The Donor Substrate Spectrum of Recombinant Sucrose Synthase 1 from Potato for the Synthesis of Sucrose Analogues

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Supporting information for this article is available on the WWW under http://asc.wiley-vch.de or from the author.

Abstract: The donor substrate spectrum of recombinant sucrose synthase 1 (SuSy1) from potato was studied in order to synthesise novel sucrose analogues. With D-fructose as acceptor substrate SuSy1 accepts a variety of UDP-activated sugars, e.g., UDP-*N*-acetyl-α-D-glucosamine (UDP-GlcNAc, **2**) (100%), UDP-α-D-glucuronic acid (UDP-GlcA, **3**) (32%), UDP-α-D-xylose (UDP-Xyl, **4)** (39%), UDP-α-D-galactose (UDP-Gal, **5)** (23%), and UDP-*N*-acetyl-α-D-galactosamine (UDP-GalNAc, **6)** (23%). The kinetic analyses revealed that the non-natural donors **2** (k_{cat}/K_m 1.2 s^{-1} mM^{-1}) and **5** (k_{cat}/K_m 0.01 s^{-1} mM^{-1}) were relative poor substrates compared to UDP-Glc **1** (k_{cat}/K_m 310.4 s^{-1} mM^{-1}). UDP-GlcNAc was used in a preparative synthesis to produce 188 mg (0.5 mmol) 2-acetamido-2-deoxy-D-glucopyranosyl-β-D-fructofuranoside (**9**). The sucrose analogue **9** was not hydrolysed by invertase.

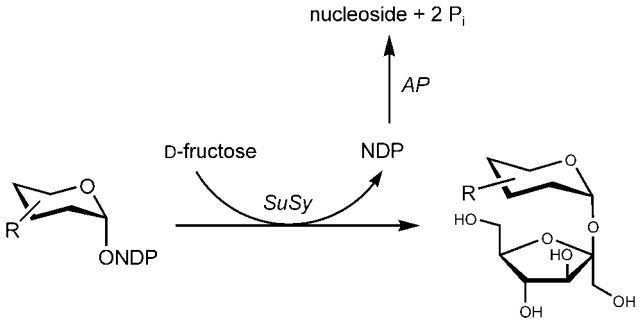
Keywords: enzyme catalysis; glycoconjugates; glycosides; sucrose analogues; sucrose synthase

The plant glycosyltransferase sucrose synthase (SuSy, EC 2.4.1.13) plays an important role in plant metabolism. SuSy provides the plant cell with activated sugar precursors for the biosynthesis of starch[1] and cellulose[2] by the cleavage of sucrose with nucleoside diphosphates. Among the Leloir glycosyltransferases, SuSy represents a unique enzyme because it catalyses *in vitro* also the readily reversible reaction, the synthesis of sucrose from UDP-Glc and D-fructose. SuSy shows a wide specificity for nucleoside diphosphates in the sucrose cleavage direction[3] and in the direction of sucrose synthesis.[4,5] This ability was recently utilised in the synthesis of the sucrose analogues 1′-deoxy-1′-fluoro-β-D-fructofuranosyl-α-D-glucopyranoside, [$^{13}C_1$]-β-D-fructofuranosyl-α-D-glucopyranoside, α-D-glucopyranosyl-α-L-sorbofuranoside, and α-D-glucopyranosyl-α-D-lyxopyranoside with recombinant SuSy1 from potato.[5]

In the present work, the donor substrate spectrum of recombinant SuSy1 from potato was explored for the synthesis of sucrose analogues (Scheme 1).

Figure 1 illustrates that the UDP-activated sugars and dTDP-Glc **7** were converted with D-fructose as acceptor. UDP-GlcNAc **2** was found to be the best non-natural donor substrate when compared with **1**. The relative activities for the other tested donor substrates were much lower. However, also UDP-Gal **5** and UDP-GalNAc **6**, the C4-epimers of **1** and **2**, as well as UDP-GlcA **3** were accepted. Previous data on SuSy from rice grains revealed only **2** and UDP-Xyl **4** as donor substrates.[6] SuSy from wheat germ accepted **4** and **5**.[7] The acceptance of dTDP-Glc **7** was already demonstrated for SuSy from various plants.[8] In our experiments the conversion of D-fructose with **7** as donor substrate was approx. half of that for **1** after 6 h (see supporting information). Obviously, the enzyme activity is lower when dTDP-activated sugars are used as donor substrates. In contrast, dTDP-6-deoxy-4-keto-Glc **8**, which was synthesised as described elsewhere,[9] does not serve as donor substrate for recombinant SuSy1 from potato in the synthesis direction. These results suggest that apart from uronic acids (**3**) and pentoses (**4**), deoxyhexoses are not accepted. In addition, the keto group in the C4-position of **8** also contributes to the lack of conversion.

The kinetic properties of the donor substrates **2** and **5** are summarised in Table 1. The *N*-acetyl group of **2** has only a weak influence on the enzyme affinity as



Scheme 1.

