A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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Accepted Article

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To be cited as: *ChemBioChem* 10.1002/cbic.201700215

Link to VoR: http://dx.doi.org/10.1002/cbic.201700215

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10.1002/cbic.201700215

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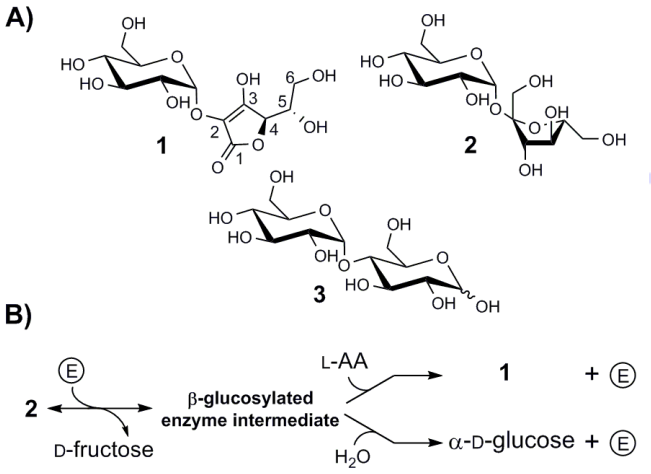
Walking a fine line with sucrose phosphorylase: efficient single-step biocatalytic production of L-ascorbic acid 2-glucoside from sucrose

Rama Krishna Gudiminchi[a] and Bernd Nidetzky*[a,b]

Abstract: The 2-*O*-α-D-glucoside of L-ascorbic acid (AA-2G) is a highly stabilized form of vitamin C, with important industrial applications in cosmetics, foods and pharmaceuticals. AA-2G is currently produced through biocatalytic glucosylation of L-ascorbic acid from starch-derived oligosaccharides. Sucrose would be an ideal substrate for AA-2G synthesis, but it cannot be used lacking a suitable transglycosidase. We show here that in a narrow pH window between 4.8 and 6.0, with sharp optimum at pH 5.2, select sucrose phosphorylases catalyzed 2-*O*-α-glucosylation of L-ascorbic acid from sucrose with high efficiency and perfect site-selectivity. Optimized synthesis with the enzyme from *Bifidobacterium longum* at 40°C gave a concentrated product solution (155 g/L; 460 mM) from which pure AA-2G was readily recovered in ~50% overall yield, so providing the basis for an advanced production process. The peculiar pH dependence was suggested to arise from a "reverse-protonation" mechanism in which the catalytic base Glu^{232} on the glucosyl-enzyme intermediate must be protonated for attack on the anomeric carbon from the 2-hydroxyl of the ionized L-ascorbate substrate.

Its high antioxidative activity causes L-ascorbic acid (L-AA) to be an inherently unstable molecule.[1] This restricts important applications, in cosmetics and foods for example, in which a long-lasting effect from L-AA is demanded. Derivatization of the 2-OH obtains stabilized forms, but releasing the free L-AA again can be problematic.[1a-b] The 2-*O*-α-D-glucoside (AA-2G; **1**, Scheme 1a) of L-AA presents a practical compromise between stability and bioavailability in humans, for it can be hydrolyzed slowly by an α-glucosidase widespread in the epithelial tissues.[2] AA-2G is an industrially produced fine chemical with established uses in skin-care cosmetic products.[3] Another important use, among a number of interesting applications, is that of a vitamin C supplement in foods.[4]

