Applied Biocatalysis

DOI: 10.1002/anie.200803562

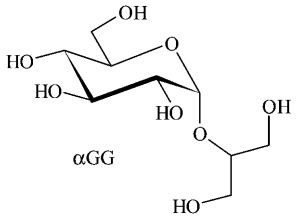
A High-Yielding Biocatalytic Process for the Production of 2-*O*-(α-D-glucopyranosyl)-*sn*-glycerol, a Natural Osmolyte and Useful Moisturizing Ingredient**

*Christiane Goedl, Thornthan Sawangwan, Mario Mueller, Alexandra Schwarz, and Bernd Nidetzky**

Dedicated to Professor Herfried Griengl on the occasion of his 70th birthday

Compatible solutes constitute a diverse class of small organic molecules, many of which have a glycosidic chemical structure. They are found ubiquitously in nature, where they serve an essential function in protecting cells against high salt concentration, extremes of temperature, and other forms of external stress. Their physiological effectiveness[1] and technological performance[2] can be traced back to their ability to regulate the cellular water balance, prevent protein denaturation, and stabilize supramolecular biological structures, such as those of lipid membranes.

Glycosyl glycerols are powerful osmolytes that are produced by various plants, algae, and bacteria in adaptation to salt stress and drought.[3] Among them, 2-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (αGG), the main compatible solute in

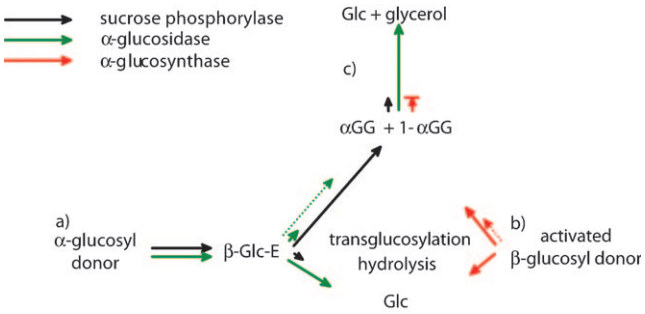


αGG

photosynthetic bacteria,[4] has attracted special attention as a promising moisturizing agent in cosmetics,[5] but also as a low-calorie sweetener for the prevention of tooth decay.[6] Possible therapeutic applications based on the ability of αGG to stabilize proteins and cells are currently under evaluation.[7]

However, the development of industrial applications for αGG is severely restricted by compound availability. Reported synthetic procedures are not technologically mature as a result of insufficient yield, selectivity, or productivity, or a combination of these problems.[6,8] We describe herein a new biocatalytic process which overcomes the chemical and technological challenges associated with the production of stereochemically pure αGG as an industrial chemical.

Scheme 1 summarizes the key features of the enzymatic synthesis of αGG by transglucosylation to glycerol from sucrose. Under natural conditions, sucrose phosphorylase (EC 2.4.1.7) catalyzes the reversible conversion of sucrose and phosphate into α-D-glucose 1-phosphate (αG1P) and



Scheme 1. Comparison of enzymatic routes for the production of αGG by a) transglucosylation and b) a hypothetical "α-glucosynthase"-catalyzed reaction. The relative flux through each step is indicated by arrow length and derived from kinetic data from this study and reference [6]. Available kinetic data for a β-glucosynthase-catalyzed reaction were used for path b.[25] The problems of regioselectivity (dotted lines) and secondary product hydrolysis (c) are indicated. 1-αGG, 1-*O*-(α-D-glucopyranosyl)-*sn*-glycerol.

