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Characterization of a novel *Salmonella* Typhimurium chitinase which hydrolyzes chitin, chitooligosaccharides and an *N*-acetyllactosamine conjugate

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Salmonella **contain genes annotated as chitinases; however, their chitinolytic activities have never been verified. We now demonstrate such an activity for a chitinase assigned to glycoside hydrolase family 18 encoded by the *SL0018 (chiA)* gene in *Salmonella enterica* Typhimurium SL1344. A C-terminal truncated form of *chiA* lacking a putative chitin-binding domain was amplified by PCR, cloned and expressed in *Escherichia coli* BL21 (DE3) with an N-terminal $(His)_6$ tag. The purified enzyme hydrolyzes 4-nitrophenyl *N*,*N*′-diacetyl-β-D-chitobioside, 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose and carboxymethyl chitin Remazol Brilliant Violet but does not act on 4-nitrophenyl *N*-acetyl-β-D-glucosaminide, peptidoglycan or 4-nitrophenyl β-D-cellobioside. Enzyme activity was also characterized by directly monitoring product formation using ^{1}H-nuclear magnetic resonance which showed that chitin is a substrate with the release of *N*,*N*′-diacetylchitobiose. Hydrolysis occurs with the retention of configuration and the enzyme acts on only the β-anomers of chitooligosaccharide substrates. The enzyme also released *N*-acetyllactosamine disaccharide from Galβ1 → 4GlcNAcβ-*O*-$(CH_2)_8$CONH $(CH_2)_2$NHCO-tetramethylrhodamine, a model substrate for LacNAc terminating glycoproteins and glycolipids.**

Keywords: Enterobacteriaceae / family 18 hydrolase / kinetics / *N*-acetyllactosamine / NMR

Introduction

Chitin, an insoluble linear polymer of β1–4-linked *N*-acetylglucosamine (GlcNAc), is the second most abundant biopolymer in nature and the most abundant carbohydrate in the aquatic biosphere. Chitin is found in the cell walls of fungi, the cuticles of arthropods and the peritrophic membranes of annelids and some arthropods (Gooday 1990; Cauchie 2002). Many bacteria produce enzymes that hydrolyze chitin and the majority of these belong to glycoside hydrolase family 18 (GH-18; www.cazy.org).

Bacterial chitinases play important roles in chitin cycling especially in the marine environment. *Vibrio cholerae* and other *Vibrio* spp. chitinases are involved in nutrient acquisition and promote increased environmental survival (Keyhani and Roseman 1999; Meibom et al. 2004). Bacterial chitinases can, however, also contribute to human and animal infections as shown for *Legionella pneumophila* where secreted chitinase promotes bacterial infection in the lung of mice (DebRoy et al. 2006). *Listeria monocytogenes* chitinase expression is upregulated in the mouse intestine after infection (Toledo-Arana et al. 2009) and is under the control of a central virulence regulator (Larsen et al. 2010). For the *Salmonella* SL1344 strain, a micro array study has revealed that the expression of the GH-18 *SL0018* gene is strongly upregulated during the infection of murine mouse macrophage cells (Eriksson et al. 2003). However, chitinase activity has not yet been demonstrated for *Salmonella* (Clarke and Tracey 1956; O'Brien and Colwell 1987; Norhana et al. 2009). Using bioinformatics, we now show that several *Salmonella* strains contain a gene for a chitinase that is a member of GH-18 though the gene product lacks a signal peptide. To gain a better understanding of the possible biological roles of this chitinase, we cloned the corresponding SL1344 gene *SL0018 (chiA)* to verify the chitinolytic activity of the gene product and to investigate its reaction specificity with a panel of substrates.

Results

Examination of bacterial chitinase activity

No chitinolytic activity was observed with the *Salmonella* Typhimurium SL1344 strain incubated on a chitin agar medium under aerobic or anaerobic conditions at 30°C for up to 12 days.

Phylogenetic analyses of chiA

We identified a putative chitinase gene, *SL0018* (*chiA*) (http://xbase.bham.ac.uk/colibase/genome.pl?id=1846), in

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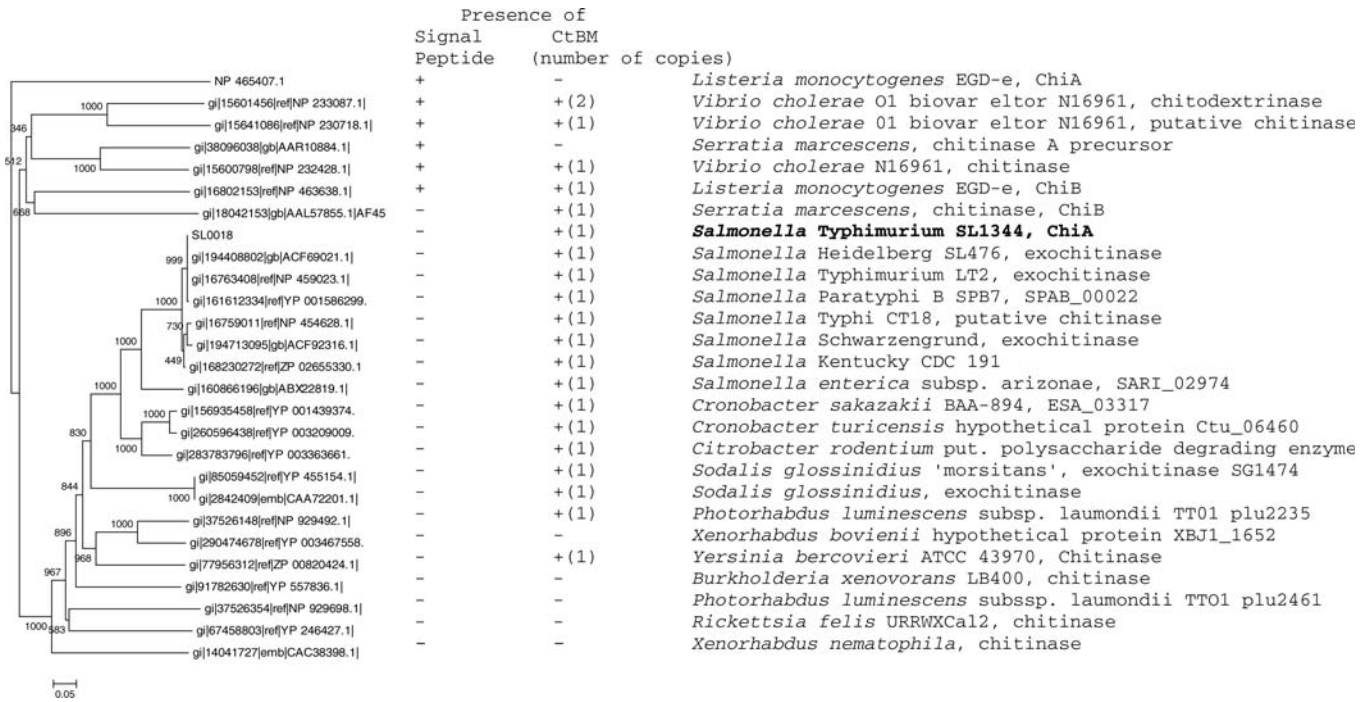

Fig. 1. Alignment of the amino acid sequence of *Salmonella* Typhimurium SL1344 ChiA GLH18 domain (in bold) with representative sequences for similar domains in other species and strains.