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indicated by the 2-fold higher K_m value in comparison to the natural donor **1**. However, the maximum velocity of the enzyme is significantly decreased resulting in a 300-fold decreased catalytic efficiency (k_{cat}/K_m). Surprisingly, the kinetic analysis of **5** revealed a 5-fold lower K_m value, however, the catalytic efficiency is decreased by a factor of 30,000 due to the very low enzyme activity. In contrast to **1**, both non-natural donors do not show any substrate inhibition. As a first conclusion, these data demonstrate the importance of the C4 configuration for the catalytic activity of SuSy1.

In a preparative synthesis the donor substrate **2** was converted with D-fructose as acceptor yielding 2-acetamido-2-deoxy-α-D-glucopyranosyl-β-D-fructofuranoside (**9**; Figure 2). With a synthesis yield of 98% and a space-time yield of 1.03 g L^{-1} d^{-1}, 0.83 mmol of the crude disaccharide product was obtained. The enzyme productivity was 0.8 mg U^{-1} of recombinant SuSy1. Product isolation gave 188 mg (0.5 mmol) of **9** with an overall yield of 59%. The product was characterised by ^1H and ^{13}C NMR. In comparison, **9** was previously synthesised chemically starting from sucrose. From sucrose in seven steps, an overall yield of 12.5% was obtained.[10] The sucrose analogue **9** was not hydrolysed by invertase, which makes it valuable for studies on signal transduction pathways[11] and sugar transporter[12] in plants.

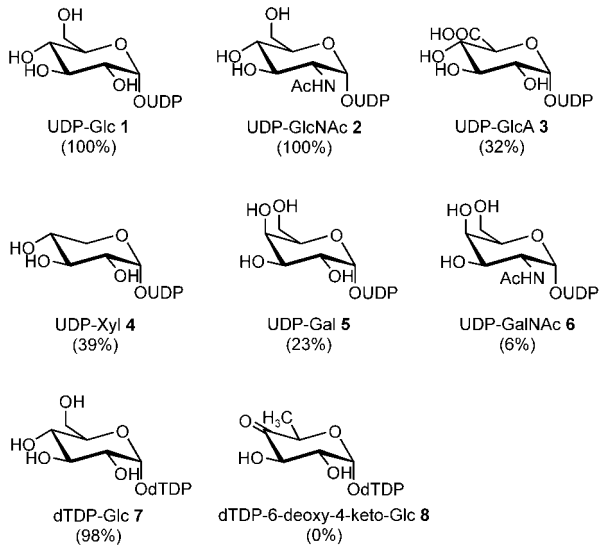

Figure 1.

In summary, our studies demonstrate that the repertoire of accessible sucrose analogues can be significantly extended by the broad donor substrate spectrum of recombinant SuSy1 from potato.

Experimental Section

Variation of Donor Substrates

Different UDP-activated sugars (**1** – **6**) (1 mM) were tested as donor substrates in the synthesis reaction of SuSy1 with D-fructose (2 mM) as acceptor substrate. The assay mixtures containing 1 U mL^{-1} SuSy1 and 1 U mL^{-1} alkaline phosphatase were incubated in 200 mM HEPES buffer at pH 8.0 for 16 h at 30 °C. The reaction was stopped by heating for 5 min at 95 °C. The formation of disaccharides was monitored by HPLC analysis as described by Römer et al.[5] The formation of UDP, UMP and uridine was observed by reversed-phase ion-pair HPLC[13] and capillary zone electrophoresis (CZE),[14] respectively.

The dTDP-activated sugars (**7**, **8**) (1 mM) were tested as described above. The assay mixtures, containing 1 U mL^{-1} SuSy1 and 1 U mL^{-1} alkaline phosphatase, were incubated in 100 mM Tris buffer, pH 8.0 at 30 °C. The course of reaction was followed by CZE analysing the formation of thymidine, dTMP and dTDP. The conditions for CZE are described in the supporting information.

Kinetic Data

All kinetic measurements in the synthesis direction were performed in 200 mM HEPES buffer, pH 8.0 at 30° C. The kinetic constants for **1** were determined as described by Römer et al.[5] The kinetic data of **2** and **5** were determined at a constant concentration of 10 mM D-fructose in the presence of

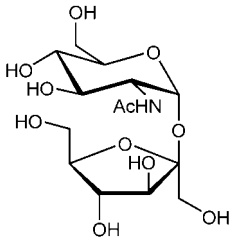

Figure 2.

Table 1. Kinetic constants of UDP-Glc **1**, UDP-GlcNAc **2**, and UDP-Gal **5** for the synthesis reaction of recombinant SuSy1 from potato. The kinetic data for **1** were taken from Römer et al.[5]

Substrate	K_m [mM]	K_{iS} [mM]	V_{max} [U mg^{-1}]	k_{cat} [s^{-1}]	k_{cat}/K_m [s^{-1} mM^{-1}]
1[a]	0.46	2.30	21.76	142.8	310.4
2[b]	0.76	–	0.14	0.9	1.2
5[b]	0.11	–	0.02	0.001	0.01

[a] The activity assay was performed without addition of alkaline phosphatase.
[b] The activity assay was performed with addition of alkaline phosphatase.

