Listeria monocytogenes has a functional chitinolytic system and an active lytic polysaccharide monooxygenase

Dafni Katerina Paspaliari[1,*], Jennifer Sarah Maria Loose[2,*], Marianne Halberg Larsen[1] and Gustav Vaaje-Kolstad[2†]

[1]Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Stigbøjlen 4, DK-1870 Frederiksberg C., Denmark

[2]Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, P. O. Box 5003, N-1432 Ås, Norway

*These authors contributed equally to this work

†Corresponding author. Email gustav.vaaje-kolstad@nmbu.no.

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ABSTRACT

Chitinases and chitin-active lytic polysaccharide monooxygenases (LPMOs) are most commonly associated with chitin metabolism, but are also reported as virulence factors in pathogenic bacteria. *Listeria monocytogenes*, a well-known virulent bacterium, possesses two chitinases (ChiA and ChiB) and a multi-modular lytic polysaccharide monooxygenase (*Lm*LPMO10). These enzymes have been related to virulence, but their role in chitin metabolism is poorly understood. It is thus of interest to functionally characterize the individual enzymes in order to shed light on their roles *in vivo*. Our results demonstrate that *L. monocytogenes* has a fully functional chitinolytic system. Both chitinases show substrate degradation rates similar to those of the non-processive endo-chitinase *Sm*ChiC from *Serratia marcescens*. Compared to the *S. marcescens* LPMO CBP21, *Lm*LPMO10 shows a similar rate, but different product profiles depending on the substrate. In LPMO-chitinase synergy experiments, CBP21 is able to boost the activity of both ChiA and ChiB more than *Lm*LPMO10. Product analysis of the synergy assays revealed that the chitinases were unable to efficiently hydrolyse the LPMO products (chitooligosaccharide aldonic acids) with a degree of polymerization below four (ChiA and *Sm*ChiC) or three (ChiB). Gene transcription and protein expression analysis showed that *Lm*LPMO10 is neither highly transcribed nor abundantly secreted during growth of *L. monocytogenes* in a chitin-containing medium. The chitinases on the other hand are both abundantly secreted in the presence of chitin. Although *Lm*LPMO10 is shown to promote chitin degradation in tandem with the chitinases *in vitro*, the secretome and transcription data question whether this is the primary role of *Lm*LPMO10 *in vivo*.

INTRODUCTION

Listeria monocytogenes is a Gram-positive food-borne pathogenic bacterium, which upon ingestion can cause listeriosis, a disease of varying severity that can prove fatal for susceptible patient groups such as infants and the elderly. Outside human and animal hosts, the bacterium is known to inhabit terrestrial and marine environments, mainly adopting a saprophytic lifestyle [1-3]. Indeed, the *L. monocytogenes* genome harbors an array of carbohydrate-active enzymes that may be harnessed to degrade complex polysaccharide structures found in both plants and animals [4]. Although few studies have been conducted to investigate the activity of these enzymes towards plant polysaccharides, some effort has been made to elucidate the activity of the chitin-degrading enzymes. Chitin is a linear polysaccharide constituted by β-1,4 linked *N*-acetylglucosamine units (GlcNAc). This recalcitrant and insoluble carbohydrate is predominantly found in the exoskeletons of crustaceans and insects and in the cell walls of fungi.

GH18 chitinases depolymerize chitin chains by cleaving the β-1,4 glycosidic linkages through a hydrolytic reaction mechanism (Fig. 1A) yielding $(GlcNAc)_2$ and GlcNAc as the major end products. The *L. monocytogenes* genome encodes two chitinases (ChiA and ChiB) that belong to the family 18 of the glycoside hydrolases (GH18). ChiA only consists of a single GH18 catalytic module, whereas ChiB is a multi-domain chitinase containing an N-terminal GH18 catalytic module, followed by a long linker region attached to an FnIII-like module and a C-terminal CBM5/12 chitin-binding module (Fig. 1B). Both chitinases have been shown to be actively transcribed during growth of *L. monocytogenes* in media containing chitin [5] and the bacterium has also been shown to degrade chitin [6]. ChiA is known to be catalytically active [7] and both chitinases have been deemed important for the long-term survival of *L. monocytogenes* in minimal medium containing chitin [5, 8]. Furthermore, ChiA and ChiB are both induced during growth in soil, where chitin is a common carbohydrate, indicating a metabolic role for these enzymes [9]. So far, neither of the chitinases have been biochemically characterized towards the natural, insoluble polymeric form of the chitin.

In addition to ChiA and ChiB, the *L. monocytogenes* genome harbors a gene (*lmo2467*) that encodes a lytic polysaccharide monooxygenase (*Lm*LPMO10) belonging to the auxiliary activity family 10 (AA10) of the carbohydrate-active enzymes (CAZy; [10]). The AA10 family contains enzymes previously classified in family 33 of the carbohydrate-binding modules (CBM33), and members of this family have also been referred to as chitin-binding proteins (CBPs, see [10] for details on

reclassification). *Lm*LPMO10 contains four domains; an N-terminal family LPMO10 catalytic module, followed by a linker region connected to an FnIII-like module trailed by two C-terminal family CBM5/12 chitin-binding modules (Fig. 1B).

Lytic polysaccharide monooxygenases (LPMOs) represent a new enzyme family that is important for the efficient degradation of recalcitrant polysaccharides like chitin by a variety of bacterial species [11-16]. The enzymes are copper-dependent and cleave polysaccharide chains embedded in the crystalline regions of the substrate that are generally inaccessible to glycoside hydrolases [13, 17-21]. LPMO activity contributes to both substrate depolymerization and increased accessibility of the substrate to enzymes. Cleavage of the glycosidic bond is achieved by oxidation of the C1 or C4 carbon, which results in the generation of an aldonic acid or 4-ketoaldose, respectively [13, 22, 23]. Chitin-targeting LPMOs have hitherto only been observed to oxidize the C1 carbon ([13, 15, 24, 25]; Fig. 1A).

The roles of glycoside hydrolases and LPMOs have mostly been described in the context of biomass conversion. In parallel to this metabolic aspect, there is an emerging body of literature that associates bacterial chitinases and LPMOs with virulence (see [26] for a comprehensive review). Specifically, the deletion of chitinases and LPMOs has been found to decrease bacterial adhesion to host epithelial cells, as well as attenuate infection in *in vivo* models in a number of pathogenic bacteria [26-31]. This is also the case for *L. monocytogenes*, for which both chitinases and *Lm*LPMO10 have been identified as virulence factors. *L. monocytogenes* single-gene mutants lacking the genes encoding ChiA, -B and *Lm*LPMO10 showed significantly reduced ability to colonize murine liver and spleen compared to the wild type [32]. A recent study showed that the role of ChiA is directly related to its enzymatic activity; successful colonization of mice was shown to depend on a catalytically active ChiA, which through an unknown mechanism, achieved downregulation of inducible nitric oxide synthase (iNOS), an important enzyme in the innate immune system [33]. The involvement of these proteins in the pathogenic lifestyle of *L. monocytogenes* raises the question of whether their main role is as chitin-assimilation facilitators, virulence factors or both.

In order to evaluate the performance of the *L. monocytogenes* chitinolytic enzymes in a metabolic context, we have analyzed the chitin-degrading abilities of ChiA, ChiB and *Lm*LPMO10 and compared them to those of a well-characterized endo-chitinase, ChiC (henceforth referred to as

115 *Sm*ChiC), and an LPMO, CBP21, from *Serratia marcescens*. Transcription of *Lm*LPMO10 and
116 secretion of the chitinolytic enzymes has also been evaluated by analysis of *L. monocytogenes*
117 cultures grown with chitin as a carbon source.

RESULTS AND DISCUSSION

Characterization of LmLPMO10

Sequence analysis shows that homologues of *Lm*LPMO10 are present in a variety of bacterial species. Several of these enzymes are annotated as GlcNAc-binding proteins (abbreviated Gbp) due to sequence similarity to the *Vibrio cholerae* protein "GbpA" (henceforth called *Vc*GbpA) that was given this name because of its GlcNAc-binding properties [30]. *Vc*GbpA is the only "Gbp"-type protein that is biochemically characterized [25, 34]. The protein contains an N-terminal LPMO10, followed by two bacterial surface-binding modules and a C-terminal CBM5/12 [34] and is primarily thought to play a role in virulence by mediating host-bacterium contact/adhesion [27, 30, 34-36]. The presence of the catalytic LPMO10 module in combination with chitin-binding modules raises the question whether *Vc*GbpA, *Lm*LPMO10 and other "Gbp"-type proteins actually are enzymes that play a role in metabolism or have other catalytic functions.

In order to investigate the role of *Lm*LPMO10 in chitin degradation, substrate binding and activity assays were performed with various substrates. The binding preference of *Lm*LPMO10 proved to be relatively broad as strong binding was observed to α-, β-chitin and cellulose (Fig. 2A), and irreversible binding to chitin beads prevented the use of this chromatographic medium for protein purification. Binding to insoluble substrates has been demonstrated previously for two of the module types represented in *Lm*LPMO10, namely LPMO10s and CBM5/12s; LPMO10 modules have been shown to bind specifically to one of the chitin allomorphs [37-39], both chitin allomorphs [12, 15, 40] or cellulose [41]. CBM5/12s occur regularly in chitinases where chitin binding has been demonstrated [42], but are also encountered in cellulases, where cellulose-binding has been shown [43]. It is thus likely that the broad binding specificity of *Lm*LPMO10 is caused by the combined action of the LPMO10 module and the tandem CBM5/12 module. For comparison, *Vc*GbpA, which as previously noted also contains an LPMO10 and a CBM5/12 module, was shown to bind strongly to chitin, but only weakly to cellulose [34]. It should be noted that the occurrence of CBMs in tandem often results in an enhanced binding capacity [44], which would explain the stronger binding of *Lm*LPMO10 to cellulose compared to *Vc*GbpA.

Upon incubation of *Lm*LPMO10 with α-, β-chitin and cellulose and an external electron donor, soluble reaction products could be observed for both chitin substrates (Fig. 2B), but not for cellulose

(results not shown). Even though *Lm*LPMO10 binds cellulose, the lack of activity towards this substrate is not surprising since enzymes (and CBMs) that bind crystalline polysaccharides like chitin and cellulose often bind well to both substrates, as exemplified by Moser *et al.* [45]. The masses observed for the products (Fig. 2B) combined with HILIC analysis (Fig. 3A) verified that the products were the aldonic acid forms of fully acetylated chitooligosaccharides that result from cleavage of the glycosidic bond through oxidation of the C1 carbon [13]. These product profiles are essentially identical to those observed for CBP21 on the same substrates [16], and also show a lack of high DP products for α-chitin compared to β-chitin. This has been suggested to be caused by the tighter packing of chitin chains in the α-allomorph of these substrates, which prevents release of long oligosaccharides due to adhesion to the insoluble substrate [16].

Through HILIC analysis, base line separation was obtained for all soluble aldonic acids resulting from LPMO activity on β-chitin (Fig. 3A), enabling the estimation of relative progress curves (Fig. 3B-F). The relative rate of *Lm*LPMO10 was similar to that of the well-characterized LPMO, CBP21 [11, 13, 16, 38, 46] from *Serratia marcescens (*Fig. 3B-F). The only clear difference between the enzymes was that *Lm*LPMO10 gave a slightly higher abundance of low DP products (tetramer and pentamer; Fig. 3B&C) and lower abundance of the hexamer (Fig. 3D). Since the active site and putative substrate-binding residues of *Lm*LPMO10 are essentially identical to those of CBP21 (Fig. S1), it is reasonable to speculate that the tandem CBM5/12 module has influence on the/this product profile. The tight binding of the protein to the substrate may allow more catalytic events to take place on a limited region of the substrate, thereby yielding more products with low DP.

The presence of the tandem CBM5/12 module on *Lm*LPMO10 prompted investigation of the ability of this LPMO to depolymerize an amorphous substrate like colloidal chitin, a substrate not reported as substrate for chitin active LPMOs in existing literature. Indeed, activity was confirmed by a time course assay and, intriguingly, also for CBP21, which was used as a control (Fig. 4; CBP21 only contains a single LPMO10 module). Compared to activity on β-chitin, initial rates were lower for all products, even though a higher substrate concentration was used (7.5 mg/ml for colloidal chitin, 5.0 mg/ml for β-chitin). This is likely due to the low crystalline and highly amorphous nature of colloidal chitin, which gives/offers fewer sites that allow productive binding of LPMOs. This is in contrast to chitinases, which are generally known to be highly efficient in degrading colloidal chitin due to its amorphous nature. Comparison of the product profiles resulting from LPMO activity on

β- and colloidal chitin shows that for the latter substrate there is less dominance of even-numbered products. The dominance of even-numbered products observed in LPMO product profiles has previously been attributed to the fact that they act on substrate-crystal surfaces, where the two-fold screw axis of the polysaccharide chains mediate productive binding to only every second sugar unit [13]. Thus, the difference in the dominance of even-numbered products between β- and colloidal chitin may be related to the morphological differences of the two substrates.

