

## The Non-catalytic Chitin-binding Protein CBP21 from *Serratia marcescens* Is Essential for Chitin Degradation\* $\blacklozenge$

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The Gram-negative soil bacterium *Serratia marcescens* uses three different family 18 chitinases to degrade chitin, an abundant insoluble carbohydrate polymer composed of  $\beta$ (1,4)-linked units of *N*-acetylglucosamine. We show that efficient chitin degradation additionally depends on the action of a small non-catalytic protein, CBP21, which binds to the insoluble crystalline substrate, leading to structural changes in the substrate and increased substrate accessibility. CBP21 strongly promoted hydrolysis of crystalline  $\beta$ -chitin by chitinases A and C, while it was essential for full degradation by chitinase B. CBP21 variants with single mutations on the largely polar binding surface lost their ability to promote chitin degradation, while retaining considerable affinity for the polymer. Thus, binding alone is not sufficient for CBP21 functionality, which seems to depend on specific, mostly polar interactions between the protein and crystalline chitin. This is the first time a secreted binding protein is shown to assist in the enzymatic degradation of an insoluble carbohydrate via non-hydrolytic disruption of the substrate. Interestingly, homologues of CBP21 occur in most chitin-degrading microorganisms, suggesting a general mechanism by which chitin-binding proteins enhance chitinolytic activity. Homologues also occur in chitinase-containing insect viruses, whose infectiousness is known to depend on chitinase efficiency.

Chitin is degraded by chitinases (EC 3.2.1.14) that belong to families 18 and 19 of the glycoside hydrolases (Refs. 2 and 3; afmb.cnrs-mrs.fr/CAZY). Chitinolytic enzymes have been detected in many microorganisms in both terrestrial and aquatic ecosystems. For example, the Gram-negative soil bacterium *Serratia marcescens*, known as one of the most effective microbial chitin degraders (4), produces three family 18 chitinases, ChiA, ChiB, and ChiC (5–8). Like cellulases (9, 10), chitinases, including ChiA, ChiB, and ChiC, often contain one or more non-catalytic domains that are known to help in binding and degrading the insoluble substrate (11–14). Such domains are often referred to as carbohydrate-binding modules (CBMs)<sup>1</sup> and have been classified in the CAZY data base (afmb.cnrs-mrs.fr/CAZY).

In addition to being important for biomass turnover, chitin degradation is essential in a variety of biological processes. For example, plants are known to produce chitinases in response to attack by chitin-containing fungi (15), whereas some non-pathogenic fungi such as *Trichoderma* species are considered as “biocontrol” agents because of their ability to inhibit other, chitin-containing, fungi (16). While chitin-containing organisms may be inhibited by exogenous chitinolytic activity, they do need some endogenous chitin turnover as part of morphological and developmental processes (17). The malaria parasite *Plasmodium falciparum* needs family 18 chitinases to cross the chitinous peritrophic matrix of the mosquito host (18). Analogous processes are thought to occur in other protozoan parasites, e.g. *Leishmania* (19). A final example concerns insect viruses, which produce chitinases to disintegrate the peritrophic matrix of their target organism, thereby increasing virus infectiousness (20).

In addition to three chitinases, *S. marcescens* secretes a 18.8-kDa protein, CBP21, when grown on chitin (21). This protein belongs to CBM family 33 and is known to bind to  $\beta$ -chitin (21, 22). Its three-dimensional structure has recently been solved (22). Homologues of the *cbp21* gene can be found in the genomes of most microorganisms which possess chitinase genes. Chitin binding has been demonstrated for a few of these homologues (21, 23–26), including a study showing that binding is not limited to the surface, but also involves intrusion into the chitin structure (27). Homologues also occur in insect viruses (named GP37 or fusolin proteins in baculoviruses and entomopoxviruses, respectively; see Ref. 28 and the references therein). It is known that these virus homologues are important for infectiousness (29–32), and in one case chitin binding has been demonstrated (33). Currently, the precise biological function of CBP21-like microbial and virus proteins is not

Each year more than one billion tons of chitin, a linear polymer of  $\beta$ (1–4)-linked *N*-acetylglucosamine, is produced in the biosphere, mainly by insects, fungi, crustaceans, and other marine organisms (1). Like cellulose, chitin is an abundant insoluble linear polymer with considerable mechanical and chemical strength. Three crystalline forms of chitin have been described in terms of the arrangement of the chitin chains:  $\alpha$ -chitin (chains run anti-parallel),  $\beta$ -chitin (chains run parallel), and  $\gamma$ -chitin (mixed parallel/antiparallel chains).

Despite its resilience, insolubility, and abundant production, chitin does not accumulate in most ecosystems, suggesting that nature has developed efficient processes for chitin degradation.

