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Characterization and application of recombinant sucrose synthase 1 from potato for the synthesis of sucrose analogues

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Abstract

The characteristics and application of recombinant sucrose synthase 1 (SuSy1) from potato for the synthesis of sucrose analogues are described. With UDP-Glc as donor substrate SuSy1 accepts a variety of ketoses, e.g. 1-deoxy-1-fluoro-D-fructose (100%), D-psicose (7%), L-sorbose (18%) and D-xylulose (7%), as well as aldoses, e.g. D-lyxose (22%) and L-mannose (16%). The enzyme also shows a flexible the donor substrate spectrum with acceptance of UDP-GlcNAc (100%), UDP-GlcA (32%), UDP-Gal (23%), UDP-GalNAc (6%) and UDP-Xyl (39%). The kinetic analysis revealed a substrate inhibition by UDP-Glc with a K_m- and K_{iS}-value of 0.46 mM and 2.25 mM, respectively and a V_{max} of 21.76 U/mg. A substrate inhibition was also obtained for D-fructose with a K_m- and a K_{iS}-value of 2.05 mM and 35.88 mM, respectively, and a V_{max} of 10.63 U/mg at a constant concentration of 2 mM UDP-Glc. Susy1 was used for the preparative synthesis of 1´-deoxy-1´-fluoro- b-D-fructofuranosyl-a-D-glucopyranoside and [$^{13}C_1$]-b-D-fructofuranosyl-a-D-glucopyranoside. Both products were characterized by ^{1}H-, ^{13}C-NMR and in case of of 1´-deoxy-1´-fluoro-b-D-fructofuranosyl-a-D-glucopyranoside also with ^{19}F-NMR.

Introduction

The plant glycosyltransferase sucrose synthase (SuSy, EC 2.4.1.13) plays an important role in the metabolism of sucrose and its conversion to polysaccharides. SuSy catalyzes the cleavage of sucrose with nucleoside diphosphates and provides the plant cell with activated sugar precursors for sucrose-starch transformation (Zrenner *et al.* 1995) and for cell wall synthesis (Armor *et al.* 1995). SuSy represents an unique case among the Leloir glycosyltransferases by catalyzing *in vitro* the cleavage of sucrose with nucleoside diphosphate yielding D-fructose and nucleotide-activated D-glucose and the readily reversible reaction. It has been shown for developing potato tubers and other plant tissues that the reaction is also reversible *in vivo* (Geigenberger and Stitt, 1993). In contrast to other enzymes of the sucrose metabolism SuSy shows a wide specificity for nucleoside diphosphates in the sucrose cleavage direction (Pontis, 1977). In direction of sucrose synthesis the enzyme shows also a wide specificity (Elling *et al.*, 1993, Bean and Hassid, 1955). This ability was utilized for the synthesis of the sucrose analogues 2O-6-deoxy-a-L-sorbofuranosyl-D-glucose (Peters et al. 1993), a-D-glucopyranosyl-b-D-xylulofuranoside and a-D-glucopyranosyl-a-D-lyxopyranoside (Grothus *et al.*, 1994) with SuSy purified from rice grains. Card *et al.* (1984, 1986) used sucrose synthase in the course of studies on a sucrose carrier protein for the synthesis of fluoro- and azido-analogues of sucrose.

The present paper presents the kinetic data of recombinant sucrose synthase 1 from poatato for the synthesis direction and the substrate spectrum with different acceptor and donor substrates. Two sucrose analogues, 1´-deoxy-1´-fluoro-b-D-fructofuranosyl-a-D-glucopyranoside **1** and [$^{13}C_1$]-b-D-fructofuranosyl-a-D-glucopyranoside **2** were synthesized following the scheme in **Figure 1**. Both products were characterized by ^{1}H and ^{13}C-NMR. Work is in progress to utilize **1** and **2** for studies on sugar sensing in plants (Sinha *et al.*) as well as on sugar transport in plants by *in vivo*-NMR techniques (Köckenberger, in preparation).

