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Enzymatic N-acylation Step Enables the Biocatalytic Synthesis of Unnatural Sialosides

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Abstract: Reversible and chemoselective: Chitin is one of the most abundant and cheaply available biopolymers in Nature. Chitin has become a valuable starting material for many biotechnological products through manipulation of its N-acetyl functionality, which can be cleaved under mild conditions using the enzyme family of de-N-acetylases. However, the chemo-selective enzymatic re-acylation of glucosamine derivatives, which can introduce new stable functionalities into chitin derivatives, is much less explored. Here we describe an acylase (CmCDA from *Cyclobacterium marinum*) that catalyzes the N-acylation of glucosamine with a range of carboxylic acids under physiological reaction conditions. This biocatalyst closes an important gap in allowing for the conversion of chitin to complex glycosides, such as C5-modified sialosides, through the use of highly selective enzyme cascades.

Carbohydrate active enzymes (CAZy) have become valuable catalysts for the highly selective production of complex natural and unnatural glycans and their use in one-pot enzyme cascades is particularly attractive [1]. In particular, glucosamine derivatives are fundamental building blocks for the synthesis of a range of hexosamines and sialylated glycoconjugates [2] and as bioorthogonal chemical reporters for metabolic glycan labelling and engineering [3] as illustrated in Scheme 1. Glucosamine (2) itself is a valuable food additive that can be generated from chitin through chemical or enzymatic depolymerization to 1 and de-acylation [4]. Subsequent epimerases (to generate the galactose and mannose derivatives 4 and 5 respectively) and further elaboration to sialic acids (6) and glycoconjugates can also be achieved enzymatically. However, there remains a gap in the enzyme toolkit in that the acylation of glucosamine 2 needs to be performed chemically, requiring activation of carboxylic acids or stoichiometric coupling agents and protecting strategies [5].

Hence, the enzymatic synthesis of N-acylated glucosamine derivatives directly from glucosamine and unprotected carboxylic acids would allow for the generation of target structures 3-6 and their derivatives using enzyme cascades directly from chitin (Scheme 1). In biosynthesis, N-acylation is often mediated through the generation of acetyl precursors such as acetyl coenzyme A or acetyl phosphate, or acetyladenine [6] and cannot directly utilize glucosamine 2, but require activated glucosamine-6-phosphate as the acceptor substrate [7]. A more attractive strategy is the reversible enzymatic amine acyl exchange of amines and free carboxylic acids (Scheme 1, compound 2 to 3 using free acid as the acylating agent), thus directly replacing the chemical step. Such reactions, with more reactive (protonated) carboxylic acids stabilised in the active site of enzymes, have been previously described for N-acylation reactions of peptides, urea, or ammonia [8], but to our knowledge, there is no example of the N-acylation of glucosamine. An enzyme-catalyzed approach would not require activation of the carbonate and in addition has the potential to be highly selective for glucosamine in the presence of other biogenic amines.

Scheme 1. Current enzyme cascades for the conversion of chitin from biomass to complex glycoconjugates, which still require chemical acylation strategies for amino sugars. Here we address the challenge of achieving selective acylation by enzyme catalysis.

In the absence of any obvious candidate enzymes from the literature, we looked for promiscuous activities of known biocatalysts. We previously described a recombinant chitin de-N-acylase CmCDA that is able to specifically and efficiently deacetylate N-acylglicosamine derivatives (including GlcNAc), whereas no activity towards other carbohydrates such as chitobiose, ManNAc or GalNAc was detected [4]. As we were curious how CmCDA de-N-acylates GlcNAc, we reviewed the postulated mechanism of a reported peptidoglycan...
Deacetylase [9]. The relevant catalytic amino acids are conserved in both enzymes, including the His-His-Asp zinc-binding triad coordinating the N-acetyl group, as well as the respective histidine and aspartic acid residues acting as the catalytic acid and base. The active site residues suggest acylation via the described tetrahedral oxyanion intermediate which would also allow for the reverse the reaction, i.e. the N-acetylation reaction of glucosamine (1) with a carboxylic acid (Scheme 2).

