Efficient Acylation of Sugars and Oligosaccharides in Aqueous Environment Using Engineered Aciyltransferases

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ABSTRACT: A major challenge for the enzymatic synthesis of sugar esters is the low solubility of sugars in anhydrous, often toxic, organic solvents. We overcame this limitation by using acyltransferases for efficient acetylation of sugars in water. Selective 6-O-acetylation of glucose, maltose, and maltotriose with conversions of up to 78% was achieved within 15 min using engineered acyltransferases (4 μM). Moreover, we identified EstA as a promiscuous acyltransferase preferentially acetylating sugars instead of hydrophobic acyl acceptors. This expands the applicability of promiscuous acyltransferases to sugar modifications and contributes to the understanding of how to adapt acyltransferases to hydrophilic substrates.

KEYWORDS: acyl transfer, enzyme catalysis, promiscuous acyltransferase, protein engineering, sugar ester

Ampiphilic sugar esters have interesting physical properties, are useful as biosurfactants,1−3 and are of interest for pharmaceutical,4,5 cosmetic,6,7 agricultural,8,9 and food industries.2,10 They can be generated from cheap renewable materials and are completely biodegradable, not harmful to the environment, and nontoxic.11,12 Selective acylation of sugars can be catalyzed by lipases or proteases in organic solvents,11,13 or ionic liquids,14,15 obviating the need for the complex protection and deprotection strategies,16,17 required for chemical synthesis. For example, porcine pancreatic lipase was applied in pyridine as a solvent.18 Moreover, a “solid-phase” system, where a fatty acid and a sugar are mixed with a small amount of acetone or t-butanol as a phase transfer adjuvant, was reported employing the lipase CAL-B.19,20 Nevertheless, long reaction times, difficult upscaling,19,20 less environmentally friendly or toxic solvents,5,18 and the challenging separation of the product from expensive ionic liquids are downsides of these processes.14,15,21,22 Moreover, enzymatic sugar acylation can be performed in acyl donor-saturated aqueous biphasic systems,23,24 but the low acyltransferase specificity of the biocatalysts limits efficiency and applicability. Recently, enzymatic N-acetylation of glucosamine in bulk water was achieved using CmCDA from Cyclobacterium marinum, an enzyme catalyzing deacetylation of N-acetylglucosamine in nature via a tetrahedral oxyanion intermediate.25 Unfortunately, the acylase activity of CmCDA is restricted to its natural sugar substrate/product.25 More information on different mechanisms and principles of enzymatic acylation can be found in the literature.26−29

A solution to these issues could be the use of promiscuous acyltransferases which increasingly attract attention because of their ability to efficiently catalyze acyl transfer in monophasic aqueous environments, thereby overcoming the need for organic solvents, harsh reaction conditions, and long reaction times.30−32 For example, CAL-A from Pseudozyma antarctica and other enzymes of the CAL-A superfamily can be applied in bulk water for the production of biodiesel33 or in complex cascade reactions for the formation of oligocaprolactone from cyclohexanol.34 To date, the most widely studied promiscuous acyltransferase is MsAcT from Mycobacterium smegmatis, which has been used for the formation of various esters35 or in complex amides,36 including flavor compounds37 and high-value tryptamine derivatives.42 Recently, two protein engineering studies successfully increased the acyltransferase efficiency, substrate specificity, and enantioselectivity of MsAcT, further enhancing its potential for synthetic applications.43,44 The promiscuous acyltransferase Est8 catalyzes the formation of oligomers from dimethyl carbonate and diols.45 On the basis of the crystal structure of Est8 and a sequence-based prediction
technique, we identified several promiscuous acyltransferases in the bacterial hormone-sensitive lipase (bHSL) family.46

Product formation catalyzed by promiscuous acyltransferases is kinetically controlled, which means that a maximum product concentration is rapidly reached before the product is hydrolyzed due to the thermodynamic equilibrium.46,47 It is believed that hydrophobicity in the active site of promiscuous acyltransferases and high affinity toward the acyl acceptor are key requirements for facilitating binding of the acyl acceptor for acyl transfer and preventing water from hydrolyzing the acyl-enzyme intermediate.46,48,49 Unfortunately, their highly hydrophobic active sites46 (Figure S1) so far mostly enable acyl transfer to hydrophobic, aromatic substrates and limit their applicability for sugar acylation in aqueous reaction systems.

In this study, we applied MsAcT, 59 MsAcT variants,43 Est24, a close homologue of MsAcT with a more flexible structure,45,50 and six Est24 variants for acetylation of glucose, using ethyl acetate as an acyl donor (2.5 equivalents, monophasic system). Only 0.3% ± 0.1% conversion to glucose monooacetate could be achieved using wild-type MsAcT and 1.96% ± 0.08% for the best variant, MsAcT-N94S (Figures S2 and S3). For Est24, 0.62% ± 0.02% (wild type) and 0.87% ± 0.01% (N96S variant) conversion to glucose acetate was observed (Figure S4).