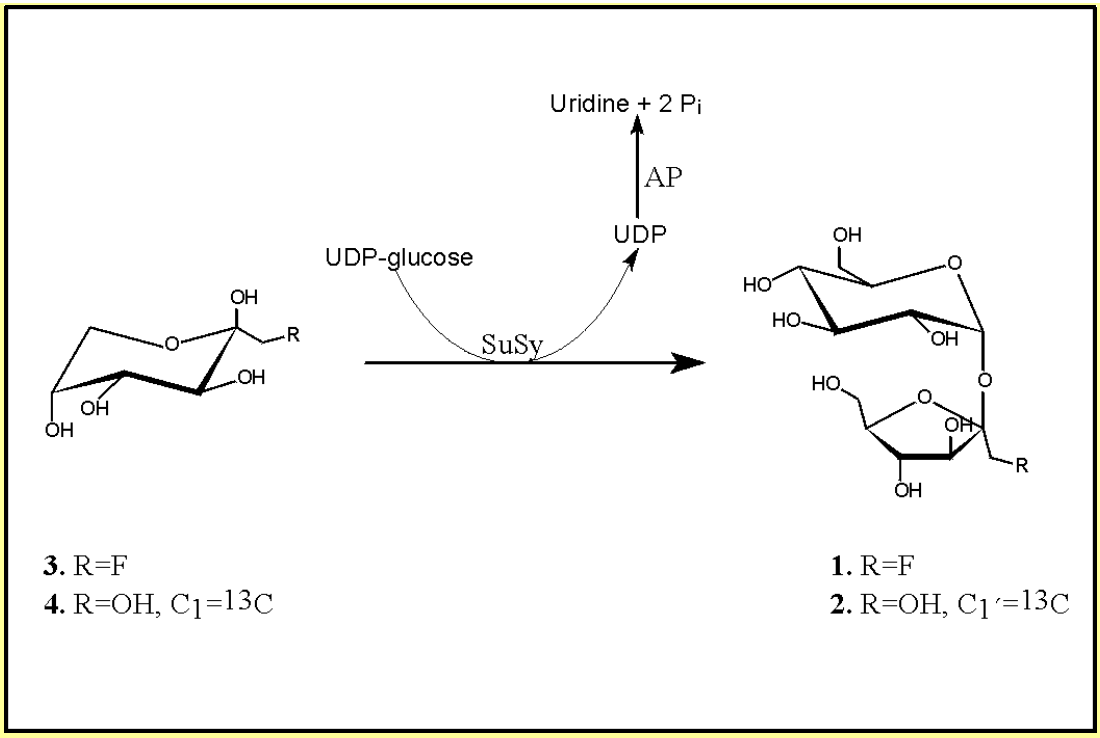

Figure 1. Enzymatic synthesis of 1´-deoxy-1´-fluorosucrose **1** and [$^{13}C_1$]-b-D-fructofuranosyl-a-D-glucopyranoside **2** with recombinant SuSy1 from potato and alkaline phsphatase (AP).

Materials and methods

Recombinant sucrose synthase 1 from potato was obtained by constitutive expression of the *sus1* gene from potato in *Saccharomyces cerevisiae*. The production, purification, and biochemical characterization of SuSy1 will be described elsewhere (Römer *et al.*, manuscript in preparation).

Enzyme assay

The enzyme units used for the synthesis of sucrose analogues refer to enzyme activity determination in the direction of sucrose cleavage. In a total volume of 1 ml HEPES buffer (50 mM), pH 7.6, recombinant SuSy1 from potato was incubated with 2 mM UDP and 500 mM sucrose for 10 min at 30° C (Zervosen *et al.* 1998, Zervosen and Elling 1999). The reaction was stopped by heating at 95° C for 5 min. The formation of UDP-glucose was determined by HPLC (Ryll and Wagner, 1991).

Kinetic data

In the synthesis direction the kinetic constants for UDP-glucose at a constant concentration of 10 mM D-fructose and for D-fructose at a constant concentration of 2 mM UDP-Glc were determined in 50 mM HEPES buffer, pH 8.0 at 30° C, respectively. Initial rate measurements were obtained by allowing a maximum conversion of 10% of the variable substrate following detection of formed UDP by HPLC (Ryll and Wagner, 1991). The kinetic constants were calculated by a non-linear regression analysis of the data using the kinetic equation for a substrate inhibition: $V=(V_{max} \bullet S)/(S+K_m+S^2/K_{iS})$ and the program MicroMath®Scientist®, Version 2.0 (MicroMath, Inc.).

