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Engineering chitinases for the synthesis of chitin oligosaccharides: Catalytic amino acid mutations convert the GH-18 family glycoside hydrolases into transglycosylases

Eduardo Andres Martinez[a], Harry Boer[b], Anu Koivula[b], Eric Samain[a], Hugues Driguez[a], Sylvie Armand[a], Sylvain Cottaz[a,*]

[a] Centre de Recherches sur les Macromolécules Végétales (CERMAV-CNRS), BP53, 38041 Grenoble cedex 9, France[1]
[b] VTT Technical Research Centre of Finland, P.O. Box 1000, FI 02044 VTT, Finland

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ABSTRACT

Two family GH-18 chitinases mutated on the key catalytic amino acids were evaluated as "glycosynthases" for the coupling of oxazoline activated donor to chitin oligosaccharide (COs) acceptors obtained from microbial fermentation. *Bacillus circulans* WL-12 chitinase A1 (*Bc* ChiA1) and *Trichoderma harzanium* chitinase 42 (*Th* Chit42) were individually mutated on the three conserved carboxylic acids, all suggested to have a role in catalysis: the general acid/base glutamate and the two aspartates, defined as the putative stabilizer and its assistant. The mutants D200A and D202A of *Bc* ChiA1, together with D170N and to a lesser extent D170A of *Th* Chit42 proved to be active for chitinbiose oxazoline polymerization, and also for coupling reaction between Gal($\beta1\rightarrow4$) chitinbiose oxazoline and chitinpentaose at neutral pH. These mutants have additionally retained the ability to catalyze transglycosylation reaction on natural COs, whereas their hydrolytic activity is abolished. Such mutants can be considered as chitin transglycosylases, verifying also the fact that the two conserved aspartic acids, the stabilizer and its assistant, are not a prerequisite for the formation of oxazolinium intermediate by acetamido anchimeric assistance, i.e. for the substrate-assisted catalysis mechanism of family GH-18 chitinases.

1. Introduction

Chitin oligosaccharides (COs, $\beta(1\rightarrow4)$-linked GlcNHAc oligomers) and related compounds are an important class of signalling molecules involved in plant-biosphere cell–cell interactions [1–5]. COs in the cell wall of pathogen fungi elicit plant defence reactions by inducing the biosynthesis of reactive oxygen species, cell wall lignification and the release of pathogenesis-related proteins such as chitinases and $\beta(1\rightarrow3)$-glucanases [6–8]. In addition, it has been also reported that COs induce defence response in mammalians and are potentially useful as immunopotentiating, antitumor, antifungal and antibacterial agents [9,10].

COs mixtures are marketed as human dietary supplement to boost the immune system. Despite their biological interest and potential agronomical usefulness, COs with well-defined structure remain poorly accessible because of their very limited abundance and heterogeneity from natural sources. Smaller COs with DP ranging from 2 to 6 can be obtained from chitin by chemical or enzymatic depolymerization [11–13], while higher oligomers can be prepared from chitosan (partially de-*N*-acetylated chitin polymer) oligomers followed by re-*N*-acetylation [14]. Most biological activities require COs larger than the tetrasaccharide [5,6], a length hardly accessible by depolymerization methodologies, mostly because of poor water solubility of COs with DP $\geq$ 7. Enzymatic synthesis of COs has been a matter of research by exploiting the transglycosylation activity of retaining, family 18 chitinases (GH-18; see also http://www.cazy.org/Glycoside-Hydrolases.html). GH-18 chitinases do not display a classical double-displacement molecular mechanism of retaining hydrolases but apply a substrate-assisted catalytic mechanism ([15–18], see also Fig. 2). They have a α/β TIM-barrel fold, which provides a long substrate-binding groove composed of several subsites for binding the chitin chain. Chitinases purified from *Trichoderma reesei* or *Nocardia orientalis* have been used to convert chitintetraose to the corresponding penta, hexa-, and heptasaccharide using high salt concentration [19,20].

Abbreviations: *Bc* ChiA1, *Bacillus circulans* WL-12 chitinase A1; *Th* Chit42, *Trichoderma harzanium* chitinase 42; COs, chitin oligosaccharides; CO-*n*, chitin oligosaccharide of DP = *n*; GalCO-*n*, β-D-galactopyranosyl-(1 → 4)-chitin oligosaccharide of DP = *n*; DP, degree of polymerization; GH, glycoside hydrolase; IMAC, immobilized metal ion affinity chromatography; PBS, phosphate buffer saline.

* Corresponding author. Tel.: +33 476 037 603; fax: +33 476 547 203.
E-mail address: cottaz@cermav.cnrs.fr (S. Cottaz).
[1] Affiliated with Université Joseph Fourier Grenoble 1, and member of the Institut de Chimie Moléculaire de Grenoble.

