Substrate Selectivity of the Sublancin S-Glycosyltransferase

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Supporting Information

ABSTRACT: SunS is a novel S-glycosyltransferase involved in the biosynthesis of the antimicrobial peptide sublancin. It selectively modifies Cys22 in a 56 amino acid peptide substrate SunA and can accept a variety of NDP sugars. This study reports the substrate selectivity with regard to the peptide substrate and the antimicrobial activity of the resulting sublancin analogues. The results suggest that SunS recognizes an α-helix N-terminal of the Cys to be glycosylated, which is present in a flexible linker. Interestingly, when Cys22 is mutated, sugar attachment is not required for sublancin antimicrobial activity. Furthermore, the sublancin-producing strain Bacillus subtilis 168 also becomes susceptible to such mutants. These data suggest that S-glycosylation may be important for self-resistance.

Figure 1. Post-translational modifications during sublancin biosynthesis. The leader peptide of the SunA precursor peptide is shown in red, and the double-glycine-type proteolytic cleavage site is underlined. The predicted helix-forming regions are underlined with blue bars. The cysteine residues that are not glycosylated are shown in green, and the glycosylated residue Cys22 is shown in red. The order of glycosylation and disulfide bond formation in vivo is unknown and is arbitrarily shown with glycosylation occurring first. Residue numbering is based on the core peptide of SunA.

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### Table 1. Partial Sequences of SunA Peptides (Residues 11–30)

| Substrate            | Sequence                        |
|----------------------|---------------------------------|
| SunA                 | WLGKCALGTTGCGGGAVACQN            |
| SunA-G21K            | WLGKCALGTTGCGGGAVACQN            |
| SunA-G23K            | WLGKCALGTTGCGGGAVACQN            |
| SunA-G21F            | WLGKCALGTTGCGGGAVACQN            |
| SunA-G23F            | WLGKCALGTTGCGGGAVACQN            |
| SunA-G21A            | WLGKCALGTTGCGGGAVACQN            |
| SunA-G23A            | WLGKCALGTTGCGGGAVACQN            |
| SunA-G21E            | WLGKCALGTTGCGGGAVACQN            |
| SunA-G23E            | WLGKCALGTTGCGGGAVACQN            |
| SunA-Xa-T19C         | WLGKCALGTTGCGGGAVACQN            |
| SunA-Xa-A120         | WLGKCALGTTGCGGGAVACQN            |
| SunA-Xa-S16-G17insAAA| WLGKCALGTTGCGGGAVACQN            |
| SunA-Xa-Q13G-A15G    | WLGKCALGTTGCGGGAVACQN            |

*Mutations are highlighted in yellow. Cysteines that were glucosylated by SunS are shown in red underlined font.*

In one peptide, SunA-Xa-A120, Ile20 was deleted, bringing Cys22 one residue closer to the N-terminus. In a second peptide, SunA-Xa-(S16-G17insAAA), three Ala residues were inserted between Ser16 and Gly17 to position the Cys three residues further away from the N-terminus. In both SunA mutant peptides, a Factor Xa cleavage site was engineered to allow removal of the leader peptide and evaluation of the bioactivities of the sublancin analogues. Incubation of these substrate analogues with SunS resulted in glucosylation of both peptides at the third Cys residue from the N-terminus (equivalent to Cys22 in wt SunA), as determined by ESI-MSMS. Thus, SunS tolerates changes in the position of the targeted Cys residue toward either the N- or C-terminus without compromising its regio- and chemoselectivity (Figure S2).

The observation that SunS can selectively modify Cys residues at positions other than the 22nd amino acid of the core peptide encouraged exploration of its capability to conjugate multiple sugar moieties. First, the mutant peptide SunA-Xa-T19C was produced to introduce an additional Cys in the stretch of amino acids between Cys14 and Cys29 that encompasses Cys22 (Table 1). After incubation with SunS, only a monoglycosylated peptide was observed by MALDI-TOF-MS. However, ESI-MSMS analysis showed that the enzyme does not display a strong preference for the N- or C-terminus without compromising its regio- and chemoselectivity (Figure S2).

Interestingly, incubation of SunS with a SunA mutant in which the flanking residues of Cys14 (one of the residues not modified in wt SunA) were mutated to glycines (SunA-Xa-Q13G-A15G, Table 1) resulted in a mixture of mono- and bisglycosylated products (Figure S4a). Subsequent ESI-MSMS analysis showed that both the Cys14 and Cys22 residues were modified (Figure S4b,c). The observation that SunS can modify two Cys residues in SunA-Xa-Q13G-A15G but not in SunA-Xa-T19C indicates that a minimal distance between two Cys residues is required in order to accommodate the initially formed monoglycosylated peptide into the active site for a subsequent second glycosylation.

Whereas it is clear that SunS has substantial tolerance toward mutations in its peptide substrate, the basis of its substrate recognition mechanism is still largely unknown. Previous studies showed that the leader peptide of SunA is not required for enzymatic processing by SunS in vitro. Therefore, the core peptide must provide sufficient binding affinity for substrate recognition and processing. Given the outcome of our mutagenesis studies, we wondered whether perhaps secondary structure elements in SunA would be recognized by SunS. Structure prediction tools (PSIPRED) anticipated two α-helical regions in the reduced core peptide spanning residues 3–15 and 26–36 (Figure 1). Circular dichroism experiments support the existence of α-helical character in both reduced (no disulfides) and native sublancin and in the reduced, linear His_{6}-SunA peptide (data not shown). In addition, the recently solved NMR structure of the S-linked glycopeptide bacteriocin glycocin F, which shares structural similarity with sublancin (Figure S5), consists of two α-helices held together by a pair of disulide bonds. The two α-helices in glycocin F project well onto regions 7–14 and 29–36 of SunA, although the two peptides have no sequence identity except for the four cysteines (Figure S5). If such a helical structure provides the basis for enzyme–substrate recognition, it offers a possible explanation for the observed regioselectivity of SunS. The four Cys residues in SunA that are not modified by SunS are all inside the proposed helical regions, whereas the Cys that is modified (Cys22) is in a flexible coil region connecting the helices. This hypothesis is supported by the double glycosylation observed in SunA-Xa-Q13G-A15G. The PSIPRED structure prediction program suggests that because of the helix-breaking residues Gly13 and Gly15, Cys14 is no longer within the N-terminal α-helix in this mutant, possibly explaining why Cys14 was glucosylated by SunS.

