

Conversion of α -chitin substrates with varying particle size and crystallinity reveals substrate preferences of the chitinases and lytic polysaccharide monooxygenase of *Serratia marcescens*.

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1 ABSTRACT

2 Industrial depolymerization of chitinous biomass generally requires numerous steps and the
3 use of deleterious substances. Enzymatic methods provide an alternative, but fundamental
4 knowledge that could direct potential development of industrial enzyme cocktails is scarce.
5 We have studied the contribution of mono-component chitinases (ChiA, -B, and -C) and the
6 lytic polysaccharide monooxygenase (LPMO) from *Serratia marcescens* on depolymerization
7 of α -chitin substrates with varying particle size and crystallinity that were generated using a
8 converge mill. For all chitinases activity was positively correlated to a decline in particle size
9 and crystallinity. Especially ChiC, the only non-processive endo-chitinase from the *S.*
10 *marcescens* chitinolytic machinery, benefited from mechanical pretreatment. Combining the
11 chitinases revealed clear synergies for all substrates tested. CBP21, the chitin-active LPMO
12 from *S. marcescens*, increased solubilization of substrates with high degrees of crystallinity
13 when combined with each of the three chitinases, but this synergy was reduced upon decline
14 in crystallinity.

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17 KEYWORDS

18 α -chitin, mechanical pretreatment, chitinase, GH18, lytic polysaccharide monooxygenases,
19 LPMO, AA10, CBP21, *Serratia marcescens*

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22 INTRODUCTION

23 Chitin is a linear homopolysaccharide composed of GlcNAc units covalently connected by β -
24 1, 4 glycosidic linkages and is an abundant biomass, synthesized in nature at a rate of 10^{11}
25 tons per year (1). The biological role of chitin is foremost to provide mechanical strength and
26 chemical resistance and it is predominantly found in exoskeletons of crustaceans, cuticle of
27 insect and fungal cell walls. There is a rising interest in GlcNAc, soluble
28 chitooligosaccharides and their deacylated derivatives (glucosamine and chitosan oligomers,
29 respectively) as these biomolecules have uses in applications ranging from food to medicine
30 and agriculture. GlcNAc is especially interesting for use in food as it is chemically stable and
31 has a refreshing and sweet taste (2). The most abundant product of enzymatic chitin
32 hydrolysis by chitinases is $(\text{GlcNAc})_2$, which can be readily hydrolyzed to GlcNAc by *N*-
33 acetylhexosaminidases. $(\text{GlcNAc})_2$ represents a useful product itself as e.g. an inducer for
34 production of chitinolytic enzymes (3) or as a donor substrate for enzymatic
35 transglycosylation for production of chitooligosaccharides (4). Chitin and chitosan oligomers
36 are known for eliciting plant defense responses and it is expected that chitin oligomers can be
37 used in many applications, including biopesticides or foods (5, 6).

38 A major challenge in the industrial enzymatic depolymerization of insoluble polysaccharides
39 (like chitin) is their innate recalcitrance, chemical stability and crystalline nature which
40 prevent efficient hydrolysis. So far, few studies have described enzymatic chitin
41 depolymerization in an industrial context, but the analogous field of cellulose saccharification
42 is plentiful of such studies (see e.g. (7) and references within). Traditional enzyme cocktails
43 used for the purpose of recalcitrant biomass conversion contain a series of complementary
44 enzyme activities like processive enzymes acting from either the reducing or non-reducing
45 end of the polysaccharide chains and non-processive endo type enzymes that act randomly on

46 amorphous parts of the substrate. The recent discovery of a new family of carbohydrate
47 active enzymes that specifically target crystalline parts of the substrate (lytic polysaccharide
48 monooxygenases; LPMOs; (8-11)) has provided a new enzyme activity to the existing
49 cocktails promoting more efficient substrate conversion. LPMO driven increased substrate
50 conversion, has indeed has been shown for the Novozymes “Cellic” enzyme products (12).
51 However, the use of a non-enzymatic pretreatment of the biomass is still needed for obtaining
52 the best possible starting point for efficient enzymatic depolymerization. For chitin,
53 mechanical pretreatment (milling) has been shown increase the rate downstream enzymatic
54 conversion through the reduction of particle size and crystallinity (13).