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<sup>1</sup> The abbreviations used are: CBM, carbohydrate-binding module; CBP, chitin-binding protein.

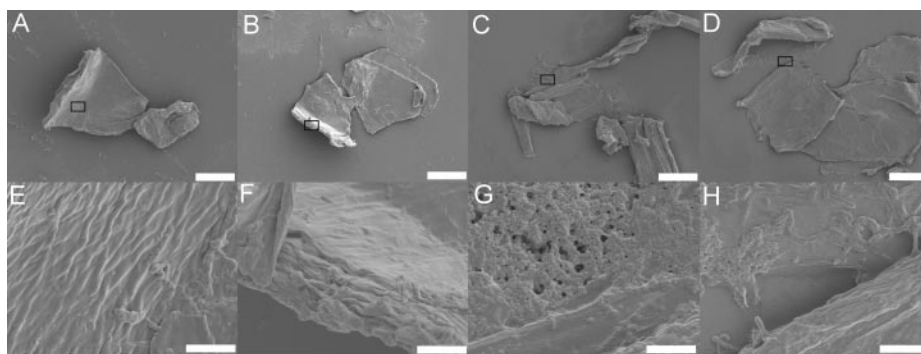


FIG. 1. **Scanning electron micrographs of  $\beta$ -chitin particles.** The figure shows representative pictures of structures observed in the absence or presence of CBP21. Control particles (no CBP21 added) are shown in A and B ( $\times 400$  magnification) with close ups ( $\times 5000$  magnification) of the respective particles shown in E and F, respectively. Particles incubated with CBP21 are shown in C and D, with close ups ( $\times 5000$  magnification) shown in G and H, respectively. The black frame drawn on the  $\times 400$  magnified images indicate the area targeted for the pictures taken at 5000x magnification. The scale bars in A–D and E–H represent 50 and 5  $\mu\text{m}$ , respectively.

clear, but it has been hypothesized that they may play a role in microbial attachment to chitin or to enhance substrate availability by disruption of crystalline chitin (25). Auxiliary CBMs in cellulases (34–37) and amylases (38) have also been suggested to work at least in part by disrupting substrate structure, but experimental evidence in support of this hypothesis is scarce.

In contrast to the majority of the CBMs, which use solvent exposed aromatic residues to interact with substrates, CBP21 lacks an aromatic surface region (22), and the mode of CBP21 substrate binding is not known. Here, we show that binding of CBP21 to chitin leads to disruption of the crystalline substrate structure and to a dramatic increase in chitinase efficiency. We show that CBP21 exerts this effect through specific polar interactions, which are not only important for binding, but also for alteration of the substrate structure. The implications of these findings for microbial chitin degradation and the potential roles of CBP21-like proteins in insect viruses are discussed. Apart from providing new insights into natural chitin degradation, the results also indicate ways to improve already established methods in e.g. plant disease control, which are based on the action of chitinolytic enzymes.

#### EXPERIMENTAL PROCEDURES

**Protein Expression and Purification**—ChiA (6), ChiB (7), and ChiC<sup>2</sup> (Synstad *et al.*, GenBank<sup>TM</sup> accession number AJ630582) from *S. marcescens* BJJ200 were overexpressed in *Escherichia coli* and purified from periplasmic extracts (39) using a two-step procedure. The first step consisted of standard ion-exchange chromatography using Q-Sepharose Fast Flow (Amersham Biosciences, Uppsala, Sweden) at pH 9.4 to separate the chitinases from the majority of proteins in the periplasmic extracts. The second step consisted of hydrophobic interaction chromatography using a phenyl superpose 5/5 column (Amersham Biosciences), as described elsewhere (39). His-tagged ChiG from *Streptomyces coelicolor* A3 (2) (GenBank<sup>TM</sup> accession number AB017013) was cloned behind a T7 promoter into the pETM11 (Günter Stier, EMBL, Heidelberg, Germany) expression vector. The protein was produced in isopropyl  $\beta$ -D-thiogalactopyranoside-induced *E. coli* BL21 DE3 cells, cells were lysed by sonication, and the protein was purified using a nickel column (5  $\times$  2 cm, Qiagen), under standard conditions. All proteins were dialyzed into 20 mM Tris-HCl, pH 8.0, before use and stored at 4  $^{\circ}\text{C}$ . Wild type CBP21 and the CBP21 mutants were produced and purified as described previously (22).

