

# Structure-activity relationships in the peptide antibiotic nisin: antibacterial activity of fragments of nisin

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Received 10 May 1996; revised version received 3 June 1996

**Abstract** The post-translationally modified peptide antibiotic nisin has been cleaved by a number of proteases and the fragments produced purified, characterised chemically, and assayed for activity in inhibiting the growth of *Lactococcus lactis* MG1614 and *Micrococcus luteus* NCDO8166. These results provide information on the importance of different parts of the nisin molecule for its growth-inhibition activity. Removal of the C-terminal five residues leads to approximately a 10-fold decrease in potency, while removal of a further nine residues, encompassing two of the lanthionine rings, leads to a 100-fold decrease. There are some differences between analogous fragments of nisin and subtilin, suggesting possible subtle differences in mode of action. Cleavage within, or removal of, lanthionine ring C essentially abolishes the activity of nisin. The fragment nisin<sup>1–12</sup> is inactive itself, and specifically antagonises the growth-inhibitory action of nisin. These results are discussed in terms of current models for the mechanism of action of nisin.

**Key words:** Nisin; Lantibiotic; Peptide antibiotic; Proteolysis; Structure-activity relationships

## 1. Introduction

The post-translationally modified peptide antibiotics known as 'lantibiotics' contain cyclic structures formed by lanthionine or 3-methyl-lanthionine residues, and often also dehydroalanine and/or dehydrobutyrine residues [1]. The first lantibiotic to be characterised was nisin, produced by strains of *Lactococcus lactis* carrying a transposon containing genes coding for the nisin precursor and for proteins involved in nisin biosynthesis and resistance [2–5]. Nisin has been quite widely used as a food preservative, notably in cheese and other dairy products and in canned vegetables, for some 30 years [6,7]. It inhibits the growth of a wide range of Gram-positive organisms, and also inhibits the germination and/or outgrowth of spores of *Bacillus* and *Clostridium* species [6]. The growth-inhibitory activity of nisin, and that of other 'type A' lantibiotics such as subtilin, appears to be due to the for-

mation of voltage-dependent pores in biological membranes [8–10], although recent evidence indicates that the inhibition of the outgrowth of spores by nisin and subtilin takes place by a different mechanism [11,12]. We have been studying the structure-activity relationships in nisin and subtilin, by both genetic and chemical modification of the structure [12–20], with a view to understanding their mechanism of action and to developing new derivatives with desirable properties. We now report the preparation, characterisation and anti-bacterial activity of a number of proteolytic fragments of nisin and subtilin, which allow us to define the parts of the molecule most important for biological activity.

## 2. Materials and methods

Nisin and subtilin were prepared as previously described [14,15]; [Ser<sup>33</sup>]-nisin, in which the serine residue at position 33 has escaped processing to a dehydroalanine residue, was isolated as a minor component of commercial nisin. All proteases were obtained from Sigma Chemical Co., Poole, Dorset, UK.

### 2.1. Production and purification of nisin and subtilin fragments

The sites of cleavage of nisin by the proteases used are indicated in Fig. 1. The procedures used for each proteolytic digestion were as follows: in each case, the progress of the reaction was followed by HPLC of aliquots. **Trypsin:** 10 mg nisin was dissolved in 10 ml buffer (25 mM sodium acetate, 5 mM Tris acetate, 5 mM CaCl<sub>2</sub>, pH 7.0), 1 mg trypsin was added and the reaction mixture stirred at 30°C. Further aliquots of 0.5 mg trypsin were added after 24 and 48 h. After 3–4 days, the mixture was acidified and lyophilised. **Subtilin<sup>1–19</sup>** and **subtilin<sup>3–29</sup>** were prepared by digestion of subtilin with trypsin in a similar way. **Chymotrypsin:** 5 mg nisin was dissolved in 5 ml buffer (25 mM Tris acetate, pH 7.5), 0.6 mg  $\alpha$ -chymotrypsin was added and the reaction mixture incubated at 30°C; a further 0.3 mg chymotrypsin was added after 24 h. After 72 h, the reaction was stopped by acidification and lyophilisation. **Thermolysin:** 4 mg nisin was dissolved in 5 ml buffer (25 mM Tris acetate, 5 mM CaCl<sub>2</sub>, pH 7.8), 0.8 mg thermolysin added, the mixture incubated at 35°C for 8 h and then acidified and lyophilised. This provided nisin<sup>1–29</sup>; for more extensive thermolysin digestion to obtain additional cleavage in ring C, the incubation at 35°C was extended to 24 h; at this time, an additional 0.5 mg thermolysin was added, and the reaction mixture left at room temperature for an additional 60 h before acidification and lyophilisation. Nisin<sup>1–29</sup> was also obtained by exhaustive digestion of [Ser<sup>33</sup>]-nisin with carboxypeptidase Y for 4–5 h at 30°C.