55 To increase the understanding of how the physiochemical properties of chitin influence
56 enzymatic degradability, we have evaluated the solubilization of crab α -chitin with a variable
57 degree of mechanical pretreatment by mono-component enzymes from the well characterized
58 *S. marcescens* chitinolytic system (14). The *S. marcescens* enzymes include the family GH18
59 chitinases, ChiA, -B and -C (15-19), the LPMO called CBP21 and chitobiase, a family GH20
60 *N*-acetylhexosaminidase. ChiA and B are processive exo-chitinases moving in opposite
61 directions (20) while ChiC is a non-processive endo-chitinase (18, 21). CBP21 is a family
62 AA10-type (auxiliary activity family 10) LPMO that specifically targets crystalline chitin (11,
63 22). All enzymes, except chitobiase, were assayed individually and combined in order to
64 determine the limiting factors of chitin depolymerization.

65

66 MATERIALS AND METHODS

67 *Preparation of the substrates*

68 Initial particle size reduction of the crab α -chitin flakes (Yaizu Suisankagaku Industry Co.
69 Ltd.) was accomplished by shearing the particles for 60 s in a 300 cc type cutter mill
70 (Hikikko; Tokyo unicom Co. Ltd.) run at maximum velocity. This chitin was named C0.
71 Converge milling was conducted by milling 20 g α -chitin samples at 800 rpm with zirconia
72 balls (10 mm in diameter) using a converge mill (Makabe giken Co. Ltd.; (23, 24)). The
73 volume of balls used in the experiment represented 10% of the sample volume. Samples were
74 milled for 2, 5, 10 or 30 minutes (Table 1) and named C2, C5, C10 and C30, respectively.

75

76 *Property determination of the substrates*

77 The average particle size (median size D_{50}) was determined by a particle size distribution
78 analyzer (Nikkiso, HRA [X-100]) using methanol to disperse the particles. Equatorial
79 diffraction profiles were obtained using Cu-K α radiation from a powder X-ray generator
80 (Japan Electronic Organization Co. Ltd., JDX-3530) operating at 30kV and 30mA. The
81 crystallinity index was calculated from normalized diffractograms according to the protocol
82 described in (13). The Fourier transform infrared (FTIR) spectroscopy of each substrate was
83 measured with a Nicolet iZ10 spectrometer with OMNIC software (Thermo Fisher Scientific
84 Inc.).

85

86 *Enzyme production and purification*

87 Recombinant enzymes used in the chitin degradation reactions (ChiA, B, C and CBP21) were
88 cloned and expressed as previously described (15, 16, 19, 25). All enzymes were purified by
89 chitin affinity chromatography using the protocol developed for CBP21 (25). In short,
90 periplasmic extracts of *E. coli* cultures containing the enzyme of interest prepared by cold
91 osmotic shock according to (15), were applied directly on a 20 ml chitin beads (New England
92 Biolabs) column equilibrated with 20 mM Tris-HCl pH 8.0 binding buffer. Following elution
93 of non-bound proteins and stabilization of the base line, the enzymes were eluted by applying
94 20 mM acetic acid (elution buffer). Collected enzymes were immediately adjusted to pH 8.0
95 with Tris-HCl pH 8.0 and concentrated with Vivaspin protein concentration devices (GE
96 Healthcare), followed by buffer change to 20 mM Tris-HCl pH 8.0 using the same device. All
97 enzymes were kept at 4°C until use.

98

99 *Enzyme reactions*

100 Chitin degradation reactions were conducted in 1.5 mL sample tubes containing 4.0 mg/mL
101 chitin, 0.2 μM chitinase and/or 1.0 μM CBP21 in total volume of 0.5 ml in 50 mM
102 ammonium acetate buffer (pH 6.3). In reactions containing CBP21, ascorbic acid was added
103 to a final concentration of 1.0 mM (external electron donor). To avoid microbial
104 contamination, substrates were autoclaved before use. All reactions were incubated statically
105 at 37°C. Samples (60 μL) were taken for analysis after 0, 2, 4, 6, 8, 24 and 48 h of incubation,
106 mixed with an equal volume of 50 mM H₂SO₄ in order to terminate the reactions and stored at
107 -20°C until analysis. All reactions were run in triplicates. Although the end product of chitin
108 hydrolysis is GlcNAc and (GlcNAc)₂, only (GlcNAc)₂ was used as a measure of chitinase
109 activity since the generation of GlcNAc was less than 10% of the total soluble sugar released

110 in all reactions. Toluene was added to all reactions (0.5% v/v) in order to prevent microbial
111 contamination.