To investigate further the importance of each of the two proposed helices in the core peptide of SunA for substrate recognition by SunS, two peptides spanning residues 1–27 (SunA-1–27) and 16–26 (SunA-16-26) were synthesized by solid-phase peptide synthesis (Table S2). A third peptide, SunA-17-37, was obtained by expression of SunA-S16F in Escherichia coli, digestion of the peptide with endoprotease GluC, and HPLC purification. Incubation of these three peptides with SunS under the standard assay conditions resulted in glycosylated SunA-1-27, whereas SunA-16-26 and SunA-17-37 were not recognized as substrates. These results suggest that the presence of the N-terminal helical region (residues 3–15) of the core peptide is required for substrate recognition whereas the C-terminal helix (residues 26–36) is dispensable and the flexible linker connecting the helices (residues 16–26) is not sufficient. As a further test of the hypothesis that the N-terminal helix in the core peptide of SunS is important for substrate recognition, the helix-breaking residue Pro was introduced at position 11 of SunA by site-directed mutagenesis. The peptide fragments SunA-(S3-33)-W11P and SunA-(S-33) were then generated by trypsin digestion of SunA-W11P and wt SunA, respectively (Table S2). Incubation of these peptides with SunS showed that the proline mutant SunA-(S-33)-W11P peptide was not a substrate whereas SunA-(S-33) was still glycosylated (Figure S6). Therefore, the N-terminal helix appears to be essential for substrate recognition by SunS.
The relaxed substrate specificity of SunS observed herein also provided a convenient tool for generating a series of sublancin analogues. The leader peptides were removed from glucosylated SunA-Xa-G23E, SunA-Xa-G23F, SunA-Xa-ΔI20, and SunA-Xa-(S16-G17insAAA) using Factor Xa. Subsequently, these peptides were subjected to the previously reported oxidative folding procedure to facilitate the formation of the correct disulfides.11 Interestingly, all of the reconstituted sublancin analogues exhibited similar growth inhibitory activity against B. subtilis 6633 as reconstituted wt sublancin (Figure 2), suggesting that neither the residues flanking the glucosylated Cys nor the precise position of glucose attachment are critical for its antimicrobial potency.

In our previous study, we reported that glycosylation of SunA is essential for antimicrobial activity of sublancin.11 Further investigation revealed that without glycosylation, the correct disulfide bonds of sublancin are not formed during the oxidative folding process (Figure S7). Presumably, the free thiol of unmodified Cys22 disrupts the formation of the correct disulfide bridges by thiol-disulfide exchange.16 Hence, the importance of the actual glycosylation for antimicrobial activity has not been tested. To provide additional information regarding this question, the mutants SunA-Xa-C22S and SunA-Xa-C22A were generated. Surprisingly, after proteolytic removal of the leader peptide and subsequent oxidative folding, both sublancin-C22S and sublancin-C22A exhibited similar growth inhibitory activity against B. subtilis 6633 as reconstituted wt sublancin (Figure 2B). Similar to the observations for sublancin, removal of the leader peptide was required for the bioactivity of both sublancin analogues. These results indicate that the presence of sugar moieties at position 22 is not required for the antimicrobial activity of sublancin. The observation that the glucose is not important for bioactivity also explains why sublancin analogues with a variety of different sugars attached to Cys22 all exhibit similar antimicrobial activity11 and why analogues with the glucosylated Cys at different positions in the loop connecting the two α-helices are also all active (Figure 2). It therefore appears that the two helices held together by the disulfide bonds are most important for biological activity. To rule out the possibility that the sublancin C22S mutant acts by a different mechanism of action, we tested the compound against a B. subtilis 6633 mutant strain that acquired resistance upon exposure to wt sublancin. Neither wt sublancin nor the C22S mutant displayed antimicrobial activity against this mutant strain at concentrations of up to 50 μM, suggesting that the C22S mutant acts by the same mechanism of action as wt sublancin.

We also tested the activity of sublancin-C22S against the sublancin producing strain B. subtilis 168. As expected, neither autogenous nor reconstituted sublancin showed inhibitory activity against B. subtilis 168 (Figure 3C), presumably because of the self-resistance mechanisms in the producer organism mediated by SunI.17 Surprisingly, however, B. subtilis 168 was susceptible to sublancin-C22S (Figure 3C), suggesting the possibility that glycosylation is related to a self-resistance mechanism, possibly in combination with SunI. Future studies will focus on further evaluation of this possibility.

In summary, we have demonstrated that a novel S-linked glycosyl transferase, SunS, exhibits high promiscuity in regard to its peptide substrates without losing its chemo- and regioselectivity. The data presented here suggest that the enzyme recognizes an α-helix that spans approximately residues 3–15. A variety of sublancin analogues were generated by utilizing SunS to modify mutant substrates. These mutants show that glucose attachment is not required for the antimicrobial activity of sublancin.
ASSOCIATED CONTENT

Supporting Information. Molecular biology procedures, protein purifications, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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