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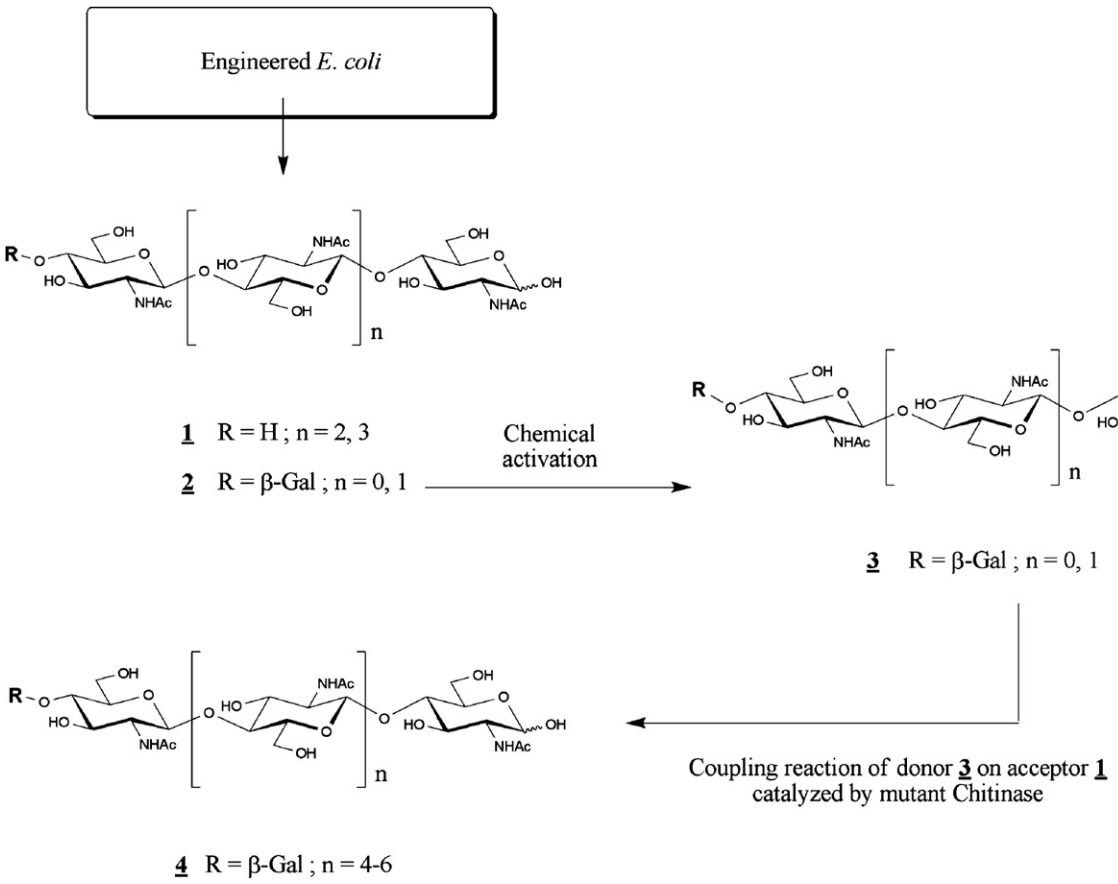

Fig. 1. General strategy for COs synthesis by mutant chitinases. Galactosylated chitin oligosaccharides produced by metabolically engineering *E. coli* are chemically activated and used as oxazoline donor substrate for coupling reaction with chitin oligosaccharides. The presence of a galactosyl unit at the non-reducing end prevents the reaction of self condensation of the oxazoline activated substrate.

However, as wild type enzyme preparates were employed in these studies, complex mixtures of starting material and various reaction products were obtained due to the remaining hydrolytic activity of the biocatalysts and uncontrollable random transglycosylation reactions. A similar strategy leading to the same results was investigated using GH-20 β-hexosaminidase from *Aspergillus oryzae*, also displaying a substrate-assisted mechanism [21]. The use of chitinbiose oxazoline substrate, a transition state analog of GH-18 chitinases, together with commercial *Bacillus* sp. chitinases and *Bacillus circulans* WL-12 chitinase A1 (*Bc* ChiA1) preparates constituted an important development in enzymatic COs synthesis. These chitinolytic enzymes have under basic pH a reduced hydrolytic activity but are still active for coupling[2] reaction, i.e. transfer of the chitinbiosyl moiety onto suitable acceptors, in good yield [22,23]. The combination of oxazoline activated donor with an engineered chitinase having low hydrolytic activity constitutes an even more sophisticated approach, leading to chitinases with improved coupling activity [23–26].

For the enzymatic synthesis of long COs, the availability of starting building blocks (COs with DP ≤ 6) is also an issue, which we have solved by expression of rhizobial nodulation (*nod*) genes in *Escherichia coli* [27–29]. This technology of microbial production allows the production of unprecedented amounts of COs building blocks ranging from chitinbiose to chitinpentaose. These combined with the coupling activity of mutated chitinases appear as a tool of choice to connect donor and acceptor blocks in order to get COs with extended backbone (Fig. 1). We report here the mutagenesis of two GH-18 glycoside hydrolase, *B. circulans* WL-12 chitinase A1 (*Bc* ChiA1) and *Trichoderma harzanium* chitinase 42 (*Th* Chit42), and usage of *E. coli* produced building blocks to generate long COs. The mutagenesis followed in principal the "glycosynthase technology" introduced originally by Withers and his co-workers [30,31]. This approach gives, upon mutation of catalytic residues of classical retaining glycoside hydrolases, biocatalysts able to perform transglycosylation reaction with no remaining hydrolytic activity. In our work the conserved catalytic residues of *Bc* ChiA1 and *Th* Chit42 have been mutated individually, and each mutant screened for its transglycosylation behaviour.

2. Experimental

2.1. General procedures

Chitinbiose (CO-II), β-D-galactopyranosyl-(1 → 4)-chitinbiose (GalCO-II) and chitinpentaose (CO-V) were produced using an engineered *E. coli* strain [27,28]. β-D-Galactopyranosyl-(1 → 4)-chitinbiose oxazoline (GalCO-IIoxa) was prepared from GalCO-II according to standard procedure [22,23]. 4-Nitrophenyl β-chitinbioside was commercially available (Sigma–Aldrich). MALDI-MS spectra were recorded on a MALDI-TOF-TOF Autoflex Speed (Bruker Daltonics), equipped with a laser emitting at 355 nm with a frequency of 250 Hz. 2,5-Dihydroxybenzoic acid (DHB) was used as a matrix at a concentration of 50 g L^{-1} in water. COs samples were

[2] For the sake of clarity along this manuscript the term *coupling* will be devoted to a transglycosylation reaction based on oxazoline activated donor. The term *disproportionation* will refer to a transglycosylation reaction based on "natural" oligosaccharidic substrate (see also Fig. 3).

dissolved in DMSO at a concentration of approx. 200 μmol L^{-1}, and mixed with the same volume of matrix solution before air drying at room temperature on the MALDI sample plate.