The desired fragments were purified from the acidified and lyophilised reaction mixtures by reversed phase HPLC. In most cases, a Kromasil KR100-5C8 (250 × 8 mm) column was used, but for purification of nisin<sup>1–12</sup> a Hypersil Pep C18 column was used. Elution was with a linear gradient from 0.06% aqueous trifluoroacetic acid to 0.06% trifluoroacetic acid in 90% acetonitrile, 10% water.

Nisin<sup>1–31</sup> was isolated as a minor component from a fermentation producing the mutant Dha<sup>33</sup> → Ala nisin<sup>(1)</sup> (Dodd, Horn, Chan, Clark, Gasson, Bycroft and Roberts, unpublished work). Nisin<sup>1–32</sup> and [des-Dha<sup>5</sup>]-nisin<sup>1–32</sup> were prepared as previously described [13].

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<sup>1</sup>Abbreviations used for post-translationally modified residues are defined in the caption to Fig. 1.

## 2.2. Characterisation of nisin and subtilin fragments

Each of the fragments was checked for purity by analytical HPLC and structurally characterised by amino acid analysis and plasma desorption mass spectrometry (Bioion 20), and in some cases by  $^1\text{H}$  NMR spectroscopy (Bruker AM500); these methods are described elsewhere [12].

## 2.3. Assays of biological activity

Assays of inhibition of growth of *L. lactis* MG1614 (a nisin-sensitive strain lacking the transposon carrying the *nis* operon [20]) and of *M. luteus* NCDO8166 were carried out either by agar diffusion or by following growth in suspension by measuring absorbance at 600 nm. The results of all assays are presented as the mean of duplicate experiments which agreed to within less than 20%. To determine the effect of pre-incubation of cells with nisin $^{1-12}$  on the growth-inhibitory effect of nisin, two 0.4-ml samples of a culture of *L. lactis* MG1614 in GM17 broth were taken and the cells harvested. One sample was incubated in 1 ml GM17 broth containing 20  $\mu\text{g/ml}$  nisin $^{1-12}$  for 30 min, while the control sample was incubated for the same period in broth alone. Cells from both samples were harvested, washed three times with GM17 broth, resuspended in broth and aliquots used to determine growth ( $A_{600}$ ) in the presence of 0–150 ng/ml nisin.

## 3. Results and discussion

### 3.1. Production and characterisation of nisin fragments

The structure of nisin [14,22] is shown in Fig. 1; it can be seen to contain five rings formed by lanthionine or methyl-lanthionine residues. Subtilin has exactly the same ring structure, but has 12 amino acid substitutions relative to nisin, and two deletions in the C-terminal 'tail'. The five lanthionine rings constrain the conformation of the molecule [18,23,24] and, presumably primarily as a consequence of these constraints, nisin and subtilin are generally resistant to attack by proteolytic enzymes. However, we have found that prolonged exposure to trypsin, chymotrypsin or thermolysin, as described in Section 2, can lead to rather specific cleavages of the molecule, as shown in Fig. 1, and these can be used to prepare fragments of the molecule.

Trypsin cleaves the Lys $^{12}$ -DAbu $^{13}$  bond of nisin to give the nisin $^{1-12}$  fragment. It is not known whether the Lys $^{22}$ -DAbu $^{23}$  bond is also cleaved, since the other cleavage product(s) were not isolated; cleavage at Lys $^{22}$ -DAbu $^{23}$  in the absence of cleavage at Lys $^{12}$ -DAbu $^{13}$  does not occur to a significant extent, since the nisin $^{1-22}$  fragment is not a major product. The only aromatic amino-acid residues in nisin are His $^{27}$  and His $^{31}$ , and chymotrypsin cleaves, slowly and somewhat unexpectedly, at Asn $^{20}$ . Initial cleavage by thermolysin (0.2 mg enzyme/mg nisin for 8 h) is at Ser $^{29}$ ; more prolonged digestion

