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A Novel Thermophilic Glycosynthase That Effects Branching Glycosylation

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Abstract—A novel thermophilic glycosynthase that effects branching glycosylation has been obtained by mutation of the nucleophile in the active site of the glycosidase from *Sulfolobus solfataricus*. Two methods for the use of this mutant are reported.

Glycosyl hydrolases are recognized as valid and less expensive tools for the synthesis of oligosaccharides.[1] However, with the aid of site-directed mutagenesis it is possible to obtain enzymes with useful properties by replacing the active site nucleophile involved in the catalytic mechanism. We recently reported that the activity of two mutants in the nucleophile (Glu to Ala/Gly) of the β-glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus* (Ss-β-Gly) can be restored by the use of sodium azide or formate as external nucleophiles.[2] The mutated glycosidases can act as *glycosynthases* since they cannot hydrolyze the transglycosylation products which accumulate in the reaction. Up to now three other *glycosynthases* have been used for the synthesis of different linear oligosaccharides[3–6] possessing mainly β-1,4 linkage.

However, Ss-β-Gly is the only thermophilic representative known so far among these new biocatalysts and possess interesting catalytic activity for the synthesis of branched oligosaccharides, an important class of molecules possessing great potential in pharmaceutical and food science.

The aim of this communication is to present stereochemical details of the transglycosylation reactions performed by the mutant thermophilic Glu387Gly Ss-β-Gly. A preliminary comparison of two methods which can be used by this mutant enzyme is also reported (Scheme 1).[7]

The mutant Glu387Gly Ss-β-Gly was prepared by site-directed mutagenesis as reported previously.[2] The acceptance of easy-to-handle and inexpensive 2-NP-based substrates, with respect to more activated derivatives used by the mesophilic counterparts,[2] allowed the use of thermophilic glycosynthase in the synthesis of oligosaccharides by supplying reaction mixture with different 2-NP-based substrates (β-D-gluco-, β-D-fuco, β-D-galacto and β-D-xyloside) and sodium formate (pathway 1, Scheme 1) thus forming a transient[8] intermediate able to transfer the carbohydrate molecule.[2] The β-D-galacto and β-D-xyloside substrates showed lower activity and were not used in this work. The alternative approach (pathway 2, Scheme 1), which consists in supplying the reaction mixture with an appropriate "glycosyl intermediate" such as α-glucosyl fluoride, is the only one used with mesophilic glycosynthases and has also been tested. In Table 1 the reactions performed by the use of both methods are reported.[9]

The glycosylated products obtained by reaction 1[10] (Table 1) are composed of 50% of disaccharides, 40% of trisaccharides and 10% of tetrasaccharides as judged by HPLC profile. Within the class of disaccharides, compound **1** is the most abundant (80%) followed by **2** (18%) and **3** (2%).[11] Two major trisaccharides are obtained, namely **4** (70%) and **5** (14%) both sharing the disaccharide substructure **1**.[11] The remaining 16% is composed by at least four trisaccharides which are not obtained as single pure compounds. The structures of **4** and **5** were further confirmed by the analysis of products (TLC, HPLC) of their enzymatic hydrolysis[12] by

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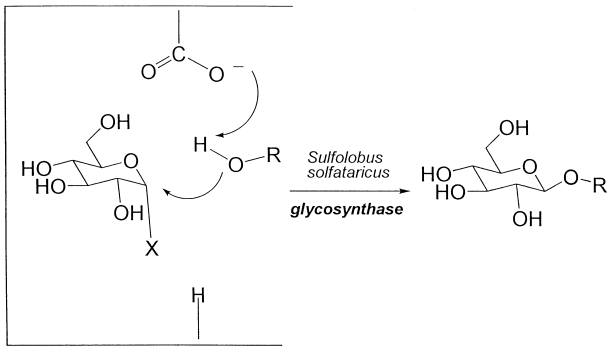

Scheme 1.

Table 1. Reactions performed by the thermophilic glycosynthase

Entry	Donor	Acceptor	Nucleophile	Products	Yields[9] (%)
1	A[a]	2-NP-β-D-Glc	Formate	1–6	85[b]
2	B[c]	2-NP-β-D-Fuc	Formate	7	N.D.
3	A	Model diol[d]	Formate	See text	50
4	A	4-NP-β-D-lactose	Formate	8[e]	12
5	C	Model diol	—	See text	50
6	C	2-NP-β-D-Glc	—	1	80
7	C[c]	4-NP-β-D-lactose	—	8[f]	N.D.

