Mechanism and Engineering of Bacterial 1,3-1,4-β-Glucanases: From Glucan Hydrolase to Glycosynthases in Enzymatic Oligosaccharide Synthesis

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Abstract: Bacillus 1,3-1,4-β-glucanases are retaining endo-glycosidases of family 16 GH. The hydrolytic mechanism of the Bacillus licheniformis enzyme follows a double-displacement reaction involving a catalytic triad with Glu134 as enzyme nucleophile, Glu138 as general acid-base, and a third residue, Asp136, that assists in catalysis. It is shown that the carboxylate side chain of Asp136 participates in both glycosylation and deglycosylation steps contributing to the pKa modulation of the general acid-base during the enzyme cycle. Upon mutation of the catalytic nucleophile to alanine, the B. licheniformis 1,3-1,4-β-glucanase is a highly efficient endo-glycosynthase, with strict specificity for formation of β-1,4 glycosidic bonds. It is shown that Glu 138 acts as a general base in the transglycosylation mechanism. Strategies to control donor-acceptor condensation, donor self-condensation, elongation and polymerization reactions have been devised, and examples are here shown for their application in oligosaccharide assembly or polysaccharide synthesis.

Key words: glucanase, mechanism, glycosynthase

1,3-1,4-β-Glucanases (or lichenases, EC 3.2.1.73) are retaining endo-glycosidases that catalyze the depolymerization of mixed-linked 1,3-1,4-β-glucans such as lichens and cereal β-glucans. The plant enzymes are responsible for the break down of the endosperm cell wall of cereals during germination. Not only plants produce lichenases, but also a number of microorganisms (bacteria and fungi) secrete 1,3-1,4-β-glucanases, involved in the degradation of polysaccharides that can be present in their natural environment and be used as an energy source. The plant and microbial enzymes have neither amino acid sequence similarity nor related three-dimensional structure, being an example of convergent evolution towards the same substrate specificity. Whereas the plant enzymes belong to family 17 of glycosyl hydrolases with an (R/a)8 barrel three-dimensional structure, the bacterial enzymes are members of family 16 with a jellyroll β-sandwich structure.

1,3-1,4-β-glucanases exhibit a strict substrate specificity for cleavage of β-1,4 glycosidic bonds in 3-O-substituted glucopyranose units. The main final hydrolysis products from barley β-glucan are the trisaccharide 3-O-β-cellobiosyl-D-glucopyranose and the tetrasaccharide 3-O-β-cellobiosyl-D-glucopyranose.

Bacillus 1,3-1,4-β-glucanases are the prototype of family 16 GH which have been analyzed in our group for several years, and for which a number of mechanistic, protein engineering and structural studies have unraveled many details of their catalytic action and specificity. The enzyme has an open binding side cleft composed of six subsites (−4 to +2), with subsite −3 having the larger contribution to transition state stabilization. Protein-carbohydrate interaction defining substrate specificity have been dissected through a detailed mutational analysis of all residues that may interact with an hexasaccharide substrate spanning the entire binding site cleft on the basis of a modeled E•S complex.

As retaining glycosidases, the mechanism of bacterial 1,3-1,4-β-glucanases involves a double-displacement reaction assisted by general acid-base catalysis (Scheme 1). The catalytic residues have been identified by mutational analysis, and X-ray crystallography. For the B. licheniformis enzyme, Glu138 is the general acid-base residue, and Glu134 the catalytic nucleophile, their functional role being assessed by a chemical rescue methodology of inactive alanine mutants on each essential residue. Azide as exogenous nucleophile reactivated the mutants in a concentration-dependent manner using an activated 2,4-dinitrophenyl glycoside substrate. The E138A mutant yields the β-glycosyl azide product arising from nucleophilic attack of azide to the glycosyl-enzyme intermediate, thus proving that Glu138 is the general acid-base residue. By contrast, azide reactivates the E134A mutant through a single inverting displacement to give the α-glycosyl azide product, consistent with Glu134 being the catalytic nucleophile.