112

113 *High-performance liquid chromatography (HPLC)*

114 Quantities of (GlcNAc)₂ released from the chitin degradation reactions were determined by an
115 isocratic liquid chromatography using a Shimadzu Prominence HPLC system equipped with a
116 Rezex RFQ-Fast acid H⁺ (8%) 7.8 x 100 mm (Phenomenex) column with a Carbo-H, 4 x 3.0
117 mm guard column and Rezex RFQ-Fast Acid H⁺ (8%) 7.8 x 50 mm fitted in front. The mobile
118 phase was composed of 5 mM H₂SO₄ and was run at a flow of 1.0 mL/min. Eluted (GlcNAc)₂
119 was detected by monitoring the absorbance at 195 nm and calibration standards were run
120 routinely.

121

122 *MALDI-TOF MS of oxidized chitooligosaccharides*

123 Activity of CBP21 was determined by MALDI-TOF MS analysis of products generated by
124 1.0 μM CBP21 combined with 1.0 mM ascorbic acid, 1.0 mg/mL milled α-chitin (C0, C2 and
125 C10) or β-chitin from squid (France Chitin, Marseille) in 20 mM Bis-Tris pH 6.3. The
126 MALDI-TOF MS protocol was identical to that used in (11).

127

128 *Binding assays*

129 The substrates (C0, C2 and C10) were washed prior to the binding assay by suspending the
130 chitin in 100 volumes of 20 mM acetic acid, followed by sedimentation by centrifugation at

131 5000 g. After decanting off the supernatant the chitin pellet was resuspended in 0.5 ml 50 mM
132 ammonium acetate buffer pH 6.3 (binding buffer) followed by sedimentation by
133 centrifugation. The washing step was repeated three times in order to ensure removal of all
134 acetic acid. The concomitant substrate binding assays were performed by mixing 1 μ M
135 enzyme with 10 mg washed substrate suspended in 50 mM ammonium acetate buffer pH 6.3
136 (100 μ L total volume) in 1.5 mL test tubes, followed by 1 h static incubation at 37°C. After
137 sedimentation of the chitin by centrifugation, the substrates containing the bound proteins
138 were washed three times with 0.2 ml binding buffer. After the final washing step, 0.2 ml
139 elution buffer (20 mM acetic acid) was added and in order to release the proteins from the
140 substrate. After 10 minutes of incubation, proteins released from the chitin by the elution
141 buffer were analyzed by SDS-PAGE. Enzymes not eluted by the elution buffer, but still
142 bound to the chitin were analyzed by resuspending the chitin in 20 μ l of loading buffer,
143 followed by 10 min boiling and subsequent analysis by SDS-PAGE. The gel was stained by
144 Coomassie brilliant blue R-250 (CBB).

145

146

147 RESULTS

148 *Properties of milled chitin*

149 Converge mill grinding of α -chitin resulted in a time dependent reduction of mean particle
150 size and crystallinity (Table 1, Fig. 1A). From the initial size of the particles of 2.0 mm (C0
151 sample), mean particle size converged at 21 μm after 30 min grinding (Table 1). In addition to
152 size reduction, the degree of crystallinity was reduced from 94% in the C0 sample to 40% in
153 the sample milled for 30 minutes. The reduction in crystallinity was accompanied by a
154 decrease in the crystallite size being 7.0 nm in the C0 sample and 6.6, 6.1, 5.2, and 2.7 nm in
155 the C2, C5, C10 and C30 samples, respectively. Milling also gave an increase in d -spacing of
156 the (020) lattice compared to ground state (C0), whereas essentially no shift in the (110)
157 lattice could be observed. Finally, FTIR analysis of the C2, C5 and C10 substrates showed
158 changes in the 1640 cm^{-1} region of the spectra (Fig. 1B and C), which represents signals of
159 amide group or carbonyl groups.

160 Putative morphological changes to the C2 sample upon treatment with $1.0\ \mu\text{M}$ CBP21 in the
161 presence of $1.0\ \text{mM}$ ascorbic acid was also investigated with FTIR. Compared to the
162 unreacted C2 chitin, the CBP21 treatment increased absorption in the lower cm^{-1} ($1530\text{-}1000$)
163 of the spectrum (Fig. 1D).

164

165 *Enzymatic degradation of α -chitin*

166 α -chitin milled with the converge mill for either 0 (C0), 2 (C2), 5 (C5), 10 (C10) or 30 (C30)
167 minutes were subjected for hydrolysis by the individual components of the *S. marcescens*
168 chitinolytic system. There was essentially no difference in degradation rate between of C10

169 and C30 and the latter substrate was therefore not included in the degradation studies
170 described below.