2.2. Strains, plasmids and culture conditions

2.2.1. Bacillus circulans ChiA1

E. coli BL21 (Invitrogen. F^-, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, nupG, recA1, araD139, Δ(ara-leu)7697, galE15, galK16, rpsL(StrR), endA1, λ-) was used both for molecular biology and protein expression purposes of the *Bc* ChiA1 chitinase. The *chiA1* gene was kindly provided by Prof. T. Watanabe as pHT002 plasmid [32]. The gene was PCR amplified from pHT002 (*Bc* ChiA1a: 5′ CAG CGT *GAA TTC* GAT TCT TAT AAA ATC GTT GG 3′. *Bc* ChiA1β: 5′ TGA ATT CT*G CGG CCG C*TT GAA GCT GCC ACA AGG C 3′. Restriction sites in italics), removing signal peptide and stop codon. Digested PCR product was subcloned into pET20b(+) vector (Invitrogen) between EcoRI and NotI to obtain the His_6-tagged ChiA1 chitinase. Final expression construct was verified by DNA sequencing. Unless specified, all cultures were done at 37 °C in LB supplemented with ampicillin (50 μg/mL).

2.2.2. Trichoderma harzanium Chit42

E. coli strain LMG194 (Invitrogen) was used as expression host for the pBAD/gIIIA (Invitrogen) based expression plasmid. Plasmid pBADchit42 used for the periplamic expression of *T. harzianum* is described in Ref. [32]. SB medium (pH 7.0) used for the cultivation of the Chit42 expressing strain contained 30 g tryptone, 20 g yeast extract, 10 g MOPS, per litre. *E. coli* strain LMG194 is both streptomycin (10 μg/mL) and tetracycline (12.5 μg/mL) resistant. Unless specified, all cultures were done at 37 °C until OD_{600} = 0.6 was reached. Chitinase expression was induced by adding 0.02% L-arabinose and the cells were harvest after 3–4 h of growth after induction.

2.3. Construction of site-directed mutants

2.3.1. Bacillus circulans ChiA1 mutants D200A, D202A and E204Q

The three carboxylic acids were mutated by Quick Change Mutagenesis® methodology using the following primers: 5′ GAC GGG GTA **GCT** CTG GAC TGG 3′ and 5′ CCA GTC CAG **AGC** TAC CCC GTC 3′ for D200A. 5′ GGT AGA TCT **GGC** CTG GGA GTA CC 3′ and 5′ GG TAC TCC CAG **GCC** AGA TCT ACC 3′ for D202A. 5′ CTG GAC TGG **CAG** TAC CCG GTA TC 3′ and 5′ GA TAC CGG GTA **CTG** CCA GTC CAG 3′ for E204Q (Mutated codon in bold, base pair substitution in italic.) according to standard protocol. All sequences were confirmed by DNA sequencing.

2.3.2. Trichoderma harzanium Chit42 mutants D170N, D170A and E172Q

The carboxylic acids were mutated by Quick Change Mutagenesis® methodology essentially as described in Ref. [33].

2.4. Enzymes production and purification (including assay of residual hydrolytic activity)

2.4.1. Bacillus circulans ChiA1 wild type and mutants

E. coli BL21 (Invitrogen) were used for the expression of all *Bc* ChiA1 variants. As the pET expression system (containing the T7 promoter) was found to be too strong and all protein to fall into insoluble fraction when standard expression conditions were used, modifications were made to help the correct folding of the expressed chitinase. Freshly transformed *E. coli* cells were used to inoculate 1 L LB supplemented with 50 μg/mL ampicillin and 2% glucose. Grown at 37 °C until $OD_{600} \approx 0.3–0.4$, the culture was subjected to a heat shock (42 °C, 30 min) to induce the formation of *E. coli* chaperones, and subsequently changed to 1 L LB supplemented with 50 μg/mL ampicillin and 1 mM IPTG. The induction was done for overnight at lowered temperature of 25 °C for expression. Protein was recovered from the periplasmic fraction according to standard osmotic shock procedure. Clarified lysate was loaded onto His-Trap resin (containing Ni-affinity matrix), equilibrated with PBS (20 mM sodium phosphate pH 7.5, 250 mM NaCl). The column was washed with PBS and the *Bc* ChiA1 was eluted with 100 mM imidazole in PBS. Imidazole was removed and protein was concentrated with Centricon YM-10® to afford a final yield of 3–5 mg/L culture.

Hydrolytic activity of the purified mutants was measured through 4-nitrophenol released upon hydrolysis of 4-nitrophenyl β-chitinbioside. For this, each ChiA1 variant was added to a 200 μL PBS solution containing 1 mM 4-nitrophenyl β-chitinbioside to a final concentration of 2 μg/mL, incubated at 37 °C and following the absorbance at 415 nm with a Bio-Rad 680 microplate reader.

2.4.2. Trichoderma harzianium Chit42 mutants D170N, D170A and E172Q

Th Chit42 was expressed in the periplasmic space of *E. coli* and recovered by an osmotic shock procedure as described previously [33]. The purification procedure is described there as well. Briefly, for purification of *Th* Chit42, the pH of the osmotic shock fluid was first adjusted by adding 10 mL of 1 M KP_i, pH 7.5, and the sample was loaded onto a DEAE Sepharose FF column (Pharmacia; 2.5 cm × 15 cm), which was equilibrated with 25 mM KP_i pH 7.5, at a flow rate of 1 mL/min. After washing the column until the A_{280} of the effluent reached the value of the equilibration buffer, the protein was eluted with a linear gradient (2× 300 mL) of 0–1 M NaCl in the equilibration buffer. Fractions were collected and *Th* Chit42 was detected by measuring the residual activity on 4-methylumbelliferyl-β-chitinbioside. The *Th* Chit42 containing fractions were pooled, concentrated and the buffer exchanged by ultrafiltration (PES membrane with 10 kDa cut-off, Vivascience) into 25 mM KP_i pH 6.5. This procedure resulted in pure *Th* Chit42 enzyme and the yield was about 1 mg per litre culture.

2.5. The transglycosylation activity assay with Bacillus circulans ChiA1 mutants using HPLC ESI-MS

To study the coupling activity of the *Bc* ChiA wild-type and mutant enzymes, transglycosylation of GalCO-IIoxa onto CO-II was used as standard reaction. For each reaction 4.7 mg (8.27 μmol) GalCO-IIoxa and 5.25 mg CO-II (1.5 eq.) were mixed and freeze dried in 1.5 mL microtubes. Substrate mixture was dissolved in Tris/Carbonate buffer (50 mM) at pH 7.0 and 10.0 depending on the setup. Reaction at 37 °C started with the addition of the enzyme to a final 1–5% (w/v) concentration in 200 μL reaction. Samples (15 μL) were heat-inactivated (90 °C, 20 min) and freeze dried. Analyses were performed on a LC–MS system including a Waters ZQ mass spectrometer coupled with a Beckmann LC-152S liquid chromatography equipped with a diode array detector. LC separations were performed with an 4.6 mm × 150 mm aminopropyl column using isocratic elution at 3:1 acetonitrile–water, and single ion quantification of $[M+Na]^+$ adduct of each species.

