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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ABSTRACT

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

KEYWORDS

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

INTRODUCTION

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

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

MATERIALS AND METHODS

Preparation of the substrates

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

Property determination of the substrates

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

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^{o}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 H_2SO_4 in order to terminate the reactions and stored at
107 -20^{o}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

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

High-performance liquid chromatography (HPLC)

Quantities of $(GlcNAc)_2$ released from the chitin degradation reactions were determined by an isocratic liquid chromatography using a Shimadzu Prominence HPLC system equipped with a Rezex RFQ-Fast acid H^+ (8%) 7.8 x 100 mm (Phenomenex) column with a Carbo-H, 4 x 3.0 mm guard column and Rezex RFQ-Fast Acid H^+ (8%) 7.8 x 50 mm fitted in front. The mobile phase was composed of 5 mM H_2SO_4 and was run at a flow of 1.0 mL/min. Eluted $(GlcNAc)_2$ was detected by monitoring the absorbance at 195 nm and calibration standards were run routinely.

MALDI-TOF MS of oxidized chitooligosaccharides

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

Binding assays

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

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

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 μM CBP21 in the
161 presence of 1.0 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} (1530-1000)
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

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

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

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

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

An impression of the synergy obtained by combining the complementary enzyme activities 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).

206

207

DISCUSSION

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

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

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

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

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

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

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

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

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

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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 CO_2; 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 μM 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 μM ChiA (A), -B (B) and -
420 C (C) in the presence and absence of 1.0 μM 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 μM enzyme in
439 total, 0.2 μM of each enzyme) in the presence and absence of 1 μM 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 ~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 μL 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

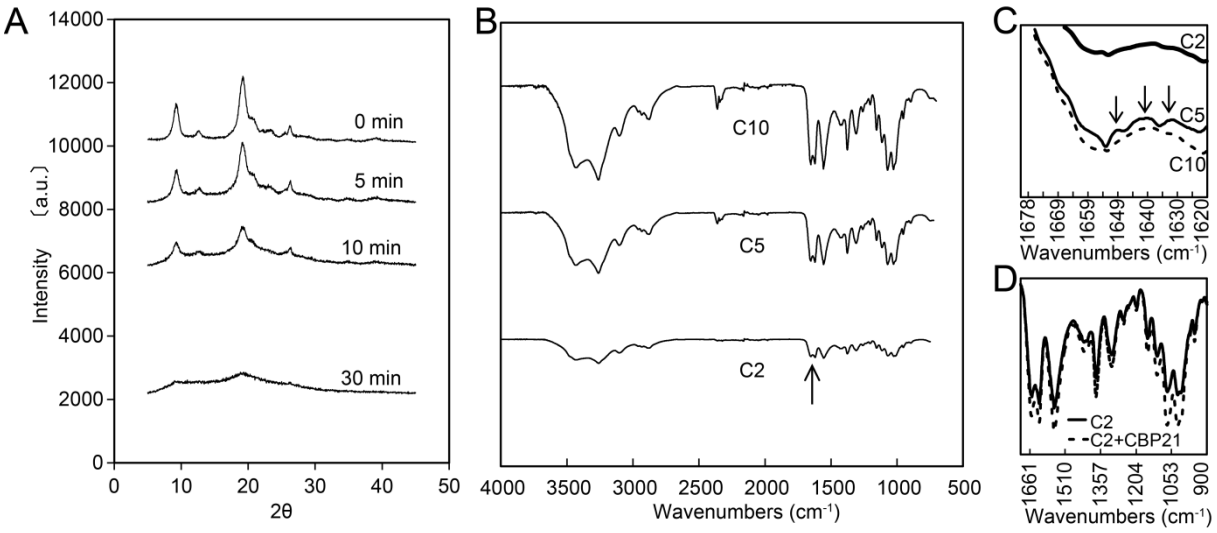

Figure 1.

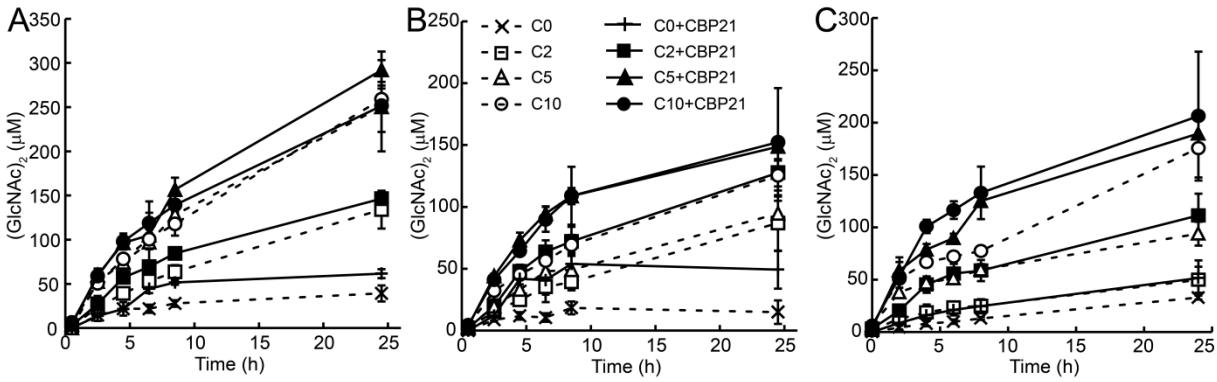
A
B
C
(GlcNAc)2 (µM)
Time (h)
C0
C2
C5
C10
C0+CBP21
C2+CBP21
C5+CBP21
C10+CBP21

Figure 2.