171 A general trend observed was that decrease in particle size and crystallinity was correlated
172 with an increase in hydrolysis rate and yield by all chitinases (Fig. 2). However, the level of
173 substrate milling needed for optimal degradation was different for the three chitinases. For
174 ChiA, maximum rate was achieved for C5 (Fig. 2a), whereas ChiB and ChiC showed
175 maximum rate for C10 (Fig. 2b and c). Amongst the chitinases, ChiA appeared to be the
176 fastest enzyme, whereas ChiB showed the slowest rate. Furthermore, ChiA also gave the
177 highest product yield among the chitinases. ChiB and ChiC gave highest yields from the C10
178 substrate, whereas ChiA produced approximately equal amounts for C5 and C10.

179 The presence of CBP21 and an external electron donor (for activation of CBP21) showed
180 different effects on the chitinase performances (Fig. 2). ChiA was generally not influenced by
181 CBP21 activity, although a little effect could be observed for C0 and C5 (Fig. 2A). ChiB and
182 ChiC, on the other hand, were clearly boosted by the presence of CBP21 (Fig. 2B and C),
183 although the effect decreased with declining crystallinity of the substrates. Incubation of
184 CBP21 with C2 in the absence of chitinases showed that this LPMO also was able to
185 individually depolymerize the substrate producing soluble oxidized chitooligosaccharides
186 (Fig. 3).

187 Combination of the three chitinases in the presence and absence of CBP21, showed an
188 increase in degradation rate and yield correlating to the degree of pretreatment similar to what
189 was observed for the individual chitinases (Fig. 4A). However, the contribution of CBP21
190 was only significant for the non-milled chitin.

191 An impression of the synergy obtained by combining the complementary enzyme activities
192 can be demonstrated by comparing the sum of products generated by the individual enzymes

193 (data from Fig. 2) to the amount of product formed by the enzymes when combined in a
194 reaction (data from Fig. 4A). For the three chitinases and CBP21, conversion of C2 and C10
195 was indeed more efficient by the enzymes in combination, thus showing synergy between the
196 enzymes (Fig. 4, panels B and C). The contribution of CBP21 is minimal for the low
197 crystallinity substrate (C10), but significant for the high crystallinity substrate (C2).

198

199 *Chitin binding assay*

200 The binding of each enzyme to C2 (high crystallinity) and C10 (low crystallinity) were
201 investigated by binding assays. ChiA showed somewhat stronger binding to both substrates
202 than the rest of the enzymes, especially for C10 (Fig. 5). CBP21, on the other hand, showed
203 little binding to either C2, C10 (Fig. 5) or C0 (data not shown). CBP21 was the only of the
204 enzymes that could be close to fully unbound from the substrates by the elution buffer (20
205 mM acetic acid).

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208 DISCUSSION

209 It has previously been shown that chitin can be efficiently ground by a converge mill and that
210 the resulting chitin powder is readily degraded by commercially available chitinases (13).
211 However, in the former study milling times were long (30 to 60 minutes) and the components
212 of the enzyme cocktail were unknown. In order to improve the strategy for efficient chitin
213 conversion, α -chitin was processed by a converge-mill in times ranging from 2 to 30 minutes
214 and degradability was assayed with pure, recombinant mono-components enzymes from the *S.*
215 *marcescens* chitinolytic machinery. The short milling times showed a substantial effect on the
216 size and crystallinity of the chitin (Table 1) and after 10 minutes the substrate properties were
217 altered sufficiently to yield maximum degradation rates by the chitinolytic enzymes (Fig. 2).
218 This result differs from those of Nakagawa *et al.* (13), where milling times up to 60 minutes
219 were needed to obtain maximum enzyme conversion rate. This difference in optimal milling
220 time is most likely related to the choice of enzyme system. It seems that the commercial
221 chitinase system used by Nakagawa *et al.* was more optimal for amorphous chitin than
222 crystalline, whereas the *S. marcescens* system appears to handle the recalcitrance of the
223 substrate with higher residual crystallinity better. Thus, tailoring an enzyme cocktail to match
224 the properties of the substrate may be important for obtaining a maximum rate of
225 solubilization.