1 U mL^{-1} alkaline phosphatase (Roche Diagnostics, Mannheim). Initial rate measurements were obtained by allowing a maximum conversion of 10% of the variable substrate followed by HPLC detection of formed UDP or UMP and uridine in the presence of alkaline phosphatase. The kinetic constants were determined by a non-linear regression analysis of the data using the Michaelis-Menten equation: $V = (V_{max} \times S)/(S + K_m)$ or the kinetic equation for substrate inhibition: $V = (V_{max} \times S)/(S + K_m + S^2/K_{iS})$.

Synthesis of 2-Acetamido-2-deoxy-D-glucopyranosyl-β-D-fructofuranoside (9)

The synthesis of **9** was performed in a final volume of 130 mL using the fed-batch-technique. The reaction mixture containing 2 mmol of D-fructose and 0.05 mmol of **2** in 200 mM HEPES buffer, pH 8.0, was gently stirred at 30 °C after the addition 400 U recombinant SuSy1 and 800 U alkaline phosphatase. The course of reaction was monitored by HPLC analysis of the disaccharide as described above. After incubation for 0.5 h 0.8 mmol of **2** was added to the reaction mixture by a linear feed over 39 h. The batch was subsequently stirred for 16 h. The reaction was terminated by separating the enzymes from the reaction mixture by ultrafiltration. The synthesis yield for **9** was 0.83 mmol (98%). The raw product solution was adjusted to pH 8.6 and loaded onto an anion exchange column filled with Dowex 1x8 resin (Cl^- form, 100–200 mesh, 167 mL bed volume, Serva, Heidelberg), which was equilibrated with distilled water. The elution with distilled water (linear velocity: 30.5 cm h^{-1}) resulted in a product pool which was concentrated by evaporation under vacuum to a final volume of 15 mL. The disaccharide (aliquots of 5 mL product solution) was further purified by gel filtration on a Bio-Gel P2 resin column (extra fine, 500 mL bed volume, BioRad, Munich). Elution with 10 mM ammonium formate, pH 7.0, (linear velocity: 2.4 cm h^{-1}) gave the disaccharide containing fractions which were pooled and lyophilised. For the sucrose analogue **9** an overall yield of 59% (0.50 mmol, 188 mg) was obtained. ^{1}H NMR(500 MHz, D_2O; Bruker, DRX 500): $\delta = 1.89$ (CH_3 from Ac, s); 3.35 (1′-H_a, d), 3.44 (1′-H_b, d), 3.89 (2′-H, t), 3.87 (3′-H, t), 4.06 (4′-H, d), 3.61–3.73 (5′-$H_{a,b}$, 6-$H_{a,b}$, 2-H, 3-H, 5-H, 6-$H_{a,b}$, m), 5.22 (1-H, d), 3.39 (4-H, d); $^3J_{1,2} = 3.7$ Hz, $^3J_{3,4} = {}^3J_{4,5} = 8.9$ Hz, $^3J_{3',4'} = {}^3J_{4',5} = 9.1$ Hz, $^4J_{1',1'} = 12.1$ Hz; ^{13}C NMR (125 MHz, D_2O): $\delta = 91.09$ (C-1), 81.68 (C-5′), 76.01 (C-3′), 74.10 (C-4′), 72.79 (C-5), 70.63 (C-3), 69.97 (C-4), 62.59 (C-6), 61.20 (C-1′), 60.43 (C-2′), 54.16 (C-2), 21.21 (CH_3 from Ac).

Invertase Assay

The sucrose analogue **9** was tested as a substrate for yeast invertase (Sigma, Deisenhofen) and compared to the natural substrate sucrose. Invertase catalyses the hydrolysis of sucrose to yield D-glucose and D-fructose. A photometric invertase assay was used where D-glucose and D-fructose are determined by a combined enzymatic test with hexokinase, phosphoglucose isomerase and glucose 6-phosphate dehydrogenase.[15] The conversion of $NADP^+$ to $NADPH + H^+$ was measured at 340 nm. The assay was carried out by incubation of 80 U mL^{-1} invertase (Sigma, Deisenhofen), 3 U mL^{-1} hexokinase (Roche Diagnostics, Mannheim), 3.3 U mL^{-1} phosphoglucose isomerase (Roche Diagnostics, Mannheim) and 1.5 U mL^{-1} glucose 6-phosphate dehydrogenase (Roche Diagnostics, Mannheim) in 0.1 M MOPS (final volume 130 μL) containing 5 mM $MgCl_2$, 1 mM ATP and 0.4 mM $NADP^+$, and 0.1 mM sucrose or sucrose analogue at pH 6.0 and 25 °C. The linear increase of absorption over time (ΔE min^{-1}) was observed at 340 nm giving initial rate measurements. The half initial rate of invertase for sucrose as substrate was set to 100% and compared to the substrate **9**.

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