

# Characterization of two novel bacterial type A *exo*-chitobiose hydrolases having C-terminal 5/12-type carbohydrate-binding modules

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**Abstract** Type A chitinases (EC 3.2.1.14), GH family 18, attack chitin ((1 → 4)-2-acetamido-2-deoxy-β-D-glucan) and chito-oligosaccharides from the reducing end to catalyze release of chitobiose (*N,N'*-diacetylchitobiose) via hydrolytic cleavage of *N*-acetyl-β-D-glucosaminide (1 → 4)-β-linkages and are thus “*exo*-chitobiose hydrolases.” In this study, the chitinase type A from *Serratia marcescens* (SmaChiA) was used as a template for identifying two novel *exo*-chitobiose hydrolase type A enzymes, FbalChi18A and MvarChi18A, originating from the marine organisms *Ferrimonas balearica* and *Microbulbifer variabilis*, respectively. Both FbalChi18A and MvarChi18A were recombinantly expressed in *Escherichia coli* and were confirmed to exert *exo*-chitobiose hydrolase activity on chito-oligosaccharides, but differed in temperature and pH activity response profiles. Amino acid sequence comparison of the catalytic β/α barrel domain of each of the new enzymes showed individual differences, but ~69% identity of each to that of SmaChiA and highly conserved active site residues. Superposition of a model substrate on 3D structural models of the catalytic domain of the enzymes corroborated *exo*-chitobiose hydrolase type A activity

for FbalChi18A and MvarChi18A, i.e., substrate attack from the reducing end. A main feature of both of the new enzymes was the presence of C-terminal 5/12 type carbohydrate-binding modules (SmaChiA has no C-terminal carbohydrate binding module). These new enzymes may be useful tools for utilization of chitin as an *N*-acetylglucosamine donor substrate via chitobiose.

**Keywords** Chitinase · *Ferrimonas balearica* · *Microbulbifer variabilis* · C-terminal CBM

## Introduction

Chitin, a β-(1,4)-linked polymer of *N*-acetylglucosamine moieties (GlcNAc), is the second most abundant natural polysaccharide in the world besides cellulose. Chitin serves as the structural component in the exoskeleton of the arthropods (including crustaceans such as crabs and shrimp) and is also present in the cell walls of fungi and yeast (Synowiecki and Al-Khateeb 2003; Rinaudo 2006). Partial deacetylation of chitin results in chitosan; in contrast to chitin, chitosan is soluble in acidic aqueous solution. Both chitin and chitosan are of commercial interest due to their functional properties as well as their presumed biocompatibility and have recently been reported to have potential in applications ranging from biomaterials to drug delivery (Anitha et al. 2014). Since chitin is abundantly available, chitinases are interesting as processing aids for production of chito-oligosaccharides which present several possible medical applications, including inhibition of tumor growth (Shen et al. 2009) and of TH2-induced inflammation in asthma (Elias et al. 2005). In addition, chitobiose was recently shown to act as an *N*-acetylglucosamine donor substrate for enzymatic *trans*-glycosylation reactions to produce human milk oligosaccharide mimics (Nyffenegger et al.

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2015). Chitinases (EC 3.2.1.14) are glycoside hydrolases that catalyze hydrolytic depolymerization of chitin into oligosaccharides by catalyzing the cleavage of the  $\beta$ -(1,4) linkages in the polymer backbone (Cohen-Kupiec and Chet 1998). Based on the similarity of amino acid sequences and their catalytic mechanism, chitinases can be divided into two glycosyl hydrolase (GH) families: GH family 18 and 19 (Lombard et al. 2014). Chitinases of family 18 are distributed among microbes, animals, and other organisms while GH 19 chitinases were first found in higher-order plants, and somewhat later, the first bacterial GH 19 chitinase was identified in *Streptomyces griseus* (Ohno et al. 1996). Up to now (January 2017), according to the CAZy database, chitinases of family 19 have been found in bacteria, eukaryotes, and viruses (Cantarel et al. 2009). Most of the available knowledge of microbial GH 18 chitinases is based on detailed work done on the three *Serratia marcescens* chitinases, ChiA (SmaChiA), ChiB (SmaChiB) (Fuchs et al. 1986; Van Aalten et al. 2000; Horn et al. 2006a, b), and ChiC (SmaChiC) (Suzuki et al. 1999; Synstad et al. 2008). In addition to the GH18 SmaChiA, SmaChiB, and SmaChiC, *S. marcescens* produces a lytic polysaccharide monoxygenase, CBP21, active on chitin (Suzuki et al. 1998; Vaaje-Kolstad et al. 2005). SmaChiA and SmaChiB are described as processive exolytic enzymes that catalyze the release of chitobiose from crystalline chitin acting from the reducing and non-reducing ends, respectively (Hult et al. 2005), whereas SmaChiC is an *endo*-acting enzyme attacking randomly in amorphous regions of the chitin backbone (Horn et al. 2006a, b; Sikorski et al. 2006). SmaChiA and SmaChiB are known as multidomain enzymes both having a central catalytic domain including a  $(\beta/\alpha)_8$  triosephosphate isomerase (TIM) barrel fold structure containing the key catalytic motif DXXDXDXE (Van Aalten et al. 2001) and minimum one chitin-binding domain: SmaChiA has an *N*-terminal chitin-binding module, whereas SmaChiB has a C-terminal carbohydrate-binding module (CBM) 5/12 type chitin-binding module (and thus SmaChiA does not have a C-terminal CBM module and SmaChiB does not have an *N*-terminal chitin-binding module) (Vaaje-Kolstad et al. 2013).

The objective of the present work was to identify new chitinases able to catalyze the release of chitobiose from chitin as part of our quest to produce designed human milk oligosaccharides via enzymatic reactions on natural substrates, i.e., including using chitin derivatives as a source of GlcNAc moieties (Nyffenegger et al. 2015). In addition, the objective of the work was to improve the understanding of the action of chitinases on chito-oligosaccharides, and not least to investigate the chitin-binding domains (or modules) of GH18 chitinases. The SmaChiA enzyme was used as a sequence template in a genomic database mining approach to identify putative chitinases. On this basis, we report here the identification and characterization of two novel bacterial chitinases

from marine microbes. Both enzymes are able to cleave the chito-oligosaccharides to produce chitobiose, and both enzymes contain the classic GH18 chitinase catalytic domain in addition to a C-terminal CBM with only low homology to SmaChiB.

## Materials and methods

### Chemicals

Chitin (from shrimp shells), isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG), and imidazole were purchased from Sigma-Aldrich (Steinheim, Germany). 4-Nitrophenyl-*N*-acetyl- $\beta$ -D-glucosamine (pNP-GlcNAc), 4-nitrophenyl *N,N'*-diacetyl- $\beta$ -D-chitobioside (pNP-GlcNAc<sub>2</sub>), and 4-nitrophenyl *N,N',N''*-triacetyl- $\beta$ -D-chitotriose (pNP-GlcNAc<sub>3</sub>) were purchased from Carbosynth (Compton, UK). *N,N'*-Diacetyl chitobiose, *N,N',N''*-triacetyl chitotriose, *N,N',N'',N'''*-tetraacetyl chitotetraose, *N,N',N'',N''',N''''*-pentaacetyl chitopentaose, and *N,N',N'',N''',N''''*-hexaacetyl chitohexaose were purchased from Megazyme (Wicklow, Ireland). Restriction enzymes were purchased from Thermo Fischer Scientific (MA, USA).

### Strains and genes

The SmaChiA amino acid sequence (GenBank accession no. AAA26551.1) was used as a sequence template for alignment analysis (blast) to select putative GH18 chitinases or GH18 hypothetical proteins of bacteria in the GenBank database. Selection of putative chitinases was focused on candidates that had 40–70% sequence similarity with SmaChiA and which were from non-pathogenic sources. Clustal Omega (Sievers et al. 2011) was used to construct the multiple alignment for the selected putative chitinases (20 different sequences) and further used to generate the neighbor-joining phylogenetic tree using SeaView (Gouy et al. 2010). Two bacterial chitinases from *Ferrimonas balearica* (original GenBank accession no. ADN76700.1 and new GenBank accession no. KY131984 (codon-optimized sequence for *Escherichia coli* expression)) and *Microbulbifer variabilis* (original GenBank accession no. WP\_051089467.1 and new GenBank accession no. KY100262), respectively, were selected based on their close distance in the phylogenetic tree (Fig. S1) to the benchmark enzyme SmaChiA from *Serratia marcescens* (GenBank accession no. AAA26551.1) (Brurberg et al. 1994). These two new chitinase enzymes are referred to as FbalChi18A and MvarChi18A, respectively.

