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review

Biocatalytic Process for Production of α-Glucosylglycerol Using Sucrose Phosphorylase

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Summary

Glycosylglycerols are powerful osmolytes, produced by various plants, algae and bacteria in adaptation to salt stress and drought. Among them, glucosylglycerol (2-*O*-α-D-glucopyranosyl-*sn*-glycerol; GG) has attracted special attention for its promising application as a moisturizing agent in cosmetics. A biocatalytic process for the synthesis of GG as industrial fine chemical is described in which sucrose phosphorylase (from *Leuconostoc mesenteroides*) catalyzes regioselective glucosylation of glycerol using sucrose as the donor substrate. The overall enzymatic conversion, therefore, is sucrose+glycerol→GG+D-fructose. Using a twofold molar excess of glycerol acceptor in highly concentrated substrate solution, GG yield was 90 % based on ≥250 g/L of converted sucrose. Enzymatic GG production was implemented on a multihundred kg-per-year manufacturing scale, and a commercial product for cosmetic applications is distributed on the market under the name Glycoin®. Technical features of the biotransformation that were decisive for a successful process development are elaborated. Stabilization of proteins is another interesting field of application for GG.

Key words: glucosylglycerol, sucrose phosphorylase, transglucosylation, glycerol, Glycoin®, protein stabilization, osmolytes

Introduction

Microbial life under extremes of temperature, osmotic pressure and pH is facilitated by the intracellular accumulation of a diverse class of small molecules that are often referred to as compatible solutes (*1–4*). A conserved characteristic for many of these compounds is a glycosidic chemical structure (Scheme 1). Compatible solutes (also termed osmolytes) are found ubiquitously in nature where they serve a conserved function in protecting cells against high salt concentration, extremes of temperature and other forms of external stress. Their physiological effectiveness (*5–7*) and technological performance (*3,8*) can be traced back to the ability to regulate the cellular water balance and to the prevention of denaturation of proteins and the stabilization of supramolecular biological structures like those of lipid membranes.

Glycosylglycerols are powerful osmolytes that are produced by various plants, algae, and bacteria in adaptation to salt stress and drought (*9*). Among them, 2-*O*-α-D-glucopyranosyl-*sn*-glycerol (GG; Scheme 1), which is the main compatible solute in photosynthetic bacteria (*10*), has attracted special attention for its promising application as a moisturizing agent in cosmetics (*11–13*), but also for use as low-calorie sweetener that does not cause tooth decay (*14*). Possible therapeutic applications of GG that are inferred from its ability to stabilize proteins and cells are currently being evaluated (*15,16*). However, the development of industrial applications for GG (and related carbohydrate-based compatible solutes)

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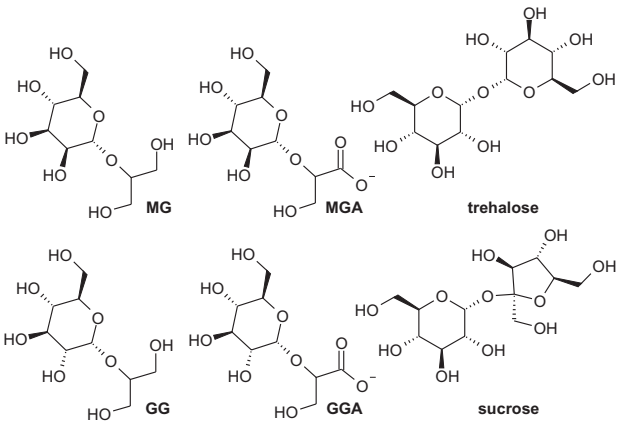


Scheme 1. Chemical structures of mannosylglycerol (MG), mannosylglycerate (MGA), glucosylglycerol (GG), glucosylglycerate (GGA), α,α-trehalose and sucrose

was severely restricted by compound availability. Previously reported synthetic procedures were not technologically mature due to difficult problems of insufficient yield, selectivity, productivity, or a combination of these major limitations (*14,17–19*). We have described a new biocatalytic process which overcomes the chemical and technological challenges of the production of stereochemically pure GG as an industrial chemical.

