processive chitinases, such as SmChiA, SmChiB, and BcChiA1, contain one or two additional domains. (Fig. 7.6a, b, d). Removal of these extra domains reduces the biological activities of the crystalline forms of chitin. Aromatic residues such as tryptophan that are localized in the substrate-binding clefts function as flexible hydrophobic sheaths for binding of the polymer chain and thereby, improve the processivity of the chitin. The polymer chain can slide during the processive action in the sheath. The W97A mutation at

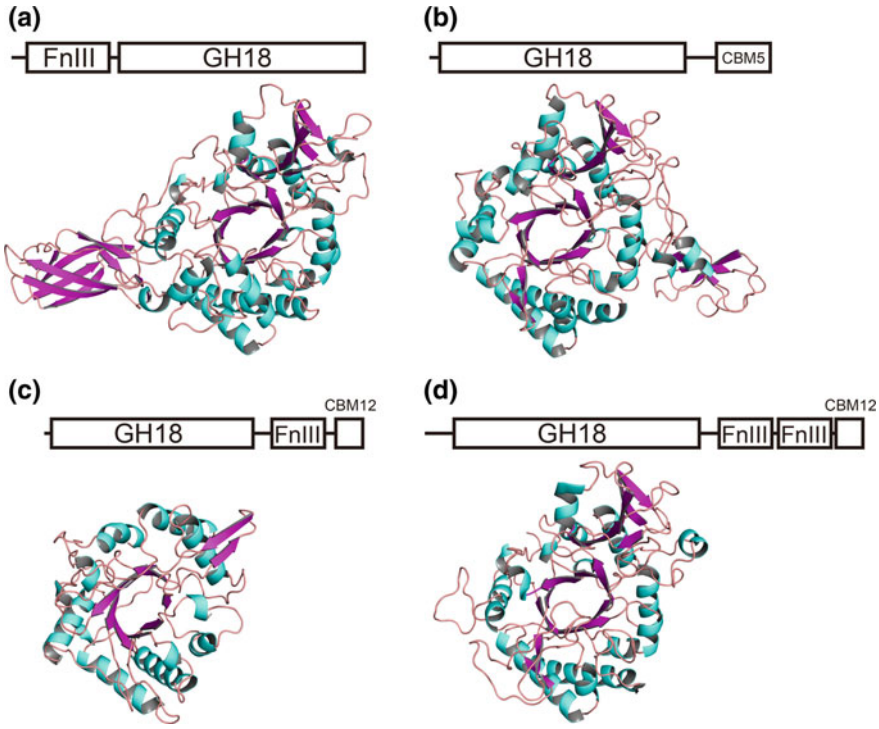

Fig. 7.6 Three-dimensional structures of bacterial GH18 chitinases. **a** SmChiA has a FnIII domain (β-sandwich) at the N-terminus, except for the GH18 catalytic domain (TIM-barrel). GH18 of SmChiA has one additional domain, CID, protruding from the core barrel. **b** SmChiB has a CBM5 domain at the C-terminus, except for the GH18 catalytic domain. GH18 of SmChiB also has a CID. **c** The GH18 catalytic domain of SmChiC. Although SmChiC has FnIII and CBM12 domains at the C-terminus, the crystal structure was determined without these auxiliary domains. **d** The GH18 catalytic domain of BcChiA1. Auxiliary domains of BcChiA1, such as two FnIII and CBM12 domains, are absent in the crystal structure. GH18 of BcChiA1 also has a CID

the subsite +1 of SmChiB reduces the processivity and the degradation activity for crystalline chitin. However, W97A mutation also causes a 29-fold increase in the degradation rate of single chains (Hamre et al. 2014). The processivity seems to be counterbalanced by the catalytic efficiency of the reaction. On the other hand, Watanabe et al. reported that the W433 and Y279 mutants of BcChiA1 exhibited reduced hydrolytic activity against crystalline chitin, colloidal chitin, and $(GlcNAc)_5$ (Watanabe et al. 2001, 2003). The W433 residue interacts with GlcNAc at subsite −1 through a hydrophobic stacking interaction and holds the residue at this position during the catalytic reaction. The Y279 residue assists the formation of an oxazolinium ion intermediate. In addition to the amino acid residues in the substrate-binding cleft, there are two extra tryptophan residues, W122 and W134, on the surface of BcChiA1. These tryptophan residues are located at the extension of the nonreducing end side of the substrate. Mutations at both these sites reduce the

