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Cysteine *S*-glycosylation, a new post-translational modification found in glycopeptide bacteriocins

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ABSTRACT

_O_-glycosylation is a ubiquitous eukaryotic post-translational modification, whereas early reports of _S_-linked glycopeptides have never been verified. Prokaryotes also glycosylate proteins, but there are no confirmed examples of sidechain glycosylation in ribosomal antimicrobial polypeptides collectively known as bacteriocins. Here we show that glycocin F, a bacteriocin secreted by _Lactobacillus plantarum_ KW30, is modified by an _N_-acetylglucosamine β-_O_-linked to Ser18, and an _N_-acetylhexosamine _S_-linked to C-terminal Cys43. The _O_-linked _N_-acetylglucosamine is essential for bacteriostatic activity, and the C-terminus is required for full potency (IC_{50} 2 nM). Genomic context analysis identified diverse putative glycopeptide bacteriocins in Firmicutes. One of these, the reputed lantibiotic sublancin, was shown to contain a hexose _S_-linked to Cys22.

1. Introduction

Glycosylation is a ubiquitous eukaryotic post-translational modification, the glycans being typically linked to asparagine (*N*-linked) or serine/threonine (*O*-linked) sidechains and fulfilling physicochemical and/or molecular recognition roles [1]. There is also a growing appreciation of the extent and functional significance of bacterial glycoproteomes [2,3].

Prokaryotes secrete bacteriocins, ribosomally synthesised antimicrobial polypeptides that often have a narrow phylogenetic range of toxicity determined by specific interactions with receptor molecules and/or targets of inhibitory action [4]. Some bacteriocins exhibit unusual post-translational modifications [5,6], for example the C-terminal glycosyl ester linkage in microcin E492m [7], but there are no confirmed reports of bacteriocins with glycosylated sidechains.

Glycocin F (GccF, formerly plantaricin KW30), secreted by *Lactobacillus plantarum* KW30, is stable between pH 2-10, and at 100 °C for 2 h, and is unaffected by incubation with α-amylase and lysozyme [8]. These properties, deduced from studies on culture supernatant, are typical of bacteriocins from *L. plantarum* [9] and other lactic acid bacteria.

Here we present the molecular characterisation of purified GccF and show that it contains post-translational modifications not described previously in bacteriocins, including peptide cysteine *S*-glycosylation which is without verified biological precedent. Earlier accounts of naturally occurring cysteine (*S*-linked) glycopeptides [10,11] are almost forty-years old and in doubt because the peptide sequences (GenBank accessions P02728, P02729) have not been found in the human proteome.

Genomic context analysis indicated the sporadic occurrence of diverse putative *glyco*peptide bacterio*cins* (*glycocins*) in Firmicutes. We confirmed that one of these, the reputed lantibiotic sublancin 168 [12], is in fact an *S*-linked glycopeptide bacteriocin. These discoveries expand our awareness of the array of post-translational modifications available to confer antimicrobial and other properties on peptide scaffolds, and support the contention that bacteriocin diversity is greater than is currently recognised [13].

Abbreviations: GccF, glycocin F; ^{deOGlcNAc}GccF, *O*-deglycosylated GccF; $GccF_{1-41}$, peptide fragment 1–41 of GccF; GlcNAc, *N*-acetylglucosamine; HexNAc, *N*-acetylhexosamine; CD, circular dichroism; FT-ICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; IC_{50}, concentration that decreases growth rate by 50%; ECD, electron capture dissociation; TFA, trifluoroacetic acid

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2. Materials and methods

2.1. Bacterial strains and culture conditions

L. plantarum strains (KW30, ATCC 8014) were grown at 30 °C in MRS medium (Merck). For GccF production KW30 was grown at 22 °C. For sublancin production *Bacillus subtilis* BR151 (BGSC, Ohio State University) was grown as described by Paik et al. [12].