2.6. The transglycosylation assays for Bacillus circulans and Trichoderma harzanium mutants using MALDI-MS

In a typical procedure chitinase mutant (at a final concentration of 1–2 mg/mL) was added to a 50–100 mM substrate solution in Tris/HCl–PBS (50 mM, pH 7.4), and incubated on an orbital shaker at 37 °C for 18 h. The resulting suspension was centrifuged at 13 000 rpm for 5 min, and the pellet was washed by resus-

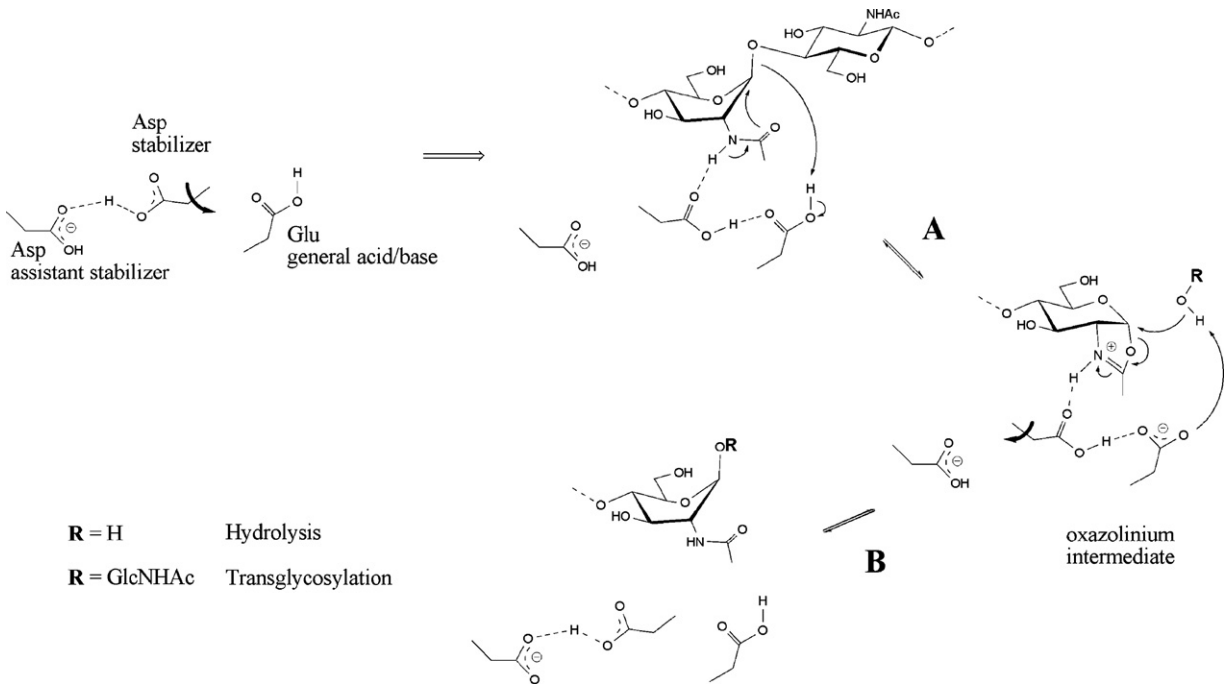

Fig. 2. Proposed catalytic mechanism of GH-18 chitinases involving a substrate-assisted mechanism of hydrolysis. In the resting enzyme the two aspartic acids are coupled via a hydrogen bond. Upon substrate binding, the stabilizing Asp rotates towards the general acid/base (Glu). In step (A) the Glu acts as a general acid and protonates the glycosidic oxygen. Leaving group departure is stimulated by a concomitant nucleophilic attack by the C2 acetamido group of the GlcNHAc at subsite −1, which leads to formation of an oxazolinium ion intermediate. The Asp stabilizes the positively charged oxazolinium intermediate. In the second step (B) the Glu acts as a general base and catalyzes the incoming hydroxyl attack on the intermediate. Hydrolysis or transglycosylation depend on the nature of the R–OH incoming group.

pension in water in an ultrasound bath for 2 min. The pellet washing was repeated two times, and the resulting solid was freeze-dried before MALDI-MS analysis. Polymerization reactions were conducted with chitinbiose oxazoline (CO-IIoxa) as substrate, coupling reactions were conducted using an equimolar mixture of β-D-galactopyranosyl-(1 → 4)-chitinbiose oxazoline (GalCO-IIoxa) and chitinpentaose (CO-V), disproportionation reactions were conducted with chitinpentaose (CO-V) as unique substrate.

3. Results and discussion

3.1. General strategy for the design of mutant chitinases

The GH-18 retaining chitinases contain a conserved DXDXE sequence motif, where the glutamate has been shown to act as the general acid/base in the substrate-assisted catalysis mechanism [15–18]. The catalysis is suggested to include all three conserved carboxylic acids of the DXDXE sequence (Fig. 2). In the resting enzyme the two conserved aspartic acids, defined here as the putative stabilizer and its assistant, are coupled through a hydrogen bond. Substrate binding causes rotation of the stabilizing aspartate towards the catalytic acid/base glutamate. The acid/base glutamate acts as a proton donor in activating the glycosidic bond between −1 and +1 subsites. The stabilizer is believed to play a triple role by (1) orientating the 2-acetamido group acting as a nucleophile to form the oxazolinium intermediate, (2) stabilizing the oxazolinium transition state, (3) lowering the pK_a of the general acid/base by donating a proton. The main role of the second aspartate, the assistant stabilizer, is suggested to raise the pK_a of the stabilizer (Fig. 2A). It is of importance to note that the substrate must adopt a distorted boat or skew-boat conformation at −1 subsite in order to facilitate the nucleophilic attack by the *N*-acetyl oxygen. In a second step the oxazolinium intermediate is opened by the nucleophilic attack of a water molecule, positioned and activated by the deprotonated glutamate acting as a general base, to yield a new reducing end with an overall retention of the configuration at the anomeric center (Fig. 2B).