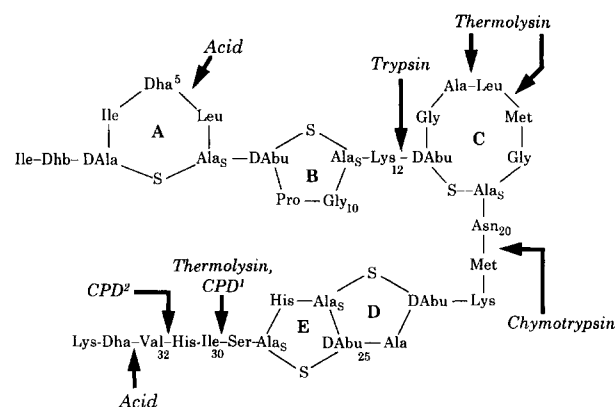


Fig. 1. Structure of nisin, indicating the points of cleavage which generate the fragments discussed in the text. Dehydroalanine and dehydrobutyryne residues are abbreviated Dha and Dhb, respectively. *Meso*-lanthionine and  $\beta$ -methyl-lanthionine residues are indicated by DAla-S-Ala $_8$  and DAbu-S-Ala $_8$  respectively (where the amino-terminal moieties have the D configuration). For details of cleavage conditions, Section 2. CPD $^1$ : exhaustive cleavage by carboxypeptidase Y of [Ser $^{33}$ ]-nisin. CPD $^2$ : cleavage, presumably by endogenous carboxypeptidases, of the Dha $^{33}$ →Ala mutant of nisin.

leads to additional cleavages of the Ala $^{15}$ -Leu $^{16}$  and then the Leu $^{16}$ -Met $^{17}$  bonds in ring C, with release of Leu $^{16}$ . Nisin is particularly resistant to carboxypeptidase action, but this resistance can be decreased by alterations to the primary structure near the C-terminus. Thus [Ser $^{33}$ ]-nisin, in which the serine residue at position 33 has escaped processing to a dehydroalanine residue, can be isolated as a minor component of commercial nisin, and digestion of this with carboxypeptidase Y yields nisin $^{1-29}$ . Similarly, nisin $^{1-31}$  was isolated as a minor component, presumably resulting from the action of endogenous carboxypeptidases, from a fermentation producing the mutant (Dha $^{33}$ →Ala) nisin (Dodd, Horn, Chan, Clark, Gasson, Bycroft and Roberts, unpublished work). Each of the fragments produced by these digestions was rigorously purified by HPLC and structurally characterised by amino acid analysis and plasma desorption mass spectrometry; in each case the measured masses were in excellent agreement with those expected from the proposed structure (Table 1).

### 3.2. Growth-inhibitory activity of fragments of nisin

The activities of the various fragments of nisin as inhibitors

Table 1  
Inhibition of bacterial growth by nisin and its fragments

Compound	Molecular ion		MIC ( $\mu\text{g/ml}$ )	
	Calc.	Found	<i>L. lactis</i> MG1614	<i>M. luteus</i> NCDO8166
Nisin	3355.12	3354.8	0.08	0.07
[Ser $^{33}$ ]-Nisin	3373.13	3373.6	0.12	0.08
Nisin $^{1-32}$ amide <sup>a</sup>	3156.90	3157.0	0.04	0.05
Nisin $^{1-31}$	3058.76	3060.9	0.70	nd <sup>b</sup>
Nisin $^{1-29}$	2808.45	2808.0	1.28	0.79
Nisin $^{1-20}$	1881.31	1881.2	9.21	3.50
[Ala $^{15}$ -OH, H-Leu $^{16}$ ] Nisin $^{1-29}$	2826.46	2828.1	> 400	~ 100
[des-Leu $^{16}$ , Ala $^{15}$ -OH, H-Met $^{17}$ ] Nisin $^{1-29}$	2713.30	2715.8	> 400	~ 200
Nisin $^{1-12}$	1151.43	1151.7	> 200	16.5
[des-Dha $^5$ ] nisin $^{1-32}$ amide <sup>a</sup>	3174.89	nd	> 200	4.48

<sup>a</sup>Data from [2].

<sup>b</sup>Not determined.

of the growth of *Lactococcus lactis* and *Micrococcus luteus* are summarised in Table 1. These can most simply be discussed by considering the progressive removal of residues from the C-terminus of the molecule. Acid treatment of nisin leads to hydrolysis at Dha<sup>33</sup>, forming nisin<sup>1–32</sup> amide [13] whose activity is very similar to that of nisin itself. The non-essential nature of Dha<sup>33</sup> is further demonstrated by the essentially full activity of [Ser<sup>33</sup>]-nisin (Table 1) and of the Dha<sup>33</sup> → Ala mutant (Dodd, Horn, Chan, Clark, Gasson, Bycroft and Roberts, unpublished work). Further removal of Val<sup>32</sup>, in nisin<sup>1–31</sup>, leads to a significant (~10-fold) decrease in activity against *L. lactis*, suggesting either that this valine residue makes a significant contribution to activity, or that the presence of a negative charge at this point in the molecule is unfavourable. Removal of His<sup>31</sup> and Ile<sup>30</sup> to give nisin<sup>1–29</sup>, the shortest fragment which retains all the lanthionine rings, gives a molecule with 7% the activity against *L. lactis* and 10% the activity against *M. luteus* of the intact nisin molecule, showing that the C-terminal 'tail' of nisin plays a significant, but far from dominant, role in its antibacterial activity.