In the framework of our structure/function studies and protein engineering of Bacillus 1,3-1,4-β-glucanases, we here report some new features of the enzyme active site and their role in catalysis as well as the engineering of the enzyme hydrolase activity to a synthase activity (glycosynthase) summarizing specificity, mechanism and synthetic applications of endo-glycosynthases.
RESULTS AND DISCUSSION

1) Mechanism: Asp136 as the third residue in a catalytic triad.

Environment of the catalytic residues involves various auxiliary amino acid residues proposed to modulate their pKa and to stabilize their conformation during the catalytic cycle. In particular we address here the role of Asp136 as part of a catalytic triad with E134 (nucleophile) and E138 (general acid-base) proposing the function of pKa modulator.

The X-ray structure of the wild-type B. licheniformis and Bacillus macerans enzymes\(^1,13\) show a third carboxylate residue (Asp136 in the B. licheniformis numbering) in the active site that hydrogen-bonds with the catalytic nucleophile G1u134, which, together with Trp132, positions the nucleophile side chain in its proper orientation for catalysis. This Asp residue is strictly conserved in clan-B of glycosyl hydrolases (families 16 and 7).

Mutation D136A removes the carboxylate side chain, and the mutant enzyme reduces \(k_{cat}\) 60- and 2500-fold (for G4G3G-MU and G4G3G-2,4DNP substrates, respectively) relative to the wild-type enzyme but maintains the same \(K_M\) values (Table 1). It indicates that Asp136 contributes to catalysis providing transition state stabilization but it does not have a particular role in substrate binding. These effects are consistent with the previous results on kinetics of the D136N mutant with barley \(\alpha\)-glucan as substrate, where \(k_{cat}\) was 0.5% of that of wild type and \(K_M\) did not change.\(^10\)

The substrates G4G3G-MU and G4G3G-2,4DNP share the same glyconic part and differ in the aglycon leaving group. A Hammett analysis on the wild-type enzyme using a series of substituted aryl glycosides with different phenol-leaving group abilities revealed that the glycosylation step is rate-determining in the double displacement mechanism of this retaining glycosidase, and that, for substrates with a pKa of the aglycon lower that 7.5 (which includes both substrates used here), the Hammett \(\beta_{lg}\) value of \(-0.8\) reflects a large degree of negative charge accumulation on the phenolate oxygen, indicative of a late transition state with extensive C-O bond breaking and relatively little protonation.\(^14\) The different reduction of \(k_{cat}\) values in the D136A mutant with the two substrates is significant to the leaving group abilities of the aglycon in the mutant relative to the wild-type enzyme. This reflects a reduced sensitivity to the leaving group abilities of the aglycon in the mutant relative to the wild-type enzyme. This reflects a reduced sensitivity to the leaving group abilities of the aglycon in the mutant relative to the wild-type enzyme.

Removal of the carboxylate side chain of Asp136 does not significantly modify the kinetic pKa value on the acidic limb in the pH dependence of \(k_{cat}/K_M\) (5.4 for wt, 5.6 for D136A, Fig. 1), but induces a large effect on the pKa on the basic limb (7.0 for the wt, 8.6 for D136A). D136 is hydrogen bonding to the nucleophile in the free (uncomplexed) enzyme.\(^10\) This hydrogen bond is lost in the mutant but it apparently does not modify the pKa of the nucleophile. The major effect of the mutation is exerted on the general acid/base residue. The kinetic pKa on the basic limb (7.0 for the wt enzyme) was assigned to E138 on the basis of kinetics of pH dependence of enzyme inactivation by a water-soluble carbodiimide.\(^6\) This high pKa value for a glutamate residue in the active site is attributed to the hydrophobic environment of the carboxylic acid side chain and to the electrostatic destabilization of its conjugate base by the close negative charge of the catalytic nucleophile. The increase of the pKa of the general acid by 1.6 pH units upon mutation of Asp136 can be interpreted as the result of increasing this electrostatic destabilization of the conjugate base of the general acid due to deshielding the negative charge on the nucleophile as a consequence of losing the hydrogen bond Glu134...
Asp136. The same trends on pKₐ shifts are observed on the kcat vs. pH profile.

