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Glycosynthase-Catalysed Syntheses at pH below Neutrality

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Abstract—The use of the new glycosynthase Ta-β-GlyE386G and of the already known Ss-β-GlyE387G for the synthesis of interesting 4-methylumbelliferyl disaccharides and for the galactosylation of α- and β-xylosides of 4-penten-1-ol is reported. The results show satisfactory yields of reaction in presence of low excesses of acceptors and demonstrated that the high activity of these enzymes at pH below neutrality is applicable in the transfer of glucose as well as of galactose from the preferred 2-NP-based donors.

Chromophoric oligosaccharides such as nitrophenyl and 4-methylumbelliferyl glycosides are of widespread interest for the kinetic analysis of hydrolytic activities and to define the mode of action of particular enzymes (i.e., *exo*- or *endo*-glycosidases). These compounds have proved to be a valuable tools in different field of applicative interest such as clinical, biological and food chemistry.[1–3] *n*-Pentenyl glycosides are stable to a wide range of reagents and are readily activated by halonium ion, thus they are excellent substrates for a wide variety of reactions occurring at anomeric center in oligosaccharide synthesis in the so called Fraser–Reid methodology.[4] The enzymatic synthesis of these sugar building blocks could then shorten the synthetic routes by coupling enzymatic and chemical methodologies thus improving overall yield in a synthetic plan.[4] One of the synthetic strategies for these molecules generally rely upon the availability of the appropriate carbohydrate sequence as found in natural sugar polymers and applying in turn the aglycones by chemical methods.[5]

The enzymatic approach using glycoside hydrolases is based on reverse hydrolysis or transglycosylation. The latter is performed by the action of two active-site carboxylic acids, one acting as nucleophile, the other as an acid-base catalyst. The major drawback of this approach is that the reaction products are substrates for the enzyme and can be hydrolysed back thus reducing yields of transfer products.

Recently the synthesis catalysed by glycosynthases emerged as significant development in in this field.[6,7] By using these enginereed enzymes, which lack the nucleophile residue in their active site, the products can accumulate in the reaction mixture due to the loss of the hydrolytic activity of the biocatalyst for the products. Among glycosynthases, the thermophilic representatives can be used efficiently in inverting (with α-glycosyl fluoride as donors) or retaining (with aryl β-glycosides as donors) reactions. In the latter case, while mesophilic members can react only with highly activated 2,4-dinitrophenyl glycosides, stable 2-nitrophenyl glycosides can be used by the thermophilic biocatalysts with sodium formate (2–4 M) acting as external biomimicking nucleophile. In addition different biodiversity in terms of regioselectivity of the reaction conducted with thermophilic enzymes is observed. These biocatalysts are also characterized by a high degree of reactivation compared to the mesophilic counterparts.[6] Moreover, it has been recently observed that at pH's below neutrality in diluted (50 mM) sodium formate buffers, the k_{cat} values of three thermophilic glycosynthases (Ss-β-GlyE387G, Ta-β-GlyE386G, CelBE372A) can be rescued at levels comparable to the wild type enzymes or 17-fold higher than the k_{cat} values observed at high concentration of sodium formate, thus improving the efficiency of the hydrolytic reaction in terms of reaction time and amounts of enzyme used.[8] In these new reaction

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conditions, the three mutants (Ss-β-GlyE387G, Ta-β-GlyE386G, CelBE372A) produced 2-nitrophenyl oligoglucosides with different selectivity and degree of polymerization when 2-nitrophenyl-β-glucoside was used as substrate (donor and acceptor).[8] The thermophilic nature of these biocatalysts coupled with their high activity in diluted buffers are interesting issues considering the improved solubility of donors and acceptors and some practical benefits, in the monitoring and purification procedures, due to the absence of molar concentration of nucleophiles. Practical application for long reaction times is secured by the good stability expressed by these thermophilic proteins at 65 °C in 50 mM sodium formate buffer, pH 4.[8]

In this communication we report on the preparation of chromophoric oligosaccharides and on the galactosylation of xylosides of 4-penten-1-ol exploiting the new glycosynthase from *Thermosphaera aggregans* and the already known Ss-β-GlyE387G[6] in these new reaction conditions.[9]