[a]A = 2-NP-β-D-Glc, pathway 1 (Scheme 1); B = 2-NP-β-D-Fuc; C = α-F-Glc pathway 2 (Scheme 1).
[b]The calculated efficiency of glycosynthase (15% of free glucose at the end of reaction) is in agreement with preparative yield of reaction on half-gram scale;[9] in the other cases the yields are expressed with respect to the substrate present in lower amount (see text). Starting from 1.54 mmol of 2-NP-β-D-Glu (acting as donor and acceptor) ca. 60% of *o*-nitrophenol is recovered in the glycosylated mixture of products (294 mg). N.D. = not detected.
[c]Reactions conducted on analytical scale.
[d](*R,S*)-3-*O*-benzyloxy-1,2-propanediol (11 fold molar excess).
[e]Compound **1** is also present as product.
[f]Conducted at a donor molar excess of 2.

the wild type Ss-β-Gly which possess exoglucosidase activity and a strong but not exclusive preference for the hydrolysis of 3-O-β-linkage. The remaining 10% of the methanol fraction is composed by a mixture of five tetrasaccharides. The 54% of this class is constituted by a single component, **6**, obtained as pure compound after acetylation and chromatographic purification. The body of results from two-dimensional NMR and enzymatic analysis[11,13] firmly suggest that the tetrasaccharide **6** was based on **4** with the additional glucose molecule 3-*O*-β-linked as indicated in Figure 1.

When using 2-NP-β-D-Fuc as donor and acceptor (entry 2, Table 1) in a reaction conducted on analytical scale (0.035 mmol) the disaccharide **7**[11] was the most abundant compound although trace amount of two other disaccharides were observed in TLC analysis of reaction mixture. Interestingly enough, higher homologues are not present as found instead in reaction 1.

The reaction using the methodology in pathway 1 has been performed, also with alcohols of different structures, such as the example with a model diol reported in entry 3 obtaining a mixture of glycosylated derivatives.

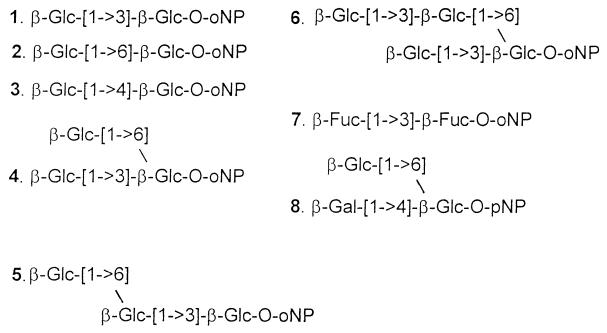

Figure 1. Oligosaccharides formed by thermophilic glycosynthase.

The reaction (entry 4), using 4-NP-β-D-lactopyranoside as acceptor was conducted as described for entry 1 and adding a stoichiometric amount of the acceptor. At total consumption of the donor (2-NP-β-D-Glc), TLC analysis of the reaction mixture indicates the presence of products such as the disaccharide **1** and of a mixture of trisaccharides. Chromatographic purification of the latter afford ca. 12% isolated yield of 4-NP-based trisaccharides. The most abundant component is **8**.[11] After acetylation of this material a mixture of two TLC spots was obtained. The NMR of the major acetylated component confirms the structure **8** for this trisaccharide. The minor spot in TLC of acetylated material was composed of at least two other trisaccharides, as indicated by NMR spectra, not obtained as pure material. No further efforts were made to increase the yield of this reaction. The use of such an acceptor is a unique characteristic of the mutant enzyme because this molecule will be hydrolyzed by the wild type.

The alternative approach (Scheme 1, pathway 2) in the use of glycosynthases consists in incubating the enzyme with α-glucosyl fluoride.[6] This reaction (entry 5) has been conducted at two different temperatures and, at total donor consumption (24 h at 40 °C or 6 h at 60 °C), ca. 50% yield of the same mixture of glycosylated derivatives of the acceptor in reaction 3 was obtained. Much more interestingly, when 2-NP-β-D-Glc was the acceptor (entry 6, Table 1) used in a 5-fold molar excess, both the yield and speed of the resulting reaction increased (80% in 1 h at 60 °C), thus showing the increased affinity of the enzyme for the pyranose-like acceptor with respect to the diol molecule. The main (>90%) product of this reaction is the disaccharide **1**. Disaccharides **2** and **3** are present only in trace amounts. This selectivity lowered in experiments conducted at stoichiometric amount of 2-NP-β-D-Glc thus obtaining **1** (65%), **2** (26%), **3** (trace) and **4** (8%). In entry 7 of Table 1 is reported the result of the reaction using the 4-NP-β-D-lactopyranoside as the acceptor, obtaining the same mixture of trisaccharides as described in entry 4 with **8** as the most abundant compound. This approach avoids in this case the formation of disaccharide **1** observed in entry 4.