Characterization of the listerial chitinases

Sequence analysis of the *L. monocytogenes* chitinases to other well-characterized GH18 chitinases shows that ChiA closely resembles *Sm*ChiC from the chitin-degrading bacterium *S. marcescens* (63% sequence identity for the catalytic modules). *Sm*ChiC is a non-processive endo-chitinase with a shallow substrate binding cleft that cleaves chitin at random positions on the polymer chains [46-49]. The catalytic module of ChiB is similar to processive exo-chitinases like *Bs*ChiA from *Bacillus circulans* (32% sequence identity) and *Sm*ChiB from *S. marcescens* (30% sequence identity). Processive chitinases have deep substrate binding clefts that allow attachment to the ends of the substrate chains, followed by continuous hydrolytic processing of the chains without release of the polymer [50-52]. Functional chitinolytic systems usually contain both exo- and endo-acting chitinases, as these have complementing activities and give synergistic chitin degradation [46]. Based on the sequence analysis of the *L. monocytogenes* chitinases, it is conceivable that these enzymes are part of a chitinolytic machinery.

The two *L. monocytogenes* chitinases have previously been shown to be secreted when the bacterium grows in the presence of colloidal (amorphous) chitin [5, 53]. Whereas no activity data has been published for ChiB, the enzymatic properties of ChiA towards soluble substrate analogues have been elucidated [7]. No data exist that describe the activity of these chitinases towards polymeric, insoluble chitin, which is a more realistic substrate for the enzymes to encounter *in vivo*. The activities of ChiA and ChiB were therefore evaluated using crystalline chitin as a substrate and compared to the activity of *Sm*ChiC. Both chitinases showed degradation rates comparable to the *S. marcescens* chitinase (Fig. 5; ChiA, ChiB and ChiBΔ produced 0.7, 0.5 and 0.2 fold the amount of $(GlcNAc)_2$ compared to *Sm*ChiC, respectively, after 8 h of incubation), indicating that the enzymes indeed are capable of rapid chitin degradation. Previously obtained data for ChiA activity towards the substrate trimer analogue $(GlcNAc)_2$-p-Nitrophenol indicated poor substrate binding and a

resulting low catalytic efficiency (K_m=1.6 mM, k_{cat} 22 s^{-1}; [7]). However, our results show that the hydrolysis rate of polymeric chitin is comparable to that of *Sm*ChiC, indicating that the estimated kinetic parameters of ChiA may have been misleading due to the artificial nature of the substrate used. The putatively processive ChiB shows a lower substrate conversion rate than the putatively non-processive ChiA. The substrate-binding modules of processive enzymes have been proposed to play an important role in keeping the enzymes in close proximity to the substrate [54, 55], thus facilitating hydrolysis. This hypothesis is indeed valid for ChiB, which shows a dramatic drop in activity when the CBM5/12 binding module is removed from the enzyme (Fig. 5C).

The influence of LmLPMO10 on chitinase efficiency

Since the discovery of LPMOs, several studies have documented their important contribution to biomass-degrading enzyme machineries [13, 20, 21, 41, 56]. Enzyme database surveys show that most aerobic bacteria that target recalcitrant substrates such as cellulose or chitin harbor one or more LPMOs in their genome. The *L. monocytogenes* genome is no exception, containing two chitinases with complementary processive/ non-processive activities and one LPMO10. Bacteria having similar chitinolytic systems do indeed show a synergistic interplay between the chitinases and the LPMO [11-15]. The same is observed for the *L. monocytogenes* chitinolytic system as *Lm*LPMO10 increases the rate of chitin depolymerization by both ChiA and ChiB (Fig. 5). Interestingly, CBP21 boosts the activity of the chitinases more than *Lm*LPMO10 (Fig. 5 A&B), despite having similar relative rate compared to the *L. monocytogenes* LPMO (Fig. 3).

The chitinases seem to depolymerize chitin with an efficiency that lies in the range of chitinases (*Sm*ChiC) that are devoted to this role (Fig. 5A, B&D), indicating that a metabolic role can be argued for these enzymes. This also corresponds well with the fact that transcription of both *chiA* and *chiB* is induced upon exposure of *L. monocytogenes* to chitin [5] and secretion of the corresponding enzymes is increased under similar conditions (see experiments described below).

The activities of ChiA, ChiB and *Sm*ChiC towards chitin yield GlcNAc and $(GlcNAc)_2$ as products (Fig. 6A). However, in the presence of an LPMO, ion-exclusion chromatography revealed the existence of products of higher DP in the reaction mixture (Fig. 6A). In order to resolve the nature of these products, samples were also analyzed by HILIC, revealing the presence of oligomeric native and oxidized chitooligosaccharides (Fig. 6B), albeit in substantially lower concentrations than

$(GlcNAc)_2$. Two possible explanations may be that the chitinases either are fully bound to the substrate, thus unable to process soluble chitooligosaccharides, or that the free chitinases are prevented from efficient hydrolysis due to non-productive binding of the oxidized chitooligosaccharides to the chitinase active site. Treatment of solubilized high DP reaction products with freshly added chitinase resulted in complete degradation of the native chitooligosaccharides, but only partial degradation of the oxidized products (Fig. 6B). It seems that ChiA and *Sm*ChiC are incapable of degrading $(GlcNAc)_{1\text{-}3}$GlcNAc1A. ChiB shows a slightly different product profile, being only inactive towards $(GlcNAc)_{1\text{-}2}$GlcNAc1A. These data indicate that the presence of oligomeric products may be the result of a combination of low concentration of free enzymes and product inhibition. The ability of ChiB to depolymerize $(GlcNAc)_2$GlcNAc1A (in contrast to ChiA and *Sm*ChiC) may be related to ChiB having an exo-processive function, as opposed to ChiA and *Sm*ChiC, which are non-processive.

Induction and expression of chitinolytic enzymes

Although *L. monocytogenes* possesses chitinolytic enzymes that are capable of efficient chitin depolymerization (Fig. 5), the bacterium has not been reported to utilize chitin as a sole carbon source [8]. The only conditions where *L. monocytogenes* has been observed to actively degrade chitin is in cultivation experiments using LB medium supplemented with chitin as a polysaccharide source [5, 6, 53]. In these studies, both the *chiA* and c*hiB* genes were shown to be upregulated and the expressed proteins were also identified in the culture supernatant. In the current study, we re-examined these conditions using both colloidal chitin and β-chitin as substrates and investigated the transcript abundance of the *Lm*LMPO10A gene (*lmo2467*) as well as the presence of *Lm*LMPO10A and the chitinases in the culture supernatant.

The presence of chitin appeared to marginally increase transcription of *lmo2467* in the stationary phase (~2-fold; Fig. 7). However, compared to the abundant up-regulation observed for the chitinase genes (>17-fold for *chiB*; [5, 53]), induction of *lmo2467* does not seem to be part of a response to growth on chitin as a carbon source. To exclude that induction of *lmo2467* necessitates chitin of crystalline structure, we carried out transcript abundance analysis using α- and β-chitin instead of colloidal chitin. Addition of α-chitin particles did not influence transcription of *lmo2467* (results not shown). Addition of β-chitin particles lead to partial RNA degradation in the samples, for unknown reasons, and therefore quantitative comparison was not possible.

Identification of chitin binding proteins secreted by *L. monocytogenes* during growth in media with and without chitin was achieved using a chitin bead pull-down assay. Both ChiB and ChiA were identified in the supernatant when the bacterium was grown in the presence of chitin (Fig. 8; proteins were identified by trypsination and MALDI-TOF MS or by comparison with the profiles of isogenic deletion mutants as described in [53]). *Lm*LPMO10, on the other hand, could not be detected by MALDI-TOF MS (or SDS-PAGE) in any of the conditions analyzed. The highest abundance of the chitinases was observed when the bacterium was grown in the presence of β-chitin. A third prominent protein (~65 kDa) observed in all conditions was by MALDI TOF MS identified to be the virulence factor p60 (Uniprot ID: P21171), whose affinity towards chitin most likely stems from its two LysM (CBM50) modules that are generally known for chitin-binding properties [57]. Whether this protein is involved in chitin degradation is unknown, but existing literature has shown this protein to be an autolysin that participates in cell division and hydrolysis of peptidoglycan [58-60].

In order to identify proteins secreted by *L. monocytogenes* that were below the threshold of detection by SDS-PAGE and subsequent MALDI-TOF MS analysis (see above), a more comprehensive and highly resolved analysis was obtained by Orbitrap LC-MS. ChiA and ChiB were identified in the stationary phase in the presence and absence of chitin, whereas *Lm*LPMO10 only in the absence of chitin (Table 1). The strong binding of *Lm*LPMO10 to chitin (Fig. 2A) may have prevented its identification in the supernatant from the chitin-containing samples. None of the three enzymes were detected in the mid-exponential phase in any of the conditions.

All in all the chitin pull-down assay, northern blot and secretome analysis results indicate that the presence of either crystalline chitin or amorphous chitin (both excellent substrates for all three enzymes) initiates a markedly higher level of secretion for both ChiA and ChiB, but not *Lm*LPMO10. This is in agreement with the fact that deletion of the *Lm*LPMO10 gene (*lmo2467*) does not impair chitin hydrolysis during growth of *L. monocytogenes* in solid medium containing chitin [6] and may indicate that *Lm*LPMO10 is not part of the *L. monocytogenes* chitinolytic system. On the other hand, it is interesting to note that the activity of ChiA has also been deemed important for *L. monocytogenes* host colonization [33]. It is thus plausible that ChiA is a bi-functional enzyme.

Conclusions

The *L. monocytogenes* chitinolytic system appears to be fully functional and can likely be utilized by the bacterium to exploit chitin as a source of carbon and nitrogen. Secretome analysis clearly shows that the chitinases are expressed during stationary phase and with increased abundance when chitin is present as a carbon source. We importantly show that the *L. monocytogenes* LPMO is an active enzyme. Although *Lm*LPMO10 shows activity on chitin and also acts in synergy with the *L. monocytogenes* chitinases, our secretome and transcriptional data indicate that the role of this enzyme may be uncoupled from the chitinases. Evidence from the literature points towards a possible alternative role in virulence, but the exact mechanism of action in such a case remains unclear.

MATERIALS AND METHODS

Bacterial strains and culturing conditions

Listeria monocytogenes EGD-e was kindly provided by Dr. W. Goebel (Biozentrum, University of Würzburg, Germany).

For analysis of secreted proteins, *L. monocytogenes* EGD-e was cultured aerobically at 30°C in 50 ml LB medium containing 3.3 g/L colloidal chitin, 10 g/L β-chitin or no additional carbon source (control). For the chitin pull-down assay (described below), cultures were grown overnight prior to analysis. For Orbitrap LC-MS analysis (described below), cultures were grown to OD_{600} 0.7 (exponential phase) and OD_{600} 1.2 (stationary phase) before harvesting. At the appropriate time/ OD, cultures were centrifuged for 10 min at 6000 g in order to sediment cells and remaining chitin particles. Subsequently, culture supernatants were sterile filtered and subjected to the chitin pull-down assay or the MALDI-TOF MS and Orbitrap LC-MS analysis.

*Cloning of lmo0105 (*ChiB*) and lmo2467 (Lm*LPMO10)

Chromosomal DNA was obtained from overnight-grown *L. monocytogenes* EGD-e using the DNeasy blood and tissue kit (Qiagen). The genes *lmo0105 (*Genebank ID: CAC98320.1*;* protein name proposed in this study; ChiB) and *lmo2467* (Genebank ID: CAD00545.1, protein name proposed in this study; *Lm*LPMO10) were amplified from genomic DNA using the forward and reverse primer pairs listed in Table 2. A truncated variant of ChiB lacking the C-terminal CBM 5/12 module (corresponding to amino acid residues 711-755) was made using the wild-type gene forward primer and a reverse primer entering a stop codon at amino acid position 710. The truncated ChiB variant was named "ChiBΔ".