In Table 1 the specificity of mutant and wild type enzymes is reported for different substrates. 2-Nitrophenyl glucoside is efficiently cleaved by the glycosynthase of *T. aggregans* as well as by the glycosynthase of *Sulfolobus solfataricus*. The loss of activity is 80–90% by changing the position of nitro group in the aglycon (2-NP-Glc vs 4-NP-Glc). Moreover on 4-methylumbelliferyl β-D-glucoside, Ta-β-GlyE386G retains only 3.6% of the activity expressed on 2-NP-Glc (Table 1). It is interesting to note, as a basic foundation of glycosynthase activity of these biocatalysts, that the ratio of activities of wild types versus mutant enzymes for 2-NP-Glc and cellobiose respectively, shifts from ca. 2 to 332 for *T. aggregans* and from 4 to 52 for *S. solfataricus*. These findings lead to the benefit of using different aryl-based structures as acceptors thus obtaining useful compounds in moderate-high yield. The substrates we chose for the reactions[10,11] are 4-methylumbelliferyl β-D-glucoside and α- and β-xylosides of 4-penten-1-ol; the use of acceptors with β-glycosidic linkages is a unique characteristic of mutant enzymes in that these molecules are completely hydrolyzed by the wild type. Blank experiments (GOD analysis) secured that no significant (<1% in 3 h and ca. 11% in 24 h) chemical hydrolysis of aryl-donor occurred in the reaction time intervals. The reaction[10] conducted using the mutant of Ta-β-Gly and 2-NP-glucoside as donor and 4-methylumbelliferyl β-D-glucoside as acceptor, furnished two disaccharides: 4-methylumbelliferyl laminaribioside, **1** (4-MU-Lam, 40%) and 4-methylumbelliferyl cellobioside, **2** (4-MU-Cell, 60%) (Table 2).

Table 1. Substrate specificity (U mg^{-1}) of *Thermosphaera aggregans* glycosynthase and Ss-β-GlyE387G in 50 mM sodium formate buffer, pH 3 as compared to the wild types

Substrates	Ta-β-Gly		Ss-β-Gly	
	wt	m	wt	m
2-NP-Glc	72.2	129.8	252.6	63.3
2-NP-Gal	140.9	6.3	218.6	2.7
2-NP-Xyl	10.4	6.4	19.1	2.5
4-NP-Glc	61.7	12.0	149.8	14.9
4-MUG	33.5	4.7	88.2	9.7
Cellobiose	33.8	0.39	61.9	1.2

Aryl-glycosides and cellobiose substrates were assayed at 5 and 200 mM final concentration, respectively, in 50 mM sodium formate buffer pH 3.0 (see ref 8 for details); wt, wild type; m, mutant.

The synthesis was conducted at three different pH in 50 mM sodium formate/formic solutions. The donor conversion at pH 5 is not significant even after 96 h; at pH 3, 75% conversion of donor was reached in 3 h and the overall yield of the disaccharides **1** and **2** is 65% after purification and acetylation for NMR spectroscopy study.[12] At pH 4 the conversion reached 93% after 9 h and the isolated yield of **1** and **2** is 60%. Unreacted 4-MUG is recovered during product purification. These data are in agreement with the reported increase of enzymatic activity observed at acidic pH.[8] The yields are remarkably high considering the low, 2-fold molar excess of the acceptor used. Trace amounts of glucose and of the disaccharides of the donor are observed. No trisaccharidic product formation was observed in TLC analysis of the reaction mixtures. Elongation to trisaccharides was investigate using as acceptors: *p*-nitrophenyl-β-D-lactoside, the disaccharide **1** and phenyl thiocellobioside[13] (Table 2). No reaction was observed in the first case, while poor yields of trisaccharides were obtained for the phenyl thiocellobioside. Using 4-MU-Lam (**1**) as acceptor, 16% yield of two trisaccharides (**3a** and in lesser amount **3b**) was obtained in a reaction conducted at equimolar donor/acceptor ratio. The analysis of ^{1}H NMR spectra, COSY and ^{1}H–^{13}C NMR correlations permit to establish the trisaccharidic nature of products and the interglycosidic linkages for **3a** as β-Glc-(1-4)-β-Glc-(1-3)-β-Glc-MU.[12]