Sucrose Phosphorylase

Sucrose phosphorylase (sucrose:phosphate α-D-glucosyltransferase; EC 2.4.1.7) catalyzes the reversible conversion of sucrose (α-D-glucopyranosyl-1,2-β-D-fructofuranoside) and phosphate into α-D-glucopyranosyl phosphate (G1P) and D-fructose. The equilibrium constant for the phosphorylase reaction at pH=7.0 and 30 °C is approx. 44 (*20*). The enzyme has been characterized from a relatively small number of bacterial species, namely *Bifidobacterium adolescentis* (*21*), *Leuconostoc mesenteroides* (*20,22–24*), *Pseudomonas saccharophila* (*25–27*) and *Streptococcus mutans* (*28–30*). However, genes encoding putative sucrose phosphorylases have been detected in many other species, mainly in members of the bacterial class of *Bacillus*. The enzyme is about 500 amino acids long and was found to occur as a functional monomer or dimer. Its activity is not dependent on cofactors or cosubstrates. A crystal structure has been determined for the homodimeric sucrose phosphorylase from *B. adolescentis* (*31*). The enzyme adopts the canonical $(\beta/\alpha)_8$-barrel fold (Fig. 1a) of glycoside hydrolases and transglycosidases of family GH13 (the 'α-amylase family') of the glycoside hydrolase families. Therefore, in spite of its EC categorization as a glycosyltransferase, sucrose phosphorylase is structurally related to glycoside hydrolases. The active site residues, a triad of two aspartic acids and one glutamic acid, are highly conserved in both sequence and structure (Fig. 1b).

Biochemical studies of sucrose phosphorylase go back in time to the 1940s. The enzyme was first discovered in *L. mesenteroides* (*32*) and *P. saccharophila* (*33*). Mieyal and Abeles (*34*) summarized evidence from early mechanistic work on sucrose phosphorylase that led to the perceptive proposal of a double displacement-like reaction mechanism involving a catalytically competent covalent enzyme intermediate. A β-configured *O*-glycosidic linkage between the glucosyl moiety and a carboxylate side chain from Asp/Glu was demonstrated (*35*). The proposed covalent intermediate has now also been seen crystallographically (*36*). Residues involved in catalysis were probed and their proposed function confirmed by kinetic characterization of relevant site-directed mutants (*37–39*). The catalytic mechanism of sucrose phosphorylase is summarized in Scheme 2.

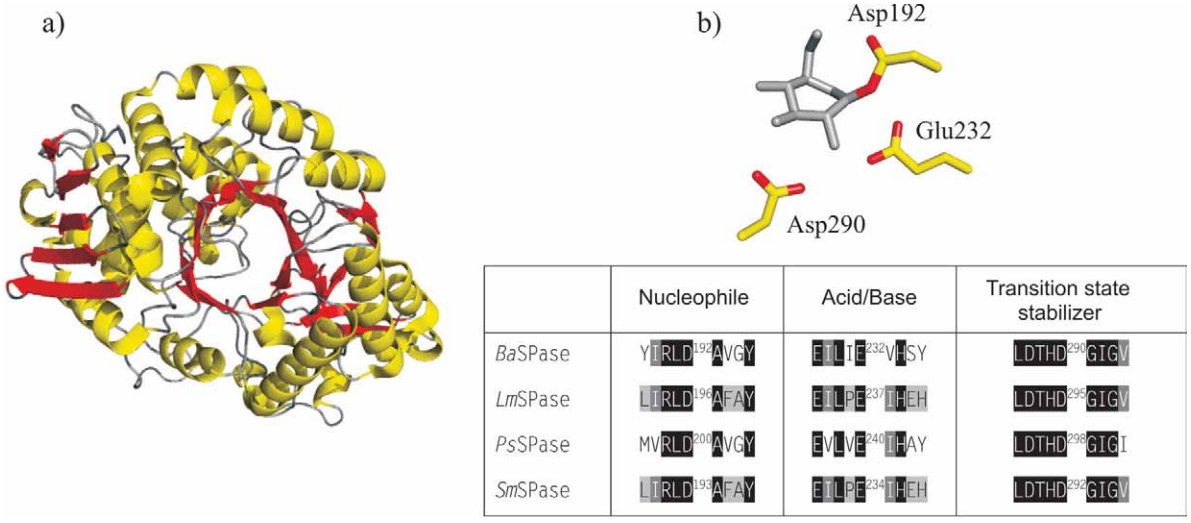
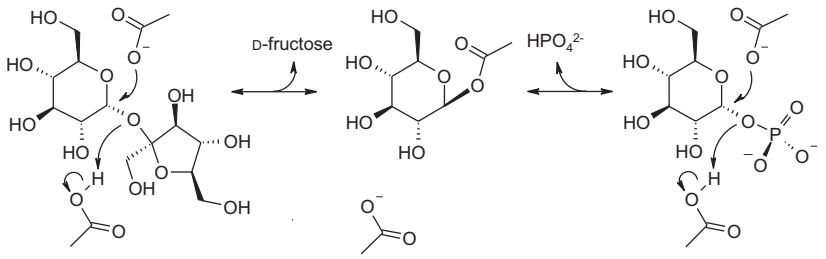