Salmonella Typhimurium SL1344 encoding a chitinase assigned to GH-18. This gene is widely distributed among genome-sequenced *Salmonella* strains (Figure 1).

The gene product of *chiA* include a 412 amino acid GH-18 domain (amino acids 53–465) and a 50 amino acid C-terminal chitin-binding module (CtBM; amino acids 646–696) but no signature sequence for *sec*-dependent protein export. The sequence of the putative catalytic region embedded in the GH-18 domain could not readily be arranged within any of the glycoside hydrolase 18 groups as outlined by Svitil and Kirchman (1998). The GH-18 domain of ChiA is, however, related to GH-18 domains in gene products annotated as chitinases in other members of the Enterobacteriaceae family, including *Citrobacter rodentium*, *Cronobacter sakazakii*, *Cronobacter turicensis*, *Photorhabdus luminescens*, *Sodalis glossinidius*, *Xenorhabdus bovienii*, *Xenorhabdus nematophila* and *Yersinia bercovieri* as well as the other bacterial groups including *Burkholderia xenovorans* and *Rickettsia felis* (Figure 1). As was seen for *chiA*, the related genes in all these bacteria also lack a signal peptide sequence.

Cloning and expression of chiAΔCtBM

A truncated form of ChiA was constructed without the putative CtBM (amino acids 646–696) and three additional C-terminal amino acids (amino acids 697–699). The 1971 bp DNA fragment encoding the truncated ChiA chitinase and a $(His)_6$ tag at the N-terminus was cloned into a pET-46 Ek/LIC expression vector and expressed in *Escherichia coli* BL21 (DE3) cells. The predicted mass of the tagged protein ChiAΔCtBM is 71,365 Da. Expression levels of the recombinant protein were very good and 80–100 mg enzyme/L of cell culture could be obtained after chromatography on HisTrap columns (Figure 2).

Activity, kinetic parameters, pH and temperature effects

The activity spectrum of ChiAΔCtBM was investigated by the use of the chitin pseudo-substrates, 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (pNP-GlcNAc), 4-nitrophenyl *N*,*N*′-diacetyl-β-D-chitobioside (pNP-$(GlcNAc)_2$) and 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose (pNP-$(GlcNAc)_3$). The recombinant *Salmonella* enzyme demonstrated chitinase activity toward the substrate pNP-$(GlcNAc)_2$. Product amounts per 1 nM enzyme were 0.09 and 0.14 μM for 0.21 and 0.82 mM substrates, respectively, after incubation at 30°C for 30 min. The enzyme also showed activity toward pNP-$(GlcNAc)_3$ with product amounts of 0.4 and 0.15 μM for 0.21 and 0.82 mM substrates, respectively. Thus, substrate inhibition was seen for higher concentrations of pNP-$(GlcNAc)_3$. No activity was measured toward pNP-GlcNAc, the cellulose pseudo-substrate 4-nitrophenyl β-D-cellobioside or peptidoglycan, whereas there was low activity toward carboxymethyl chitin. In the latter case, the specific activity of ChiA was 0.000053 ($\Delta OD550\ min^{-1}$, per nmol enzyme). The *Salmonella* chitinase has a pH optimum of pH 6.1 and relatively high activity from pH 4 to more than pH 7.25 (Figure 3). The temperature optimum was at 37°C and the enzyme remained active down to 4°C (Figure 4). The k_{cat} for pNP-$(GlcNAc)_2$ was 0.7 ± 0.07/s with a K_m of 0.73 ± 0.23 mM at pH 5 where the activity is nearly as high as observed at the optimal pH 6.1 value (Figure 3).

The *Salmonella* chitinase showed activity toward Galβ1 → 4GlcNAcβ-*O*-$(CH_2)_8CONH(CH_2)_2NHCO$-tetramethylrho

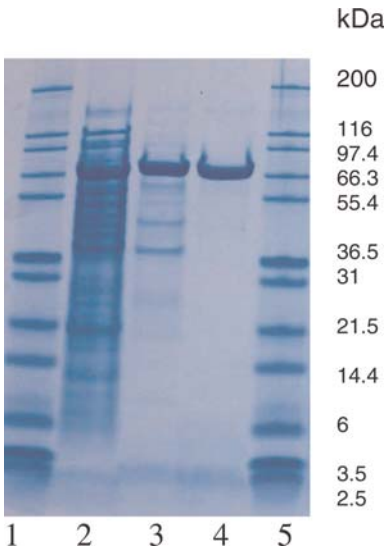

Fig. 2. SDS–PAGE of *Salmonella* chitinase at different stages of purification. Lanes 1 and 5 standards, lane 2 crude extract, lane 3 HisTrap column eluted with 0.5 M imidazole and lane 4 HisTrap with a gradient of imidazole from 5 to 250 mM.

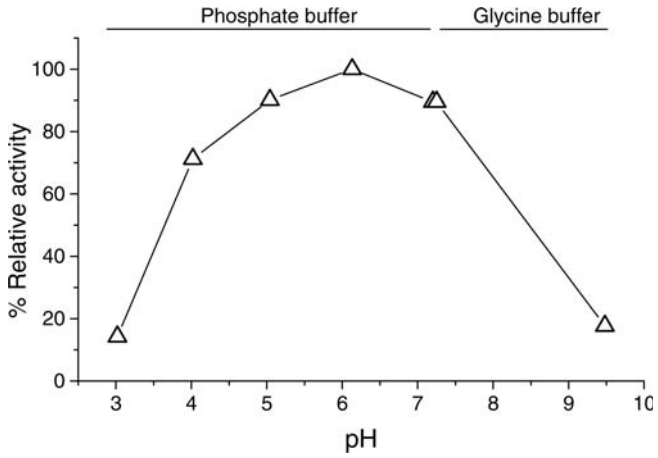

Fig. 3. pH profile at 30°C for activity of ChiAΔCtBM toward 0.41 mM pNP-$(GlcNAc)_2$.

damine (LacNAc-TMR) when examined by thin layer chromatography releasing the linking arm HO-$(CH_2)_8$CONH $(CH_2)_2$NHCO-tetramethylrhodamine (Figure 5). This activity was inhibited by the addition of 1.8 mM hexa-*N*-acetylchitohexaose where only a trace of linking arm was observed suggesting that the activity is due to the chitinase rather than a contaminating enzyme. *Trichoderma viride* chitinase did not act on LacNAc-TMR but was very active on GlcNAc-β-*O*-$(CH_2)_8$CONH$(CH_2)_2$NHCO-tetramethylrhodamine (GlcNAc-TMR) with the complete release of the linking arm. The LacNAc isomer, Galβ1 → 3GlcNAcβ-*O*-$(CH_2)_8$CONH$(CH_2)_2$NHCO-tetramethylrhodamine (Type I-TMR), was not a substrate for either enzyme (data not shown). Kinetic constants were determined by capillary electrophoresis (CE) analysis of reactions at different

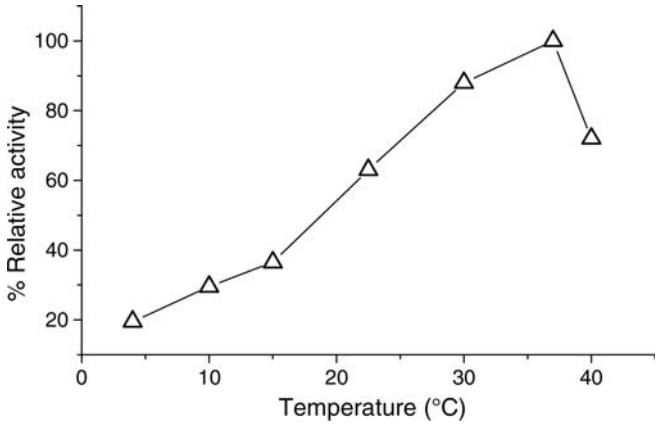

Fig. 4. Temperature profile for activity of ChiAΔCtBM toward 0.41 mM pNP-$(GlcNAc)_2$.