The amplified gene products were cloned into the pET-46 Ek / LIC vector using the ligation-independent cloning kit (Novagen). The vector controls gene expression by a T7 promoter and includes an N-terminal hexa-histidine tag separated from the target gene by a sequence encoding an enterokinase (Ek) cleavage site. The position of the Ek site allows removal of the hexa-histidine tag with the Enterokinase protease, leaving no non-native amino acids on the target protein. The integrity of the gene constructs was confirmed by sequencing (Macrogen, Korea). Finally, all constructs were transformed into *E. coli* BL21 (DE3) for protein expression.

The second *L. monocytogenes* chitinase (ChiA, GenBank ID: CAC99961.1) was also included in this study. The cloning of this gene has been described previously [7].

Protein expression and purification

E. coli BL21 (DE3) strains containing the expression vectors encoding ChiA, ChiB and ChiBΔ were grown in LB supplemented with 100 μg/mL carbenicillin at 30°C to an OD_{600} of 0.4, at which point protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Following overnight incubation, the cells were harvested by centrifugation and resuspended in 20 mM MOPS, pH 7.2, containing 0.5 M NaCl and 5 mM imidazole. Cells were disrupted with the aid of a Constant Systems cell disruptor at 4°C at a pressure of 1.36 Kbar. The lysate was centrifuged at 4°C for 1.5 h at 48.000 *g* and the filtered supernatant was applied to a 1 mL Ni-NTA agarose column (Qiagen) operated by a peristaltic pump with a flow rate of 1.0 mL/min at 4 °C. The column was washed with 100 mL of 20 mM MOPS, pH 7.2, containing 0.5 M NaCl and 5 mM imidazole, and proteins were eluted in 100 mM MOPS, pH 7.8, containing 0.5 M NaCl and 0.5 M imidazole. The eluates were dialyzed against 50 mM sodium phosphate buffer, pH 6.0, at 4°C and concentrated in a Vivaspin (GE Healthcare) with a cutoff of 30 and 10 kDa for ChiB and ChiA, respectively.

For purification of *L. monocytogenes Lm*LPMO10, the *E. coli* BL21 (DE3) strain was grown in LB supplemented with 100 μg/mL ampicillin at 37°C to an OD_{600} of 0.4, followed by addition of IPTG to a final concentration of 1 mM for induction of protein expression. Incubation of the induced cells was continued for 4 h followed by cell-harvesting by centrifugation. The cell pellet was resuspended in 50 mM Tris-HCl, pH 8.0, containing 20 mM imidazole, followed by cell disruption with a sonication probe adjusted to 27% intensity and a sonication cycle of 0.5 s on 0.5 s off for 30 seconds, using a Vibra cell Ultrasonic Processor (Sonics, Newton, CT, USA). The cells were kept on ice at all times. The lysate was centrifuged for 10 min at 30000 *g* at 4°C and the resulting supernatant was filtered using a 0.2 micron sterile filter (Millipore). Using an Äkta Purifier (GE Healthcare) protein purification system, the filtrate was applied on a 5 mL Ni-NTA agarose column (Qiagen) equilibrated with binding buffer (50 mM Tris-HCl, pH 8.0, 20 mM imidazole) at a flow rate of 1 mL/min. His-tagged protein bound to the column was eluted with 50 mM Tris-HCl, pH 8.0 containing 0.5 M imidazole, collected and concentrated using an Amicon Ultra-centrifugal filter unit with a 10 kDa

cutoff (Millipore). The same centrifugal device was used to exchange the elution buffer to 50 mM Tris-HCl, pH 8.0. The Hexa-histidine tag was removed by adding 0.5 units EKMax™ enterokinase (Life Technologies) per mg His-tagged protein in 1 mL reactions containing 50 mM Tris-HCl pH 8.0 and 1 mM $CaCl_2$. After incubation for 16 h at 37°C, the reaction mixture was applied on a Ni-NTA column equilibrated with 50 mM Tris-HCl, pH 8.0 as binding buffer, in order to isolate the uncleaved proteins and the free hexa-histidine tags. The flow-through, containing the native form of *Lm*LPMO10 and the EKMax™ enterokinase, was concentrated using an Amicon Ultra centrifugal filter unit and applied on a Superdex 75 HiLoad 16/60 (GE Healthcare) size exclusion chromatography (SEC) column operated by an Äkta Purifier in order to separate the two proteins (*Lm*LPMO10~50 kDa, EKMax™~22 kDa). The running buffer was composed of 50 mM Tris pH 8 and was applied at a flow rate of 0.5 mL/min. The fraction containing *Lm*LPMO10 was collected, sterile filtered using a 0.2 micron filter, concentrated to approximately 2.0 mg/mL with an ultra-centrifugal filter unit with a 10 kDa cutoff (Millipore) and stored at 4°C until use.

CBP21 and *Sm*ChiC were expressed and purified as previously described by Vaaje-Kolstad *et al.* [38] and Synstad *et al.* [48], respectively,

Cu(II) saturation of LPMOs

Before use in activity assays, *Lm*LPMO10 and CBP21 were saturated with Cu(II) according to the protocol described by Loose *et al.* [25]. Briefly, the enzymes were incubated for 30 minutes at room temperature with $Cu(II)SO_4$ at a 1:3 molar ratio (enzyme:copper). After saturation, the enzymes were run through a PD MidiTrap G-25 (GE Healthcare) desalting column using 20 mM Tris HCl pH 8.0 as running buffer to remove excess $Cu(II)SO_4$.

Preparation of chitin substrates

Colloidal chitin was prepared by stirring 5 g shrimp shell α-chitin particles (C9213, Sigma-Aldrich) overnight in 50 ml 36-38% HCl. Following this treatment, the pH was adjusted to ~8 by addition of NaOH. In order to wash the chitin, the suspension was pelleted by centrifugation followed by decanting of the supernatant and resuspension of the chitin in MilliQ water. The washing step was repeated seven times and the pure colloidal chitin pellet was stored at 4°C until use. Pure β-chitin particles were purchased from France Chitin (Orange, France) and was also used to generate the β-chitin nano-fibres according to the protocol described by Fan *et al.* [61]. In short, 75 mg β-chitin was

suspended in 1.8 mM acetic acid to a final concentration of 10 mg/mL and sonicated at 35% amplitude for 4 minutes, using a Vibra Cell Ultrasonic Processor (Sonics). For enzyme reactions, all chitin variants were used in concentrations high enough to ensure substrate saturation, but at the same time enabling appropriate mixing and pipetting.

Chitin-binding assays

Binding of *Lm*LPMO10 was assayed in 100 μL reactions containing 10 mg/mL of substrate (α-chitin (Chitinor, Norway), β-chitin (France Chitin) or Avicel cellulose (Sigma)) and 0.2 mg/mL purified *Lm*LPMO10 in 20 mM Bis-Tris pH 6.3. Control reactions were identical, but contained no substrate. The reactions were incubated at 37°C for 3 h. Subsequently, the substrate was sedimented by centrifugation at 16000 g for 5 min. The supernatant (containing the non-bound protein) was decanted off and kept on ice until analysis. The pellet was washed twice with 1 mL buffer (20 mM Bis-Tris pH 6.3), followed by resuspension in 50 μL SDS-PAGE sample buffer (Invitrogen) and 10 min incubation at 99°C. 7.5 μL of supernatant and 15 μL of the proteins that had remained bound to the pellets were analyzed using SDS-PAGE Mini-Protean Stain-free 10% gels (Bio-Rad).

LPMO and chitinase activity assays

Enzyme activity assays were conducted by incubating 10 mg/mL β-chitin or α–chitin with 0.1 μM chitinase in 500 μL reactions buffered by 50 mM Bis-Tris pH 6.0, in the presence or absence of 1.0 μM LPMO. 1.0 mM ascorbate was included as an external electron donor in all assays. Assays determining LPMO activity were conducted by incubating 5 mg/mL sonicated β-chitin (chitin nano-fibrils) or 7.5 mg/mL colloidal chitin with 1.0 μM LPMO in 500 μL reactions buffered by 50 mM Tris pH 8.0 and 1.0 mM ascorbate. Reactions were incubated vertically at 37°C in an Eppendorf Thermomixer shaking continuously at 1000 rpm. Samples of the reaction mixtures were taken at regular intervals for determination of product profiles and quantities. LPMO reactions were stopped by separating the insoluble substrate from the products by filtration using a 96-well filter plate (Millipore) operated by a Millipore vacuum manifold. Chitinase reactions were stopped by boiling for 10 min, followed by the filtration procedure described above. Soluble products formed by LPMO activity were analyzed qualitatively by MALDI-TOF MS and quantitatively (and qualitatively) by hydrophilic interaction chromatography (HILIC) using an Agilent Technologies 1290 Infinity equipped with an Acquity UPLC BEH Amide 150 mm column. The specific details of both protocols have been published previously [13]. In short, MALDI-TOF MS was conducted by mixing 1 μL

sample with 2 µL 9 mg/mL 2,5-dihydroxybenzoic acid (DHB) in 30 % Acetonitrile on a MTP 384 target plate (Bruker Daltonics), followed by drying and analysis with an Ultraflex MALDI-TOF/TOF instrument (Bruker Daltonics GmbH) operated in positive mode. Separation of the oxidized chitooligosaccharides by HILIC was accomplished by running a linear gradient running from 74% to 62% acetonitrile/15 mM Tris-HCL, pH 8.0. Products were detected by monitoring absorption at 205 nm. Standards of chitooligosaccharide aldonic acids ranging in DP from 1 to 6 were generated according to the protocol described in [25].

Quantification of the dominant product from the chitinase reactions ($(GlcNAc)_2$; represents >75% of the total products formed) was achieved by ion-affinity chromatography, using a Dionex Ultimate 3000 UPLC system equipped with a Rezex column heated to 80°C, using 5 mM H_2SO_4 as the mobile phase at 1.0 mL/min. Chitooligosaccahrides were separated by isocratic chromatography and detected by monitoring absorption at 194 nm. Standards were run regularly to ensure precise quantification of the analytes.

Identification of chitinolytic proteins in L. monocytogenes culture supernatants (pull down assay)

10 mL of sterile-filtered supernatants acquired from ON-grown *L. monocytogenes* cultures was mixed with 50-70 µL magnetic chitin beads and incubated overnight at 200 rpm and 30°C to allow binding of proteins with affinity to the beads. Thereafter, the beads were separated with the use of a magnet and washed twice with 1 mL 50 mM Tris-HCl pH 8.0. In order to release proteins bound to the chitin beads, 20 µL SDS-PAGE sample buffer was added and the suspension was incubated for 10 min at 99°C. The samples were loaded on a 10% SDS-PAGE gel (Invitrogen) for protein separation. SDS-PAGE gels were stained with SYPRO Ruby or SimpleBlue Safestain (Invitrogen) following the manufacturer's instructions.

Protein identification by MALDI-TOF MS

Proteins of interest identified from the chitin pull-down assay by SDS-PAGE (see above) were analyzed by MALDI-TOF MS. Sample preparation and identification was carried out as described in Berner *et al.* (2013), with a modified protocol. The modification entailed inclusion of a reduction and alkylation step after dehydration, involving incubation with 10 mM DTT at 56°C for 45 min, followed by addition of 55 mM iodoacetamine and incubation in the dark at room temperature for 30 min.

Peptides identified were analyzed using the MASCOT software suite equipped with the NCBI prokaryotic genomes database as a search database.

Protein identification by Orbitrap LC-MS

Supernatants obtained from *L. monocytogenes* cultures were transferred to centrifugal ultrafiltration units with a 10 kDa cutoff (Millipore) and centrifuged for 14 min at 4500 g to concentrate proteins in the sample. Next, samples were diluted 10-fold in 50 mM Tris-HCl pH 7.5, followed by re-concentration in order to remove salts and other small MW medium components. The desalting procedure was repeated six times and samples were stored at -20°C until further analysis.

Proteins were prepared for trypsination by adjusting 20 µL of each sample to 50 mM NH_4HCO_3 and 10 mM DTT, followed by incubation at 56ºC for 30 min. After cooling down to room temperature, samples were adjusted to 50 mM iodoacetamide followed by 30 min incubation in the dark, addition of DTT to 50 mM and incubation for 15 min at room temperature. For proteolytic digestion, 2.5 µL immobilized trypsin (Poroszyme, Life Technologies) was equilibrated using 25 mM NH_4HCO_3/ 5 % acetonitrile, followed by application of the protein solution. The resulting peptides were collected by a C18 membrane (Empore) that was conditioned with $MeOH_{absolut}$ and equilibrated with NH_4HCO_3/5 % acetonitrile prior to use. The bound peptides were washed twice with 10 µL 0.1 % TFA and eluted with 5 µL 70 % acetonitrile/ 0.1 % TFA. The eluent was dried using a vacuum centrifuge.

