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Mutation of fungal endoglucanases into glycosynthases and characterization of their acceptor substrate specificity

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Abstract

Humicola insolens mutant Cel7B E197A is a powerful *endo*-glycosynthase displaying an acceptor substrate specificity restricted to β-D-glucosyl, β-D-xylosyl, β-D-mannosyl and β-D-glucosaminyl in +1 subsite. Our aim was to extend this substrate specificity to β-D-*N*-acetylglucosaminyl, in order to get access to a wider array of oligosaccharidic structures obtained through glycosynthase assisted synthesis. In a first approach a trisaccharide bearing a β-D-*N*-acetylglucosaminyl residue was docked at the +1 subsite of *H. insolens* Cel7B, indicating that the mutation of only one residue, His209, could lead to the expected wider acceptor specificity. Three *H. insolens* Cel7B glycosynthase mutants (H209A, H209G and H209A/A211T) were produced and expressed in *Aspergillus oryzae*. In parallel, sequence alignment investigations showed that several cellulases from family GH7 display an alanine residue instead of histidine at position 209. Amongst them, *Trichoderma reesei* Cel7B, an endoglucanase sharing the highest degree of sequence identity with *Humicola* Cel7B, was found to naturally accept a β-D-*N*-acetylglucosaminyl residue at +1 subsite. The *T. reesei* Cel7B mutant nucleophile E196A was produced and expressed in *Saccharomyces cerevisiae*, and its activity as glycosynthase, together with the *H. insolens* glycosynthase mutants, was evaluated toward various glycosidic acceptors.

Keywords: Glycosynthase; Protein engineering; Cellulase; Oligosaccharide synthesis; Enzymatic synthesis

1. Introduction

Glycosynthases are mainly mutated retaining glycoside hydrolases in which one of the two catalytic amino acids (the nucleophile) is mutated to a non-carboxylic Ala, Gly or Ser residue [1,2]. These enzymes condense in high yield glycosyl fluorides with the appropriate acceptor molecules without remaining hydrolytic activity. This concept has been developed first by engineering a β-glucosidase from *Agrobacterium* [3,4], and has later been applied successfully by us and others to a variety of *endo*- and *exo*-glycoside hydrolases [5–10]. *Endo*-glycosynthases (obtained from *endo*-glycanases) are particularly attractive because of their regiospecificity, and their ability to condense oligosaccharidic donor substrates to glycosidic acceptors, giving access to large oligosaccharides [11–13] and to polysaccharides [6,9,14]. *Humicola insolens* (*Hi*) Cel7B endoglucanase (family GH7, CAZy, http://www.cazy.org) is a retaining β-D-glycoside hydrolase secreted by the fungus during the process of cellulose fermentation, and is produced for specific industrial applications like detergency and pulp and paper processes [15]. This enzyme has also found laboratory applications for the chemo-enzymatic synthesis of cellodextrin derivatives, by catalysing the condensation reaction of β-glycosyl fluorides to glycosidic acceptors in hydro-organic medium [16]. However the size of oligosaccharides synthesized was limited by the inherent side reactions of transglycosylation starting with

Abbreviations: wt, wild-type; *Tr*, *Trichoderma reesei*; *Hi*, *Humicola insolens*; PGK, phosphoglycerate kinase

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donor or acceptor substrates longer than disaccharides [16]. This limitation has been circumvented by mutating the nucleophilic catalytic Glu197 into a non-carboxylic alanine or serine residue, making *Hi* Cel7B E197A and E197S mutants the most powerful *endo*-glycosynthases to date, with a turnover number (k_{cat}) of more than 18 s^{-1} for the serine mutant catalysing the condensation reaction between α-lactosyl fluoride and 4-nitrophenyl cellobioside [17]. *Hi* Cel7B glycosynthase has been described as an extremely efficient catalyst for the synthesis of artificial low-DP cellulose [6], for the controlled oligomerization of α-cellobiosyl fluoride into cellodextrins [11], and has given access to a new bifunctionalized fluorescence quenched substrate for cellulases [13]. The donor and acceptor substrate specificity of *Hi* Cel7B glycosynthase is well established, particularly with the help of 3D X-ray structure studies revealing exquisite details related to the electrostatic and hydrophobic interactions between the glycosynthase and its substrates [6,17]. The +1 subsite (subsite nomenclature according to [18]) is extremely versatile with its capability to accommodate not only β-D-glucosyl but also β-D-mannosyl, β-D-xylosyl, and β-D-glucosaminyl residues [6]. However, *N*-acetyl β-D-glucosaminyl residues, which are potential acceptor substrate for the synthesis of complex carbohydrates of interest, are not glycosylated by the glycosynthase. Encouraged by the recent success of other research groups in modulating the specificity of carbohydrate acting enzyme by rational redesign [19–23], we have thus embarked on the modification of +1 subsite specificity toward *N*-substituted β-D-glucosaminyl residues by engineering of the *Hi* Cel7B E197A glycosynthase. In addition, the highly homologous *Trichoderma reesei* (*Tr*) endoglucanase Cel7B, which naturally accepts a β-D-*N*-acetylglucosaminyl residue at +1 subsite, was tested as a glycosynthase.

2. Experimental

2.1. General procedures

4-Nitrophenyl glycosides were commercially available.

α-Lactosyl fluoride was prepared from β-lactose octa-acetate according to ref. [24], followed by deprotection with catalytic sodium methoxide in MeOH.

N^{I}-Acetyl-chitobiose and chitinbiose (N^{I},N^{II}-diacetyl-chitobiose) were produced using an engineered *Escherichia coli* strain [25].

β-D-Galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose (lactosyl-β(1 → 4)-chitinbiose) was prepared as described in [6].

2.2. Strains, plasmids and culture conditions

The strains of *E. coli* used in this study were JM109 (F^{-}, recA1, endA1, gyrA96, thi-1, supE44, relA1, Δ(lac Δ proAB), (F' traD36, proAB, lacIq lacZΔM15), mcrA) and MC1061 (MhsdR mcrB araΔ139 Δ(araABC-leu) 7679 ΔlacX74 galU galK rpsL thi). *Saccharomyces cerevisiae* yeast strain INVSc1 (his3D1/his3D1, leu2/leu2, trp1-289/trp1-289, ura3-52/ura3-52) (Invitrogen) was used for the expression of the mutated *Tr* Cel7B enzyme. The *E. coli* plasmid pCE10 contains the region of cDNA encoding the catalytic domain of the *Hi* glycosynthase Cel7B E197A and signal sequence cloned into pCR4 TOPO vector [J. Vind (Novozymes), unpublished results]. The yeast expression plasmid pMP311 contains the *Tr* Cel7B cDNA and signal sequence cloned under the constitutive phosphoglycerate kinase (PGK) promoter, and a *LEU2* marker gene for selection [26]. Recombinant *E. coli* strains were cultured in Luria broth supplemented with 100 μg/mL ampicillin, at 37 °C with shaking (200 rpm). For the yeast cultivations synthetic complete (SC) medium without leucine, buffered at pH 6.0 with 160 mM succinate and supplemented with 2% glucose was used [27].