D-fructose.[9] In the absence of phosphate, glycerol can intercept the β-glucosyl enzyme intermediate of the reaction with sucrose to produce αGG; hydrolysis of the glucosylated enzyme can also occur as a side reaction.[10] Although the overall strategy of the transglycosidase-catalyzed synthesis of glycosides is well established in carbohydrate chemistry,[11] several peculiarities make this biocatalytic process to our knowledge unique. First of all, the active site of sucrose phosphorylase provides splendid control over the regioselectivity of glucosyl transfer. The regioselectivity of this process is often insufficient with other transglycosidases (see reference [12] for the general case and reference [13] for the

[*] C. Goedl, T. Sawangwan, M. Mueller, Dr. A. Schwarz, Prof. Dr. B. Nidetzky
Institute of Biotechnology and Biochemical Engineering
Graz University of Technology
Petersgasse 12/1, 8010 Graz (Austria)
Fax: (+43) 316-873-8434
E-mail: bernd.nidetzky@tugraz.at
Homepage: http://www.biote.tugraz.at

[**] Financial support from the FWF Austrian Science Fund (DK Molecular Enzymology W901-B05) and the OEAD is gratefully acknowledged. We thank Prof. Dr. Lothar Brecker, University of Vienna, for the NMR spectroscopic analysis.

synthesis of αGG). Second, with the enzyme sucrose phosphorylase, the competing reaction with water is suppressed kinetically to the extent that hydrolysis of the substrate is prevented completely in the presence of glycerol as an acceptor at a suitable concentration. Third, the use of sucrose as a high-energy glucosyl donor, in combination with the substantial kinetic hindrance to the degradation of αGG by sucrose phosphorylase, provides a large driving force for an essentially unidirectional reaction, which gives the product in almost quantitative yield (on the basis of converted substrate). Therefore, the biocatalytic process for the production of αGG unites the main synthetic advantages of transglycosidases (with respect to the relative simplicity of the reaction system and the use of cheap substrates), glycosyltransferases (with respect to product uniformity due to the high regioselectivity of glucosyl transfer), and glycosynthases (with respect to the kinetic stability of the glycosidic product).[14,15]

Steady-state kinetic assays were performed to study the competing reactions of the glucosyl enzyme intermediate of sucrose phosphorylase with glycerol and bulk water. Depending on the glucosyl donor used as the substrate, the rate of fructose (V_{Fru}; sucrose) or phosphate (V_{Pi}; αG1P) release was measured along with the rate of glucose formation (V_{Glc}). The ratios V_{Fru}/V_{Glc} and V_{Pi}/V_{Glc} were determined at varying concentrations of glycerol (Figure 1a). With each donor substrate, the rate ratio increased with an increasing concentration of glycerol, as expected if glycerol competes with bulk water to react with the glucosylated enzyme (Scheme 1). Fits of straight lines to data obtained with sucrose and αG1P gave slope values of 7.9 M^{-1} and 2.8 M^{-1}, respectively. These values reflect the kinetic partition coefficient of the glucosylated enzyme under the conditions used and indicate clearly that, in contrast to previous findings,[16] glycerol takes part in the enzymatic reaction as an acceptor of the glucosyl residue transferred from the enzyme. Importantly, the leaving group of the donor substrate influenced the overall efficiency of glucosyl transfer to glycerol, a result which is not accounted for by Scheme 1. We ascribe the observed effect tentatively to conformational flexibility at the acceptor-binding site of sucrose phosphorylase. Such flexibility is apparent from high-resolution X-ray structures of the enzyme (from *Bifidobacterium adolescentis*).[17]