Orbitrap LC-MS was carried out using a QExactive/Ultimate 3000 RSLCnano (ThermoFisher) setup, and was performed as follows: the dried peptides were dissolved in loading solution (0.05 %TFA, 2% ACN in water), loaded onto a trap column (Acclaim PepMap100, C18, 5 µm, 100 Å, 300 µm i.d. x 5 mm) and then backflushed onto a 50 cm x 75 µm analytical column (Acclaim PepMap RSLC C18, 2 µm, 100 Å, 75 µm i.d. x 50 cm, nanoViper). A 90 min gradient from 4 to 40 % solution B (80 % ACN, 0.1% formic acid) was used for separation of the peptides, at a flow rate of 300 nL/min. The Q-Exactive mass spectrometer was set up as follows (Top10 method): a full scan (300-1600 m/z) at R=70.000 was followed by (up to) 10 MS2 scans at R=35000, using an NCE setting of 28. Singly charged precursors were excluded for MSMS, as were precursors with z>5. Dynamic exclusion was set to 20 seconds. Raw files were converted to mgf format using the msconvert module of ProteoWizard (http://proteowizard.sourceforge.net/). The resulting mgf files were submitted to database search (automatic decoy option) against the *L. monocytogenes* EGD-e database (downloaded

from UniprotKB) on an in-house Mascot (v.2.4) server using 10 ppm/ 20mamu tolerance for MS and MS/MS, respectively, and allowing for up to 2 miscleavages. Carbamidomethylated cysteine and oxidized methionine were selected as fixed and variable modifications, respectively.

RNA extraction and Northern blot analysis

Bacterial cultures intended for transcriptional analysis were grown aerobically in LB at 30°C with shaking at 190 rpm overnight. Subsequently, the cultures were diluted to an OD_{600} of 0.05 in 30 mL of LB supplemented with 0.05% glucose, and thereafter grown at 30°C with shaking at 190 rpm until late-exponential phase (OD_{600} = 0.7). At this point 15 mL of each culture was transferred to new sterile flasks containing either colloidal chitin, α-chitin, β-chitin or water (control for β-chitin), to reach a final concentration of 3.3 g/L for the colloidal and α-chitin (C9213, Sigma-Aldrich) and 1.33 g/L for the β-chitin. Both induced and uninduced cultures were incubated further at 30°C with shaking and samples for RNA extraction were harvested after 15 min and 2 h, corresponding to late exponential and stationary phase, respectively. RNA extraction and Northern blot analysis were carried out as described previously by Larsen *et al.* [5] using the primer pair lmo2467P_F: CGACAAATTTAGCAGCGACA and lmo2467P_R: CCGATTTCCAGGTGTTCAGT for the amplification of the DNA probe.

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AUTHOR CONTRIBUTIONS

D.K.P: Planned experiments, performed experiments, analyzed data, wrote the paper. J.S.M.L.: Planned experiments, performed experiments, analyzed data, wrote the paper. M.H.L.: Planned experiments, analyzed data, wrote the paper. G.V-K: Planned experiments, analyzed data, wrote the paper.

REFERENCES

1. Freitag, N. E., Port, G. C. & Miner, M. D. (2009) *Listeria monocytogenes* - from saprophyte to intracellular pathogen. *Nat Rev Microbiol* **7**, 623-628.
2. Gray, M. J., Freitag, N. E. & Boor, K. J. (2006) How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect Immun* **74**, 2505-2512.
3. Sauders, B. D. & Wiedmann, M. (2007) Ecology of Listeria species and *L. monocytogenes* in the natural environment, 3 edn, CRC Press
4. Nelson, K. E., Fouts, D. E., Mongodin, E. F., Ravel, J., DeBoy, R. T., Kolonay, J. F., Rasko, D. A., Angiuoli, S. V., Gill, S. R., Paulsen, I. T., Peterson, J., White, O., Nelson, W. C., Nierman, W., Beanan, M. J., Brinkac, L. M., Daugherty, S. C., Dodson, R. J., Durkin, A. S., Madupu, R., Haft, D. H., Selengut, J., Van Aken, S., Khouri, H., Fedorova, N., Forberger, H., Tran, B., Kathariou, S., Wonderling, L. D., Uhlich, G. A., Bayles, D. O., Luchansky, J. B. & Fraser, C. M. (2004) Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. *Nucleic Acids Res* **32**, 2386-2395.
5. Larsen, M. H., Leisner, J. J. & Ingmer, H. (2010) The chitinolytic activity of *Listeria monocytogenes* EGD is regulated by carbohydrates but also by the virulence regulator PrfA. *Appl Environ Microbiol* **76**, 6470-6476.
6. Leisner, J. J., Larsen, M. H., Jorgensen, R. L., Brondsted, L., Thomsen, L. E. & Ingmer, H. (2008) Chitin hydrolysis by Listeria spp., including *L. monocytogenes*. *Appl Environ Microbiol* **74**, 3823-3830.
7. Leisner, J. J., Larsen, M. H., Ingmer, H., Petersen, B. O., Duus, J. O. & Palcic, M. M. (2009) Cloning and comparison of phylogenetically related chitinases from *Listeria monocytogenes* EGD and *Enterococcus faecalis* V583. *J Appl Microbiol* **107**, 2080-2087.
8. Premaratne, R. J., Lin, W. J. & Johnson, E. A. (1991) Development of an improved chemically defined minimal medium for *Listeria monocytogenes*. *Appl Environ Microbiol* **57**, 3046-3048.
9. Piveteau, P., Depret, G., Pivato, B., Garmyn, D. & Hartmann, A. (2011) Changes in gene expression during adaptation of *Listeria monocytogenes* to the soil environment. *PLoS One* **6**, e24881.
10. Levasseur, A., Drula, E., Lombard, V., Coutinho, P. M. & Henrissat, B. (2013) Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. *Biotechnol Biofuels* **6**, 41.
11. Vaaje-Kolstad, G., Horn, S. J., van Aalten, D. M. F., Synstad, B. & Eijsink, V. G. H. (2005) The non-catalytic chitin-binding protein CBP21 from *Serratia marcescens* is essential for chitin degradation. *J Biol Chem* **280**, 28492-28497.
12. Vaaje-Kolstad, G., Bunæs, A. C., Mathiesen, G. & Eijsink, V. G. H. (2009) The chitinolytic system of *Lactococcus lactis* ssp. *lactis* comprises a nonprocessive chitinase and a chitin-binding protein that promotes the degradation of alpha- and beta-chitin. *FEBS J* **276**, 2402-2415.
13. Vaaje-Kolstad, G., Westereng, B., Horn, S. J., Liu, Z. L., Zhai, H., Sørlie, M. & Eijsink, V. G. H. (2010) An oxidative enzyme boosting the enzymatic conversion of recalcitrant polysaccharides. *Science* **330**, 219-222.
14. Purushotham, P., Arun, P. V., Prakash, J. S. & Podile, A. R. (2012) Chitin binding proteins act synergistically with chitinases in *Serratia proteamaculans* 568. *PLoS One* **7**, e36714.
15. Vaaje-Kolstad, G., Bøhle, L. A., Gåseidnes, S., Dalhus, B., Bjørås, M., Mathiesen, G. & Eijsink, V. G. H. (2012) Characterization of the chitinolytic machinery of *Enterococcus faecalis* v583 and high-resolution structure of its oxidative CBM33 enzyme. *J Mol Biol* **416**, 239-254.
16. Nakagawa, Y. S., Eijsink, V. G. H., Totani, K. & Vaaje-Kolstad, G. (2013) Conversion of alpha-chitin substrates with varying particle size and crystallinity reveals substrate preferences of the

595 chitinases and lytic polysaccharide monooxygenase of *Serratia marcescens*. *J Agric Food Chem* **61**,
596 11061-11066.
597 17. Aachmann, F. L., Sørlie, M., Skjåk-Bræk, G., Eijsink, V. G. H. & Vaaje-Kolstad, G. (2012) NMR
598 structure of a lytic polysaccharide monooxygenase provides insight into copper binding, protein
599 dynamics, and substrate interactions. *Proc Natl Acad Sci U S A* **109**, 18779-18784.
600 18. Hemsworth, G. R., Davies, G. J. & Walton, P. H. (2013) Recent insights into copper-containing
601 lytic polysaccharide mono-oxygenases. *Curr Opin Struct Biol* **23**, 660-668.
602 19. Hemsworth, G. R., Taylor, E. J., Kim, R. Q., Gregory, R. C., Lewis, S. J., Turkenburg, J. P.,
603 Parkin, A., Davies, G. J. & Walton, P. H. (2013) The copper active site of CBM33 polysaccharide
604 oxygenases. *J Am Chem Soc* **135**, 6069-6077.
605 20. Phillips, C. M., Beeson, W. T., Cate, J. H. & Marletta, M. A. (2011) Cellobiose dehydrogenase
606 and a copper-dependent polysaccharide monooxygenase potentiate cellulose degradation by
607 *Neurospora crassa*. *ACS Chem Biol* **6**, 1399-1406.
608 21. Quinlan, R. J., Sweeney, M. D., Lo Leggio, L., Otten, H., Poulsen, J. C., Johansen, K. S., Krogh,
609 K. B., Jorgensen, C. I., Tovborg, M., Anthonsen, A., Tryfona, T., Walter, C. P., Dupree, P., Xu, F.,
610 Davies, G. J. & Walton, P. H. (2011) Insights into the oxidative degradation of cellulose by a copper
611 metalloenzyme that exploits biomass components. *Proc Natl Acad Sci U S A* **108**, 15079-15084.
612 22. Beeson, W. T., Phillips, C. M., Cate, J. H. & Marletta, M. A. (2012) Oxidative cleavage of
613 cellulose by fungal copper-dependent polysaccharide monooxygenases. *J Am Chem Soc* **134**, 890-
614 892.
615 23. Isaksen, T., Westereng, B., Aachmann, F. L., Agger, J. W., Kracher, D., Kittl, R., Ludwig, R.,
616 Haltrich, D., Eijsink, V. G. H. & Horn, S. J. (2014) A C4-oxidizing lytic polysaccharide
617 monooxygenase cleaving both cellulose and cello-oligosaccharides. *J Biol Chem* **289**, 2632-2642.
618 24. Forsberg, Z., Røhr, A. K., Mekasha, S., Andersson, K. K., Eijsink, V. G. H., Vaaje-Kolstad, G.
619 & Sørlie, M. (2014) Comparative study of two chitin-active and two cellulose-active AA10-type lytic
620 polysaccharide monooxygenases. *Biochemistry* **53**, 1647-1656.
621 25. Loose, J. S. M., Forsberg, Z., Fraaije, M. W., Eijsink, V. G. H. & Vaaje-Kolstad, G. (2014) A
622 rapid quantitative activity assay shows that the *Vibrio cholerae* colonization factor GbpA is an active
623 lytic polysaccharide monooxygenase. *FEBS Lett* **588**, 3435-3440.
624 26. Frederiksen, R. F., Paspaliari, D. K., Larsen, T., Storgaard, B. G., Larsen, M. H., Ingmer, H.,
625 Palcic, M. M. & Leisner, J. J. (2013) Bacterial chitinases and chitin-binding proteins as virulence
626 factors. *Microbiology* **159**, 833-47.
627 27. Bhowmick, R., Ghosal, A., Das, B., Koley, H., Saha, D. R., Ganguly, S., Nandy, R. K., Bhadra,
628 R. K. & Chatterjee, N. S. (2008) Intestinal adherence of *Vibrio cholerae* involves a coordinated
629 interaction between colonization factor GbpA and mucin. *Infect Immun* **76**, 4968-4977.
630 28. DebRoy, S., Dao, J., Soderberg, M., Rossier, O. & Cianciotto, N. P. (2006) *Legionella*
631 *pneumophila* type II secretome reveals unique exoproteins and a chitinase that promotes bacterial
632 persistence in the lung. *Proc Natl Acad Sci U S A* **103**, 19146-19151.
633 29. Kawada, M., Chen, C. C., Arihiro, A., Nagatani, K., Watanabe, T. & Mizoguchi, E. (2008)
634 Chitinase 3-like-1 enhances bacterial adhesion to colonic epithelial cells through the interaction with
635 bacterial chitin-binding protein. *Lab Invest* **88**, 883-895.
636 30. Kirn, T. J., Jude, B. A. & Taylor, R. K. (2005) A colonization factor links *Vibrio cholerae*
637 environmental survival and human infection. *Nature* **438**, 863-866.
638 31. Tran, H. T., Barnich, N. & Mizoguchi, E. (2011) Potential role of chitinases and chitin-binding
639 proteins in host-microbial interactions during the development of intestinal inflammation. *Histol*
640 *Histopathol* **26**, 1453-1464.