This two step mechanism implies a secondary transglycosylation activity aside of hydrolysis. This arises when the oxazolinium intermediate is opened by an incoming hydroxyl group different than water. Moreover, when this acceptor is a *N*-acetyl-D-glucosaminyl residue, the newly formed bound inherits the β(1 → 4) specificity. In normal conditions the hydrolytic activity largely prevails upon transglycosylation. But under certain circumstances, like early stages of hydrolysis when large quantities of oxazolinium intermediate may accumulate, transglycosylation products are transiently formed [34,35].

In first published transglycosylation attempts a commercial mixture of chitinases from *Bacillus sp.* [22] and later the purified wild type *Bc* ChiA1 [25] were used under basic pH conditions in order to deprotonate the general acid/base glutamate and inactivate hydrolytic activity. Under these conditions, chitinbiose oxazoline was polymerized, acting both as donor and acceptor of transglycosylation to give artificial chitin. The chitin product of the reaction from *Bacillus sp.* was characterized by CP/MAS ^{13}C NMR, X-ray diffraction, and low-resolution MALDI-MS which was not sufficient to give the precise molecular structure of synthesized oligomers [22,36]. In our hands duplication of this experiment with the commercial *Bacillus sp.* chitinase preparate gave a mixture of even and odd-numbered chitin oligomers, indicating that besides coupling reaction from chitinbiose oxazoline, also disproportionation reaction from initially produced, longer COs was occurring (Fig. 3). Thus, the use of wild type chitinases in basic medium was not applicable for the synthesis of pure COs due to uncontrollable disproportionation reaction. This observation prompted our investigations of mutant chitinases directed to catalytic amino acids, where the catalytic machinery would be disrupted enough to abolish hydrolytic and disproportionation activities, but still operational for coupling reaction using transition state analog substrates.

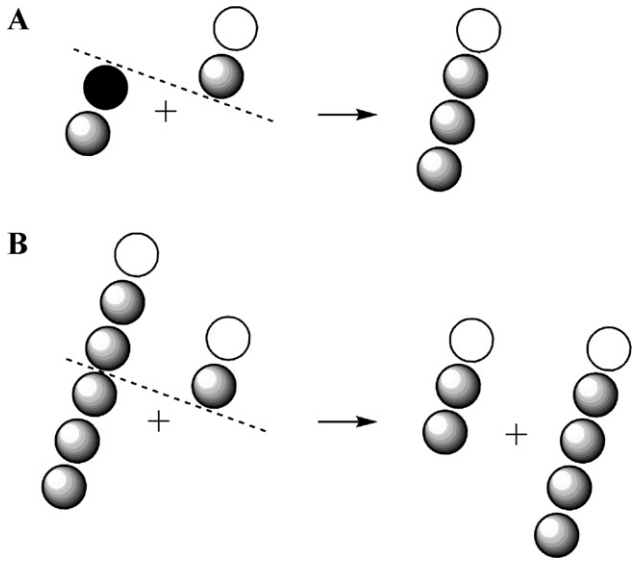

Fig. 3. Schematic representation of coupling and disproportionation reactions. (A) The condensation of an oxazoline activated donor on an acceptor substrate is termed coupling reaction; (B) the cleavage and transfer of a chitin oligomer to an acceptor substrate is termed disproportionation reaction. Both coupling and disproportionation reactions can be regarded as transglycosylation reactions. Shaded circles: β(1 → 4) *N*-acetylglucosaminyl unit; white circles: reducing end *N*-acetylglucosamine; black circles: *N*-acetylglucosamine oxazoline.

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CHIA_SERMA/301-330   -LQTWKFFDGVD311ID313WE315FPGGKGANPNLGSPQ-
CHIA1_BACCI/190-219  FLRKYN-FDGVD200LD202WE204YPVSGGLDGNSKRPE-
CHIB_SERMA/130-159   IMKDYG-FDGVD140ID142WE144YP-QAAEVDGFIAALQ
CHI4_TRIHA/162-191   FMKDWG-FDGID168ID170WE172YPADATQASNMILLL-
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Fig. 4. Multiple alignments of family GH-18 chitinases. Sequence alignment of the catalytic sites from ChiA and ChiB from *Serratia marcescens* for which most crystallographic studies and mechanistic proposals have been made together with *Bc* ChiA1 and *Th* Chit42 that have been studied in the present work. Conserved DXDXE motif can be observed in all cases.

Only few previous studies have been published on the usage of catalytically deficient GH-18 chitinases as glycosynthases. *Bc* ChiA1 E204Q mutant (E204 is assumed to be the catalytic general acid/base) has been reported earlier to have no remaining hydrolytic activity but still to be active in coupling reaction with oxazoline donor [25]. However, this finding was revised later with no reference to the previous work [23]. In the same report *Bc* ChiA1 D202N (which is assumed to be the stabilizer from sequence alignment studies, see also Fig. 4) was also produced and partially characterized, indicating that mutation of the stabilizer could lead to a good catalyst for coupling reaction with oxazoline donor. *N*-Acetyllactosamine oxazoline was found to be an appropriate donor for coupling reaction on various acceptors such as *N*-acetylglucosaminides, chitinbiosides and interestingly on cellobiosides [23]. The non-reducing galactosyl unit, which can be accommodated at −3 and −2 subsites by *Bc* ChiA1 but not at +1, plays a role of protecting group preventing uncontrolled polymerization of the donor, and remains selectively removable by β-galactosidase catalyzed hydrolysis [23,24].

We wanted to re-investigate the behaviour of catalytically mutated chitinases and able to perform coupling reactions under neutral conditions towards the synthesis of COs with extended backbone. This concept is studied here on two selected GH-18 chitinases easily expressed in *E. coli*: *B. circulans* chitinase A1 (*Bc* ChiA1) and *Trichoderma harzianum* chitinase 42 (*Th* Chit42). These enzymes share about 34% sequence identity, but show conservation of the DXDXE sequence motif as shown in Fig. 4. The well defined catalytic triad homology as well as earlier published structure–function studies enabled the identification of the putative assistant stabilizer, stabilizer and general acid/base as D200, D202 and E204 in *Bc* ChiA1 and D168, D170 and E172 in *Th* Chit42, respectively.