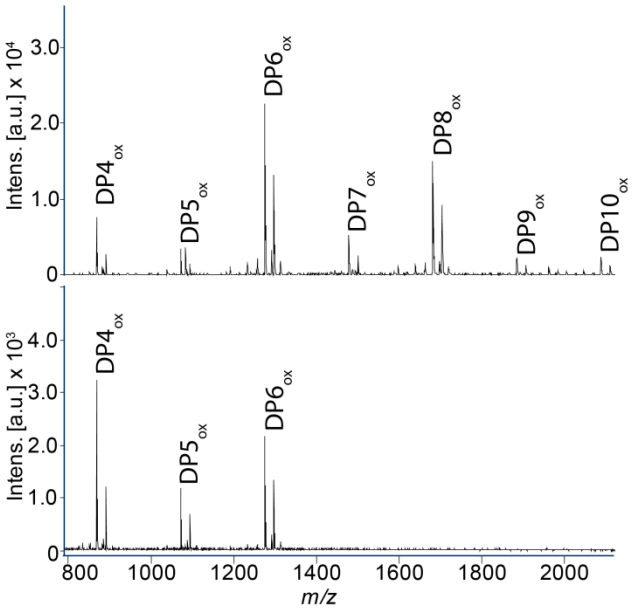
Intens. [a.u.] x 10^4
3.0
2.0
1.0
0
DP4ox
DP5ox
DP6ox
DP7ox
DP8ox
DP9ox
DP10ox
Intens. [a.u.] x 10^3
4.0
3.0
2.0
1.0
0
DP4ox
DP5ox
DP6ox
800
1000
1200
1400
1600
1800
2000
m/z

Figure 3.

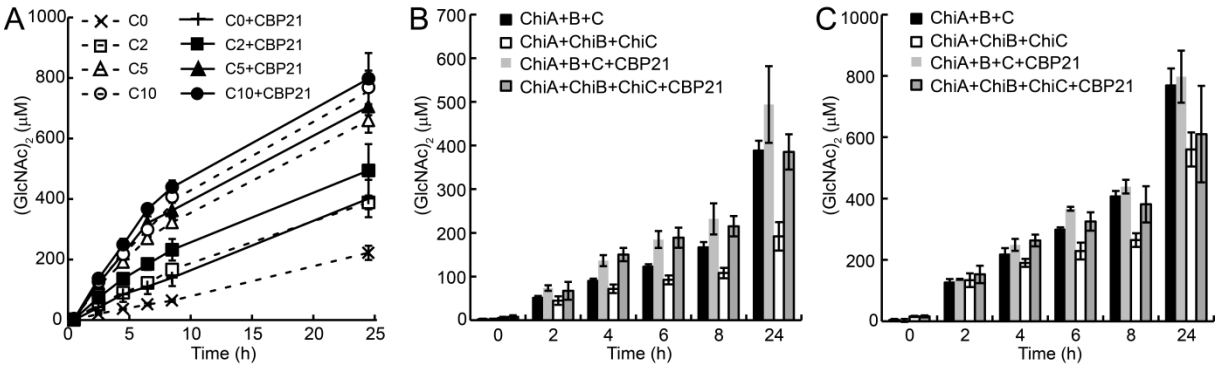
A
B
C
C0
C2
C5
C10
C0+CBP21
C2+CBP21
C5+CBP21
C10+CBP21
ChiA+B+C
ChiA+ChiB+ChiC
ChiA+B+C+CBP21
ChiA+ChiB+ChiC+CBP21
(GlcNAc)2 (μM)
Time (h)

Figure 4.

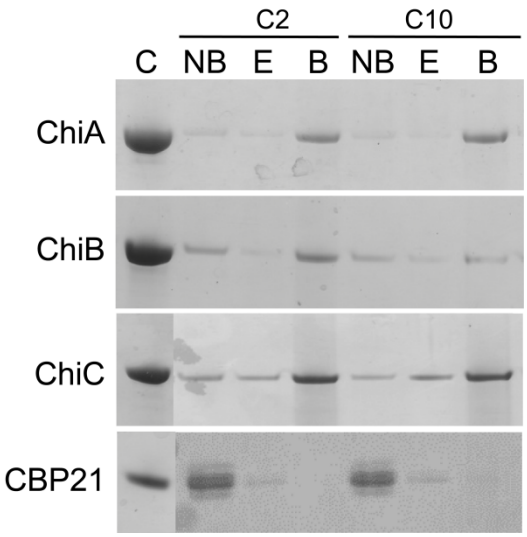

Figure 5.

452

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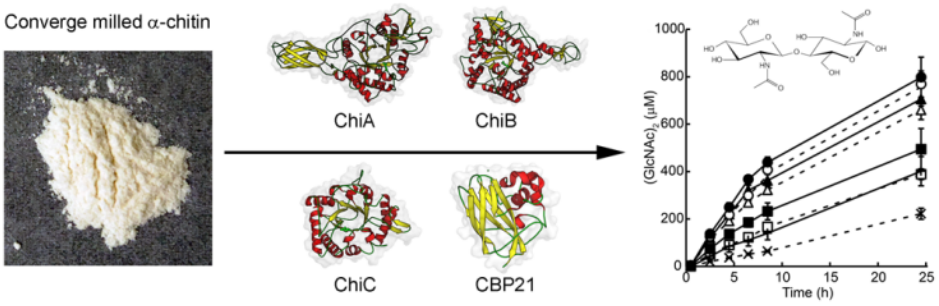
454



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