AA-2G is synthesized via a biocatalytic transglucosylation in which starch-derived cyclic or linear oligosaccharides are reacted with L-AA in the presence of a suitable glucanotransferase.[3b,5] In spite of the absolute stereo-selectivity and the usually also high site-selectivity of the enzymes used, the glucanotransferase process inherently suffers from a low yield based on the oligosaccharide substrate utilized.[5, 6] There are limitations due to the enzyme, attaching oligoglucosyl chains to L-AA, which have to be trimmed to a single glucosyl unit later, or having hydrolase side activity; but also others due to the substrate itself. From a thermodynamic point of view,[7] sucrose (**2**) would be a highly suitable glucosyl donor, for it exhibits an exceptionally energy-rich disaccharide structure, lacking in maltose (**3**) and short-chain malto-oligosaccharides. Thus, sucrose should be capable of promoting glucosylation of L-ascorbic acid far more efficiently than the reported donor substrates. Sucrose is furthermore cost-efficient, more so even than some donor substrates currently in use, cyclodextrins in particular. However, to benefit from sucrose in AA-2G synthesis, a suitable transglucosidase is needed.



Scheme 1. A) Structures of AA-2G (**1**), sucrose (**2**) and maltose (**3**) B) Kinetic scheme of synthesis of **1** via sucrose phosphorylase catalyzed transglucosylation from **2**.

In a variant of its natural reaction, that is α-glucosyl transfer from sucrose to phosphate, sucrose phosphorylase (EC 2.4.1.7) utilizes hydroxyl group-containing acceptors with broad specificity to form the corresponding α-glucosides (Scheme 1b).[8a] Several studies show that the enzyme, *combined* with sucrose as the donor substrate, constitutes an extremely efficient transglucosylation system, suitable for synthetic use across scales,[8] up to the industrial production.[8b] Examined for AA-2G synthesis, sucrose phosphorylase or engineered variants thereof did form small amounts of **1** but the enzymes were poor in utilizing the L-AA[9] and so would not be of practical use. Besides sucrose phosphorylase, glucansucrase (EC 2.4.1.5) could be considered for a synthesis of **1** via Scheme 1b in

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principle. However, its intrinsic preference to synthesize α-glucan polymers renders glucansucrase unlikely useful for efficient production of **1**.[10]

Investigating the reaction of sucrose phosphorylase (from *Bifidobacterium longum*) with L-AA in more detail, we discovered an unusual pH dependence of the enzyme's selectivity. At pH 7.0-7.5 where the enzyme was optimally active in phosphorolysis, hardly any AA-2G was formed (Figure 1a) and sucrose was utilized mainly via hydrolysis (Scheme 1b), so confirming the literature.[9] Enzyme site selectivity was also insufficient under these conditions: the regioisomeric 6-*O*-α-glucoside (AA-6G) accumulated to about 15-20% of the total glucosylation product from L-AA (Supporting Information, Figure S1). However, on decreasing the pH to 6.0 or lower, a strong activity of AA-2G synthesis appeared and AA-6G formation was suppressed below detection. The activity increased sharply on lowering the pH, reaching a distinct maximum at pH 5.2, as shown in Figure 1 (panel a). Further decrease in the pH resulted in strong activity loss, apparently reflecting the limit of tolerance to low pH in the enzyme used. A specific activity of 1.92 and 0.36 U/mg was determined for AA-2G formation at the optimum pH of 5.2 and pH 7.5, respectively. This can be compared with the enzyme's specific activity of 40 and 70 U/mg in phosphorolysis of **2** at pH 5.2 and the optimum pH of 7.5, respectively.