**Scanning Electron Microscopy**—A 0.1 mg/ml  $\beta$ -chitin (France Chitin, Marseille) suspension in 50  $\mu\text{M}$  phosphate buffer, pH 6.3, was preincubated for 48 h at 37  $^{\circ}\text{C}$  in Eppendorf tubes with either 0.1 mg/ml bovine serum albumin or 0.1 mg/ml bovine serum albumin and 0.1 mg/ml CBP21, applied onto an object glass (10 mm; 25  $\mu\text{l}$  drops), and dried at 37  $^{\circ}\text{C}$  to fix the sample. The object glasses containing the samples were glued onto scanning electron microscopy aluminum studs with carbon

tape and sputter-coated with gold-palladium. Scanning was performed in a JEOL JSM 6400 scanning electron microscope at 5 kV.

**Chitin Degradation Assays**—Determination of chitinolytic activity was done using  $\beta$ -chitin from a squid pen (France Chitin),  $\alpha$ -chitin isolated from shrimp shells (Hov-Bio, Tromsø, Norway), or crab shells (Sigma) or microparticulate  $\beta$ -chitin (Seikagaku Corp.) as substrate. Standard reaction mixtures contained varying concentrations of chitinase and CBP21, 0.1 mg/ml purified bovine serum albumin, 0.1 mg/ml chitin powder (unless stated otherwise), in 50 mM sodium phosphate buffer, pH 6.3. Reaction mixtures were incubated at 37  $^{\circ}\text{C}$  for up to 2 weeks. No agitation was used since the insoluble substrate easily adheres to the dry inner walls of the Eppendorf tubes, which would affect the substrate concentration. At time points ranging from 2 to 400 h, 60  $\mu\text{l}$  of the reaction mixture was transferred to an Eppendorf tube containing 60  $\mu\text{l}$  of 70% acetonitrile, to stop the reaction. Before taking samples, reaction mixtures were resuspended by gentle pipetting to leave the chitin concentration unaltered. All reactions were run in triplicate and all samples were stored at  $-20^{\circ}\text{C}$  until further analysis.

Samples were analyzed by isocratic high performance liquid chromatography using an Amide-80 column (Tosoh Bioscience, Montgomeryville, PA), coupled to a Gilson Unipoint high performance liquid chromatography system (Gilson). The liquid phase consisted of 70% acetonitrile, with a flow rate of 0.7 ml/min. 20- $\mu\text{l}$  samples were injected using a Gilson 123 autoinjector. Eluted oligosaccharides were monitored by recording absorption at 210 nm. Chromatograms were collected and analyzed using the Gilson Unipoint software (Gilson). Since in all cases (GlcNAc)<sub>2</sub> represented more than 95% of the total amount of degradation products on a molar basis, only (GlcNAc)<sub>2</sub> peaks were subject for data analysis and used for quantification of the extent of chitin degradation. A standard solution containing 0.25 mM (GlcNAc)<sub>2</sub> was analyzed at the start, in the middle, and at the end of each series of samples, and the resulting average value (displaying standard deviations of less than 3%) was used for calibration.

#### RESULTS

**Scanning Electron Microscopy of  $\beta$ -Chitin Fragments**—Fig. 1 shows scanning electron micrographs of untreated and CBP21-treated  $\beta$ -chitin particles. The edges and surfaces of the untreated particles are discrete in shape, with smooth surfaces (Fig. 1, A, B, E, and F). In contrast, the edges and surfaces of the CBP21-treated particles showed an amorphous and porous character (Fig. 1, C and G), along with areas of disassembled chitin fibrils (Fig. 1, D and H).

**Degradation of  $\beta$ -Chitin with Different Chitinases**—Degradation of  $\beta$ -chitin with the family 18 chitinases ChiA, ChiB, or ChiC showed biphasic kinetics, with an initial fast linear phase, followed by a slower, hyperbolic phase (Fig. 2, A–C). Initial degradation rates were determined by linear regression, whereas the hyperbolic second phase only allowed end point analysis as a rate descriptor (Table I). In the absence of CBP21 ChiA and ChiC had similar activities toward chitin, both in terms of initial rate and the time needed to fully degrade the substrate ( $t_{\text{full}}$ ), whereas ChiB displayed a  $\sim 3$ -fold slower initial rate and never managed to fully digest the substrate (Fig.

<sup>2</sup> B. Synstad, S. J. Horn, G. Vaaje-Kolstad, and V. G. H. Eijsink, manuscript in preparation.

