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Introducing Transglycosylation Activity into Human Salivary α-Amylase (HSA)†

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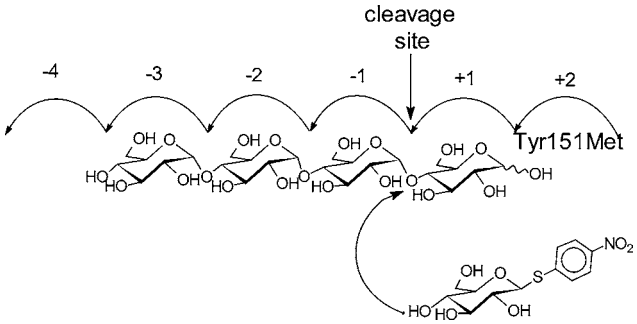
ABSTRACT



Synthesis of 4-nitrophenyl 1-thio-β-D-maltoside, maltotrioside, and maltotetraoside in yields up to 60% has been achieved by a Tyr151Met (Y151M) mutant of human salivary α-amylase. Y151M is capable of transferring maltose and maltotriose residues from a maltotetraose donor onto different *p*-nitrophenyl glycosides. ^{1}H and ^{13}C NMR studies revealed that the mutated enzyme preserved the stereo- and regioselectivity. The glycosylation took place at position 4 of the glycosyl acceptor, forming the α(1–4)glycosidic bond, exclusively.

Enzyme-catalyzed synthesis of oligosaccharides is a very attractive method because it allows the formation of well-defined oligosaccharides selectively without using any protection of hydroxyl groups. In addition, on the basis of the advances in genetic engineering, it is becoming possible to produce a wider range of enzymes on a large scale, expanding the number of enzymes available for synthetic reactions. Considerable effort has been made to study the application of transglycosylation ability of retaining glycosidases as an alternative approach for the synthesis of chromogenic oligosaccharide substrates.[1−3] Eneyskaya et al.[2] reported the regio- and stereoselective synthesis of *p*-nitrophenyl β-(1−4)-D-xylooligosaccharides from 2−7 sugar units (DP 2−7) using a β-D-xylosidase from *Aspergillus* sp. Usui and co-workers investigated the transglycosylation reaction of a maltotriose-forming amylase from *Streptomyces griseus*.[3] One potential limitation of this approach, however, is that the products of transglycosylation may themselves be hydrolyzed by the enzyme during the course of reaction. The introduction of glycosynthases, mutant glycosidases, which lack a catalytic nucleophile and therefore are incapable of carrying out substrate hydrolysis in the presence of glycosyl fluoride donors, has overcome this problem for the synthesis of a number of β-[4,5] and α-aryl[6] oligosaccharides.

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The great majority of the syntheses published in the literature are catalyzed by exo-acting glycosidases. The ability of endo-acting enzymes to synthesize novel oligosaccharides has rarely been tested.[2,3] This paper reports the synthesis of oligosaccharide glycosides catalyzed by an endoenzyme, the mutant Y151M of HSA.

Despite the increasing work carried out with glycosidases, little is known about the structural requirements for the binding of a sugar acceptor to the enzyme, which is essential to improve the synthetic utility of this methodology.

Recently, the substrate binding sites of human salivary α-amylase (HSA) and its Tyr151Met mutant[7] (Y151M) were characterized in our laboratory. The first subsite maps were described for both amylases; the numbers of subsites, the position of cleavage sites, and apparent subsite energies were determined. The product distributions revealed that in the mutant maltose changed to glucose as the minimal leaving group due to the presence of Met at subsite (+2), which is not advantageous to a polar glucose residue. The subsite maps confirmed that the binding region of Y151M was composed of four glycone- and two aglycone-binding sites. The subsite maps showed that Y151M had strikingly decreased binding energy at subsite (+2), where the mutation had occurred (−2.6 kJ/mol), compared to the binding energy at subsite (+2) of HSA (−12.0 kJ/mol). The methionine at position 151 would not provide the stacking interaction and the water-mediated hydrogen-bonding interaction provided by the tyrosine residue of HSA.[8] It was envisaged that the structural change at the aglycone binding site could improve the synthetic activity of HSA and *p*-nitrophenyl-glycosides would be better acceptors for Y151M than for HSA. Use of PNP as an aglycon is very advantageous because it makes detection and differentiation of reaction products more sensitive and unambiguous.