Table 1. CmCDA catalysed N-acetylation of 1 and transacylation of 2 from carboxylic acids 8-13.[a]

<table>
<thead>
<tr>
<th>Sugar substrate</th>
<th>Carboxylic acid</th>
<th>Amide product</th>
<th>Conversion [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlcNH2 (1)</td>
<td>2</td>
<td>60±13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>56±12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>59±14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>49±8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>75±11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>80±8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>66±10</td>
<td></td>
</tr>
<tr>
<td>GlcNAc (2)</td>
<td>4</td>
<td>37±2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>38±5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>41±10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>41±8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>31±15</td>
<td></td>
</tr>
</tbody>
</table>

[a] 1 mL reactions consisting of 40 mM 1 or 2, 200 mM 7-13 (in combination with 1, or 8.9.11.12.13 with 2), 420 mM Na2HPO4 buffer (pH 8.0), 0.4 U CmCDA, 37°C, 20 h. [b] Conversion determined by 1H NMR.

To our delight CmCDA (expressed recombinantly in E. coli) could indeed catalyse N-acetylation reactions with a series of carboxylic acids: in the presence of 5 equivalents of acetic acid (7), propionic acid (8), butyric acid (9), hexanoic acid (10), azidooacetic acid (11), glycolic acid (12) and thiolglycolic acid (13). Glucosamine (1) yielded the corresponding N-acetylglucosamines 2 and 14-19, respectively (Table 1).

Under the same conditions, no product formation was observed using benzoic acid as carboxylic acid donor, suggesting a steric or electronic limitation of CmCDA for bulkier aromatic organic acids. As expected, the amide formation requires an excess of the carboxylic acid in the reaction mixture: whereas 1 equivalent of acid gave low yield, the addition of 5 equivalents shifted the equilibrium to the formation of the amide bond.

To demonstrate the selectivity of CmCDA for glucosamine in the presence of other amines, 1 was reacted with an excess nonactivated, nonprotected γ-aminobutyric acid (20). Whereas no formation of N-acetyl glucosamine 21 could be observed using the inactive variant CmCDA-Asp21Asn [11], the formation of 21 catalysed by native CmCDA could be observed in a time-course experiment by mass spectrometry (Scheme 3).

Given that CmCDA also catalyses the hydrolysis, we then investigated whether it would be feasible to generate N-acetyl glucosamine derivatives via N-transacylation directly from GlcNAc (1). Given the stability of the N-acyl bond, this had been achieved chemically before only by using highly fluorinated anhydrides: the exchange of the N-acetyl group of unprotected and peracetylated GlcNAc to a N-perfluoroacyl group using trifluoroacetic anhydride or heptafluorobutanoic anhydride [13]. Further N-transacylation attempts used oleoyl chloride and lauryl chloride as acyl donors of protected GlcNAc in pyridine under reflux [14]. Examples of direct N-transacylation are limited, require harsh conditions and, commonly, the protection of all hydroxyl groups, and are restricted to fluorinated or long-chain acylation reagents. Performing these transacylation reactions with unprotected GlcNAc (1) and non-activated carboxylic acids enzymatically would extend the scope to acyl donors which are of interest in the synthesis of sialic acid derivatives, such as azido, hydroxyl or thiol groups [15].
Applying the reaction conditions established for the N-acetylation reaction of glucosamine (adding 5 equivalents of the acid donor), the overall efficiency of the N-transacylation reaction was comparable to the conversion rates of the N-acetylation reactions (Table 1). Propionic acid (8), butyric acid (9), azidoacetic acid (11), glycolic acid (12) and thiglycolic acid (13) were explored as acyl donors and showed between 37-46% conversion to the corresponding N-acyl glucosamines [16]. ^1H NMR analysis of the reaction mixtures showed the complete disappearance of 2 in all cases whereas 1 could be observed as product of the deacetylation reaction, indicating that this enzymatic amine acyl exchange consists of two independent deacylation and re-acylation steps.