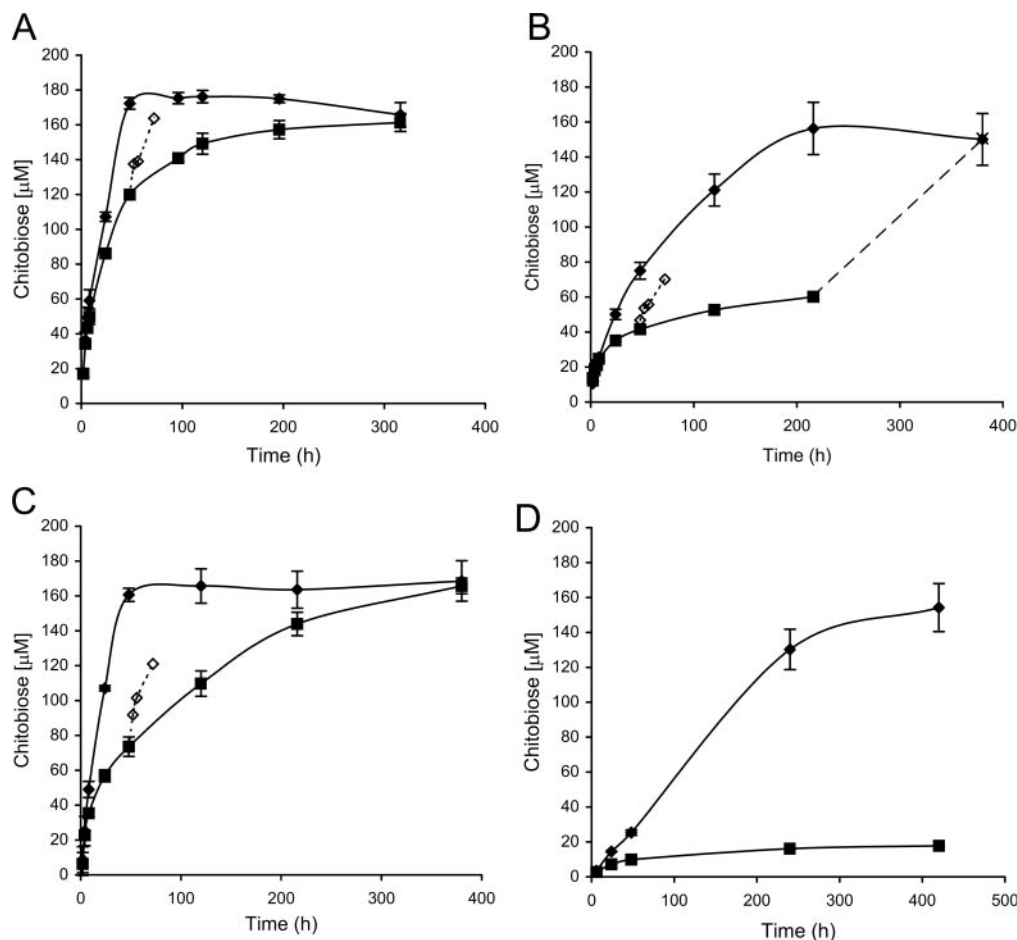


FIG. 2. **Degradation of  $\beta$ -chitin in the absence or presence of CBP21.** Reaction mixtures contained 0.1 mg/ml  $\beta$ -chitin, 0.2  $\mu$ M enzyme, and 5  $\mu$ M CBP21, added at  $t = 0$  unless stated otherwise. The lines connecting the points are drawn for illustration purposes only. A, ChiA (squares), ChiA + CBP21 (closed diamonds), or ChiA + CBP21 added at  $t = 48$  h (open diamonds). B, ChiB (squares), ChiB + CBP21 (closed diamonds), ChiB + CBP21 added at  $t = 48$  h (open diamonds) or ChiB + CBP21 added at  $t = 216$  h (squares connected by a dashed line; see “Results” for details). C, ChiC (squares), ChiC + CBP21 (closed diamonds), or ChiC + CBP21 added at  $t = 48$  h (open diamonds). D, 0.3  $\mu$ M ChiG (squares) or 0.3  $\mu$ M ChiG + 5  $\mu$ M CBP21 (diamonds).

TABLE I

Initial rates (calculated for the first four time points: 2, 4, 6, and 8 h) and reaction end points ( $t_{full}$ ) for the degradation of  $\beta$ -chitin with 0.2  $\mu$ M ChiA, ChiB, or ChiC, in the absence or presence of CBP21

The  $R$ -square values from the linear regression analyses are indicated in brackets. The data are derived from the curves shown in Fig. 2. ND, not determined.

Chitinase	Initial rate		End point ( $t_{full}$ )	
	-CBP21	+CBP21	-CBP21	+CBP21
	$mM (GlcNAc)_2/h$		$h$	
ChiA	2.7 (0.95)	3.4 (0.98)	~360	~48
ChiB	0.9 (0.98)	1.3 (0.99)	ND	~200
ChiC	2.3 (0.93)	3.3 (0.99)	~360	~48

2, Table I). The addition of CBP21 had only minor effects on the initial rates but large effects on the slower second phase (that is, on  $t_{full}$ ). For ChiA and ChiC  $t_{full}$  decreased  $\sim$ 7-fold, whereas for ChiB addition of CBP21 led to complete degradation of the substrate, albeit still at a slower rate than in the case of ChiA and ChiC (Table I).