hydrolyzing activity against highly crystalline β-chitin microfibrils (Watanabe et al. 2003). The direction of the sliding action of processive chitinases differs in each enzyme. For instance, SmChiA cleaves disaccharides from the reducing end of chitin, while SmChiB does so from the nonreducing end (Hult et al. 2005; Horn et al. 2006; Sikorski et al. 2006).

7.9 Structure and Function of GH18 Non-processive Endo-Chitinases

The GH18 endo-acting non-processive chitinases have a tendency to randomly hydrolyze glycosidic linkages in disordered (or amorphous) regions of crystalline chitin (Payne et al. 2012). The processive chitinases have closed tunnels or deep clefts with highly conserved aromatic residues, in particular, tryptophan residues, that interact with the ligand for the processive action (described above in 7.8). In contrast, the endo-acting non-processive chitinases exhibit open (or shallow) clefts lacking the CID subdomain and have a few aromatic amino acids on the cleft surfaces. Both the processive chitinases and the endo-acting non-processive chitinases are often multi-modular. For instance, SmChiC has two extra domains, CBM12 (for chitin binding) and FnIII domain (Fig. 7.6c). These CBMs of the non-processive enzymes may loosely associate with the substrate for assisting the activity of the endo-acting non-processive enzymes.

7.10 Structure and Function of the Carbohydrate-Binding Modules of GH18 Chitinases

In addition to the catalytic domain, bacterial GH18 chitinases often contain single or multiple CBMs or FnIII domains, as described above. These extra domains can be found either in the N-terminal or in the C-terminal of the enzyme (Fig. 7.6). The structures and functions of the CBMs responsible for binding crystalline polysaccharides, such as chitin and cellulose, are well studied (Boraston 2005; Georgelis, et al. 2012). Although their contribution to the efficiency of catalysis is not yet completely understood, CBMs associate with the crystalline polysaccharide substrates and position them correctly to the catalytic domains. The four types of roles that CBMs have in chitinolysis include: (a) targeting effect, where CBMs target the enzymes to appropriate regions on their substrates (reducing end, nonreducing end, or internal of polysaccharide chains); (b) proximity effect, where CBMs increase the concentrations of the enzymes in close proximity to their substrates and lead the catalytic domains to efficiently act on their substrates; (c) disruptive effect, where some CBMs bind to polysaccharides and disrupt the surface of tightly packed chains, thereby, loosening and exposing the packed substrates to be attacked by their catalytic domains; and (d) adhesion, where some

CBMs have functions to adhere enzymes onto the surfaces of bacterial cell walls. The catalytic domains break the neighboring substrate polymer chains. Cell walls are complex structures; thus, the binding targets of CBMs are not always the intrinsic substrate polysaccharides for their catalytic domains. Some CBMs have broad specificity for various polysaccharides in the cell walls.

The CBM5 and CBM12 modules consist of 40–60 residues and are often found in GH18 chitinases (Fig. 7.6). Both the families have similar amino acid sequences and have conserved aromatic tryptophan residues that interact with the substrate on the protein surface. The presence of either of these modules increases the substrate affinity and the efficiency of chitin hydrolysis, especially for crystalline chitin (Watanabe et al. 1994; Hashimoto et al. 2000; Uni et al. 2012).

All the FnIIIs domains consist of 80–100 residues and share amino acid sequence similarities. Each FnIII domain folds into a typical immunoglobulin-like (β-sandwich) fold with three and four strands (Fig. 7.6a). The FnIII of SmChiA (also referred to as chitinase A N-terminal domain) contains exposed aromatic residues responsible for substrate binding, thereby, improving the catalytic efficiency of the enzyme (Uchiyama et al. 2001). In contrast, the FnIII domain of BcChiA1 does not have surface-exposed aromatic residues and is not directly involved in the chitin binding (Jee et al. 2002). Studies suggest that the latter types of FnIII domains serve as linker domains for the adequate stabilization of other domains or of the overall structure of the enzyme for a competent degradation.