Fig. 1. Sucrose phosphorylase structure: a) three-dimensional fold of the subunit of sucrose phosphorylase from *B. adolescentis* derived from the crystal structure of the dimeric enzyme (PDB entry 1r7a), b) close-up structure of the active site in the β-glucosyl enzyme intermediate of the phosphorylase (PDB entry 2gdv, molecule A). Residues of the catalytic triad, Asp192 (nucleophile; glucosylated in the structure), Glu232 (acid–base), and Asp290 (transition state stabilizer) are indicated. Partial multiple sequence alignment of primary structures of sucrose phosphorylase enzymes from *B. adolescentis* (*Ba*SPase, GenBank AAO33821), *L. mesenteroides* (*Lm*SPase, GenBank BAA14344), *P. saccharophila* (*Ps*SPase, GenBank AAD40317), and *S. mutans* (*Sm*SPase, GenBank AAA26937). The alignment was performed with the Vector NTI program using the AlignX-modul with the blosum62mt2 scoring matrix



Scheme 2. Proposed catalytic mechanism for sucrose phosphorylase. The enzymatic reaction proceeds in two catalytic steps *via* a covalent enzyme intermediate. For reasons of clarity, only the catalytic nucleophile (top) and the general acid–base (bottom) are shown

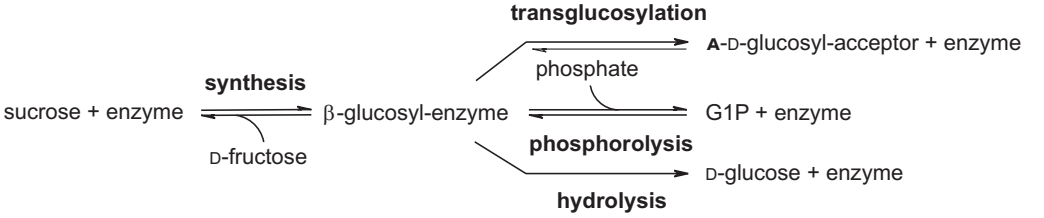
Sucrose phosphorylase is known to catalyze three types of overall reaction, as shown in Scheme 3: glucosyl transfer to and from phosphate, often termed phosphorolysis and synthesis; hydrolysis; and transglucosylation. Hydrolysis and transglucosylation are reactions of the glucosylated phosphorylase when phosphate is lacking. Hydrolysis occurs at a rate of ≥50 times more slowly than glucosyl transfer to phosphate. External acceptors differ in reactivity towards glucosylation by the phosphorylase (*40*).

Sucrose, G1P and α-D-glucopyranosyl fluoride (G1F) are highly reactive donor substrates for the enzyme. The range of acceptor substrates is broad (*34,41*), defining the scope for sucrose phosphorylase as a useful transglucosylation catalyst. The crystallographically observed conformational flexibility of the acceptor binding site in sucrose phosphorylase (from *B. adolescentis*) may be the key to the accommodation of a diverse group of compounds to become glucosylated (*40*).

