Bioengineering of the model lantibiotic nisin

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The lantibiotics are a class of bacterially produced antimicrobial peptides (bacteriocins) that contain several unusual amino acids resulting from enzyme-mediated post-translational modifications. They exhibit high specific activity against Gram-positive targets, including many antibiotic-resistant pathogens, and consequently have been investigated with a view to their application as antimicrobials in both the food and medical arenas. Importantly, the gene-encoded nature of lantibiotics makes them more amenable to bioengineering strategies to further enhance their antimicrobial and physicochemical properties. However, although the bioengineering of lantibiotics has been underway for over 2 decades, significant progress has only been reported in recent years. This review charts recent developments with regard to the implementation of bioengineering strategies to enhance the functional characteristics of the prototypical and most studied lantibiotic nisin.

Introduction

Ribosomally synthesized antimicrobial peptides have been observed in virtually every living organism, and those produced by bacteria, termed bacteriocins have become the focus of many biomedical and food-related research groups. Although bacteriocins comprise a large heterogenous group, it is the highly modified lantibiotics (derived from lanthionine-containing antibiotics), a potent class of bioactive peptides produced by Gram positive bacteria that hold most promise due to their high specific activity against pathogenic food and clinical targets and the fact that they exhibit distinctly different mechanisms of action to currently used chemotherapeutics.1,2 The defining structural feature of these peptides is the presence of one or more non-standard residues including lanthionine and/or methyl lanthionine that are initiated by a series of enzyme-mediated post-translational modifications. While other peptide-modifying enzymes may also be present, these extensive modifications confer not only structure and function to the peptide, but also provides increased proteolytic resistance3,4 and greater tolerance to oxidation.5 Indeed, these and a range of other desirable features make them suitable for potential use in human and veterinary medicine and also in the pharmaceutical, biochemical, agricultural and food industries.6 However, lantibiotics present certain drawbacks for widespread use, including instability and/or insolubility at physiological pH and susceptibility to the action of intestinal proteases. Therefore, attention has focused on the bioengineering of these peptides to increase their potency and stability and generate derivatives with potentially superior pharmacokinetic properties. The present review will focus on recent developments with regard to the implementation of bioengineering strategies to enhance the functional characteristics of nisin, the oldest known and most widely studied lantibiotic.

Nisin, the Archetypical Lantibiotic Peptide

Nisin is the first, and so far only lantibiotic to have found commercial use. Discovered in 1928,7 it exhibits a broad spectrum of inhibition against Gram positive bacteria including spoilage and food-borne pathogens such as staphylococci, bacilli, clostridia and Listeria. Nisin has been extensively used in the food industry as a food preservative for decades gaining both EU (E234) and Food and Drug Administration (FDA) approval8,9 and has been employed in a wide variety of foods, for reviews see refs.10,11 Nisin is synthesized as a biologically inactive pre-peptide consisting of an N-terminal leader peptide attached to the C-terminal pro-peptide (Fig. 1A). Serine and threonine residues of the unmodified core peptide (with N-terminal leader peptide attached) are dehydrated by NisB, forming dehydroalanine (Dha) and dehydrobutyrine (Dhb) respectively (Fig. 1B). NisC then catalyzes the other bond formation in subsequent intramolecular addition reactions involving nearby cysteine residues and the double bonds of Dha and Dhb to form lantionine (lan) and methyllanthionine (Melan), respectively (Fig. 1C). Modified nisin with the leader peptide still attached is subsequently transported via the dedicated ABC-type transporter NisT. Only after proteolytic cleavage of the N-terminal leader sequence, mediated by the extracellular serine protease NisP is the mature bioactive nisin peptide released (Fig. 1D). The antimicrobial nature of nisin dictates that a producing strain must be immune to the effects of its own product. Immunity is conferred by 2 different systems consisting of a lipoprotein NisI and an ABC transporter NisFEG.12 Furthermore, the biosynthesis of nisin is regulated (in order to maintain a proper balance between production and immunity) via a 2-component system that consists of a receptor histidine kinase
(NisK) and a transcriptional response regulator (NisR). Several elegant studies have shown that Nisin exerts its antimicrobial activity both by pore formation and by inhibition of cell wall synthesis through specific binding to lipid II, an essential precursor of the bacterial cell wall. This combined action is mediated by 2 structural domains located at the N- and C-termini. The N-terminal domain comprising 3 lanthionine rings (A, B and C) is linked to the C-terminal rings (D and E) by a flexible hinge region (Fig. 1D) consisting of 3 amino acids (Asn20-Met21-Lys22). It has been established that the A, B and C rings form a ‘cage-like’ enclosure that facilitates binding of the pyrophosphate moiety of lipid II, thus inhibiting cell wall synthesis. This binding enhances the ability of the C-terminal segment, containing rings D and E, to form pores in the cell membrane, resulting in the rapid efflux of ions and cytoplasmic solutes. The combined effect of both activities results in minimum inhibitory values in the nanomolar range against sensitive bacterial targets. Notably, lipid II is also the molecular target for the glycopeptide antibiotic vancomycin. However, as nisin binds lipid II at a site distinct from vancomycin, it retains activity against vancomycin-resistant Gram positive pathogens.

