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Characterization of a chitinase from the cellulolytic actinomycete *Thermobifida fusca*

Yasser Gaber^a, Sophanit Mekasha^b, Gustav Vaaje-Kolstad^b, Vincent G.H. Eijsink^b, Marco W. Fraaije^{c,*}

^a Beni-Suef University, Microbiology Department, Faculty of Pharmacy, Beni-Suef 62514, Egypt

^b Norwegian University of Life Sciences, NMBU, Department of Chemistry, Biotechnology and Food Science, P.O. Box 5003, NO-1432 Aas, Norway

^c University of Groningen, Molecular Enzymology Group, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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ABSTRACT

Thermobifida fusca is a well-known cellulose-degrading actinomycete, which produces various glycoside hydrolases for this purpose. However, despite the presence of putative chitinase genes in its genome, *T. fusca* has not been reported to grow on chitin as sole carbon source. In this study, a gene encoding a putative membrane-anchored GH18 chitinase (Tfu0868) from *T. fusca* has been cloned and overexpressed in *Escherichia coli*. The protein was produced as SUMO fusion protein and, upon removal of the SUMO domain, soluble pure TjChi18A was obtained with yields typically amounting to 150 mg per litre of culture. The enzyme was found to be relatively thermostable (apparent $T_m = 57.5^\circ\text{C}$) but not particularly thermoactive, the optimum temperature being 40–45 °C. TjChi18A bound to α - and β -chitin and degraded both these substrates. Interestingly, activity towards colloidal chitin was minimal and in this case, substrate inhibition was observed. TjChi18A also cleaved soluble chito-oligosaccharides and showed a clear preference for substrates having five sugars or more. While these results show that TjChi18A is a catalytically competent GH18 chitinase, the observed catalytic rates were low compared to those of well-studied GH18 chitinases. This suggests that TjChi18A is not a true chitinase and not likely to endow *T. fusca* with the ability to grow on chitin.

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1. Introduction

Chitin is a polymer with high similarity to cellulose. It is naturally found as a supportive material in the shells of marine creatures such as crustaceans and also in the cell walls of fungi. Chitin can be referred to as the marine-equivalent of cellulose and is composed of *N*-acetyl-D-glucosamine units linked via β 1–4 glycosidic bonds. Chitin occurs in two major forms classified according to the arrangement of the polymeric chains, namely α -chitin, a more recalcitrant form with anti-parallel chain orientation, and β -chitin, a less recalcitrant form with parallel chain orientation [1].

Chitin-degrading enzymes are classified in the Carbohydrate-Active enZymes (CAZy) database based on their amino acid sequences and occur primarily in Glycoside Hydrolase (GH) families GH18, GH19 and GH20 [2,3]. The GH18 family includes chitinases from bacteria, fungi, higher eukaryotes and viruses. GH18 enzymes have a characteristic TIM barrel-fold [4,5] with a conserved glutamate residue, which is part of a conserved DXXDXDXE sequence motif, acting as catalytic acid. Many GH18 chitinases contain an insertion between beta-strand 7 and alpha-helix 7 of the TIM barrel that comprises 50–70 residues and is sometimes referred to as the $\alpha + \beta$ or α/β domain. This insertion,

which typically lacks in GH18 enzymes known to be non-processive and endo-acting, contributes to shaping deep-active site clefts and to substrate-binding [6,7]. In addition to the catalytic domain many GH18 enzymes contain one or more carbohydrate-binding modules (CBMs) that contribute to substrate-binding. Notably, this basic architecture shows quite some variation. In particular, there is large variation in the occurrence and length of surface loops, which relates to the interaction between the catalytic domain and varying CBMs in varying orientations, as well as to the shape of the substrate-binding surface or cleft. It has been well-documented that the active site clefts of GH18 enzymes are tailored for different and often complementary chitinolytic functionalities; family members differ in terms of preferred substrate binding mode, endo- vs exo-activity, as well as processivity and its directionality [7].

Thermobifida fusca is a well-known cellulolytic actinomycete whose cellulases have been extensively studied [8]. Generally, *T. fusca* is known as a good source of robust biocatalysts [9]. *T. fusca* is a moderately thermophilic actinobacterium that grows at 50–55 °C and lives in a wide pH range [10]. *T. fusca* secretes multiple extracellular glycoside hydrolases and can efficiently degrade cellulosic biomass using enzymes such as cellulases, xylanases, and lytic polysaccharide monooxygenases [11, 12]. Despite the presence of two putative GH18-encoding genes in its genome and its known production of a chitin-active LPMO called E7 [11,12], *T. fusca* is not capable to grow on chitin as single carbon source

* Corresponding author.

