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Transglucosylation potential of six sucrose phosphorylases toward different classes of acceptors

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ABSTRACT

In this study, the transglucosylation potential of six sucrose phosphorylase (SP) enzymes has been compared using eighty putative acceptors from different structural classes. To increase the solubility of hydrophobic acceptors, the addition of various co-solvents was first evaluated. All enzymes were found to retain at least 50% of their activity in 25% dimethylsulfoxide, with the enzymes from *Bifidobacterium adolescentis* and *Streptococcus mutans* being the most stable. Screening of the enzymes' specificity then revealed that the vast majority of acceptors are transglucosylated very slowly by SP, at a rate that is comparable to the contaminating hydrolytic reaction. The enzyme from *S. mutans* displayed the narrowest acceptor specificity and the one from *Leuconostoc mesenteroides* NRRL B1355 the broadest. However, high activity could only be detected on L-sorbose and L-arabinose, besides the native acceptors D-fructose and phosphate. Improving the affinity for alternative acceptors by means of enzyme engineering will, therefore, be a major challenge for the commercial exploitation of the transglucosylation potential of sucrose phosphorylase.

1. Introduction

Glycosylation can significantly influence the properties of a chemical compound.[1] Indeed, a glycosyl moiety can improve the solubility, stability, flavor, and pharmacokinetic behavior of a molecule, or can simply be crucial for its biological activity. Glycosides are synthesized either chemically or by enzymatic glycosylation with glycosyl transferases, transglycosidases, glycoside phosphorylases, and glycoside hydrolases.[2] Among the glycoside phosphorylases, sucrose phosphorylase (SP, E.C. 2.4.1.7) is an attractive biocatalyst because of its broad acceptor promiscuity. The enzyme is classified in the α-amylase family (GH-13) and catalyzes the reversible phosphorolysis of sucrose into α-D-glucose-1-phosphate (α-Glc-1-P) and D-fructose.[3–6] Biochemical studies have revealed that SP follows a double displacement mechanism, passing through a covalent glucosyl-enzyme intermediate with final retention of the anomeric configuration.[7–10] In addition, the crystal structure of the SP from *Bifidobacterium adolescentis* has been elucidated[11,12] and the catalytic residues have been identified by mutational analysis.[13–17]

The acceptor promiscuity of SP was first recognized in 1944 by Doudoroff and colleagues, who could use the enzyme for the synthesis of α-glucopyranosyl L-sorboside.[18] Since then, it has been shown that SP can transfer a glucosyl moiety to a wide variety of mono-, di- and trisaccharides.[19,20] Furthermore, non-carbohydrate molecules like phenolic compounds and furanones can also be used as an acceptor.[21,22] In contrast to its broad range of acceptors, SP is highly specific for the transfer of a glucosyl moiety and does not tolerate structural modifications on the glucopyranosyl ring.[23] Besides sucrose and α-Glc-1-P, only α-D-glucose-1-fluoride is known to be an efficient glucosyl donor for this enzyme.[8,24]

A major limitation of the already reported data about the acceptor promiscuity of sucrose phosphorylase is that the transglucosylation activity is usually expressed as transfer ratio or yield, which is the ratio of the transfer product (mol) formed against the initial amount of acceptor (mol).[25] This parameter does, however, reflect the thermodynamic equilibrium of the substrate and product, and does not provide any information about the efficiency of the enzyme itself. To address this problem, the initial reaction rate of SP on a variety of acceptors has been determined in this study. Six different enzymes have been compared to allow an evaluation of their respective acceptor promiscuities. Because several of the acceptors do not dissolve well in water, the activity of the SP enzymes in the presence of various co-solvents has also been determined.

2. Results and discussion

2.1. Enzyme expression and characterization

In the carbohydrate-active enzyme (CAzy) database, around 280 putative sucrose phosphorylases have been assigned to the family

Abbreviations: SP, sucrose phosphorylase; LmSP1, *L. mesenteroides* NRRL B1149 SP; LmSP2, *L. mesenteroides* ATTC 12291 SP; LmSP3, *L. mesenteroides* NRRL B1355 SP; SmSP, *S. mutans* SP; LaSP, *L. acidophilus* SP; BaSP, *B. adolescentis* SP.

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Table 1
The UniProt IDs for the different SP enzymes

Enzyme	Strain	UniProt ID	Reference
LaSP	*Lactobacillus acidophilus* LMG 9433	Q7WWP8	51
SmSP	*Streptococcus mutans* LMG 14558^T	P10249	52
BaSP	*Bifidobacterium adolescentis* LMG 10502^T	Q84HQ2	38
LmSP1	*Leuconostoc mesenteroides* NRRL B1149	Q14EH6	20
LmSP2	*Leuconostoc mesenteroides* ATTC 12291	Q59495	53
LmSP3	*Leuconostoc mesenteroides* NRRL B1355	—	—

GH-13, subfamily 18.[26] These mainly originate from lactic acid bacteria, but also include soil bacteria and inhabitants of the gastro intestinal tract like *Bifidobacterium*. Six of them have been recombinantly expressed in *Escherichia coli* and purified by His_6-tag chromatography (Table 1). To the best of our knowledge, LmSP3 is described here for the first time, although its sequence differs in only five amino acids (K138R, G225D, P236S, S390Y and M488N) from the SP isolated from *Leuconostoc mesenteroides* NRRL B742 (UniProt ID: B2BS85).[27] The enzymes from the five lactic acid bacteria share a sequence identity of more than 65%, which even increases to 86% for the three LmSP enzymes. In contrast, the similarity between BaSP and the other SP enzymes is only 35% (Fig. 1).

For all six enzymes, the apparent kinetic parameters have been determined for sucrose in the phosphorolytic reaction and for fructose in the synthetic reaction (Table 2). All of these experiments were performed at 37 °C and pH 7 because these conditions will also be used for the screening of the acceptor promiscuity. In general, K_m for the acceptor fructose is significantly larger than that for the donor sucrose, which could be explained by the different sizes of the molecules. The highest acceptor affinity is observed in SmSP and BaSP, with a K_m for fructose of 8.3 and 10.1 mM, respectively.