Table 1
Kinetic parameters of wt and mutant *Bc* ChiA1 chitinases.

Enzyme	*Km* (mM)[a]	k_{cat} (min^{-1})[a]
ChiA (wild type)	2.2 ± 0.5	378 ± 65
ChiA1 D200A	nd	Activity 2.1% of the wild type[b]
ChiA1 D202A	nd	Activity 1.7% of the wild type[b]
ChiA1 E204Q	nd	Activity 1.1% of the wild type[b]

[a] Results obtained from chitinbiose-PNP hydrolysis ranging from 1 μM to 2.5 mM in Tris HCl/Carbonate buffer 0.05 mM pH 6.9 at 37 °C.
[b] The residual hydrolytic activity (% of the wild type) was measured at one substrate concentration of 2.5 mM.

3.2. *Bacillus circulans ChiA1 site-directed mutagenesis, expression and purification*

Each of the three conserved carboxylic acids of *Bc* ChiA1 was individually mutated according to its putative catalytic role. The assistant stabilizer and stabilizer aspartates, expected to affect to the oxazolinium intermediate formation, were changed into alanine mutants as these Asp's should become unnecessary once the synthetic oxazoline transition state analog is supplied. By contrast, the general acid/base glutamate may be needed for hydroxyl acceptor activation, and for this reason it was changed into glutamine. This mutation eliminates the proton exchange capability but keeps the possibility of H-bonding the incoming hydroxyl group to catalyze the oxazoline ring opening. The expression conditions for the *Bc* ChiA1 mutants in *E. coli* BL21 were first optimised as explained in more details under Section 2. Each *Th* ChiA1 variant was purified from freshly transformed *E. coli* cultures using Ni-IMAC purification. The purification was first tested on periplasmic fraction and total soluble lysate. Whole lysate purification gave better yields of purified protein and activity (ten times the amount of protein and 4.5 times the activity units), but also resulted in a less pure chitinase. Periplasmic purification yields ranged between 3 and 5 mg/L culture. This amount was considered sufficient for the basic biochemical characterization and transglycosylation activity analysis of each of the chitinase variants.

3.3. *Transglycosylating activity of Bacillus circulans E204Q, D200A, D202A ChiA1 mutants*

Kinetic properties of wild type *Bc* ChiA1 and residual hydrolytic activity of ChiA1 mutants were evaluated using 4-nitrophenyl β-chitinbioside as susbtrate (Table 1). As can be seen each perturbation of the catalytic machinery caused a fall in hydrolytic activity, which dropped to 1–2% of the wild type activity, depending on the mutated position. This included the assistant stabilizer D200 mutant, pointing out the importance of this residue in the catalysis.

Then the different *Bc* ChiA1 mutants were tested for a standard coupling reaction. The designed reaction was the coupling of β-D-galactopyranosyl-(1 → 4)-chitinbiose oxazoline (GalCO-IIoxa) donor to chitinbiose as acceptor, to form β-D-galactopyranosyl-(1 → 4)-chitintetraose (GalCO-IV). Under these conditions, each ChiA1 mutant was used to perform this reaction at neutral and basic pH, and the reaction course was followed by HPLC–ESIMS (Fig. 5).

As expected, the wild type *Bc* ChiA1 showed the strongest activities at initial time. That was better observed when basic pH was used. The slowed hydrolysis allowed the formation of large amount of GalCO-IV that reached its maximum after only 5–6 h of incubation. When neutral pH was used, this whole process occurred during the first minutes of reaction, and both donor and the transient product reaction disappeared after only 15 min of incuba-

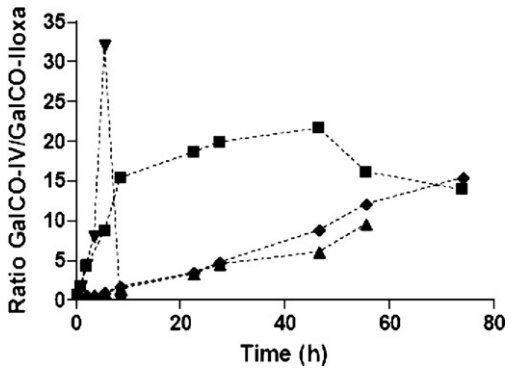

Fig. 5. Time course coupling reaction between β-D-galactopyranosyl-(1 → 4)-chitinbiose oxazoline and chitinbiose. Coupling of β-D-galactopyranosyl-(1 → 4)-chitinbiose oxazoline (GalCO-IIoxa) on chitinbiose (CO-II) to form β-D-galactopyranosyl-(1 → 4)-chitintetraose (GalCO-IV) was catalyzed by the *Bc* ChiA1 mutants at neutral and basic pH. Reaction course is expressed as HPLC–ESIMS integrals ratio between product and donor reactant. *Bc* ChiA1 mutants and pH used: (■) D202A pH 7; (▲) D202A pH 7; (▼) wild type pH 10; (♦) D200A pH 10.

tion (not shown). The general acid/base mutant *Bc* ChiA1 E204Q was completely inactive and no coupling product was observed after 4 days of incubation. This observation confirms the essential role of this residue presumably for correct positioning and activation of the incoming acceptor hydroxyl group. This result also confirms the work reported in Ref. [23] in contradiction with earlier study [25]. *Bc* ChiA1 E204Q was neither able to hydrolyze long COs (DP 6–10) after five days of incubation. This behaviour contrasts with the kinetic results obtained with 4-nitrophenyl β-chitinbioside, pointing towards a potential artefactual effect of measuring hydrolytic activities against a synthetic substrate containing an aglycone that is better leaving group than the natural sugar chain.

Bc ChiA1 D200A and D202A mutants offered the most interesting behaviours. Both mutants showed a similar reaction pattern, reaching maximum yields of coupling product after 24–30 h of reaction. *Bc* ChiA1 D200A yielded the highest amounts of product, but its concentration slowly decreases after long incubation times (above 48 h). This diminution was not observed when the same mutant was used in basic pH, but the coupling yield was lower. *Bc* ChiA1 D202A mutant behaved similarly and gave lower coupling yields even at neutral pH, but no late decreasing was detected either.