[8,11,13]. This observation raises the question whether the putative GH18 chitinases represent functional enzymes. We report on the cloning, expression and characterization of one of these putative chitinases (Tfu_0868), with UniProt accession number Q47RL1 and hereunder referred to as *TfChi18A*. Judged by its sequence, *TfChi18A* is a single domain GH18 chitinase that is secreted via the Tat-pathway.

2. Material and methods

2.1. Sequence analysis, homology modelling and docking

For Tat-signal peptide analysis (TatP), and prediction of transmembrane membrane protein topology (TMHMM) servers at www.cbs.dtu.dk/services were used. BLAST searches were performed at NCBI (blast.ncbi.nlm.nih.gov/Blast.cgi). The YASARA homology modelling function with default settings was used for modelling the structure of *TfChi18A* [14]. Coordinates for chitoheptaose were extracted from the PDB (2PI8; [15]) and the ligand was subjected to the default energy minimization function in YASARA employing the AMBER99 force field. Docking was done using AutoDock Vina via the PyRx interface [16,17]. A structure-based multiple sequence alignment was generated using PROMALS3D [18].

2.2. Cloning, expression, and purification of *TfChi18A*

T. fusca YX was grown on Hågerdal medium at 50 °C. Genomic DNA was extracted using the Bacterial Genomic Miniprep Kit from Sigma Aldrich following the provided protocol. The gene encoding *TfChi18A* (NCBI reference sequence, NC_007333.1, gene ID, 3579555, protein, Tfu_0868) was amplified by PCR in such a way that the region encoding the signal peptide was removed. The signal peptide was determined by sequenced-based predictions and by comparing a structural model of *TfChi18A* to the crystal structure of a related bacterial chitinase (PDB:1KFW, [19]). A serine residue was introduced at the N-terminus of the chitinase to allow easy cleavage of the N-terminally fused SUMO protein, equipped with an N-terminal His-tag, by the SUMO-specific protease. More details on construct design are provided below and in Table S1. The primers used for amplification of the chitinase gene were 5'TCTCGCCTGGGGAAGTGGTGGACCA3' (forward) and 5'TCAGCGCAGCGACTCCGCCAT3' (reverse). PCR reactions were performed using the Expand long template PCR kit from Roche Diagnostics (Mannheim, Germany). Reaction mixtures contained expand long template buffer (10 µL), 2.5 mM dNTP (2.5 µL), 10 µM forward primer (1.5 µL), 10 µM reversed primer (1.5 µL), genomic DNA (2.0 µL), DMSO (3.0 µL), polymerase blend (0.75 µL), and water (up to 50 µL). The PCR conditions were as follows: 95 °C, 3 min; 30 × [95 °C, 1 min; 59 °C, 1 min; 68 °C, 1 min]; 68 °C, 7 min. *Taq* polymerase (5 U) and dATP (0.6 mM) purchased from New England BioLabs were used to add 3'-A overhangs to the obtained PCR product. This DNA was ligated into the pET-SUMO vector as described in the Champion pET SUMO Expression System user manual (Invitrogen, Carlsbad, CA). The plasmid was transformed into *Escherichia coli* One Shot[®] Mach1[™]-T1[®] and a few colonies were selected for verifying the correct construct and the gene sequence using DNA sequencing (GATC, Konstanz, Germany).

For overexpression, the newly generated expression vector was transformed into *E. coli* BL21 (DE3) CodonPlus. The resulting colonies were pre-inoculated into TB broth supplemented with kanamycin (50 µg/mL) and grown overnight at 37 °C. Such overnight cultures were used to start 750 mL cultures in 2-litre Erlenmeyer flasks at 37 °C. When the A_{660} reached 0.4–0.6, isopropyl β -D-1-thiogalactoside was added to a final concentration of 1 mM for the induction of expression, followed by further growth at 24 °C for 16 h. Cells were harvested by centrifugation and the cell pellets were resuspended in PBS buffer, pH 7.5. Cell disruption was performed with using a Vibra-cell sonicator (Sonics and Materials INC Danbury, CT, USA), 10 min and 50% on/off operation. Ammonium sulphate was added to the obtained cell lysate to a