2.2. Solvent stability

Many of the interesting targets for glycosylation do not dissolve well in water, a problem that can be alleviated by the addition of organic co-solvents.[28] To identify the most suitable medium for these reactions, the effect of five different co-solvents on the activity of SP has been investigated (Fig. 2). In accordance with their higher temperature optima,[25,29–31] BaSP and SmSP were found to be the most stable enzymes in the majority of solvents. The addition of increasing amounts of DMF and acetonitrile generally resulted in the sharpest drop in activity, while ethanol, isopropanol, and DMSO seem to have a less drastic effect. For DMSO, all enzymes except BaSP display a similar C_{50}-value of around 25%, which is the concentration at which the enzymes retain half of their initial activity. Furthermore, the decrease in activity is more gradual in this solvent, which renders the screening more robust and less sensitive to errors. In addition, DMSO has the highest boiling point, mixes well with water, and does not interfere with the assay procedures. Therefore, 25% DMSO has been selected as the reaction medium to test the lipophilic acceptors.

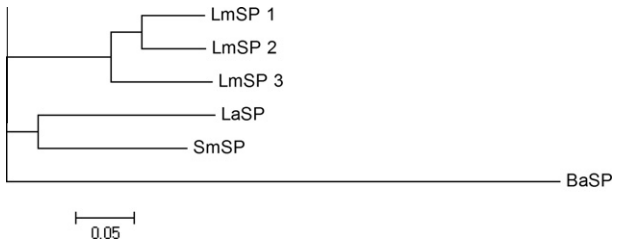

Figure 1. Phylogenetic tree of the different SP enzymes.

Table 2
Apparent kinetic parameters for sucrose and fructose at 37 °C and pH 7

Enzyme	Sucrose[a]			Fructose[b]		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
LmSP1	14.1 ± 0.9	28 ± 5	2.0	22.7 ± 1.9	14 ± 2.5	0.6
LmSP2	7.3 ± 0.5	62 ± 13	8.5	21.9 ± 2.0	22 ± 5	1.0
LmSP3	3.0 ± 0.2	4 ± 1	1.4	17.4 ± 2.0	1 ± 0.3	0.05
SmSP	0.8 ± 0.1	16 ± 1	20.3	8.3 ± 1.0	5 ± 1	0.6
LaSP	2.3 ± 0.2	14 ± 3	0.6	17.4 ± 1.3	7 ± 1	0.4
BaSP	1.4 ± 0.1	78 ± 14	55.9	10.1 ± 0.6	16 ± 3	1.6

[a] Using 75 mM phosphate as co-substrate.
[b] Using 50 mM Glc-1-P as co-substrate.

2.3. Acceptor screening

2.3.1. Setup of the screening procedure

Sucrose phosphorylase is able to transfer a glucosyl moiety from either sucrose or α-Glc-1-P, resulting in the release of fructose or inorganic phosphate, respectively (Fig. 3). Because of the different properties of the screened acceptors, two different assays had to be developed by exploiting both glycosyl donors. Indeed, the detection of released phosphate cannot be applied to aromatic acceptors because these precipitate under the acidic conditions of the Gawronski-assay. In turn, the detection of released fructose cannot be applied to reducing acceptors because these interfere with the BCA-assay.

During transfer reactions, the glucosyl-enzyme intermediate can also be intercepted by water, resulting in hydrolysis instead of transglucosylation of the substrates (Fig. 3). As found by the Nidetzky group, the rate of hydrolysis and consequently the transglucosylation efficiency is influenced dramatically by the concentration of both the donor and acceptor substrates. By increasing the donor concentration from 0.3 to 0.8 M, they could reduce the hydrolysis from 44% to 9% for the transglucosylation of *R*-glycerate, and similar results were obtained for 3-ethoxy-1,2-propanediol.[32,33] In addition, a linear relationship was observed between the rate ratio of transglucosylation/hydrolysis and the concentration of glycerol, arabitol and sorbitol as acceptors.[34,35] The resulting kinetic partition coefficient is a good measure of the enzyme's preference for an acceptor over water. Although high donor and acceptor concentrations favor transglucosylation, several limitations had to be taken into account in our experiments. On the one hand, the concentration of α-Glc-1-P could not exceed 30 mM because the traces of inorganic phosphate generated a considerable background in the screening assay. On the other hand, aromatic acceptors do not dissolve at high concentrations in aqueous media, even when 25% DMSO is added as co-solvent. Therefore, the acceptor concentration has been set to 65 mM for all acceptors, to allow an unambiguous comparison.

As the different SP enzymes display different specific activities (Table 2), the transglucosylation rates are not reported here as absolute values but with reference to the hydrolysis rate ($v_{Acceptor}/v_{Water}$). Although the kinetic partition coefficient, that is, the slope of the linear regression between the rate ratio v_{Fru}/v_{Glc} (sucrose) or v_{Pi}/v_{Glc} (Glc-1-P) and the acceptor concentration, would be the most complete measure of an enzyme's transglucosylation efficiency,[35] that would require too much measurements for a screening experiment. We have, therefore, performed our tests at a single acceptor concentration and have used the activity in the absence of any acceptor besides water as single reference. The rate of hydrolysis was found to be 2–5% of the native activity for all enzymes. Interestingly, a linear correlation could be observed between the ratios obtained by measuring the hydrolysis in the absence ($v_{Acceptor}/v_{Water}$) and presence (v_{Fru}/v_{Glc}) of the acceptor (Fig. 4).