641 32. Chaudhuri, S., Bruno, J. C., Alonzo, F., Xayarath, B., Cianciotto, N. P. & Freitag, N. E. (2010)
642 Contribution of chitinases to *Listeria monocytogenes* pathogenesis. *Appl Environ Microbiol* **76**, 7302-
643 7305.
644 33. Chaudhuri, S., Gantner, B. N., Ye, R. D., Cianciotto, N. P. & Freitag, N. E. (2013) The *Listeria*
645 *monocytogenes* ChiA chitinase enhances virulence through suppression of host innate immunity.
646 *mBio* **4**, e00617-12.
647 34. Wong, E., Vaaje-Kolstad, G., Ghosh, A., Hurtado-Guerrero, R., Konarev, P. V., Ibrahim, A. F.
648 M., Svergun, D. I., Eijsink, V. G. H., Chatterjee, N. S. & van Aalten, D. M. F. (2012) The *Vibrio*
649 *cholerae* colonization factor gbpa possesses a modular structure that governs binding to different host
650 surfaces. *Plos Pathog* **8**.
651 35. Jude, B. A., Martinez, R. M., Skorupski, K. & Taylor, R. K. (2009) Levels of the secreted *Vibrio*
652 *cholerae* attachment factor GbpA are modulated by quorum-sensing-induced proteolysis. *J Bacteriol*
653 **191**, 6911-6917.
654 36. Zampini, M., Pruzzo, C., Bondre, V. P., Tarsi, R., Cosmo, M., Bacciaglia, A., Chhabra, A.,
655 Srivastava, R. & Srivastava, B. S. (2005) *Vibrio cholerae* persistence in aquatic environments and
656 colonization of intestinal cells: involvement of a common adhesion mechanism. *FEMS microbiol lett*
657 **244**, 267-273.
658 37. Suzuki, K., Suzuki, M., Taiyoji, M., Nikaidou, N. & Watanabe, T. (1998) Chitin binding protein
659 (CBP21) in the culture supernatant of *Serratia marcescens* 2170. *Biosci Biotechnol Biochem* **62**, 128-
660 135.
661 38. Vaaje-Kolstad, G., Houston, D. R., Riemen, A. H. K., Eijsink, V. G. H. & van Aalten, D. M. F.
662 (2005) Crystal structure and binding properties of the *Serratia marcescens* chitin-binding protein
663 CBP21. *J Biol Chem* **280**, 11313-11319.
664 39. Zeltins, A. & Schrempf, H. (1997) Specific interaction of the Streptomyces chitin-binding protein
665 CHB1 with alpha-chitin--the role of individual tryptophan residues. *Eur J Biochem* **246**, 557-564.
666 40. Chu, H. H., Hoang, V., Hofemeister, J. & Schrempf, H. (2001) A *Bacillus amyloliquefaciens*
667 ChbB protein binds beta- and alpha-chitin and has homologues in related strains. *Microbiology* **147**,
668 1793-803.
669 41. Forsberg, Z., Vaaje-Kolstad, G., Westereng, B., Bunæs, A. C., Stenstrøm, Y., Mackenzie, A.,
670 Sørlie, M., Horn, S. J. & Eijsink, V. G. H. (2011) Cleavage of cellulose by a CBM33 protein. *Protein*
671 *Sci* **20**, 1479-1483.
672 42. Hashimoto, M., Ikegami, T., Seino, S., Ohuchi, N., Fukada, H., Sugiyama, J., Shirakawa, M. &
673 Watanabe, T. (2000) Expression and characterization of the chitin-binding domain of chitinase A1
674 from *Bacillus circulans* WL-12. *J Bacteriol* **182**, 3045-3054.
675 43. Brun, E., Gans, P., Marion, D. & Barras, F. (1995) Overproduction, purification and
676 characterization of the cellulose-binding domain of the *Erwinia chrysanthemi* secreted endoglucanase
677 Egz. *Eur J Biochem* **231**, 142-148.
678 44. Boraston, A. B., Bolam, D. N., Gilbert, H. J. & Davies, G. J. (2004) Carbohydrate-binding
679 modules: fine-tuning polysaccharide recognition. *Biochem J* **382**, 769-781.
680 45. Moser, F., Irwin, D., Chen, S. L. & Wilson, D. B. (2008) Regulation and characterization of
681 Thermobifida fusca carbohydrate-binding module proteins E7 and E8. *Biotechnol Bioeng* **100**, 1066-
682 1077.
683 46. Vaaje-Kolstad, G., Horn, S. J., Sørlie, M. & Eijsink, V. G. H. (2013) The chitinolytic machinery
684 of *Serratia marcescens* - a model system for enzymatic degradation of recalcitrant polysaccharides.
685 *FEBS J* **280**, 3028-3049
686 47. Payne, C. M., Baban, J., Horn, S. J., Backe, P. H., Arvai, A. S., Dalhus, B., Bjoras, M., Eijsink,
687 V. G., Sorlie, M., Beckham, G. T. & Vaaje-Kolstad, G. (2012) Hallmarks of processivity in glycoside

688 hydrolases from crystallographic and computational studies of the Serratia marcescens chitinases. *J*
689 *Biol Chem* **287**, 36322-36330.
690 48. Synstad, B., Vaaje-Kolstad, G., Cederkvist, H., Saua, S. F., Horn, S. J., Eijsink, V. G. H. & Sørlie,
691 M. (2008) Expression and characterization of endochitinase C from *Serratia marcescens* BJL200 and
692 its purification by a one-step general chitinase purification method. *Biosci Biotechnol Biochem* **72**,
693 715-723.
694 49. Suzuki, K., Taiyoji, M., Sugawara, N., Nikaidou, N., Henrissat, B. & Watanabe, T. (1999) The
695 third chitinase gene (chiC) of *Serratia marcescens* 2170 and the relationship of its product to other
696 bacterial chitinases. *Biochem J* **343 Pt 3**, 587-96.
697 50. Horn, S. J., Sikorski, P., Cederkvist, J. B., Vaaje-Kolstad, G., Sørlie, M., Synstad, B., Vriend, G.,
698 Vårum, K. M. & Eijsink, V. G. H. (2006) Costs and benefits of processivity in enzymatic degradation
699 of recalcitrant polysaccharides. *Proc Natl Acad Sci U S A* **103**, 18089-18094.
700 51. Horn, S. J., Sørbotten, A., Synstad, B., Sikorski, P., Sørlie, M., Vårum, K. M. & Eijsink, V. G.
701 H. (2006) Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia*
702 *marcescens*. *FEBS J* **273**, 491-503.
703 52. Sørbotten, A., Horn, S. J., Eijsink, V. G. H. & Vårum, K. M. (2005) Degradation of chitosans
704 with chitinase B from *Serratia marcescens*. Production of chito-oligosaccharides and insight into
705 enzyme processivity. *FEBS J* **272**, 538-549.
706 53. Paspaliari, D. K., Mollerup, M. S., Kallipolitis, B. H., Ingmer, H. & Larsen, M. H. (2013)
707 Chitinase expression in *Listeria monocytogenes* is positively regulated by the *agr* system. *Plos One*
708 **9**, e95385.
709 54. Herve, C., Rogowski, A., Blake, A. W., Marcus, S. E., Gilbert, H. J. & Knox, J. P. (2010)
710 Carbohydrate-binding modules promote the enzymatic deconstruction of intact plant cell walls by
711 targeting and proximity effects. *Proc Natl Acad Sci U S A* **107**, 15293-15298.
712 55. Linder, M. & Teeri, T. T. (1997) The roles and function of cellulose-binding domains. *J*
713 *Biotechnol* **57**, 15-28.
714 56. Westereng, B., Ishida, T., Vaaje-Kolstad, G., Wu, M., Eijsink, V. G. H., Igarashi, K., Samejima,
715 M., Stahlberg, J., Horn, S. J. & Sandgren, M. (2011) The putative endoglucanase pcgh61d from
716 *Phanerochaete chrysosporium* is a metal-dependent oxidative enzyme that cleaves cellulose. *Plos*
717 *One* **6**.
718 57. Ohnuma, T., Onaga, S., Murata, K., Taira, T. & Katoh, E. (2008) LysM domains from Pteris
719 ryukyuensis chitinase-A: a stability study and characterization of the chitin-binding site. *J Biol Chem*
720 **283**, 5178-5187.
721 58. Lenz, L. L., Mohammadi, S., Geissler, A. & Portnoy, D. A. (2003) SecA2-dependent secretion
722 of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. *Proc Natl Acad Sci U S A* **100**,
723 12432-12437.
724 59. Pilgrim, S., Kolb-Maurer, A., Gentschev, I., Goebel, W. & Kuhn, M. (2003) Deletion of the gene
725 encoding p60 in *Listeria monocytogenes* leads to abnormal cell division and loss of actin-based
726 motility. *Infect Immun* **71**, 3473-3484.
727 60. Wuenscher, M. D., Kohler, S., Bubert, A., Gerike, U. & Goebel, W. (1993) The iap gene of
728 *Listeria monocytogenes* is essential for cell viability, and its gene product, p60, has bacteriolytic
729 activity. *J Bacteriol* **175**, 3491-3501.
730 61. Fan, Y., Saito, T. & Isogai, A. (2008) Preparation of chitin nanofibers from squid pen beta-chitin
731 by simple mechanical treatment under acid conditions. *Biomacromolecules* **9**, 1919-1923.
732
733

734 **TABLES**

735

736 Table 1. Chitinolytic enzymes identified in the culture supernatants of *L. monocytogenes* grown to
737 stationary phase by Orbitrap LC-MS. No chitinolytic enzymes could be detected in the mid-
738 exponential phase.

Medium	Protein (UniProt ID)	Id. probability	Unique peptides	Unique spectra	Sequence coverage
LB	ChiA (Q8Y619)	100 %	10	12	36%
LB+β-chitin	ChiA (Q8Y619)	100 %	11	14	39%
LB+ colloidal chitin	ChiA (Q8Y619)	100 %	16	23	55%
LB	ChiB (Q8YAL3)	100 %	10	11	18%
LB+β-chitin	ChiB (Q8YAL3)	100 %	11	12	21%
LB+ colloidal chitin	ChiB (Q8YAL3)	100 %	16	19	32%
LB	*Lm*LPMO10 (Q8Y4H4)	100%	5	6	15%
LB+β-chitin	*Lm*LPMO10 (Q8Y4H4)	Not detected	-	-	-
LB+ colloidal chitin	*Lm*LPMO10 (Q8Y4H4)	Not detected	-	-	-

739

740

741 Table 2. Primers used for cloning *chiB* and *lmo2467*. Primer sequences are shown from 5' to 3'.

Gene	Protein	Primer	Sequence
lmo0105	ChiB	*lmo0105_F*	GACGACGACAAGGAGCCAAAACGGGCGAAAG
		lmo0105_R	GAGGAGAAGCCCGGTTTAATTTATTAACAACCAAG
		lmo0105_ΔCBM_R	GAGGAGAAGCCCGGTTTATGCTGGTGGTGTTGCCGCGTC
lmo2467	*Lm*LPMO10	*lmo2467_F*	GACGACGACAAGCATGGATACATATCAAAACCG
		lmo2467_R	GAGGAGAAGCCCGGTTTAATTTAATAATGTCCAAATG

742

743

FIGURE LEGENDS

Figure 1. Catalytic activities of the *L. monocytogenes* chitin-degrading enzymes and their domain structures. (A) Reactions catalyzed by chitin-cleaving GH18s (hydrolysis of the glycosidic bond) and LPMO10s (lytic oxidation of the glycosidic bond). The end-products resulting from chitin hydrolysis by GH18 are $(GlcNAc)_2$ and GlcNAc. The soluble end-products observed from chitin oxidation by LPMO10s are chitooligosaccharide aldonic acids, $(GlcNAc)_nGlcNAc1A$, where "n" ranges from 1 to approximately 11. (B) The *L. monocytogenes* genome contains three putative chitinolytic enzymes, two family GH18 chitinases (ChiA, ChiB) and one family AA10 LPMO (*Lm*LPMO10A). The size of the rectangles representing the individual domains is not related/indicative of/scaled to domain size (number of amino acids).