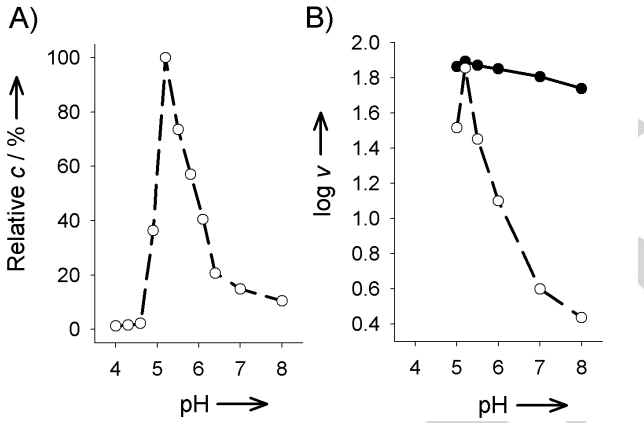


Figure 1. A) The pH dependence of synthesis of **1** from **2** (80 mM) and L-AA (120 mM) by *B. longum* sucrose phosphorylase is shown. The relative concentrations were compared with respect to the concentration of AA-2G achieved (15 mM) at pH 5.2. B) The pH-rate profile of synthesis of **1** from α-glucosyl fluoride (80 mM) using wild-type (○) and E232Q variant (●) sucrose phosphorylases. The reaction rate (*V*) is given in μM/min. Reaction conditions: purified enzyme (36 mg/L; 0.63 μM); 40°C; 24 h (a) or 45 min (b) reaction time. Reactions were performed in 50 mM sodium citrate buffer (pH 4.0-6.0) or in 50 mM MES buffer (pH 5.0-8.0).

To examine if AA-2G synthesis at low pH was a feature more generally found in sucrose phosphorylases, we analyzed 4 additional enzymes (*B. adolescentis*, *Streptococcus mutans*, *Lactobacillus acidophilus*, *Leuconostoc mesenteroides*), representing by and large the sequence diversity within the protein family (glycoside hydrolase family GH-13, subfamily 18).[9b] The phosphorylase from *B. adolescentis* was chosen in particular because its crystal structure was known.[11] Note that thermophilic enzymes[12] were not considered because issues of L-AA stability were expected at substantially elevated temperatures (≥55°C). Furthermore, the sucrose/sucrose 6'-phosphate phosphorylase from *Thermoanaerobacterium thermosaccharolyticum* was described with extremely low activity for using L-AA as the acceptor substrate.[12b] The feature that AA-2G was formed at pH 5.2 while it was essentially lacking at pH 7.5 was present in all phosphorylases tested. However, whereas the phosphorylases from *La. acidophilus* and *Le. mesenteroides* released AA-6G in substantial proportion (~20%) of total product, the site-selectivity of the enzymes from *B. adolescentis* and *S. mutans* was absolute with no AA-6G formed above the detection limit (Supporting Information, Figure S2). The pH dependence of AA-2G synthesis by the enzyme from *S. mutans* was almost superimposable on that of *B. longum* sucrose phosphorylase (Figure 1), suggesting a general trend. Enzyme comparison in preliminary synthesis experiments revealed the superior performance of the phosphorylase from *B. longum* (Supporting Information, Figure S3), which was therefore used further on. To summarize briefly, we showed that glucosylation of L-AA by sucrose phosphorylase exhibits a distinctive pH dependence which it is absolutely essential to consider for AA-2G synthesis. Requirement to use pH conditions far below the normal pH optimum and the consequent effects on activity and selectivity also made choice of the enzyme critical.

For the development of an efficient synthesis of **1** according to Scheme 1b, hydrolysis of **2** was a problem requiring special attention. Besides reaction optimization, enzyme engineering was considered right at the outset. A Q345F variant of *B. adolescentis* sucrose phosphorylase was recently shown to exhibit strongly decreased hydrolysis of **2** in transglucosylation reactions with polyphenolic acceptors.[8h,13] The *B. longum* variant harboring the analogous site-directed replacement was therefore prepared and evaluated for synthesis of **1** (Supporting Information, Figure S4). However, the Q345F variant had lost the high site-selectivity of the wild-type enzyme, producing ~30% AA-6G of total glycosylated product. Its specific reaction rate was also lowered substantially, almost 9-fold. The wild-type phosphorylase was therefore used further on.