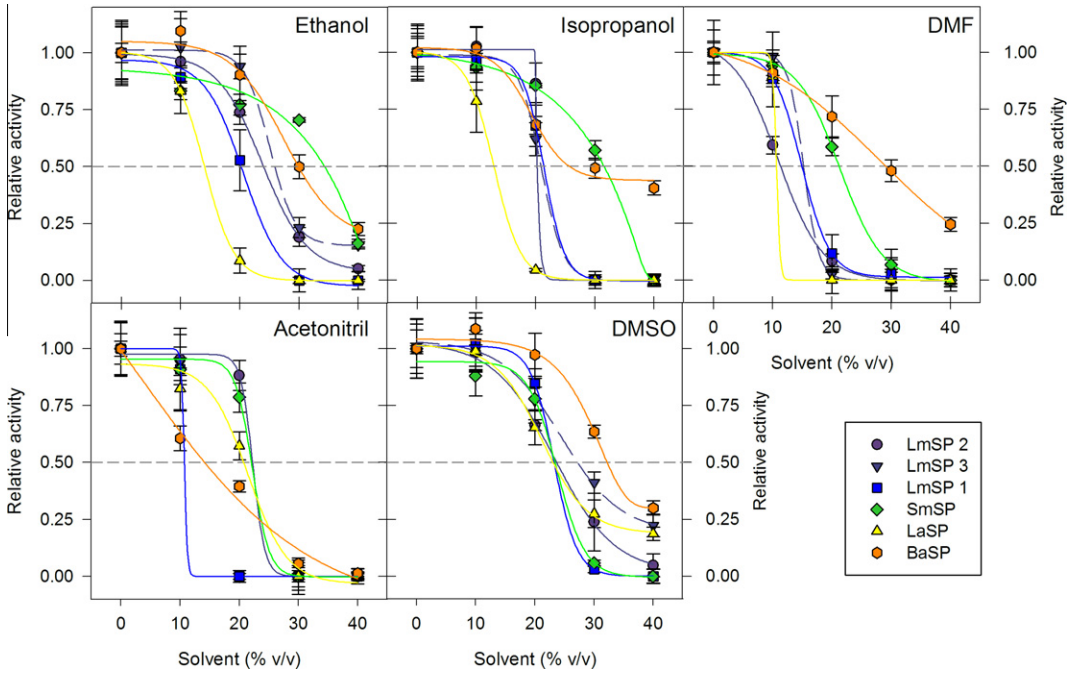

Figure 2. Relative activities for the different SP enzymes in 0–40% (v/v) solvent/water mixture for ethanol, isopropanol, DMF, acetonitrile and DMSO.

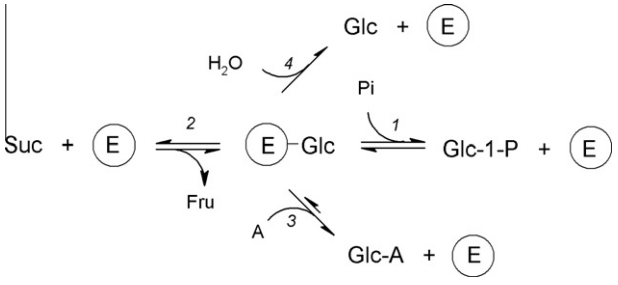

Figure 3. Reaction scheme for SP with the four possible reactions: (1) phosphorolysis, (2) synthesis, (3) transglucosylation, and (4) hydrolysis. Suc: sucrose, Glc-1-P: α-glucose-1-phosphate, E: enzyme, Fru: fructose, P_i: inorganic phosphate, A: acceptor.

The concurrent detection of glucose and either fructose or phosphate is thus not necessary for the evaluation of the acceptor promiscuity of SP, which drastically reduces the screening effort.

2.3.2. Acceptor promiscuity

The acceptor promiscuity of different SP enzymes has been determined previously by different research groups. Although this information is valuable and excellently reviewed by Goedl et al.,[25] the data are heterogeneous and difficult to compare since the experiments were performed with varying concentrations of enzyme, donor, and acceptor, or at a different pH and temperature. In this study, we have compared the transglucosylation activity of six different SP enzymes on 80 putative acceptors under the same reaction conditions. To the best of our knowledge, the acceptor specificity of LaSP and LmSP3 has not yet been analyzed and is

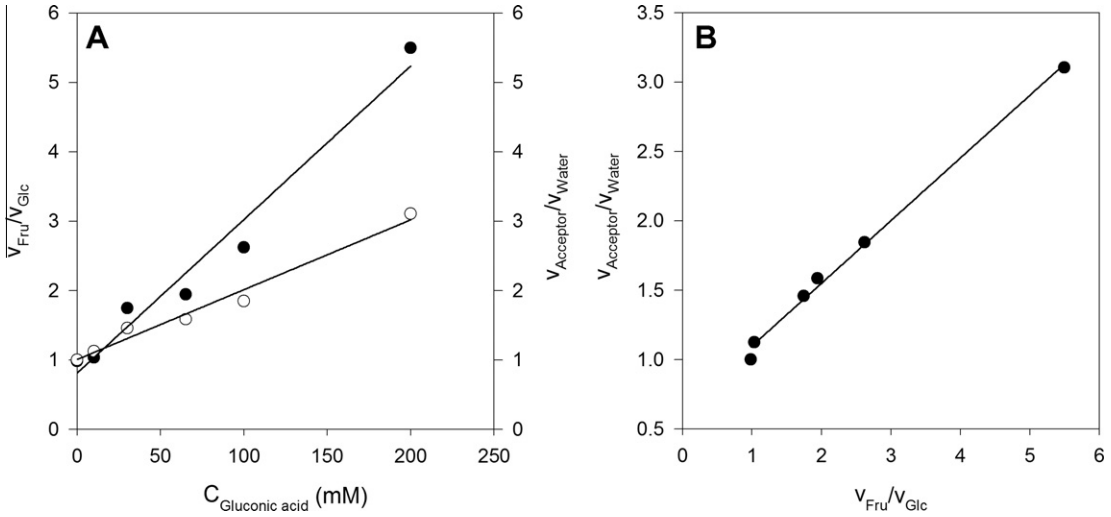

Figure 4. Comparison of different methods to describe the transglucosylation of gluconic acid by BaSP. (A) (●) v_{Fru}/v_{Glc} and (○) $v_{Acceptor}/v_{water}$ in function of acceptor concentration; (B) relationship between $v_{Acceptor}/v_{water}$ and v_{Fru}/v_{Glc}. The experiments have been performed at pH 7.0 and 37 °C, using 50 mM sucrose as donor.

thus described here for the first time. For SmSP, a detailed evaluation was only available for carboxylic acceptors.[36,37]

In contrast to what might be assumed from the high transfer ratios that have previously been published,[19,20,38] most acceptors are poor substrates in terms of catalytic activity (Table 3). As can be seen from the box plots in Figure 5, at least 75% of the tested acceptors do not result in a transglucosylation activity that is considerably higher than the hydrolytic activity ($<1.5 \times v_{water}$). It can also be seen that SmSP has the narrowest acceptor specificity and LmSP3 the broadest. Besides fructose, only two good acceptors could be identified for SmSP. Interestingly, this enzyme was also found to have the lowest K_m-value for fructose of all sucrose phosphorylases (Table 2), which might be correlated with its strict specificity. In contrast, the LmSP enzymes display a higher K_m-value and looser specificity, which could indicate a more 'relaxed' +1 subsite.