Although not every product of the reaction in entry 1 has been identified, compounds **1**–**6** afford for ca. 90% of the reaction mixture and some conclusion concerning the regioselectivity of the glycosynthase can be drawn.

Previous studies[14] indicated a strong preference of wild type enzyme in transglycosylation reactions towards primary hydroxyl groups. The elimination of a hydrolytic pathway in the mutant enzyme reflects in the isolation of 3-O-based disaccharide **1** as the most abundant product and, most interestingly, allowed the production of tri- and tetrasaccharides by using pathway 1. Higher oligosaccharides have never been observed in reactions performed with wild type Ss-β-Gly. Their synthesis can be obtained by this new glycosynthase in a high regioselective manner with preference for the branching to the internal primary hydroxyl group (**4** and **8**). To the best of our knowledge, this result has never been reported before. The structure of the tetrasaccharide **6** indicates that the regioselectivity of the enzyme switches back to the C3-secondary hydroxyl group of external glucose moiety. This switch deserves further investigation on different acceptor molecules. These observations are confirmed by the the results obtained using 2-NP-β-D-Fuc, a substrate without primary hydroxyl groups. In this case only disaccharides are formed, the most abundant, compound **7**, possessing the same interglycosidic linkage of **1** thus confirming the analysis of regioselectivity and securing that in the active site of the enzyme, β-D-fucose should have interactions similar to those of glucose despite the presence of axial C-4 hydroxyl which prevents acceptor role with a mesophilic glycosynthase.[6]

The use of α-glucosyl fluoride as a donor (pathway 2, Scheme 1), will allow the design of products by supporting the reaction with different acceptors (entries 6 and 7)[6] and tuning donor/acceptor ratios to achieve reasonable yields and selectivity. Using this approach ca. 80% yield was achieved with acceptor molar excess of 5 with high selectivity and speed of reaction[4,6] towards disaccharide **1**. Trisaccharide **8** is the most abundant compound formed when 4-NP-β-D-lactopyranoside is used as acceptor. No further efforts were made to optimize reaction conditions in this case.

In conclusion, we demonstrated that both approaches are possible with this thermophilic glycosynthase widening the potentiality of these new biocatalysts and encouraging their exploitation in the synthesis of target oligosaccharides. In fact, the branching functionalization represents a unique characteristic of this thermophilic enzyme for the preparation of molecules of practical interests. For example, the same structural motif of compound **4** is present in antitumoral β-(1-6)-branched (1-3)-β-D-glucan from the alkaline extract of *Amanita muscaria*[15] and glycosyl-lactose derivatives have recently gained interest in the pharmaceutical field and in food science.[16] Moreover the branched products could be useful substrates to widen the studies of glycosidases.

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9. The efficiency of glycosynthase activity of the mutant enzyme has been firmly established by following enzymatically (GOD) the glucose content of the reaction mixture up to the complete consumption of the substrate as judged by a close monitoring (TLC) of reactions. The stereospecificity of the enzymatic reaction has been evaluated by determining structures (FAB, NMR, enzymatic cleavage) of different regioisomers formed and their relative proportion (HPLC μBondapak-NH_2). NMR studies of purified compounds (^1H, ^{13}C and DEPT experiments) were conducted in CD_3OD and those of acetylated derivatives (COSY, TOCSY, ^1H-^{13}C correlations) in $CDCl_3$ or benzene-d_6.
10. To 1.54 mmol of 2-NP-β-Glc dissolved in 40 mL phosphate buffer were added 10.8 g of sodium formate and 2.4 mL of mutant enzyme (1.7 mg/mL). After 1 h at 60 °C the reaction was stopped by cooling and eluting on RP-8 column with water and methanol. The methanol extract was subjected to different chromatographic procedures (RP-18, silica gel, HPLC) to obtain pure compounds.
11. Diagnostic downfield shifted signals (glycosylated positions) were found at δ: 87.31 (**1**); 69.94 (**2**); 79.89 (**3**); 86.86 (CH) and 69.91 (CH_2) (**4**); 89.26 (CH) and 70.17 (CH_2) (**5**); 86.20 and 85.37 (CH) and 69.54 (CH_2) (**6**); 84.54 (**7**); 79.76 (CH) and 70.01 (CH_2) (**8**), other signals in the spectra of this compound are compared to those in the spectrum of disaccharide substrate 4-NP-β-D-lactoside. Remaining signals in each NMR spectra of native and acetylated derivatives, and FAB–MS spectra are in accord with the proposed structures. Acetylated derivatives: **1** (^1H (^{13}C) NMR signals, benzene-d_6): δ 4.49 (101.4), 5.42 (72.5), 3.84 (78.7), 4.92 (68.6), 3.34 (72.1), 4.09–4.14 (62.04) (internal glucose); 4.56 (100.8), 5.12 (72.2), 5.30 (73.34), 5.13 (68.4), 3.25 (71.6), 4.35-3.94 (61.8) (external glucose). **2** (^{13}C NMR signals, $CDCl_3$) δ 100.2, 72.7, 72.04, 68.7, 74.0, 68.1 (internal glucose) 100.6, 72.3, 71.1, 68.2, 70.4, 61.7 (external glucose). **3** (anomeric ^1H and ^{13}C) NMR signals, $CDCl_3$) δ 4.55 (100.8, 72.2, 72.07, 75.92, 70.48, 61.45) (internal glucose) 5.14 (99.2, 72.8, 72.9, 67.7, 70.9, 61.45). **4** (^1H and (^{13}C) NMR signals, $CDCl_3$) δ 4.92 (100.2), 5.28 (72.0), 3.95 (78.3) 4.82 (68.0), 3.85 (74.1) 3.68–3.91 (68.4) (aryl linked glucose); 4.54 (100.6), 5.05 (71.8), 5.14 (71.5), 5.06 (68.1), 3.69 (70.8), 4.37–4.05 (61.7) (6-O-β-linked glucose);