Previous studies show that synthesis of α-D-glucosides by sucrose phosphorylase is conducted best at high concentrations of both the acceptor substrate and sucrose (≥ 0.5 M), for under these conditions the enzyme's activity toward hydrolyzing **2** is almost completely suppressed. This is usually not the case at low substrate concentrations.[8] The Supporting Information (Table S1) and Figure 2 (panel A) summarize the evidence from a systematic evaluation, thus establishing reaction conditions (0.8 M of **2**, 1.2 M of L-AA) under which both the end concentration (~460 mM in 72 h) and the yield of **1** (~40%, based on L-AA) were maximized. The reaction temperature of 40°C was a compromise between optimum reaction rate (50°C) and stability of L-AA and enzyme. A typical time course of the optimized conversion (Figure 2, panel b) comprised a fast initial reaction phase (up to ~10 h) in which about 50% of the total **1** was released. The second phase involved a gradual slowdown

of the **1** formation and the synthesis levelled out after about 72 h. A close mass balance between substrate **2** consumption (~70%) and product formation (~60% **1**; ~10% D-glucose) was obtained at this point. No isoforms of **1** were detectable. The molar ratio of **1** and glucose was approximately constant during the reaction. Its value of ~7.0 expresses the reaction selectivity of sucrose phosphorylase, transglucosylation compared to hydrolysis, under the conditions used. Additional dosing of fresh sucrose phosphorylase after 24 h and 48 h, when the reaction had almost come to a halt, caused the conversion to continue, however, only at a relatively low rate that suggested inhibition of the enzyme under these conditions.

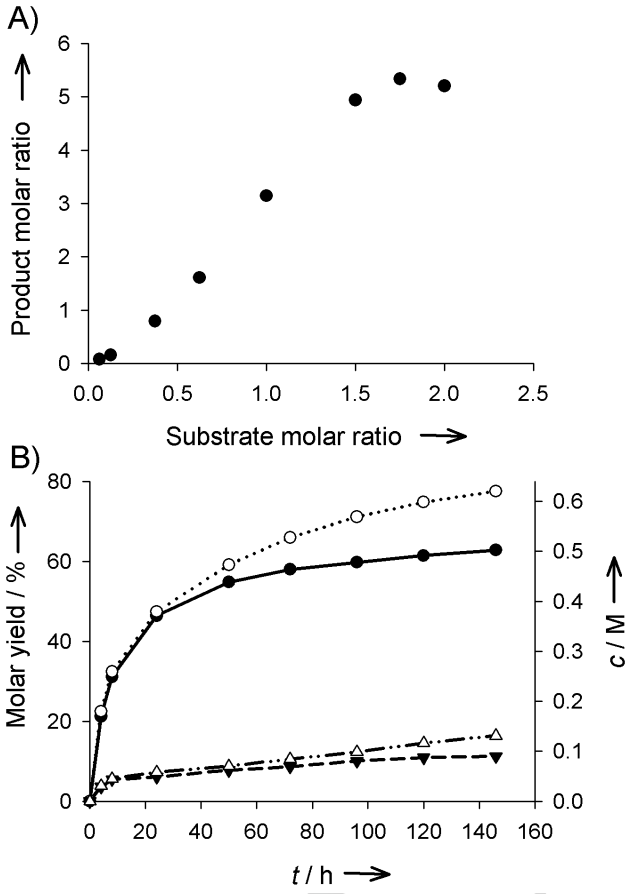
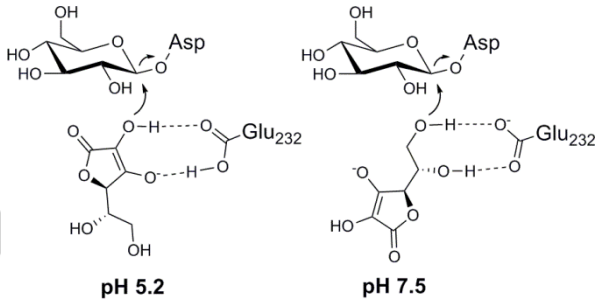


Figure 2. Optimization of synthesis of **1** by sucrose phosphorylase from *B. longum* is shown. A) Effect of the L-AA/**2** substrate ratio on the **1**/D-glucose product ratio. The L-AA concentration was varied (0.05-1.8 M) at a constant concentration of **2** (0.8 M). B) Time course of synthesis of **1** from **2** (0.8 M) and L-AA (1.2 M). Circles show **1**, triangles show D-glucose. Open and closed symbols show reactions with and without additions of enzyme (30 U/mL) after 24 h and 48 h. The molar yield is based on **2** converted. Reaction conditions: 40°C, pH 5.2, 30 U/mL purified enzyme (428 mg/mL; 7.5 μM).