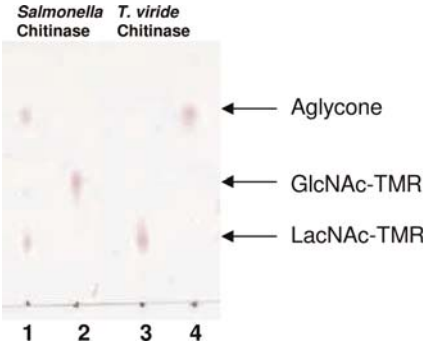

Fig. 5. Thin layer chromatography of reactions with fluorescent acceptors. LacNAc-TMR (lanes 1 and 3) or GlcNAc-TMR (lanes 2 and 4) were incubated with 5 μL of *Salmonella* ChiA chitinase (1.7 mg/mL) or *T. viride* chitinase (1.7 mg/mL) for 30 min. LacNAc-TMR and GlcNAc-TMR were substrates for the *Salmonella* and *T. viride* chitinases, respectively, releasing the aglycone HO-$(CH_2)_8$CONH$(CH_2)_2$NHCO-tetramethylrhodamine completely from GlcNAc-TMR in the case of *T. viride* enzyme.

concentrations of LacNAc-TMR; k_{cat} was 0.05 ± 0.005/s with a K_m of 10 ± 1.4 mM at pH 6.

Nuclear magnetic resonance studies with chitin, pNP-$(GlcNAc)_2$, chitooligosaccharides $(GlcNAc)_3$ and LacNAc-TMR

Chitin was shown to be a substrate for the *Salmonella* chitinase with the slow release of *N*,*N*′-diacetylchitobiose ($(GlcNAc)_2$) as monitored by ^{1}H-nuclear magnetic resonance (NMR) (Figure 6). The same slow release was seen for colloidal chitin (data not shown). The stereochemical course of hydrolysis of pNP-$(GlcNAc)_2$ was studied at 15°C to reduce the rate of anomerization of the released saccharides (Figure 7). At 800 MHz, all the *N*-acetyl groups of pNP-$(GlcNAc)_2$, $(GlcNAc)_2$ and GlcNAc have distinct chemical shifts (Figure 7B). After 15 min, new *N*-acetyl signals for the β-anomer of $(GlcNAc)_2$ are observed confirming the reaction occurs with the retention of configuration and after 20 min half of the substrate had been depleted. This is followed by the slow mutarotation of the released $(GlcNAc)_2$ giving

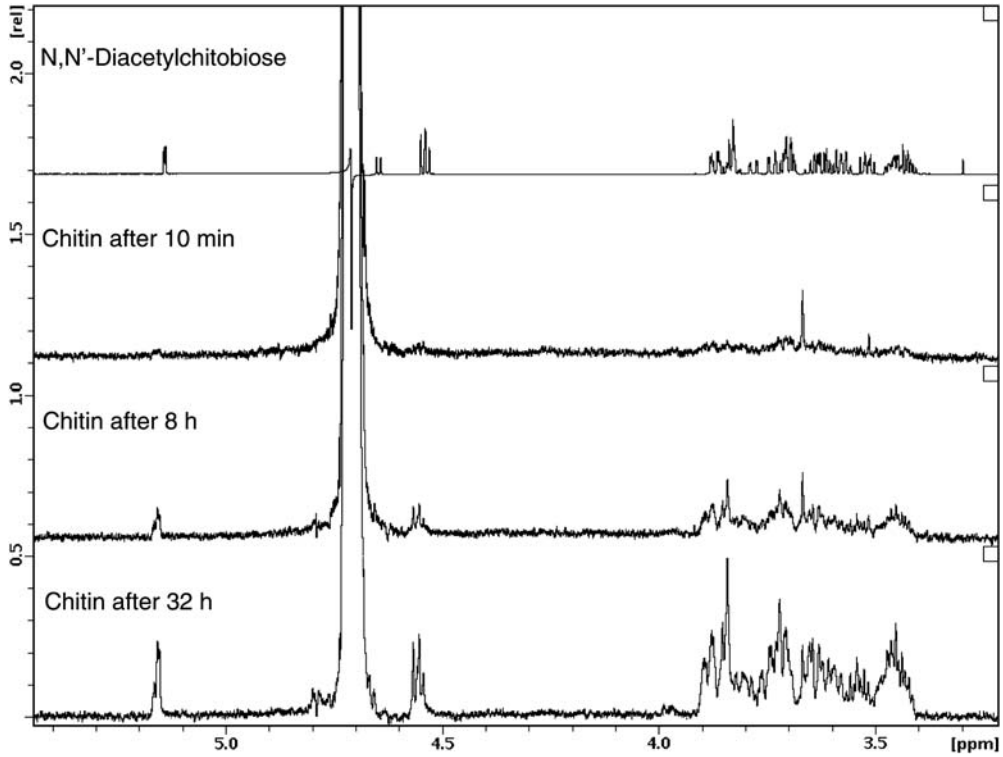

Fig. 6. Hydrolysis of chitin by *Salmonella* ChiA chitinase. NMR spectra at 25°C showing the conversion of crab shell chitin (2 mg) to $(GlcNAc)_2$ over time; the top spectrum is that of a $(GlcNAc)_2$ reference, whereas the other spectra were acquired after addition of 0.1 mg of enzyme.

the α-anomer after 1 h. After 15 min, traces of β-GlcNAc and pNP-GlcNAc are also produced. These can arise from the hydrolysis of $(GlcNAc)_2$ and/or the terminal GlcNAc of pNP-$(GlcNAc)_2$. pNP-GlcNAc is not a substrate for *Salmonella* chitinase; thus, this signal remains constant after 3 h when all of the pNP-$(GlcNAc)_2$ has been depleted. At 15 min, a small amount of transglycosylation products have been produced which are hydrolyzed. The amount of GlcNAc at 3h20min is greater than that of pNP-GlcNAc demonstrating that $(GlcNAc)_2$ has also been hydrolyzed to GlcNAc. In all cases, reactions occur with the retention of configuration since β-anomers are the only products of enzyme-catalyzed steps.