In some cases, the addition of acceptor to the reaction was found to lower the overall activity of the SP enzymes, which seems strange at first sight. These compounds must, therefore, interact with the enzyme either as inhibitor or as substrate of very low reactivity. To gain a better understanding of their mode of action, the reaction of BaSP with L-ascorbic acid ($v_{Acceptor}/v_{Water} = 0.3$) was analyzed in more detail by HPLC. The formation of glucosylated product could be clearly observed, although the transglucosylation rate was about 100 times lower than the rate of hydrolysis. In addition, the synthesis of sucrose was found to be hardly inhibited by L-ascorbic acid, corresponding to a K_i of about 0.6 M, that is, much higher than the acceptor concentrations used in our screening experiments (65 mM). Compounds with a relative transglucosylation rate below 1 should thus be described as slow acceptors and not as inhibitors. It is, however, not exactly clear why the hydrolytic background reaction is suppressed in those cases, but could be the result of an induced fit mechanism that has been proposed based on crystallographic analysis of enzyme–substrate complexes.[34]

2.3.3. Transfer to carbohydrates

Sixteen monosaccharides have been tested as acceptor, using α-Glc-1-P as donor substrate (Table 3). Unsurprisingly, the native acceptor D-fructose generates the highest activity, which is 16–48 times higher than the hydrolysis rate. Besides D-fructose, L-arabinose and L-sorbose were among the best acceptors for all SP enzymes. These measurements do, however, paint a different picture than the transfer ratios that have previously been reported. Indeed, with several other monosaccharides, higher yields (thermodynamic equilibrium) can be obtained, while we clearly show that fructose is the best substrate (kinetic activity). For LmSP1, for example, a transfer ratio of 67% has been obtained with galactose compared to 12% with fructose,[20] although the reaction is now found to proceed almost 40 times slower (Table 3). Similarly, BaSP displays a higher transfer ratio but lower activity toward D-arabinose than toward D-fructose.[38]

A remarkable observation is that D-psicose and D-tagatose, the C3- and C4-epimers of fructose, are poorly accepted by all SP enzymes. In contrast to fructose, tagatose mainly occurs as a pyranose ring in solution but this is not the case for psicose.[39] Specific interactions in the active site can thus be expected to be responsible for this discriminative behavior. In a recent study of BaSP, structure–activity relationships have been established for the C1–OH and C6–OH of fructose but not for its C3–OH or C4–OH.[40] Inspection of the enzyme's crystal structure[11] has now revealed that these hydroxyl groups are within hydrogen-bonding distance of His234 and Asp342, respectively. Based on our kinetic experiments, both residues should be considered to be crucial for the native activity of SP.

Substitution of the monosaccharides does not seem to have a dramatic effect on the transglucosylation activity (Table 3). Alkyl groups can be efficiently accommodated at the anomeric carbon of glucose, although the result depends on the size of the substituent as well as on the configuration of the glucosidic bond. Indeed, lower activity is observed on methyl β-glucoside than on methyl α-glucoside and the activity further decreases when octyl β-glucoside is used as acceptor. The presence of an aminogroup at the C2-position of galactose lowers the activity of all SP enzymes, but the effect is more variable for glucose and mannose.

Among the tested disaccharides, four showed significant transglucosylation rates, that is, difructose anhydride III, trehalose, isomaltose, and turanose (Table 3). Difructose anhydride III (α-D-fructofuranose-β-D-fructofuranose-2′,1:2,3′-dianhydride) is a cyclic disaccharide consisting of two fructose moieties linked at their anomeric carbons,[41] and can serve as acceptor for LmSP3 with a reaction rate that is only six times slower than for D-fructose. In addition to these disaccharides, LmSP1 and especially LmSP3 also display good activity toward several trisaccharides (Table 3), which could indicate that these enzymes have a more spacious active site.

In general, sugar alcohols can be transglucosylated at a rate significantly higher than 1, although SmSP seems to be less efficient in that respect (Table 3). For LmSP1, the transfer of a glucosyl moiety to glycerol proceeds only slightly faster than the transfer to water. Nevertheless, this reaction has been developed into a commercial process by the careful engineering of the reaction conditions.[34] Using 0.8 M sucrose and 2.0 M glycerol as substrates, the competing hydrolytic reaction could be eliminated almost completely, resulting in a product yield of 90%. Interestingly, the transglucosylation product could hardly serve as substrate, revealing that secondary hydrolysis is neglectable during the production of glucoglycerol.

2.3.4. Transfer to non-carbohydrates

Non-carbohydrate molecules have also been tested as acceptors for the SP enzymes (Table 3). The glucosylation of such molecules is of significant importance, as it allows to drastically increase their solubility in water, and hence their bio-availability.[28,42] For the vast majority of aromatic acceptors, a transglucosylation activity lower than 1 was observed, which means that they are poorer acceptors than water. Nevertheless, a few phenolic compounds like (+)-catechin and epigallocatechin gallate have been successfully glucosylated by LmSP2, allowing the characterization of the corresponding glucosides.[22] It should, however, be mentioned that the product yields were low, even when a large excess of donor and a high enzyme concentration was applied. In our experiments, the highest transglucosylation activity was detected for LmSP3 and 4-phenoxyphenol as acceptor, displaying a reaction rate that is about 2.5 times higher than the hydrolytic reaction.