Table 2. Syntheses conducted with Ta-β-GlyE386G and Ss-β-GlyE387G

Donor	Acceptor	Enzyme	Products	Yield (%)
2-NP-Glc	4-MUG	Ta-β-GlyE386G	**1**, **2**	60–65
2-NP-Glc	PNP-Lac	Ta-β-GlyE386G	—	—
2-NP-Glc	Ph-S-Cell	Ta-β-GlyE386G	—	Poor
2-NP-Glc	**1**	Ta-β-GlyE386G	**3a**, **3b**	16
2-NP-Gal	β-Xyl-4P	Ta-β-GlyE386G	**4**	34
2-NP-Gal	α-Xyl-4P	Ss-β-GlyE387G	**5a**, **5b**	33
2-NP-Gal	β-Xyl-4P	Ss-β-GlyE387G	**4**	40

2-NP, 2-nitrophenyl; Glc, glucose; Gal, galactose; 4-MUG, 4-methylumbelliferyl β-D-glucoside; PNP-Lac, *p*-nitrophenyl-β-D-lactoside; Ph-S-Cell, phenyl thio-cellobioside; **1**, 4-methyl umbelliferyl-β-D-laminaribioside; **3a**, β-Glc-(1-4)-β-Glc-(1-3)-β-Glc-MU; **3b**, β-Glc-β-Glc-β-Glc-MU; β-Xyl-4P, 4-penten-1-yl β-D-xylopyranoside; α-Xyl-4P, 4-penten-1-yl α-D-xylopyranoside; **4**, β-Gal-(1-3)-β-Xyl-4P; **5a**, β-Gal-(1-3)-α-Xyl-4P; **5b**, β-Gal-(1-4)-α-Xyl-4P.

The glycosynthases from Ta-β-Gly and Ss-β-Gly remain active also on 2-NP-Gal (Table 1) and we used this potential to investigate the transfer of galactose to the xylosides of 4-penten-1-ol[13] for the synthesis of the disaccharidic unit (or regioisomers) present in xyloglucan oligosaccharides possessing several biological activities.[14] The reactions[11] were conducted at 1–5 molar excess of acceptor by adding aliquots of the substrate to the reaction mixtures. Conversions are still satisfactory being ca. 90 and 60% for *S. solfataricus* and *Thermosphaera* enzyme, respectively in 24 h reaction time. The yields, calculated on the substrate converted, are similar for both enzymes, in the range 35–40%. Much more interesting is the change in regioselectivity for the two anomers of the xyloside used. Essentially a single[12] (**4**, β-Gal-(1-3)-β-Xyl-4P > 95%) regioisomer was obtained when using β-Xyl-4P: while a 33% yield of two disaccharides **5a** and **5b** was obtained using α-Xyl-4P by the use of the glycosynthase Ss-β-glyE387G in 19:81 ratio, respectively (Table 2). Using *Thermosphaera* mutant enzyme a similar yield of compound **4** is obtained when using β-Xyl-4P. The difference of regioselectivity observed for the Ss-β-glyE387G with respect to the anomeric configuration of the acceptor (β-Xyl-4P/α-Xyl-4P) is for the first time observed for a glycosynthase and confirms the well known phenomenon revealed for the wild type glycoside hydrolases.[15]