$(GlcNAc)_3$ was also studied at 15°C since the rate of anomerization in spectra acquired at 25°C was too rapid to determine anomeric preference. Figure 8B shows the rapid decrease in the *N*-acetyl signals for the β-trisaccharide with 50% depletion in 40 min and with its complete depletion at 150 min. The rate of decrease in the α-trisaccharide anomer is subject to a lag followed by a slow constant decrease due to its anomerization to β $(GlcNAc)_3$, which is the preferred substrate for the enzyme (Figure 8B). For up to 50 min, there is formation of equal amounts of β-$(GlcNAc)_2$ and β-GlcNAc along with a lag in the formation of α-$(GlcNAc)_2$ and α-GlcNAc, which are only formed by anomerization as expected for hydrolysis with the retention of configuration. However, at the end of the reaction, there is a somewhat larger amount of both α- and β-GlcNAc than α- and β-$(GlcNAc)_2$, which is attributed to the slow hydrolysis of $(GlcNAc)_2$. All enzymatic and nonenzymatic steps for $(GlcNAc)_3$ hydrolysis are shown in Figure 8A.

The hydrolysis of LacNAc-TMR also occurs with the release of LacNAc with the retention of configuration followed by the slower mutarotation of β-LacNAc to α-LacNAc (Figure 9). This reaction occurs with 50% conversion to product in 280 min. LacNAc-$(CH_2)_8CO_2CH_3$ was also hydrolyzed releasing disaccharide though at a much slower rate than LacNAc-TMR taking 24 h for 50% conversion (results not shown).

Discussion

In this study, we were unable to show chitinolytic activity for the *Salmonella* Typhimurium SL1344 strain using a standard assay. Indeed, while *Salmonella* Typhimurium LT2 has been reported to be able to hydrolyse $(GlcNAc)_2$ and to use this substrate for growth (Keyhani and Roseman 1997), genuine chitinolytic activity by this species has not been demonstrated previously (Clarke and Tracey 1956; O'Brien and Colwell 1987; Norhana et al. 2009). *Salmonella* encode, however, both GH-18 and GH-19 enzymes annotated as chitinases. A chitinolytic phenotype has been readily observed by the use of similar assays for chitinolytic bacteria such as *Vibrio* spp. for which this activity is important for survival in the environment (Keyhani and Roseman 1999; Meibom et al. 2004). We speculated whether the *Salmonella* Typhimurium SL1344 ChiA enzyme is not expressed under conditions related to the environmental survival and more particularly whether the biological role of the ChiA chitinase might instead be associated with the infection of insects and/or vertebrates. Indeed, *Salmonella* is associated with chitin-containing insects, such

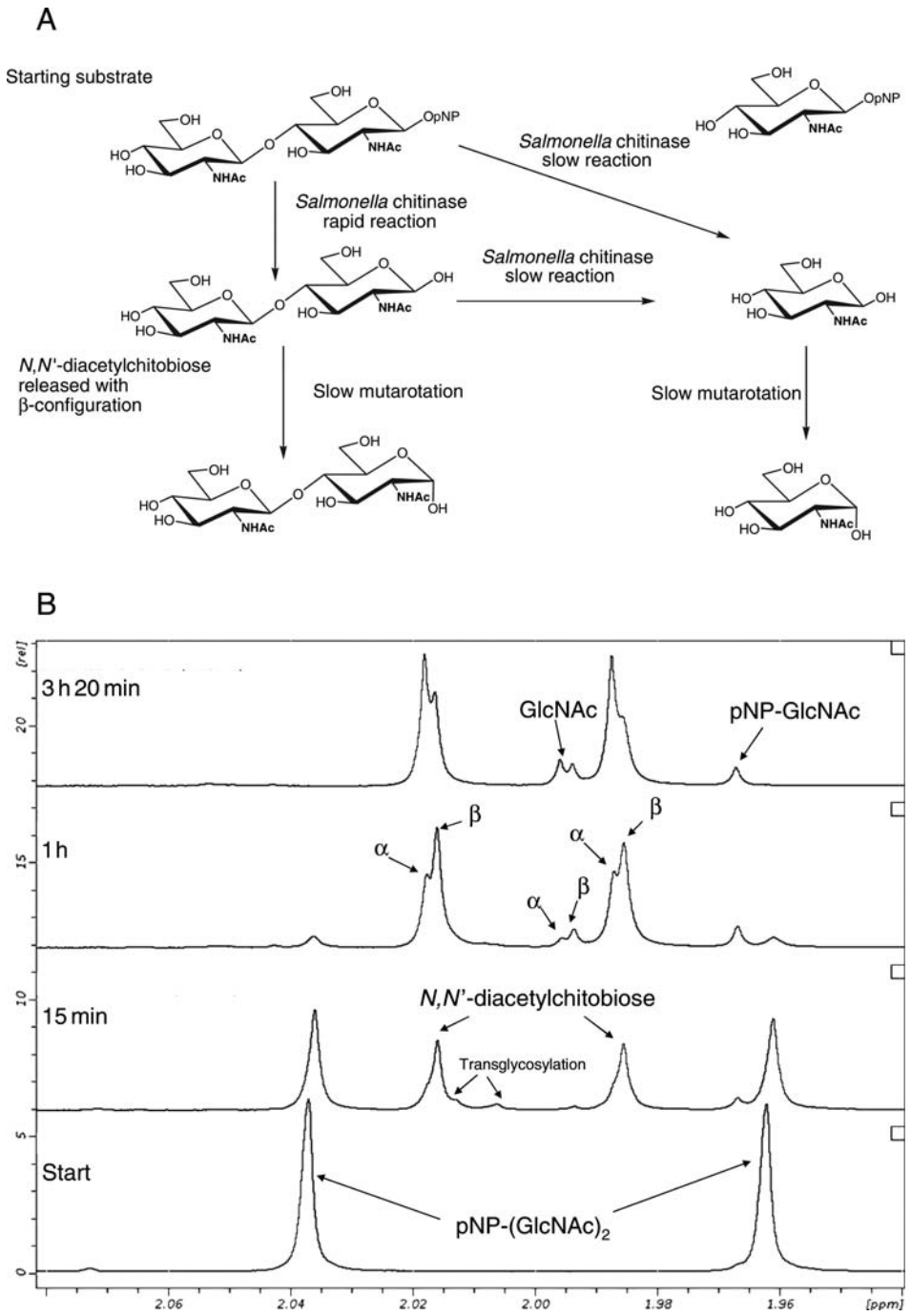

Fig. 7. (**A**) An overview of the products formed during the hydrolysis of pNP-$(GlcNAc)_2$ by *Salmonella* ChiA chitinase. (**B**) Partial ^{1}H-NMR spectra of the *N*-acetyl groups of substrate (1 mg) and products during hydrolysis by 0.14 mg of enzyme monitored at 15°C.

as cockroaches, chironomus midges and flies, and these hosts may carry the bacteria as possible dispersal vectors (Kopanic et al. 1994; Olsen and Hammack 2000; Moore et al. 2003; Holt et al. 2007). In addition, *Salmonella* Typhimurium is a well-known infectious pathogen of many vertebrate species including humans (Jay 2000).