Variation of acceptor substrates

Different aldoses and ketoses (10 mM) were tested as acceptor substrates in the synthesis reaction of SuSy1 with UDP-Glc(1 mM) as donor substrate. The assay mixtures containing 200 mU/ml SuSy1 and 1 U/ml alkaline phosphatase (Roche Diagnostics, Mannheim) were incubated in 50 mM HEPES buffer, pH 8.0, for 16 h at 30° C. The reaction was stopped by heating for 5 min at 95° C. The formation of disaccarides was detected by HPLC analysis using an Aminex HPX-87C column (300 x 7.8 mm, BioRad, Munich) and elution with distilled water at 85° C. The formation of UDP, UMP and uridine was also followed by HPLC with the method of Ryll and Wagner (1991).

Variation of donor substrates

Different UDP-activated sugars (1 mM) were tested with the natural acceptor d-fructose (2 mM). The assay mixtures containing 1 U/ml SuSy1 and 1 U/ml alkaline phosphatase in 50 mM HEPES buffer, pH 8.0, were incubated for 16 h at 30° C. The assays and appropriate controls were

followed by HPLC analyses as described above.

Synthesis of 1´-deoxy-1´-fluorosucrose (1-deoxy-1-fluorofructofuranosyl- a-D-glucopyranoside) 1

1-Deoxy-1-fluoro-D-fructose **3** was obtained by the reaction described by Card and Hitz (1984). In brief, the readily available 2,3:4,5-di-o-isopropylidene-D-fructopyranose was converted into the trifylate by the procedure described by Binkley *et al*. (1980). Tryfylate was fluorinated by TASF (tris(dimethylamino)sulfur(trimethylsilyl)-difluoride, Aldrich Chemicals) in refluxing tetrahydrofuran. After removal of the isopropylidene protection groups, 1-deoxy-1-fluoro-D-fructose was obtained as a syrup in 75% yield. The synthesis of 1'-deoxy-1'-fluorosucrose was carried out by the repetitive-batch-technique (Kragl *et al.* 1993). The reaction mixture (100 ml) containing 0.96 mmol 1-deoxy-1-fluorofructose (176 mg) and 1 mmol UDP-a-D-glucose (Sigma, Deisenhofen) in 200 mM HEPES buffer, pH 8,0, was gently stirred at 30 °C after the addition of 40 U recombinant SuSy1 from potato and 200 U alkaline phosphatase (Roche Diagnostics, Mannheim). The course of the reaction was controlled by HPLC analysis of the product with an Aminex HPX-87C column as described above. After 48 h the enzymes were recovered by ultrafiltration and used in a second and third batch, respectively, by the addition of new substrates. The yield of the combined product solutions was 2.9 mmol (100%) for 1´-deoxy-1´-fluorosucrose. Prior to isolation the product solution was adjusted to pH 8.6 and loaded onto an anion exchanger column ($HCOO^-$-form) filled with AG 1-X8 resin (100-200 mesh, 122 ml bed volume, BioRad, Munich), which was equilibrated with distilled water. Elution with distilled water (linear flow-rate: 56.5 cm/h) gave a product pool, which was concentrated by *in vacuo* evaporation to a final volume of 5 ml. The disaccharide was further purified by chromatography on a AG 50W-X8 resin column (200-400 mesh, Ca^{2+}-form, 1532 ml bed volume, BioRad, Munich). Elution with distilled water (linear flow rate: 3 cm/h) gave the fractions containing the disaccharide, which were pooled and lyophilized. The dry product was dissolved in 10 ml absolute methanol and crystallized at 25° C. 1´-deoxy-1´-fluorosucrose was obtained in an overall yield of 85% corresponding to 2.5 mmol (860.5 mg) with a HPLC purity of 89%. NMR spectroscopy (11.7T) of 1'-deoxy-1'-fluorosucrose revealed the typical couplings between ^{19}F and ^{1}H or ^{13}C: ^{1}H-NMR (D_2O): H_1, d:4.39 ppm, m, J_{1H-19F}: 46.6 Hz, J_{1H-1H}: 10.4 Hz; ^{19}F-NMR (D_2O): d_{CFCl3}: -229.4 ppm, m, J_{19F-1H}: 46.6 Hz.; ^{13}C-NMR (D_2O): C_1, d:80.7 ppm, d, $J_{13C-19F}$: 174.2 Hz; C_2, d:101.8 ppm, d, $J_{13C-19F}$: 19.6 Hz.