It is important to mention that the mutation decreased the specific activity for the hydrolysis of starch by 26.5% and the hydrolytic activity for shorter oligosaccharides, as well. In addition, an increase in K_m value for the mutant was observed, suggesting that the ground-state binding is altered, which may arise as a result of the decrease or absence of aromatic stacking interaction at subsite (+2). The decrease in the k_{cat} observed for Y151M is due to the destabilization of the transition state.[8]

Our interest was directed to explore the application of this modified novel enzyme for the synthesis of PNP 1-*S*-β-glucosides using maltotetraose[9] as donor and PNP 1-*S*-β-Glc as acceptor catalyzed by the mutant Y151M of HSA.

Initially, a wide range of glycosyl acceptors, such as *p*-nitrophenyl α-D-glucopyranoside (PNP α-Glc), β-D-glucopyranoside (PNP β-Glc), 1-thio-β-D-glucopyranoside (PNP 1-S-β-Glc), α-D-mannopyranoside (PNP α-Man), and α-D-xylopyranoside (PNP α-xyl), were utilized in transglycosylation experiments by Y151M. The PNP α-Glc, PNP β-Glc, and PNP 1-S-β-Glc acceptor products were formed with similar good yield. These demonstrated the versatility of this system for the aqueous-phase synthesis of oligosaccharides (data not shown). PNP 1-S-β-Glc was selected for further detailed investigations because of its stability against the hydrolysis activity of Y151M enzyme as well as the potential for chemical activation at the anomeric position in subsequent glycosylation reactions. In this regard, thioglycosides have proven to be powerful glycosylating agents in chemical syntheses by activation with specific thiophilic reagents.[10,11]

Detailed enzymological studies revealed that temperature was the most important variable for control of synthesis.

The temperature range covered in our investigations was between 37 and 8 °C, and the transfer products were monitored by HPLC. Distribution of products is shown in Table 1.

Table 1. Effect of Temperature on Transglycosylation[a]

	product distribution (area %)[b]			
temp (°C)	dimer	trimer	tetramer	conversion (%)
37	**59**	1		60
25	30	15	3.6	48.6
15	2	**16**	1.6	**19.6**
8	1.5	1	**15**	17.5

[a] Reaction conditions: 10 mM maltotetraose and 15 mM PNP 1-S-β-Glc; 25 mM Na-glycerophosphate buffer (pH 6.0), 6 h, 20 nM Y151M enzyme. [b] Area % measured by HPLC.

Enzymatic transglycosylation at 37 °C resulted in the dimer glycoside as the main product (59%). Dimer glycoside is an unlikely primary transfer product of the transglycosylation reaction; it might be a secondary hydrolysis product of the oligosaccharide glycosides as shown in Scheme 1. We determined that transglycosylation using Y151M mutant at 37 °C for 6 h is optimal for obtaining large quantity of PNP 1-thio-β-maltoside (PNP 1-S-β-Glc_2).

Reactions performed at 25 °C still favored the hydrolysis of the transglycosylated products, as suggested by the presence of significant amounts of PNP 1-S-β-Glc2. However, the release of PNP 1-S-β-Glc_3 and PNP 1-S-β-Glc_4 suggests the transfer of maltose and maltotriose units into the acceptor molecule. Figure 1 shows the typical HPLC profile of transfer products at 25 °C.