This hypothesis prompted us to clone and overexpress the enzyme in a heterologous host with a view to purify it and test the catalytic activity toward substrates representing models of chitin as well as GlcNAc-containing glycans found on or in eukaryotic cells. We employed a C-terminal truncated version of the protein omitting the chitin-binding domain in order to facilitate a kinetic comparison with an *L. monocytogenes* ChiA GH-18 enzyme with no chitin-binding domain that was simultaneously cloned, expressed and purified in our laboratory (Leisner et al. 2009). Some of the chitinases related to ChiA produced by other bacteria do also not contain such a domain (Figure 1).

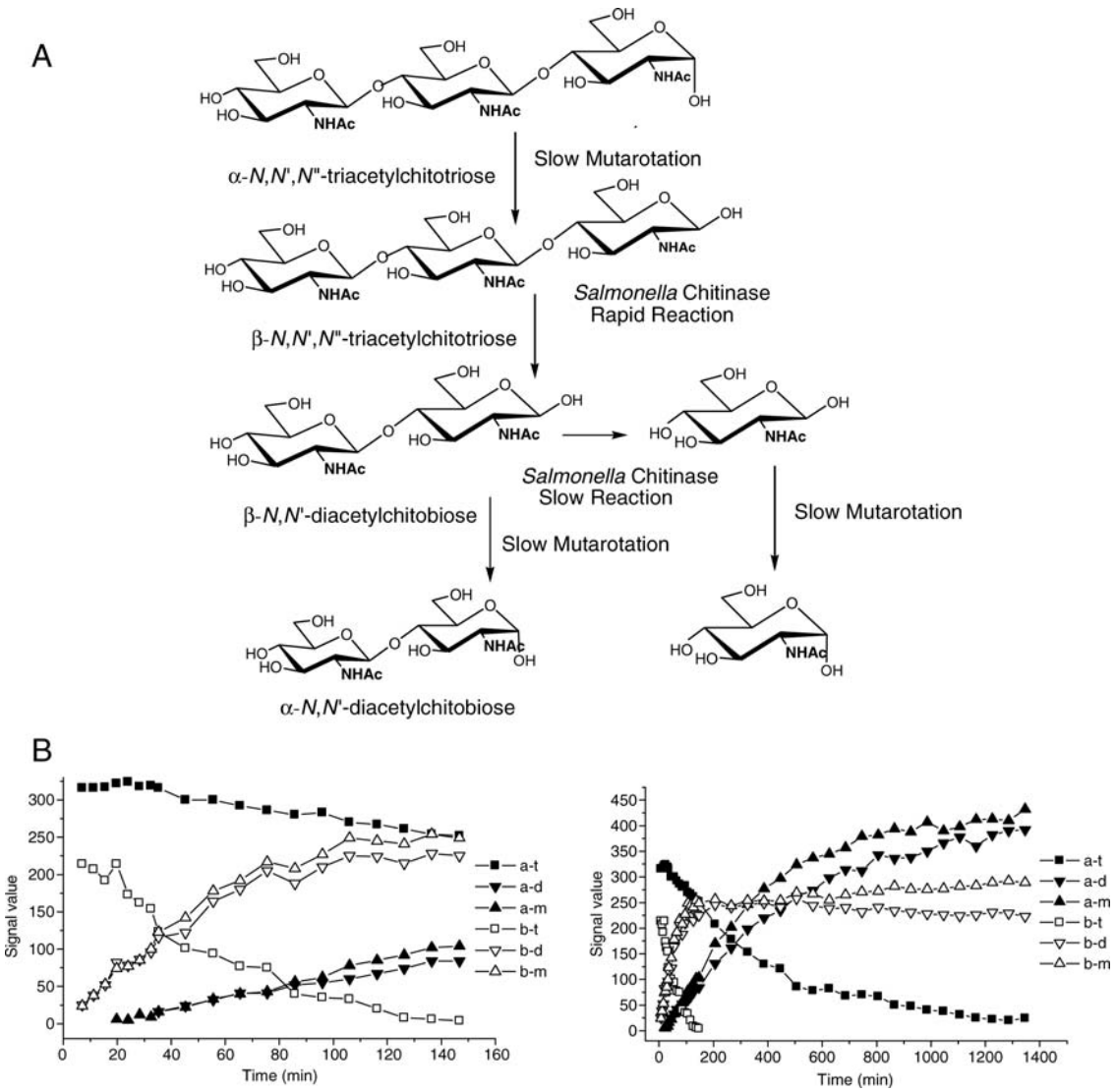

Fig. 8. (**A**) An overview of the enzymatic products of the hydrolysis of $(GlcNAc)_3$ by *Salmonella* ChiA chitinase. At the start of the reaction, there is a 60:40 ratio of α- to β-$(GlcNAc)_3$; however, only the β-anomer is a substrate for the enzyme. (**B**) Plots of the changes in *N*-acetyl signals of α-$(GlcNAc)_3$ (a-t) and β-$(GlcNAc)_3$ (b-t) during the hydrolysis of 1 mg of substrate by 0.14 mg of S*almonella* chitinase monitored at 15°C. Up to 40 min, the only products are β-$(GlcNAc)_2$ (b-d) and β-GlcNAc (b-m). α-$(GlcNAc)_2$ (a-d) and α-GlcNAc (a-m) are slowly formed upon anomerization of their corresponding β-anomers.

The recombinant *Salmonella* enzyme showed activity toward both pNP-$(GlcNAc)_2$ and pNP-$(GlcNAc)_3$ in agreement with the hydrolysis reaction starting two and three units from the nonreducing end of the chitin pseudo-substrate as shown for an *Aeromonas caviae* chitinase (Wang et al. 2003) but without a markedly reduced hydrolysis at the three unit position. The activity toward pNP-$(GlcNAc)_3$ was lowest at the highest concentration, which may indicate some kind of substrate inhibition of the enzyme. Such a result has been reported by Den Tandt and Scharpe (1997), when measuring the activity of a human plasma specific chitinase toward methylumbelliferyl-tetra-*N*-acetyl-β-D-chitotetraoside, a chitin pseudo-substrate. For this reason, we only measured kinetic values for pNP-$(GlcNAc)_2$ and found that the observed K_m values were similar to other chitinases, whereas the k_{cat} value was at the lower end (www.brenda-enzymes.info/, Department of Bioinformatics and Biochemistry, Technical University of Braunschweig, Germany; Leisner et al. 2009). The effect of the carbohydrate-binding domain on the activity of the ChiAΔCtBM enzyme remains to be studied. This type of domain appears to support the activity of other chitinases toward α-chitin and colloidal chitin (Watanabe et al. 1994; Svitil and Kirchman 1998; Wang et al. 2003) but it was not entirely necessary for activity toward chitin in our study as verified by monitoring the slow release of $(GlcNAc)_2$ by ^{1}H-NMR (Figure 6) and the hydrolysis of carboxymethyl chitin Remazol Brilliant Violet (CM-Chitin-RBV) solution. In the latter case, the specific activity of ChiA was, however, markedly less than observed for *Serratia marcescens* A, B and C1 chitinases (Δ0.0035–0.066 OD550 min^{-1}, per pmol enzyme, Synstad et al. 2008), although it should be noted that incubation temperature (30 vs. 37°C) and substrate