Bioengineering and Rational Design-Generating More Effective Nisins

The remarkable commercial success of nisin has positioned it at the pinnacle of bacteriocin research not only in terms of the wealth of knowledge attained with respect to genetics, structure, mode of action and chemical properties but also for the scope and variety of its practical applications. Several recent studies highlight the multiple ways in which nisin could potentially be used as a therapeutic agent. For instance, it has been shown that nisin has immunomodulatory properties analogous to those described for many human defense peptides giving rise to the suggestion of a possible role for nisin as a novel immunomodulatory therapeutic. Additionally, nisin was shown to be effective in the treatment of head and neck squamous cell carcinoma. Notably, this is the first time a bacteriocin has been used to prevent the growth of cancer cells. Furthermore, the in vivo effectiveness of nisin has been demonstrated while a range of multi-drug resistant bacteria have all been shown to be susceptible. Although nisin has found certain applications in the veterinary field due to its efficacy against the Gram positive pathogens responsible for bovine mastitis, it is only in the last few years that its potential for use in the treatment of human mastitis infections has been studied. However, despite exhibiting high efficacy against clinically relevant pathogens, in addition to its lack of cytotoxicity and the absence of observed resistance development in routine practice, the widespread use of nisin as a therapeutic entity has not yet been fulfilled, in part due to its low solubility and stability at physiological pH, effectively hampering its systemic use. Consequently, the pharmacological and physicochemical properties of the peptide must be augmented for nisin to achieve its maximum therapeutic potential and suitability for use in a wide variety of settings. Fortunately, the gene-encoded nature of nisin permits strategies that can modify the structure of the peptide in a more direct fashion than is possible for other classes of antimicrobials, and generate variants with potentially beneficial biological, chemical and physical properties. The simplest systems involve in vivo expression of the modified NisA structural gene in a variant of the original producer strain, either in cis by replacing the original NisA gene or in trans by complementing an inactivated copy of the original structural gene. These approaches are limited to generating novel pre-peptides that are compatible with the native biosynthetic, transport and immunity machinery. Indeed, the...
existence of a number of natural nisin variants with differing properties offered some evidence that the generation of novel derivatives was both possible and likely to be auspicious. Several natural variants of nisin have so far been identified. Nisin A, nisin Z, nisin Q and nisin F are produced by *Lactococcus lactis* species while nisin U and nisin U2 are produced by *Streptococcus uberis*. In a study that directly compared the activities of Nisin A, F, Q and Z against a range of *Staphylococcus aureus* targets, it was apparent that Nisin F was the most active. Recently, a novel nisin variant produced by *L. lactis* has been described in which valine replaces isoleucine at position 4 (I4V) and alanine replaces leucine at position 16 (L16A). Further analysis of the associated structural gene suggests the strain may be able to produce a nisin analog (designated Nisin P) that has similarities to nisin U. Interestingly, a nisin homolog geobacillin I, was identified in the genome of the thermophile *Geobacillus thermodenitrificans* NG80-2 that displayed superior stability at pH 7 and 8 and high temperatures when compared to nisin A. The diversity of these natural variants and related homologues highlights the extreme tolerance of certain residues and domains within the molecule to change. To further explore this diversity, several PCR-based approaches have been utilised, permitting site-directed, random and site-saturation mutagenesis to be applied. Indeed, when coupled with high-throughput screening technologies, large banks of nisin derivatives can be generated that possess all possible point mutations or even several simultaneous mutations, providing a blueprint of which residues are essential for structure-function relationships as well as for their impact on production. Moreover, it allows for screening and detection of derivatives with potentially improved functional properties including antimicrobial activity, solubility, protease and/or pH stability. The most notable successes achieved as a consequence of employing these various strategies in recent years are summarised below.