The crystal structure of a covalent enzyme-inhibitor complex showed that the H-bond between Asp136 and the nucleophile Glu134 observed in the free enzyme is disrupted in the complex. Instead, the carboxylate side chain of Asp136 moves away from the nucleophile and it is at a hydrogen bonding distance of the carboxylic acid of the general acid Glu138. If this geometric arrangement is also obtained in the glycosyl-enzyme intermediate of the hydrolytic reaction, the newly established H-bond between Glu138 and Asp136 may contribute to lowering the pKₐ of the general acid residue to act as a general base in the deglycosylation step. This pKₐ shift during the normal enzyme cycle of retaining glycosidases has been demonstrated on the B. circulans xylanase, and it was mainly attributed to the formation of the covalent bond between the substrate and the nucleophile residue which eliminates the net negative charge on the nucleophile thus reducing the electrostatic destabilization of the conjugate base of the general acid with the effect of lowering its pKₐ in the intermediate. In the case here discussed, in addition to this phenomena, Asp136 may also contribute to lower the pKₐ of Glu138 through H-bonding to the carboxylate side chain in the glycosyl-enzyme intermediate.

Following this rationale, Glu138 would become a worst general base in the D136A mutant when the glycosyl-enzyme is reached, and hydrolysis of the covalent intermediate will have less general base assistance with the concomitant reduction of the rate of the deglycosylation step. While glycosylation has been shown to be rate determining in the wild-type enzyme with aryl glycoside substrates, the deglycosylation step may become partially rate-determining in the D136A mutant. This is in agreement with the reduction in the β₁g value for the mutant which means that the reaction is less sensitive to the leaving group ability of the aglycon, effect that would be expected if deglycosylation becomes partially rate determining.

2) Engineering the mechanism from hydrolase to glycosynthase.

Retaining glycosidases have been extensively used in enzymatic oligosaccharide synthesis by reversal of their normal hydrolytic activity either by displacing the equilibrium towards glycosidic bond formation (thermodynamically controlled synthesis) or by using activated glycosyl donors (kinetically controlled transglycosylation). However, the major drawback of wild-type retaining glycosidases in glycoside synthesis is hydrolysis of the newly formed glycosidic linkage, and condensation or transglycosylation yields rarely exceed 50%.

A novel strategy based on the redesign of the enzyme’s catalytic machinery is currently being developed. The glycosynthase concept was introduced in 1998 by the Withers’ group on an exo-glycosidase and extended to endo-glycosidases by our group. A glycosynthase is a specifically mutated retaining glycosidase in which site-directed mutation of the catalytic nucleophile by a non-catalytic residue (Ala, Gly or Ser) renders a hydrolytically inactive enzyme, yet able to catalyze the transglycosylation of glycosyl fluoride donors having the opposite anomeric configuration of that of the normal substrates of the parental wild-type enzyme. The transglycosylation products formed are not hydrolyzed, and production yields are as high as 95–98% in some cases. Some other few glycosynthases have been reported up to date, derived from β-glycosidases, and just recently the first example of one derived from a α-glycosidase.