Synthesis of [$^{13}C_1$]-b-d-fructofuranosyl-a-D-glucopyranoside 2

The synthesis was also carried out with the repetitive-batch-technique. The reaction mixture (100 ml) containing 1 mmol [$^{13}C_1$]-b-D-fructofuranoside **4** (Euriso-top, Gif-Sur-Yvette, France) and 1 mmol UDP-a-D-glucose (Sigma, Deisenhofen) in 200 mM HEPES buffer, pH 8.0, was gently stirred at 30 °C after the addition of 20 U recombinant SuSy1 from potato and 200 U alkaline phosphatase. The course of reaction was controlled by HPLC. After 24 h the enzymes were recovered by ultrafiltration and used in the following three batches, respectively, by the addition of new substrates. The yield of the combined product solutions was 3.78 mmol (94%) for [$^{13}C_1$]-b-D-fructofuranosyl-a-D-glucopyranoside. Prior to isolation the product solution was adjusted to pH 8.6 and loaded onto an anion exchanger column (Cl^--form) filled with Dowex 1X2 resin (100-200 mesh, 400 ml bed volume (Serva, Heidelberg)), which was equilibrated with distilled water. Elution with distilled water (linear flow-rate: 22 cm/h) gave a product pool, which was concentrated by *in vacuo* evaporation to a final volume of 16 ml. The disaccharide (aliquots of 2 ml product solution) was further purified by chromatography on a Bio-Gel P2 resin column (extra fine, 500 ml bed volume, BioRad, Munich). Elution with distilled water (linear flow rate: 5 cm/h) gave the disaccharide containing fractions, which were pooled, combined and lyophilized. [$^{13}C_1$]-b-D-fructofuranosyl-a-D-glucopyranoside was obtained in an overall yield of 55% corresponding to 2.07 mmol (712 mg) with a HPLC purity of 89%. NMR: ^{13}C-NMR(D_2O): C_1, d:62,2 ppm, s; ^{1}H-NMR(D_2O): H_1, d:3.56ppm, dd, J_{1H-13C}:144 Hz; H_1 d:5.33ppm, dd, J_{1H-13C}:169 Hz;

Results and Discussion

Kinetic constants

The kinetic constants for the sucrose synthesis reaction of SuSy1 from potato are summarized in **Table 1**. Both substrates shows a substrate inhibition (**Figure 2**, **Figure 3**). The K_{iS} for D-fructose and UDP-Glc is 35.88 mM and 2.3 mM, respectively. The V_{max} value for D-fructose as variable substrate was only half of the value determined for UDP-Glc, because SuSy1 is already inhibited at 2 mM UDP-Glc.

Table 1. Kinetic constants for recombinant SuSy1 from potato for the sucrose synthesis direction.

	K_m [mM]	**K_{iS} [mM]**	**V_{max} [U mg^{-1}]**
UDP-glucose	**0.46± 0.04**	**2.30± 0.22**	**21.76± 4.74**

D-fructose	**2.05 ± 0.17**	**35.88± 2.31**	**10.63± 0.31**

In comparison to enzyme preparations from potato (**Table 2**) recombinant SuSy1 shows a 3.5 – 13.5 fold higher affinity for UDP-Glc. The K_m-value for D-fructose is comparable to those determined by Pressey (1969) and Murata (1972), however, a substrate inhibition for the substrates were not found. Doehlert (1987) described a substrate inhibition of D-fructose above 20 mM for SuSy from maize endosperm. Nakai *et al.* (1997) found for a recombinant sucrose synthase from mung bean seedlings in *E. coli* a K_m-value for D-fructose of 7.72 mM and for UDP-Glc a K_m of 0.4 mM.