At first glance the slow decrease in coupling product could be attributed to residual hydrolytic activity, but a possible disproportionation reaction on GalCO-IV as we observed for wild type ChiA1 chitinbiose oxazoline polymerization could not be ruled out. We decided to check this possibility first by incubating the *Bc* ChiA1 D200A and D202A mutants with chitinbiose oxazoline solely. Chitinbiose oxazoline polymerization could be externally followed as insoluble products above chitinhexaose were formed. In fact this observation indicated an expected better behaviour of assistant stabilizer D200A mutant, which turned reaction media into a cloudy gel after 4 h of reaction, much earlier than the stabilizer D202A mutant. After 24 h of incubation, a white precipitate was formed, and it was recovered by centrifugation following several washing with water and freeze-drying. The solid was analyzed by MALDI-MS, and it revealed to be a mixture of COs, the two mutants giving the same pattern of polymerization with even and odd numbered COs with DP ranging from 3 to 13 (Fig. 6A). It is of importance to note that D200A and D202A reactions showed large amounts of polymerization products remaining after as much as five days of incubation.

In a second set of experiments we incubated each of the two mutants with β-D-galactopyranosyl-(1 → 4)-chitinbiose oxazoline (GalCO-IIoxa) as donor and an equimolar amount of chitinpentaose as acceptor. Again a white precipitate was formed after 18 h of incubation and its MALDI-MS analysis indicated a mixture of GalCO-VII as main product, but contaminated with CO-VI to CO-XII, and also GalCO-VIII to GalCO-XIII (Fig. 6B). This result strongly suggests that the two mutants were able to perform not only coupling reaction from oxazoline activated donor, but also disproportionation reaction on higher DP COs.

In a third set of experiment the two mutants were incubated with chitinpentaose solely, applying the same conditions that for precedent polymerization and coupling reactions. This time the reaction solution became turbid after 18 h of incubation, and the whole reaction medium was freeze-dried before MALDI-MS analysis. Beside the remaining chitinpentaose, a series of COs with DP ranging from 2 to 12 was detected in the reaction mixture.

This observations confirmed that Asp to Ala mutation on the stabilizer or its assistant does not prevent *Bc* ChiA1 to perform synthesis reaction starting from native COs, also called as a disproportionation reaction which is often encountered in polysaccharide transglycosylases such as cyclodextrin glycosyl transferase (CGTase, GH-13) or xyloglucan *endo*-transglucosylase (XET, GH-16).

Taken all together these three experiments demonstrate clearly that (1) the residual hydrolytic activity on COs is abolished or negligible when the D202 stabilizer or its D200 assistant in *Bc* ChitA1 is mutated to alanine residues; (2) the D200A or D202A mutation turn *Bc* ChiA1 into a powerful catalyst for coupling reaction between oxazoline activated donor and COs acceptor; (3) the coupling activity of D200A and D202A mutants is interconnected with the disproprotionation activity, which diminishes their usefulness for the synthesis of large, water insoluble COs, yielding an inextractible mixture of products.

Having these results in hand, we turned our attention to *T. harzanium* Chit42, a fungal GH-18 chitinase, questioning whether such behaviour would be a generality among GH-18 chitinases.

3.4. Transglycosylating activity of Trichoderma harzanium E172Q, D170N, D170A Chit42 mutants

Our earlier mutagenesis studies have shown that the *Th* Chit42 E172Q (general acid/base) and D170N (stabilizer) mutants have 0.3% and 0.6% activity left, respectively, when compared to the wild type activity [33], confirming their essential roles in catalysis. Under the same conditions D170A displayed only 1.3% activity from the wild type expressed in *E. coli*.

In order to study the transglycosylation, the three *Th* Chit42 mutants were first incubated with chitinbiose oxazoline solely, for polymerization reaction. The general acid/base mutant *Th* Chit42 E172Q was completely inactive and no coupling product was observed after 4 days of incubation. *Th* Chit42 D170N mutant behaved like *Bc* ChiA1 D2002A, yielding a mixture of even- and odd-numbered COs. *Th* Chit42 D170A mutant gave a mixture of mainly even-numbered COs with DP ranging from 4 to 10 (Fig. 6C).

Encouraged by this result, *Th* Chit42 D170A mutant was tested for coupling reaction between GalCO-IIoxa as donor and an equimolar amount of chitinpentaose as acceptor. The reaction product was MALDI-MS analyzed, indicating a mixture of CO-V as main component, together with GalCO-VII and very low amount of higher DP COs.

Although it is not possible to precisely evaluate the transglycosylation activity for reactions yielding insoluble products, unfortunately D170A mutant was the less active catalyst so far tested. We suspect that the apparent abolition of disproportionation activity in *Th* Chit42 D170A mutant is more of an effect of a poor