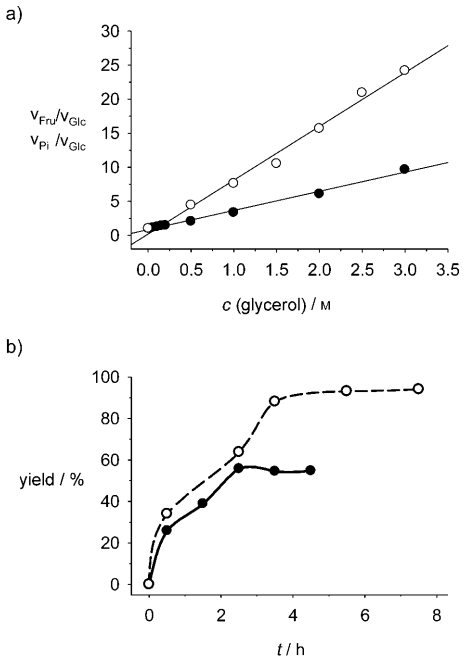


Figure 1. Comparison of sucrose and αG1P as glucosyl-donor substrates for the synthesis of αGG. a) Kinetic partitioning analysis with sucrose (0.8 M, ○) and αG1P (0.1 M, ●) in the presence of sucrose phosphorylase at concentrations of 20 U mL^{-1} (○) and 3 U mL^{-1} (●). b) Formation of αGG during the reaction of sucrose (0.3 M, ○) or αG1P (0.1 M, ●) with glycerol (2.0 M) in the presence of sucrose phosphorylase (20 U mL^{-1}) at 30 °C and pH 7.0.

The ramifications of the kinetic evidence for the synthesis of αGG are shown in Figure 1b, in which the course of product formation over time is compared for the enzymatic conversion of sucrose and αG1P. The yield of transfer product as determined by HPLC was much higher with sucrose (ca. 95% with respect to conversion of the donor substrate) than with αG1P. The formation of free glucose serves as a measure of the fraction of glucosyl residues not transferred to glycerol. The data in Figure 1b corroborate the hypothesis that hydrolysis competes more strongly with glucosyl transfer when sucrose is replaced with αG1P as the donor substrate. NMR spectroscopic analysis of the product mixture obtained after the complete conversion of sucrose revealed that, within the limits of detection of the methods used (0.02%), only the desired regioisomer of α-glucosylglycerol had been formed.

Table 1 summarizes the results of experiments carried out to optimize the product and space–time yields of the enzymatic conversion of sucrose and glycerol. Considering the broad optimum pH range (5.0–8.0) for the enzymatic formation of αG1P from sucrose,[18] there was an unexpectedly narrow operating range with respect to the pH value for the synthesis of αGG. The optimum pH value is 7.0. By varying the initial concentrations of sucrose and glycerol systematically, we established conditions (0.8 M sucrose, 2.0 M glycerol) under which the specific space–time yield (STY, based on the amount of enzyme activity used per unit volume) was highest and, at the same time, the product yield was around 90%. A further increase in the concentrations of sucrose and glycerol did not lead to significant improvements in either the STY or the product yield. To determine the required purity of the soluble biocatalyst, we compared the performance of the crude *Escherichia coli* cell extract from which recombinant sucrose phosphorylase was obtained with that of the isolated enzyme. There was no detectable difference between the two enzyme preparations (Table 1). However, the use of whole bacterial cells that express sucrose phosphorylase led to a substantial decrease in product yield as compared to the same reaction with the free enzyme.

Figure 2 shows the complete course of αGG synthesis over time under optimized reaction conditions. Importantly, the hydrolysis of sucrose occurred to only a very small extent throughout the reaction. The transfer product (0.7 M) was isolated with an estimated purity of $\geq 98\%$ (HPLC) and in an overall yield of about 63% by a single-step chromatographic

Table 1: Optimization of the synthesis of αGG with respect to the product yield and specific space–time yield (STY).[a]

c(sucrose) [M]	*c*(glycerol) [M]	Transfer yield [%]	STY [M h^{-1} 1000 kU]
0.3	2.0	93	0.54
0.5	2.0	93	0.97
0.8	2.0	85	1.41
1.0	2.0	80	1.11
1.5	2.0	55	1.15
2.0	2.0	47	1.31
0.8	0.5	47	0.59
0.8	1.0	60	0.75
0.8	1.5	73	0.91
0.8	2.0	88[b]/63[c]	1.11
0.8	2.5	79	0.98
0.8	3.0	87	1.09
0.8	2.0	85	1.41
0.8	2.0	86[d]	1.43
0.8	2.0	33[e]	–
0.3	2.0	30[f]	0.19
0.3	2.0	68[g]	0.43
0.3	2.0	95[h]	0.59
0.3	2.0	63[i]	0.39
0.3	2.0	39[j]	0.25
0.1[k]	3.0	55	0.68

[a] Unless otherwise mentioned, the isolated enzyme was used in MES buffer (50 mM, pH 7.0). [b] Analytical yield. [c] Yield of the isolated product. [d] A cell-free extract was used. [e] Whole *E. coli* cells (3.25 g) were used. [f] The reaction was carried out at pH 5.5. [g] The reaction was carried out at pH 6.5. [h] The reaction was carried out at pH 7.0. [i] The reaction was carried out at pH 7.5. [j] The reaction was carried out at pH 8.5. [k] αG1P was used.