The putative chitinase-encoding genes for FbalChi18A and MvarChi18A were synthesized by DNA 2.0© (Newark, CA, USA) with a C-terminal His<sub>6</sub> tag and codon-optimized for *E. coli* expression. The expression vector pJ414 was used as

provided by DNA 2.0©. The *E. coli* strain DH5 $\alpha$  (Invitrogen® Life Technologies, Thermo Fisher Scientific, MA, USA), was used as cloning host and *E. coli* BL21 (DE3), C41 (DE3), C43 (DE3), TOP10 (DE3), and TUNER (DE3) (Novagen and Lucigen, USA) were used as transformation and expression hosts of the putative chitinases.

### Expression and purification of novel chitinases

Enzyme expression was done principally as described previously (Nyffenegger et al. 2015). The *E. coli* transformants (BL21 (DE3) turned out to be best) harboring the individual recombinant chitinase genes (FbalChi18A and MvarChi18A, respectively) were induced for overexpression with 1 mM IPTG as the cell culture reached an OD<sub>600</sub> = 0.6 and were grown further overnight at 30 °C (for FbalChi18A) and 25 °C (for MvarChi18A) in LB medium with 100  $\mu\text{g ml}^{-1}$  ampicillin. Cells were harvested by centrifugation, and the pellets were resuspended in binding buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH 7.8) before being disrupted by sonication and further centrifugation (20,000 $\times$ g, 20 min at 4 °C) for removal of cell debris. The supernatant obtained by centrifugation was then filtered through a 0.22- $\mu\text{m}$  filter and applied to a 5-ml Ni<sup>2+</sup> Sepharose HisTrap HP column (GE Healthcare, Uppsala, Sweden) which was equilibrated with binding buffer using an Äkta purifier (GE Healthcare, Uppsala, Sweden). Proteins were eluted by a linear gradient of elution buffer (20 mM sodium phosphate buffer, 500 mM NaCl, 500 mM imidazole, pH 7.8). The eluted fractions were analyzed by SDS-PAGE to assess the purity, and the homogenous fractions were pooled.

### Protein determination

Protein concentration was determined according to the bicinchoninic acid assay using bovine serum albumin as the standard (Smith et al. 1985).

### Enzyme activity assays

Chitinase activity was assayed using 50 mM phosphate citrate buffer at pH 6.0 at 25 °C with 0.15 mM pNP-GlcNAc<sub>2</sub> as substrate. The reaction was terminated by adding Na<sub>2</sub>CO<sub>3</sub> (to 0.4 M) after 30 min of reaction, and the absorbance was then read at 410 nm in an Infinite 200 microplate reader (Tecan, Grödig, Austria). One unit of enzyme activity was defined as released 1  $\mu\text{mol}$  of pNP per minute per milliliter at 25 °C, pH 6, in the assay. The optimal temperature for the enzymes was determined by assaying in 50 mM phosphate citrate buffer (pH 6) at different temperatures from 20 to 70 °C; analogously, the optimal pH for each enzyme was determined using 50 mM phosphate citrate buffer at different pH values ranging from 2.2 to 9 at 25 °C, in both cases using

0.15 mM pNP-GlcNAc<sub>2</sub> as substrate, and the assay was run according to the method mentioned above. Thermal stability was determined by pre-incubation of the individual enzymes at different temperatures ranging from 30 to 55 °C for 5, 10, 15, and 20 min. The residual activity was then measured as the initial rate using 0.15 mM pNP-GlcNAc<sub>2</sub> in 50 mM phosphate citrate buffer (pH 6) at 25 °C. Reactions were terminated by adding Na<sub>2</sub>CO<sub>3</sub> (to 0.4 M), and the absorbance was read at 410 nm as described above. All reactions were run as true replicate runs ( $n = 2$ ).

### Substrate degradation

Colloidal chitin was used as the insoluble chitin substrate and prepared according to Rojas Avelizapa et al. (1999), briefly as follows: 10 g chitin was dissolved in 100 ml 85% (v/v) phosphoric acid, and the homogenized mixture was incubated overnight at 4 °C, then Milli-Q water was added in excess until visible chitin precipitation occurred; the mixture was then filtered through a cheesecloth, and the chitin precipitate was washed repeatedly (minimum 4 times) with Milli-Q water until acid removal was complete (neutral pH). The enzymatic hydrolysis of the colloidal chitin (1% (w/v)) was then performed in 50 mM sodium acetate buffer, pH 6, at 25 °C with 0.6  $\mu\text{M}$  FbalChi18A or 0.9  $\mu\text{M}$  MvarChi18A for 18 h. The reaction was stopped by heat inactivation, and the reaction mixture was applied to a Vivaspin 500 centrifugal concentrator with 5000 MWCO (Sartorius, Goettingen, Germany) for enzyme removal. The permeate was analyzed by mass spectrometry (see below).

One millimole of each chito-oligomer (chitobiose, chitotriose, chitotetraose, chitopentaose, and chitohexaose) was dissolved individually in 50 mM sodium acetate buffer, pH 6 at 25 °C, and reactions were initiated by addition of 0.02  $\mu\text{M}$  FbalChi18A or MvarChi18A; during reaction, aliquots of 100  $\mu\text{l}$  were taken at 5, 10, 15, 30, and 45 min and immediately heat inactivated for 10 min in 100  $\mu\text{l}$  of Milli-Q water at 100 °C. After inactivation, the enzyme was removed by filtration using a Vivaspin 500 centrifugal concentrator with 5000 MWCO (Sartorius, Goettingen, Germany). The permeate was then further analyzed by mass spectrometry (see below).

### Mass spectrometry analysis

Identification and quantification of chito-oligosaccharides and their degradation products were performed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) on an amaZon SL ion trap (Bruker Daltonics, Bremen, Germany) coupled to an UltiMate 3000 UHPLC from Dionex (Sunnyvale, CA USA). Five-microliter samples were injected on a porous graphitized carbon column (Hypercarb PGC, 150 mm  $\times$  2.1 mm; 3  $\mu\text{m}$ , Thermo Fisher Scientific,

Waltham, MA, USA). The chromatography was performed at  $0.4 \text{ ml min}^{-1}$  at  $70 \text{ }^\circ\text{C}$  on a two-eluent system with eluent A (0.1% formic acid in water) and eluent B (acetonitrile). The elution profile was as follows (time indicated in min): 0–1, 0% B; 1–28, linear gradient to 27.5% B; 28–31, linear gradient to 60% B; 31–35, isocratic 60% B; 35–40, isocratic 0% B. The electrospray was operated in positive mode with ultrascan mode and a scan range from 100 to 1500  $m/z$ , smart parameter setting of 500  $m/z$ , capillary voltage at 4.5 kV, end plate offset 0.5 kV, nebulizer pressure at 3.0 bar, dry gas flow at  $12.0 \text{ l min}^{-1}$ , and dry gas temperature at  $280 \text{ }^\circ\text{C}$ . Chitinase insertion domain fragmentation was performed using SmartFrag enhanced amplitude ramping from 80 to 120% and fragmentation time 20 ms. Quantification was performed based on manual MS/MS fragmentation of selected masses corresponding to chito-oligosaccharides DP 2–6 using the Compass QuantAnalysis 2.2 Program (Bruker Daltonics, Bremen, Germany).