Although the total conversion decreased at 15 °C to only 19.6%, the major chemical product was PNP 1-S-β-Glc_3 (16%), which is significant considering the fact that chemical synthesis of PNP 1-S-β-Glc_3 is a multistep process leading to a moderate yield. The reaction conditions established here

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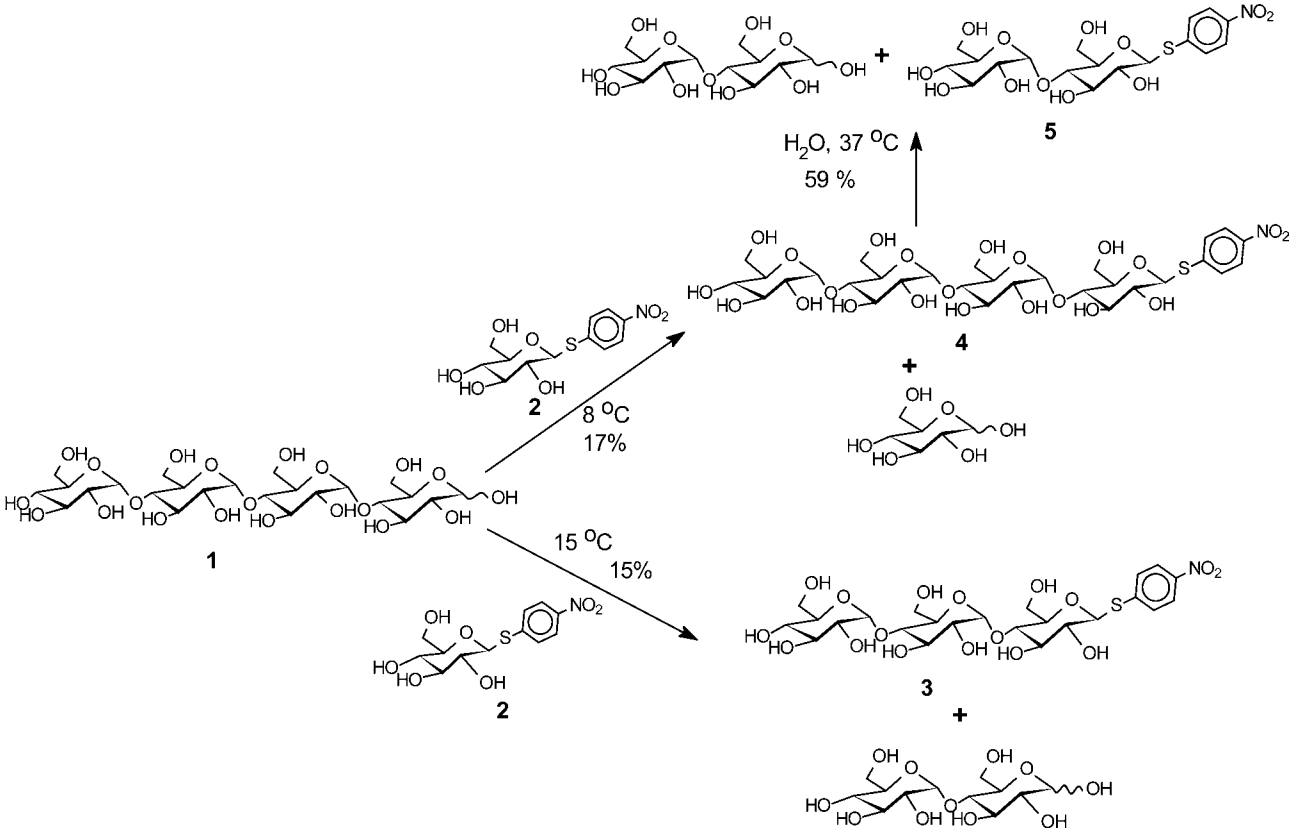
Scheme 1. Transglycosylation Procedure Catalyzed by Y151M[a]