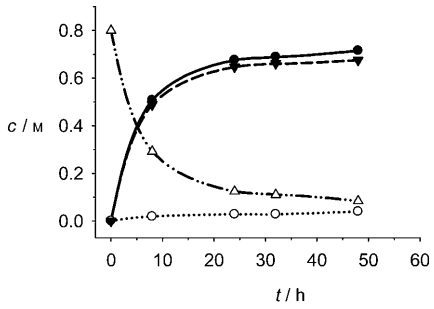


Figure 2. Synthesis of αGG under optimized reaction conditions: 0.8 M sucrose, 2.0 M glycerol, 20 U mL^{-1} enzyme, pH 7.0. ● D-fructose, ○ D-glucose, ▼ αGG, △ sucrose.

workup (see Experimental Section). The chemical structure of the product was confirmed by NMR spectroscopy.[*]

[*] Assignment of the chemical shifts in αGG: ^{1}H NMR: glucose: 1-H, 5.21; 2-H, 3.66; 3-H, 3.84; 4-H, 3.52; 5-H, 3.93; 6-H^a, 3.95; 6-H^b, 3.83 ppm; glycerol: 1′-H^a, 3.80; 1′-H^b, 3.75; 2′-H, 3.91 ppm. ^{13}C NMR: glucose: C1, 98.2; C2, 71.9; C3, 73.3; C4, 69.9; C5, 72.4; C6, 61.0 ppm; glycerol: C1′, 63.8; C2′, 79.2 ppm.[8a] Chemical shifts are referenced to external acetone: $\delta(^1H) = 2.22$ ppm, $\delta(^{13}C) = 31.5$ ppm.

Secondary product hydrolysis frequently presents a major restriction to transglycosidase-catalyzed glycoside synthesis. For this reason, the enzymatic reaction must usually be performed in a tightly controlled kinetic regime, and product yields are often only around 10–20%.[19] Concerns about hydrolytic breakdown of the product have driven the development of the glycosynthase concept. Glycosynthases are engineered glycosidases that cannot promote secondary hydrolysis because of structural changes in their active site.[20] However, the successful uncoupling of product synthesis and hydrolysis hinges on the use of highly activated glycosyl donors, which are often chemically unstable.[14] In light of the state of transglycosidase-catalyzed glycoside synthesis, it was intriguing that the formation of αGG appeared to be equilibrium controlled (see Figures 1b and 2), which implies an unusual kinetic stability of the product under the transglucosylation conditions used. We therefore measured the activity of sucrose phosphorylase by using purified αGG as the glucosyl-donor substrate and phosphate, water, or fructose as the acceptor. The turnover frequency was extremely low in these reactions ($k_{cat} \leq 5 \times 10^{-4}$ s^{-1}) and can be compared with the k_{cat} values of about 100 s^{-1} and 2 s^{-1} for the conversion of sucrose into αG1P and D-glucose, respectively. Thus, the replacement of D-fructose with glycerol as the leaving group increased the stability of the α-glucoside towards hydrolysis catalyzed by sucrose phosphorylase by nearly four orders of magnitude.

In conclusion, an exceptionally efficient and selective transglycosidase process has been developed. This process should provide the basis for the production of αGG as an industrial chemical.[21] We expect that it will also promote the development of projected applications of this compound, particularly as an ingredient for cosmetic formulations.