### 3D structure modeling and substrate superposition

A 3D structure model for both identified enzymes was generated by the I-TASSER modeling tool (Yang et al. 2015) through homology modeling based on the 3D structure of SmaChiA (PDB code 1X6L) (Aronson et al. 2006). Structure comparisons of the active site amino acids were done by using PyMOL (PyMOL Molecular Graphics System, Delano Scientific, Portland, USA). Substrate superposition was based on 3D structure models for the catalytic domain for each of the identified enzymes generated by the ModWeb modeling web server (Pieper et al. 2011) through homology modeling based on the 3D structure of SmaChiA (PDB code 1EDQ; the 1X6L chitinase A is the W167A mutant of the (wild-type) 1EDQ chitinase A) (Papanikolaou et al. 2003). For substrate superposition, the crystal structure (i.e., PDB code 1NH6; SmaChiA active site mutant E315L co-crystallized with chitohexaose) was aligned with the homology model of the catalytic domains of either FbalChi18A or MvarChi18A using YASARA 16.8.19 (YASARA Biosciences GmbH, Vienna, Austria). The model substrate was superposed by deleting the SmaChiA backbone structure and water molecules and joining the chitohexaose and the respective homology model into one object.

## Results

### Sequence analysis

Both FbalChi18A and MvarChi18A had a sequence similarity of 67 and 63%, respectively, with SmaChiA (Uniprot: P07254) (Perrakis et al. 1994). The neighbor-joining phylogenetic tree showed that FbalChi18A and MvarChi18A were

close in distance to SmaChiA, the benchmark enzyme (indicated by black arrows, Fig. S1). SmaChiA was chosen as the benchmark enzyme as it has been well studied and characterized by others and is known to catalyze release of chitobiose from chitin (from the reducing end of chitin) (Horn et al. 2006a, b; Sikorski et al. 2006). Based on the similarity of amino acid sequences and their catalytic mechanism, both FbalChi18A and MvarChi18A are classified into family 18 of glycosyl hydrolases (GH18), the GH family harboring most chitinases identified so far (Lombard et al. 2014) (the more detailed sequence alignment analysis and structural prediction will be discussed further below).

### Enzyme expression and purification

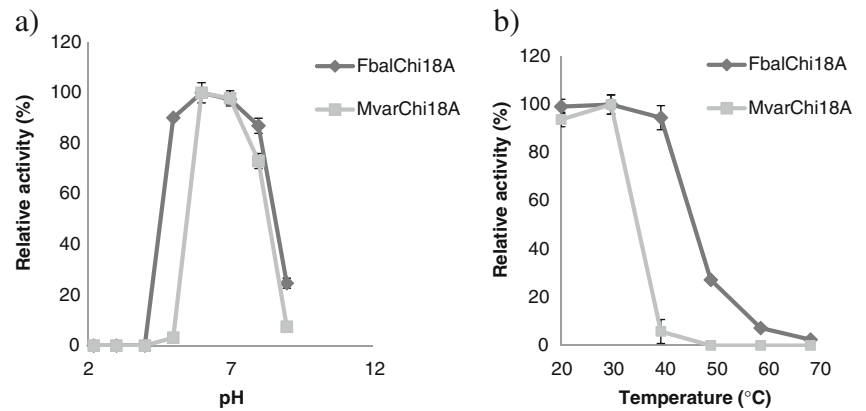
The two recombinant enzymes, FbalChi18A and MvarChi18A, were successfully expressed in *E. coli* strain BL21 (DE3) and purified (Fig. S2) (the enzymes were also expressed successfully in the TUNER (DE3) strain, data not shown). The yields of the purified FbalChi18A and MvarChi18A were approximately 275 and 860  $\text{mg l}^{-1}$  of *E. coli* cell culture, respectively.

The two chitinases, FbalChi18A and MvarChi18A, are encoded by genes with length of 2610 and 2565 bp which correspond to 870 and 855 amino acids, respectively. The predicted molecular weight from the deduced amino acid sequence plus the His-tag of FbalChi18A and MvarChi18A were 93.8 and 92.5 kDa, respectively. The purified protein FbalChi18A and MvarChi18A correspondingly had a molecular weight of approx. 95 and 100 kDa using SDS-PAGE as shown in Fig. S2 (interpretation of the minor discrepancies between the predicted molecular weight and the molecular weight read off from the SDS-PAGE gels may require further analysis by amino acid sequencing).

### Catalytic characterization of the two novel chitinases

Both FbalChi18A and MvarChi18A were found to have optimum temperature at  $30 \text{ }^\circ\text{C}$  and optimum pH at 6 (Fig. 1). FbalChi18A was active in a broader temperature range ( $20\text{--}40 \text{ }^\circ\text{C}$ ) and a broader pH range (pH 5–8) than MvarChi18A ( $20\text{--}30 \text{ }^\circ\text{C}$ ; pH 6–7.5). Regarding thermal stability, FbalChi18A was found to be stable from room temperature up to  $45 \text{ }^\circ\text{C}$  with a  $t_{1/2}$  of 75 min at  $45 \text{ }^\circ\text{C}$ , but unstable at  $55 \text{ }^\circ\text{C}$  with a  $t_{1/2}$  of 9 min (Fig. 2). MvarChi18A was stable from room temperature up to  $35 \text{ }^\circ\text{C}$  with a  $t_{1/2}$  of 52 min, but unstable at  $45 \text{ }^\circ\text{C}$  and above with a  $t_{1/2}$  of 1.5 min at  $45 \text{ }^\circ\text{C}$  (Fig. 2). The specific activities for the purified FbalChi18A and MvarChi18A on the assay substrate pNP-GlcNAc<sub>2</sub> were 0.128 and 0.056  $\text{U}\cdot(\mu\text{mol enzyme}^{-1})$ , respectively (none of the enzymes had activity on pNP-GlcNAc, data not shown).

**Fig. 1** Relative activity of FbalChi18A and MvarChi18A **a** at different pH values (2.2–9) and **b** different temperatures (20–70 °C). Results are presented as means  $\pm$  standard error ( $n = 2$ ), and 100% activities of FbalChi18A and MvarChi18A are 0.1 and 0.06 U  $\mu\text{mol}^{-1}$ , respectively



### Substrate degradation of the two novel chitinases

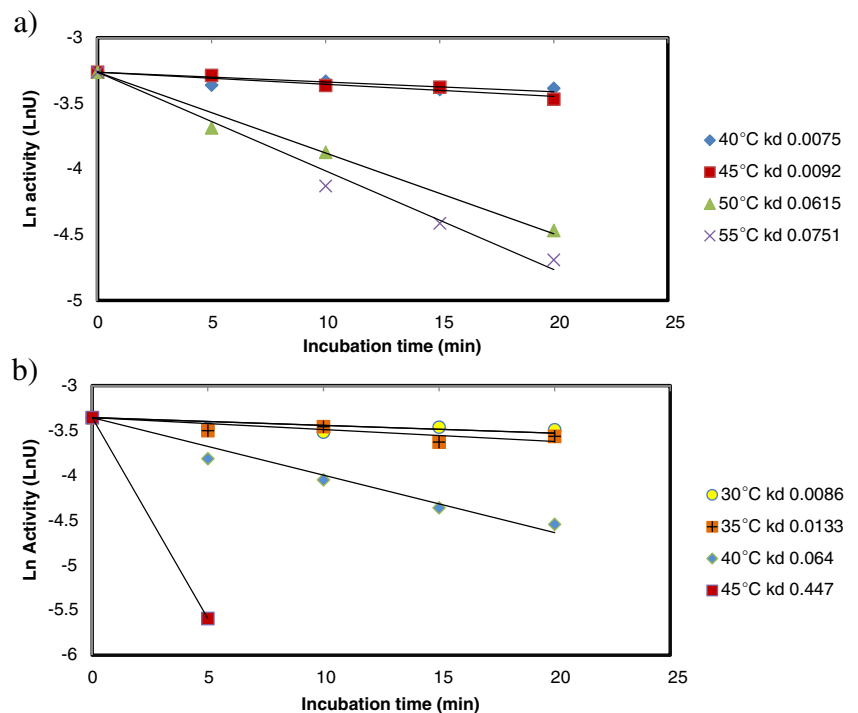
The end products from the enzymatic hydrolysis of the chitooligosaccharides by the two new chitinases were determined by mass spectrometry. From the trimer ( $\text{GlcNAc}_3$ ), tetramer ( $\text{GlcNAc}_4$ ), pentamer ( $\text{GlcNAc}_5$ ), and hexamer ( $\text{GlcNAc}_6$ ), the enzymes both catalyzed release of the dimer ( $\text{GlcNAc}_2 = \text{chitobiose}$ ) (Fig. 3a–d), but none of the two chitinases were able to cleave the  $\text{GlcNAc}_2$  dimer (data not shown). Analysis of colloidal chitin hydrolysis by both enzymes confirmed catalytic production of the dimer ( $\text{GlcNAc}_2$ ), besides indicating some trimer releasing activity, but at a 15- and 6-fold lower rate of the dimer release for FbalChi18A and MvarChi18A, respectively (Table 1). No products of higher chain length were detected. The final

dimer/trimer ratios are different between the two enzymes as it is affected by the specific binding preferences of the individual enzymes (Horn et al. 2012).