To verify that the enzymes remained active during the reactions, a series of experiments were carried out, in which CBP21 was added after 48-h preincubation with the chitinases. Fig. 2 shows that addition of CBP21 increased reaction rates to levels comparable to those observed in reactions where the CBP21 was present from  $t = 0$ . In an additional control experiment with ChiB, CBP21 was added after 216 h, which led to full

degradation of the substrate (Fig. 2B). Control reactions containing CBP21 without enzyme did not yield detectable amounts of soluble chitooligosaccharides. Taken together, these results demonstrate that CBP21 facilitates the degradation of  $\beta$ -chitin by family 18 chitinases in a non-enzymatic manner.

To investigate whether the effects of CBP21 on the efficiency of family 18 chitinases from *S. marcescens* were due to specific enzyme-CBP21 interactions, we also conducted experiments with ChiG, a family 19 chitinase from *S. coelicolor*. The results (Fig. 2D) show that CBP21 increased ChiG efficiency, suggesting that CBP21 has a general effect on substrate availability and does not act through specific interactions with particular enzymes.

Dose-response studies of the effect of CBP21 on ChiC efficiency showed that ChiC displays maximum degradation rates at CBP21 concentrations  $\geq 50$  nM, regardless of the enzyme concentration (Fig. 3). Thus, the beneficial effect of CBP21 does not seem to be caused by a stoichiometric interaction with the enzyme.

**$\beta$ -Chitin Degradation Using Combinations of ChiA, -B, and -C and CBP21**—It is generally assumed that ChiA and ChiB are exochitinases, while ChiC is an endochitinase (8, 40, 41), and synergistic effects between these enzymes have been observed in studies with colloidal chitin and  $\alpha$ -chitin (8, 39). In agreement with previous experiments, Fig. 4 shows that the three *S. marcescens* chitinases act synergistically on  $\beta$ -chitin

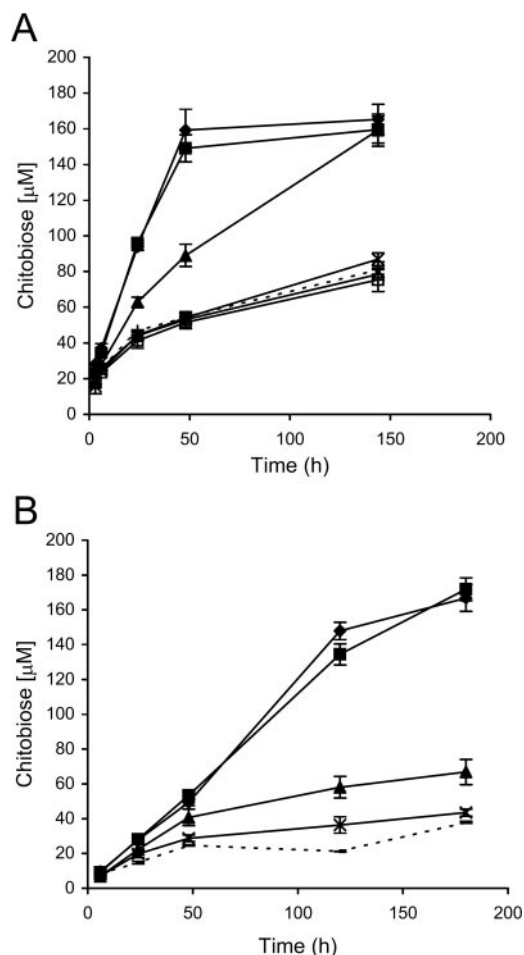


FIG. 3. Dose-response effects for ChiC. Reaction mixtures contained 0.1 mg/ml  $\beta$ -chitin, 50 nM (A) or 5 nM (B) ChiC, and 500 (diamonds), 50 (squares), 5 (triangles), 0.5 (crosses), 0.05 (open squares), 0.005 (open triangles), or 0 nM CBP21 (dotted line).

too. In all cases, CBP21 increased the degradation efficiency. The highest efficiency was obtained when combining all three enzymes in the presence of CBP21.