[a] Selected spectroscopic data for **5**. Characteristic ^{1}H NMR data: δ 5.36 (d, 1H, H-1′, $J_{1'2'} = 3.5$ Hz); 5.02 (d, 1H, H-1, $J_{1,2} = 10.3$ Hz); 3.87 (m, H, H-3); 3.76 (m, 1H, H-4); 3.71 (m, 1H, H-3′); 3.60 (m, 1H, H-2′); 3.53 (m, 1H, H-2); 3.45 (m, 1H, H-4′). ^{13}C NMR data: δ 99.22 ppm (C-1′); 85.14 (C-1); 78.18 (C-4); 77.25 (C-3); 74.22 (C-3′); 71.25 (C-2′); 71.15 (C-2); 68.00 (C-4′); 60.38 (C-6); 60.05 (C-6′). MALDI-TOF MS: $(M + Na)^+$ $C_{18}H_{25}O_{12}NSNa$ calcd 502.10, measured 502.14.

provide an efficient, alternative way to obtain shorter PNP maltooligomers with a DP of 2−4. It is important to mention that PNP 1-S-β-Glc$_3$ is not a good substrate for Y151M and its hydrolysis at 15 °C is very slow.

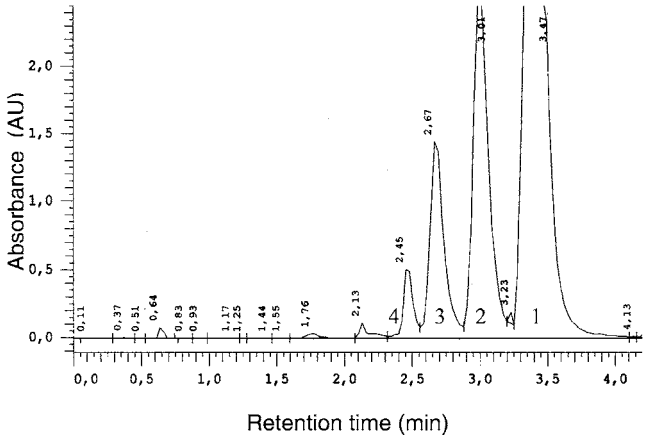


Figure 1. HPLC separation of transglycosylation products. Numbers 1, 2, 3, 4: DP of compounds. Reaction conditions: see Table 1. Chromatographic conditions: column ODS2, eluent MeCN/water 13:87, detection 302 nm.

To shift the transfer toward the synthesis of PNP 1-S-β-Glc$_4$ and reduce the secondary hydrolysis of transfer products, incubation was carried out at a lower temperature (8 °C) for 6 h.

At this lower temperature (8 °C), PNP 1-S-β-Glc$_4$ was formed in a yield of 15% as the major transfer product. Interestingly, the shorter oligomers were formed in much lower yields. The preferred formation of PNP 1-S-β-Glc$_4$ over PNP 1-S-β-Glc$_3$ could be explained by our earlier observations (data not shown) that temperature affects the cleavage pattern. Unlike room-temperature studies, the major productive binding mode at 8 °C is one in which subsite (+1) is occupied rather than (+2). Such a binding mode will release glucose rather than maltose from the aglycone binding site.[7] These results show that transglycosylation catalyzed by Y151M mutant is a highly preferable alternative method for the synthesis of PNP 1-S-β-Glc$_4$ substrate, as well.

The fate of the products of transglycosylation over time was analyzed, and the summary of the results is given in Figure 2.

Six hours of incubation time resulted in the tetrasaccharide as main product (16.5%). When the transglycosylation was allowed to proceed for a longer period of time (24 h), the reaction mixture contained a small amount of PNP 1-S-β-

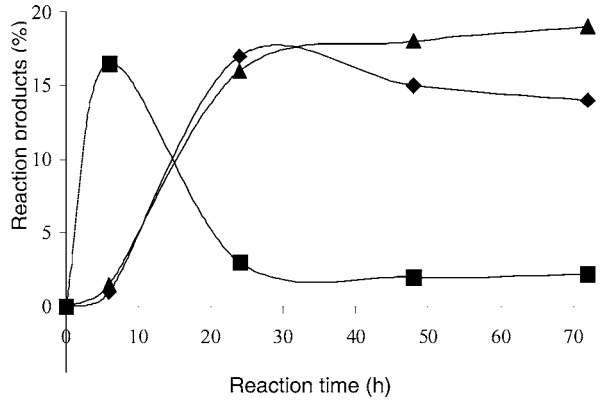


Figure 2. Time course of transglycosylation at 8 °C: (■) PNP 1-S-β-Glc_4, (♦) PNP 1-S-β-Glc_3, (▲) PNP 1-S-β-Glc_2.