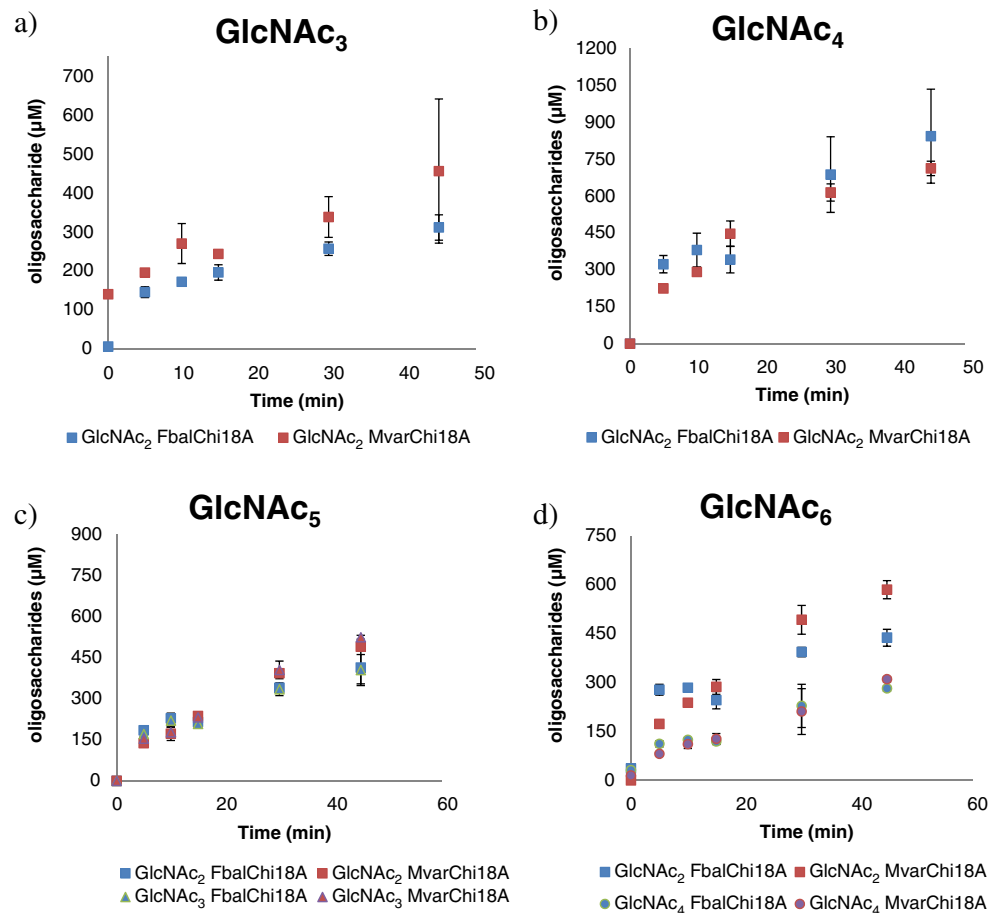
### Functional domains of the two novel chitinases

Most enzymes in the GH18 family exhibit a multidomain architecture with different distribution or arrangement of the domains. Based on sequence homology and tertiary structure prediction (via the web-based analysis tool InterPro program (<http://www.ebi.ac.uk/interpro/>) (Mitchell et al. 2015)), FbalChi18A and MvarChi18A were both predicted to each have four major domains: (1) the chitinase A *N*-terminal domain, (2) the catalytic  $\beta/\alpha$  barrel domain (classified as glycoside hydrolase family 18; GH18), (3) a polycystic kidney

**Fig. 2** Thermal inactivation for both chitinases under different temperature ranging from 30 to 55 °C. Both enzymes, FbalChi18A (**a**) and MvarChi18A (**b**), were incubated for 5, 10, 15, and 20 min before the residual activity was determined. *kd* is the inactivation constant at the specific temperature as indicated



**Fig. 3** Reaction products of both FbalChi18A and MvarChi18A during incubation with **a** GlcNAc<sub>3</sub>, **b** GlcNAc<sub>4</sub>, **c** GlcNAc<sub>5</sub>, and **d** GlcNAc<sub>6</sub>. Results are presented as means  $\pm$  standard error ( $n = 2$ )



disease (PKD) domain, and (4) a C-terminal chitin-binding domain (Table 2). For FbalChi18A, the sequence identity of the chitinase A *N*-terminal domain (amino acids 24–128) to that of SmaChiA was 62%, whereas it was only 50% for MvarChi18A (amino acids 19–153) (Table 2). The similarity to SmaChiA of the catalytic  $\beta/\alpha$  barrel structure, indicated as  $\beta 1$ – $\beta 8$  in Fig. 4, was 69% for both enzymes (Table 2) (discussed further below). The PKD domain in each enzyme was found to consist of tandem repeats of about 85–90 amino acid residues with the two individual “repeat” stretches having 38% intra-identity for FbalChi18A and 49% identity for MvarChi18A (residues 569–659, 668–752 in FbalChi18A and residues 566–655, 662–747 in MvarChi18A, Table 2). Similarly, the C-terminal chitin-binding domain was also

found to consist of tandem repeats, each repeat being  $\sim 44$  residues, and having 43 and 44% identity among them for FbalChi18A and MvarChi18A, respectively.

### Domain analysis

Sequence alignment comparison with SmaChiA with recognition of the  $\alpha$ -helix and  $\beta$ -strand structures indicated that in both of the enzymes, FbalChi18A and MvarChi18A, the GH 18 catalytic  $\beta/\alpha$  barrel domain consisted of a  $(\beta/\alpha)_8$  TIM barrel fold with a small  $\alpha+\beta$  domain insertion between  $\beta 7$  and  $\beta 8$  (Fig. 4). The catalytic domain of family 18 can be classified into three subfamilies A, B, and C based on the amino acid sequence similarity (Watanabe et al. 1993, 1994; Suzuki et al. 1999). The main structural differences between these three subfamilies is the existence of a small  $\alpha+\beta$  domain insertion between  $\beta 7$  and  $\beta 8$  for subfamily A, as this insertion domain is absent in subfamilies B and C (Suzuki et al. 1999). This kind of insertion, which is also known as chitinase insertion domain, is usually composed of five or six anti-parallel  $\beta$ -strands and one  $\alpha$ -helix (Li and Greene 2010). The occurrence of a chitinase insertion domain in the catalytic  $(\beta/\alpha)_8$  barrel domain of chitinases has been proposed to increase the depth of the substrate-binding cleft (Perrakis et al.