7.11 Cell Surface-Expressed Multi-modular Chitinase ChiW from *Paenibacillus* sp. FPU-7

The Gram-positive bacterium *P.* str. FPU-7 effectively hydrolyzes chitin with several secreted chitinases, as described above. This bacterium produces a unique chitinase, ChiW that has two catalytic domains. This multi-modular enzyme is expressed on the cell surface and has a high activity toward various chitins, including crystalline chitins (Itoh et al. 2013, 2014, 2016). The cell surface-expressed enzyme, ChiW, enhances chitin degradation when it acts in combination with other secreted chitinases of *P.* FPU-7 (Itoh et al. 2013). ChiW contains 1,418 amino acids with a secretory signal peptide (150 kDa). The unique multi-modular architecture of ChiW allows it to function efficiently on the cell surface via three surface layer homology domains (SLH), a right-handed β-helix domain (CBM54), a Gly-Ser-rich loop, two immunoglobulin-like fold domains (Ig-1 and Ig-2), and two GH18 catalytic domains (Figs. 7.7 and 7.8).

The SLH domains are absent in the ChiW crystal structure. Typical SLH domains are composed of three repeats of highly conserved amino acid sequences (∼18 kDa) and bind noncovalently to glycan backbones of the peptidoglycan of Gram-positive bacteria. Thus, the cell wall is surrounded by proteins congregated with SLH domains as a cell envelope or surface layer (Schneewind and Missiakas 2012).

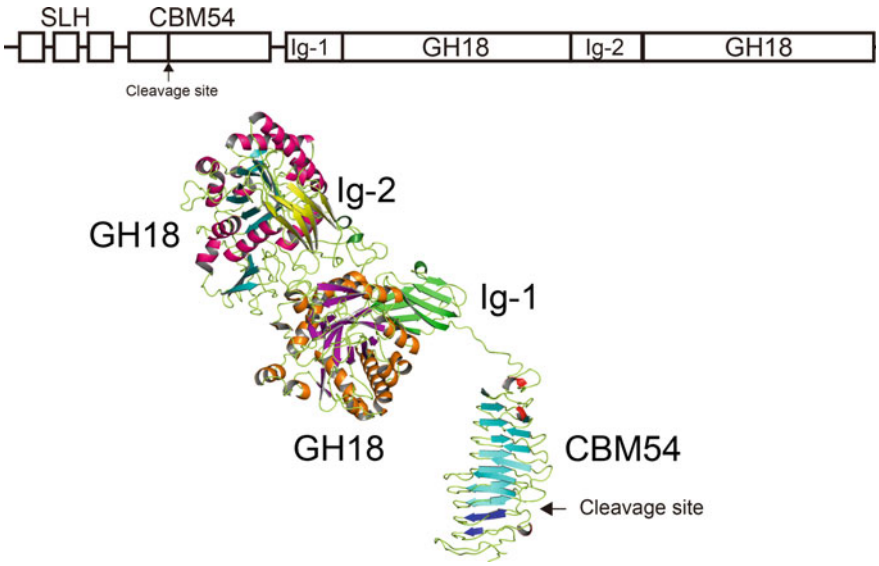

Fig. 7.7 Overall structure of *P.* str. FPU-7 ChiW. The structure is multi-modular with a CBM54 domain and a catalytic region (Ig-1, Ig-2, and two GH18 domains). ChiW is specifically cleaved between Asn282 and Ser283 at the CBM54 domain. The position is pointed out as the cleavage site. SLH domains are absent in the crystal structure

The CBM54 flexibly links to the catalytic region (two Ig-like domains and two GH18 domains) of ChiW via the Gly-Ser-rich loop. The structure of CBM54 consists of a right-handed parallel β-helix fold with 12 coils (Fig. 7.8a). There are 34 β-strands that form 3 parallel β-sheets, named SB1 (made of 12 β-strands), SB2 (made of 12 β-strands), and SB3 (made of 10 β-strands), making 3 distorted faces. Although this fold is often found in enzymes such as carbohydrate lyases, CBM54 of ChiW displays no detectable carbohydrate degradation activities. The domain possesses diverse substrate specificities and can therefore, bind to several cell wall polysaccharides, including chitin, chitosan, β-1,3-glucan, xylan, and cellulose. This domain putatively helps in an efficient decomposition of the cell wall chitin through the contact surface. However, the molecular surface of CBM54 has no distinct cleft or patch surrounded by aromatic residues.