226

227 The contribution of each chitinase to chitin conversion was analyzed for four chitin variants
228 with variation in particle size and crystallinity. The activity of all chitinases showed a positive
229 correlation with decrease in particle size and crystallinity (Fig. 2). Among the three chitinases,
230 ChiC was especially responsive to the mechanical substrate treatment. This observation is in
231 line with the non-processive endo-activity that has been demonstrated for this enzyme (14,
232 21), which implies favoring of an amorphous/ non-crystalline substrate. The most efficient

233 enzyme was ChiA, which showed an increase in activity for C5 compared to C2, but no
234 increase in activity for C10 compared to C5 (Fig. 2). This may indicate that ChiA activity is
235 less dependent on chitin crystallinity and particle size for efficient substrate solubilization
236 compared to ChiB and ChiC. When all chitinases were combined, a clear synergy was
237 observed (Fig. 4), demonstrating the complementary activities of the three chitinases. Such
238 synergy has been reported before for this enzyme system (17, 26, 27), but not for a well
239 characterized powder substrate such as the one used in this study.

240

241 The maximum turnover rate of all chitinases was obtained for the substrate with lowest
242 particle size and degree of crystallinity (C10; particle size 24.4 μm , and crystallinity index =
243 75%), indicating that the optimal particle size/ degree of crystallinity for the *S. marcescens*
244 chitinolytic machinery is higher than for the commercial chitinase cocktail applied on the
245 same type of substrate by Nakagawa *et. al* ((13); optimal particle size was 19.5 μm). This may
246 imply that the *S. marcescens* enzymes are more optimized for crystalline material
247 (crystallinity is correlated with particle size/ milling time) and that pretreatment time may be
248 reduced compared to the optimum suggested by Nakagawa *et. al* ((13); 60 minutes milling by
249 converge mill). Such enzymatic properties may be favorable in an industrial chitin conversion
250 setup where an efficient and time saving treatment of the raw material is advantageous.

251

252 An important contribution to the field working on the enzymatic degradation of recalcitrant
253 polysaccharides was the recent discovery of the LPMOs (9-11, 22). LPMOs have an activity
254 that is complementary to the processive and non-processive endo- and/or exo-acting glycoside
255 hydrolases as these enzymes induce chain breaks in polysaccharide chains that are “locked
256 up” in a crystalline arrangement. *S. marcescens* secretes one LPMO (named CBP21) that has
257 been shown to be essential for efficient degradation of chitin by the bacterium (11, 26).

258 CBP21 has previously been shown to bind specifically to β -chitin and only show weak
259 binding to the α -chitin allomorph (25, 28). This correlates well with the observations from the
260 binding data obtained in this study, where only weak binding of CBP21 to C2 and C10 is
261 observed (Fig. 5). Nevertheless, in the presence of an external electron donor, CBP21 was
262 able to cleave this substrate (Fig. 3A). Interestingly, only oxidized chitooligosaccharides with
263 a degree of polymerization (DP) up to 6 were observed, whereas when β -chitin is used as a
264 substrate, products up to DP10 can be observed (Fig. 3B and ref. (11)). It is likely that this is
265 caused by the tighter interaction of the chitin chains in α -chitin compared to β -chitin, making
266 high-DP chitooligosaccharides unable to dissociate from the crystalline arrangement.
267 Furthermore, CBP21 activity seems to have an effect on the substrate morphology, illustrated
268 by changes in the FTIR spectrum (1660-1000 cm^{-1} region) for C2 incubated with CBP21 (Fig.
269 1D). Moreover, the activity of CBP21 also increased conversion rates of chitin by the
270 chitinases, although the effect declines with the decrease in particle size and crystallinity (Fig.
271 2). Thus the activity of CBP21 correlates with the degree of crystallinity of the substrate,
272 which agrees with the hypothesis that LPMOs mainly target the crystalline areas of the
273 insoluble substrates and that this crystallinity is inhibiting for many glycoside hydrolases.

274

275 When comparing the effect of CBP21 for the individual chitinases, the results show large
276 differences. Firstly, it seems that the activity of ChiA is not influenced by the activity of
277 CBP21 except for the highly crystalline C0 substrate (Fig. 2A). This indicates that ChiA
278 targets a different region on the substrate than CBP21 and a plausible explanation may be that
279 ChiA and CBP21 target different faces of the chitin crystal, similar to what has been observed
280 for various cellulose binding modules (29-31). Despite boosting the activity of ChiA only
281 marginally, both ChiB and ChiC activity benefitted from CBP21 activity (Fig. 2B&C),
282 indicating that these enzymes act on the same physical landscape of the substrate. Although

283 ChiC is endo-type and non-processive chitinase and ChiB is an exo-type processive enzyme,
284 they both share a C-terminal family 5/12 chitin binding domain (14). This suggests targeting
285 of the same physical parts of the substrate, which correlates well with the increase in velocity
286 when combined with CBP21.