**Table 1** Enzymatic hydrolysis of colloidal chitin by FbalChi18A and MvarChi18A

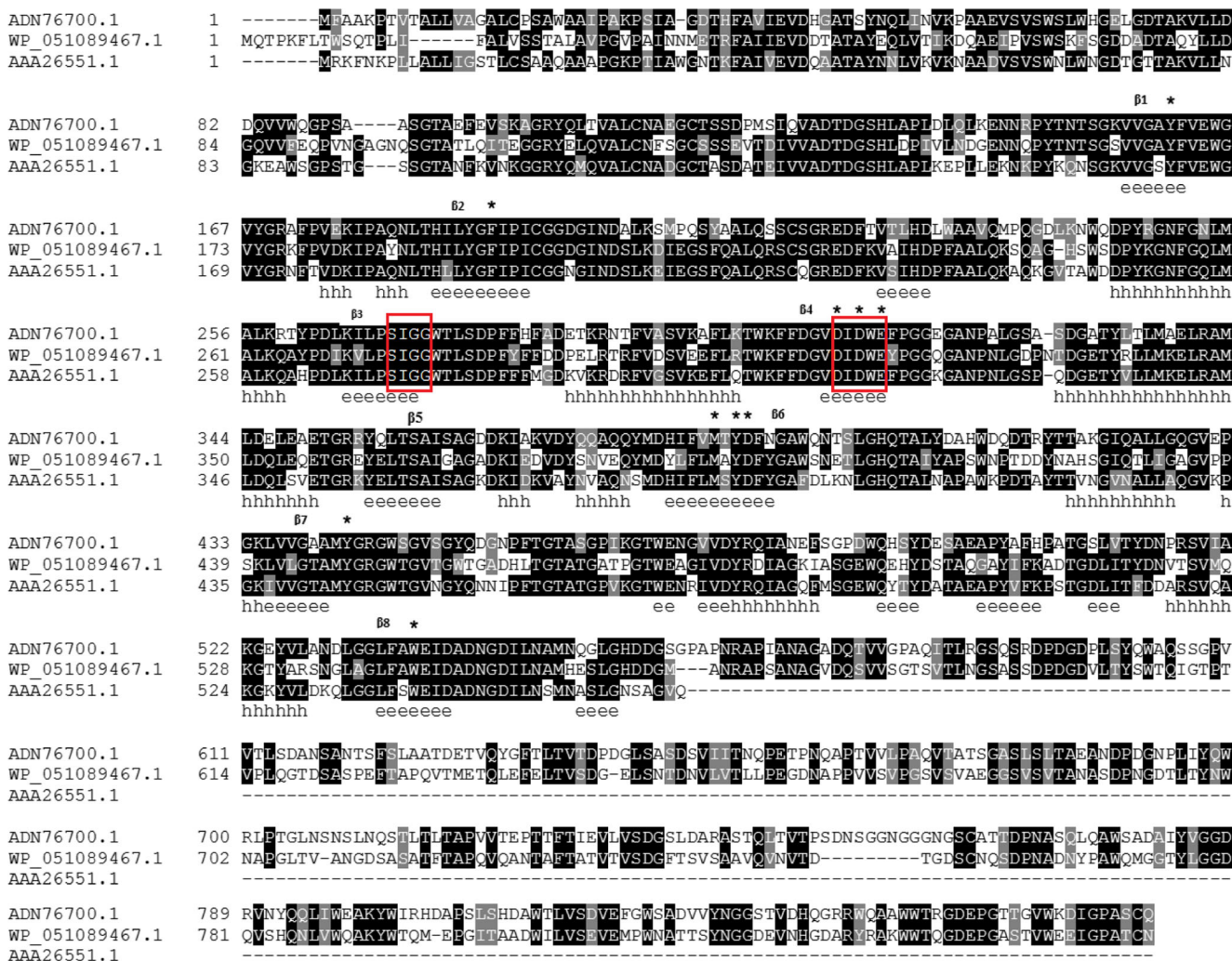
Enzymes	Final product levels of colloidal chitin hydrolysis ( $\mu\text{M}$ )	
	Trimer (GlcNAc <sub>3</sub> )	Dimer (GlcNAc <sub>2</sub> )
FbalChi18A	9.0 $\pm$ 0.4	139.8 $\pm$ 2.5
MvarChi18A	8.8 $\pm$ 0.1	51.8 $\pm$ 2.1

Results are presented as means  $\pm$  standard error ( $n = 2$ )

**Table 2** Detailed distribution of predicted domains in both FbalChi18A and MvarChi18A and the identical percentage of these domain towards domain in SmaChiA

Domains	Position of amino acids	Identical to SmaChiA (%)
FbalChi18A		
Chitinase A N-terminal domain	23–153	62
Catalytic β/α barrel	156–542	69
PKD1 domain	569–659, 668–752	–
C-terminal chitin-binding domain	775–818, 824–864	–
MvarChi18A		
Chitinase A N-terminal domain	19–153	50
Catalytic β/α barrel	158–542	69
PKD1 domain	566–655, 662–747	–
C-terminal chitin-binding domain	760–803, 809–849	–

1994; Suzuki et al. 1999). Hence, the chitinase insertion domain appears to play a role in substrate specificity by promoting the orientation and binding of the enzyme to longer substrates (Li and Greene 2010). It is proposed that both



**Fig. 4** Sequence alignment of GH18 chitinases from *Ferrimonas balarica* (FbalChi18A; ADN76700.1), *Microbulbifer variabilis* (MvarChi18A; WP\_051089467.1), and *Serratia marcescens* (SmaChiA; AAA26551.1). The secondary structure of all the chitinases is indicated at the bottom of the sequence alignment by e (β-strand) and h

(α-helix). Red boxes indicate the most conserved motifs, the SXGG and DXDXE motifs, which are the fully conserved motifs in the GH18 family of chitinases, while the asterisk symbol indicates the ten conserved residues in GH18 chitinases (color figure online)

of the newly identified chitinases, FbalChi18A and MvarChi18A, are classified into subfamily A.

The two short conserved motifs of family 18 chitinases, SXGG and DXDXE, which are located at the third and fourth  $\beta$ -strands were observed in the amino acid sequence of both FbalChi18A and MvarChi18A (Fig. 4). The conserved glutamate residue, E313 in FbalChi18A and E312 in MvarChi18A (E315 in SmaChiA), in the DXDXE motif has been predicted to act as a catalytic residue where it facilitates the cleavage of the glycosidic bond via the protonation of the glucosidic oxygen (Vaaje-Kolstad et al. 2013).

The PKD domain which was observed in both FbalChi18A and MvarChi18A but not present in SmaChiA was first identified in human polycystin-1. The PKD domain is known to be common in hydrolases from marine bacteria and has been reported found in proteases, cellulases, and other chitinases in, e.g., *Alteromonas* sp. O-7 (Miyamoto et al. 2002), *Stenotrophomonas maltophilia* (Kobayashi et al. 2002), and *Vibrio proteolyticus* (Itoi et al. 2007). This PKD domain consists of a  $\beta$ -sandwich fold which is similar to Ig-like folds found in proteins and the fibronectin III (FnIII) superfamily (Bycroft et al. 1999). The PKD domain has been suggested to facilitate the binding of chitinase towards chitin (Orikoshi et al. 2005) besides may support the protein-protein interactions (Frederiksen et al. 2013). However, the importance of such functionality for bacterial chitinases has yet to be investigated.

The tandem “repeats” (Table 2) in the C-terminal regions of both chitinases were predicted as two putative chitin-binding domains encoding a CBM (See also Fig. 5). Further verification using the dbCAN annotation server (<http://csbl.bmb.uga.edu/dbCAN/>) (Yin et al. 2012) also confirmed the existence of two putative CBM5/12 in the C-terminal region of both chitinases. Alignment of these predicted C-terminal chitin-binding domains of FbalChi18A and MvarChi18A with those of other bacterial chitinases being in close proximity in the phylogenetic tree, and with the C-terminal chitin-binding domain sequence of SmaChiB, showed some homology, but also some key differences: (1) Whereas the sequences grouped together in the phylogenetic tree search (based on SmaChiA) contained some hallmark repeats of the signature sequence AKWWTQ or AKYWTQ (Fig. 6), it was evident that

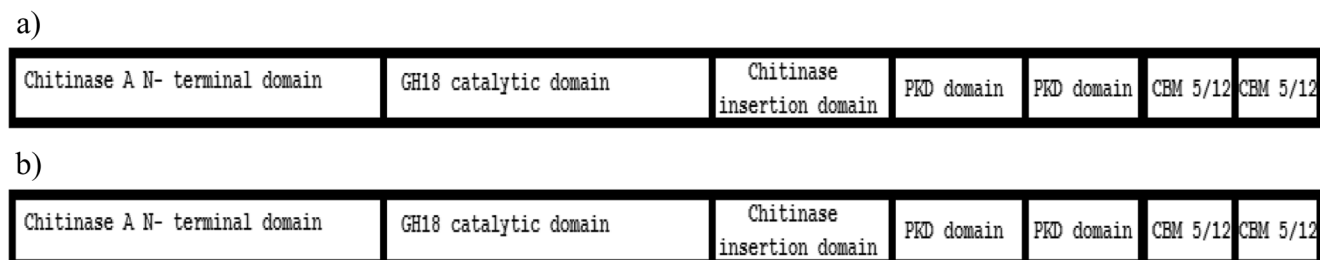
FbalChi18A was slightly different in this otherwise conserved region, but also that this segment differed from that of SmaChiB. Nevertheless, based on this analysis, we propose that the two newly identified chitinases FbalChi18A and MvarChi18A do contain a CBM 5/12 type chitin-binding domain in the C-terminus.