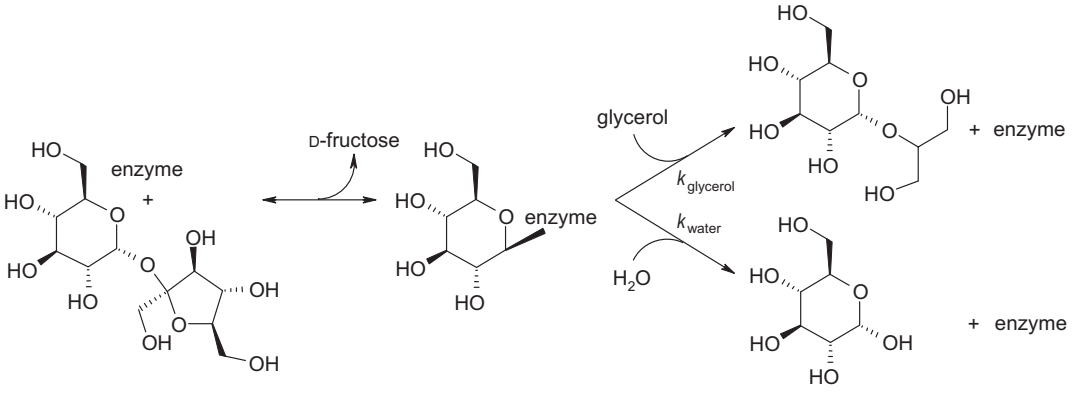
Biocatalytic Synthesis of GG

The enzymatic reaction used for the synthesis of GG is shown in Scheme 4. Sucrose phosphorylase promotes transglucosylation in two steps where the β-glucosyl-enzyme intermediate formed in the first half-reaction (enzyme glucosylation) is intercepted by the 2-OH of glycerol in the second half-reaction to yield the α-configured glucosidic product (enzyme deglucosylation). Hydrolysis of the intermediate potentially competes with group transfer to the acceptor substrate. Furthermore, hydrolysis of a product would constitute an undesired secondary reaction catalyzed by the enzyme (see later).

Glucosyl transfer from sucrose to glycerol was characterized kinetically. An assay was used in which the rate of the overall reaction, measured as the release of fructose (V_{Fru}), was compared to the hydrolysis rate, measured as the release of glucose (V_{Glc}). A ratio V_{Fru}/V_{Glc} that is substantially greater than the unity indicates glucosylation of acceptor substrate. Experiments performed



Scheme 3. Three types of overall reactions catalyzed by sucrose phosphorylases. The β-glucosyl-enzyme intermediate can react with phosphate (phosphorolysis) and fructose (synthesis), with water (hydrolysis) or with various other acceptors (transglucosylation). Double arrows indicate reversible reactions



Scheme 4. Enzymatic reaction used for the synthesis of GG

under the conditions in which glycerol concentration varied in the range of 0.02–3.0 mol/L while the sucrose concentration was constant at 0.8 mol/L revealed a linear dependence of V_{Fru}/V_{Glc} on the acceptor substrate concentration. The slope of this linear dependence yields the kinetic partition coefficient ($k_{glycerol}/k_{water}$) for the reaction of covalent enzyme intermediate with glycerol and water. It had a value of 7.9 L/mol. The comparison of $k_{glycerol}/k_{water}$ for sucrose phosphorylase with kinetic partition coefficients for transglycosylation by other enzyme systems for which a similar analysis has been performed suggests that glycerol should be a synthetically useful acceptor substrate to become glucosylated from sucrose.

Based on the available kinetic evidence, synthesis experiments were designed (*e.g.* 0.3 mol/L sucrose, 2.0 mol/L glycerol) in which the formation of glucosyl transfer product was monitored by HPLC as well as NMR analysis of samples taken at certain times. It was found that transglucosylation occurred with excellent efficiency (approx. 95 % of donor substrate converted), implying that competition from the hydrolysis reaction was nearly absent under the used conditions. Structural characterization of the product revealed glucosyl transfer with high selectivity for the 2-OH of glycerol. Within limits of detection of the used analytical method, no glucosylation of the 1-OH was observed.