Experimental Section

Synthesis of αGG: Sucrose (0.3–2.0 M) or αG1P (0.1 M) was incubated with glycerol (0.5–3.0 M) and recombinant sucrose phosphorylase from *Leuconostoc mesenteroides*[22] (3–80 U mL^{-1}) in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (50 mM, pH 5.5–8.5) at 30 °C (agitation rate: 550 rpm) for up to 72 h. The reaction time depended on the quantity of the enzyme. Samples taken after appropriate time intervals were inactivated by heating and centrifuged. The isolated enzyme was purified according to a reported procedure.[10]

Analytical methods: The concentration of D-fructose was determined in an enzymatic assay by using recombinant mannitol dehydrogenase from *Pseudomonas fluorescens*.[23] A colorimetric glucose oxidase/peroxidase assay was used to determine the concentration of D-glucose. Glucose oxidase from *Aspergillus niger*, horseradish peroxidase, and *o*-dianisidine were purchased from Sigma. The quantity of inorganic phosphate present was determined colorimetrically at 850 nm.[24] HPLC analysis was performed on an aminex HPX-87C column (Bio-Rad) at 85 °C and at a constant flow rate of 0.6 mL min^{-1} with deionized water as the mobile phase. The concentration of αGG was determined directly by HPLC or indirectly from the difference in the concentrations of D-fructose (or phosphate) and D-glucose.

Isolation of αGG: The product mixture (16.2 g αGG in 90 mL) was loaded onto an XK 50/60 column. The column material (1 L) consisted of a 1:1 mixture of activated charcoal Norit (type Norit SX ultra, Sigma) and calcined Celite 501 (Sigma). A four-step elution gradient was used: The products were eluted with deionized water

(4 L) followed by 2% ethanol/water (4 L), 15% ethanol/water (2 L), and 25% ethanol/water (1 L) at a constant flow rate of 20 mL min^{-1}. The fractions in 2% ethanol, which contained αGG (10.2 g, 40 mmol), were pooled, concentrated under vacuum, lyophilized, and stored at −21 °C.

Received: July 22, 2008
Published online: November 21, 2008

Keywords: enzymatic synthesis · glycosides · kinetics · regioselectivity · transglucosylation

[1] a) H. Santos, P. Lamosa, T. Q. Faria, N. Borges, C. Neves in *Physiology and Biochemistry of Extremophiles* (Eds.: C. Gerday, N. Glansdorff), ASM Press, Washington, DC, **2007**, pp. 86–103; b) N. Empadinhas, M. S. da Costa, *Int. Microbiol.* **2006**, *9*, 199–206; c) M. S. da Costa, H. Santos, E. A. Galinski, *Adv. Biochem. Eng./Biotechnol.* **1998**, *61*, 117–153.

[2] a) G. Lentzen, T. Schwarz, *Appl. Microbiol. Biotechnol.* **2006**, *72*, 623–634; b) "Orally used compatible solute containing agents": T. Schwarz, G. Lentzen, J. Krutmann, Germany, WO 2006097263, **2006**.

[3] D. K. Hincha, M. Hagemann, *Biochem. J.* **2004**, *383*, 277–283.

[4] S. Fulda, J. Huckauf, A. Schoor, M. Hagemann, *J. Plant Physiol.* **1999**, *154*, 240–249.

[5] a) "α-D-Glucopyranosyl glycerol derivatives as antiallergic agents, health foods, and cosmetics": K. Yoshida, A. Takenaka, T. Nitta, M. Iki, Japan, JP 2007137862, **2007**; b) "Cosmetic preparations containing glucosyl glycerides and one or more acrylamidomethyl propylsulphonic acid polymers": U. Breitenbach, V. Kallmayer, T. Raschke, C. Scherner, W. Siefken, S. Viala, Germany, WO 2006122669, **2006**; c) "Cosmetic preparations with an effective amount of glycosylglycerides as skin moisturizers": J. Thiem, O. Scheel, G. Schneider, Germany, EP 770378, **1997**.

[6] F. Takenaka, H. Uchiyama, *Biosci. Biotechnol. Biochem.* **2000**, *64*, 1821–1826.