The rationale of the methodology is depicted in Scheme 2. In the kinetically-controlled transglycosylation by the wild-type enzyme, the covalent glycosyl-enzyme intermediate is the actual donor for glycosyl transfer to an acceptor or hydrolysis by transfer to water. Even though the hydroxyl group of the sugar acceptor is a better nucleophile than water and transglycosylation is kinetically favored, this step is reversible and the process is shifted towards hydrolysis. In the glycosynthase (Scheme 2b), mutation of the catalytic nucleophile disables the enzyme as a hydrolase because no glycosyl-enzyme intermediate can be formed; the same glycosyl donors that would be used with the wild-type enzyme (i.e., aryl β-glycosides or α-glycosyl fluorides for a β-glycosidase) will not react with the glycosynthase. But an activated glycosyl donor with an anomeric configuration opposite to that of the donor substrate in the wild-type reaction (i.e., an α-glycosyl fluoride for a β-glycosidase) would mimic the glycosyl-enzyme intermediate and then be able to react with an acceptor. The cavity created in the active site by mutation of the carboxylate residue acting as nucleophile in the wild-type enzyme by a smaller residue allows binding of the glycosyl fluoride with opposed anomeric configuration. As with the wild-type enzyme, transglycosylation is kinetically favored but the transglycosylation reaction is now irreversible because of the lack of the catalytic nucleophile; the product is no longer hydrolyzed and accumulates to give high transglycosylation yields.

Our first insight into the glycosynthase mechanism arose from the chemical rescue experiments of inactive mutants at the catalytic residues by the action of exogenous nucleophiles. As presented in the Introduction, the E134A mutant of B. licheniformis 1,3-1,4-β-glucanase (nucleophile-less mutant) is hydrolytically inactive (kcat 10²-fold lower than that of the wild-type enzyme), but the
enzyme activity was rescued by azide as a donor leading to the α-glycosyl azide product (an inverted reaction). When formate, which resembles more closely the structure of the excised glutamate side chain in the E134A mutant, was added as exogenous nucleophile to the reaction with an activated aryl β-glycoside, it restored the hydrolytic activity of the enzyme leading to the normal hydrolysis product (β-anomer), thus resulting in an overall retention reaction. But remarkably, a transient intermediate with a half-life of more than 2-hours was detected and identified by 1H-NMR and MS as being an α-glycosyl formate (Scheme 3). This transient intermediate mimics the covalent glycosyl-enzyme intermediate in the reaction mechanism of a retaining glycosidase. When a glycosyl acceptor was added to a pre-incubated mixture of the E134A mutant, 2,4-dinitrophenyl-α-glycoside, and sodium formate, transglycosylation products of different degree of polymerization were readily formed. Product yields, however, were rather low due to the competing hydrolysis (spontaneous and enzymatic) of the α-glycosyl formate adduct. Detection of the intermediate and its ability to transglycosylate informed us of two concepts:

a) an α-glycoside is able to bind into the active site of the nucleophile-less E134A mutant: an α-substituent as large as a formyl group can be accommodated into the cavity left by removal of the carboxyl side chain in the E134A mutant,  

b) a mimic of the glycosyl-enzyme intermediate is able to act as a glycosyl donor.

The E134A mutant 1,3-1,4-β-glucanase was shown to be an efficient glycosynthase catalyzing the condensation of α-laminaribiosyl fluoride with glucosides, cello- and laminaribiosides in excellent yields. The new glycosidic bond formed is exclusively β-1,4 in all cases, illustrating the higher regioselectivity of the endo-acting enzymes as compared to the exo-acting glycosynthases so far reported.

### Mechanism

It is commonly assumed that the mechanism of the glycosynthase reaction proceeds with general base catalysis, and that the same residue that acted as general acid-base in the hydrolase mechanism of the wild-type enzyme, now plays the role of general base catalyst increasing the nucleophilicity of the glycosyl acceptor (Scheme 2b). We provide here experimental proofs of this mechanism by analyzing the pH dependence of the glycosynthase reaction, and the properties of the double mutant E134A/E138A (where E138 was the general acid-base in the wild-type hydrolase reaction).