Table 2. Kinetic constants for SuSy isolated from potato tubers.

	K_m [mM]	
	UDP-Glc	**D-fructose**
Slabnik *et al.* (1968)	**1.65**	**5.9**
Pressey (1969)	**6.2**	**1.6**
Murata (1972)	**2.0**	**1.4**

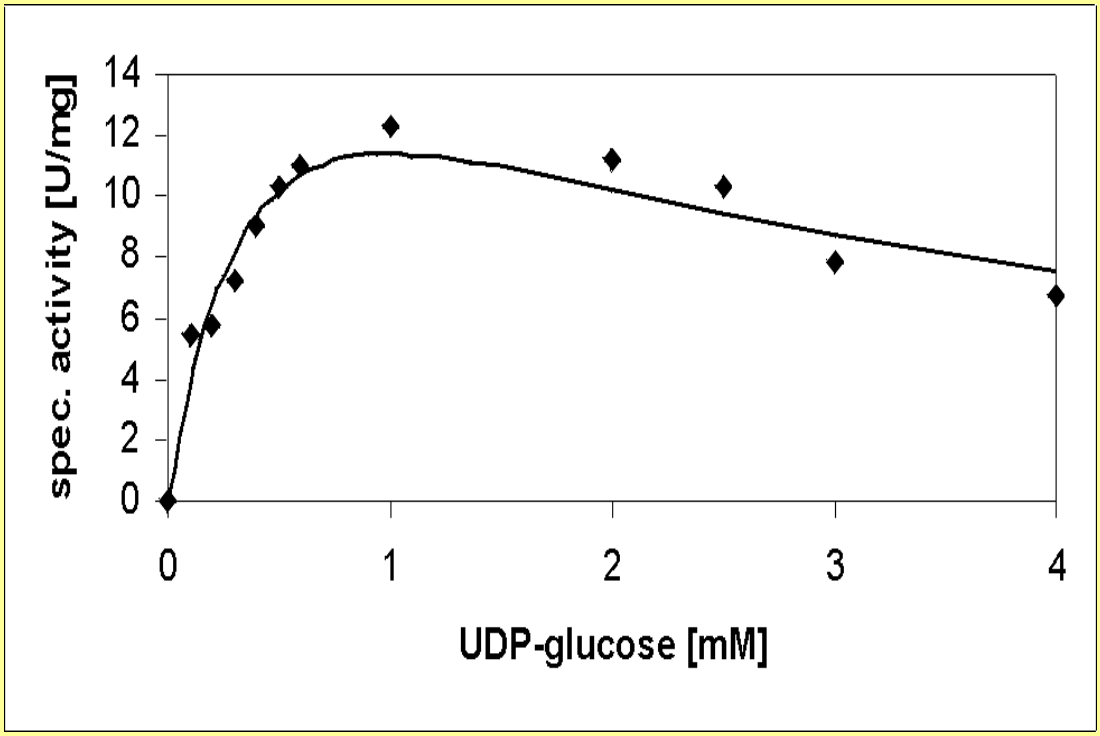

Figure 2. UDP-Glucose kinetic of recombinant SuSy1 from potato with d-fructose (10 mM) and 3.63 U mg^{-1} enzyme.