Further interesting candidates for sugar acylation are the recently discovered, exceptionally efficient promiscuous acyltransferases from the family VIII carboxylesterases, showing up to 20-fold higher acyl transfer to hydrolysis ratios toward benzyl alcohol than MsAcT.51 In contrast to MsAcT or the bHSLs, these enzymes do not require extensive hydrophobic regions for efficient acyl transfer but only small hydrophobic areas near the catalytic center.51 By having a wide and partially hydrophilic substrate-binding cavity, the family VIII carboxylesterase EstCE1, adopting a β-lactamase-like fold, appeared to be more likely to bind polar compounds like sugars (Figures S1 and S5). Therefore, we investigated wild-type EstCE1, previously designed W339 variants51 and 27

Figure 1. (A) Crystal structure of EstCE1 (PDB code: 7ATL)31 with catalytic serine (orange sticks), residues L125, L239, F243, V342, and W339 (blue sticks), substrate binding site (white surface), and hydrophobic regions within the substrate binding site (yellow surfaces) shown. (B) Conversions for acetylation of glucose (200 mM) with ethyl acetate (500 mM) catalyzed by purified wild-type MsAcT, MsAcT-N94S, wild-type Est24, Est24-N96S, wild-type EstCE1, and selected EstCE1 variants (4 μM) in ammonium acetate (200 mM, pH 8.2) after 1 h reaction time. Results for all EstCE1 variants (Figure S6) and all measured time points (Figure S7, Table S1) can be found in the Supporting Information.

Figure 2. Acetylation of glucose (100 mM), maltose (50 mM), and maltotriose (25 mM) catalyzed by wild-type EstCE1 and EstCE1-MAA (4 μM each) using ethyl acetate (EtOAc, 500 mM) or isopropenyl acetate (IPA, 200 mM) as acyl donors in ammonium acetate (200 mM, pH 8.2). Maximum product formation determined from multiple time samples (15–240 min, Figure S9) and conversions for scaled-up reactions (180 mg glucose/171 mg maltose/126 mg maltotriose) with EstCE1-MAA are given.

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additional rationally designed EstCE1 variants for the acetylation of glucose. The rational design strategy aimed to increase acyltransferase activity toward sugars by targeted replacement of bulky residues in the active site by either smaller residues or residues often involved in the substrate binding cavities of proteins that bind nonacidic sugars\(^{52}\) (Figure S5). As expected, wild-type EstCE1 catalyzed glucose acetate formation (0.81% ± 0.10% conversion) about 2.7-fold more efficiently than wild-type MsAcT under identical reaction conditions (Figure 1B, Table S1). W339 variants (including W339Y and W339F), which increased acyl transfer to hydrolysis ratios using benzyl alcohol as an acceptor up to 5-fold,\(^{51}\) did not have any positive effect on the formation of glucose acetate (Figure S6, Table S1). Tryptophan is often found in the substrate-binding cavities of proteins that bind nonacidic sugars,\(^ {52}\) perhaps explaining why wild-type EstCE1 was best for glucose acetylation. By testing the 27 additional EstCE1 variants (Figures S5−S7) for glucose acetylation, we identified L125, L239, F243, and V342 (Figure 1A) as hotspots for increasing glucose acetate formation. The best single mutant (F243H) increased the formation of glucose acetate 6.7-fold to 5.4% (Figure 1B, Table S1). Subsequently, we designed seven combinatorial variants, of which the L239M/F243A/V342A variant (EstCE1-MAA) performed best with 17.1% ± 0.3% glucose acetate formation after 1 h (Figure 1B and Table S1). EstCE1-MAA thus yielded 5.9% glucose acetate after 1 h, 77.5% ± 0.04% maltose acetate, and 5.0% ± 0.04% maltotriose acetate after 2 h, could be achieved (Figure 2 and Table S2).

Due to the simple reaction conditions, the transformations could be easily scaled up without a loss of performance (Figure 2, black bars). We purified the monoacetylated glucose produced by EstCE1-MAA via acetonitrile precipitation and column chromatography, yielding 64.9 mg (36% isolated yield) of a product which was determined to be 6-O-acetyl glucose by NMR spectroscopy (Table S3 and Figures S12−S24). Analogously, the products derived from maltose and maltotriose were purified, and their structures were elucidated by in-depth NMR (1D, 2D analyses) analysis. Confirmation of monoaetylation at the 6-O-positions of each sugar, and quantification of conversion in crude reaction mixtures, was relatively straightforward. The unambiguous determination of the exact position of acetylation, however, required full assignment of all three homologous products relying on extensive 2D-NMR analyses, including TOCSY, HSQC-TOCSY, and HMBC experiments (Tables S4−S7 and Figures S16−S24). Selective 6-O-acylation of sugars, to form sugar esters, which are important as biosurfactants\(^{53,54}\) and in the food industry,\(^ {20,53,57}\) from simple substrates, has so far mostly been achieved in organic media or solvent-free systems.\(^ {20,53,57}\) A possible application for the 6-O-acetyl glucose we prepared is