It can be added that part of the CBM 5/12 sequence, i.e., the second part of the C-terminal repeated sequence of FbalChi18A (amino acids 824–864), showed sequence similarities to those of chitinases from *Aeromonas* sp. No. 10S-24 (41% identity) (Shiro et al. 1996), *Clostridium paraputrificum* (38% identity) (Morimoto et al. 1997), and *Alteromonas* sp. O-7 (33% identity) (Hiroshi et al. 1998) (data not shown). While for MvarChi18A, a similar analysis for the CBM 5/12-type chitin-binding domain (amino acids 809–849) showed sequence similarities of 51, 50, and 43% identity with *Aeromonas* sp. No. 10S-24, *Cl. Paraputrificum*, and *Alteromonas* sp. O-7, respectively (data not shown).

The highly conserved stWWst motif in the CBM 5/12-type domain is presumed to play a specific role in substrate binding. Binding of substrate to the enzyme via the CBM 5/12-type domain is thus believed to occur through hydrophobic interactions between the two exposed aromatic residues (WW) and the chitin substrate moieties (Brun et al. 1997; Suzuki et al. 1999). Chitinases from *Aeromonas hydrophila*, *Bacillus circulans*, and *Pyrococcus kodakaraensis* lacking a CBM have been shown to lose their binding capacity and activity towards insoluble chitin (Wu et al. 2001; Hashimoto et al. 2000; Tanaka et al. 1999). The CBM-type 5/12 is also considered as contributing to maintain the bound enzyme to the substrate during progressive reaction (Aronson et al. 2003).

### Modeling of 3D structure

The 3D models for the catalytic domains of both FbalChi18A and MvarChi18A (Fig. 7a, b) appeared as being accurate as evaluated by Qualitative Model Energy ANalysis (QMEAN) (Benkert et al. 2011), Z-score analysis, and PROCHECK (Laskowski et al. 1993). The visual impression of the catalytic domain showed a characteristic barrel-type structure with a hollow center surrounded by  $\beta$ -sheets and with eight  $\alpha$ -



**Fig. 5** Schematic representation of the domain structures for the two identified enzymes. **a** FbalChi18A. **b** MvarChi18A. Each contains a chitinase A N-terminal domain, GH18 catalytic domain, repetitive PKD domain, and repetitive CBM 5/12 domain



```

ADN76700.1|775-818      -----LQAWSADAIYVGGDRVNYCQLTWFAKWVIRHDAPSLSHDAWTLV-----
ADN76700.1|824-864      -----GWSFDVVYNGGSTVDHCGRRWCFAWWTFCDEPGTIG-VWKDI-----
WP_051089467.1|760-809  -----SDPNADNYPAWOMGGTYLGGDQVSHQNLVWCAKWWTCQE-PGITAADWILV-----
WP_051089467.1|815-855  -----PWNATTSYNGGDEVNHDGARDYFAKWWTCGDEPGAST-VWEEI-----
WP_050901667.1|863-905  -----FPAWOSGSTYLGGDQVSHEQLVWCAKWWTCQE-PSFTSADWQLV-----
WP_050901667.1|909-951  -----EVPWASTAYNNGGDEVNHDGKRYFAKWWTCGQEPSTSS-DWEEI-----
WP_050659730.1|765-808  -----YPAWEASTVYTGDKVSYQGLVWCAKWWTCGDTSPQADGPPALV-----
WP_050659730.1|812-854  -----AQTWQAGVAYNNGGDEVNHDGKRYFAKWWTCGEEPGVAS-VWVDI-----
WP_035481260.1|770-813  -----FPAWDSAAVYVGGAKVSYQNLVWFAKWWTCGNTSPQADGPPKLV-----
WP_035481260.1|817-859  -----AQQWQASVAYNNGGDEVNHDGKRYFAKWWTCNEEPGIAA-VWEDI-----
BAA31568.1|438-498      LRYTGVGPGNLPIMTAPAVVPGTTYAQQGALVSYQGYVWCFKGYITSAFGSDS-AWLKVGRL

```

**Fig. 6** Comparison of predicted C-terminal CBM5/12 of two identified chitinases, FbalChi18A and MvarChi18A, with other closely related putative GH18 chitinases from the phylogenetic tree which have a repetitive

CBM 5/12. The red box indicates the two aromatic residues (tyrosine and tryptophan) that are responsible for the interaction of the binding domain and chitin/carbohydrate molecules (color figure online)

helices forming the barrel (Fig. 7a, b). The QMEAN Z-score for both FbalChi18A and MvarChi18A were  $-0.86$  and  $-0.36$ , respectively (Fig. S3). The Ramachandran plot generated through the PROCHEK program which evaluated the stereochemical quality of the protein structures showed that for FbalChi18A, 92.3% of the residues were in the most favored region while 7.7% of the residues were in allowed regions (Fig. S4). While for MvarChi18A, 92.5% of the residues were in most favored regions while 7.5% of the residues in allowed regions (Fig. S5). Both of the evaluation, QMEAN Z-score and PROCHECK, indicated the favorability of these two models. Hence, this analysis showed that the active site cores of both enzymes agreed highly with the active site structure of SmaChiA and also that the active site regions of the two enzymes (as defined by the selected 10 amino acids) were conserved among the two new chitinases in terms of overlay (Fig. 7c, d).

### Modeling of substrate binding

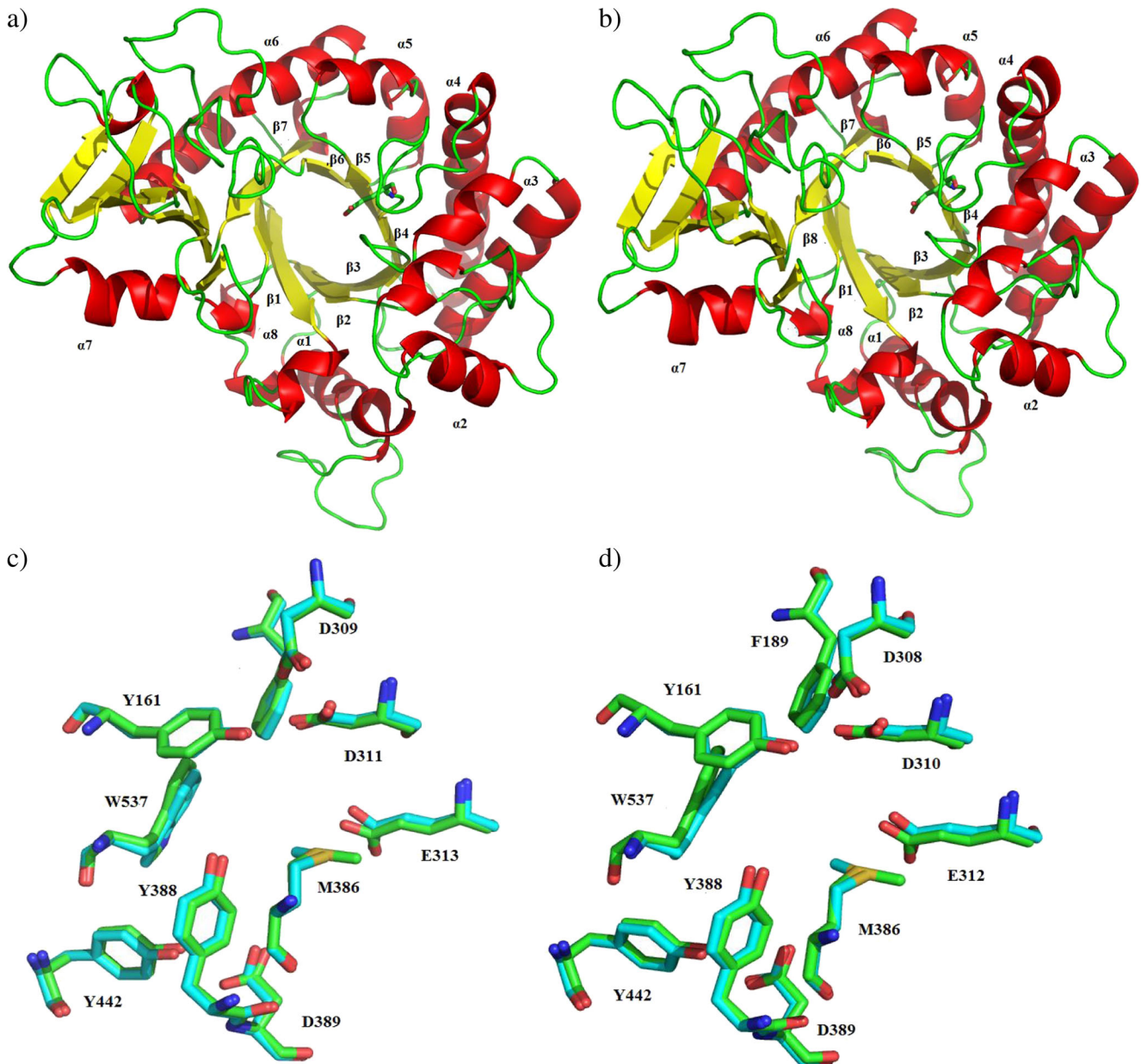
The possible binding mode for the hexamer substrate (GlcNAc<sub>6</sub>) to each of the enzymes FbalChi18A and MvarChi18A was predicted via superimposing the model of the catalytic domain of each enzyme onto the 3D crystal structure model of the catalytic domain of SmaChiA with its chitohexaose substrate (PDB code 1NH6). This superimposition modeling suggested that both FbalChi18A and MvarChi18A had a narrow substrate cleft that spanned across the whole side of the catalytic domain and that the cleft, as expected, was aligned with aromatic residues (Fig. 8). This type of substrate binding cleft is one of the characteristics for subfamily A in the catalytic domain of GH18 (Suzuki et al. 1999). Such substrate-binding clefts, fitting the substrate, are also observed for both SmaChiA and SmaChiB and are due to the presence of small so-called  $\alpha+\beta$  chitinase insertion domains in the catalytic domain—appearing in FbalChi18A from residues 444–516 and in MvarChi18A from residues 442–514. The modeling of FbalChi18A with its substrate was more mischievous than the modeling of MvarChi18A,