Dosing of just sucrose at these times was ineffective. We furthermore showed in separate experiments that **1** was not hydrolyzed by sucrose phosphorylase within 48 h, so explaining the high kinetic stability of the **1** released in the synthesis. The decline in the **1** formation rate and the incomplete conversion of substrate **2** was due to a combination of effects of relatively strong product inhibition by **1** (K_i = ~50 mM) and enzyme inactivation. Enzyme stabilization to improve the total turnover number therefore constitutes a relevant target of further process optimization but was left for consideration in the future. Both enzyme engineering[13, 14] and immobilization[15] strategies have been used successfully in the past to develop stabilized preparations of sucrose phosphorylase.

Synthesis of **1** was performed at the gram scale (Supporting Information) and the product (~6 g) was recovered from the reaction mixture at ≥98% purity and in a yield of ≥50% using a two-step chromatographic work-up.[5] The expected product structure was confirmed by ^{1}H and ^{13}C NMR (Supporting Information, Figure S5).



Scheme 2. The proposed "reverse protonation" scenario of O2 glucosylation of L-AA at the optimum pH of 5.2 is shown. The pK_a of the catalytic residue Glu^{232} is ~5.8.[16] At pH 5.2, when Glu^{232} is largely protonated, the anionic L-AA may bind in the way shown to enable site-selective glucosylation at O2. At pH 7.5, when Glu^{232} is deprotonated and thus poised for canonical function as catalytic base for the glucosylation of non-ionic acceptor substrates, an alternative binding-mode of L-AA becomes favored. Glucosylation of L-AA is therefore no longer site-selective and occurs at both O2 and O6. Coordination of Glu^{232} to the relevant diol moieties of L-AA is hypothetical, however, in agreement with the binding pose of glycerol computationally docked to the glucosyl enzyme intermediate[19a] as well as with the way the fructosyl moiety of sucrose binds in the crystal structure of a Glu^{232}→Gln variant of sucrose phosphorylase from *B. adolescentis*.[11b] For further explanation, see text.

We hypothesized that the unusual pH dependence of **1** formation could have its origin in the requirement of sucrose phosphorylase to react with an *anionic* acceptor substrate. We also noted that glucosylation of acetic acid by the enzyme[17] exhibits a similar pH dependence as glucosylation of L-AA. The L-AA ionizes due to deprotonation of its enediol moiety above an apparent pK_a of ~4.2.[18] The phosphorylase performing a transglucosylation utilizes a double displacement-like mechanism in which a β-glucosyl enzyme intermediate, formed from sucrose and Asp^{192} (amino acid numbering of the enzyme from *B. longum*) in the first catalytic step, is intercepted by the acceptor substrate. The reactive hydroxyl group of the acceptor is normally not ionized and its attack occurs under base catalytic assistance from Glu^{232} (Scheme 2).[11, 19] An apparent pK_a of ~5.8 was determined for this Glu in the covalent β-glucosyl enzyme intermediate.[16] Under pH conditions in which Glu^{232} and L-AA both are ionized (e.g., pH 7.5), repulsion of like charges might disturb acceptor substrate binding, catalysis or both, with consequent effects on reactivity. At pH 5.2, however,