Optimization of the biocatalytic transformation involved systematic variation of the acceptor/donor ratio at different levels of sucrose. We found that 0.8 mol/L of sucrose gave the best transfer yield (85 %) under the conditions when excess of glycerol (2.0 mol/L) was present. Further increase of the sucrose concentration resulted in lowering of yield and productivity. The use of a glycerol/sucrose ratio of ≥2 promotes glucosyl transfer in high yield (≥85 %). However, a process development that integrates biocatalytic reaction and downstream processing might sacrifice a few percent of yield in favour of a reduced amount of unreacted glycerol that needs to be removed during product isolation. We were able to decrease the concentration of glycerol to 1.0 mol/L without seriously compromising the synthesis of GG from 0.8 mol/L of sucrose (72 % yield).

Fig. 2 shows a time course of glucosylation of glycerol (2.0 mol/L) from sucrose (1.0 mol/L) using a crude preparation of *L. mesenteroides* sucrose phosphorylase as the catalyst. The enzyme used in the reaction was recombinantly produced in *E. coli* and can be obtained in useful yield and productivity from a bioreactor culture of the organism (*20*). It was determined that the performance of sucrose phosphorylase in the synthesis of GG was not dependent on biocatalyst purity (*42*). The isolated enzyme could be replaced by crude *E. coli* cell extract containing the same amount of phosphorylase activity without introducing a negative effect on either product yield or productivity. However, the use of whole bacterial cells expressing sucrose phosphorylase was accompanied by a substantial decrease in product yield as compared to the same reaction employing the free enzyme.

Analysis using HPLC and NMR showed that stereochemically pure GG was obtained in about 90 % yield, based on the converted donor substrate. The final product concentration was close to 1 M (250 g/L) and with the enzyme loading of 20 000 U/L, productivity of about 1.5 mol/(L·h) was reached. Considering that synthesis of GG is a kinetically controlled reaction (see Scheme 4) for which it would be expected that the overall conversion eventually approaches a thermodynamic equilibrium favouring hydrolysis of all glycosidic compounds present, the time course in Fig. 2 is quite interesting. Most of the transformation of sucrose into GG happens in the first 24 h of reaction and after this time, an apparent equilibrium appears to have been reached where the concentration of all measured compounds remains (nearly) constant. By way of comparison, a biocatalytic transglycosylation in which the glycosidic product undergoes rapid secondary hydrolysis by the enzyme typically results in a reaction time course where product concentration first increases to a transient maximum value but then decays at extended incubation times. The technological advantage of a time course like that seen for GG production in Fig. 2 is that residence time in the (industrial) reactor needs not be tightly controlled, which it has to be for kinetically 'unstable' products. We could also show that there is a marked hindrance for hydrolysis of GG by sucrose phosphorylase, providing a plausible explanation for the unusual (kinetic) stability of GG under the conditions used for the synthesis. The turnover frequency (k_{cat}) of the enzyme for GG was of the order of $5{\cdot}10^{-4}$ s^{-1} and can be compared with a k_{cat} of about 2 s^{-1} for the hydrolysis of sucrose. The mechanistic basis for the poor reactivity of sucrose phosphorylase with GG is not fully clear but it seems that replacement of D-fructosyl (in sucrose) by glycerol (in GG) as the leaving group of the donor substrate causes enzyme glucosylation to become an extremely slow step of the overall catalytic cycle of the phosphorylase.

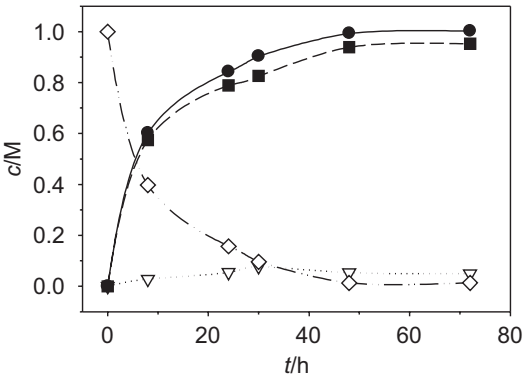


Fig. 2. Time course of synthesis of GG. Following conditions were used: sucrose 1.0 M, glycerol 2.0 M, sucrose phosphorylase 20 U/mL (crude *E. coli* cell extract containing the recombinant enzyme) at 30 °C, pH=7.0. ● D-fructose, ▽ D-glucose, ■ GG, ◇ sucrose

The product mixture obtained in the conversion shown in Fig. 2 contained (in g/L): GG 224, D-fructose 172, glycerol 103, sucrose 13 and D-glucose 13. The isolation of GG at a purity of 95 % or greater was accomplished on laboratory scale by elution chromatography using a packed bed of activated charcoal. GG was obtained by gradient elution using increasing concentrations of ethanol in water. The yield of the purification

was about 60 %, leaving clear room for improvement of the overall production process in the downstream processing.