**Hydrolysis of  $\beta$ -Chitin with ChiC in the Presence of CBP21 Mutants**—Combination of the structure of CBP21 with a multiple sequence alignment of bacterial CBPs has previously revealed conserved surface residues, whose mutation to alanine decreased chitin affinity 3–8-fold (Ref. 22; Fig. 5). These mutants, as well as two control CBP21 variants with wild type binding characteristics (A152R and Q161A (22); Fig. 5), were used in hydrolysis studies with ChiC. Experiments with a CBP21 concentration of 50 nM, which gives maximum effects on ChiC efficiency in the case of wild type CBP21 (Fig. 3), showed that CBP21 mutants Y54A, E55A, E60A, H114A, and D182A had lost their functionality, while the N185A, A152R, and Q161A mutants showed wild type-like functionality (Fig. 6). The deleterious effects of the Y54A, E55A, and H114A mutations on CBP21 function were only slightly negated by increasing the CBP21 concentration as much as 100-fold (results not shown; E60A and D182A were not tested).

**Hydrolysis of Other Chitin Forms**—The three family 18 chitinases from *S. marcescens* can degrade several chitin forms for which CBP21 has low affinity, for example  $\alpha$ -chitin from crab shells and shrimp shells. Experiments similar to the ones described above showed that addition of CBP21 at concentrations up to as high as 5  $\mu$ M did not affect of the efficiency of ChiA, ChiB, and ChiC toward these substrates (results not shown).

## DISCUSSION

The present results show that CBP21 from *S. marcescens* interferes with the crystalline structure of  $\beta$ -chitin (Fig. 1), which leads to increased enzymatic turnover of the substrate, regardless of which chitinase(s) is/are present (Figs. 2 and 4). The results also show that this effect of CBP21 is not due to formation of specific stoichiometric CBP21-chitinase complexes but rather to a more general effect of CBP21 on substrate availability (Figs. 2 and 3).

More than 5 decades ago, it was proposed that degradation of insoluble cellulose was governed by two consecutive phases, an initial non-hydrolytic phase making the substrate accessible for hydrolytic enzymes, followed by the actual hydrolysis phase (42). In the period of time following this postulation, two groups have shown that, indeed, the isolated auxiliary CBMs of two cellulases can disrupt the structure of crystalline cellulose (34, 35). In 1994 Din *et al.* (36) showed that the auxiliary CBM of CenA, an endoglucanase from *Cellulomonas fimi*, increases the substrate availability for the catalytic domain, thus increasing the efficiency of CenA. While chitinases such as ChiA, ChiB, and ChiC also contain auxiliary CBMs, most microorganisms containing chitinase genes also contain a gene encoding for a homologue of CBP21, that is, a non-catalytic chitin-binding protein. Although these proteins have been hypothesized to assist chitinases in chitin degradation, possibly by disrupting chitin (25), this has so far not been shown experimentally.

ChiA, ChiB, and ChiC differ in their efficiency toward  $\beta$ -chitin (Fig. 1) and other chitin forms,<sup>3</sup> which may in part be due to the fact that these enzymes have different types of auxiliary CBMs (ChiA contains a fibronectin type III-like CBM, ChiB contains a family 5 CBM, and ChiC contains a family 12 and an fibronectin type III-like CBM; see afmb.cnrs-mrs.fr/CAZY/for family classification). In all cases though, CBP21 had beneficial effects on enzyme efficiency. The most dramatic effects were observed with ChiB and the family 19 chitinase ChiG (a chitinase without auxiliary CBMs), for which the presence of CBP21 was essential to obtain full substrate conversion. The degradation of  $\beta$ -chitin appeared biphasic, indicating the presence of two substrate forms with different degrees of accessibility. Addition of CBP21 hardly affected the fast first phase of degradation, while it had large effects on the slow second phase. It is conceivable that the fast phase represents degradation of easily accessible amorphous regions in the substrate, while the slow, CBP21-sensitive phase is likely to represent degradation of more recalcitrant crystalline regions.

CBMs from cellulases or chitinases with known structures all have binding surfaces containing several aromatic residues, often tryptophans. Remarkably the structure of CBP21 showed that the surface of CBP21 is devoid of such an extended aromatic binding surface but contains a surface patch of largely hydrophilic residues that are conserved (Fig. 5; note that this patch does contain the only aromatic residue on the CBP21 surface, Tyr<sup>54</sup>). It had already been shown that individual mutation of these conserved residues reduces affinity for chitin 3–8-fold (22). Here, we show that five out of six mutants with reduced affinity for chitin have a strongly reduced ability to promote chitinase activity (Fig. 6). Strikingly, while these mutants only showed a 3–8-fold reduction in binding affinity (22), their functionality could not be fully restored by increasing the CBP concentration by as much as 100-fold. Thus, the effects of individual mutations on chitin binding are modest compared with the effects on the ability to increase substrate accessibility and turnover. Interestingly, the opposite situation was also

<sup>3</sup> G. Vaaje-Kolstad, S. J. Horn, and V. G. H. Eijsink, unpublished observations.