The pH dependence of kcat/Km(donor) for the glycosynthase reaction of E134A is presented in Fig. 2. At acidic pH (< 5) the enzyme is inactive and activity increases at higher pH values up to a maximum at pH 7-7.2. It corresponds to a general base catalysis with a kinetic pKa of 5.9. According to the proposed mechanism in Scheme 2b, the carboxylate of Glu138 is the candidate to act as a base, and the pKa of 5.9 will reflect its ionization. In the wild-type hydrolase mechanism, Glu138 has a pKa of 7.0 as a general acid. The 1.1 pH-units drop in the E134A mutant and shape of the pH profile as general base is attributed to the lack of the negatively charged nucleophile in the mutant, as already argued for the pKa cycling of the general acid-base residue in the wild type en-

### Specificity

The donor and acceptor specificity and enzyme kinetics have been analyzed for this glycosynthase. For a series of donors with increasing degree of polymerization ([Glcβ1,4]nGlcβ1,3GlcεF, n=0–2), reaction rates are higher for the tetrasaccharide than for the disaccharide donor, as expected for an endo-glycosynthase, and in agreement with subsite mapping analysis of the wild-type enzyme. The acceptor specificity was analyzed with mono- and disaccharides. Methyl β-glucoside is a poor acceptor whereas 4-methylumbelliferyl β-glucoside reacts readily. Therefore subsite +1 has low affinity and the acceptor must occupy subsite +II, as also seen from the higher rates with disaccharides. α-Glucosides, sucrose and maltose do not react at all, indicating that an α-linkage is not accepted. As expected, galactosides and 2-acetamide-2-deoxy glucosides are not glycosylated on disaccharides, cellobiosides are better acceptors than laminaribiosides, probably as a consequence of a competition of the laminaribioside acceptor with the α-donor for the same subsites −II/−I, rather than higher affinity of the cellobiosides for subsites +1/+II; subsite mapping analysis on the wild-type enzyme has shown that subsites +1/+II can accommodate both laminaribiosyl and cellobiosyl units with similar affinities.
zyme when the covalent glycosyl-enzyme intermediate is formed (the same residue acting as general acid in the glycosylation step then becomes a base in the deglycosylation step). At higher pH values, a pH-independent glycosynthase activity of the E134A mutant would be expected (a plateau in the $k_{cat}/K_M$ vs. pH profile) since the general base remains in its active ionization state. However, a decrease in activity following a ionization of $pK_a$ 7.9 down to a residual 70% activity is observed. It indicates that the ionization of another residue, although not essential, affects the catalytic efficiency. We tentatively propose it to be Asp136, the third residue of the catalytic triad in the wild-type enzyme: in the glycosynthase-substrates complex, Asp136 may be close to Glu138 to stabilize the ionized carboxylate to behave as a general base, as proposed in the deglycosylation step of the wild-type hydrolytic reaction (see above). Ionization of Asp136 at high pH may render a less than optimal ionization/charge distribution of the active site that is reflected in a lower, but still important, glycosynthase efficiency.

To assign Glu138 as the actual general base in the glycosynthase mechanism, the double mutant E134A/E138A was prepared. First, its residual hydrolase activity was analyzed using a highly reactive 2,4-dinitrophenyl $\beta$-glycoside substrate. The double mutant is inactive as a glycosidase with a residual $k_{cat}$ value of the same order of magnitude than that obtained for the single E134A mutant. Likewise, activity was rescued upon addition of azide as exogenous nucleophile, the product being the $\alpha$-glycosyl azide as in the case of the chemical rescue of the single E134A mutant. It demonstrates that the active site topology in the E134A/E138A mutant is maintained, and that cleavage of the $\beta$-glycosidic bond of the activated 2,4-dinitrophenyl glycoside does not require protons assistance by a general acid in the enzyme.

The E134A/E138A double mutant has no glycosynthase activity with $\alpha$-laminaribiosyl fluoride as donor and 4-methylumbelliferyl $\beta$-cellobioside as acceptor. Glu138 is therefore an essential residue for the condensation reaction. Addition of azide to the reaction mixture of the E134A/E138A enzyme and $\alpha$-laminaribiosyl fluoride restores enzyme activity, and $\beta$-laminaribiosyl azide is obtained. The $\alpha$-fluoride donor reacts with azide in the absence of enzyme, but the reaction is one to two orders of magnitude faster in the presence of the E134A/E138A mutant, the rate of $\beta$-laminaribiosyl azide formation being enzyme-concentration dependent. These results support the role of Glu138 as the actual general base in the glycosynthase mechanism: removal of the carboxylate side chain in the double mutant eliminates the general base assistance to increase the nucleophilicity of the hydroxyl group of the saccharide acceptor, and no condensation product is formed. But a stronger nucleophile that does not require general base assistance such as azide, is able to react with the $\alpha$-donor leading to a product with the same anomeric configuration as that produced in the normal glycosynthase reaction.