2.2. Bacteriocin purification

Supernatant from a three-day 8 L KW30 culture was adjusted to pH 7 with NH_4OH and stirred overnight with 1 L phenyl Sepharose (FF, low substitution, GE Healthcare). The resin was packed into a glass column, washed with 3 L 2% NH_4HCO_3 and eluted with 2 L 2% NH_4HCO_3, 40% ethanol. Fractions (10 ml) containing active GccF were pooled, concentrated to ~12 ml by rotary evaporation, then purified by RP-HPLC (Jupiter 5 μm C_{18} 300 Å, 250 × 10 mm, Phenomenex; 1 ml injection, 5 ml min^{-1}, 25 min 0–50% B linear gradient. A: H_2O, 0.1% trifluoroacetic acid (TFA); B: acetonitrile, 0.08% TFA). GccF, eluting at ~40% B, was lyophilised (Fig. S1). The yield of purified GccF was 0.5–1 mg L^{-1} of culture. Active sublancin 168 was purified as described by Paik et al. [12].

2.3. Enzymatic dissection

GccF (1 mg ml^{-1} in 1 ml 1% NH_4HCO_3, pH 8.1) was incubated with 10 μg trypsin (Promega) overnight at room temperature. The digest was fractionated by RP-HPLC (Jupiter 5 μm C_{18} 300 Å, 250 × 4.6 mm, Phenomenex; 1 ml min^{-1}, gradient as in Section 2.2) to purify $GccF_{1-32}$ and $GccF_{33-43}$. A chymotryptic digest under similar conditions with a GccF:protease ratio of 50:1 by weight was incubated for 4.5 h and fractionated as above to purify peptide fragment 1–41 of GccF ($GccF_{1-41}$) and 'HX' (His42Cys43-*N*-acetylhexosamine (HexNAc)). GccF and $GccF_{1-32}$ (each 1 mg ml^{-1} in 1 ml 50 mM sodium acetate buffer, pH 4.5, 5 mM EDTA) were *O*-deglycosylated by adding 0.5 mg purified recombinant *N*-acetyl-β-D-glucosaminidase GcnA [14] and incubating overnight at room temperature. Reaction samples were analysed using MALDI-TOF MS (Micromass m@ldi) to detect the loss of *O*-linked HexNAc, and *O*-deglycosylated peptides purified as described for proteolytic fragments.

2.4. Analytical methods

GccF, reduced GccF, reduced and alkylated GccF, *O*-deglycosylated GccF (^{deOGlcNAc}GccF), $GccF_{1-41}$, $GccF_{1-32}$, $^{deOGlcNAc}GccF_{1-32}$, $GccF_{33-43}$, C-terminal peptide His42Cys43-HexNAc and sublancin were characterised by tandem mass spectrometry (PE SCIEX API 300 LC/MS/MS, PE Sciex Instruments; micrOTOF-Q, Bruker Daltonics; 9.4T APEX-Q Ultra Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), Bruker Daltonics). Circular dichroism (CD) spectra were collected on a Chirascan™ spectrometer (Applied Photophysics, U.K.). Experimental details of MS and CD spectroscopy, Edman and DNA sequencing, and bioinformatic methods are given in Supplementary data. Nucleotide sequence of the *gcc* cluster (6789 bp) was deposited in GenBank, accession number GU552553.

2.5. GccF activity assays

MRS medium containing 1% bacteriological agar was autoclaved then cooled to 40 °C. ATCC 8014 cells were added to ~3×10^6 cells ml^{-1} and 15 ml of this indicator agar was poured immediately into 7.2 cm diameter Petri dishes. Samples (2.5 μl) were pipetted onto indicator plates and incubated overnight at 30 °C. To detect antibacterial activity associated with peptides analysed by tricine SDS–PAGE, destained gels were overlaid with indicator agar. For IC_{50} determinations, the optical density (O.D. 600 nm) of 3 ml suspensions of ~3×10^7 cells ml^{-1} in MRS medium at ~30 °C was monitored (Hitachi U-1100 spectrophotometer) after addition of GccF and peptide fragments. IC_{50} is the concentration that halves the rate of O.D. 600 nm increase excluding the lag phase prior to inhibition. Tricine SDS–PAGE and LIVE/DEAD® cell assay methods are described in Supplementary data.