![Image](https://dx.doi.org/10.1021/acscatal.1c00048)
as a precursor in the biosynthesis of the high-intensity sweetener sucralose.\textsuperscript{10,55}

Because of the exceptional sugar acetylation activity of the EstCE1 variants, we studied additional family VIII carboxylesterases,\textsuperscript{51} using isopropenyl acetate as an acyl donor (Figure S26). This led to the discovery that EstM2, a close homologue of EstCE1, can also acetylate glucose. More remarkably, wild-type EstA from \textit{Arthrobacter nitroguajacolicus} converted glucose to glucose monoacetate (42.2% \pm 0.7% conversion) and glucose diacetate (0.67% \pm 0.03% conversion, Figure 3A). These conversions are comparable to those for EstCE1-MAA and are approximately 60-fold higher than the conversion achieved with wild-type EstCE1 under the same reaction conditions.

We previously identified a three-amino acid motif which was postulated to be a substrate and specificity switch in family VIII carboxylesterases.\textsuperscript{51} While EstCE1 and EstM2 (WGG motif) display high acetyltransferase activity using pNPA and benzyl alcohol, EstA (HDG motif) lacks acetyltransferase activity toward these substrates.\textsuperscript{51} Interestingly, the HDG motif seems to enable acetyl transfer to sugars but not to hydrophobic acceptors like benzyl alcohol. This is supported by docking studies of glucose into the acetyl-enzyme complex of EstA showing polar contacts between D323 of the HDG motif and the 6-O-position of glucose (Figure S30).

In the reaction of glucose and ethyl acetate, only 0.65% \pm 0.06% glucose acetate formation could be observed for EstA, which is even less than for wild-type EstCE1 (Figure 3A). That clearly shows that EstA’s acetyltransferase activity significantly depends on the acyl donor and that EstA has a preference for acetylation of sugars and not hydrophobic compounds, unlike previously described promiscuous acyltransferases. Acetylation of the larger sugars maltose and maltotriose with isopropenyl acetate led to 6.6\% \pm 0.2\% and 2.0\% \pm 0.2\% product formation. This indicates that EstA is more suitable for glucose acetylation while wild-type EstCE1 showed the highest conversion toward maltotriose and EstCE1-MAA toward maltose.

Structural comparison of EstA (PDB code: 3ZYT)\textsuperscript{58} and EstCE1 revealed that the EstCE1 hotspot residues L125 and F243 correspond to smaller residues (A221 and A325) in EstA. Furthermore, the extended Ω-loop containing EstCE1 hotspot residues L239 and F243 is not present in EstA (Figure 3B). Moreover, hydrophobic regions in EstA are not as close to the catalytic serine as in EstCE1 (Figure 3B). Therefore, we assume that increased space in the EstA active site and a less hydrophobic environment around the catalytic serine have a positive impact on the binding of glucose, resulting in the remarkable glucose-acetylation activity of wild-type EstA.

We here reported the selective acetylation of glucose oligomers in aqueous media using promiscuous acyltransferases. We could identify the family VIII carboxylesterase EstCE1 as a biocatalyst which could be substantially improved by rational protein engineering. Thus, the EstCE1 variant L239M/F243A/V342A enabled the selective formation of 6-O-acetyl glucose, 6′-O-acetyl maltose, and 6″-O-acetyl maltotriose in high yields within short reaction times (15 min to 2 h) at room temperature. This expansion of EstCE1’s substrate scope toward sugar substrates together with its broad acyl donor acceptance and the ability to catalyze ester, amide, carbonate, and carbamate formation\textsuperscript{51} creates great potential for biocatalytic processes. Furthermore, we discovered the remarkable glucose acetylation activity of EstA—a promiscuous acyltransferase preferentially catalyzing acyl transfer to highly polar acceptor substrates. Our work demonstrates the usefulness of promiscuous acyltransferases for selective acylation of sugars in aqueous reactions, which is hard to achieve by chemical methods.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acscatal.1c00048.

Experimental methods; comparison of active site hydrophobicities; results for sugar acetylations catalyzed by wild-type and variants of MsAcT, Est24, and EstCE1; NMR analysis of sugar products; HPLC-ELSD standard curves; and mutagenesis primers (PDF)

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**Author Contributions**

U.T.B. conceived and directed the project. S.P.G. designed and performed most experiments supported by C.P.S.B. and H.M. H.M. performed EstA docking studies. C.St. and C.Su. conducted purifications and NMR analysis of the products and reaction mixtures. S.P.G. drafted the manuscript to which all authors contributed. All authors have approved the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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**Abbreviations**

MsAcT, *Mycobacterium smegmatis* acyltransferase; CAL-A, *Pseudozyma antarctica* lipase A; bHSL, bacterial hormone-sensitive lipase

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