**Competing reactions.**

As observed with most of the current glycosynthases, the donor-acceptor condensation reaction is often accom-panied by other side reactions: the glycosyl fluoride donor and the transglycosylation product may also act as acceptors provided that the configuration of the condensable hydroxyl group on the non-reducing end of the donor has the same stereochemistry as the normal acceptor, then leading to self-condensation of the donor or elongation of the transglycosylation product, respectively. It is therefore important to modulate these different reactions for an efficient use of the glycosynthase methodology in preparative synthesis of target oligosaccharides.

Different strategies to control these undesired reactions have been designed and here tested with the E134A glycosynthase, as they had also been successfully used with another endo-glycosynthase, the E197A mutant of cellulase Cel7B from *Humicola insolens*:

(a) Addition of an excess of acceptor to decrease the probability of the donor to act as an acceptor. Simple addition of a large excess of the acceptor reduces self-condensation, polymerization and elongation reactions, but the success of this approach highly depends on the acceptor molecule and the relative binding affinity of donor and acceptor for the same acceptor site. Moreover, it may be inappropriate when the acceptor is an expensive or elaborated molecule, and a chromatographic step is required to remove it from the reaction product.

(b) Selection of a donor with a different configuration of the hydroxyl group that normally acts as acceptor. A trisaccharide analogue containing a galactosyl unit on the non-reducing end proved to be a good substrate of *W. licheniformis* 1,3-1,4- $\beta$-glucanase, showing that subsite $-3$ in the binding side cleft of the enzyme can accommodate a glucosyl or a galactosyl residue with almost the same affinity. Using $\alpha$-galactosyl-$\beta$-1,4-laminaribiosyl fluoride (Gal$\beta$1, 4Glc$\beta$1, 3Glc$\alpha$F) as donor in the E134 glycosynthase reaction, single condensation products with mono- and disaccharide acceptors are obtained, whereas the gluco-trisaccharide analogue (Glc$\alpha$1, 4Glc$\beta$1, 3Glc$\alpha$F) produces an insoluble polysaccharide in the absence of acceptor, or a mixture of condensation and elongation products when an acceptor is present in a 1 to 1 molar donor/acceptor ratio.

(c) Use of a temporary protecting group on the polymerizable hydroxyl group of the donor. As shown for the E197A cellulase Cel7B from *H. insolens*, a 4$\alpha$-tetrahydropyranyl(THP)-protected $\alpha$-cellobiosyl fluoride is a good donor that is unable to self-condense, and the protecting group has been applied to the E134A 1,3-1,4-$\beta$-glucanase, showing that subsite $-3$ of the hydroxyl group on the non-reducing end of the donor has a good acceptor for the same acceptor site. Moreover, it may be inappropriate when the acceptor is an expensive or elaborated molecule and the relative binding affinity of donor and acceptor for the same acceptor site. In this case, the galactosyl unit in the non-reducing end of the donor prevents self-condensation and elongation reactions (‘capped-donor’).

Use of a temporary protecting group on the polymerizable hydroxyl group of the donor. As shown for the E197A cellulase Cel7B from *H. insolens*, a 4$\alpha$-tetrahydropyranyl(THP)-protected $\alpha$-cellobiosyl fluoride is a good donor that is unable to self-condense, and the protecting group has been applied to the E134A 1,3-1,4-$\beta$-glucanase, showing that subsite $-3$ of the hydroxyl group on the non-reducing end of the donor has a good acceptor for the same acceptor site. Moreover, it may be inappropriate when the acceptor is an expensive or elaborated molecule.