Scheme 4. One-pot five enzyme cascade for the synthesis of sialosides 23–25 from glucosamine and X-gal (22).

The enzymatic synthesis of C5-substituted sialosides requires N-acetylglucosamine or N-acetylmannosamine derivatives as precursor molecules [17]. The enzymatic synthesis of these compounds would allow the integration of the N-acetylation reaction in established enzymatic cascade syntheses for unnatural sialosides, which currently consists of the following one-pot enzyme cascade. In a first step, chemically N-acetylated glucosamine analogues are enzymatically epimerized to the corresponding N-acetylmannosamines and transformed into the sialic acid derivatives via an enzymatic aldol addition with pyruvate. These intermediates can be then used for the synthesis of sialyloligosaccharides via enzymatic activation by CMP-sialic acid synthase and sialyltransferases. Previously, we had been successful in using 5-bromo-4-chloro-indolyl-β-D-galacto-pyranoside (X-Gal, 22) as an acceptor substrate for sialylation reactions, generating valuable labelled oligosaccharides. In the case of incomplete sialylation reactions, residual 22 could simply be removed by treating the reaction mixture with β-galactosidase. The released 5-bromo-4-chloro-3-indole dimerizes to form an insoluble indigo dye. Thus, sialosides containing N-acetylenuraminic acid (Neu5Ac), N-propanoylneuraminic acid (Neu5Prop) and N-glycolylneuraminic acid (Neu5Gc) were synthesized using a five-enzyme one-pot reaction (Scheme 3) by only providing glucosamine (1), pyruvate, and acyl donors 7, 8 and 12 as sialic acid precursors, cytidine triphosphate (CTP) for the activation of the sialic acid analogues, and 22 as the acceptor substrate for the sialylation reaction (Scheme 3). Sialosides 23 and 24 were obtained in quantitative yield, whereas 25 yielded 29% [18].

In conclusion, we have demonstrated that CmCBA is able to carry out a range of N-acetylation and N-transacylation reactions to generate N-acetylglucosamines on preparative scale. This enzyme is key for the development of comprehensive and extended enzymatic cascades, as demonstrated for the first enzymatic total synthesis of sialosides using glucosamine as sialic acid precursor, opening up new avenues in the chemoenzymatic synthesis of glycoconjugates and non-natural analogues.

Acknowledgements

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Keywords: N-acetylation • enzymatic synthesis • unnatural sialosides • glycosylation • chitin de-N-acylases


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[10] Initial experiments the CmCDA-catalysed acylation was examined in the presence of 1, 2, and 5 equivalents of acetic acid, which resulted in 15%, 28%, and 53% conversion, respectively (Supporting Information).

[11] Details of the generation of the CmCDA Asp21Asn enzyme variant are described in the Supporting Information.

[12] 1 mL reaction were performed using 25 mM GlcNH₂ (1), 125 mM aminobutyric acid (20), 200 mM phosphate buffer, and 0.4 U CmCDA at 37 °C. Further details in the Supporting Information.


[16] CmCDA catalysed conversion of GlcNAc (2) in the presence of propionic acid (8), butyric acid (9), azidoacetic acid (11), malonic acid (12) and thioglycolic acid (13) into the corresponding N-acetylglucosamine derivatives 14, 15, and 17-19 were monitored using H NMR (Supporting Information).


Reversible and chemoselective: The enzyme *Cyclobacterium marinum* chitobiose deacetylase, which is highly selective for monomeric GlcNAc as a substrate, was found to catalyze the N-acylation of glycosamine with carboxylic acids under physiological reaction conditions. This biocatalyst was applied in the first fully enzymatic synthesis of a series of C5-modified sialosides starting from glucosamine and a series of non-activated carboxylic acids.