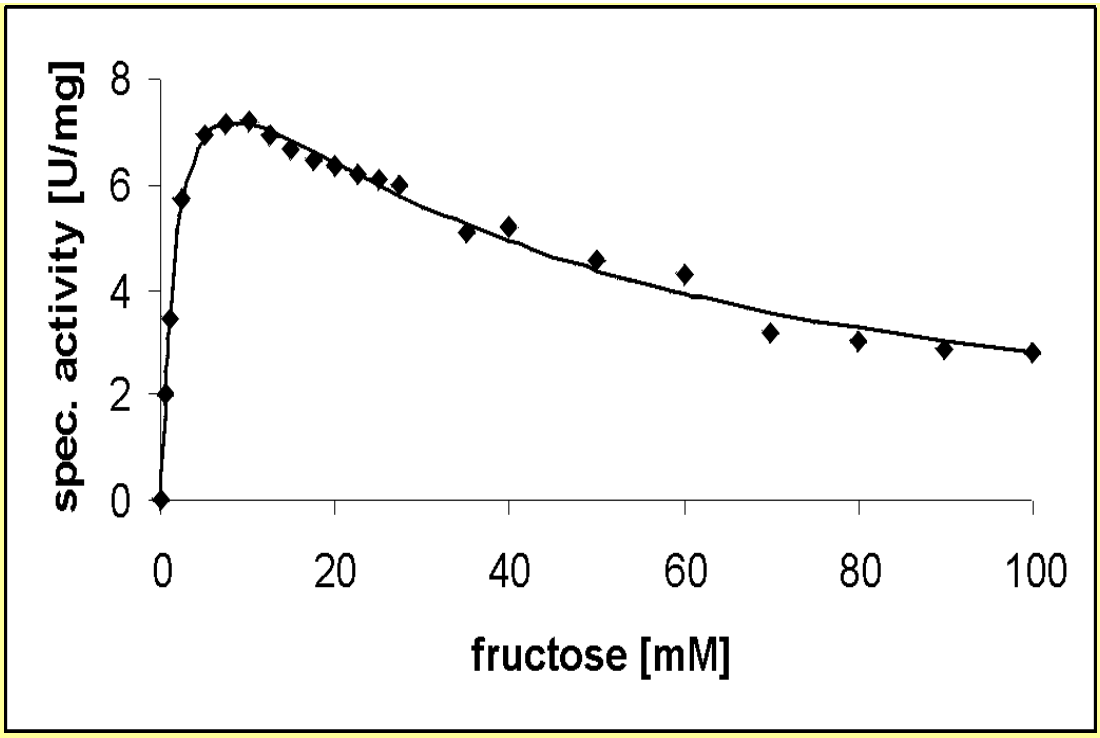

Figure 3. D-Fructose kinetic of recombinant SuSy1 from potato with UDP-Glc (2 mM) and 18.18 U mg^{-1} enzyme.

Variation of the acceptor substrate

Different ketoses and aldoses were tested as acceptor substrates for SuSy1 with the natural donor UDP-Glc. **Figure 4** shows that 1-deoxy-1-fluoro-D-fructose, D-psicose, L-sorbose, D-xylulose and D-tagatose are accepted. Previous studies with SuSy isolated from rice grains gave similar results (Elling *et al.*, 1993).

