Biocatalysis

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# Creating Space for Large Acceptors: Rational Biocatalyst Design for Resveratrol Glycosylation in an Aqueous System**

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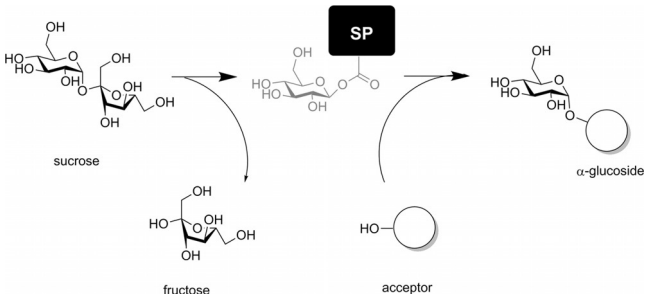
***Abstract:*** *Polyphenols display a number of interesting properties but their low solubility limits practical applications. In that respect, glycosylation offers a solution for which sucrose phosphorylase has been proposed as a cost-effective biocatalyst. However, its activity on alternative acceptor substrates is too low for synthetic purposes and typically requires the addition of organic (co-)solvents. Here, we describe the engineering of the enzyme from Thermoanaerobacterium thermosaccharolyticum to enable glycosylation of resveratrol as test case. Based on docking and modeling studies, an active-site loop was predicted to hinder binding. Indeed, the unbolted loop variant R134A showed useful affinity for resveratrol ($K_m = 185$ mM) and could be used for the quantitative production of resveratrol 3-α-glucoside in an aqueous system. Improved activity was also shown for other acceptors, introducing variant R134A as promising new biocatalyst for glycosylation reactions on bulky phenolic acceptors.*

Polyphenols are natural products with important applications in the cosmetic, pharmaceutical and food industry.[1] In particular, resveratrol (3,5,4′-trihydroxystilbene) is seen as a very interesting compound, as several studies state it to be highly beneficial for human health and nutrition.[2] Glycosylation of resveratrol can dramatically improve its solubility and bioavailability, but the exact effect depends on the position and configuration of the glycosidic bond.[3] For stereo- and regiospecific glycosylation, the advantages of biocatalysis over chemical processes are obvious.[4]

Most glycosyl transferases require nucleotide-activated donor substrates (e.g. UDP-Glc), which are rather expensive for large-scale applications.[5] However, a few enzymes are able to catalyze transglycosylation reactions starting from simple sugars like maltodextrins (e.g. CGTase) or sucrose (e.g. glucansucrase).[4b] Similarly, sucrose phosphorylase (SP, EC 2.4.1.7) can transfer the glucose moiety of sucrose to a variety of acceptors through a double displacement mechanism




***Scheme 1.*** Sucrose phosphorylase (SP) can produce α-glucosides through a transglycosylation mechanism with sucrose as donor substrate.

(Scheme 1).[6] High yields can be obtained, as has been reported for the production of 2-*O*-α-D-glucopyranosyl-*sn*-glycerol, a moisturizing agent in cosmetics commercialized under the tradename Glycoin.[6a]

Unfortunately, the activity of SP on (poly)phenolic acceptors is very low, even barely detectable in most cases.[7] It has been suggested that the main problem is the enzyme's low affinity for such compounds, which cannot be compensated by increasing the acceptor concentrations because of their low solubility.[7a,b] Although increased transglycosylation rates have been reported upon addition of organic solvents,[7b,c] saturation of the enzyme has never been achieved and productivity has generally remained too low for practical applications. With resveratrol, for example, the best result has been obtained in 20% of the ionic liquid AMMOENG 101, but that only generated 3 mM of resveratrol 3-α-glucoside.[7b] Therefore, we aimed to engineer SP for improved binding of polyphenols, using resveratrol as test case.

Two (thermo)stable SP enzymes have so far been identified, one in *Bifidobacterium adolescentis* (BaSP) and one in *Thermoanaerobacterium thermosaccharolyticum* (TtSPP).[8] The latter actually prefers sucrose 6′-phosphate ($K_m = 13$ mM) as substrate (EC 2.4.1.329) but can still efficiently use sucrose as glycosyl donor ($K_m = 77$ mM).[8b] Neither of these enzymes display significant activity on resveratrol as acceptor but, interestingly, the latter is considerably more active on the smaller building blocks resorcinol (benzene-1,3-diol) and orcinol (5-methylbenzene-1,3-diol) (Figure 1). Thus, TtSPP was chosen as template for engineering. Its gradual decrease in activity with increasing acceptor size points to a limitation in the active site pocket, which is a useful clue for developing a mutagenesis strategy.