2.3. Docking simulation of N-acetylglucosamine in the +1 subsite of the H. insolens Cel7B active site

Protein–ligand docking studies were performed using Autodock 3.05 [28] on the basis of the crystal structures of *Hi* endoglucanase Cel7B (pdb entry 2A39) and *Tr* cellobiohydrolase Cel7A complexed with cellononaose in −7 to +2 subsites (pdb entry 8CEL). Mutation of residue 209 from histidine to alanine was introduced in the *Hi* Cel7B model. Hydrogen atoms were added to the protein using WHAT IF [29,30] and partial charges were assigned using all-atoms charges of the AMBER force field [31]. Polar hydrogens were differentiated from non-polar hydrogens to account for hydrogen bonds by using different Lennard-Jones parameters (12-10 hydrogen bonding parameters for polar hydrogen and 12-6 parameters for non-polar hydrogen) from AutoDock Version 1.

Ligands were created from cellononaose modeled in *Tr* cellobiohydrolase Cel7A. Hydrogens were added to ligands using WHAT IF and partial atomic charges were generated using PIM parameters for the TRIPOS force field [32] or with MOPAC 7.0 (Quantum Chemistry Program Exchange, Bloomington, Indiana) for *N*-acetyl-D-glucosamine. For the *N*-acetyl-D-glucosamine moiety, initial conformers bearing the four possible orientations of the acetamido group [33] were defined.

For the docking experiments, and to allow the ligand to search the entire active site, a cubic box of 22.5 Å × 22.5 Å × 22.5 Å was centered on the O4 hydroxyl group of the glycosyl unit in +1 subsite. In the definition of the grid, a sigmoidal distance-dependent dielectric function is used to model solvent screening [34]. The search for possible ligand binding sites was performed within this box at the default grid point spacing of 0.375 Å. All the rotatable ligand bonds were defined using the Autotors module of Autodock. Each single docking experiment consisted of 100 runs employing a Lamarckian genetic algorithm [28]. All other docking parameters were set to their default values. The resulting protein–substrate complexes were clustered employing an RMS deviation tolerance of 1 Å. Only clusters representing productive protein–ligand complexes were further analyzed, which were in most cases the highest populated cluster or the cluster with the lowest binding energy, respectively.

2.4. Construction of site-directed mutants

Site-directed mutagenesis was carried out according to the manufacturer's instructions (Quick-Change Mutagenesis kit, Stratagene). Appropriate mutations were generated by using the following primers (sense and antisense) to synthesise the DNA *in vitro*: for *Hi* Cel7B H209A 5′-GCC AAC TCG CGA GCC TCG **GC**C GTG GCT CCC CAC-3′ and 5′-GTG GGG AGC CAC G**GC** CGA GGC TCG CGA GTT GGC-3′; for *Hi* Cel7B H209G 5′-GCC AAC TCG CGA GCC TCG **GG**C GTG GCT CCC CAC-3′ and 5′-GTG GGG AGC CAC G**CC** CGA GGC TCG CGA GTT GGC-3′, for *Hi* Cel7B A211T 5′-GGT GTG GGG AG**T** CAC GGC CGA GGC TCG CGA ATT CGC CTC CC-3′ and 5′-GGG AGG CGA ATT CGC GAG CCT CGG CCG TG**A** CTC CCC ACA CC-3′; for *Tr* Cel7B E196A 5′-CCT CCA GGA TAT CCA T**GG** CGT TGC AGC AGA AGC CC-3′ and 5′-GGG CTT CTG CTG CAA CG**C** **C**AT GGA TAT CCT GGA GG-3′. The mutated nucleotides are in bold. Thermocycling parameters were 95 °C for 30 s, 15 cycles (95 °C for 30 s, 55 °C for 1 min, 68 °C for 23 min). The PCR reaction products were digested with *Dpn*I prior to transformation of competent cells (JM109 or MC1061) to remove templates. Plasmids were extracted from overnight cultures of transformed *E. coli* cells and verified by DNA sequencing (GENOME express). To ensure that only the desired mutations had occurred, the complete sequences of *cel7B* derivatives containing the appropriate mutation were determined.

2.5. Enzymes production and purification

Hi Cel7B wild-type endoglucanase, glycosynthase (Cel7B E197A) and three mutated glycosynthases were expressed using the *A. oryzae* expression system. In order to transfer the constructed cel7B variants into an *Aspergillus* expression vector, a PCR reaction was made using plasmid containing the variant as template, the two primers 5′-CTG AGG GAT CCA CCA TGG CTC GCG GTA CCG CTC TC-3′ and 5′-CTG AGA GAT CTT CAG TGG TGG TGG TGG TGGT GCT C-3′, and Phusion DNA polymerase as recommended by manufacturer (Finnzymes). The resulting PCR fragment was cut with the restriction enzymes *Bam*HI and *Bgl*II and ligated into the *Aspergillus* expression plasmid pENI2516 (PCT Publication No. WO 2004/069872), cut with the same restriction enzymes. The three constructed expression plasmids were transformed into *A. oryzae* (PCT Publication No. WO 2004/069872). The best Cel7B producing *A. oryzae* transformants were identified on SDS-PAGE [35], inoculated in a shake flask containing G2-gly media (18%, w/v yeast extract, 2%, w/v glycerol, 0.1%, w/v Dowfax 63N10) and grown overnight at 30 °C with shaking at 250 rpm. Two millilitres of the overnight culture were transferred to 10 new shake flasks each containing 200 mL 2mdu-2bp media (4.5% maltose, 0.1% magnesium sulphate, 0.1% sodium chloride, 0.2% potassium sulphate, 1.2% potassium dihydrogenphosphate, 0.7% yeast extract, 0.01% Dowfax 63N10, trace metals) and grown 72 h at 30 °C with shaking at 250 rpm.

Secreted proteins were recovered from cultivation broths and purified by column chromatography on AKTA Explorer (Amersham Pharmacia) as described below. Solid ammonium sulphate was added to the culture supernatant to reach a final concentration of 1.2 M and the pH was adjusted to 8. The supernatant was then applied on a Phenyl Sepharose column (Amersham Pharmacia) equilibrated with 1.2 M ammonium sulphate. Unbound material was washed out with 1.2 M ammonium sulphate pH 8 until an A_{280} below 0.05 was reached. The bound proteins were then eluted with 50 mM Tris–acetate buffer pH 8. Collected fractions were analysed by SDS-PAGE and those containing proteins with expected molecular weight were pooled and diluted with distilled water to adjust the conductivity below 4 mS. The pooled fractions were then applied on an anion exchange FFQ Sepharose column (Amersham Pharmacia) equilibrated with 50 mM Tris–acetate pH 8. After washing the unbound material, the bound proteins were eluted using a linear sodium chloride gradient in the same buffer. Collected fractions were analysed and pooled as described above.