Enzymatic production of GG (*43*) was implemented on industrial scale by the company bitop AG (Witten, Germany) and GG was commercialized under the tradename Glycoin®. A product termed Glycoin Extremium is available on the market and will be used as active ingredient for cosmetic formulations (*44*).

Technical Features Decisive for Successful Process Development

Transglycosylation efficiency and yield

Sucrose phosphorylase catalyzes glucosyl transfer with retention of the α-anomeric configuration of the donor substrate in the resulting glucosidic product. The enzyme shares its double displacement-like catalytic mechanism (Scheme 2) with the vast majority of glycoside hydrolases and transglycosidases that catalyze their reactions *via* configurational, α→α or β→β retention (*45*). Interception of the glycosyl enzyme intermediate by external acceptors is the common mechanistic basis for transglycosylation by these enzymes (*46*). Transfer to acceptor usually occurs under strong competition with the reaction of water. The transglycosylation product is by definition a substrate of the enzyme and can therefore be degraded by secondary hydrolysis. An 'ideal' biocatalytic system for transglycosylation would efficiently prevent primary hydrolysis of the glycosylated enzyme and also show low reactivity towards the glycosyl transfer product (secondary hydrolysis). Both primary and secondary hydrolysis decrease the atom economy of the biotransformation. Furthermore, the hydrolysis product may inhibit the enzyme and it can serve as an unwanted glycosyl acceptor, leading to contamination of the glycosidic product derived from transglycosylation. The glycosyl donor substrate should be a chemically stable and benign ('green') compound. However, its reactivity towards the enzyme should be sufficient to provide a high steady-state concentration of the covalent intermediate. Thermodynamically, the donor substrate should drive the overall transglycosylation towards the product. Results (see Fig. 2) show that the system 'sucrose phosphorylase and sucrose' differs from other transglycosylation catalysts in that it fulfills these requirements very well.

In terms of catalytic efficiency (k_{cat}/K_M measured at a saturating concentration of phosphate), sucrose is a 5.4-fold better donor substrate for glucosylation of sucrose phosphorylase (from *L. mesenteroides*) than G1P (*38*). Considering the equilibrium constant for phosphorolysis of sucrose (K_{eq}~44) (*20*), the overall thermodynamic driving force for glucosylation to proceed from sucrose will be substantially higher than that for the same transformation starting from G1P. Kinetic studies in which sucrose and G1P were compared as substrates for glucosylation of glycerol have revealed that the covalent intermediate of *L. mesenteroides* sucrose phosphorylase reacted more efficiently with the acceptor when sucrose instead of G1P was employed as glucosyl donor (*42*). Enhanced transglucosylation efficiency could result from a truly increased reactivity of the glucosylated enzyme towards glycerol when sucrose is present or it could be apparent and reflect (partial) suppression of the reaction with water under these conditions. Whatever explanation is correct, in practical terms, however, sucrose is the clearly preferred choice of donor substrate for the enzyme.