The compounds classified as specialties are glucosylated at varying rates by the different SP enzymes. Sawangan et al.[43] have optimized the transglucosylation reaction of LmSP2 for the production of glucosyl glycerate, obtaining a yield of 91% with 0.8 M sucrose and 0.3 M *R*-glycerate as substrate. At equimolar concentrations of sucrose and glycerate (0.3 M), however, roughly equal activities toward glycerate and water were observed, which is in good agreement with our result (transglycosylation activity of 1.4) under slightly different conditions. Kwon et al.[44] have reported on the synthesis of glucosyl ascorbic acid with the SP from *Bifidobacterium longum*, using a substrate solution containing 30% (w/v) sucrose and 0.5% (w/v) L-ascorbic acid. Nevertheless, none of the enzymes tested here display a transglycosylation activity higher than 1 on L-ascorbic acid as acceptor.

3. Concluding remarks

Sucrose phosphorylase is a promising biocatalyst for the glucosylation of a wide variety of acceptor molecules, but was found to display a rather low transglucosylation activity toward the majority of

Table 3
Transglucosylation activity at 37 °C and pH 7, expressed with hydrolysis as reference ($v_{Acceptor}/v_{Water}$)

Acceptor	LmSP1	LmSP2	LmSP3	SmSP	LaSP	BaSP
Water[A,B]	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.11	1.00 ± 0.13	1.00 ± 0.14	1.00 ± 0.12
Inorganic phosphate[B]	40.39 ± 4.87	22.47 ± 1.32	18.14 ± 1.03	33.77 ± 5.4	36.77 ± 6.08	44.4 ± 6.05
Monosacharides						
L-Arabinose[A]	3.01 ± 0.23	3.88 ± 0.34	4.40 ± 0.30	8.00 ± 0.37	4.11 ± 0.17	4.91 ± 0.29
D-Arabinose[A]	1.03 ± 0.05	1.10 ± 0.10	1.18 ± 0.07	1.20 ± 0.04	0.99 ± 0.04	1.22 ± 0.09
D-Xylose[A]	1.47 ± 0.05	0.96 ± 0.10	1.5 ± 0.17	0.99 ± 0.03	1.09 ± 0.08	1.13 ± 0.09
D-Ribose[A]	1.58 ± 0.16	1.26 ± 0.10	1.15 ± 0.1	1.15 ± 0.05	1.10 ± 0.05	1.73 ± 0.15
L-Ribose[A]	1.57 ± 0.32	1.46 ± 0.06	1.86 ± 0.13	1.32 ± 0.01	1.30 ± 0.01	1.42 ± 0.08
2-Deoxy-L-ribose[A]	1.84 ± 0.20	1.23 ± 0.18	2.19 ± 0.23	1.21 ± 0.06	1.19 ± 0.06	1.20 ± 0.06
L-Rhamnose[A]	1.19 ± 0.19	1.00 ± 0.07	1.26 ± 0.08	1.00 ± 0.02	1.01 ± 0.03	1.16 ± 0.07
D-Lyxose[A]	1.51 ± 0.10	1.13 ± 0.04	1.72 ± 0.28	1.02 ± 0.06	0.99 ± 0.08	1.23 ± 0.15
L-Fucose[A]	1.53	1.2	NA	0.97	1.05	NA
D-Fructose[A]	48.64 ± 3.06	43.50 ± 4.58	18.53 ± 0.95	22.90 ± 0.89	16.7 ± 1.46	38.36 ± 4.29
D-Psicose[A]	2.01 ± 0.26	1.30 ± 0.12	1.75 ± 0.15	1.06 ± 0.04	1.25 ± 0.05	1.27 ± 0.13
D-Tagatose[A]	1.34 ± 0.09	1.54 ± 0.14	0.43 ± 0.04	0.92 ± 0.07	0.88 ± 0.08	1.01 ± 0.07
L-Sorbose[A]	2.64 ± 0.04	3.06 ± 0.11	3.26 ± 0.11	8.86 ± 0.26	2.99 ± 0.06	8.17 ± 0.36
D-Galactose[A]	1.20 ± 0.08	1.31 ± 0.07	1.27 ± 0.08	1.55 ± 0.13	1.33 ± 0.18	1.36 ± 0.03
D-Glucose[A]	0.93 ± 0.04	1.06 ± 0.09	0.84 ± 0.05	1.02 ± 0.05	0.96 ± 0.08	1.03 ± 0.13
D-Mannose[A]	1.59 ± 0.10	1.37 ± 0.07	1.50 ± 0.10	1.07 ± 0.07	1.10 ± 0.05	1.43 ± 0.06
Substituted monosaccharides						
Methyl α-D-glucopyranoside[B]	1.34 ± 0.12	1.16 ± 0.12	1.16 ± 0.06	0.97 ± 0.09	0.53 ± 0.06	1.37 ± 0.06
Methyl β-D-glucopyranoside[B]	1.23 ± 0.05	0.99 ± 0.06	1.09 ± 0.03	0.83 ± 0.11	0.56 ± 0.06	0.88 ± 0.05
Hexyl β-D-glucopyranoside[B]	0.74 ± 0.10	0.60 ± 0.10	0.53 ± 0.01	1.15 ± 0.17	0.54 ± 0.02	0.62 ± 0.07
Octyl β-D-glucopyranoside[B]	0.73 ± 0.09	0.72 ± 0.09	0.60 ± 0.03	0.42 ± 0.16	0.70 ± 0.18	0.80 ± 0.12