**Modulation of the Antimicrobial Activity and Spectrum of Nisin**

The bioengineering of nisin commenced over 2 decades ago. However, it is only in recent years that researchers armed with a greatly enhanced understanding of lantibiotic biology and an ability to carry out bioengineering on a larger scale, have finally begun to achieve some notable success. The first promising derivatives were enhanced against non-pathogenic bacteria. Nisin Z T2S, and nisin Z M17Q/G18T (Fig. 2B), and 2 mutants of nisin A at positions 3–5 corresponding to KSI and KFI (Fig. 2A) demonstrated increased activity against a limited number of non-pathogenic indicator strains (*M. flavus, S. thermophilus, P. pentosaceus, L. johnsonii, L. mesenteroides* and *M. luteus*). The first instance of a bioengineered derivative with enhanced activity against pathogenic bacteria was reported by Yuan and coworkers who established that nisin Z N20K and M21K (located within the flexible hinge-region) (Fig. 2B) exhibited increased activity toward Gram negative strains *Shigella, Pseudomonas* and *Salmonella* spp. Shortly after, nisin derivatives with enhanced activity against gram positive pathogens were identified. Nisin A K22T (Fig. 2A) displayed superior activity against *Streptococcus agalactiae* ATCC13813, a pathogen associated with early perinatal human infections and bovine mastitis, nisin A N20P, a derivative displaying enhanced activity against *S. aureus* (including an MRSA

![Figure 2. Overview of enhanced bioengineered nisin derivatives. Structures of (A) nisin A and (B) nisin Z. Derivatives with increased specific activity as a result of the introduction of single amino acid substitutions are denoted by broken arrows/light green, or a combination of amino acid substitutions by braced/purple. Enhancement of physicochemical properties as a result of single amino acid substitutions are denoted by orange/broken line or by a combination of amino acid substitutions by braced/light blue. Post translational modifications are indicated as follows: Abu: 2-aminobutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3-methyllanthionine) Dha: dehydroalanine, Dhb: dehydrobutyryne.](#)
isolate), nisin A M21V that exhibits superior anti-listerial activity compared to wild-type nisin, and nisin A K22S, with improved activity against bovine mastitis associated S. aureus and S. agalactiae.\textsuperscript{44} Notably, the variants also displayed greater anti-mycobacterial activity compared to nisin A as determined by a microtitre alamar blue assay.\textsuperscript{45} Further studies have emphasized the enhanced potency of the derivative nisin A M21V, subsequently renamed Nisin V against a wide range of targets, including medically significant pathogens such as methicillin-resistant S. aureus (MRSA), vancomycin-resistant enterococci (VRE), Clostridium difficile and L. monocytogenes.\textsuperscript{44,46} This enhanced activity was apparent in food model experiments\textsuperscript{46} as well as in vivo mouse model experiments\textsuperscript{47} against L. monocytogenes. As the majority of these hinge derivatives transpired as a result of strategies in which one or 2 of the hinge residues were manipulated, a more intense approach through randomization of all 3 hinge residues simultaneously within nisin A was undertaken.\textsuperscript{48} Notably, this study revealed a pattern whereby many producers of derivatives containing small chiral amino acids within the hinge displayed enhanced bioactivity. This inspired the rational design of additional derivatives not identified from within the random bank, yielding peptides containing AAA and SAA hinges, which were particularly notable with respect to the extent to which bioactivity was enhanced relative to the wild type producer.\textsuperscript{48} Furthermore, the advantage of decreasing or extending the hinge region and the subsequent impact on activity and spectrum has recently been investigated. Notably, both truncated hinge variants (\(-1\) amino acid) and elongated mutants (\(+2\) amino acids) displayed improved bioactivity against one or more indicator targets including L. monocytogenes, E. faecalis and B. subtilis othermodurans in deferred antagonism assays when carried out under a range of different temperatures.\textsuperscript{49} Outside of the hinge region, several nisin A derivatives were described that displayed increased potency against a range of bacterial targets. Replacing serine at position 29 (serine 29) with all the other available standard amino acids resulted in the generation of variants (Fig. 2A) that displayed enhanced activity against a range of Gram positive bacterial targets, with S29G and S29A representing the first nisin derivatives to exhibit enhanced activity against both Gram-positive and Gram-negative bacteria.\textsuperscript{50} Indeed this strain variable nature provides further evidence that nisin derivatives can be generated with distinct target specificities. Additionally, derivatives bioengineered at lysine 12 (K12), located in a flexible region located between rings B and C of the peptide led to the discovery of several derivatives of interest with one in particular, K12A, displaying enhanced specific activity against numerous Gram-positive microorganisms of food and/or clinical significance.\textsuperscript{51} These studies show that bioengineering can successfully generate nisin derivatives that possess not only enhanced antimicrobial activity but also a broader target specificity. Significantly, the data generated can also provide insight into structure-function relationships as well as substrate specificity of the maturation enzymes and immunity that can also be applied successfully in the rational design of enhanced variants.