Figure 2. Substrate-binding of *Lm*LPMO10 and characterization of its LPMO activity. (A) The amount of *Lm*LPMO10 (0.2 mg/ml) bound to 10 mg/ml α-chitin (α), β-chitin (β), and cellulose (cel) in 20 mM Bis-Tris pH 6.3 is depicted in comparison to the non-bound protein remaining in the supernatant after 3h of incubation with the substrates at 37°C. NC refers to the control sample with no substrate. The faint band observed in the (NC) indicates that *Lm*LPMO10 binds the inner walls of the test tube (protein precipitation was not observed). Results were reproduced in at least two independent experiments. (B) Product profiles generated by the activity of 1.0 μM *Lm*LPMO10 (A) towards 10 mg/ml α-chitin or 5 mg/ml β-chitin nano-fibers were identified by MALDI-TOF MS. All reactions were performed in 20 mM Bis-Tris pH 6.3, incubated at 37°C, using 1.0 mM ascorbic acid as electron donor. Each chitooligosaccharide aldonic acid product was identified by two masses representing the $[M+Na^+]$ and $[M+K^+]$ adducts. The masses observed were 869.1/ 885.1 ($DP4_{ox}$), 1072.2/ 1088.2 ($DP5_{ox}$), 1275.2/ 1291.2 ($DP6_{ox}$), 1478.3/ 1494.3 ($DP7_{ox}$) and 1681.4/ 1697.4 ($DP8_{ox}$). DPn_{ox} indicates the degree of polymerization (DP) of the C1 oxidized chitooligosaccharide (e.g. $DP6_{ox}$ refers to $(GlcNAc)_5GlcNAc1A$, where GlcNAc1A is the aldonic acid form of GlcNAc). The experiment was repeated multiple times with essentially identical outcomes.

Figure 3. Separation and relative quantification of oxidized chitooligosaccharides from β-chitin nano-fibers. (A) HILIC chromatogram representing products generated by incubation of 1.0 μM *Lm*LPMO10 with 5 mg/ml β-chitin nano-fibers in 50 mM Bis-Tris pH 8.0, incubated for 3 h at 37°C using 1.0 mM ascorbic acid as electron donor. The identities of the oligosaccharides were determined by peak fractionation and concomitant MALDI-TOF MS. The chromatogram was obtained by

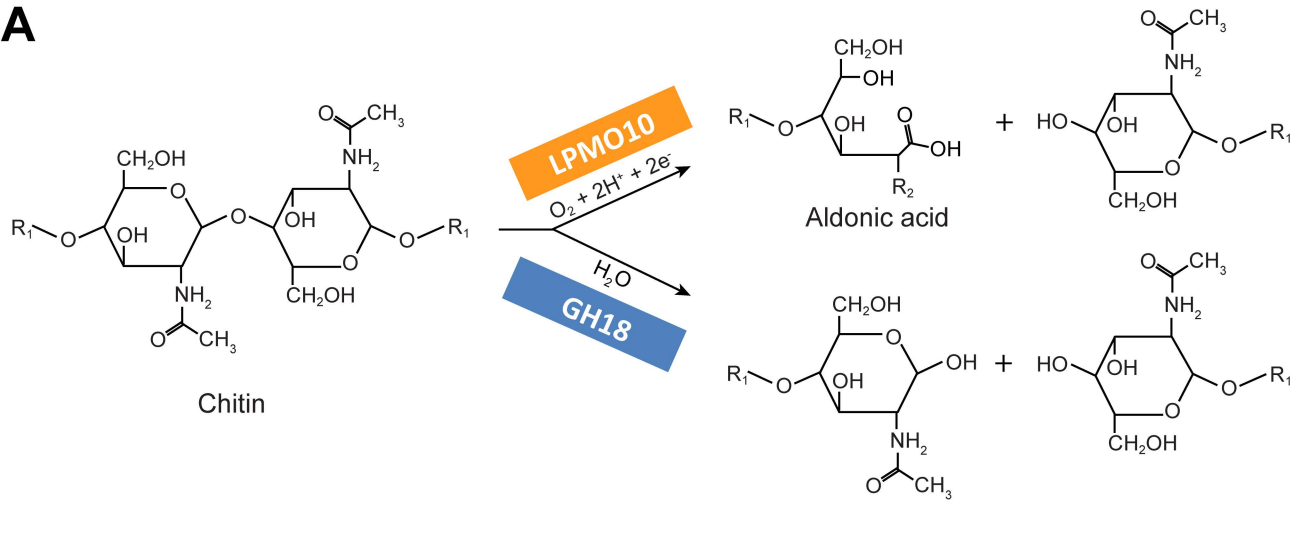
recording absorption at 195 nm. The relative rates of 1.0 μM *Lm*LPMO10 (squares on complete line) and CBP21 (diamonds on dashed line) towards β-chitin nano-fibers (same conditions as described for the panel A experiment) are shown as the increase of $DP4_{ox}$ (B), $DP5_{ox}$ (C), $DP6_{ox}$ (D), $DP7_{ox}$ (E) and $DP8_{ox}$ (F) over time. Standard deviation is represented by error bars (n=3), which are mostly hidden by the data point symbols.

Figure 4. Separation and relative quantification of oxidized chitooligosaccharides from colloidal chitin. (A) Profile of products generated by 1.0 μM *Lm*LPMO10 incubated with 7.5 mg/mL colloidal chitin and 1.0 mM ascorbate for 100 min at 37°C analyzed by HILIC and obtained by recording absorption at 195 nM. The relative rates of 1.0 μM *Lm*LPMO10 (squares on complete line) and 1.0 μM CBP21 (diamonds on dashed line) towards colloidal chitin (reaction conditions stated above) are shown as the generation of $DP4_{ox}$ (B), $DP5_{ox}$ (C), $DP6_{ox}$ (D), $DP7_{ox}$ (E) and $DP8_{ox}$ (F) over time. Standard deviation is represented by error bars (n=3), which are mostly hidden by the data point symbols.

Figure 5. Degradation of β-chitin by chitinases in the presence or absence of LPMOs. The accumulation of the dominant product, $(GlcNAc)_2$, released from the enzymatic depolymerization of 10 mg/mL β-chitin by 0.1 μM ChiA (A), ChiB (B) and ChiBΔ (ChiB lacking the chitin binding modules; C) in 50 mM Bis-Tris pH 6.0 was quantified in the absence (dashed line on diamonds) or presence of 1.0 μM *Lm*LMPO10A (dotted line on rectangles) or 1.0 μM CBP21 (full line on triangles). For comparison the same reaction was conducted and analyzed for *Sm*ChiC (D) in the absence (dashed line on diamonds) or presence of CBP21 (full line on rectangles) or *Lm*LMPO10A (dotted line on rectangles). Error bars indicate standard deviation (n=3). Some error bares are concealed by the data point symbols.

Figure 6. Analysis of reaction products from chitin degradation experiments. (A) Soluble products from reactions containing 10 mg/mL β-chitin, 0.1 μM ChiA, ChiB or *Sm*ChiC in 50 mM Bis-Tris pH 6.0 incubated at 37°C for 8 h in the presence or absence of 1.0 μM *Lm*LPMO10 or 1.0 μM CBP21. Ascorbic acid (1.0 mM) was used as electron donor for the LPMOs and analysis was done using ion-affinity chromatography. Native and oxidized chitooligosaccharides unresolved by this chromatographic method are annotated by "Olig". (B) Samples shown in panel A containing the chitinases and CBP21 analyzed by HILIC. The reactions represented by each chromatogram are