[7] a) "Antitumor agents containing α-D-glucopyranosylglycerols and food and cosmetics containing them": T. Nitta, A. Takenaka, M. Iki, E. Matsumura, M. Sakaguchi, JP 2007262023, **2007**; b) D. Colombo, F. Compostella, F. Ronchetti, A. Scala, L. Toma, H. Tokuda, H. Nishino, *Eur. J. Med. Chem.* **2000**, *35*, 1109–1113.

[8] a) R. Suhr, O. Scheel, J. Thiem, *J. Carbohydr. Chem.* **1998**, *17*, 937–968; b) N. Ohta, K. Achiwa, *Chem. Pharm. Bull.* **1991**, *39*, 1337–1339; c) A. Roder, E. Hoffmann, M. Hagemann, G. Berg, *FEMS Microbiol. Lett.* **2005**, *243*, 219–226.

[9] a) C. Goedl, A. Schwarz, M. Mueller, L. Brecker, B. Nidetzky, *Carbohydr. Res.* **2008**, *343*, 2032–2040; b) J. J. Mieyal, R. H. Abeles, in *The Enzymes*, *Vol. 7*, 3rd ed., Academic Press, New York, **1972**, pp. 515–532.

[10] A. Schwarz, B. Nidetzky, *FEBS Lett.* **2006**, *580*, 3905–3910.

[11] D. H. Crout, G. Vic, *Curr. Opin. Chem. Biol.* **1998**, *2*, 98–111.

[12] M. Scigelova, S. Singh, D. H. G. Crout, *J. Mol. Catal. B* **1999**, *6*, 483–494.

[13] H. Nakano, T. Kiso, K. Okamoto, T. Tomita, M. B. Manan, S. Kitahata, *J. Biosci. Bioeng.* **2003**, *95*, 583–588.

[14] F. A. Shaikh, S. G. Withers, *Biochem. Cell Biol.* **2008**, *86*, 169–177.

[15] a) M. Faijes, A. Planas, *Carbohydr. Res.* **2007**, *342*, 1581–1594; b) G. Perugino, A. Trincone, M. Rossi, M. Moracci, *Trends Biotechnol.* **2004**, *22*, 31–37; c) G. J. Davies, S. J. Charnock, B. Henrissat, *Trends Glycosci. Glycotechnol.* **2001**, *13*, 105–120.

[16] S. Kitao, H. Sekine, *Biosci. Biotechnol. Biochem.* **1992**, *56*, 2011–2014.

[17] O. Mirza, L. K. Skov, D. Sprogoe, L. A. van den Broek, G. Beldman, J. S. Kastrup, M. Gajhede, *J. Biol. Chem.* **2006**, *281*, 35576–35584.

[18] a) E. J. Vandamme, J. van Loo, L. Machtelinckx, A. de Laporte, in *Adv. Appl. Microbiol.*, *Vol. 32*, Academic Press, New York, **1987**, pp. 163–201; b) A. Schwarz, L. Brecker, B. Nidetzky, *Biochem. J.* **2007**, *403*, 441–449.

[19] S. J. Williams, S. G. Withers, *Carbohydr. Res.* **2000**, *327*, 27–46.

[20] S. M. Hancock, M. D. Vaughan, S. G. Withers, *Curr. Opin. Chem. Biol.* **2006**, *10*, 509–519.

[21] "Preparation of 2-*O*-glyceryl-α-D-glucopyranoside from a glucosyl donor and a glucosyl acceptor": C. Goedl, T. Sawangwan, B. Nidetzky, M. Mueller, Austria, WO 2008034158, **2008**.

[22] C. Goedl, A. Schwarz, A. Minani, B. Nidetzky, *J. Biotechnol.* **2007**, *129*, 77–86.

[23] M. Slatner, B. Nidetzky, K. D. Kulbe, *Biochemistry* **1999**, *38*, 10489–10498.

[24] S. Saheki, A. Takeda, T. Shimazu, *Anal. Biochem.* **1985**, *148*, 277–281.

[25] Y. W. Kim, H. Chen, S. G. Withers, *Carbohydr. Res.* **2005**, *340*, 2735–2741.