D-Glucosamine[A]	0.89 ± 0.05	1.06 ± 0.06	1.04 ± 0.06	1.07 ± 0.03	1.00 ± 0.19	1.07 ± 0.08
Methyl α-D-mannopyranoside[B]	1.48 ± 0.15	1.22 ± 0.08	1.31 ± 0.03	1.01 ± 0.07	0.93 ± 0.08	1.33 ± 0.09
D-Mannosamine[A]	0.98 ± 0.05	1.31 ± 0.13	1.21 ± 0.08	1.23 ± 0.06	1.37 ± 0.06	1.32 ± 0.06
D-Galactosamine[A]	0.89 ± 0.02	1.13 ± 0.08	1.07 ± 0.03	1.05 ± 0.05	1.05 ± 0.14	1.17 ± 0.05
Disaccharides						
Difructose anhydride III[A]	1.68 ± 0.13	1.24 ± 0.14	2.86 ± 0.11	1.10 ± 0.07	1.24 ± 0.06	1.62 ± 0.06
Kojibiose[A]	1.46 ± 0.13	1.02 ± 0.09	1.4 ± 0.07	1.02 ± 0.03	1.08 ± 0.08	1.17 ± 0.06
Gentiobiose[A]	1.08 ± 0.02	1.17 ± 0.04	1.4 ± 0.07	1.05 ± 0.08	1.11 ± 0.02	1.13 ± 0.06
Trehalose[A]	1.61 ± 0.16	1.29 ± 0.10	2.93 ± 0.04	1.06 ± 0.02	1.11 ± 0.04	1.27 ± 0.20
Lactulose[A]	1.08 ± 0.05	1.25 ± 0.08	1.21 ± 0.06	1.10 ± 0.09	1.20 ± 0.16	1.25 ± 0.06
Lactose[A]	1.11 ± 0.04	1.18 ± 0.13	1.18 ± 0.07	1.13 ± 0.03	1.25 ± 0.12	1.18 ± 0.07
Isomaltose[A]	1.45 ± 0.08	1.08 ± 0.08	2.17 ± 0.09	0.94 ± 0.02	0.94 ± 0.05	1.18 ± 0.03
Cellobiose[A]	1.10 ± 0.04	1.11 ± 0.09	1.23 ± 0.02	1.10 ± 0.04	1.26 ± 0.08	1.08 ± 0.04
Sucrose[A]	1.26 ± 0.06	1.82 ± 0.11	1.21 ± 0.05	1.17 ± 0.08	1.78 ± 0.17	1.17 ± 0.15
Leucrose[A]	1.01 ± 0.07	1.09 ± 0.02	1.05 ± 0.05	1.03 ± 0.09	1.11 ± 0.11	1.15 ± 0.03
Maltulose[A]	0.99 ± 0.05	1.13 ± 0.08	1.09 ± 0.04	1.02 ± 0.04	1.00 ± 0.07	1.07 ± 0.04
Palatinose[A]	0.68 ± 0.04	0.78 ±0.11	0.63 ± 0.04	0.75 ± 0.03	0.85 ± 0.13	0.98 ± 0.03
Maltose[A]	0.87 ± 0.03	1.00 ± 0.11	0.93 ± 0.13	1.04 ± 0.04	1.15 ± 0.12	1.02 ± 0.09
Turanose[A]	1.16 ± 0.05	0.94 ± 0.06	1.45 ± 0.24	0.99 ± 0.06	1.77 ± 0.17	1.14 ± 0.01
Trisaccharides						
Maltotriose[A]	1.26 ± 0.04	1.13 ± 0.12	2.86 ± 0.11	1.04 ± 0.04	1.07 ± 0.12	1.17 ± 0.04
Raffinose[A]	1.51 ± 0.40	1.03 ± 0.07	2.87 ± 0.21	1.08 ± 0.04	1.14 ± 0.07	1.10 ± 0.05
Melezitose[A]	1.48 ± 0.01	1.13 ± 0.03	3.05 ± 0.24	1.15 ± 0.07	0.91 ± 0.02	1.21 ± 0.01
Isomaltotriose[A]	1.58	1.2	NA	1.22	0.84	NA
Panose[A]	2.22	0.2	NA	0.96	0.87	NA
Sugar alcohols						
Erythritol[B]	1.41 ± 0.02	1.37 ± 0.09	1.40 ± 0.07	0.92 ± 0.09	1.38 ± 0.08	2.01 ± 0.10
Glycerol[B]	1.71 ± 0.15	1.17 ± 0.05	1.51 ± 0.03	1.36 ± 0.08	1.28 ± 0.02	1.75 ± 0.10
D-Arabitol[B]	1.99 ± 0.19	1.35 ± 0.05	2.74 ± 0.02	1.21 ± 0.08	1.32 ± 0.05	1.60 ± 0.18
D-Xylitol[B]	1.03 ± 0.10	1.26 ± 0.07	1.17 ± 0.04	0.93 ± 0.09	1.29 ± 0.05	1.51 ± 0.12
D-Ribitol[B]	1.43 ± 0.09	0.99 ± 0.11	0.66 ± 0.23	0.75 ± 0.06	0.67 ± 0.12	1.48 ± 0.15
D-Sorbitol[B]	1.24 ± 0.10	1.83 ± 0.08	1.24 ± 0.04	0.90 ± 0.04	1.69 ± 0.09	1.10 ± 0.16
Myo-Inositol[B]	0.99 ± 0.11	1.66 ± 0.07	1.12 ± 0.08	0.94 ± 0.05	1.96 ± 0.10	1.17 ± 0.04
Meso-Inositol[B]	0.93 ± 0.06	1.88 ± 0.06	1.03 ± 0.04	1.02 ± 0.09	1.85 ± 0.12	1.13 ± 0.07
D-Mannitol[B]	0.89 ± 0.12	1.39 ± 0.09	0.95 ± 0.05	0.90 ± 0.04	1.35 ± 0.13	1.00 ± 0.03
Aromatics						
Phenol[B]	0.74 ± 0.06	0.77 ± 0.07	0.69 ± 0.06	0.88 ± 0.04	0.61 ± 0.11	0.89 ± 0.09
Catechol[B,c]	0.25 ± 0.09	0.35 ± 0.04	ND	ND	ND	ND
Resorcinol[B]	0.85 ± 0.08	0.60 ± 0.10	0.97 ± 0.07	ND	1.41 ± 0.05	ND
2-Phenylethanol[B,a]	0.75 ± 0.21	0.81 ± 0.18	0.83 ± 0.13	0.87 ± 0.26	0.75 ± 0.07	0.52 ± 0.06
2-Nitrophenol[B,a]	0.58 ± 0.11	1.14 ± 0.09	0.73 ± 0.04	0.78 ± 0.24	0.82 ± 0.02	0.61 ± 0.07
p-Nitrophenol[B]	ND	0.55 ± 0.12	1.28 ± 0.10	ND	0.76 ± 0.02	ND
2-Phenylphenol[B,a]	0.70 ± 0.09	1.31 ± 0.25	1.69 ± 0.19	0.62 ± 0.11	0.48 ± .02	0.58 ± 0.07