808 indicated by the names of the enzymes. Reactions where solubilized native and oxidized
809 chitooligosaccharides have been re-treated with 0.2 μM chitinase for 16 h at 37°C (to completely
810 solubilize all hydrolysable products) are labelled with the name of the enzyme used in bold. The top
811 and bottom chromatograms represent standards of native chitooligosaccharides ($(GlcNAc)_{1-6}$) and
812 chitooligosaccharide aldonic acids (GlcNAc1A and $(GlcNAc)_{1-5}$GlcNAc1A), respectively. The
813 HILIC method separates the α- and β- anomers of the native chitooligosaccharides (labelled "α"
814 and "β"). A blank run has been subtracted from all chromatograms (baseline subtraction) in order to
815 improve clarity. The peak labelled "Artefact" is an artefact caused by the base line subtraction and
816 does not represent an analyte.
817
818 **Figure 7**. Northern blot analysis of *lmo2467* transcription. Cells were grown in LB supplemented
819 with 0.05% glucose at 30°C and induced by the addition of colloidal chitin. Samples were collected
820 from medium with and without chitin 15 min and 2 h after addition, corresponding to late exponential
821 and stationary phases of growth, respectively. The numbers above the bands show the relative levels
822 of transcripts compared to the sample without chitin in late exponential phase (far left). The 16S and
823 23S bands serving as a loading control are shown below the corresponding bands. The results were
824 reproduced in three independent experiments.
825
826 **Figure 8.** Analysis of proteins with affinity to chitin secreted by *L. monocytogenes*. (A) Supernatants
827 from cultures grown overnight at 30°C in plain LB (lane 1) or in LB supplemented with either
828 colloidal chitin (lane 2) or β-chitin (lane 3). Arrows show the positions of identified bands. (B) SDS-
829 PAGE analysis of purified *Lm*LPMO10. The dotted arrow marks the expected position on the SDS-
830 PAGE gel analysis of the culture supernatants. The SDS-PAGE gels were stained with SYPRO Ruby.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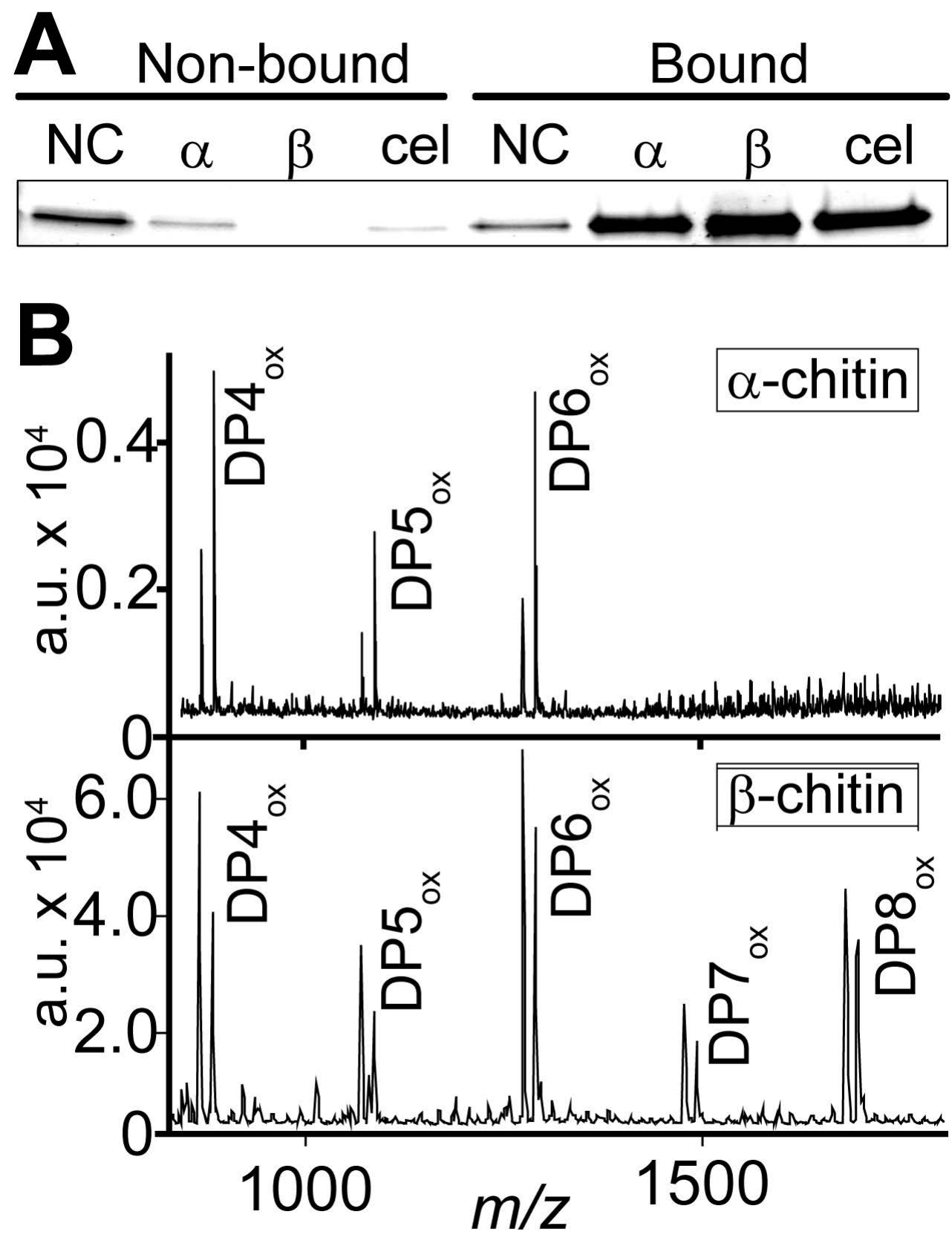
Chitin
LPMO10
$O_2 + 2H^+ + 2e^-$
H_2O
GH18
Aldonic acid
B
LmLPMO10
LPMO10
FnIII
CBM5/12
CBM5/12
ChiA
GH18
ChiB
GH18
FnIII
CBM5/12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Bound
NC
α
β
cel
NC
α
β
cel
B
α-chitin
DP4ox
DP5ox
DP6ox
a.u. x 10^4
0.4
0.2
0
β-chitin
DP4ox
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DP8ox
a.u. x 10^4
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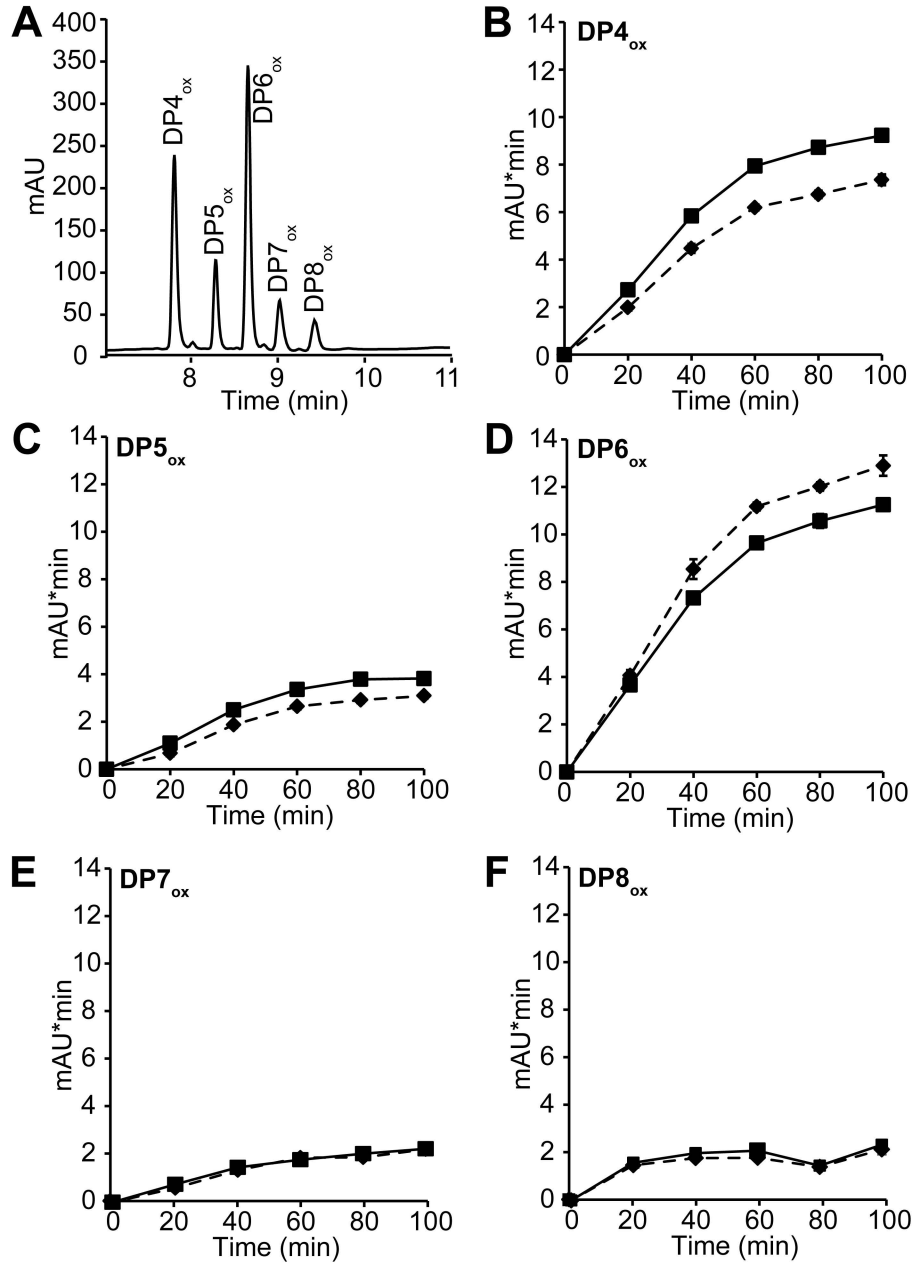
4.0
2.0
0
1000
1500
m/z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Time (min)
C
DP5ox
mAU*min
Time (min)
D
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Time (min)
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DP7ox
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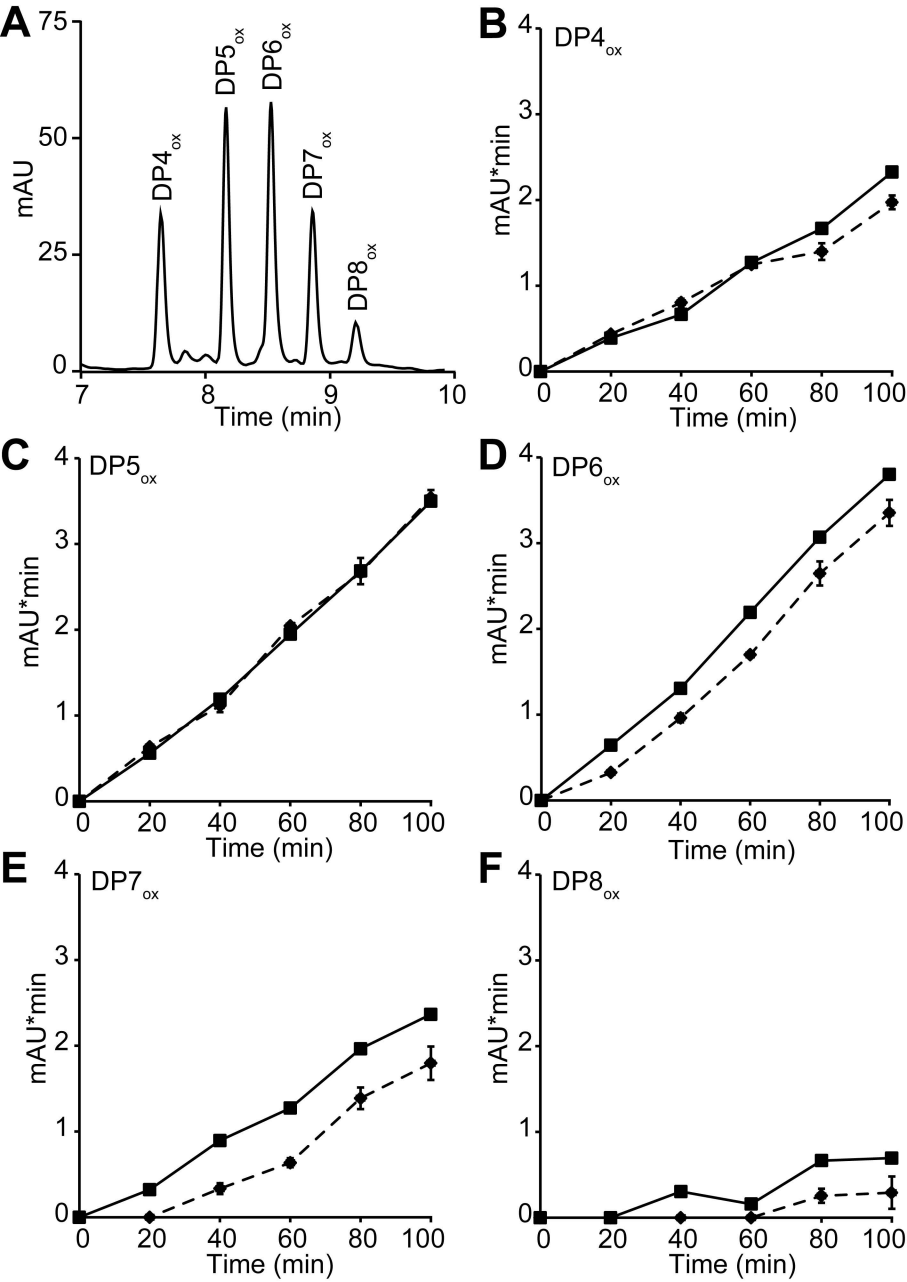
Time (min)
F
DP8ox
mAU*min
Time (min)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DP4ox
DP5ox
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DP7ox
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Time (min)
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Time (min)
C
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Time (min)
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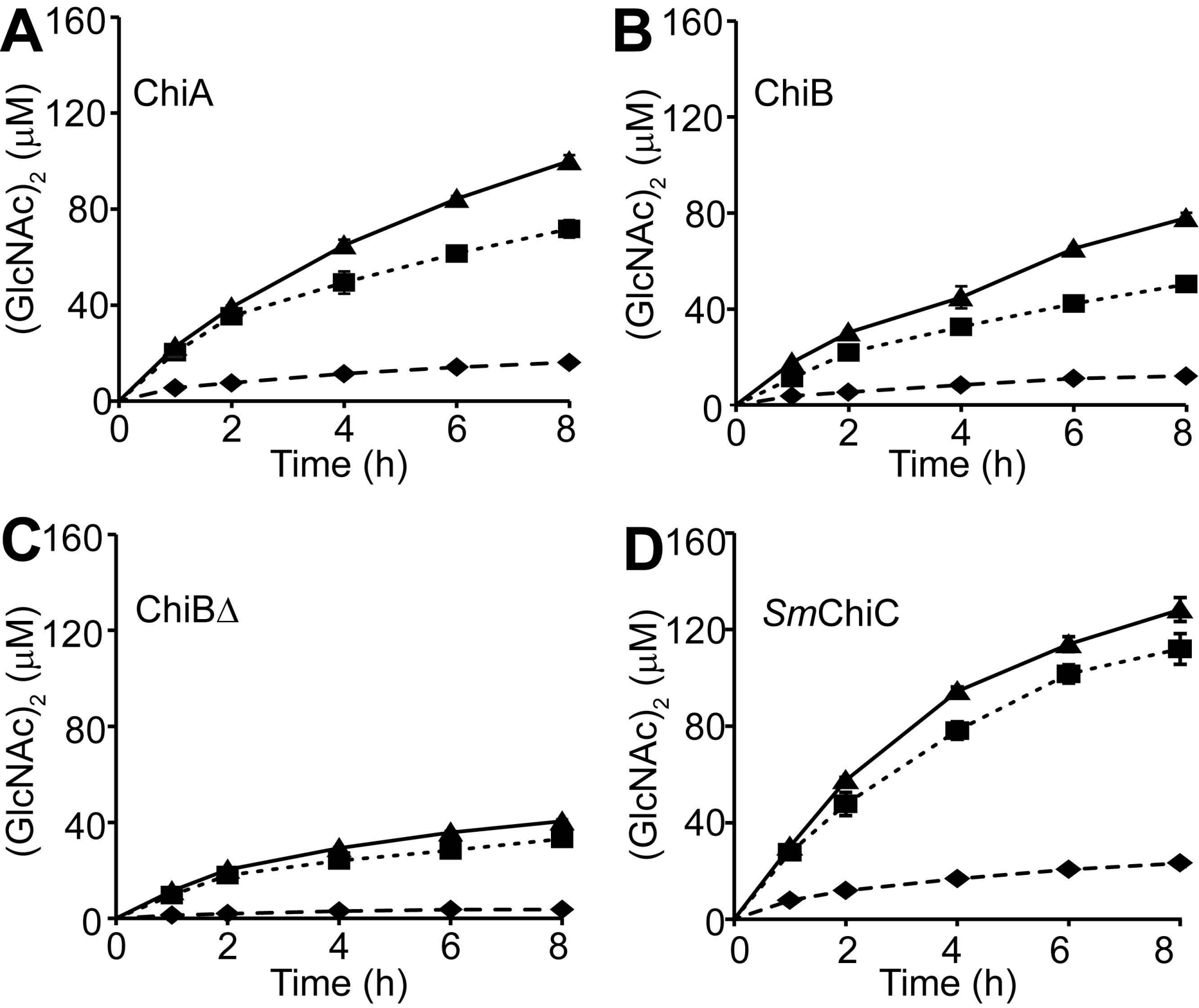
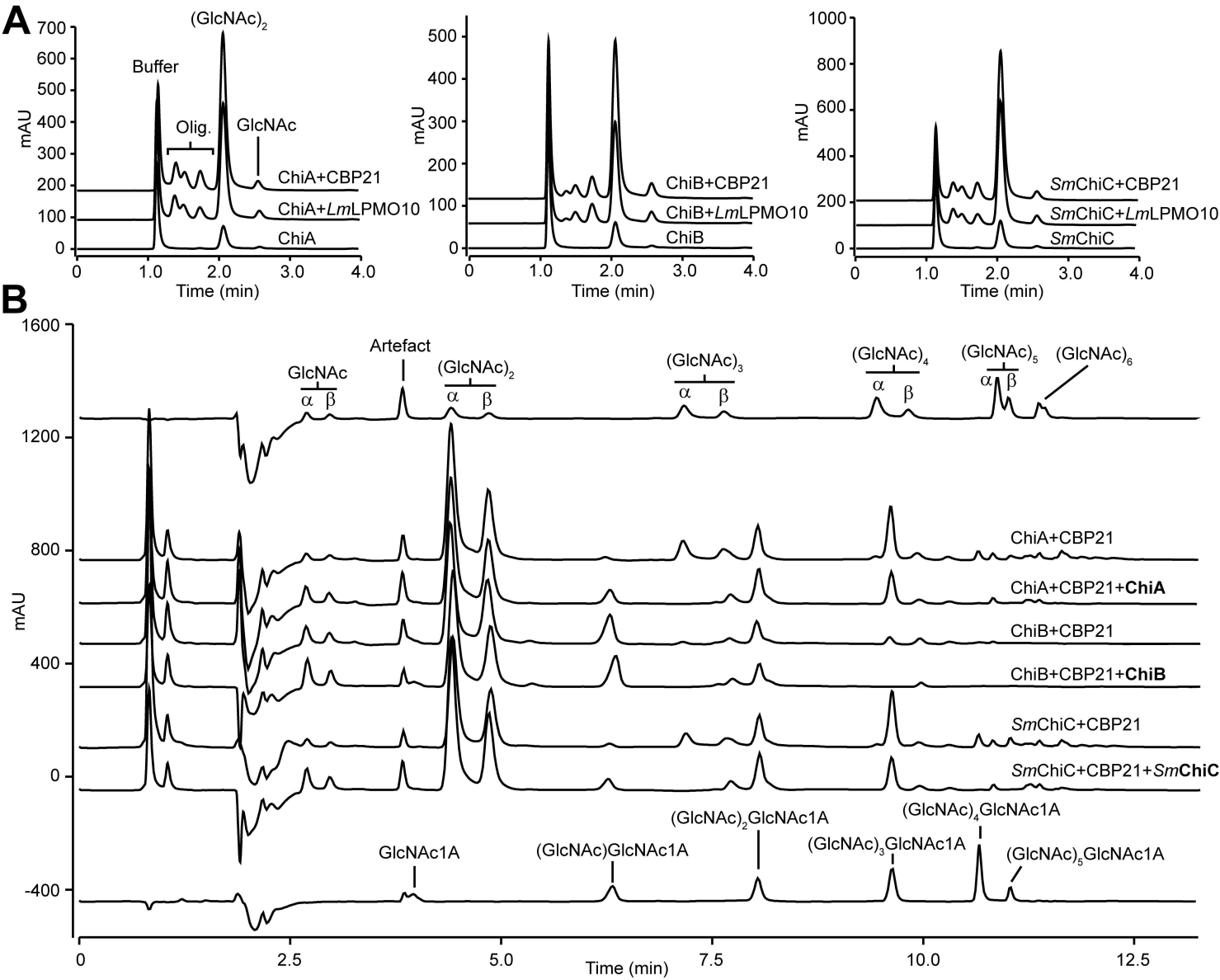
Time (min)