Glc_4 (3%) and substantial quantities of the dimer (16%) glycosides, indicating an efficient hydrolysis of the tetramer product.

However, PNP 1-S-β-Glc_3 appears to be a stable transfer product at 8 °C and more resistant to hydrolysis by Y151M, such that even after 24 h only minimal hydrolysis occurred.

Our results show clearly that α(1−4) maltooligosaccharide glycosides longer than the dimer can be formed as transfer products. These oligomers are produced by glycosylation from PNP 1-S-β-D-glucopyranoside depending on the conditions of glycosylation. These results indicate that Y151M can be used to synthesize aryl β-glycosides DP 2−4 in one step.

Also, by altering the reaction time at 8 °C, we show that one can obtain either a tetramer exclusively or a mixture of dimer and trimer using Y151M with a relatively high yield (Figure 2), making this method more practical.

The stereospecificity and regiospecificity of the reaction carried out by Y151M was determined using the dimer glycoside. Since the dimer can be obtained in a very good yield because it is not hydrolyzed by Y151M enzyme, the products were separated by preparative HPLC on reversed-phase column using acetonitrile/water (13:87) as an eluent. Structural parameters of the separated compound were established by MALDI-TOF mass spectrometric data and ^{13}C and ^{1}H NMR spectroscopy analysis.

The identity of the product was verified to be the dimer PNP 1-S-β-Glc_2 on the basis of the good agreement between the calculated (502.10 Da) and measured (502.14 Da) molecular masses from the MALDI-TOF MS spectrum.

The anomeric configuration as well as the interglycosidic bond type were determined by ^{1}H and ^{13}C NMR spectra. The ^{1}H (500 MHz) and ^{13}C NMR (125.76 MHz) spectra were recorded with Bruker DRX-500 spectrometer in D_2O using DSS as internal reference.

The $^{1}D-^{1}H$ NMR spectrum revealed the presence of two doublets with 3.5 and 10.3 Hz J1,2 coupling constants. The 10.3 Hz value can be assigned to the β-glycosidic linkage; the other one belongs to the newly formed α interglycosidic bond. These data verified that Y151M mutated enzyme retained its α-stereoselectivity and did not alter the anomeric configuration of the acceptor molecule. These conclusions were supported by the chemical shift values of the C-1 and C-1′ in their ^{13}C NMR spectrum, which resonated at 85.14 and 99.22 ppm, respectively.

Concerning the interglycosidic bond types, the J-modulated ^{13}C spin−echo spectrum showed that C-6 and C-6′ are free. Knowing the proton chemical shift from all protons from the COSY spectrum in the disaccharide, the HSQC correlated spectrum showed that the C-4 has the highest chemical shift value (78.18 ppm) among the skeleton carbons with the exception of the two anomeric carbons. This confirms the presence of α(1→4) interglycosidic bond and suggests that Y151M used for glycosylation retained not only its stereospecificity but also its regioselectivity. In conclusion, the disaccharide glycoside can be described as an α-D-Glp(1→4)-β-D-Glp-(1→SPNP).

The transglycosylation procedure is summarized in Scheme 1. The enzyme is capable of transferring maltose and maltotriose on to PNP 1-S-β-Glc acceptor, resulting in PNP 1-S-β-Glc_3 and PNP 1-S-β-Glc_4 product, respectively. Enzymatic hydrolysis of PNP 1-S-β-Glc_4 leads to the formation of PNP 1-S-β-Glc_2. Dimer and trimer products are accumulated because of their very slow hydrolysis.

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