Tr Cel7B wild-type enzyme was produced in *T. reesei* strain devoid of the genes coding for the major cellobiohydrolases Cel6A and Cel7A, and purified from the culture filtrate as described earlier [36]. In addition, the *Tr* Cel7B E196A mutant (glycosynthase) was expressed in *S. cerevisiae* INVSc1 strain. One litre buffered SC medium cultures were grown for 72 h at 30 °C. After this, the culture supernatant was obtained by centrifugation (15 min, 4000 × *g* at 4 °C) and concentrated 10 times by ultra-filtration using 10 kDa cut-off membranes. The expression of the enzyme was verified by SDS-PAGE and Western analysis using monoclonal antibody raised against Cel7B as described previously [37]. The activity of the *Tr* Cel7B E196A preparation was measured using the soluble substrate 4-methylumbelliferyl β-lactoside (MULac) in 50 mM sodium acetate buffer pH 5.0, essentially as described by van Tilbeurgh and Claeyssens [38]. Prior to glycosynthase activity assays, the yeast culture supernatant was dialysed against the appropriate buffer and concentrated using Nanosep and Microsep devices equipped with a 10 kDa cut off membranes.

Protein concentrations were determined according to Bradford [39], using bovine serum albumin as standard protein.

2.6. Hydrolysis of cellotetraose by H. insolens and T. reesei Cel7B

Thirteen millimolars of cellotetraose was incubated with 0.3 μg of *Hi* Cel7B (in 50 mM phosphate buffer pH 7), or 0.5 μg of *Tr* Cel7B (in 25 mM sodium acetate buffer pH 4.6), in a total volume of 300 μL. Initial reaction rates at 37 °C were obtained by measuring the amount of cellobiose released at regular intervals using HPLC which was carried out with a system consisting of a pump system gold LC-125S solvent module Beckmann, a μBondapak 10 μm NH_2 (300 mm × 3.9 mm) Waters column, and a rheodyne model 7725 injector, with a 20 μL sample loop. The analytes were eluted with a mixture of 80% CH_3CN in H_2O at a flow rate of 0.5 mL/min.

2.7. Hydrolysis of β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose by H. insolens and T. reesei Cel7B

Thirteen millimolars of tetrasaccharide lactosyl-β(1 → 4)-chitinbiose was incubated with 40 μg of *Hi* Cel7B (in 50 mM phosphate buffer pH 7), or 6.4 μg of *Tr* Cel7B (in 25 mM sodium acetate buffer pH 4.6), in a total volume of 200 μL. Initial reaction rates at 37 °C were obtained by measuring the amount of chitinbiose released at regular intervals using HPLC, as described in Section 2.6.

2.8. Cellobiose glycosylation by H. insolens and T. reesei glycosynthases

One hundred and forty-five millimolars of α-lactosyl fluoride (donor) and 145 mM cellobiose (acceptor) were incubated with 52 μg of *Hi* Cel7B E197A or 500 μg of *Tr* Cel7B E196A in 100 μL of 50 mM phosphate buffer pH 7 or 25 mM sodium acetate buffer pH 4.6, respectively, at 37 °C during 16 h. Twenty-five microlitres of each reaction mixture was then treated with 50 μg of *Hi* wild-type Cel7B at 37 °C during 16 h. Evolution of reactions was monitored by analytical thin-layer chromatography using silica gel 60 F254 pre-coated plates (E. Merck, Darmstadt). The analytes were eluted with a mixture of propan-1-ol/nitromethan/H_2O (5/2/2) and spots were detected by charring with an orcinol solution (0.1% orcinol in a mixture of sulfuric acid/ethanol/H_2O 3/72.5/22.5). Molecular weight of the oligosaccharide produced was confirmed by ESI-MS (Waters Micromass ZQ Spectrometer).

For the comparison of cellobiose glycosylation by the different *Hi* glycosynthase mutants, 13 mM α-lactosyl fluoride (donor) and 13 mM cellobiose (acceptor) were incubated with an appropriate amount of enzyme ranging from 28 to 180 μg (see Fig. 7 for details), in a total volume of 500 μL of 50 mM phosphate buffer pH 7. Initial reaction rates at 37 °C were obtained by measuring the amount of cellobiose consumed at regular intervals using HPLC, as described in Section 2.6.

2.9. T. reesei glycosynthase and H. insolens glycosynthase mutants activity assays on acceptors different from cellobiose

The reaction mixture contained 13 mM α-lactosyl fluoride (donor), 13 mM acceptor (excepted 4-nitrophenyl β-D-*N*-acetylglucosaminide which was used at 6.5 mM due to low solubility of the substrate) and 20–180 μg of enzyme depending on the glycosynthase tested, in 50 mM phosphate buffer pH 7 (total volume, 100 μL). The enzyme reaction was carried out at 37 °C during 16 h and 25 μL of each reaction mixture were then treated with 50 μg of *Hi* wild-type Cel7B at 37 °C during 16 h. The reaction mixture was analysed by thin-layer chromatography as described in Section 2.8, excepted that the analytes were eluted with a mixture of 75% CH_3CN in H_2O when 4-nitrophenyl glycosides were used as acceptor, and of 70% CH_3CN in 10% acetic acid with the other acceptors. Molecular weight of the oligosaccharides produced was confirmed by ESI-MS (Waters Micromass ZQ Spectrometer).

3. Results and discussion

3.1. Docking simulation of N-acetyl-β-D-glucosamine in the +1 subsite of the H. insolens Cel7B active site and sequence alignments of family GH7 enzymes

In a qualitative preliminary study, the environment around the *N*-acetyl group at position 2 of a β-D-glucosaminyl unit positioned in +1 subsite of *Hi* Cel7B was checked. To achieve this goal, we used the modeled cellononaose complexed with *Tr* cellobiohydrolase Cel7A as a reference. An *N*-acetylglucosaminyl unit was first superposed on the glucosyl unit present in +1 subsite of the enzyme. Then, this same residue was superposed in the active site of *Hi* endoglucanase Cel7B. As shown in Fig. 1, possible interactions between the enzyme and the 2-acetamido group of the glucosaminyl unit can be assumed. As a matter of fact, residues like H209 or A211 could interfere with the introduction of the side chain in the active site of the enzyme. Among those residues, histidine is a relatively large one.

To investigate the nature and variability of amino acids 209 and 211 (numbering of *Hi* Cel7B), sequence alignments of family GH7 sequences were realised using ClustalW (at the EMBL European Bioinformatics Institute, http://www.ebi.ac.uk/).