Table 3 (*continued*)

Acceptor	LmSP1	LmSP2	LmSP3	SmSP	LaSP	BaSP
4-Phenoxyphenol[B,a]	0.52 ± 0.12	0.69 ± 0.06	2.53 ± 0.07	1.57 ± 0.14	0.62 ± 0.03	0.63 ± 0.11
p-Hydroxybenzoic acid methylester[B]	0.54 ± 0.12	0.49 ± 0.08	0.58 ± 0.04	0.98 ± 0.08	0.56 ± 0.05	0.40 ± 0.03
3-Hydroxybiphenyl[B,a]	0.70 ± 0.04	0.72 ± 0.12	0.76 ± 0.03	0.83 ± 0.14	0.62 ± 0.02	0.39 ± 0.04
p-Hydroxybenzoic acid[B]	ND	1.13 ± 0.17	0.47 ± 0.10	ND	0.57 ± 0.05	ND
3,4-diHydroxybenzoic acid[B]	ND	ND	ND	ND	ND	ND
Salicylic acid[B]	0.12 ± 0.03	0.35 ± 0.05	0.85 ± 0.07	0.82 ± 0.07	0.67 ± 0.03	1.80 ± 0.14
Shikimic acid[B,d]	1.33 ± 0.08	1.32 ± 0.14	1.33 ± 0.12	0.99 ± 0.05	0.86 ± 0.08	0.78 ± 0.04
Gallic acid[B]	ND	0.65 ± 0.13	0.30 ± 0.06	ND	0.18 ± 0.15	ND
Ethyl Gallate[B,c]	0.85 ± 0.15	0.69 ± 0.05	ND	ND	ND	ND
Vanillin[B]	0.61 ± 0.01	0.74 ± 0.05	0.66 ± 0.01	0.79 ± 0.06	0.26 ± 0.04	0.82 ± 0.01
Pyridoxine[B]	NA	NA	0.99 ± 0.08	0.83 ± 0.08	0.21 ± 0.08	0.77 ± 0.12
3,4-Dimethoxybenzylalcohol[B]	0.90 ± 0.11	1.51 ± 0.08	1.37 ± 0.20	1.43 ± 0.04	1.05 ± 0.12	0.56 ± 0.06
p-Hydroxy-benzoic acid *n*-butyl ester[B,b]	0.70 ± 0.11	0.87 ± 0.12	0.60 ± 0.13	0.92 ± 0.25	ND	0.46 ± 0.08
Daidzein[B,d]	0.70 ± 0.08	1.09 ± 0.12	0.75 ± 0.33	0.79 ± 0.19	ND	0.43 ± 0.05
Specialties						
L-Hydroxyproline[B]	1.28 ± 0.15	1.31 ± 0.02	1.06 ± 0.05	0.57 ± 0.14	0.74 ± 0.09	1.04 ± 0.08
Glycerate[B]	1.31 ± 0.14	1.40 ± 0.07	0.87 ± 0.06	0.47 ± 0.15	0.55 ± 0.16	0.86 ± 0.14
Cholesterol[B]	0.76 ± 0.06	0.77 ± 0.31	0.81 ± 0.10	0.87 ± 0.24	ND	0.62 ± 0.09
D-Gluconic acid[B]	1.78 ± 0.20	0.80 ± 0.09	1.74 ± 0.08	0.47 ± 0.02	0.96 ± 0.11	1.58 ± 0.16
Ribonolactone[B]	0.99 ± 0.07	0.29 ± 0.16	0.91 ± 0.07	1.05 ± 0.14	1.13 ± 0.04	1.18 ± 0.13
L-Ascorbic acid[B]	0.16 ± 0.01	1.17 ± 0.12	0.47 ± 0.05	0.56 ± 0.04	0.33 ± 0.08	0.28 ± 0.15
L-Isoascorbic acid[B]	0.14 ± 0.03	1.13 ± 0.17	0.40 ± 0.06	0.61 ± 0.09	0.42 ± 0.06	0.24 ± 0.03
Cyclohexanol[B]	0.85 ± 0.08	1.08 ± 0.16	0.51 ± 0.04	0.63 ± 0.05	0.51 ± 0.17	ND

Acceptor concentration: [a] 15 mM, [b] 25 mM, [c] 32.5 mM, [d] 1.5 mM.
NA: Data not available, ND: activity not detectable.
[A] Using 30 mM α-Glc-1-P as donor (phosphate assay).
[B] Using 50 mM sucrose as donor (BCA assay).

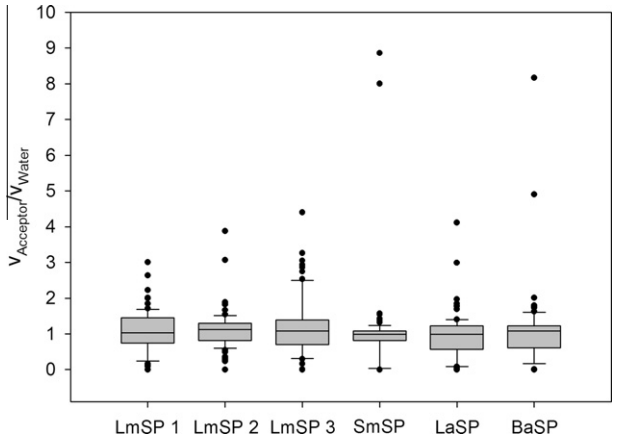

Figure 5. Boxplot of the relative transglucosylation activity ($v_{Acceptor}/v_{Water}$) of the various SP enzymes.

compounds. Consequently, the development of production processes for glycosides will require the optimization of reaction conditions to outcompete water as acceptor. Hydrophobic acceptors, however, cannot be used at high concentrations because of their limited solubility. We have shown here that organic co-solvents can be added to the reaction medium in concentrations up to 25% without too much loss of enzyme activity. Nevertheless, the solubility of several acceptors will probably still not be high enough to allow saturation of the enzyme's active site. Improving the affinity for hydrophobic acceptors by means of enzyme engineering will, therefore, be a major challenge for the commercial exploitation of the transglucosylation potential of sucrose phosphorylase.