287

288 In conclusion, the data show that degradation of chitinous substrates with a high degree of
289 residual crystallinity after pretreatment are more efficiently degraded by a chitinolytic system
290 that employs a LPMO in addition to the chitinases. In this study the LPMO (CBP21) seemed
291 primarily to promote the activity of ChiB and ChiC, indicating that the addition of a second
292 LPMO with a different substrate preference may be beneficial for optimizing the total chitin
293 solubilization efficiency of the system. Furthermore, the study also shows that a sufficient
294 reduction of particle size and crystallinity by mechanical pretreatment may eliminate the need
295 of LPMOs. However, this assumption does not consider the existence of one or more LPMOs
296 that have activity on more amorphous substrates.

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299

300 ACKNOWLEDGEMENTS

301 We thank Anne C. Bunæs, UMB and Joni Niemi, Department of Life Sciences at Turku
302 University of Applied Sciences, Finland for recombinant protein purification. We thank
303 Kenichi Koseki, INCT for the substrate milling and measurement of ash contents.

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405

406 FINANCIAL SUPPORT

407 YSN was supported by Grant for a research worker in abroad from Institute of National
408 College of Technology and Grant-in-Aid for Young Scientists (B) (#22780097). GVK and
409 VGHE were supported by grants 214138 and 196885 from the Norwegian Research Council.

410

FIGURE CAPTIONS

411 Figure 1. Properties of milled α -chitin. (A) X-ray diffractograms of samples milled by a
412 converge mill for 0 (C0), 5 (C5), 10 (C10) and 30 minutes (C30). (B) FTIR spectra of C2, C5
413 and C10 (peaks around 2300-2400 indicate CO₂; atmosphere). (C) Close-up of the 1620-1678
414 (cm⁻¹) region that represent signals for amide and carbonyl bonds. (D) Close-up of the 900-
415 1660 (cm⁻¹) region of the FTIR spectra of C2 incubated with buffer (control) and C2 treated
416 1.0 μ M CBP21 and 1.0 mM ascorbic acid. Both samples were incubated for 24 h at 37°C
417 followed by drying at 50°C for 24 h before FTIR analysis.

418

419 Figure 2. Degradation of 4.0 mg/ml C0, C2, C5 and C10 with 0.2 μ M ChiA (A), -B (B) and -
420 C (C) in the presence and absence of 1.0 μ M CBP21, measured by the release of (GlcNAc)₂.
421 All experiments were conducted in 50 mM ammonium acetate buffer (pH 6.3). When CBP21
422 was used, 1 mM ascorbic acid was included as an external electron donor. Ascorbic acid did
423 not alter the activity of the chitinases (results not shown). Error bars indicate standard
424 deviation (n = 3).

425

426 Figure 3. MALDI-TOF analysis of products liberated by CBP21 when incubated with β -chitin
427 (A) or C2 (B) in the presence of 1.0 mM ascorbic acid in 20 mM Tris-HCl buffer, pH 8.0.
428 Oxidized chitooligosaccharides (aldonic acids) are labeled according to their degree of
429 polymerization (DP). Each product is found in clusters representing sodium and potassium
430 adducts. Masses observed for both substrates, [M+Na⁺] and [M+K⁺] respectively, were 869.2
431 and 891.2 (DP4ox), 1072.3 and 1094.4 (DP5ox), 1275.3 and 1297.3 (DP6ox). Additional
432 masses ([M+Na⁺] and [M+K⁺]) only observed for β -chitin as substrate were 1478.3 and
433 1500.3 (DP7ox), 1681.4 and 1703.4 (DP8ox), 1884.4 and 1906.4 (DP9ox) and 2087.5 and
434 2109.5 (DP10ox).

435

436

437 Figure 4. Synergy of the mono-component *S. marcescens* chitinolytic enzymes. (A)
438 Degradation of 4 mg/ml α -chitin by a combination of ChiA, -B and -C (0.6 μ M enzyme in
439 total, 0.2 μ M of each enzyme) in the presence and absence of 1 μ M CBP21. Evaluation of
440 enzyme synergy was performed for substrates C2 (B) and C10 (C). The synergy is visualized
441 by comparing the amount of product formed by ChiA, -B, -C in a one pot reaction (data from
442 panel A; labeled “ChiA+B+C”) to the sum of product released by the individual chitinases
443 after 24 h incubation (data from Fig. 2 labeled “ChiA+ChiB+ChiC”), in the presence and
444 absence of CBP21. Error bars indicate standard deviation (n = 3).