Fig. 2 shows that the polypeptide sequence does not vary much in the proximity of the two catalytic residues E197 and E202. The residue 209 identified by the preliminary docking study is, in most of the family GH7 sequences, a histidine or an alanine. The alignments equally revealed that the strongly conserved histidine or alanine residues in position 209, strongly correlate with equally conserved alanine or threonine residues in position 211, by forming the pairs H209/A211 and A209/T211. After identifying these important residues, docking studies were started in order to follow the behavior of oligosaccharides bearing an *N*-acetyl-β-D-glucosaminyl residue in +1 subsite of a mutated *Hi* Cel7B H209A.

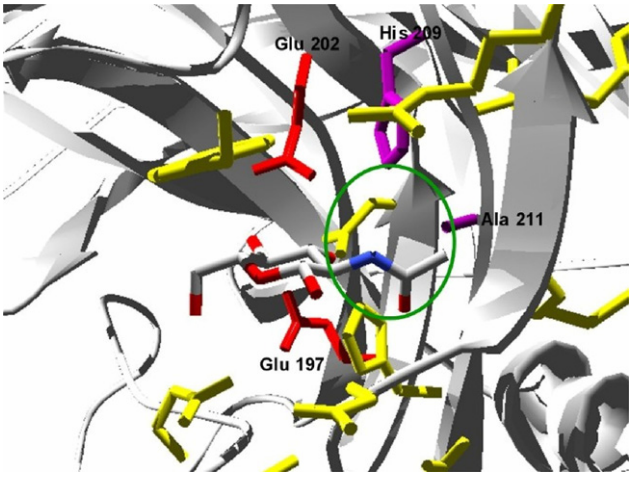


Fig. 1. Superposition of *N*-acetyl-β-D-glucosamine in +1 subsite of *Humicola insolens* endoglucanase Cel7B. Catalytic residues E197 and E202 are shown in red, residues of interest H209 and A211 in purple.

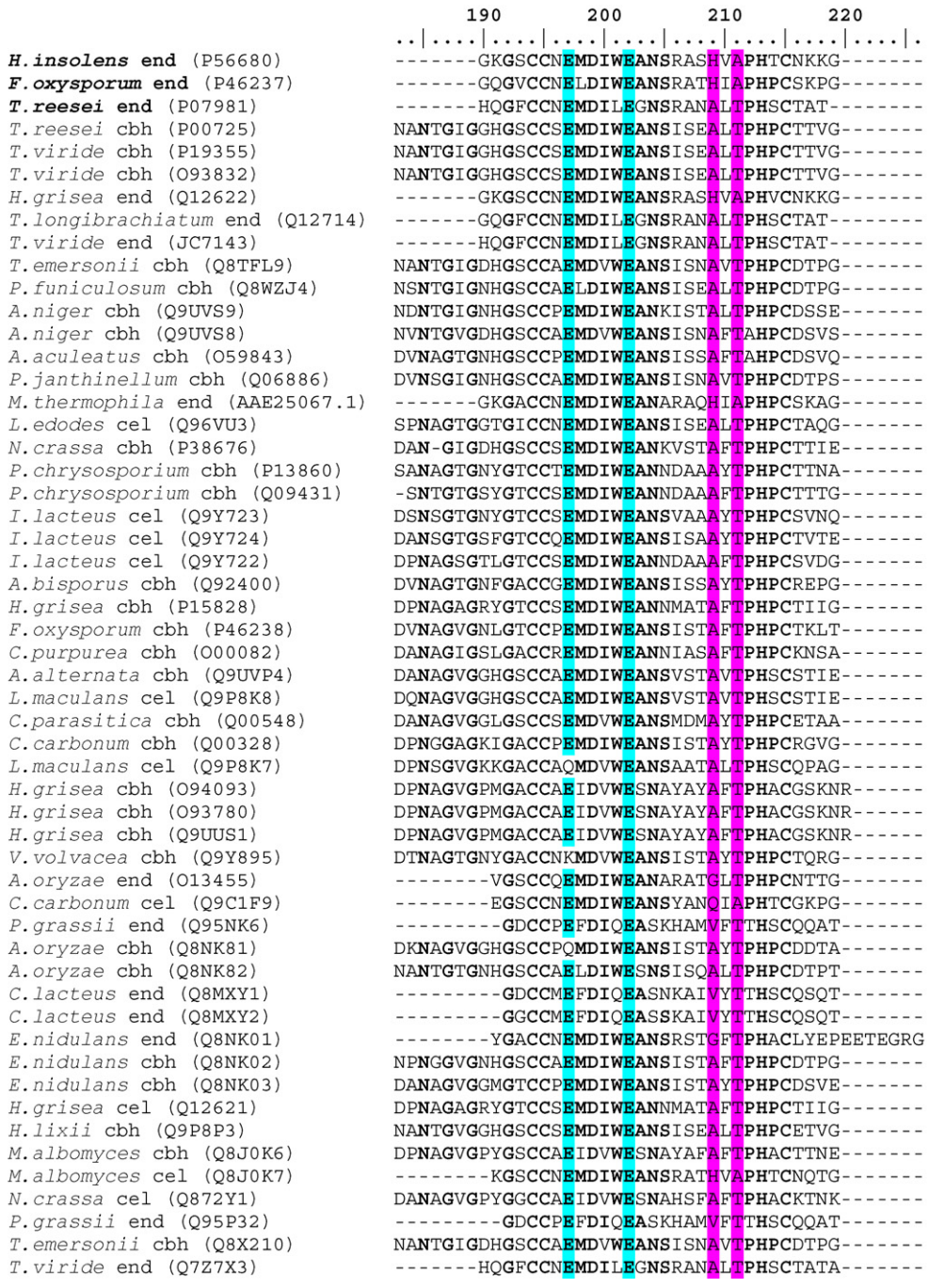


Fig. 2. Multiple alignments of family GH7 glycoside hydrolases. Residues which are identical in most of the sequences are in bold. The catalytic amino acids are coloured in turquoise and residues 209 and 211 are coloured in purple.

Two different trisaccharides were prepared so that an *N*-acetylglucosaminyl residue could be present in +1 subsite to be used in the docking studies. Furthermore, four different initial conformations for the *N*-acetyl group in the sugar ring were considered [33]. Two types of trisaccharides bearing a GlcNAc residue in +1 subsite were considered, Glc(β1,4)GlcNAc(β1,4)Glc occupying −1 to +2 subsites and Glc(β1,4)Glc(β1,4)GlcNAc from −2 to +1 subsites. All docked trisaccharide structures are summarized in Table 1.

After validating docking parameters using simple trisaccharides and tetrasaccharides derived from cellononaose modeled in *Tr* Cel7A (results not shown), docking studies of trisaccharides I-VIII were run with *Hi* endoglucanase Cel7B containing a H209A mutation. Docking solutions are shown in Table 2. Only the most significant results are listed. The best results were obtained with Glc(β1,4)Glc(β1,4)GlcNAc V-VIII positioned in −2 to +1 subsites.