Removal of the linked rings D and E, in nisin<sup>1–20</sup>, produces a further substantial drop in activity, by an overall factor of 100 against *L. lactis* and 50 against *M. luteus*. The antibacterial activity of nisin<sup>1–20</sup> is, however, still readily measurable. It is only with cleavage in, or removal of, ring C that the molecule becomes essentially totally inactive. Thermolysin cleavage of the Ala<sup>15</sup>-Leu<sup>16</sup> and subsequently the Leu<sup>16</sup>-Met<sup>17</sup> bonds in ring C of nisin<sup>1–29</sup> leads to complete loss of activity. Analogous results have been reported by Kuipers et al. [25], who introduced a tryptic cleavage site into ring C by substituting Met<sup>17</sup> with lysine; the mutant retained 50% activity, but this was decreased to ~1% on cleavage by trypsin. Complete removal of ring C, in nisin<sup>1–12</sup>, similarly leads to essentially complete loss of activity against *L. lactis* (Table 1). By contrast, this fragment retains slight but significant activity against *M. luteus*, which is very sensitive to lantibiotic action. The observation that nisin<sup>1–12</sup> is more active against this organism than the 29-residue fragments in which ring C is present but cleaved ([Ala<sup>15</sup>-OH, H-Leu<sup>16</sup>] nisin<sup>1–29</sup> and [des-Leu<sup>16</sup>, Ala<sup>15</sup>-OH, H-Met<sup>17</sup>] nisin<sup>1–29</sup>) is as yet unexplained. It may effect unfavourable interactions of the new charges in the latter molecules with a region of the nisin binding site (see below) which normally accommodates the hydrophobic ring C.

An intact ring C is thus essential for antibacterial activity of nisin. Earlier evidence has shown that this is also true for ring A; acid hydrolysis at Dha<sup>5</sup> in nisin<sup>1–32</sup> amide leads to loss of activity [13], but the Dha5Ala mutant retains full antibacterial activity [12], showing that it is the intact ring A, rather than the dehydro residue at position 5, which is required for this activity. The 'wedge' model for the formation by nisin of

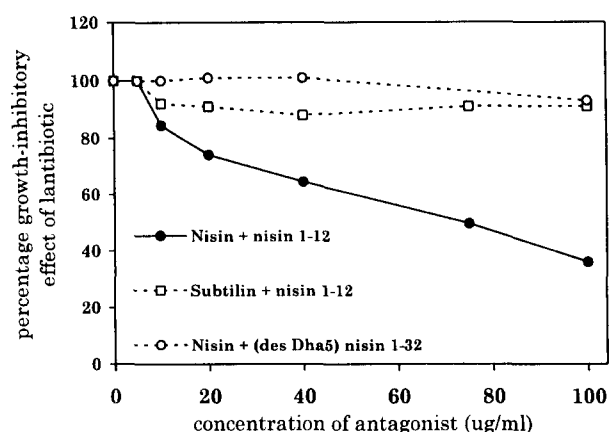


Fig. 2. Effects of nisin<sup>1–12</sup> on the growth inhibitory effects of nisin and subtilin. Inhibition produced by 5 µg/ml nisin or 5 µg/ml subtilin in an agar diffusion assay is set as 100%. The effects of nisin<sup>1–12</sup> (●, □) and of [des Dha<sup>5</sup>]-nisin<sup>1–32</sup> amide (○) on the inhibition of growth by these concentrations of nisin and subtilin are shown.

transient pores in the bacterial membrane by induction of non-bilayer structures [10,26] postulates that the N-terminal part of nisin, comprising rings A–C, is responsible for binding of nisin to the membrane surface and/or its oligomerisation, while the more cationic C-terminal part of the molecule plays a role in pore formation. The present evidence is consistent with the idea that intact rings A and C are required for this binding function, and suggests that some pore formation is possible in the absence of the cationic C-terminal segment.