The active-site loops of SP are known to undergo a conformational change upon binding of the substrate, resulting in a "closed" conformation.[9] Although molecular

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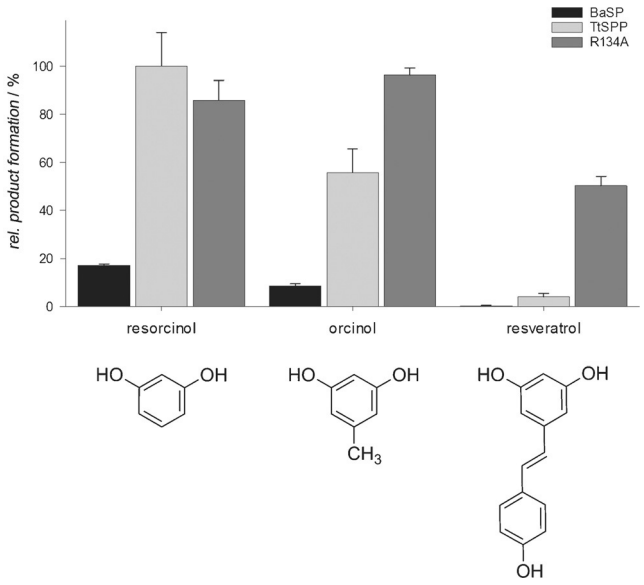


***Figure 1.*** Comparing transglycosylation activity of BaSP, TtSPP and R134A on resveratrol and its building blocks resorcinol and orcinol (10 g $L^{-1}$ each), using 1 M sucrose at pH 6.5 and 37 °C.

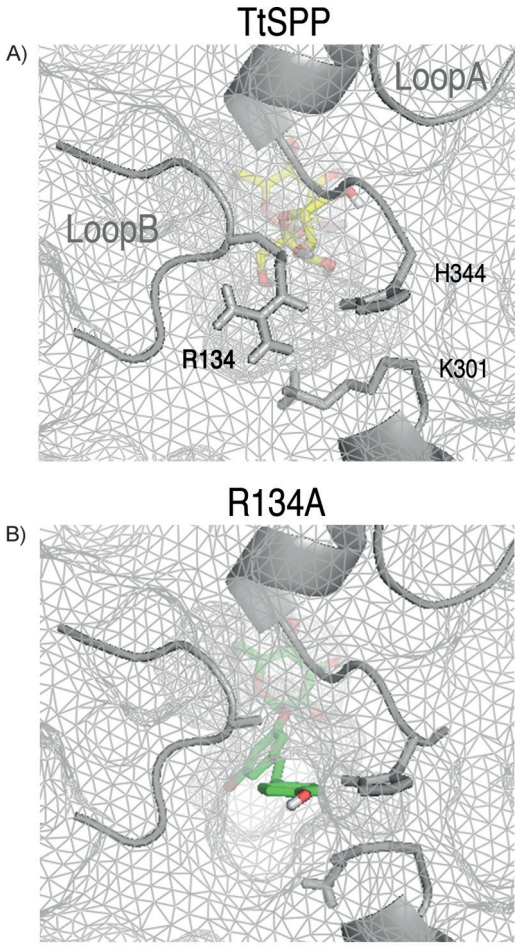


***Figure 2.*** Structure-based rationale for site-specific loop mutation at position R134. A) Positioning of active-site loops in TtSPP with sucrose (yellow) bound in the active site, showing arginine 134 to cause a closed active site. B) Variant R134A with resveratrol 3-α-glucoside (green) docked in the unbolted active site.

modeling revealed that these loops are shorter in TtSPP than in BaSP (Figure S1 in the Supporting Information), they still cover the active site completely, thereby creating a pocket that is not deep enough for binding of resveratrol (Figure 2). In particular, residue R134 seems to act as a "gatekeeper" that closes the entrance to the active site and thus limits glycosylation of large acceptors. In silico mutagenesis and docking studies indeed indicated that substitution of R134 with smaller residues (e.g. alanine) would leave an opening in the enzyme's closed conformation, enabling the second ring of resveratrol to be accommodated (Figure 2B).

To confirm these predictions, the corresponding R134A variant was produced in vitro and tested for transglycosylation activity on resveratrol and smaller building blocks (Figure 1). Effectively, the gradual decrease in activity from resorcinol > orcinol > resveratrol observed with the wild-type enzyme was almost balanced with the variant. Site-saturation mutagenesis of position 134 did not yield variants that were better than R134A, but other small amino acids like valine and threonine also offered increased activities compared to the wild-type arginine (Figure S2). This confirmed our hypothesis that a bulky side chain limits binding of large acceptors in the active site of SP. However, this only was valid for position 134, since mutating other residues at the active-site entrance (e.g. K301 or H344) to alanine did not have a similar effect (Figure S3).