Fig. 3A shows the docking solution for trisaccharide VII. The results suggest that the H209A mutation is compatible with the binding of the oligosaccharide in the active site, either as a substrate for hydrolysis by the native enzyme or as a product from the coupling reaction catalyzed by the corresponding gly-

Table 1
Different trisaccharides modeled in the active site of *H. insolens* Cel7B H209A

Type of trisaccharide (occupied subsites)	*N*-acetyl group conformation	Notation
Glc(β1,4)GlcNAc(β1,4)Glc [−1,+2]	(*Z*)-*anti*	Trisaccharide I
	(*Z*)-*syn*	Trisaccharide II
	(*E*)-*anti*	Trisaccharide III
	(*E*)-*syn*	Trisaccharide IV
Glc(β1,4)Glc(β1,4)GlcNAc [−2,+1]	(*Z*)-*anti*	Trisaccharide V
	(*Z*)-*syn*	Trisaccharide VI
	(*E*)-*anti*	Trisaccharide VII
	(*E*)-*syn*	Trisaccharide VIII

Table 2
Docking solutions of trisaccharides I–VIII in the active site of *H. insolens* Cel7B H209A[a]

Ligand	Cluster (no. of structures)	Total energy[b,c] (kcal/mol)	RMS deviation[d,e] (Å)
Trisaccharide I	1 [1/100]	−145.4 (−145.4)	2.66
	11 [1/100]	−130.2 (−130.2)	3.54
Trisaccharide II	1 [1/100]	−151.3 (−151.3)	2.25
	6 [1/100]	−136.4 (−136.4)	2.08
Trisaccharide III	1 [1/100]	−150.0 (−150.0)	2.60
	3 [1/100]	−142.6 (−142.6)	1.72
Trisaccharide IV	1 [1/100]	−149.8 (−149.8)	2.01
	3 [1/100]	−144.1 (−144.1)	1.94
	4 [2/100]	−142.3 (−140.4)	1.83
Trisaccharide V	1 [2/100]	−160.9 (−158.3)	1.55
Trisaccharide VI	1 [1/100]	−139.6 (−139.6)	2.58
Trisaccharide VII	1 [3/100]	−160.1 (−153.2)	1.53
Trisaccharide VIII	1 [1/100]	−168.8 (−168.8)	1.47
	5 [4/100]	−129.8 (−128.1)	3.43

[a] Docked structures were grouped into clusters; the best clusters being shown.
[b] Energies are calculated with Autodock.
[c] Values for the optimal structure in each cluster are to the left and cluster averages are in parentheses.
[d] RMSD compared to the structure of reference.
[e] Corresponding to the lowest energy value in the cluster.

cosynthase mutant. This mutation appears to provide enough space to accommodate the *N*-acetylglucosaminyl group at +1 subsite of the active site of the enzyme, and is likely to permit the substrate specificity modification of *Hi* endoglucanase Cel7B. In contrast, the native *Hi* enzyme cannot accommodate the *N*-acetylglucosaminyl group (Fig. 3B). When the best docking solution shown in Fig. 3A is placed in the active site of *Hi* native enzyme (Fig. 3B), the H209 makes steric contacts with the *N*-acetylglucosaminyl group.

Several studies in the literature have already shown that a larger pocket could be the key to accommodate an *N*-acetyl group, larger than a simple hydroxyl group. For example, Ramakrishnan et al. [20] showed that the *N*-acetylgalactosaminyltranferase activity of β1,4-galactosyltransferase I could be increased by mutation of residue Y289 to leucine, the tyrosine residue being involved in hydrogen bond formation with the *N*-acetyl group, and thus preventing transfer of the *N*-acetylglucosaminyl unit by the native enzyme. Also Marcus et al. [22] showed that it was possible to reverse the donor specificity of human blood group B-synthesizing α1,3-galactosyltransferase (GTB) towards UDP-GalNAc donor by a single point mutation of P234 to serine.

3.2. Comparing the hydrolytic activity of *H. insolens* and *T. reesei* Cel7B

In order to validate the modelling studies described above, we investigated the hydrolysis of the tetrasaccharide β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 4)-2-acetamido-2-deoxy-D-glucopyranose (lactosyl-β(1 → 4)-chitinbiose) by two endoglucanases belonging to the GH7 family: *Hi* Cel7B in which the residues 209 and 211 are respectively a histidine and an alanine, and *Tr* Cel7B in which the same amino acids are respectively an alanine and a threonine. Because of its structure, this oligosaccharide can accommodate in the active site of both enzymes in only one productive mode, positioning the *N*-acetylglucosamine moiety in the +1 subsite (Fig. 4).

The specific activity for the hydrolysis of the tetrasaccharide lactosyl-β(1 → 4)-chitinbiose by *Tr* Cel7B wild-type enzyme

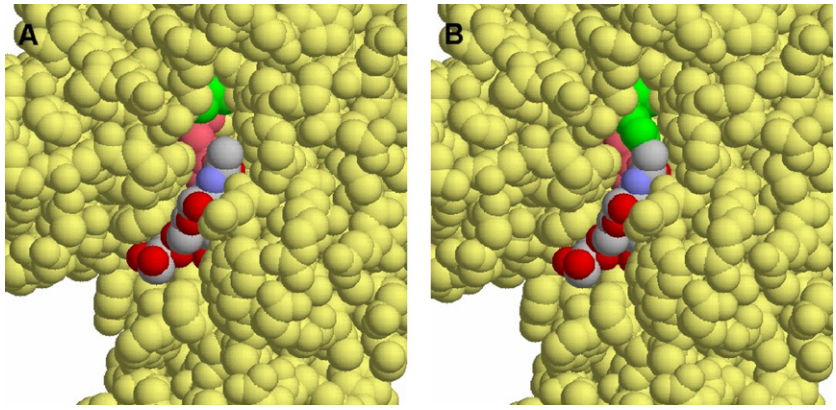


Fig. 3. (A) Trisaccharide Glc(β1,4)Glc(β1,4)GlcNAc docking solution in the substrate binding area of *H. insolens* Cel7B H209A; (B) superposition of this docking solution in the active site of native *H. insolens* Cel7B. In gold are the proteins, the trisaccharide is shown in CPK colors, catalytic residues in pink and residue 209 in green.