A
ChiA
(GlcNAc)2 (μM)
160
120
80
40
0
0
2
4
6
8
Time (h)
B
ChiB
(GlcNAc)2 (μM)
160
120
80
40
0
0
2
4
6
8
Time (h)
C
ChiBΔ
(GlcNAc)2 (μM)
160
120
80
40
0
0
2
4
6
8
Time (h)
D
SmChiC
(GlcNAc)2 (μM)
160
120
80
40
0
0
2
4
6
8
Time (h)


A
mAU
Time (min)
Buffer
(GlcNAc)2
Olig.
GlcNAc
ChiA+CBP21
ChiA+LmLPMO10
ChiA
ChiB+CBP21
ChiB+LmLPMO10
ChiB
SmChiC+CBP21
SmChiC+LmLPMO10
SmChiC
B
GlcNAc
Artefact
(GlcNAc)2
(GlcNAc)3
(GlcNAc)4
(GlcNAc)5
(GlcNAc)6
α
β
ChiA+CBP21
ChiA+CBP21+ChiA
ChiB+CBP21
ChiB+CBP21+ChiB
SmChiC+CBP21
SmChiC+CBP21+SmChiC
GlcNAc1A
(GlcNAc)GlcNAc1A
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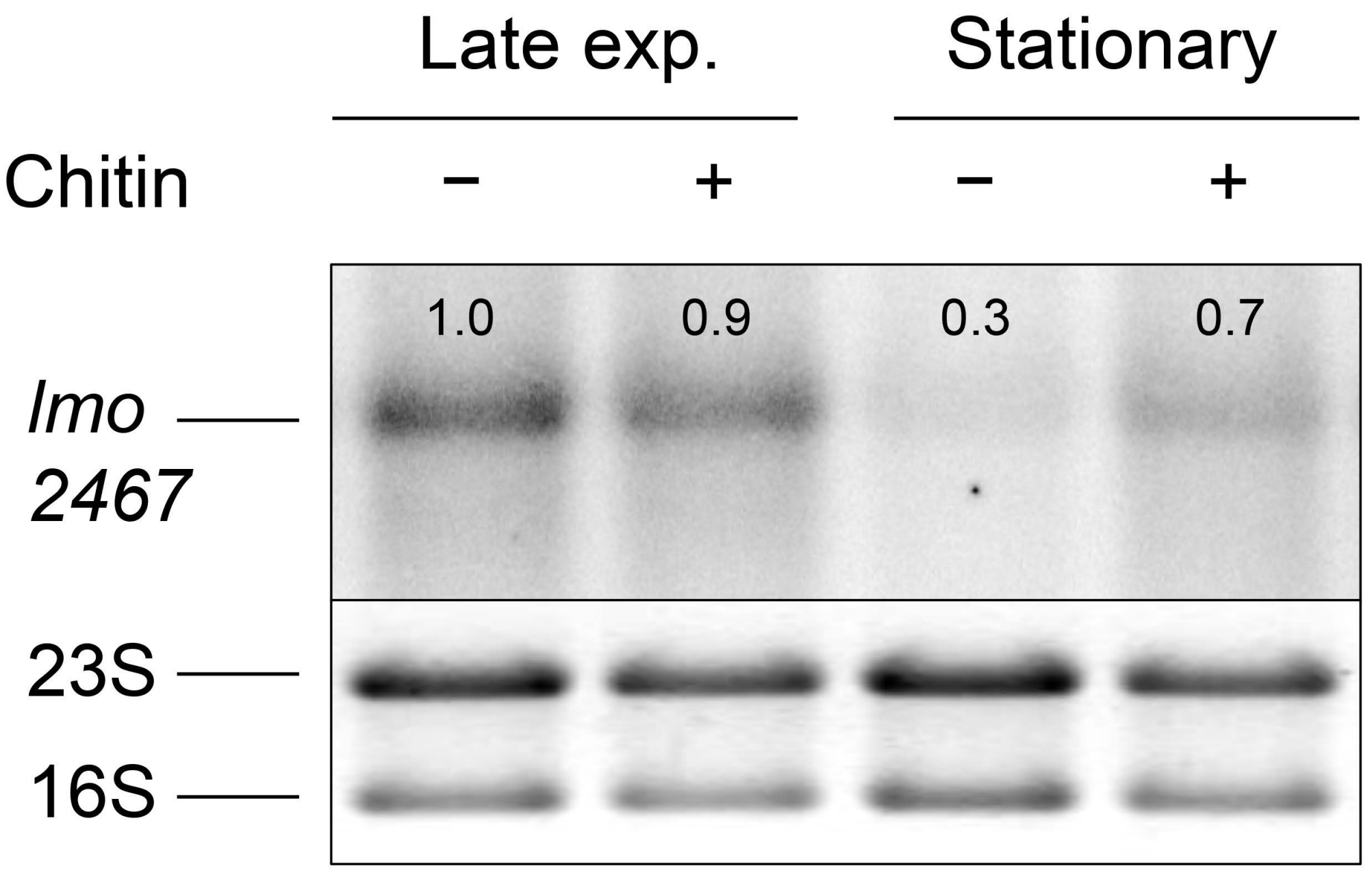
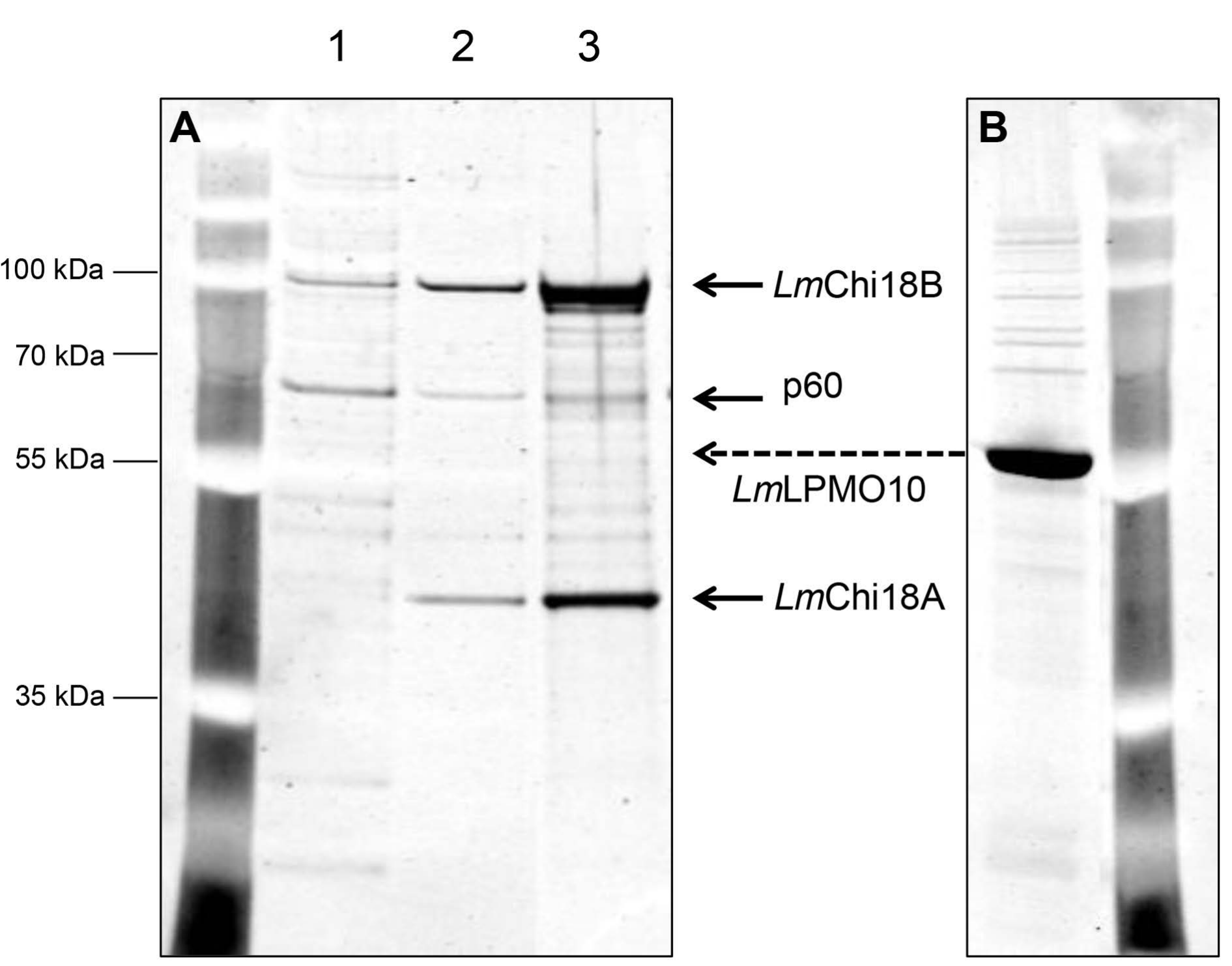

Late exp.
Stationary
Chitin
–
+
–
+
1.0
0.9
0.3
0.7
lmo
2467
23S
16S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2
3
A
B
100 kDa
70 kDa
55 kDa
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LmChi18B
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