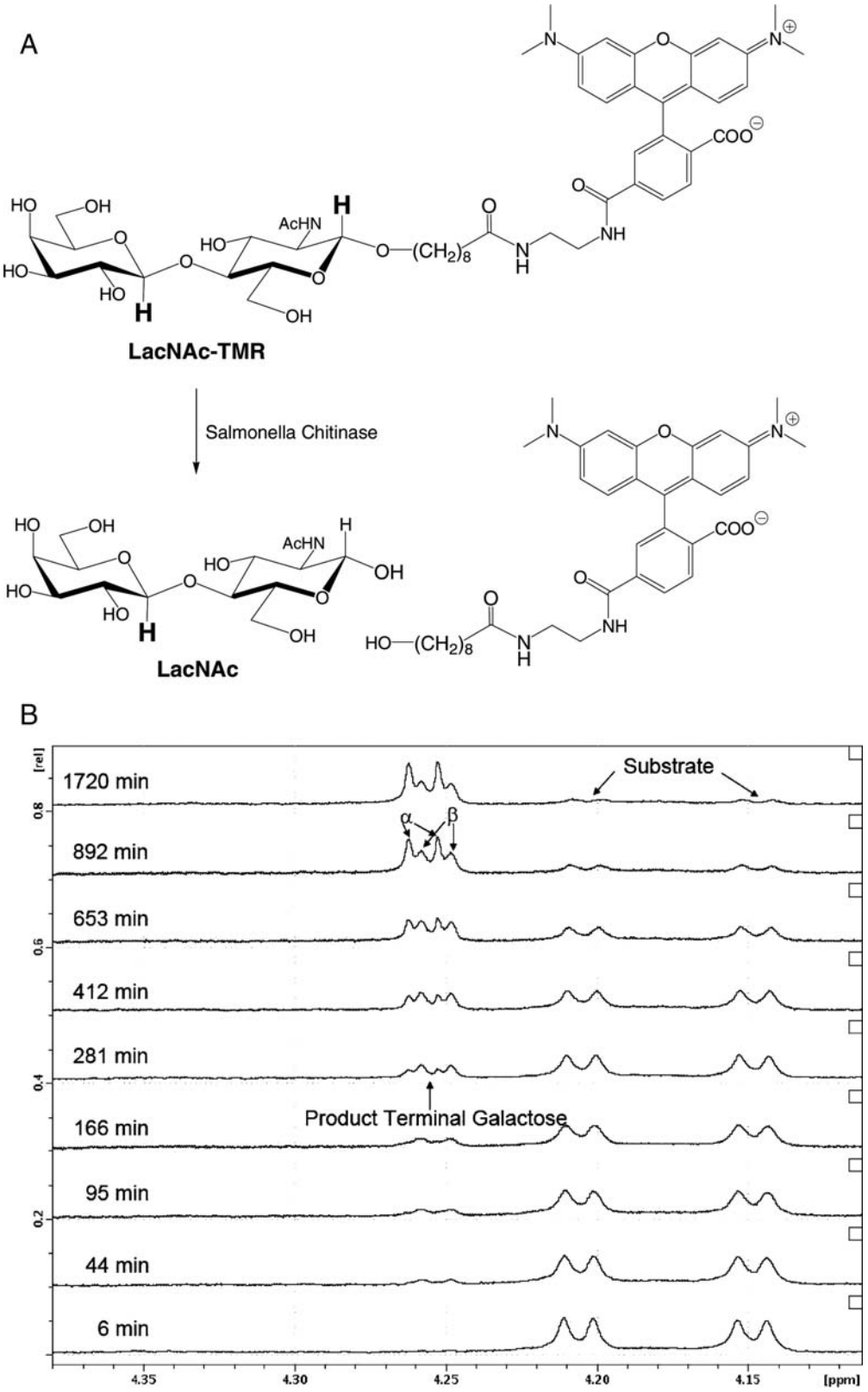

Fig. 9. (**A**) Hydrolysis of LacNAc-TMR catalyzed by *Salmonella* ChiA chitinase with the proton signals being monitored in the NMR experiment highlighted in bold. (**B**) Partial ^{1}H-NMR spectra showing the anomeric signals for the starting substrate (0.3 mg) and the terminal galactose of LacNAc that is released in the reaction. Reaction was at 15°C with 0.7 mg of enzyme.

concentration (2 vs. 0.6 mg/mL) differed. Both *S. marcescens* chitinases B and C possess chitin-binding domains contrary to the *Salmonella* ChiAΔCtBM enzyme investigated here. We intend to conduct further studies on a nontruncated version of the *Salmonella* ChiA enzyme in order to further evaluate its chitinolytic activity.

We explored further the catalytic activity of the *Salmonella* enzyme toward pNP-$(GlcNAc)_2$ by means of NMR. As expected, the enzyme exhibited a retaining mechanism (Figures 7 and 8) in accord with the classification of this enzyme as a GH-18 member. The activity toward $(GlcNAc)_3$ was somewhat lower than that toward pNP-$(GlcNAc)_2$ since the complete hydrolysis of the β-anomer of $(GlcNAc)_3$ required 150 min rather than just over 1 h for pNP-$(GlcNAc)_2$. Penta-*N*-acetylchitopentaose ($(GlcNAc)_5$) was completely hydrolyzed to $(GlcNAc)_2$ and $(GlcNAc)_3$ and a trace of GlcNAc in <7 min under identical conditions (Supplementary data, Figure S1).

The enzyme activity of *Salmonella* chitinase toward pNP-$(GlcNAc)_2$ had a broad pH optimum and a temperature range (Figures 3 and 4). The enzyme showed a relatively high pH optimum, a value in accord with gut pH values commonly observed for many but not all insects (Dow 1992; Johnson and Felton 1996; Clark 1999).

The enzyme showed no activity toward the related substrates, 4-nitrophenyl β-D-cellobioside, a cellulose pseudosubstrate and peptidoglycan. The latter result is supported by the observation that for the *Salmonella* Typhimurium LT2 strain, a gene (*STM0018*) identical to the *chiA* gene of the SL1344 strain is not essential for building of the cell wall. Thus, a mutant with this gene deleted exhibited relatively similar, although not entirely identical, growth with the wild-type strain during incubation at 30 or 37°C in Brain Heart Infusion Broth or Luria Broth (B.G. Storgaard and J.J. Leisner, unpublished results). Previously, however, bifunctional chitinases with chitinase and lysozyme activity have been reported (Wang and Chang 1997).

The ability of the *Salmonella* enzyme to release LacNAc from LacNAc-TMR is an unusual observation. The activity exhibited a low k_{cat} but a high K_m value compared with the activity toward pNP-$(GlcNAc)_2$. As expected the enzyme showed no activity toward GlcNAc-TMR, a substrate analog to pNP-GlcNAc. This type of activity has only been described for a chitinase from *Amycolatopsis orientalis*, an Actinobacteria where the disaccharide was released from pNP-LacNAc (Murata et al. 2005). Chitinases from *T. viride* (Figure 5) and *L. monocytogenes* (ChiA; data not shown) were inactive on this substrate, and none of the enzymes acted on Type I-TMR that has a Galβ1 → 3GlcNAc linkage rather than Galβ1 → 4GlcNAc of LacNAc. LacNAc-TMR is a model substrate for LacNAc terminating glycoproteins and glycolipids on vertebrate cells. Further studies are needed to elucidate whether this activity indeed fulfils a biological role but it raises the interesting possibility that the *Salmonella* enzyme is targeting LacNAc-containing glycans in the host organism. The observation that the ChiA gene expression is upregulated during the infection of murine macrophage cell lines (Eriksson et al. 2003) strongly suggest the feasibility of this possibility. Indeed, LacNAc-containing glycans are common in the human glycome (Cummings 2009). It has previously been demonstrated that LacNAc glycosides can be important for bacterial pathogenesis exemplified by *E. coli* pathogenic strains that bind to the human intestinal epithelium by LacNAc-specific lectin activity (Humphries et al. 2009).