The high rescue of hydrolytic activities of thermophilic glycosynthases,[8] obtained at low pH, coupled with high resistance to critical reaction conditions to which these biocatalysts have to face (high temperature, low pH, high concentrations of organics), prompted us to explore new strategies for the exploitation of these enzymes on different substrates. The overall results reported in this communication demonstrate that glycosynthases from *T. aggregans* and *S. solfataricus* at pH below neutrality have the ability to produce glycosylated products in presence of various acceptors. Carbohydrate elongation to tri- and higher compounds is possible for *T. aggregans* glycosynthase but the yield fall seriously down indicating steric/electronic limitations in the active site as also previously observed for this enzyme with 2-nitrophenyl β-D-glucoside.[8] However it is worth noting that despite the low molar excess of acceptors (1.9–1.0) for the synthesis of 4-methylumbelliferyl di- and trisaccharides, a ca. 65 and 16% yields, respectively, were obtained. The residual activity on 2-NP-Gal of both glycosynthases was also exploited to achieve satisfactory yields of regioisomers of Gal-Xyl present in the oligosaccharins.[14,16] It is of interest that β-configuration of the acceptor permits to obtain a single disaccharide out of three possible products. However the most abundant β-Gal-(1-4)-α-Xyl-4P could be utilized in a conceivable chemical coupling for the production of saccharides present in the region between glycosaminoglycan (GAG) chains and protein parts in serine-linked connective tissue proteoglycan.[19]

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9. The *Thermosphaera aggregans* glycosynthase (E386G) 0.43 mg/mL and Ss-β-glyE387G (0.38 mg/mL) were used as biocatalysts. They were obtained by mutation as previously described in refs 6 and 8.
10. 2-nitrophenyl β-D-glucoside (0.083 mmol) was dissolved in three different 50 mM formic acid/sodium formate buffer solutions at pH 3.02, 4.02 and 5.00 (2 mL). The 4-methylumbelliferyl β-D-glucoside, 0.165 mmol, was added and the reactions started in sealed vials at 65 °C by adding 350 μL of the enzyme. No significant (less than 5%) chemical hydrolysis of donor and acceptor was observed in these conditions by blank experiment. After 5 h, the reaction was neutralized with sodium carbonate; reverse phase RP-18 short column and preparative silica-gel TLC (EtOAc/MeOH/H_2O, 70:20:10 by vol) furnished disaccharides 1 and 2 which were in turn subjected to standard acetylation procedures (Ac_2O/pyr overnight) for NMR spectroscopy studies.
11. Donor: 2-nitrophenyl-β-D-galactoside (74 μmol). Acceptor: β-(pent-4-en-1-yl)-D-xylopyranoside (0.36 mmol). Buffer system: sodium formate 50 mM pH 4. Enzyme: Ta-β-glyE386G 96 μg. Temperature: 65 °C. The donor was converted at ca. 60% in 24 h. Yield of the reaction 34%. β-(pent-4-en-1-yl)-galactosyl-(β-1-3)-D-xylopyranoside (> 95%).
Donor: 2-nitrophenyl-β-D-galactoside (0.24 mmol). Acceptor: α-(pent-4-en-1-yl)-D-xylopyranoside (0.40 mmol). Buffer system: sodium formate 50 mM pH 4. Enzyme Ss-β-glyE387G, 95 μg. The donor was added in three aliquots of 0.08 mmol at time intervals during 4 h of reaction time to the reaction mixture to keep the molar excess of the acceptor in the range of 1–5. Temperature: 65 °C. The donor was converted at ca. 90% in 24 h. Yield of the reaction 33%. α-(Pent-4-en-1-yl)-galactosyl-(β-1-3)-D-xylopyranoside (19%), α-(pent-4-en-1-yl)-galactosyl-(β-1-4)-D-xylopyranoside (81%).
Donor: 2-nitrophenyl β-D-galactoside (0.10 mmol). Acceptor: β-(pent-4-en-1-yl)-D-xylopyranoside (0.51 mmol). Buffer system: sodium formate 50 mM pH 4. Enzyme Ss-β-glyE387G 76 μg. Temperature: 65 °C. The donor was converted at ca. 90% in 24 h. Yield of the reaction 40%. β-(Pent-4-en-1-yl)-galactosyl-(β-1-3)-D-xylopyranoside (> 95%).
12. ^1H NMR spectra, COSY and ^1H–^{13}C NMR correlations generally permit the assignments of chemical shifts as below indicated for peracetylated derivatives of compounds obtained. In the COSY spectrum of the product, following the correlations through pyranosidic protons starting from the aglycon-linked sugar, it is easy to detect the position of glyco-