final concentration of 20% saturation, to precipitate part of the background *E. coli* proteins. The insoluble debris was removed by centrifugation at 70,000 × g for 40 min at 4 °C. After filtration through a 0.45-µm syringe filter, the lysate was loaded onto a Ni²⁺-affinity column (5 mL HisTrap[™] FF, GE Healthcare) and the column was extensively washed with the binding buffer (50 mM PBS pH 7.5, 0.5 M NaCl, 20 mM imidazole). To elute the protein, a gradient of 0–500 mM imidazole in 50 mM PBS pH 7.5, 0.5 M NaCl was applied. Fractions containing SUMO-*TfChi18A* were pooled and the buffer was exchanged using an Amicon concentrator equipped with a 30 kDa filter under nitrogen pressure at 4 °C, in PBS, pH 7.5. The fusion protein was then incubated overnight at 30 °C with SUMO-protease (Invitrogen, Carlsbad, CA, USA), using approximately 2 µg of protease per mg of chitinase, to remove the N-terminal SUMO tag. Both SUMO and SUMO-protease have a His-tag and these two compounds were removed by a further Ni²⁺-affinity column purification step. For polishing the enzyme preparation, preparative size exclusion chromatography (Superdex 75 26/60, GE Healthcare) was performed in PBS, pH 7.5. Protein purity was assessed by SDS-PAGE.

2.3. Determination of apparent T_m

The apparent melting temperature of purified *TfChi18A* was determined using the ThermoFluor method [20,21]. 20 µL of a 1 mg/mL protein solution in PBS, pH 7.5, was mixed with 5 µL of 100 times diluted commercial stock solution of SYPRO Orange dye (Life Technologies, CA, USA). The samples were placed in an iQ 96-well PCR plate (Bio-Rad), and measurements were conducted using a MyiQ real-time PCR machine (Bio-Rad). The samples were heated from 20 to 95 °C at 1 °C/min and the fluorescence was recorded using a 490 nm excitation filter and a 575 nm emission filter.

2.4. Activity assays

Activity on α -chitin (Chitinor, Avaldsnes, Norway), β -chitin (France chitin, Orange, France) and colloidal chitin was tested by incubating 15 mg/mL of substrate with 1.0 µM of *TfChi18A* in 10 mM BisTris, pH 6.0, at 40 °C. Colloidal chitin was produced by treating 20 g of α -chitin (Chitinor, Norway) with 200 mL of 37% (12 M) hydrochloric acid. The mixture was incubated overnight at room temperature while mixing with a magnetic stirrer. After incubation, the gel-like slurry was diluted twice with sterile water. Hereafter, the colloidal chitin was recovered by filtering the slurry using vacuum filtration with a Pyrex glass-filter (Fischer Scientific). The pH of the colloidal chitin was adjusted by washing with sterile water until the pH reached 7, after which the material was stored at 4 °C. Product formation was analysed using HPLC (see below). Activity on soluble fully acetylated chitoooligosaccharides (A2–A6) was analysed by incubating 1 mM of substrate with 1 µM of enzyme in 10 mM BisTris, pH 6.0, at 45 °C. The temperature and pH optima for chitinase activity were determined using 0.5 mM A5 as a substrate and an enzyme concentration of 1 µM. For determination of the pH optimum for *TfChi18A*, the reaction time was 2 h and the experiment was run at 45 °C using the following buffers (50 mM ammonium acetate or BisTris or Tris or CAPS). For determination of the temperature optimum, the buffer was 50 mM BisTris pH 6.5 and the incubation time was 2 h. In all activity assays, reactions were stopped by adding sulphuric acid to a final concentration of 25 mM.

2.5. Product analysis by HPLC

Hydrolysis products were analysed by normal phase HPLC using a Rezex RFQ-Fast Acid H⁺ (100 × 7.8 mm) column with a carbo-H (4 × 3 mm) Guard column (Phenomenex) [22]. Samples (8 µL) were injected onto the column, which was operated at 85 °C, and chitoooligosaccharides were eluted isocratically at a rate of 1 mL/min in 5 mM H₂SO₄. Eluting compounds were detected with an UV detector

Fig. 1. Effect of different temperatures on overexpression of SUMO-*TfChi18A* as detected by SDS-PAGE analysis. Cultures, in TB medium (15 mL), were grown overnight at the indicated temperatures. After sonication of the cells, both the cell free-extract ("S") and the pellet ("NS") were analysed (A). Panel B shows *TfChi18A* after being cleaved from SUMO and purified to homogeneity. The calculated mass of this purified protein is 46.3 kDa. The masses of selected marker proteins are indicated in kDa.

set at 195 nm. Quantification was achieved using external standard curves constructed by injecting solutions of A2 and A3 with known concentrations.