Interestingly, the unbolted R134 variants enabled us to determine kinetic parameters for the glycosylation of resveratrol (Table 1, Figure S4), which has never been possible with the wild-type enzyme. These measurements revealed that the affinity for resveratrol is quite reasonable, with $K_m$ values in the range of 50–200 mM. As expected, the improvements in accessibility strongly influence the $k_{cat}$ value, which gradually increases to 2.4 $s^{-1}$ when smaller side chains are introduced at position 134. The highest catalytic efficiency (12.8 $M^{-1} s^{-1}$) is observed with variant R134A, which thus is the enzyme of choice for application in glycoside synthesis. Crucially, the mutation does only marginally lower the affinity towards sucrose (Table S1), although R134 is important for the binding of sucrose 6′-phosphate.[8b]

***Table 1:*** Kinetic parameters of TtSPP variants for resveratrol glycosylation.[a]

| Enzyme | $K_m$ [mM] | $k_{cat}$ [$s^{-1}$] | $k_{cat}/K_m$ [$M^{-1} s^{-1}$] |
|---|---|---|---|
| R134A | 185 ± 32 | 2.36 ± 0.25 | 12.8 ± 2.6 |
| R134V | 110 ± 9 | 0.45 ± 0.02 | 4.1 ± 0.4 |
| R134T | 56 ± 8 | 0.08 ± 0.004 | 1.4 ± 0.2 |

[a] Using as co-substrate 1 M sucrose at pH 6.5 and 55 °C.

Variant R134A was tested on a variety of phenols to evaluate its full promiscuity and potential as biocatalyst. Similar to the *meta*-benzenediol series shown in Figure 1, a gradual decrease in activity with the *ortho*-benzenediol series (catechol > 4-methyl catechol > quercetin) was again observed for the wild-type enzymes (Figure 3A). Also in this case, TtSPP showed higher transglycosylation activities than

  