when Glu^{232} is protonated and charge repulsion therefore is no longer an issue, enzyme deglucosylation by L-AA might now occur readily as proposed in Scheme 2. To examine the scenario envisaged, we replaced Glu^{232} by the isosteric but nonionizable glutamine and analyzed the pH dependence of glucosylation of L-AA by the E232Q variant, expecting none in contrast to the wild-type enzyme. Since Glu^{232} is required as a general catalytic acid during enzyme glucosylation from **2**, this substrate could not be used, for the E232Q variant was virtually inactive with it, as expected.[11b, 16] Fortunately, α-D-glucosyl fluoride is an excellent donor substrate of sucrose phosphorylase[20] and its use during enzyme glucosylation does not rely on the presence of Glu^{232} as a general catalytic acid. Using α-D-glucosyl fluoride, therefore, wild-type and E232Q sucrose phosphorylases could be evaluated relative one to another in exactly comparable reactions. A specific activity of 2.20 and 1.98 U/mg was determined for AA-2G formation with E232Q mutant and wildtype sucrose phosphorylase, respectively. Figure 1 (panel B) shows that **1** formation by the E232Q variant lacked a pH dependence whereas a pH profile almost identical to the one already observed with sucrose was received with the wild-type enzyme.[19] This evidence strongly supports a mechanistic proposal summarized in Scheme 2. Since the 2-OH in L-AA is relatively acidic (pK_a ~11.6),[18] its reliance on base catalytic assistance for reactivity with the glucosyl-enzyme intermediate is expected to be rather low; suitable positioning of the acceptor substrate in the binding pocket may therefore be sufficient to enable the glucosyl transfer to proceed efficiently.

In conclusion, a single-step biocatalytic synthesis of **1** from **2** was developed using sucrose phosphorylase. Discovery that the enzyme must be used under low-pH conditions, optimally at pH 5.2, to become both efficient and site-selective was key. The concentrations and yields of **1** are the highest ever reported. The process based on sucrose phosphorylase presents a significant advance, and so could be a good alternative, to commercial production of **1** in multiple biocatalytic steps and using substrates less convenient than **2**.

Acknowledgements

This work has been supported by the Federal Ministry of Science, Research and Economy (BMWFW), the Federal Ministry of Traffic, Innovation and Technology (bmvit), the Styrian Business Promotion Agency SFG, the Standortagentur Tirol, the Government of Lower Austria and Business Agency Vienna through the COMET-Funding Program managed by the Austrian Research Promotion Agency FFG. We thank Alexander Lepak (Graz University of Technology) for providing α-D-glucosyl fluoride, Prof. Dr. Hansjörg Weber (Graz University of Technology) for NMR analysis and Christian Schmid (acib GmbH) for technical support. We acknowledge discussions with Dr. Christiane Luley (acib GmbH).

Conflict of interest

The authors are inventors of an international patent application and have interest in developing the biocatalytic technology for industrial production.

Keywords: biocatalysis • carbohydrates • phosphorylase • glycosylation • vitamins • regioselectivity • reaction mechanism

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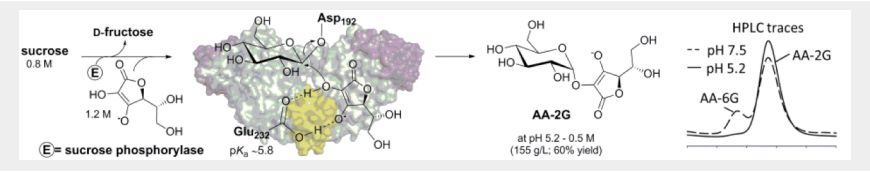

*Rama Krishna Gudiminchi, Bernd Nidetzky**

Page No. – Page No.

Walking a fine line with sucrose phosphorylase: efficient single-step biocatalytic production of L-ascorbic acid 2-glucoside from sucrose

At pH 5.2 far below the normal pH optimum of the enzyme, sucrose phosphorylase catalyzes formation of AA-2G from sucrose and L-ascorbic acid in a single-step, highly efficient and selective transglucosylation. The biocatalytic synthesis provides the basis for an advanced production for AA-2G as industrial specialty chemical. A "reverse protonation" mechanism that involves a protonated Glu^{232} underlies transglucosylation at pH 5.2.