2.6. Chitin binding

To assess enzyme binding to α - and β -chitin, *TfChi18A* (3 μ g) was mixed with chitin (3 mg) in 500 μ L 20 mM BisTris, pH 6.0. The tubes were slowly mixed using a rotation mixer (Biosan, Riga, Latvia) at room temperature for 3 h, followed by centrifugation at 16,000 rcf for 3 min using a Centrifuge 5418R (Eppendorf, Germany). The clear supernatant containing the unbound protein was collected for analysis and the pellet was washed 3 times with 500 μ L of the binding buffer. The washed chitin was then mixed with equal amounts (30 μ L) of binding buffer and 2 $\times$ SDS-PAGE sample buffer and the chitin-bound protein was released by boiling for 10 min. All samples were then analysed by SDS-PAGE. Purified Chitinase A from *Serratia marcescens* (*SmChi18A*; [23]) was used as a positive control. A recombinantly produced highly active mannanase domain, Man5A, from *Caldibacillus cellulovorans* was used as negative control [24].

Fig. 2. Determination of the optimum temperature for activity of *TfChi18A*. The reaction conditions were as follows: 0.5 mM A5 and 1 μ M enzyme in 50 mM BisTris pH 6.5, and incubation for 2 h at the indicated temperatures.

3. Results

3.1. Sequence and structural analysis

Lykidis et al. published the genome sequence of *T. fusca* and described the putative glycoside hydrolases encoded by the *T. fusca* genome [25]. This analysis revealed the presence of two genes encoding putative single-domain hydrolases belonging to the GH18 family: Tfu0580 and Tfu0868 (*TfChi18A*, 451 residues). Most GH18 family members act as chitinases, hydrolyzing *N*-acetylglucosamine- and glucosamine-based polymers. Some GH18 family members do not exhibit any catalytic activity but act as xylanase inhibitors or lectins. Both putative *T. fusca* chitinases carry sequence hallmarks of active GH18 chitinases, such as the DXXDXDXE motif, which includes the catalytic glutamate (residues in 209–216 in *TfChi18A*), and an SXGG motif upstream (residues 154–157 in *TfChi18A*). A BLAST search revealed that there are close homologues of *TfChi18A* (30 sequences with >50% sequence identity) in actinomycetes.

The N-terminus of the primary gene product contains a putative Tat-signal sequence (residues 1–16, MAGRHRILPARRRTFA↓PT), indicating

Fig. 3. Determination of the optimal pH for activity of *TfChi18A*. The reaction conditions were as follows: 0.5 mM A5 and 1 μ M enzyme in 50 mM of various buffers, and incubation for 2 h at 45 °C. The following buffers were used: ammonium acetate (pH 3.0–5.0), BisTris (pH 5.8–7.0), Tris (pH 7.5–9.0), and 3-(cyclohexylamino)-1-propanesulfonic acid (pH 9.7–11.0).

Fig. 4. Binding of *TfChi18A* to chitin. The enzymes (3 μ g) were incubated with 3 mg substrate in a total reaction volume of 500 μ L. The picture shows an SDS-PAGE gel loaded with samples from an experiment with *TfChi18A* and a positive control experiment with Chitinase A from *S. marcescens* (*SmChi18A*): lane 1, marker proteins; lane 2, purified *SmChi18A*; lane 3, *SmChi18A* in the binding reaction mixture, no substrate added; lane 4, *SmChi18A* in the binding reaction mixture after incubation with α -chitin (= unbound protein); lane 5, *SmChi18A* eluted from α -chitin (= bound protein); lane 6, *SmChi18A* in the binding reaction mixture after incubation with β -chitin; lane 7, *SmChi18A* eluted from β -chitin; lane 8, marker proteins; lane 9, purified *TfChi18A*; lane 10, *TfChi18A* in the binding reaction mixture, no substrate added; lane 11, *TfChi18A* in the binding reaction mixture after incubation with α -chitin; lane 12, *TfChi18A* eluted from α -chitin; lane 13, *TfChi18A* in the binding reaction mixture after incubation with β -chitin; lane 14, *TfChi18A* eluted from β -chitin. Control experiments carried out under the same conditions showed no binding for a recombinantly produced non-chitinolytic enzyme, Man5A from *Caldicoccus cellulosivorans*.