4.61 (100.9), 4.92 (72.9), 5.07 (72.7), 5.05 (68.5), 3.69 (71.10) 4.24–4.12 61.70 (3-O-β-linked glucose). **5** (^{13}C) NMR signals, $CDCl_3$) δ 100.5, 100.3, 99.7, 77.6, 74.7, 72.8, 72.6, 72.1, 71.9, 71.7, 71.4, 71.3, 68.5, 68.1, 68.0, 67.2, 62.0, 61.7; (^{1}H) NMR signals, $CDCl_3$) δ 5.06, 5.36, 4.07, 5.11, 4.04, 4.0-4.3 (aryl linked glucose), 4.89, 4.96, 5.15, 4.87, 3.63, 3.6–3.9 (central glucose unit), 4.64, 4.82, 5.14, 5.11, 3.73, 4.0–4.3 (external glucose). **6**, main features of ^{1}H and (^{13}C) NMR spectra: 4.91 (100.2), 5.27 (71.5) 3.95 (78.37) (aryl linked glucose); 4.61 (100.9) (glucose 3-O-β-linked to the aryl linked glucose) 4.57 (100.9) (glucose 3-O-β-linked to the 6-O-β-linked glucose); 4.35 (100.5) 5.02 (72.6), 3.85 (78.79) (central glucose unit 6-O-β-linked to the aryl linked glucose). **7**, (^{1}H NMR signals, $CDCl_3$) δ 4.55, 5.18, 4.92, 5.18, 3.74, 1.25 (external fucose unit); 4.99, 5.51, 3.94, 5.31, 3.88, 1.18 (aryl linked fucose). **8**, (^{1}H NMR signals, benzene-d_6) 4.73 (aryl linked glucose anomeric signal) 4.39 (galactose anomeric proton) 4.28 (6-O-β-linked glucose). Other ^{1}H NMR diagnostic signals of acetylated derivative of **8** overlapped each other and interglycosidic linkage determination is mainly based on the spectra of native **8** in CD_3OD/D_2O as compared to the spectra of 4-NP-β-D-lactoside used as substrate.

12. 2 mg of purified acetylated glycoside after deprotection ($MeOH/Na_2CO_3$) were dissolved in 500 μL of phosphate buffer 20 mM pH 7 and 10 μL of wild type Ss-β-Gly (2.67 mg/mL) and the reaction started at 50 °C. Samples withdrawn at different time intervals were analyzed by TLC and HPLC. The enzymatic hydrolysis of compound **4** furnished exclusively the disaccharide **2** and glucose at beginning of the reaction. Conversely, the hydrolysis of compound **5** furnished 2-NP-β-D-Glc and glucose as the two major compounds along with only trace amount of disaccharide **1**.

13. Enzymatic hydrolysis of **6** with Ss-β-Gly furnished trisaccharide **4** in the early stage of the reaction and similar pattern of enzymatic hydrolysis of **4**. The interglycosidic linkage was also confirmed by the presence of two downfield shifted signals at 78.79/3.85 and 78.37/3.95 ppm in the acetylated derivative which were similar to that found in **1** or **4** and not in **3** (75.92 ppm). The absence of signals around this chemical shift and two-dimensional NMR spectroscopy studies secured the attachment of the more external glucose unit as shown in **6**.

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