445

446 Figure 5. Binding of ChiA, B- and -C and CBP21 to C2 and C10 visualized by SDS-PAGE
447 analysis. The gel pictures show the purified enzyme before addition of substrate (“C”), protein
448 left unbound after 1 h incubation at room temperature (“NB”), protein desorbed by reduction
449 of pH to \sim 3.2 (“E”), and protein remaining attached to the chitin particles after elution by
450 acetic acid (“B”; desorbed by boiling the chitin in 20 μ L SDS-PAGE loading buffer for 10
451 minutes).

TABLES

Table 1. Property of chitin substrates used in this study.

Milling time (min)	Crystallinity index (110) (%)	d-spacing (110) (nm)	d-spacing (020) (nm)	Crystallite size (110) (nm)	Average particle size (μM)
0 (non-milled)	94	0.46	0.94	7.0	~2000
2 (C2)	93	0.46	0.95	6.6	127
5 (C5)	88	0.46	0.95	6.1	43.7
10 (C10)	74	0.47	0.95	5.2	24.3
30 (C30)	40	0.46	0.98	2.7	20.6

FIGURES

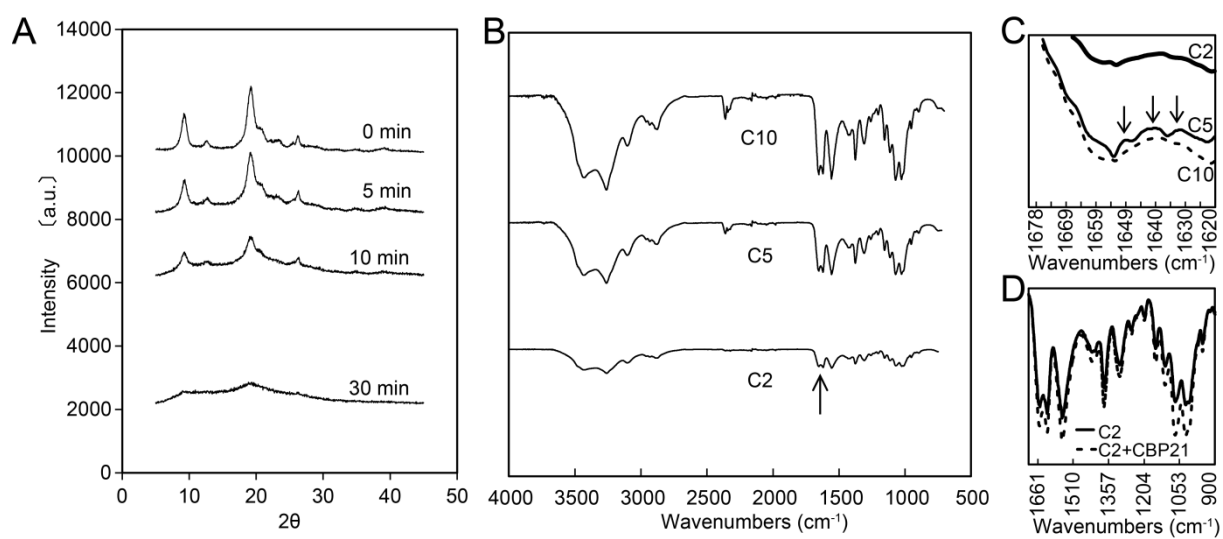


Figure 1.

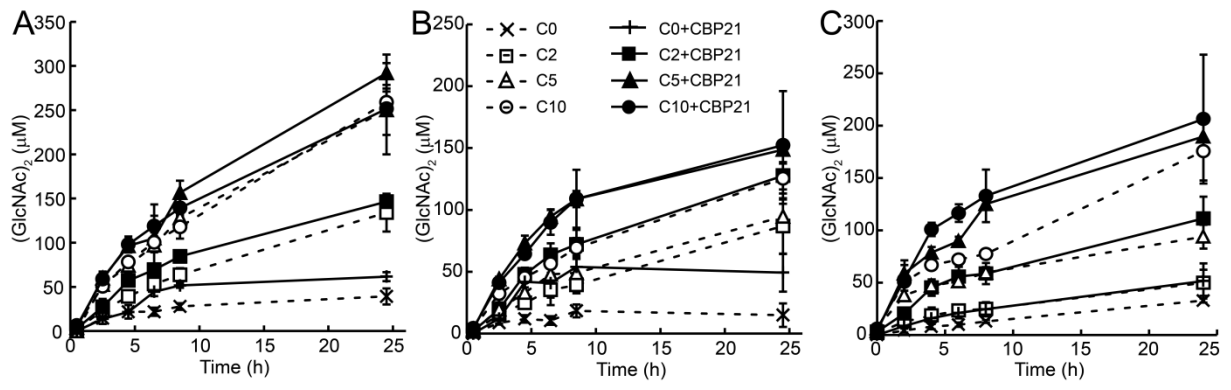


Figure 2.