3. Results

3.1. Structural characterisation of GccF

The predicted monoisotopic mass of GccF is 4796.9197 Da, whereas the mass measured by FT-ICR-MS was 5199.0488 Da (shown as $[M+H]^+$ 5200.0561 Da in Fig. S2). GccF was analysed by Edman sequencing (Table S1) and FT-ICR-MS with electron capture dissociation (ECD) (Table S2), and shown to contain two types of post-translational modifications that account for the observed mass difference (Fig. 1A, $C_{226}H_{311}N_{57}O_{72}S_7$, theoretical monoisotopic mass 5199.0472 Da). Firstly, Edman sequencing identified two nested disulfide bonds (Cys5–Cys28 and Cys12–Cys21) – the same constrained $(C–X_6–C)_2$ 'hairpin architecture' was reported for sublancin 168 [12]. Secondly, FT-ICR-MS identified two HexNAcs, one linked to Ser18 and the other to Cys43. Partially glycosylated forms of GccF were not detected. Treatment with

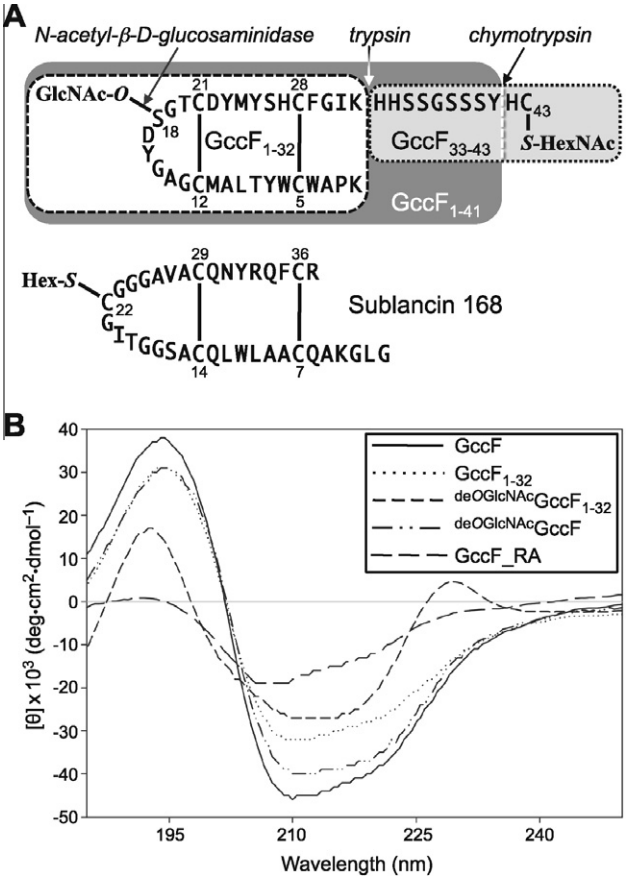

Fig. 1. (A) Schematic of GccF and sublancin 168, including enzymatic dissection of GccF. Trypsin cleavage produces $GccF_{1-32}$ and $GccF_{33-43}$, *N*-acetyl-β-D-glucosaminidase hydrolyses the *O*-glycosidic bond yielding ^{deOGlcNAc}GccF and $^{deOGlcNAc}GccF_{1-32}$, chymotrypsin cleavage yields His42Cys43-HexNAc, and partial chymotrypsin cleavage yields $GccF_{1-41}$. (B) CD spectra of GccF and fragments. GccF_RA, reduced and alkylated GccF.