4. Experimental section

4.1. Enzyme production and purification

The SP enzymes, containing a N-terminal His_6-tag, were constitutively expressed in *E. coli* Rosetta 2 under the optimal conditions, as previously described.[45] Similarly, the new SP gene from *Streptococcus mutans* LMG 14558^T was cloned into the constitutive expression vector pCXP22h using TAGCTAGCATGCCAATTACAAA TAAAACAATGTTG and TCACTGCAGTTATTCAAAGCTTATTGTTTG as forward and reverse primers, respectively. For enzyme production, 2% of an overnight culture was inoculated in 250 mL LB medium containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol in a 1 L shake flask and incubated at 37 °C with continuous shaking at 200 rpm until the beginning of exponential phase (OD_{600} = 0.6). Further expression of the SP enzymes was then performed for 2 hours at the optimal temperature,[45] with 30 °C being optimal for the SP from *S. mutans*. The produced biomass was harvested by centrifugation for 20 min at 5000*g* and 4 °C, washed with 10 mL PBS buffer (300 mM NaCl and 50 mM NaH_2PO_4 at pH 8) and the obtained cell pellets were stored at −20 °C.

The cell pellets were then thawed and dissolved in 10 mL lysis buffer (300 mM NaCl, 10 mM imidazole, 0.1 mM PMSF and 50 mM NaH_2PO_4 at pH 8) supplemented with lysozyme and DNaseI in a final concentration of 1 mg/mL and 6 U/L, respectively. This cell suspension was incubated on ice for 30 min and sonicated three times for 2.5 min (Branson sonifier 250, level 3, 50% duty cycle). The His_6-tagged proteins were purified by Ni-NTA chromatography as described by the supplier (Qiagen), after which the buffer was exchanged to 50 mM MOPS pH 7 in a Centricon YM-30 (Millipore). The protein content was analyzed with the Lowry method using bovine serum albumin as standard.[46]

4.2. Assay procedures

4.2.1. Determination of inorganic phosphate

The release of inorganic phosphate from α-Glc-1-P as glycosyl donor was measured with the method of Gawronski.[47] The color reagent is prepared by adding 2 parts of a solution containing 4% (w/v) ascorbic acid in 1 N HCl, and 1 part of a solution containing 2% (w/v) ammonium molybdate tetrahydrate (Sigma) in milliQ water. From the color reagent, 150 μL is added to 50 μL sample or standard, followed by 5 min incubation at room temperature. Subsequently, 150 μL stop solution composed of 2% (w/v) sodium citrate tribasic

dihydrate and 2% acetic acid in milliQ water is added, and the mixture is incubated for 15 min at room temperature before the absorbance is read at 655 nm.

4.2.2. Determination of reducing sugars

The release of the reducing sugar fructose from sucrose as glycosyl donor was measured with the bicinchoninic acid (BCA) assay.[48,49] The color reagent is prepared by adding 23 parts of a solution containing 130.43 mg/100 mL 4,4′-dicarboxy-2,2′-biquinoline and 6.23% (w/v) anhydrous Na_2CO_3 in milliQ water and 1 part of a solution composed of 2.33% (w/v) aspartic acid, 3.33% (w/v) anhydrous Na_2CO_3 and 0.73% (w/v) $CuSO_4$ in milliQ water and 6 parts ethanol. From the color reagent, 150 μL is added to 50 μL sample or standard, covered by a plastic foil and incubated for 30 min at 70 °C. After cooling to room temperature, the absorbance is measured at 540 nm.

4.2.3. Determination of glucose

To determine the contribution of glucose to the amount of reducing sugars, its concentration (and resulting v_{Glc}) was determined with a discontinuous coupled assay using glucose oxidase and peroxidase.[50] The coloring reagent was composed of 453 mg/L glucose oxidase (Sigma), 69.2 mg/L peroxidase (Sigma), and 500 mg/L 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) in 100 mM Tris–HCl, 5 mM NaH_2PO_4, pH 7.0, and 0.1% (v/v) Triton X100 (Sigma). To 50 μL of an inactivated sample or standard (5 min at 95 °C), 200 μL of the coloring solution was added and the reaction was incubated at 30 °C for 30 min, after which the absorbance was read at 405 nm.

4.3. Kinetic parameters

The apparent kinetic parameters for sucrose as donor and for fructose as acceptor were determined in 50 mM MOPS buffer at pH 7 and 37 °C, using 75 mM phosphate and 50 mM α-Glc-1-P, respectively, as co-substrate. The parameters were calculated by non-linear regression of the Michaelis-Menten equation using Sigma Plot 10.0.

4.4. Stability in co-solvent

The solvent stability was determined by measuring the SP activity in the presence of 0–40% (v/v) organic co-solvent, that is, ethanol (Merck), acetonitrile (Fisher), dimethylsulfoxide (DMSO) (Sigma), dimethylformamide (DMF) (Sigma) and iso-propanol (Acros). Enzyme reactions were performed in quadruple in 96-well microtiter plates using 50 mM sucrose in 50 mM sodium phosphate buffer at pH 7 and 37 °C as substrate. At regular intervals, samples were taken with a liquid-handling robot (Freedom EVO Tecan) and analyzed with the BCA assay.

4.5. Acceptor screening

Acceptors of reducing and non-reducing nature were screened with the phosphate and BCA assay, respectively (Table 3). Enzyme reactions were performed in 96-low-well microtiter plates with 65 mM acceptor and either 30 mM α-glucose-1-phosphate or 50 mM sucrose in 50 mM MOPS buffer at pH 7 and 37 °C. For the hydrophobic acceptors, 25% (v/v) DMSO was added to the reaction mixture. The purified enzymes were diluted to a level that allowed the accurate determination of their hydrolytic activity. Initial reaction rates were determined with a pipetting robot (Freedom EVO, Tecan), taking samples of 50 μl every 30 min for 2 h when α-Glc-1-P was used as donor and every 5 minutes for 20 minutes when sucrose was the donor. The transglucosylation activity and standard deviation for every compound were calculated from four repetitions. The activity in the absence of acceptor (v_{Water}) was used as reference and was measured by either phosphate determination or the BCA assay depending on the donor substrate.

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