FIG. 4. Synergistic effects in the degradation of  $\beta$ -chitin. The curves show progress in degradation of  $\beta$ -chitin with various combinations of chitinases (as indicated by combinations of the letters A–C) and CBP21. The total enzyme concentration was always 50 nM, meaning that the reactions mixtures with one, two, or three chitinases contained 50, 25, or 16.7 nM of each enzyme, respectively. The CBP21 concentration was 50 nM. For illustration purposes, the points are connected by dotted lines (single enzyme reactions), dashed lines (two-enzyme reactions), or solid lines (three-enzyme reactions). The effect of CBP21 may be evaluated by comparing curves with solid symbols (with CBP21) with curves with corresponding open symbols (same enzyme combination, no CBP21).

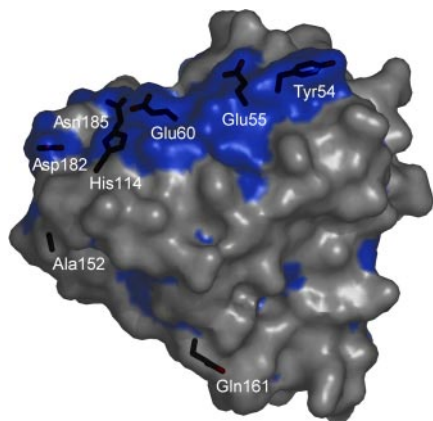
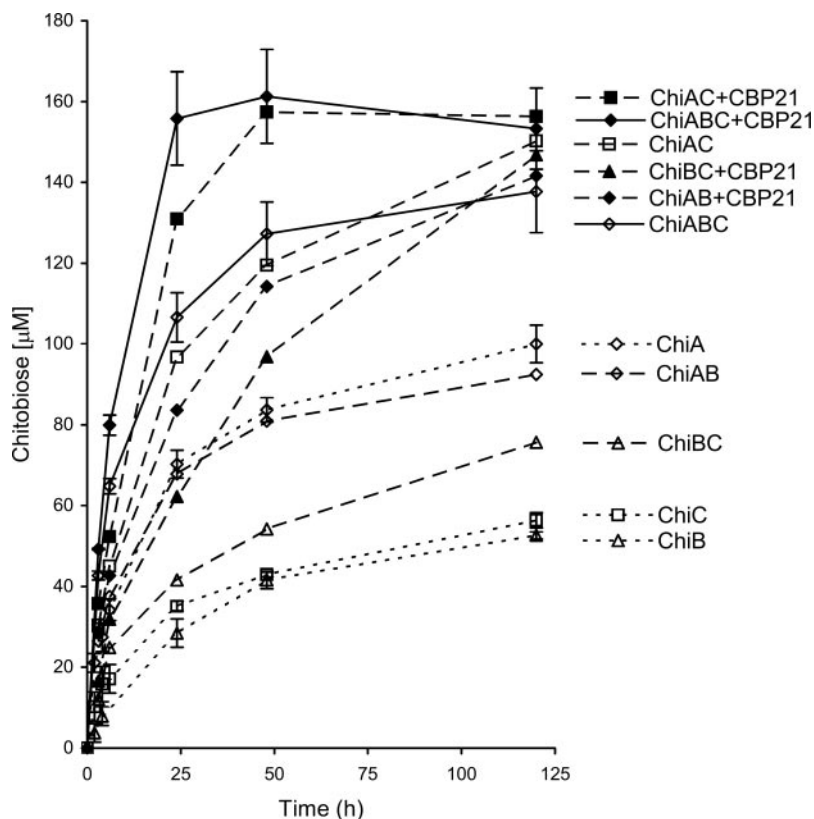


FIG. 5. Structure of CBP21. Residues more than 90% conserved in an alignment of 12 bacterial CBP sequences (22) are colored blue. The side chains of all mutated residues are shown as sticks with carbon, oxygen, and nitrogen colored gray, red, and blue, respectively. Note that Ala<sup>152</sup> and Gln<sup>161</sup> were not expected to be involved in chitin binding.