The potential role of the *Salmonella* GH-18 chitinase for the infection of insects is supported by the observation that it has an amino acid sequence similar to chitinases encoded by a range of related bacterial species (Figure 1) known to have interactions, symbiotic or pathogenic, with invertebrates, e.g. *P. luminescens* that live in nematodes (Ciche et al. 2008) and *S. glossinidius* that live as a secondary endosymbiont in the mid-gut of tsetse flies. *Sodalis glossinidius* chitinase activity is necessary for invasion of the bacterium into the insect host (Dale and Welburn 2001).

The GH-18 enzyme encoded by these organisms, including *Salmonella*, lacks a signal peptide essential for export by the general secretory pathway (Thanassi and Hultgren 2000; Figure 1) but they are still, however, predicted as secretory proteins when examined by the use of the CBS-DTU secretome 2.0 software (www.cbs.dtu.dk/services/secretomeP; results not shown). This finding suggest that these enzymes are not secreted into the environment and therefore do not play a role for survival outside a suitable host. It is possible that they instead may be effector proteins secreted by the type III secretory pathway that is employed during the infection of insects as well as mammals (Dale et al. 2001; Cornelis 2006) but further research is needed to confirm this.

This study demonstrates for the first time that a *Salmonella* GH-18 chitinase indeed possesses activity toward chitin as well as toward LacNAc-TMR. This observation raises intriguing questions regarding the potential biological roles for the enzyme that deserves further research. Thus, it will be of importance to define under which conditions and locations in relation to host cells the enzyme is expressed during the infection of insect and vertebrate host organisms. It also remains to be studied which biological substrates might be a target for the LacNAc-TMR hydrolytic activity. Finally, the potential synergism between the GH-18 chitinase included in this study and other *Salmonella* chitinases that belong to the GH-19 (www.cazy.org) remains to be clarified. Our laboratories are currently conducting research to resolve these issues.

Materials and methods

Bacterial strains, standard growth medium and examination of bacterial chitinase activities

The *Salmonella* Typhimurium SL1344 strain was obtained from Dr John Elmerdahl Olsen, Department of Veterinary Disease Biology, Faculty of Life Sciences, University of Copenhagen, whereas the *E. coli* BL21 strain was purchased from Novagen (Germany). Both bacteria were propagated in Luria Broth (Merck, Germany) at 37°C.

Investigation of the chitinolytic phenotype of *Salmonella* Typhimurium 1344 was done as described by Leisner et al. (2008) using a chitin agar medium that contained per liter 10.0 g of tryptone, 5.0 g of yeast extract, 10.0 g of NaCl, 15.0 g of agar and 2.5 g of chitin from crab shells (Sigma C9752, Germany). Cultures were incubated under aerobic and

anaerobic conditions at 30°C and scored for hydrolytic ability (clearing zones) for up to 12 days.

Identification and phylogenetic analyses of genes encoding putative chitinases in Salmonella

We used the following online resources to identify genes encoding putative chitinases in published genome sequences of *Salmonella*: www.cazy.org and http://xbase.bham.ac.uk. The translated amino acid sequence of the chitinase *chiA* gene (http://xbase.bham.ac.uk/colibase/genome.pl?id=1846) from the genome-sequenced *Salmonella enterica* Typhimurium SL1344 was used as probe to search for similar sequences using the microbial resource Blast at National Center for Biotechnology Information (ncbi; www.ncbi.nlm.nih.gov/). A selection of sequences most similar to the *chiA* gene was included for further phylogenetic analyses in addition to sequences representative of other bacterial groups. Protein domain predictions including the determination of the glycosyl hydrolase family 18 (GLH18) domains were obtained from http://smart.embl-heidelberg.de/. Signal peptide predictions were performed using SignalP 3.0 from Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark (www.cbs.dtu.dk/services/SignalP/).

GLH18 domain sequences selected from BLAST searches were aligned to the *chiA* sequence. Phylogenetic trees were constructed by the neighbor-joining method using the Clustal X version 1.81 (Thompson et al. 1997) and the *MEGA* version 4 (Tamura et al. 2007) software packages with default settings.

Cloning, recombinant expression and purification of ChiAΔCtBM

Salmonella enterica Typhimurium SL1344 DNA was purified using a commercial kit (FastDNA) according to the manufacturer (MP Biomedicals, LLC, OH). Primers designed to amplify the *chiA* gene omitting the C-terminal chitin-binding domain (amino acids 646–696) were 5′-GAC GAC GAC AAG ATC GCT ACA AGC AAA CTG ATT CAA G-3′ (forward primer) and 5′-GA GGA GAA GCC CGG TTA GTA AGG TGA CGT TTT GTC ATC TG-3′ (reverse primer). PCR was done using the Roche (Germany) expand high fidelity PCR system including the following cycle steps: initial denaturing (95°C, 30 s), 25 cycles of denaturing (95°C, 30 s), annealing (60°C, 1 min), extension (68°C, 5 min) and final extension (68°C, 10 min). The PCR product was purified using the Qiagen (Germany) MinElute PCR purification kit and the predicted sizes subsequently verified by the use of a 1.5% agarose gel. The amplified sequence was cloned into the N-terminal $(His)_6$ tag pET-46 Ek/LIC vector (Novagen) and expressed in *E. coli* BL21 (DE3) as described by the manufacturer. The presence of correct insert DNA in clones was verified by sequencing (Agowa, Germany) using T7 promoter and terminator sequences as primers. The induction of the IPTG inducible T7 promoter was verified by growing cells overnight in Luria Broth at 30°C with 2 mM IPTG and 50 μL/mL of carbenicillin and subsequent lysis using Novagen Popculture reagent as described by the manufacturer. The presence of ChiAΔCtBM was verified by SDS–PAGE as well as by enzymatic activity as described below under Enzyme assays.

For protein purification, 1 L of cells were grown at 30°C to $A_{600} = 0.4$ before induction with 2 mM IPTG. After induction, growth was continued for 21 h at 30°C. The cells were harvested by centrifugation and resuspended in 50 mL of loading buffer, 20 mM MOPS, pH 7.5, containing 0.5 M NaCl and 5 mM imidazole. The cells were disrupted using a Constant Systems cell disruptor at 4°C with the pressure of 1.36 Kbar. The lysate was centrifuged at 4°C for 1.5 h at 48,000 × *g* and the filtered supernatant applied to a 5 mL HisTrap column (GE Healthcare, UK) with a flow rate of ~5 mL/min. The column was washed with 80 mL of loading buffer before being eluted with 20 mL of 100 mM MOPS, pH 7.8, 0.5 M NaCl and 0.5 M imidazole. Fractions containing enzyme were combined, concentrated in a Vivaspin (30,000 Da cutoff), exchanged into loading buffer and re-chromatographed on a 5 mL HisTrap column with a gradient of imidazole from 5 to 250 mM imidazole. The fractions containing enzyme were dialysed against 50 mM sodium phosphate buffer, pH 6.0, at 4°C. The protein concentration in the sample was determined by the Bradford method using a commercial kit (Bio-Rad, CA) using bovine γ-globulin as a protein reference standard.