**Synthetic applications.**

Whereas *exo*-glycosynthases are useful as synthetic tools for the synthesis of disaccharides or for addition of a monosaccharyl unit on the non-reducing end of...
an oligosaccharide, *endo*-glycosynthases are better suit for the assembly of oligosaccharides. Two different synthetic applications for *endo*-glycosynthases are here proposed and illustrated with two examples: (a) oligosaccharide assembly, where the glycosynthase catalyzes the coupling of two already elaborated oligosaccharides, and where the donor-acceptor condensation is the desired reaction thus requiring strategies to prevent donor self-condensation and elongation reactions; (b) polysaccharide synthesis by letting the glycosynthase to catalyze the self-polymerization of the donor in the absence of acceptor.

(a) Oligosaccharide assembly. The potential of glycosynthases in the enzymatic synthesis of complex oligosaccharide is nicely illustrated by the synthesis of mixed-linked hexasaccharide substrates of 1,3-1,4-β-glucanases by coupling two *endo*-glycosynthases of different specificity in a ‘one-pot’ process. Because the E134A 1,3-1,4-β-glucanase and the E197A cellulase Cc17B show different specificities, towards laminaribiosyl and cellobiosyl donors, respectively, we have prepared the target hexasaccharides shown in Scheme 4 by condensation of the corresponding disaccharide building blocks through sequential addition of the glycosynthases. Both the ‘capped-donor’ and ‘temporary protecting group’ strategies have been used to prevent self-condensation and elongation reactions, and the hexasaccharide products were obtained in 80% overall yields. This stereoselective, rapid, and high-yielding multi-step coupling attests to the power of glycosynthases as synthetic tools for oligosaccharide assembly.

(b) Polysaccharide synthesis. Self-condensation of a-laminaribiosyl fluoride by the E134A glycosynthase produces a polysaccharide that precipitates from the solution. Preliminary structural characterization shows that the product obtained is an alternating β-1,3 and β-1,4 gluco-oligomer ([G3G]ₙ) with an average degree of polymerization of 12 glucosyl units. Electron microscopy and X-ray diffraction data revealed that it has a spherulite morphology with an elementary structure of platelet of 2–3 nm thickness, and the polysaccharide in an extended conformation. As opposed to β-1,3 gluco-oligomers, which adopt an helical winding conformation (obtained by an equivalent glycosynthase polycondensation catalyzed by a mutant *endo*-1,3-β-glucanase from barley), the mixed-linked polysaccharide has a structure close to cellulose-I type (Imai, Fajjes, Bulone and Planas, manuscript in preparation). Other a-glycosyl fluoride donors ([G₄]ₙ,G₃GaF, n =1,2) also undergo fast glycosynthase-catalyzed polymerizations. Further work is undertaken to fully characterize these polysaccharides that may have interesting properties and applications in material sciences.

CONCLUSIONS

The mechanism of family 16 retaining 1,3-1,4-β-glucanases involves a catalytic triad with Glu134 as enzyme nucleophile and Glu138 as general acid-base catalyst, and a third residue, Asp136, that assists in catalysis. The carboxylate side chain of Asp136 rotates through the enzyme cycle, interacting with the nucleophile in the free enzyme and contributing to positioning the nucleophile in the glycosylation step, and then moving towards the general acid-base residue in the glycosyl-enzyme intermediate and contributing to its pKa downshift to behave as a general base in the deglycosylation step.

A step forward in the use of retaining glycosidases in enzymatic oligosaccharide synthesis has been achieved by introducing the ‘glycosynthase’ concept, where engineered retaining glycosidases are efficient transglycosylases with no hydrolase activity. The E134A mutant 1,3-1,4-β-glucanase is an efficient *endo*-glycosynthase where donor-acceptor condensation, donor self-condensation and polymerization reactions can be controlled for preparative synthetic application in oligosaccharide assembly or polysaccharide synthesis. This novel methodology, still applied to a reduced number of enzymes, deserves much attention as new enzymes and synthetic applications are to be developed.

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