that the protein is secreted by *T. fusca* [26]. Analysis by TMHMM (Fig. S1) further suggested that the secreted protein may be anchored to the membrane since residues 19–41 are predicted to form a trans-membrane helix, with strong confidence. The closest homologue of *TfChi18A* with a known crystal structure is a chitinase from *Arthrobacter* sp. Tad20 (PDB:1KFW; 40% sequence identity; [19]). Using the YASARA procedure, a homology model for *TfChi18A* was built, which was a hybrid of 17 initial models that were constructed based on the following templates: 1KFW (chitinase B from *Arthrobacter* sp. Tad20), 3ARX (chitinase A from *Vibrio harveyi*), 1ITX (chitinase A1 from *Bacillus circulans*), 3B9D (chitinase A from *V. harveyi* in complex with a penta-saccharide), and 2WMO (chitinase A from *S. marcescens*). The obtained model included residues 42–451 of *TfChi18A* and the overall Z-score was -1.44 , indicating satisfactory quality [27]. The modelled structure of *TfChi18A* is discussed in more detail below.

3.2. Production and purification and initial characterization of *TfChi18A*

The sequence analysis and model building efforts described above consistently showed that the chitinase domain starts at Arg42. Therefore, for obtaining *TfChi18A* in a soluble form, the first 41 residues were replaced with His-tagged SUMO as outlined in detail in Table S1. SUMO has been shown to be effective in boosting expression of soluble protein in *E. coli*. Indeed, we observed good overexpression of the SUMO-*TfChi18A* fusion protein under various conditions, for example at 17, 24, 30 or 37 $^{\circ}$ C using LB or TB medium (Fig. 1A). The His-tagged fusion protein could be purified by affinity chromatography and after treatment with the SUMO protease highly pure *TfChi18A* was obtained (Fig. 1B). Typically, from 1 L of TB culture about 150 mg of *TfChi18A* was obtained.

To check for stability of the recombinant protein its thermal unfolding was monitored using the fluorescence-based ThermoFluor assay. This experiment yielded a typical single transition unfolding curve with a relatively high apparent melting temperature of 57.5 $^{\circ}$ C (Fig. S2). This apparent melting temperature is in good agreement with the optimal growth temperature of *T. fusca*, which is 55 $^{\circ}$ C. As part of the initial characterization of the enzyme, its temperature and pH optima were determined using A5 as a substrate. The optimum

temperature for activity at pH 6.5 was 40–45 $^{\circ}$ C and at temperatures above 50 $^{\circ}$ C activity became drastically reduced (Fig. 2). The enzyme displayed >50% of maximum activity in the pH range of 6.0 to 7.8, with a peak between 6.5 and 7.0 (Fig. 3). Initial tests of activity towards colloidal chitin using the chito-oligosaccharide oxidase-based assay [28] revealed weak chitinase activity. Binding assays (Fig. 4) showed that *TfChi18A* binds to both α - and β -chitin.

3.3. Further insight into the activity of *TfChi18A*

To obtain further insight into the activity of *TfChi18A*, reactions with several substrates were done and HPLC was used for product analysis. Fig. 5 shows the activity of *TfChi18A* on three different types of chitin. In all cases, the dominating products were A2 and A3, appearing in similar amounts (Fig. S3), which is different from many other family 18 chitinases, and in particular processive family 18 chitinases such as *SmChi18A*, whose by far most dominant product is A2 [29]. The progress curves in Fig. 5 show that *TfChi18A* is more active towards β -chitin than towards α -chitin. Higher activity towards β -chitin than towards α -chitin. Higher activity towards β -chitin is not uncommon and is ascribed to the more open and accessible structure of this chitin-form, which has less inter-chain hydrogen bonds and more hydrogen bonds with water compared to α -chitin [1,3,30,31]. Notably, while there clearly is activity, this activity is low. If we assume that each detected product is the result of two cleavages, the apparently linear progress curve for β -chitin between 6 and 23 h represents a rate in the order of two cuts per hour. The real rate is probably higher since each soluble product is likely a result of more than two cuts in the polymeric chitin chain. In any case, compared with other chitinases whose activity was quantified in the same way, the activity of *TfChi18A* is about three orders of magnitude lower [29,30]. Quite remarkably, the activity towards colloidal chitin, usually considered to be the “easiest substrate”, was very low. The progress curve flattens after 1 h, giving the impression that only a tiny subfraction of the substrate could be processed by the enzyme. Interestingly, further experiments suggested that the lack of activity on this flexible polymeric substrate is likely due to non-productive-binding (Fig. S3).