The two GH18 catalytic domains of ChiW have similar structures, consistent with their high degree of amino acid sequence similarity (56% identity) (Fig. 7.8d and e). The structures of the catalytic clefts of the two domains are also similar. Besides the core TIM-barrels, both have two additional subdomains, CID and insertion domain 2; the two subdomains protrude from the barrel and form the walls of a deep active cleft of approximately 42 Å in length and 26 Å in depth. The amino acid residues at the center of the two active sites are almost identical and well conserved in other processive GH18 chitinases. Important residues of SmChiA for saccharide binding, Trp167 at the −3 subsite, Trp539 at the −1 subsite, Trp275 at

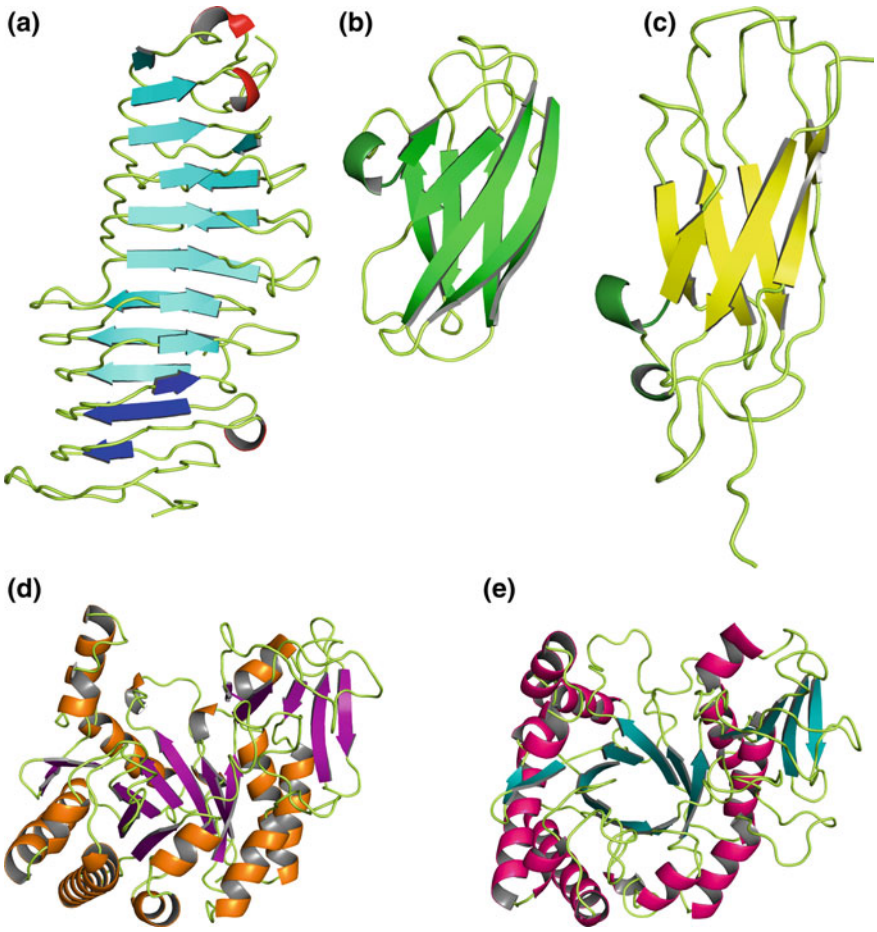

Fig. 7.8 Close-up views of the individual domain of ChiW. **a** The CBM54 domain has a right-handed parallel β-helix structure with 12 coils. **b** The Ig-1 structure is an eight-stranded β-sandwich with two four-stranded antiparallel β-sheets. **c** The Ig-2 structure is a seven-stranded β-sandwich with two antiparallel β-sheets composed of three and four β-strands. **d**, **e** The structures of the two GH18 catalytic domains. The two GH18 catalytic domains have similar structures; besides the core TIM-barrel, they have additional two subdomains, a CID and an insertion domain 2. The two subdomains protrude from the barrel and form the walls of a deep active cleft