Enzyme assays

The activity of ChiAΔCtBM were determined using pNP-GlcNAc, pNP-$(GlcNAc)_2$ and pNP-$(GlcNAc)_3$ as substrates. In a standard assay, 5 μL of enzyme (19.9 mg/mL) diluted 100- or 10,000-fold in 50 mM sodium phosphate buffer, pH 6.0, and 45 μL of either 0.21 or 0.81 mM substrate dissolved in Sigma buffer (A4855, pH 5.0) and incubated at 30°C for 30 min. The reaction was quenched by adding 100 μL of sodium carbonate. Absorbance was measured in a plate reader at 405 nm and corrected for absorption in a control sample with added sodium phosphate buffer, pH 6.0, instead of enzyme. Linearity was ensured by monitoring product formation at different time intervals and absorption values were converted into concentrations by the use of a *p*-nitrophenol (Sigma) standard curve. Kinetic parameters were determined for pNP-$(GlcNAc)_2$ by measuring initial rates of reaction at eight different substrate concentrations ranging from 0.1 to 4 mM. K_m and V_{max} were calculated using the software GraphPad Prism 4.0 (GraphPad Software) and k_{cat} estimated by dividing V_{max} by enzyme concentration.

The effect of temperature was determined using 0.41 mM pNP-$(GlcNAc)_2$ as a substrate incubated with 10,000-fold diluted enzyme (0.00199 mg/mL) for 30 min at 4, 10, 15, 22, 30, 37 and 40°C. For pH dependency measurements, the same substrate concentration and incubation temperature (30°C) in either phosphate buffer (pH 3, 4, 5, 6.1 and 7.2) or glycine buffer (pH 7.25 and 9.4) were used.

Chitinase activity was also measured using the soluble polymeric substrate CM-Chitin-RBV (LOEWE Biochemica, Munich, Germany) as described previously (Synstad et al. 2008) with the following modifications. The reaction mixture consisted of 100 μL of 2 mg/mL CM-Chitin-RBV, 90 μL of 5 mM sodium phosphate buffer, pH 6.0, and 10 μL of 1000-fold diluted enzyme (0.0199 mg/mL) incubated at 30°C for 30 min. The reaction was terminated by adding 50 μL of 1 M hydrochloric acid.

Activity toward 4-nitrophenyl β-D-cellobioside (Sigma) was measured as above for the chromogenic chitin pseudo-substrates. Lysozyme activity was measured by using *Micrococcus lysodeikticus* peptidoglycan as a substrate. The procedure was according to the manufacturer's instructions (Sigma) with an enzyme dilution of 100-fold (0.199 mg/mL) for assays with these two substrates.

Fluorescent substrates, either LacNAc-TMR (1 µL of 0.2 mg/200 µL H_2O), GlcNAc-TMR (0.5 µL of 0.4 mg/mL) or Type I-TMR (0.5 µL of a 0.4 mg/200 µL H_2O) were incubated with 5 µL of enzyme (1.7 mg/mL) at ambient temperature. Reaction progress was monitored by removing 0.5 µL aliquots for thin-layer chromatography on silica gel plates developed with $CHCl_3$/MeOH/H_2O (65/35/5).

Kinetic constants for LacNAc-TMR were determined by monitoring product formation with 10 different concentrations of substrate ranging from 0.080 to 10.5 mM at 30°C by CE. The reaction volume was 10 µL in 50 mM sodium phosphate buffer, pH 6.0. Aliquots (1 µL) were removed at 120 min, quenched with 50 µL of CE running buffer (50 mM borate, pH 9.3, and 150 mM sodium dodecyl sulfate). Analyses were performed on an automated PrinCE 560 CE system from PrinCE Technologies B.V. (Emmen, The Netherlands). Separations were carried out in an uncoated fused-silica capillary of 75 µm i.d. with CE running buffer. TMR-labeled compounds were detected and quantitated using an Argos 250B fluorescence detector (Flux Instruments, Switzerland) equipped with an excitation filter of 546.1/10 nm and an emission filter of 570 nm. All experiments were carried out at a normal polarity, i.e. inlet anodic. Data were processed by PrinCE 7.0 software.

Nuclear magnetic resonance

NMR spectra were recorded at 15 or 25°C on a Bruker Avance 800 instrument. Deuterated buffer was prepared by lyophilizing 5 mL of 50 mM sodium phosphate buffer, pH 6.0, followed by the addition of 2 mL of ^2H_2O to the dried solids, re-lyophilization and suspension in 5 mL of ^2H_2O. Substrates, either crab shell chitin (Sigma C9752), colloidal chitin (prepared as described by Larsen et al. 2010), $(GlcNAc)_5$, pNP-$(GlcNAc)_2$, $(GlcNAc)_3$ or LacNAc-TMR (0.4–1 mg) were dissolved in 0.7 mL of buffer, transferred to 5 mL NMR tubes and standard 1D ^{1}H-NMR spectra of substrates were acquired before and after the addition of 5–50 µL of enzyme. Spectra were recorded at 799.3 MHz using TMS ($\delta = 0$ ppm) as the internal standard with a 32 scan composite presaturation.

The spectra at 800 MHz were obtained on the Bruker Avance 800 spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Molecules.

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

CE, capillary electrophoresis; CM-Chitin-RBV, carboxymethyl chitin Remazol Brilliant Violet; CtBM, C-terminal chitin-binding module; GH-18, glycoside hydrolase family 18; GlcNAc, *N*-acetylglucosamine; GH-18, glycosyl hydrolase family 18; LacNAc-TMR, Galβ1 → 4GlcNAcβ-*O*-$(CH_2)_8CONH(CH_2)_2NHCO$-tetramethylrhodamine; GlcNAc-TMR, GlcNAc-β-*O*-$(CH_2)_8CONH(CH_2)_2NHCO$-tetramethylrhodamine; $(GlcNAc)_2$, *N*,*N*′-diacetylchitobiose; $(GlcNAc)_3$, *N*,*N*′,*N*″-triacetylchitotriose; $(GlcNAc)_5$, penta-*N*-acetylchitopentaose; pNP-GlcNAc, 4-nitrophenyl *N*-acetyl-β-D-glucosaminide; pNP-$(GlcNAc)_2$, 4-nitrophenyl *N*,*N*′-diacetyl-β-D-chitobioside; pNP-$(GlcNAc)_3$, 4-nitrophenyl β-D-*N*,*N*′,*N*″-triacetylchitotriose; Type I-TMR, Galβ1 → 3GlcNAcβ-*O*-$(CH_2)_8CONH(CH_2)_2NHCO$-tetramethylrhodamine.

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