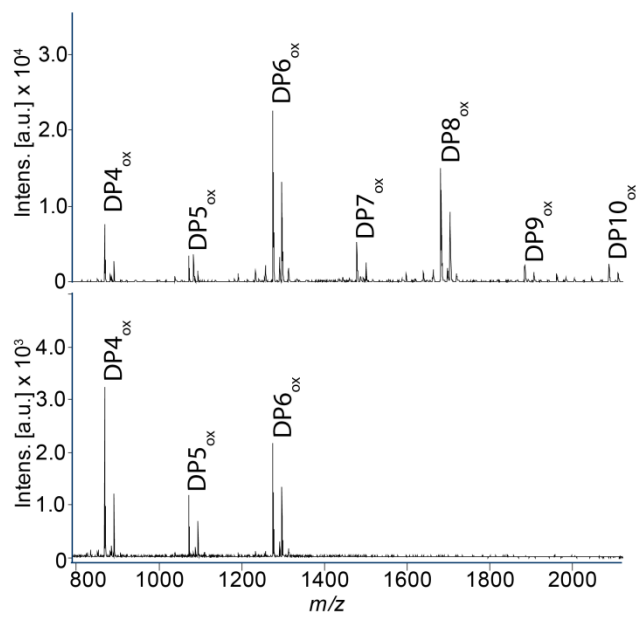


Figure 3.

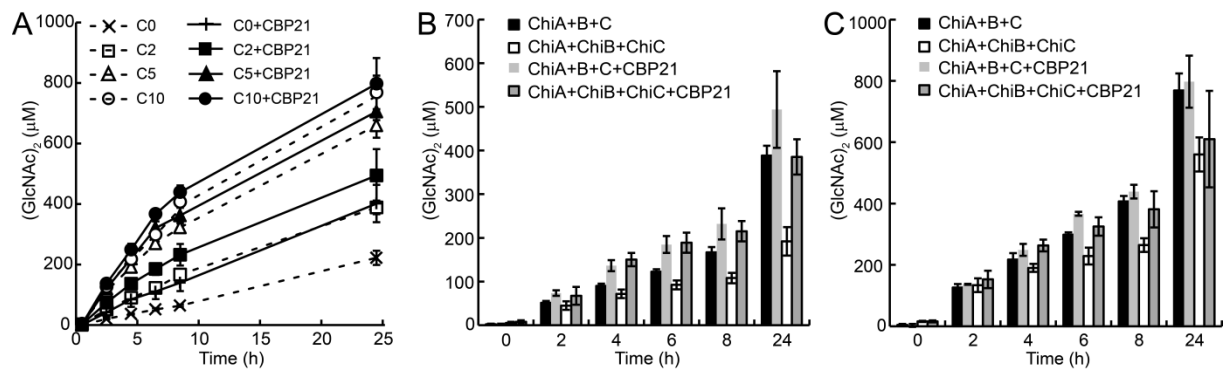


Figure 4.

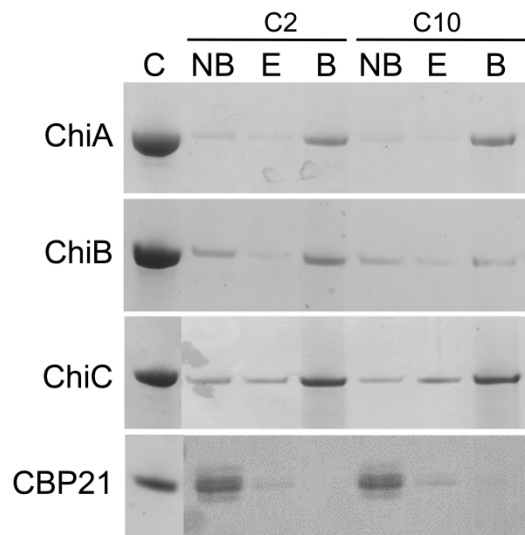


Figure 5.

Converge milled α -chitin