Regioselective glucosylation

The active site of sucrose phosphorylase provides splendid control over the regioselectivity of glucosyl transfer to glycerol such that only the desired product is obtained (*42*). By contrast, α-transglucosidases used for synthesis of GG gave a mixture of α-glucosylglycerol regioisomers in which 1-*O*-α-D-glucopyranosyl-*rac*-glycerol was the main constituent (*14,47*). It would be difficult to separate authentic GG from these other regioiso-

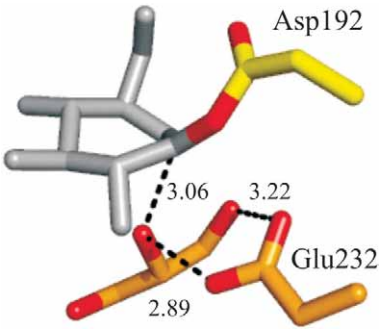
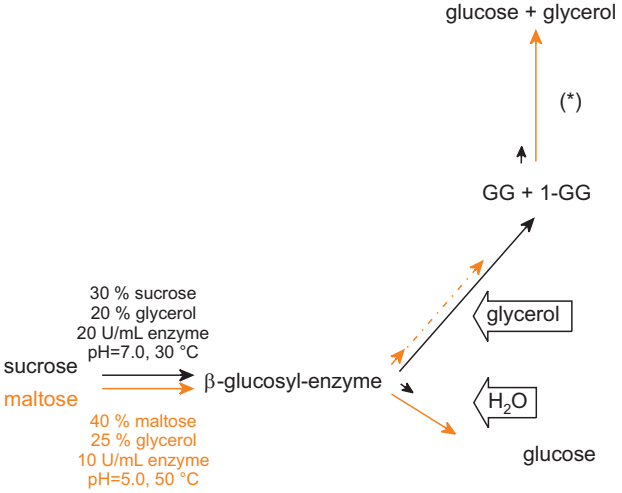


Fig. 3. Interactions of the acceptor glycerol with the β-glucosyl enzyme intermediate of sucrose phosphorylase from *B. adolescentis* (PDB-entry 2gdv, molecule A) derived from an energy-minimized flexible ligand-protein (orange) docking experiment (*48*). Distances are in Å



Scheme 5. Comparison of transglucosylation catalysts for the production of GG. The relative flux through each step of the respective enzymatic reaction was calculated using the data from literature and is indicated by arrow length (black: sucrose phosphorylase (*42*); orange: α-glucosidase (*14*)). Aspects related to regioselectivity of glucosyl transfer (dotted lines) and secondary product hydrolysis (*) are indicated. The scheme is redrawn according to Goedl *et al.* (*40*). 1-GG, 1-*O*-α-D-glucopyranosyl-*rac*-glycerol

mers. We and other authors have observed for sucrose phosphorylase that under the conditions in which a suitable 1,2-diol moiety is available for reaction in the acceptor substrate, it is generally the secondary hydroxyl that becomes glucosylated (*48*). Interactions at the acceptor binding site, suggested by the results of docking experiments based on the crystal structure of glucosylated sucrose phosphorylase from *B. adolescentis* (Fig. 3), provide a rationale for a highly regioselective glucosyl transfer by the enzyme. In Fig. 3, glycerol is positioned so that the C2-OH is ready for undergoing reaction (distance 3.06 Å) with the anomeric carbon of the β-glucosyl residue. Glu232 has a bidentate hydrogen bonding interaction with C1-OH and C2-OH of glycerol. With a distance of 2.89 Å from one of its oxygens to the oxygen of glycerol C2-OH, the (ionized) side chain of Glu232 appears to be placed suitably for providing general base catalysis to the nucleophilic attack of the acceptor substrate. The model in Fig. 3 therefore suggests that the role of C1-OH of glycerol is not just in acceptor substrate recognition. By pulling the side chain of Glu232 into the requisite position for 'activation' of C2-OH, the primary hydroxyl is expected to be directly auxiliary to the catalytic event.

Scheme 5 (*14,40,42*) summarizes the advantages of using sucrose phosphorylase for the synthesis of GG as compared to literature where a chiefly hydrolytic α-transglucosidase was employed for α-glucosylglycerol production from maltose and glycerol.