observed: the N185A mutant showed a 5-fold reduction in chitin affinity (22) but was still capable of stimulating chitin turnover (Fig. 6). While Asn<sup>185</sup> may seem to be important for binding only, the other five mutated residues are apparently involved in specific disruptive interactions with the chitin substrate. It is tempting to speculate that the hydrophilic residues engage in a series of specific hydrogen bonds with the chitin chains and that this could disrupt interchain hydrogen bonding networks. It is also conceivable that such networks specify the substrate specificity of the CBP (strictly  $\beta$ -chitin in the case of CBP21), since, for example, the surfaces of  $\alpha$ -chitin and  $\beta$ -chitin differ in terms of the accessibility and spacing of groups that may engage in hydrogen bonds (in  $\beta$ -chitin the chains are packed more “loosely” than in  $\alpha$ -chitin, and  $\beta$ -chitin has a higher water content (43, 44)). It should be noted that there are homologues of CBP21 (e.g. CHB1 from *Streptomyces*

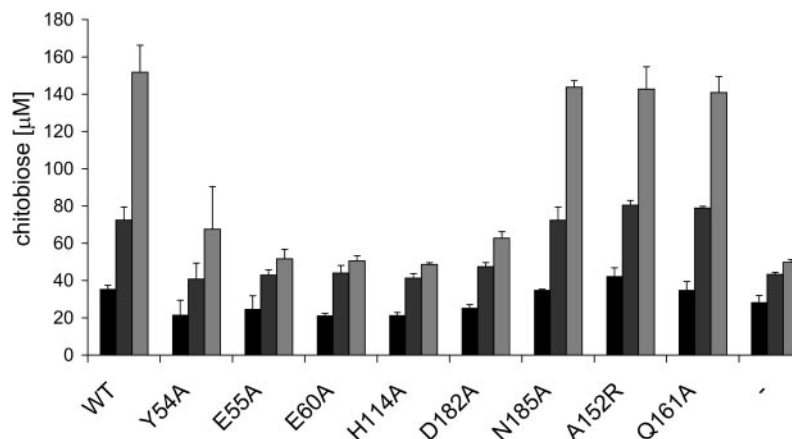
*olivaocviridis*, which is 46% identical in sequence) that bind strictly to  $\alpha$ -chitin (25).

The *cbp21* gene is located 1.5 kb downstream of the *chiB* gene in *S. marcescens*, and the CBP21 protein is produced along with ChiB and the other two chitinases, ChiA and ChiC (21, 45, 46). Fig. 4 shows that the efficiency of  $\beta$ -chitin degradation was optimal in the presence of all three enzymes and CBP21. Thus, in addition to three chitinases with apparent different roles and capabilities, CBP21 is essential for efficient  $\beta$ -chitin degradation by *S. marcescens*. Interestingly, *S. marcescens* grows fast with  $\beta$ -chitin as the sole carbon source, while growth on  $\alpha$ -chitin is slow and never yields dense cultures.<sup>4</sup> This is in accordance with the substrate specificity of CBP21.

Because chitinases degrade structural components in e.g. fungi, insects, and nematodes, they have numerous (potential) applications. For example, it has been shown that transgenic plants expressing heterologous chitinases show increased resistance toward certain plant pathogenic fungi (47–49). The present results indicate that applications of chitinases, e.g. in plague control, may in some cases be improved by not only using the enzyme(s) but also a protein such as CBP21. The fact that homologues of the *cbp21* gene seem to occur in many chitinase-producing microorganisms indicates that the use of CBP21-homologues to improve chitin turnover is a strategy commonly employed in nature. We could not detect homologues of CBP21 in *Plasmodium* and *Leishmania* which both depend on chitinases during their life cycles (18, 19). It remains to be seen whether these important parasites contain other proteins with the same function as CBP21-like proteins. Interestingly, the genomes of insect viruses contain chitinase genes as well as genes encoding homologues of CBP21, called GP37 or fusolin (20, 33, 50, 51). It has been shown that the liquefaction of larvae of *Autographa californica* by a baculovirus depends on the virus-encoded chitinase (20). Mitsuhashi and Miyamoto

<sup>4</sup> G. Vaaje-Kolstad, unpublished observations.

**FIG. 6. Degradation of  $\beta$ -chitin by ChiC in the presence of CBP21 mutants.** Degradation of 0.1 mg/ml  $\beta$ -chitin with 50 nM ChiC in the presence of 50 nM CBP21 wild-type (WT), Y54A, E55A, E60A, H114A, D182A, N185A, A152R, or no CBP21 (indicated by a *hyphen*). Total product release is shown as black bars (24 h), gray bars (48 h), and light gray bars (120 h).



(52) have suggested that a GP37/fusolin protein is involved in perforation of the peritrophic matrix of silkworm larvae upon viral infection. Additionally, Mitsuhashi *et al.* (29) have shown that fusolin increases the infectiousness of a silkworm baculovirus up to 10,000-fold when fed to silkworm larvae prior to infection. Based on the results presented above, it is tempting to speculate that GP37/fusolin proteins in insect viruses contribute to infectivity by facilitating chitinase action.

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