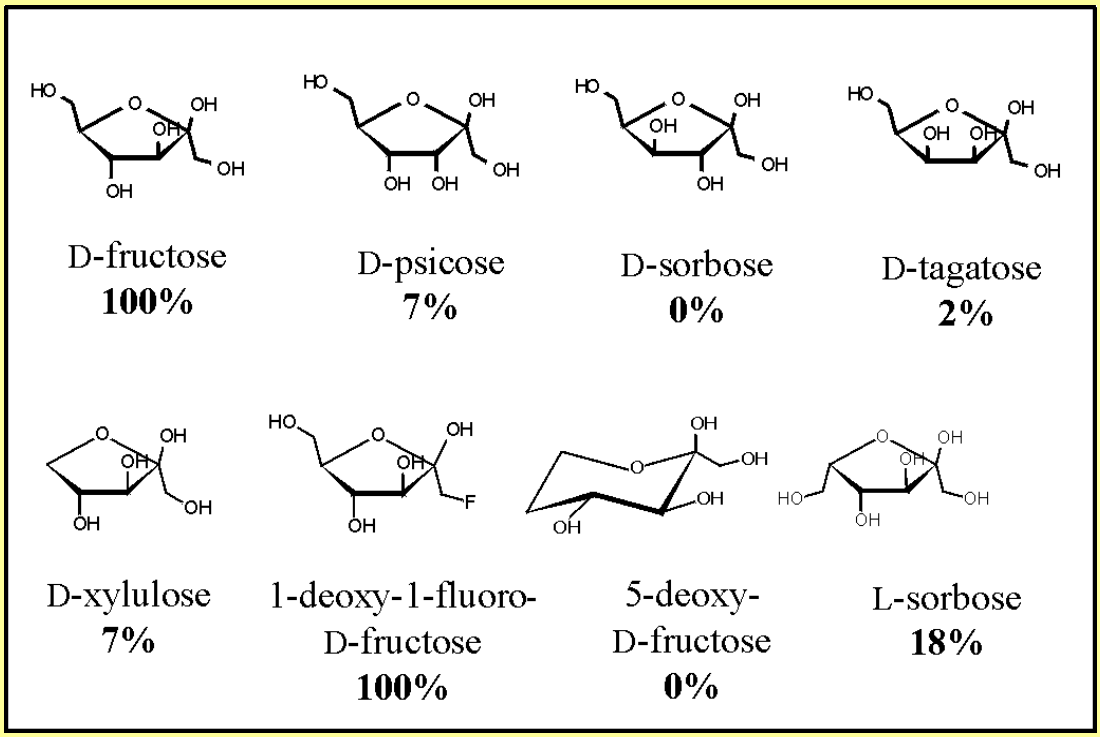

Figure 4. Acceptor substrate spectrum (ketoses) of recombinant SuSy1 from potato.

The acceptance of L-sorbose, D-xylulose and D-tagatose was described by sucrose synthase from green peas (Bean and Hassid, 1955) and sugar beet roots (Avigad and Millner, 1966). D-sorbose and 5-deoxy-D-fructose are not accepted by SuSy1 which indicate that the configuration at C_3 and C_4 of the furanose ring and the conformation of the sugars (furanose vs. pyranose) are important elements for ketoses to be accepted as substrates. In the latter case 5-deoxy-D-fructose is only present as pyranose, which is not accepted by the enzyme. This is in contrast to the fact that ketoses which are accepted by SuSy, e.g. L-sorbose and D-tagatose, are predominantly present in the pyranose form in aqueous solutions. Most interestingly, SuSy accepts also some aldoses (**Figure 5**). Apart from the anomeric centers D-lyxose and L-mannose show similar stereospecific and isosteric features when compared to the pyranose conformations of D-fructose. SuSy isolated from rice grains accepts only D-lyxose, L-arabinose and D-mannose as acceptors (Elling *et al.* 1993). With D-lyxose the non-reducing disaccharide a-D-glucopyranosyl-a-D-lyxopyranoside was obtained (Grothus *et al.*, 1994). Work is in progress to characterize the disaccharide products synthesized with recombinant SuSy1.