Activity assays with oligomeric substrates showed that *TfChi18A* does not cleave A3 (not shown), whereas it can convert A4 (to A2), A5 (to A2 + A3) and A6 (to A2, A4 and, primarily A3), as shown in Fig. 6A. The data in Fig. 6A also show that conversion of A4 was much slower than conversion of A5 and A6. Detailed kinetic analyses (Figs. 6B and C, and S4) showed an almost linear relationship between substrate concentration and catalytic turnover. The fact that saturation kinetics were not observed suggests that the K_m values for A5 and A6 are high, i.e. higher than the substrate concentrations assessed here (up to 1.5 mM for A6). The highest conversion rates were obtained at the highest tested substrate concentrations, namely 6.8 min^{-1} at 0.6 mM A5 and 26 min^{-1} at

Fig. 5. Activity of *TfChi18A* on α -, β - and colloidal chitin. Activity was monitored by quantitative analysis of the two major products, A2 and A3, in reactions containing 15 mg/mL substrate and 1 μ M enzyme in 10 mM BisTris, pH 6.0, incubated at 40 $^{\circ}$ C. Standard deviations obtained from three measurements are indicated but are mostly not visible because they were small.

Fig. 6. Activity of *TfChi18A* on chito-oligomers. (A) HPLC chromatograms showing products released after hydrolysis of A4, A5 or A6 (conditions: 1 mM of substrate, 1 μM of enzyme in 10 mM BisTris, pH 6.0, 45 °C; incubation for 6 h). The upper chromatogram shows a standard oligomer mixture. Note that conversion of A4 is far from complete, indicating that this substrate is converted much slower than A5 and A6. (B) Progress curves for conversion of A6 at different substrate concentrations (conditions: 300 nM *TfChi18A* in 20 mM BisTris pH 6.0, incubated at 45 °C; A2, which equals A4, and A3 were quantified; the number of cleavages is $2[A2]/2 + [A3]/2$). (C) A6 conversion rates at different substrate concentrations; data derived from the plots shown in panel B. Analogous data for conversion of A5 are shown in Fig. S4.

1.5 mM A6. Notably, the maximum conversion rate observed for A6 is one–two orders of magnitude lower compared to e.g. the endo- and exo-acting GH18 chitinases from *S. marcescens* [29,30].

Activity of *TfChi18A* on 65% acetylated chitosan was also assessed using a previously described experimental set-up [32]. As expected on the basis of the low activity towards colloidal chitin, activity on chitosan was low with estimated rates in the order of 1 min^{-1} , which is two–three orders of magnitude lower compared to other chitinases [32,33]. Since some members of the GH18 family are endoglycosidases that can cleave GlcNAc–GlcNAc bonds in glycoproteins but do not act on chitin, we also tested the ability of *TfChi18A* to deglycosylate RNase B, using a previously described experimental set-up [34]. We could not detect deglycosylating activity (results not shown).

4. Discussion

The data presented above show that *TfChi18A* is a catalytically competent GH18 chitinase, albeit with low activity. For practical reasons several of the activity assays were run at a suboptimal pH (6.0 instead of 6.5), but even taking into account that our data give slightly underestimated maximum rates, it is clear that the enzyme is less active than other chitinases. The kinetic data for A4–A6 show that the enzyme is more active on longer substrates. This suggests an extended substrate-binding cleft and significant enzyme–substrate interactions in subsites away from the catalytic centre. In this respect it is paradoxical that the enzyme showed low activity on polymeric substrates, in particular on the more flexible and easily accessible of these, colloidal chitin and chitosan. The fact that chitin primarily is converted to similar amounts of dimers and trimers, and not primarily to dimers, suggests that *TfChi18A* is an endo-acting non-processive enzyme. The activity of *TfChi18A* on chitin was nowhere near the activity of chitinases secreted by known efficient chitin-degrading microbes such as *S. marcescens*.