**Modulation of the Physicochemical Properties of Nisin**

The first example of nisin variants that could exhibit discrete functional characteristics was provided by the natural variant nisin Z, which differs from nisin A by just a single amino acid (asparagine rather than histidine at position 27). Despite having similar antimicrobial activity to nisin A, nisin Z displays a higher rate of diffusion,\textsuperscript{52} and is less soluble at low pH.\textsuperscript{37} Indeed, introducing lysine residues at 2 distinct locations (nisin Z N27K and nisin Z H31K) (Fig. 2B) resulted in the generation of peptides that displayed improved solubility at neutral pH while maintaining comparable antimicrobial activity and target spectrum.\textsuperscript{37} Similarly, the nisin Z derivatives N20K and M21K (Fig. 2B), in addition to realizing enhanced anti-Gram negative activity as mentioned above, also displayed improved solubility, particularly at alkaline pH values\textsuperscript{43} where the solubility of wild type nisin is particularly reduced. In addition, the N20K and M21K variants demonstrated increased stability at higher temperatures than the wild type peptide, a desirable benefit given that nisin may be subjected to thermal treatment during food processing. Importantly, bioengineering has the potential to generate peptides that are enhanced in other ways. Recently, a number of novel nisin derivatives were identified with an enhanced ability to diffuse through complex polymers.\textsuperscript{53} In the case of 2 variants, which contain the residues SVA and NAK within the hinge region (Fig. 2A), it was demonstrated that this enhanced trait enabled the peptides to surpass nisin A in restricting growth of Listeria monocytogenes in commercially produced chocolate milk containing carrageenan as a stabilizer.\textsuperscript{53} Although the generation of bioengineered nisin derivatives with superior properties has been demonstrated, one issue that remains to be tackled is that of production. Indeed, the study and application of lantibiotics including nisin is often compromised by limited production of these peptides by the native producer, a problem which is particularly notable when working with bioengineered derivatives.\textsuperscript{41} However, a recent study describes the development of a genetic system that facilitates significant overproduction of nisin.\textsuperscript{54} Such systems may also help in attaining higher yields to simplify isolation of and improve cost-efficiency of novel derivatives that are often compromised by limited production. Finally, a major drawback that has yet to be overcome with respect to therapeutic use is the sensitivity of nisin to proteolytic cleavage by intestinal enzymes. Nisin has been shown to be susceptible to the proteases trypsin and chymotrypsin.\textsuperscript{55} Although several cleavage sites are apparent e.g. Lys12, Asn20, Met21, Lys22 and His31, bioengineering strategies could be employed to replace the residues that serve as recognition sites by these and other digestive enzymes and potentially overcome the issue of its vulnerability to proteolytic breakdown in the gastrointestinal tract.

**Conclusions**

Nisin remains the only commercially available and industrially exploited bacteriocin despite the vast array of novel...
bacteriocins discovered in the last few decades. Although the primary application of nisin has been as a food preservative, the fact that nisin and other lantibiotics target what may be regarded as the ‘Achilles’ heel’ of the Gram positive cell wall; i.e. the essential cell wall precursor lipid II, makes them an attractive option as possible therapeutic agents. Consequently, broad-spectrum antimicrobials such as nisin could theoretically be of use against any clinical Gram-positive human or animal pathogen. Indeed, the emergence of drug-resistant pathogens makes the identification of such novel antimicrobials with unprecedented mechanisms of action not yet exploited in traditional therapeutics even more important. Novel lantibiotics continue to be discovered thanks to the upsurge in available bacterial genomes as well as the development of bioinformatics software and search engines designed for the identification of their biosynthetic clusters. Such in silico approaches also provide a vast array of novel biosynthetic enzymes that could be employed to introduce additional modifications to expand the range of activities of lantibiotic peptides or synthetic analogs. But perhaps it is their amenability to bioengineering that makes them exceptionally promising. Accordingly, the implementation of bioengineering strategies using existing bacteriocin structures as a blueprint provides an attractive approach to tailoring the biological activity and spectrum of inhibition of bacteriocins. In this regard nisin continues to be at the forefront of bacteriocin-related research. Mutagenesis studies in combination with high-throughput screening have been fruitful with regard to the generation of a diverse collection of derivatives with enhanced specific activities and unique antimicrobial spectra that could be of value in both food and clinical settings. Moreover, the identification of nisin derivatives with superior bioactivities other than specific activity, for example diffusion, stability, solubility, will advance even further their prospects for applications in a variety of settings. While the mechanisms underlying the enhanced antimicrobial activity of these variants have yet to be elucidated, the fact that nisin can be improved is an exciting development. Given the advances that have been made in recent years, it would seem we have already crossed an important threshold in antimicrobial peptide exploration, and it is anticipated that the tremendous potential for the development of more effective nisin-derived antimicrobials for food and medical applications will finally be realized.

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No potential conflicts of interest were disclosed.

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