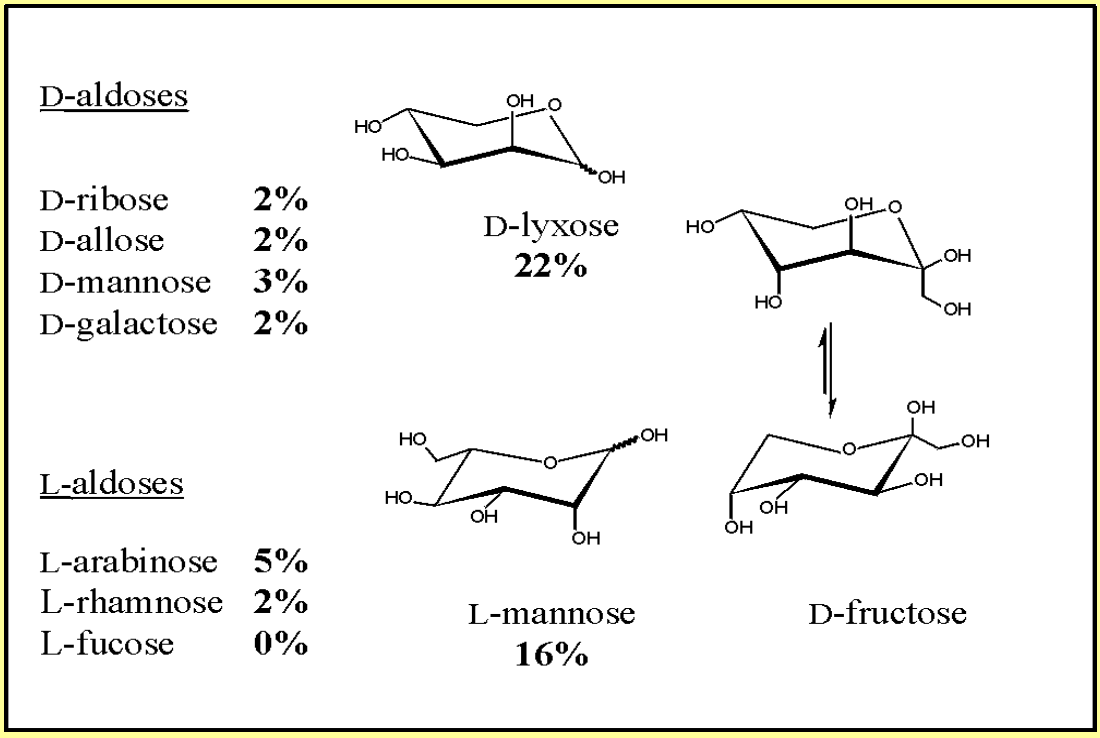

Figure 5. Acceptor substrate spectrum (aldoses) of recombinant SuSy1 from potato.

Variation of the donor substrate

Recombinant SuSy1 accepts a wide spectrum of different UDP-activated sugars (Table 3). UDP-*N*-acetylglucosamine (UDP-GlcNAc) can fully substitute the natural donor substrate UDP-Glc. SuSy from rice grains accepts also UDP-GlcNAc and UDP-xylose (Elling *et al.*, 1993). In previous studies on SuSy from wheat germ UDP-galactose and UDP-xylose were donor substrates, however, no reaction was observed with UDP-GlcNAc (Cardini *et al.*, 1955, Cardini and Recondo, 1962). Further studies are now in progress in order to determine the affinity of SuSy1 for these and other nucleotide sugars.

Table 3. Substrate specificity of recombinant SuSy1 from potato. Different UDP-activated sugars were tested with D-fructose as acceptor.

Substrate	relative activity [%]
UDP-glucose	**100**
UDP-*N*-acetylglucosamine	**100**
UDP-xylose	**39**
UDP-glucuronic acid	**32**
UDP-galactose	**23**

UDP-*N*-acetylgalactosamine	**6**

Synthesis of 1´-deoxy-1´-fluorosucrose 1 and [$^{13}C_1$]-b-D-fructofuranosyl-a-D-gluco-pyranoside 2

Card and Hitz (1984) synthesized 1´-deoxy-1´-fluorosucrose **1** with SuSy from barley seeds and obtained an overall yield of 59% (507 mg). In the present paper we could improve the synthesis with reference to the enzyme productivity by repetitive use of recombinant SuSy1 and alkaline phosphatase. The synthesis yield after three batches was 100% with reference to the acceptor substrate. After purification an overall yield of 85% (860.5 g) for 1´-deoxy-1´-fluorosucrose was obtained. The analysis by NMR confirmed the structural integrity of the product as described previously (Card and Hitz 1984). [$^{13}C_1$]-b-D-fructofuranosyl-a-D-glucopyranoside **2** was already synthesized by Duker and Serianni (1993) with an overall yield of 25% (85.6 mg) using 50 U SuSy. We could also improve this synthesis by use of the repetitive-batch-technique. With 20 U SuSy and 200 U alkaline phosphatase a synthesis yield of 94% with reference to [$^{13}C_1$]-b-D-fructofuranoside was obtained after four batches. Product isolation gave 712 mg [$^{13}C_1$]-b-D-fructofuranosyl-a-D-glucopyranoside with an overall yield of 51%. The structure was confirmed by NMR.

Conclusions

The high flexibility of recombinant Susy1 from potato for the acceptance of different acceptor and donor substrates will bring up a whole library of sucrose analogues which are valuable tools for different biochemical studies.

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