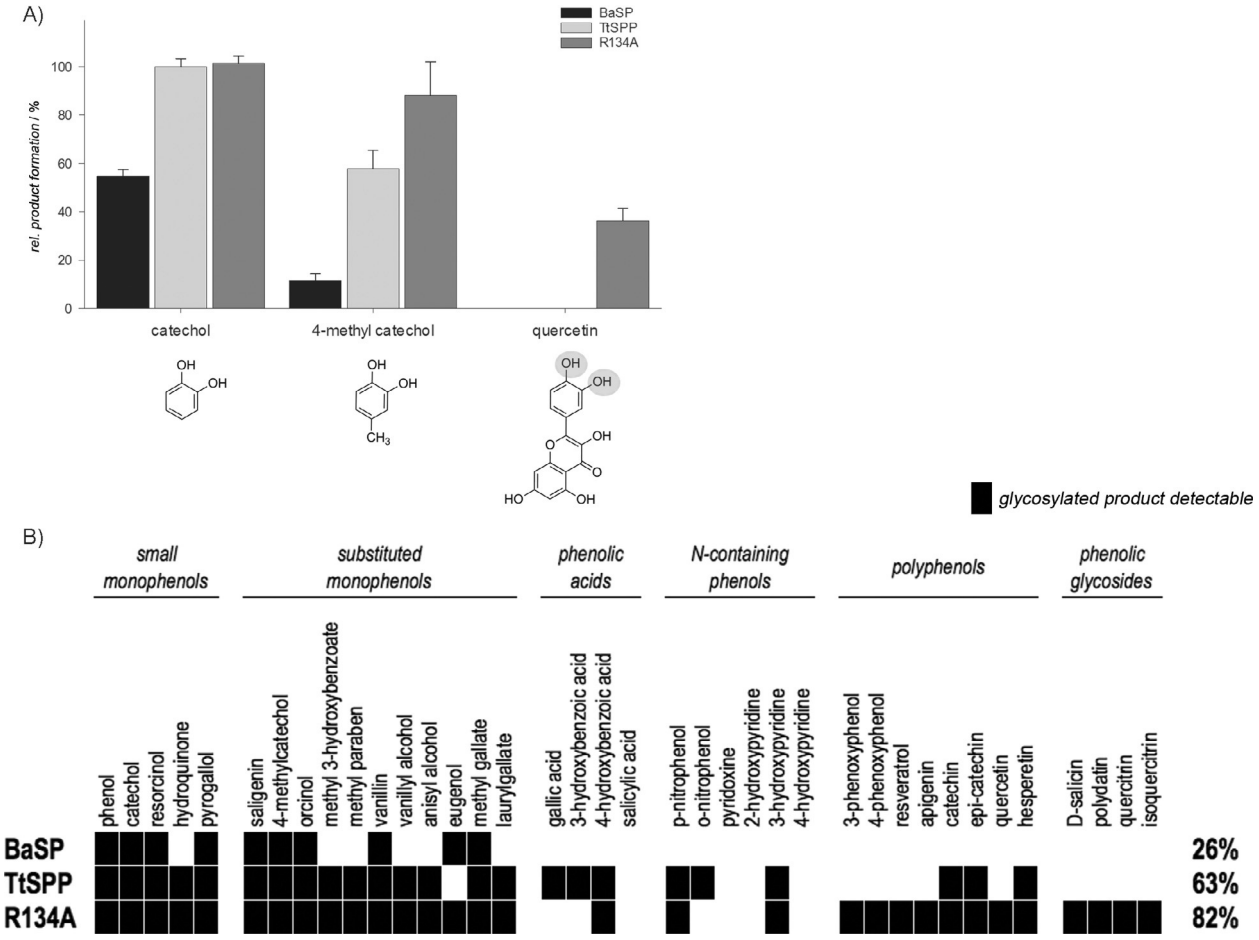


***Figure 3.*** Acceptor promiscuity of TtSPP and R134A compared to BaSP. A) Semi-quantitative comparison of transglycosylation of catechol, 4-methyl catechol and quercetin (10 g $L^{-1}$ each), using 1 M sucrose at pH 6.5 and 37 °C. B) Qualitative survey of promiscuity on 38 diverse acceptors (Table S2).

BaSP on the smaller compounds, but neither had activity on quercetin, a bulky representative of the flavonol class. In contrast, significant amounts of transglycosylation product could be detected with variant R134A, which confirms the enzyme's improved ability to bind larger acceptors thanks to its unbolted active site.

When comparing these three enzymes on 38 different acceptors, the increased promiscuity of TtSPP and especially of R134A towards large molecules became even more obvious (Figure 3B). Indeed, the latter displayed activity on 82% of the tested compounds whereas this value was only 63% and 26% for TtSPP and BaSP, respectively. This screening experiment demonstrates that variant R134A is a promiscuous biocatalyst that can be used for the glycosylation of several phenolic compounds of industrial relevance. Hence, this new enzyme is a very promising target for further engineering as it has opened up the potential to discover and exploit novel identities, properties and applications of glycosylated products.

The feasibility to use variant R134A as biocatalyst for the glycosylation of resveratrol in an aqueous system was then demonstrated by mixing the enzyme and 1M sucrose with a saturated solution of 10 g $L^{-1}$ resveratrol. After 24 h incubation at 55 °C, complete conversion of the acceptor was achieved (Figure 4) and 17.1 ± 0.6 g $L^{-1}$ of resveratrol 3-α-glucoside was produced (Figure S5, see Supporting Information for NMR results). This production process not only is selective and efficient, but can also be easily implemented at larger, commercial scales.

Recently, several studies have pointed out the importance of active-site loops[10] and enzyme gates[11] for substrate

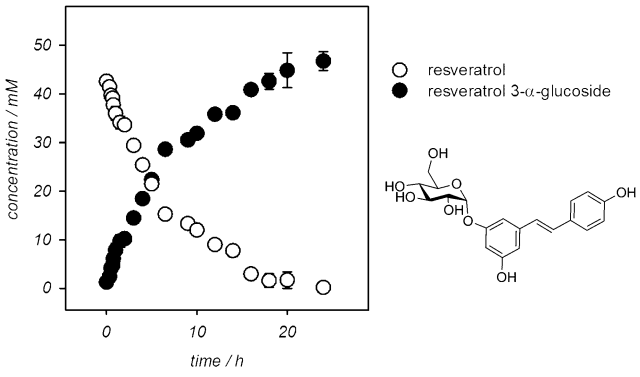


***Figure 4.*** Time course of resveratrol glycosylation by variant R134A (1 g $L^{-1}$ enzyme, 10 g $L^{-1}$ resveratrol and 1 M sucrose at pH 6.5 and 55 °C).

  

specificity, which thus have become interesting targets for enzyme engineering. Here, we report a successful case study of loop engineering, as the site-specific variant TtSPP_R134A could be used for the efficient glycosylation of resveratrol at gram scales. Indeed, this new biocatalyst is able to 1) reach full and specific conversion of resveratrol in 2) an aqueous system without the need for solvent additions and 3) using sucrose as cheap and renewable glycosyl donor. The improved performance of the designed variant was realized by a better accessibility of its active site, as was shown by both structural and kinetic analyses. An extended screening effort demonstrated that variant R134A displays high activity on a range of acceptors, thereby revealing a substrate promiscuity that should prove useful for practical applications in various industrial sectors.

**Keywords:** biocatalysis · glycosylation · protein engineering · resveratrol · sucrose phosphorylase

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