Novel Applications of GG

Proteins constitute one of the most rapidly growing classes of biotechnological products. Their major current applications are in the biopharmaceutical sector (*49–51*) and as industrial enzymes (*52–54*). Irrespective of their use, however, many of the commercialized proteins are not stable enough to fully resist denaturing effects occurring during their production (*55,56*), while being stored (*57,58*), and under the conditions of application (*52,53*). One useful strategy employed to prevent protein denaturation, and the loss of function usually associated with it, is the use of external stabilizers (*59–61*). Compared to other approaches that rely on modification of the target protein, it offers the advantages of working with the native protein structure and being potentially applicable to a number of different proteins. Due to their effects on protein preferential hydration and other direct interactions that favour the folded over the unfolded protein state (*62,63*), glycosidic solutes often serve to enhance both thermodynamic and/or kinetic stability of proteins (*59*). Mannosylglycerate (MGA; Scheme 1), a solute widely distributed in (hyper)thermophilic microorganisms, has recently been identified as a powerful stabilizer of different enzymes against thermally induced inactivation and unfolding (*64–67*). It was shown that the 'thermo-protection' conferred by MGA was usually superior to that of the widely used protein stabilizer α,α-trehalose (*66,67*).

GG was evaluated as a stabilizer of different enzymes undergoing inactivation by elevated temperature or freeze drying. The stabilizing effect was benchmarked against that of α,α-trehalose. Results are compiled in Fig. 4 for temperature stress (*68,69*) and Table 1 for freeze drying (*70*). They support the use of GG as an external reagent protecting protein preparations against denaturation (*71*).

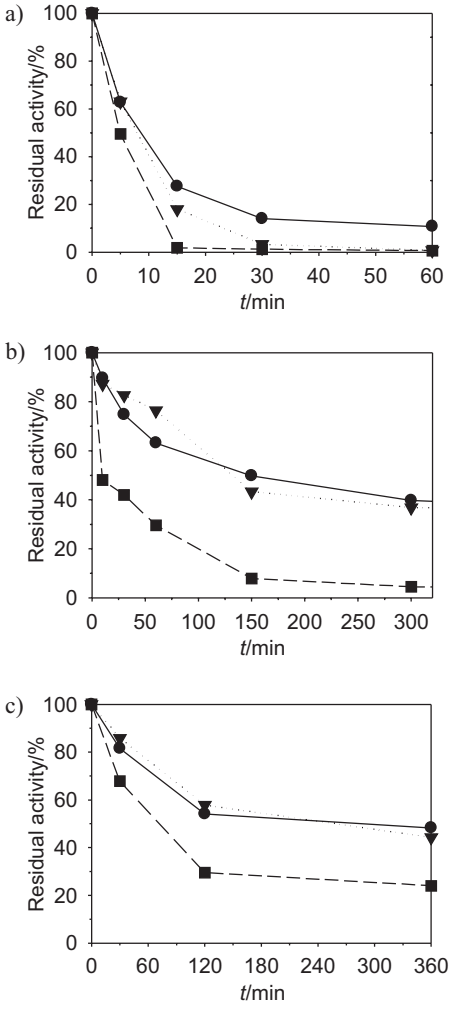


Fig. 4. Inactivation time courses at elevated temperature for: a) L-lactic dehydrogenase from rabbit muscle (Sigma) at 40 °C, b) *Corynebacterium callunae* starch phosphorylase (*68*) at 35 °C and c) *Candida tenuis* xylose reductase (*69*) at 30 °C. Experiments were performed using 50 μg of protein per mL in the absence (■) or presence of 0.5 M of GG (●) and α,α-trehalose (▼). Data taken from Sawangwan *et al.* (*71*)

Table 1. Stabilization of *Pseudomonas fluorescens* mannitol 2-dehydrogenase (*70*) during freeze drying. A protein concentration of 50 μg/mL was used

c(stabilizer)/mM	Residual activity/%	
	GG	α,α-trehalose
2.5	15	19
5	17	26
10	20	32
25	92	70
50	89	67
100	83	67
250	79	73
500	76	75
750	67	68

Conclusion

Sucrose phosphorylase is a long known enzyme whose splendid features as glucosylation catalyst may not have been fully appreciated in the past. The enzyme used together with sucrose as the donor substrate provides a highly efficient biocatalytic system for the synthesis of different α-D-glucosides linked to a range of structurally diverse *O*-aglycons. Commercialization of production of GG presents a first case of application of sucrose phosphorylase in the industrial fine chemical synthesis. The commercial product Glycoin® developed from GG is currently being evaluated for use in cosmetics. Another interesting application of GG is its use as a protein stabilizer.

Acknowledgements

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