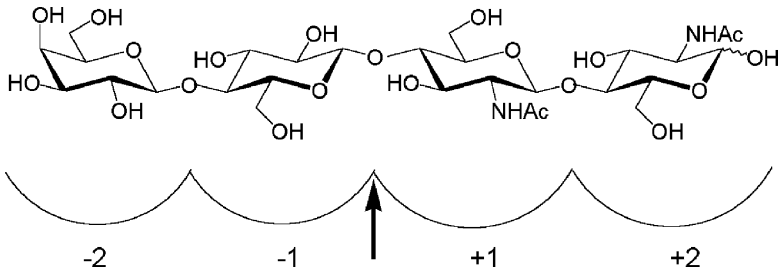


Fig. 4. Positioning of the tetrasaccharide lactosyl-β(1 → 4)-chitinbiose in the endoglucanase active site. The arrow indicates the hydrolysis site. The subsites are numbered according to [18].

was found to be 20 times higher than by the corresponding *Hi* Cel7B enzyme, while in the meantime, the specific activity for the hydrolysis of cellotetraose, a natural substrate of both endoglucanases, was only three times higher (Table 3). These results suggested that the presence of the pair alanine/threonine at positions 209/211 (numbering of *Hi* Cel7B) in the *Tr* enzyme could favour the binding of the *N*-acetylglucosamine moiety in +1 subsite, and thus enhance the specific activity for the hydrolysis of the tetrasaccharide lactosyl-β(1 → 4)-chitinbiose.

This was in accordance with the results of the modelling study, and we therefore envisaged to prepare several mutants of these enzymes. For one part, we prepared the non-nucleophile mutant of the *Tr* enzyme, the Cel7B E196A glycosynthase. For another part, two single and one double mutants of the *Hi* Cel7B E197A glycosynthase were created: Cel7B E197A/H209A, Cel7B E197A/H209G and Cel7B E197A/H209A/A211T. The enzymes were produced and purified to apparent homogeneity, as judged by SDS/PAGE (Fig. 5).

3.3. Preparation and characterisation of a novel glycosynthase: T. reesei Cel7B E196A

The results obtained with the hydrolysis of the tetrasaccharide lactosyl-β(1 → 4)-chitinbiose by the native *Tr* Cel7B led us to prepare the non-nucleophile mutant of the enzyme, the Cel7B E196A glycosynthase.

Tr Cel7B E196A mutant enzyme was expressed in the yeast *S. cerevisiae* under the control of the strong yeast PGK promoter. The expression level of the *Tr* Cel7B E196A mutant was somewhat lower (five times, based on Western blot) than that of Cel7B wild-type enzyme (not shown), and in both cases the protein band was running higher in SDS/PAGE gel as compared to that of the purified *Tr* Cel7B wild-type enzyme produced in *Trichoderma* reesei (see Fig. 5, lane F). This is apparently due to hyperglycosylation (*N*-glycosylation) of *Tr* Cel7B in yeast, as shown earlier by Penttilä et al. [26]. The concentrated and buffer exchanged yeast culture filtrate from the *Tr* Cel7B E196A glycosynthase was used in the experiments described below. The culture supernatant from the yeast strain producing the *Tr* Cel7B E196A mutant enzyme showed very low glycoside hydrolase activity on 4-methylumbelliferyl-β-lactoside MULac (approximately 1% of the yeast expressed Cel7B wild-type activity). In order to verify if this contaminating activity could interfere with the synthesis experiment, we first incubated separately α-lactosyl fluoride and cellobiose (respectively the donor and acceptor of the synthesis experiment) with the *Tr* Cel7B E196A preparation. Some glycoside hydrolases are known to catalyze the hydrolysis of a glycosyl fluoride with the "wrong" anomeric configuration [40]. When α-lactosyl fluoride was incubated with the *Tr* glycosynthase preparation, it was hydrolysed into lactose, but at a rate comparable with its spontaneous hydrolysis in water (not shown), which demonstrated that *Tr* Cel7B E196A did not possess any activity on α-lactosyl fluoride. When cellobiose was incubated with the same enzyme preparation, a significant hydrolysis into glucose was observed (Fig. 6, lane D), which demonstrated that

Table 3
Specific activities for the hydrolysis of cellotetraose and tetrasaccharide lactosyl-β(1 → 4)-chitinbiose by *H. insolens* and *T. reesei* Cel7B wild-type enzymes

Enzyme	Cellotetraose	Tetrasaccharide (lactosyl-β(1 → 4)-chitinbiose)
Specific activity (μmol substrate hydrolysed/min/mg protein)		
Hi Cel7B wt	107	0.2
Tr Cel7B wt	329	4.1

Thirteen mM of cellotetraose was incubated with 22 nM *Hi* Cel7B or 35 nM *Tr* Cel7B. Thirteen millimolars of lactosyl-β(1 → 4)-chitinbiose was incubated with 4.5 μM *Hi* Cel7B or 0.67 μM *Tr* Cel7B. Initial reaction rates at 37 °C were obtained by measuring the amount of cellobiose or chitinbiose released at regular intervals using HPLC.

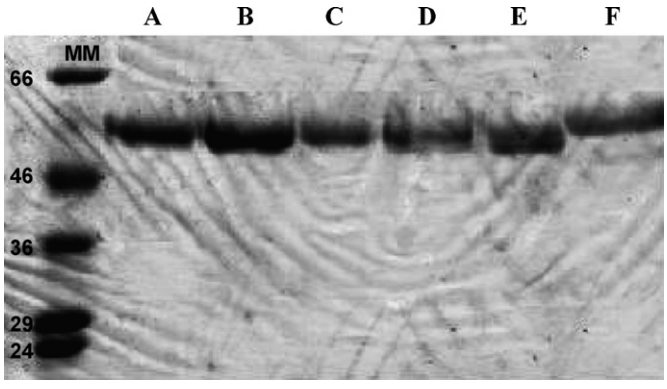


Fig. 5. SDS/PAGE of the purified wild-type and mutant forms of *H. insolens* Cel7B E197A glycosynthase and *H. insolens* and *T. reesei* wild-type Cel7B endoglucanases. *Hi* Cel7B E197A (A), *Hi* Cel7B E197A/H209A (B), *Hi* Cel7B E197A/H209G (C), *Hi* Cel7B E197A/H209A/A211T (D), *Hi* Cel7B (E) and *Tr* Cel7B (F) were subjected to SDS/PAGE together with Sigma low molecular-weight standards (lane MM), using a 10% polyacrylamide gel. Proteins were stained with Coomassie Blue reagent.