sylation for the upfield shift of the signal due to the absence of acetyl group. Diagnostic ^{13}C correlation signals are indicated. **1** ($CDCl_3$): 5.03 (H1), 5.30 (H2), 4.00 (H3 β-*O*-linked to the external sugar), 5.10 (H4), 3.87 (H5), 4.20 (H6), 4.63 (H1′), 4.92 (H2′), 5.15 (H3′), 5.13 (H4), 3.70 (H5), 4.40–4.05 (H6). ^{13}C signals: 100.9, 98.4, 78.6, 72.8, 72.5, 72.2, 71.8, 71.1, 68.0 (×2), 62.0, 61.6. **2** (C_6D_6): 4.80 (H1), 5.50 (H2), 5.39 (H3), 3.49 (H4 β-*O*-linked to the external sugar), 3.20 (H5), 4.50–4.00 (H6), 4.20 (H1′), 5.18 (H2′), 5.37 (H3′), 5.19 (H4), 3.30 (H5), 4.40–3.90 (H6). ^{13}C signals: 100.7, 98.2, 76.2, 73.2, 72.8, 72.3, 72.0, 71.5, 71.0, 67.7, 61.7, 61.5. **3a** ($CDCl_3$) carbohydrate moiety ($CDCl_3$): 5.03/98.4 (H1/C1 aryl linked glucose), 3.98/78.4 (H3/C3 aryl linked glucose), 4.60/100.8 (H1/C1 internal glucose), 3.72/75.2 (H4/C4 internal glucose), 4.50/100.7 (H1/C1 external glucose). The trisaccharidic nature of **3b** was established by the presence of three anomeric signals in the ^{13}C NMR and by the TLC R_f comparable to that of **3a**. **4** β-Gal-(1-3)-β-Xyl-4P ($CDCl_3$) pent-4-en-1-yl moiety: 5.77 (137.9), 5.04 (114.9), 3.72–3.41 (68.2), 2.10 (29.9), 1.68 (28.5); β-D-xylopyranosyl moiety H1-H5: 4.39 (100.1), 4.86, 3.84 (76.7), 4.87, 4.05–3.39; β-D-galactopyranosyl moiety H1–H6: 4.58 (101.3), 5.13, 4.99, 5.35, 3.89, 4.20-4.09. ^{13}C disaccharidic moiety: 101.3, 100.1, 76.7, 71.5, 71.0, 70.5, 69.1, 68.6, 66.9, 61.4, 61.0. **5a** β-Gal-(1-3)-α-Xyl-4P ($CDCl_3$) pent-4-en-1-yl moiety: 5.79 (137.6), 4.95 (115.1), 3.69–3.39 (67.6), 2.10 (30.1), 1.68 (28.4); α-D-xylopyranosyl moiety H1-H5: 4.90 (95.6), 4.78, 4.08 (75.8), 4.92, 3.75–3.67; β-D-galactopyranosyl moiety H1-H6: 4.66 (101.1), 5.13, 5.01, 5.38, 3.91, 4.20-4.09. ^{13}C disaccharidic moiety: 101.1, 95.6, 75.8, 72.9, 71.1, 70.6, 69.2, 69.0, 66.9, 61.0, 58.6. **5b** β-Gal-(1-4)-α-Xyl-4P ($CDCl_3$) pent-4-en-1-yl moiety: 5.79 (137.8), 4.95 (115.1), 3.69–3.39 (67.7), 2.10 (30.0), 1.68 (28.4); α-D-xylopyranosyl moiety H1–H5: 4.92 (95.8), 4.72, 5.39, 3.78 (76.6), 3.60; β-D-galactopyranosyl moiety H1-H6: 4.48 (101.1), 5.09, 5.01, 5.37, 3.89, 4.10. ^{13}C disaccharidic moiety: 101.1, 95.8, 76.6, 71.3, 70.9, 70.8, 70.2, 69.1, 66.8, 61.1, 59.1.

13. Phenyl thio-cellobioside was obtained by standard routes starting from α-D-cellobiose octacetate as described in ref 17. α- and β-xylosides of 4-penten-1-ol were obtained starting from acetylated monosaccharide (α-xylose) and using BF_3-etherate as catalyst (total yield 23%, α/β anomers 1:1) as described in ref 18.

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