indicating that despite the 69% identity of both domains to the SmaChiA domain, the FbalChi18A and MvarChi18A sequences were not 100% identical (Fig. 4). The 3D visualization nevertheless corroborated that both enzymes indeed appeared to be *exo*-chitobiose hydrolases since the structure did indicate fitting of the straight substrate backbone into a “pocket” in the right hand side of the tunnel (as opposed to being a widely open groove in the structure) (Fig. 8b, d). A closer look at the superimposition models supported that the substrate was positioned by the aromatic tryptophan residues aligning the cleft, and this positioning corresponded to subsites  $-4$  to  $+2$  in both enzymes. The enlargement of the substrate interaction with the amino acids aligning the active site-binding cleft showed how the target bond in the substrate was positioned near the catalytic glutamate (E313 in FbalChi18A and E312 in MvarChi18A) to release the dimeric chitobiose product from the reducing end (subsites  $+1$  and  $+2$ ) of the hexamer (Fig. 8a, c). Thus, this analysis supported the interpretation that both enzymes indeed seem to be type A *exo*-chitobiose hydrolases, i.e., catalyzing release of chitobiose from the reducing end of chitin and chito-oligosaccharides.

## Discussion

### Enzyme characterization

By use of the genome database mining approach using the GH18 chitinase A from *S. marcescens* as template, we managed to identify two novel chitinases, FbalChi18A and MvarChi18A, which we propose to be type A *exo*-chitobiose hydrolases. The deduced amino acid sequence of FbalChi18A has about 62% homology with chitinase from *Aeromonas molluscorum* (Uniprot: R1GWE5), 54% with chitinase from *Photobacterium angustum* (Uniprot: A0A0D8QYT5), and 53% with chitinase from *Enterobacter cloacae* (Uniprot: G8LGW3). While for MvarChi18A, its amino acid sequence has about 58% homology with chitinase from *Shewanella piezotolerans* (Uniprot: B8CII9), 56% with chitinase from



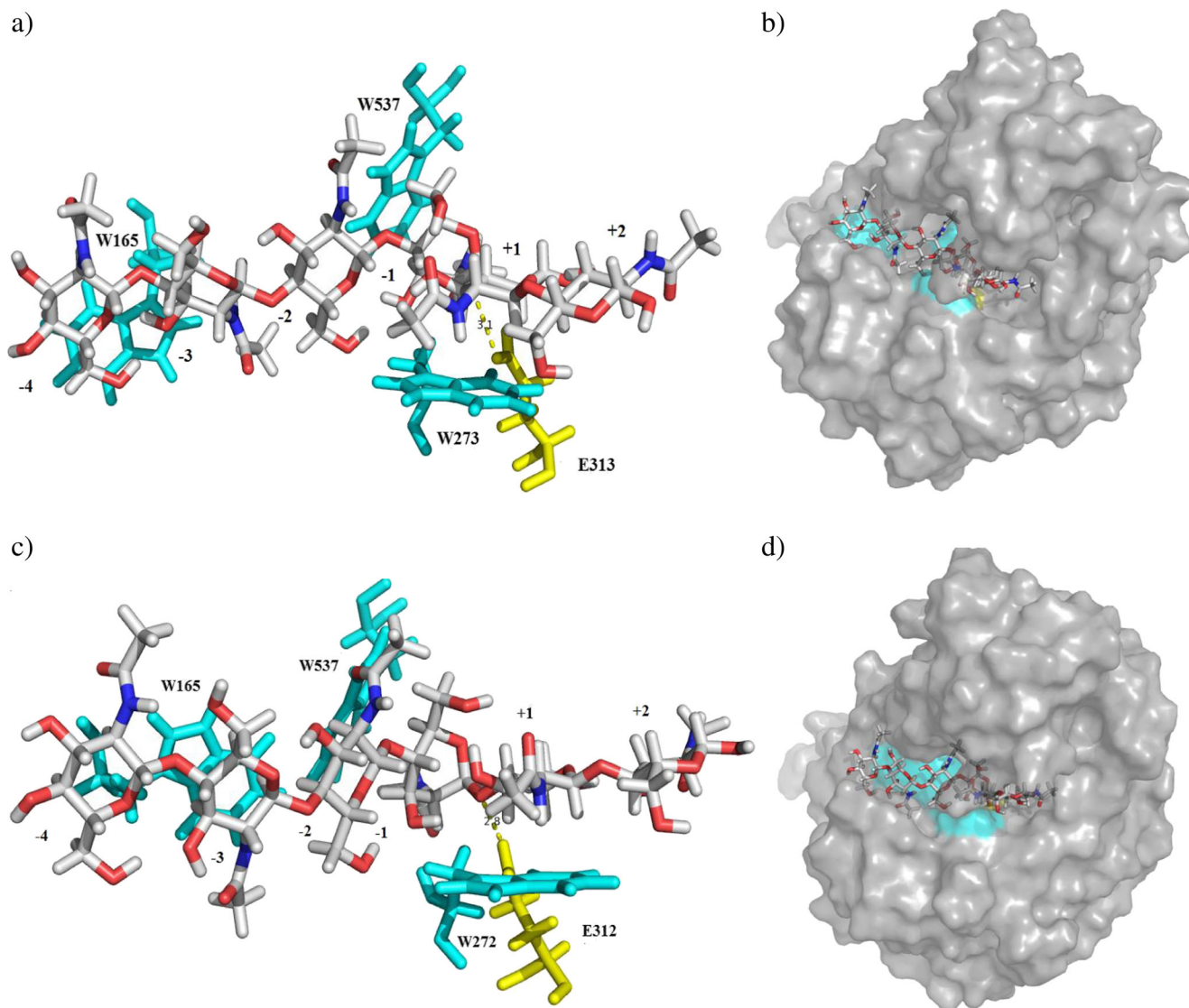
**Fig. 7** Theoretical model of a 3D structure for the catalytic domain of both identified enzymes, **a** FbalChi18A **b** and MvarChi18A, which contain the TIM barrel ( $\alpha/\beta$ )<sub>8</sub> structure. An alpha-helix is indicated by red, beta-sheet by yellow, and loop by green color. An overlay of ten

conserved active site residues for the **c** FbalChi18A **d** and MvarChi18A homology models (cyan sticks) with 1X6L chitinase from *Serratia marcescens* (green sticks) (color figure online)

*Aeromonas salmonicida* (Uniprot: T0PPB8), and 51% with chitinase from *E. cloacae* (Uniprot: G8LGW3).