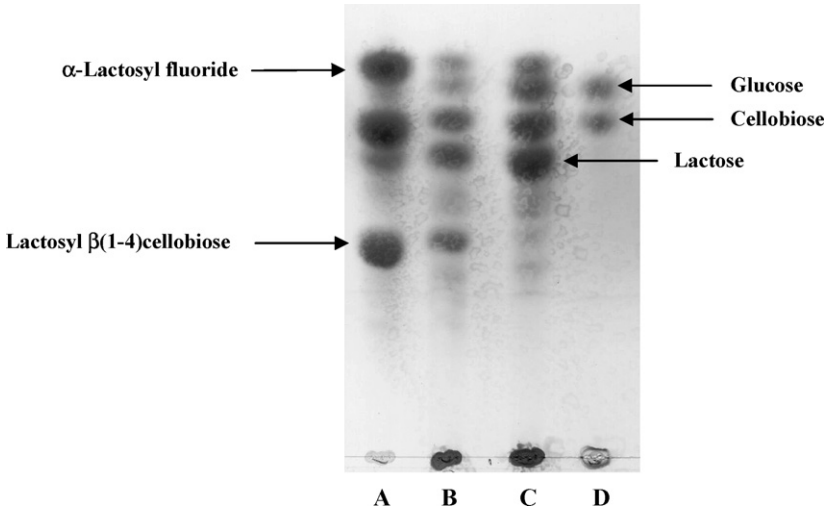


Fig. 6. Thin layer chromatography of cellobiose glycosylation by *H. insolens* and *T. reesei* Cel7B glycosynthase. One hundred and forty five millimolars of α-lactosyl fluoride and 145 mM cellobiose were incubated with 52 μg of *Hi* Cel7B E197A (A) or 500 μg of *Tr* Cel7B E196A (B) in 100 μL of 50 mM phosphate buffer pH 7 or 25 mM sodium acetate buffer pH 4.6, respectively, at 37 °C during 16 h. *Hi* wild-type Cel7B treatment of the reaction mixture from lane B (C); 145 mM cellobiose was incubated with 52 μg of *Tr* Cel7B E196A (D).

the contaminating activity is actually due to a β-glucosidase or cellobiase.

The activity of the *Tr* Cel7B E196A glycosynthase produced in yeast was first assayed using α-lactosyl fluoride and cellobiose as donor and acceptor respectively, two substrates known to be used by *Hi* Cel7B E197A glycosynthase. Mass spectrometry ($m/z = 689$ $[M + Na]^+$) and thin-layer chromatography (Fig. 6, lane B) of the reaction mixture after 16 h at 37 °C showed that a tetrasaccharide was produced, by comparison with the reaction profile of the same reaction catalyzed by the well known *Hi* Cel7B E197A glycosynthase (Fig. 6, lane A). A small amount of glucose was also produced, probably due to the β-glucosidase or cellobiase activity described above. The tetrasaccharide produced is in a 1:1 ratio with cellobiose in both reactions, but it must be noted that the amount of *Tr* glycosynthase used was 10 times higher than that of *Hi* glycosynthase. These last observations suggest that the *Tr* glycosynthase is less efficient than the *Hi* enzyme for the condensation of α-lactosyl fluoride on cellobiose. The difference may also be explained, in part, by the contaminating activity of the *Tr* Cel7B E196A which consumes part of the acceptor cellobiose and by the spontaneous hydrolysis of α-lactosyl fluoride which is more pronounced at the pH optimum of *Tr* glycosynthase (4.6) than at pH 7. In order to avoid this phenomenon, we tried to use *Tr* Cel7B E196A at pH 7, but no noticeable activity could be detected.

In order to demonstrate the stereo- and regioselectivity of the glycosidic linkage formed by *Tr* glycosynthase between lactose and cellobiose, the reaction mixture was treated with the *Hi* wild-type Cel7B. As shown, in Fig. 6, lane C, the enzymatic digestion led to the disappearance of the tetrasaccharide and only lactose and cellobiose were produced, demonstrating that a β(1 → 4) linkage was formed during the condensation reaction.

Taken together, these results clearly demonstrated that *Tr* Cel7B E196A could act as a glycosynthase and produce the tetrasaccharide lactosyl-β(1 → 4)-cellobiose from α-lactosyl fluoride and cellobiose as donor and acceptor respectively.

3.4. Cellobiose glycosylation by the different H. insolens glycosynthase mutants

In order to determine the effect of the mutations introduced on *Hi* Cel7B E197A glycosynthase, we decided to study the cellobiose glycosylation catalysed by the single and double mutants generated. Qualitative analysis of reactions by thin-layer chromatography first showed without ambiguity that all three *Hi* glycosynthase mutants are able to condense α-lactosyl fluoride on cellobiose by generating a β(1 → 4) glycosidic linkage between donor and acceptor (not shown). Even if the glycosynthase activity is still present in all the mutants, it appeared to be strongly diminished when H209 was mutated into an alanine or a glycine (Fig. 7).

Since the specific activity decreased more dramatically with the glycine mutation (H209G) than with the alanine mutation (H209A), this suggested that the absence of a lateral chain could cause a modification of the protein structure and hence lead to a wrong positioning of essential amino acids in the active site of the enzyme. However, when an additional mutation was inserted at position 211 (double mutant H209A/A211T), the glycosynthase activity was restored and approached the native one.

3.5. Substrate specificity of H. insolens mutant glycosynthases and T. reesei glycosynthase

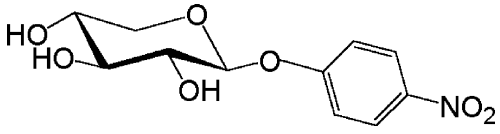
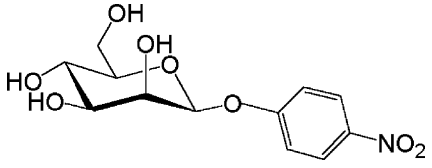
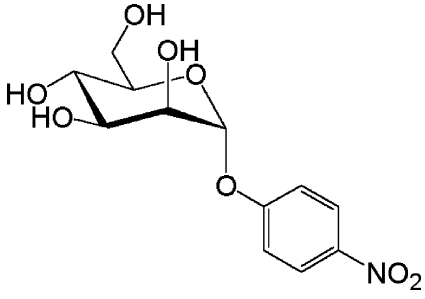
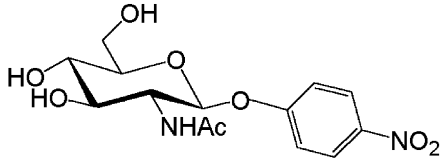
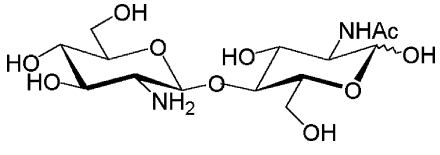
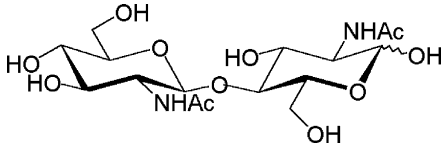
After checking that *Tr* Cel7B E196A and *Hi* Cel7B E197A mutants actually behaved as glycosynthases, we wanted to investigate the substrate specificity of these enzymes. All reaction mixtures were subjected to ESI mass spectrometry to check for the formation of a tri- or tetrasaccharide, depending on the acceptor used. To ensure that only β(1 → 4) linkages were formed during the reaction catalyzed by the glycosynthases, all new oligosaccharides were subjected to enzymatic hydrolysis by the wild-type enzyme *Hi* Cel7B and their hydrolysis products

were characterized by thin-layer chromatography using authentic samples.