Both the sequence and the structural model (Fig. 7A and B) of *TfChi18A* are in accordance with the experimental observation that the enzyme is a catalytically competent chitinase. Next to the DXXDXDXE motif (residues 209–216), also other residues known to be involved in catalysis [35,36] are present, in particular (analogous residue in the well-studied *SmChi18B* is given in parenthesis): Tyr57 (Tyr10), Ser154 (Ser93), Tyr291 (Tyr214) and Asp292 (Asp215). Aromatic residues, previously shown to be important for substrate binding are also well conserved, including Trp61 (Trp167 in the –3 subsite of *SmChi18A*), Trp158 (+1 subsite; Trp275/Trp97 in *SmChi18A/SmChi18B*), and Trp297 (+2 subsite; Phe396/Trp220 in *SmChi18A/SmChi18B*) (Figs. 7A and B, and S5). The active site of *TfChi18A*, does, however, show an unusual feature in the crucial –1 subsite where substrate distortion, in particular correct orientation of the *N*-acetyl group, needs to take place [37]. Here, a conserved methionine (Met212 in *SmChi18B*) is substituted with a glutamine (Gln289) (see Fig. S5 for a structure-based multiple sequence alignment). This substitution does occur in a few other members of the GH18 family, but the structures of these proteins, including the *Arthrobacter* chitinase (1KFW), indicate that this glutamine does not contribute to the topology of the –1 subsite. In GH18 chitinases containing the Met → Gln mutation, a phenylalanine (Phe232 in the *Arthrobacter* enzyme) takes over the subsite shaping role of the methionine, as shown in Fig. 7C. Interestingly, *TfChi18A* does not seem to have a residue that can replace the methionine structurally, which, knowing the importance of substrate distortion in the –1 subsite for catalysis could explain why the enzyme is not very active. Structural studies are needed to investigate this hypothesis.

All in all, the activity data presented above suggest that *TfChi18A* is catalytically competent, but not a true chitinase. The enzyme would likely not endow *T. fusca* with the ability to grow on chitin as a sole carbon source, an ability that indeed is lacking [8,11]. It is interesting to note that, while *T. fusca* on the one hand encodes two GH18s and two

Fig. 7. A, Model of *TjChi18A* showing the active site groove with aromatic residues (purple) putatively participating in substrate binding and the catalytic acid, Glu216 (red), which is hidden behind Trp158. B, Possible binding mode for (GlcNAc)₆, which is bound to subsites -4 to +2; sugars in the - subsites have blue carbons, sugars in the + subsites have orange carbons. C, Details of the -1 subsite in *SmChi18B* (left; PDB: 1E6N), chitinase B from *Arthrobacter* sp. Tad20 (middle; PDB: 1KFW) and *TjChi18A* (right); see text for more details. The position of the pentameric substrate, of which only four sugars binding to the -2/+2 subsites are shown, was derived from 1E6N, which is a complex of *SmChi18B_E144Q* with (GlcNAc)₅.

LPMOs (with known activity on chitin; [11,12]) it lacks genes coding for other enzymes usually involved in chitin metabolism. In particular, it lacks genes encoding the GH20 beta-hexosaminidase that is usually employed to convert chitobiose and chito-oligomers to monosugars. The GH94 family, which includes chitobiose phosphorylase, is also lacking. The low activity of *TjChi18A* thus coincides with a genomic composition that seems incomplete when it comes to chitin metabolism. At present, there is no obvious explanation for the low activity of *TjChi18A* on chitinous substrates. Further studies, both of the enzyme itself and of the regulation of its expression, are needed to unravel a possible alternative function. There are indications that Actinomycetes in particular employ GH18 enzymes in their defence against chitin-containing competitors, in particular fungi [38]. The use of chitinases to combat fungi by attacking the chitin in fungal cell walls is a well-established phenomenon in biology, but there is some recent data suggesting that some GH18 enzymes have additional roles in microbial interactions that involve catalysing the hydrolysis of non-chitin substrates [39,40]. Perhaps *TjChi18A* is part of a defence system and perhaps it acts on other components than chitin.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbapap.2016.04.010>.

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