Both FbalChi18A and MvarChi18A had an optimum of activity at pH 6 and an optimum activity at 30 °C and were stable for up to 75 min at 45 °C and 52 min at 35 °C, respectively. The sequence and structural domain comparisons between the two enzymes did not provide any obvious clues pointing towards any structural reasons for their slight temperature optimum activity divergence. The thermal optimum characteristics make FbalChi18A and MvarChi18A

mesophilic enzymes compared to the benchmark enzyme SmaChiA, which is more thermally robust and has an optimum activity between 50 and 60 °C and is stable for more than 400 h at 37 °C (Brurberg et al. 1996). Through domain predictions, FbalChi18A and MvarChi18A were both predicted to have four major domains each including the repetitive C-terminal CBM-type 5/12, and an N-terminal chitin-binding domain. The presence of a C-terminal CBM type 5/12 sets the two enzymes apart from SmaChiA which does not have a C-terminal CBM. To our knowledge, C-terminal CBM type



**Fig. 8** Substrate-binding models of FbalChi18A (a) and MvarChi18A (b) with chitohexaose (GlcNAc<sub>6</sub>) gray sticks as the substrate. The cyan sticks indicate the aromatic residues and the yellow sticks indicate the catalytic site residue (glutamate) (color figure online)

5/12 are specific for chitinases. However, several chitinases have been reported to have more than one chitin-binding domain in their structure, e.g., Chi92 from *A. hydrophila* (Wu et al. 2001), ChiC from *Salinivibrio costicola* (Aunpad and Panbangred 2003), and ChiA from *P. kodakaraensis* (Tanaka et al. 1999). Chi92 has three chitin-binding domains. In addition to two C-terminal chitin-binding domains, Chi92 also has an N-terminal chitin-binding domain (Chi92-N) that is similar to the ChiA N-terminal-binding domain of SmaChiA. Wu et al. (2001) reported that the truncated Chi92 which only contains a catalytic domain and the N-terminal Chi92 still display insoluble chitin-binding and hydrolytic activities. It was also described that the two C-terminal chitin-binding domains were functioning independently of each other (Wu et al. 2001). ChiA from *P. kodakaraensis* was also reported to have

three chitin-binding domains with one residing at the N-terminus while the other two are residing at the C-terminus. Mutants with deletion of either N-terminal or C-terminal chitin-binding domain showed that each of these chitin-binding domains was independently functional in binding insoluble chitin (Tanaka et al. 1999). Both chitinases, FbalChi18A and MvarChi18A, contained the two short conserved motifs of family 18 chitinases: SXGG and DXDXE. A mutation study of SmaChiA by Papanikolaou et al. (2001) has shown that residues Asp313 and Tyr390 together with Glu315 play a vital role in the enzyme catalysis. It was proposed that Asp313 which interacts with Asp311 will flip to an alternative position after protonation of the substrate glycosidic bond by Glu315. Since Asp313 also interacts with Glu315, it thus will force the distortion of the substrate acetamido group of the monomeric

moiety in the  $-1$  position (Papanikolau et al. 2001). As a consequence of these structural changes, the water molecule that is hydrogen-bonded to both the hydroxy group of Tyr390 and the NH of the acetamido group is displaced to a position that permits the completion of hydrolysis. Brameld and Goddard (1998) also reported that the hydrogen bonding of water molecules to Tyr390 helps to position the *N*-acetyl group prior to formation of an oxazoline ion. In contrast, the catalytic mechanism of SmaChiB (of *Serratia marcescens*) depends on the combination action of at least seven conserved active site residues: Tyr10, Ser93, Asp140, Asp 142, Glu144, Tyr 214, and Asp 215 and that Tyr10 and Ser93 are important for stabilization of the charge on Asp140 while Asp142 points towards Glu144 (Synstad et al. 2004). The partly buried Asp140 residue is essential for keeping the Asp142 protonated. Asp142 plays an interconnected role, and its rotation towards Glu144 contributes to a crucial distortion of the *N*-acetyl group of the  $-1$  GlcNAc moiety. The phenolic hydroxyl of Tyr214 contributes to the positioning of the *N*-acetyl group of the  $-1$  GlcNAc moiety. The Asp215 residue plays a role in the catalysis by being involved in the binding of the  $-1$  GlcNAc in its distorted conformation. A direct comparison of the active site residue positions in SmaChiA versus SmaChiB (Fig. 4) shows some obvious similarities.

### Substrate degradation

The enzymatic hydrolysis of the chito-oligosaccharides and colloidal chitin by each of the enzymes released mainly the dimeric product. The processivity of the family 18 chitinase may be assessed by studying the monomer/dimer ratio in product mixtures (Horn et al. 2006a, b). The product profiles thus indicated that both of them are *exo*-chitinases or *exo*-chitobiose hydrolases. The *exo*-chitinase-catalyzed hydrolysis of substrate may occur either at the reducing end or non-reducing end of the substrate. The C-terminal CBM 5/12 type in ChiB from *Serratia marcescens* helps locating the substrate-binding cleft so that the dimeric chitobiose products are released from subsites  $-1$  and  $-2$ , being the non-reducing end of the substrate. In contrast, the *N*-terminus in SmaChiA (containing the FnIII-like structure that is likely involved in interactions with the chitin chain during catalysis) provides an active site topology that locates the substrate-binding cleft at the non-reducing end of the substrate, and hence, the dimeric products are thought to be released from subsites  $+1$  and  $+2$  (Perrakis et al. 1994; van Aalten et al. 2000; Vaaje-Kolstad et al. 2013).

### Structure function analysis

The aromatic residues (notably tryptophan) in the active site, which were previously shown to be important for substrate binding in chitinases, are well conserved, and they interact

with the GlcNAc units of the bound oligomer through stacking interactions (Uchiyama et al. 2001). Three tryptophan residues, Trp167, Trp275, and Trp539 (referred to SmaChiA), in the substrate-binding cleft were identified as the aromatic residues that most likely interact with GlcNAc units of the hexamer substrate. Trp273 (FbalChi18A) and Trp272 (MvarChi18A) (corresponding to Trp275 for SmaChiA) apparently interact with the substrate at subsite  $+1$  while Trp537 (Fbalchi18A) and Trp537 (MvarChi18A) (corresponding to Trp539 for SmaChiA) interact with substrate at subsite  $-1$ . Trp165 (FbalChi18A) and Trp165 (MvarChi18A) (corresponding to Trp167 for SmaChiA) interact with the substrate at subsite  $-3$ . The significant roles of these aromatic residues towards the processive action of the enzymes have been reported by Aronson et al. (2003) and Zakariassen et al. (2009), who suggested that these aromatic residues are presumed to facilitate processivity by functioning as a flexible and hydrophobic patch, which the substrate chain can slide during the processive action. In accord with the high specific activity on the GlcNAc<sub>4</sub>, the products obtained from hydrolysis of GlcNAc<sub>5</sub> and GlcNAc<sub>6</sub> as substrates and the substrate binding analysis of the two new enzymes corroborated that FbalChi18A and MvarChi18A are most likely *exo*-chitobiose hydrolases that catalyze release of chitobiose from the reducing end of chitin and chito-oligosaccharides. The two new enzymes may find use in the production of chitobiose from chitin or merely for chitin degradation. Their use for production of chitobiose is highly relevant in an enzymatic cascade production of human milk oligosaccharides as the chitobiose product can act as a substrate for subsequent enzymatic synthesis of human milk oligosaccharide backbone structures with *N*-acetylglucosamine (Nyffenegger et al. 2015).

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### Compliance with ethical standards

**Ethical approval statement** This article does not contain any studies with human participants or animals performed by any of the authors.

**Conflict of interest** The authors declare that they have no conflict of interest.

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