We first examined the behaviour of *Hi* glycosynthase mutants on substrates already known to be acceptors in condensation reactions catalysed by the native glycosynthase, using α-lactosyl fluoride as donor (Table 4). The 4-nitrophenyl β-D-xyloside and 4-nitrophenyl β-D-mannoside (entries 1 and 2) were still glycosylated in condensation reactions catalysed by the single and double mutants, confirming that the hydroxyl groups in position 2 and 6 were not implicated in essential interactions between the substrate and the glycosynthase. The same observation was done when N^{I}-acetylchitobiose (entry 5) was used.

We secondly focused on substrates, which were not recognised as acceptors by *Hi* Cel7B E197A glycosynthase. The 4-nitrophenyl α-D-mannopyranoside (entry 3) was still not recognised as an acceptor, confirming that the glycosynthase is spe-

Table 4
The different substrates tested as potential acceptors in condensation reactions catalysed by *H. insolens* glycosynthase mutants using α-lactosyl fluoride as donor

Entry	Acceptor	Cel7B mutants tested	Activity detection	Stereo- and regio-selectivity of the glycosidic linkage formed
1		E197A	+[a]	β(1 → 4)
		E197A H209A	+	β(1 → 4)
2		E197A	+	β(1 → 4)
		E197A H209A	+	β(1 → 4)
3		E197A	n.d.[b]	
		E197A H209A	n.d.	
4		E197A	n.d.	
		E197A H209A	n.d.	
		E197A H209G	n.d.	
5		E197A	+	β(1 → 4)
		E197A H209A	+	β(1 → 4)
		E197A H209G	+	β(1 → 4)
		E197A H209A A211T	+	β(1 → 4)
6		E197A	n.d.	
		E197A H209A	n.d.	
		E197A H209G	n.d.	
		E197A H209A A211T	n.d.	

[a] +: activity detected.
[b] n.d.: activity not detected under specified experimental conditions.

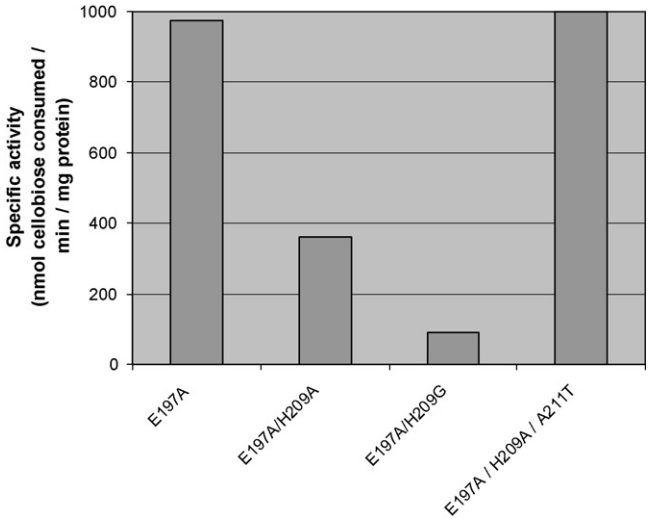


Fig. 7. Specific activities of the different *H. insolens* glycosynthase mutants for cellobiose glycosylation. Thirteen millimolars of α-lactosyl fluoride and 13 mM cellobiose were incubated at 37 °C in 50 mM phosphate buffer pH 7 with 28 μg Cel7B E197A or 180 μg Cel7B E197A/H209A or 150 μg Cel7B E197A/H209G or 180 μg Cel7B E197A/H209A/A211T.

cific for β-D-glycosides. Similarly, when 4-nitrophenyl β-D-*N*-acetylglucosaminide (entry 4) and chitinbiose (entry 6) were used as acceptors, no condensation reaction was detected, with any of the *Hi* glycosynthase mutants. Because it was hypothesised [41] and verified [42] that the presence of the histidine at position 209 adjacent to the acid/base catalyst contributes to the higher pH optimum in *Hi* Cel7B, the glycosylation of 4-nitrophenyl β-D-*N*-acetylglucosaminide and chitinbiose was also assayed at pH 5 (not shown). However, the same results were obtained. In addition, no condensation reaction could be detected with *Tr* Cel7B E196A glycosynthase, which was only tested for its ability to glycosylate chitinbiose (not shown).

These results suggested thus that neither the *Hi* glycosynthase mutants nor the *Tr* glycosynthase are able to accommodate an *N*-acetylglucosaminyl residue in their +1 subsite. However, these observations are not in accordance with the hydrolytic activity observed when the native *Tr* Cel7B endoglucanase was incubated with the tetrasaccharide described above, which suggested that *Tr* glycosynthase and possibly also *Hi* Cel7B E197A/H209A/A211T would be able to accommodate chitinbiose in their acceptor subsites. When chitinbiose is linked to a lactosyl unit as in the tetrasaccharide hydrolysed by *Tr* Cel7B, the *N*-acetyl-D-glucosaminyl moiety is accepted in the +1 subsite. However, the specific activity for the hydrolysis of the tetrasaccharide lactosyl-β(1 → 4)-chitinbiose by *Tr* Cel7B is 80 times lower than that for the hydrolysis of cellotetraose, and more than 500 times lower when *Hi* Cel7B is used for the hydrolysis (Table 3). These latter observations suggest that the tetrasaccharide lactosyl-β(1 → 4)-chitinbiose is a poor substrate for both endoglucanases and one could imagine that the affinity between the +1 subsite of the enzymes and the *N*-acetyl-D-glucosaminyl moiety is not strong enough to permit the positioning of the acceptor alone in the active site of the glycosynthases. The plasticity of the native and E196A *Tr* Cel7B active sites to simultaneously accommodate a galactosyl moiety at −2 subsite and a *N*-acetylglucosaminyl moiety at +1 subsite shows its limits. These limitations are extended for *Hi* Cel7B glycosynthase and its mutants.

4. Concluding remarks

Simple replacement of the catalytic nucleophile of a retaining glycosidase showing a high transglycosylating activity does not always lead to a glycosynthase [17]. In this study, we described the production of a new glycosynthase, *Tr* Cel7B E196A, able to glycosylate cellobiose using α-lactosyl fluoride as donor. However, in spite of the capability of the wild-type *Tr* Cel7B to hydrolyse the tetrasaccharide lactosyl-β(1 → 4)-chitinbiose, the corresponding glycosynthase did not prove to catalyse the reverse reaction between α-lactosyl fluoride and chitinbiose. The same observation was found for the three rationally designed mutants of *Hi* Cel7B E197A glycosynthase. 3D X-ray structural studies may be envisaged to help us understanding why no change in substrate specificity for *N*-acetyl-D-glucosamine as acceptor was observed with the different mutated glycosynthases.

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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.2006.08.009.

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