the +1 subsite, and Phe396 at the +2 subsite, correspond to the ChiW residues Trp568/Trp1055, Trp905/Trp1396, Trp652/Trp1138, and Trp772/Trp1258, respectively. The catalytic residues of SmChiA, Tyr390, Asp311, Asp313, and Glu315 correspond to Tyr766/Tyr1252, Asp687/Asp1173, Asp689/Asp1175, and Glu691/Glu1177, respectively. These conserved residues indicate that ChiW possesses a catalytic mechanism that is similar to that of SmChiA and other typical processive chitinases. In the processive chitinases, CBMs or FnIII domains often

locate along their catalytic domains and assist in the processive degradation of one chitin chain (Fig. 7.6). For example, SmChiA has one FnIII domain that forms a minus subsite, which leads to enzymatic degradation of chitin from the reducing ends with the production of $(GlcNAc)_2$ residues (Fig. 7.6a), whereas SmChiB, with a CBM5 on the opposite side forms a plus subsite, degrades the polymer from the nonreducing ends and also produces $(GlcNAc)_2$ residues (Fig. 7.6b). However, ChiW catalytic domains have neither such FnIII domain nor CBM, they instead have two Ig-like fold domains (Ig-1 and Ig-2). The Ig-1 structure is composed of an eight-stranded β-sandwich fold containing two four-stranded antiparallel β-sheets closely stacked upon each other (Fig. 7.8b). The structure of Ig-2 is a seven-stranded β-sandwich with two antiparallel β-sheets composed of three and four β-strands (Fig. 7.8c). Aromatic residues are located on the surface of the two Ig-like fold domains, i.e., Tyr486, Tyr537, and Phe556 on the surface of Ig-1; Tyr939, Tyr948, Tyr1000, and Phe1044 on the surface of Ig-2. The Ig-1 and Ig-2 domains might be functional substitutions of CBM. However, the two Ig-like domains are too distal from the catalytic clefts to function as a CBM; they might serve as linkers or scaffolds for the two catalytic domains (Fig. 7.7). The substrate-binding sites of ChiW, surrounded by aromatic residues, are shorter in length to those of SmChiA. Possibly, the lack of a general CBM and the short active clefts allow ChiW to transfer from chain to chain with low processivity on the cell surface.

Although ChiW is a monomer, the enzyme is cleaved between Asn282 and Ser283 at CBM54 by self-splicing (Fig. 7.7). The trigger for this self-splicing remains unresolved. The location of this cleavage site is on the SB2 face and in front of the 11th β-strand at the fourth coil from the N-terminus of CBM54. The 2 polypeptides bind tightly to each other with 13 hydrogen bonds between the third and fourth coils and they retain the β-helix fold. At the cleavage site, there are four highly conserved amino acid residues, Ser283, His285, Asp262, and Arg304. In addition to these residues, there are successive glycine residues near the site, presumably providing conformational flexibility to the cleavage site. This limited proteolysis occurs using the hydroxyl group of Ser283 as a nucleophile. The amino acid residues of this cleavage site, Asn-Ser, have been found in various self-splicing proteins (Clarke 1994; Hall et al. 1997).

On the other hand, chitinases with two GH18 catalytic domains have been found in viruses, archaea, bacteria, and insects (Hiramatsu et al. 2000; Tanaka et al. 2001; Howard et al. 2004; Arakane and Muthukrishnan 2010). Chitinase, Tk-ChiA, from the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 (Tanaka et al. 2001) and chitinase B from *Microbulbifer degradans* 2–40 (Howard et al. 2004) have an exo-chitinase at the N-terminus and an endo-chitinase at the C-terminus. In some chitinases, the two catalytic domains work synergistically, because their combined activities exhibit a significantly higher chitinolysis than the sum of their individual activities. The two clefts of ChiW are adequately positioned by the two Ig-like domains and cross each other at approximately right angles. This unique spatial arrangement may be important for the efficient degradation of chitin on the cell surface.

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