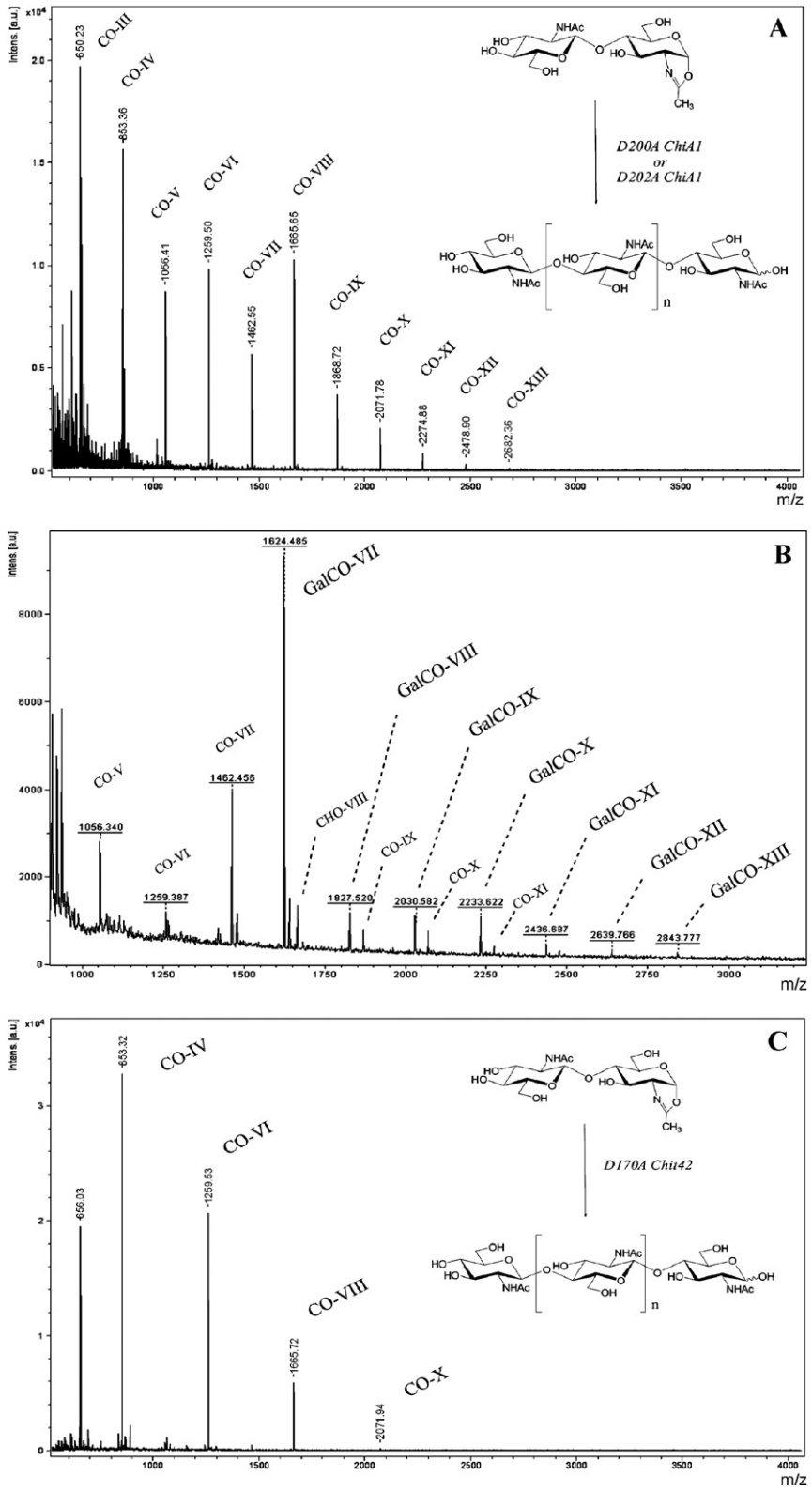

Fig. 6. Mass spectra of the products of the polymerization and coupling reaction catalyzed by mutant chitinases. (A) MALDI-MS analysis of COs mixture obtained by *Bc* ChiA1 D200A or D202A catalyzed polymerization reaction from chitinbiose oxazoline. Chitinbiose oxazoline (CO-IIoxa) is self condensed to give even numbered oligomers chitintetraose (CO-IV) to chitindodecaose (CO-XII) by coupling reaction, meanwhile odd numbered oligomers chitintriose (CO-III) to chitintridecaose (CO-XIII) are formed by disproportionation reaction; (B) MALDI-MS analysis of COs mixture obtained by *Bc* ChiA1 D200A or D202A catalyzed coupling reaction from β-D-galactopyranosyl-(1 → 4)-chitinbiose oxazoline and an equimolar amount of chitinpentaose. The expected octasaccharide β-D-galactopyranosyl-(1 → 4)-chitinheptaose (GalCO-VII) is produced as major compound by coupling reaction. It is accompagned with a mixture of chitinhexaose (CO-VI) to chitindodecaose (CO-XII) together with a mixture of β-D-galactopyranosyl-(1 → 4)-chitinoctaose (GalCO-VIII) to β-D-galactopyranosyl-(1 → 4)-chitintridecaose (GalCO-XIII) produced by disproportionation reaction from starting chitinpentaose (CO-V) and from β-D-galactopyranosyl-(1 → 4)-chitinheptaose (GalCO-VII) product; (C) MALDI-MS analysis of COs mixture obtained by *Th* Chit42 D170A catalyzed polymerization reaction from chitinbiose oxazoline. Chitinbiose oxazoline (CO-IIoxa) is self condensed to give even numbered oligomers chitintetraose (CO-IV) to chitindecaose (CO-X) by coupling reaction, odd numbered chitin oligomers formed by disproportionation reaction are virtually undetectable.

overall transglycosylation activity rather than an improvement in coupling versus disproportionation activity.

4. Conclusion

The alanine screening of the three conserved carboxylic acid residues in *B. circulans* WL-12 ChiA1 in the catalytic motif DXDXE revealed that single mutation of both aspartates D200 or D202, lead to the expected transglycosylation activity. However, this approach does not eliminate the disproportionation activity already observed with wild type chitinase at basic pH where hydrolytic activity is abolished. *T. harzanium* Chit42 mutants were also evaluated leading to the same observation, indicating that such behaviour may be inherent to all mutant chitinases displaying a substrate-assisted molecular mechanism of action. Some mechanistic implications can also be drawn from our results (see also Fig. 2). The general acid/base in GH-18 chitinases is necessary for hydrolytic, coupling and transglycosylation reactions. When either one of the conserved aspartates, i.e. the stabilizer or the assistant stabilizer, is missing, a reaction of disproportionation from COs is still observed. This indicates that upon binding COs bring enough binding energy to induce the distorted conformation (boat or skew-boat conformation in −1) leading to the formation of oxazolinium transition state by acetamido anchimeric assistance without catalytic support from the stabilizer. This binding energy is assumed to be provided by the optimal spanning of the substrate-binding groove, which contains seven or more subsites in both *Bc* ChiA1 and in *Th* Chit42 [37,38].

These Asp mutants are therefore not as such suitable for the synthesis of pure COs with large DP. One possibility in future could be to test the classical retaining glycoside hydrolases, such as GH-22 family lysozymes, as some of them have been shown to have transglycosylation ability on COs [39,40].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2011.09.003.

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