### 3.3. Comparison of the activity of nisin and subtilin fragments

By comparison to nisin, subtilin has fewer potential sites for proteolytic cleavage; Table 2 shows the growth-inhibitory activities of the two fragments we have been able to purify and characterise, subtilin<sup>3–29</sup> and subtilin<sup>1–19</sup>. Nisin<sup>1–20</sup> and subtilin<sup>1–19</sup> each consist of rings A–C. As noted above, nisin<sup>1–20</sup> has 1% activity against *L. lactis* and 2% against *M. luteus*. The very similar fragment of subtilin, subtilin<sup>1–19</sup>, however, has significantly lower activity (<0.3% against *L. lactis*, and <0.1% against *M. luteus*). It is possible that residue 20 has a crucial role in activity, although this residue is very different in the two molecules – Phe in subtilin and Asn in nisin. Alternatively, this difference may reflect subtle differences in the mechanism of pore formation by these two lantibiotics. The data in Table 2 also suggest that the N-terminus of subtilin is important for its activity; both the natural derivative of subtilin which is succinylated on its N-terminus [16] and subtilin<sup>3–29</sup> show clear decreases in activity when compared to subtilin itself.

### 3.4. Competitive antagonism of the antibacterial action of nisin by nisin<sup>1–12</sup>

As noted above, nisin<sup>1–12</sup> almost completely lacks growth-inhibitory activity against *L. lactis*. We have found that it does, however, act as a specific antagonist of the growth-inhibitory effects of nisin on this organism. Fig. 2 shows that incubation with increasing concentrations of nisin<sup>1–12</sup> decreases the size of the zone of inhibition produced by 5 µg/ml nisin in an agar diffusion assay. 50% inhibition of the effects of this relatively high concentration of nisin (6×MIC) is achieved with 75 µg/ml nisin<sup>1–12</sup> (about a third

Table 2  
Inhibition of bacterial growth by subtilin and its fragments

Compound	MIC (µg/ml)	
	<i>L. lactis</i> MG1614	<i>M. luteus</i> NCDO8166
Subtilin	1.09	0.05
N <sup>α</sup> -succinyl-Trp <sup>1</sup> subtilin <sup>a</sup>	21.3	0.34
Subtilin <sup>3–29</sup>	49.3	34.5
Subtilin <sup>1–19</sup>	> 350	80.2

<sup>a</sup>Data from [5].

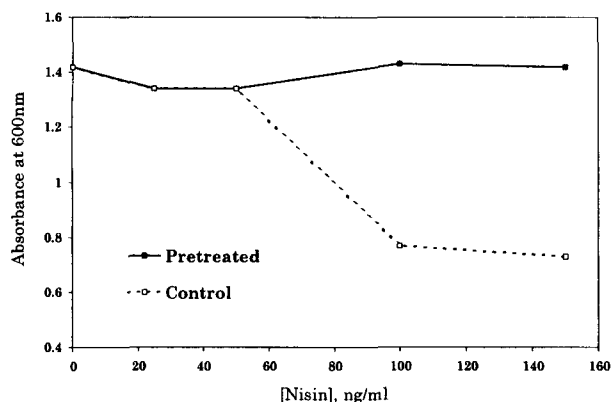


Fig. 3. Effect of preincubation with nisin<sup>1–12</sup> on the growth-inhibitory effects of nisin on *L. lactis* MG1614. *L. lactis* cells were incubated with (■) or without (□) 20 µg/ml nisin<sup>1–12</sup>, and washed before the inhibition of growth by nisin was determined.

of its own MIC). This is clearly a specific effect; as shown in Fig. 2, nisin<sup>1–12</sup> has no effect on growth inhibition by subtilin, and another inactive fragment of nisin, [des-Dha<sup>5</sup>] nisin<sup>1–32</sup> amide, does not antagonise the growth-inhibitory effects of nisin. The lack of effect on subtilin is interesting in that it provides support for the suggestion made above that there may be differences in the mechanism of action between nisin and subtilin. The antagonism of or protection against the growth-inhibitory effects of nisin by nisin<sup>1–12</sup> is also seen when the cells are preincubated with nisin<sup>1–12</sup> and then washed before being challenged by nisin (Fig. 3); in this assay, 20 µg/ml nisin<sup>1–12</sup> completely protects against the effects of 150 ng/ml nisin.

Bearing in mind the proposed mechanism of the antibacterial effect of nisin, involving the formation of pores in the cell membrane [10], several possible mechanisms of this antagonistic action of nisin<sup>1–12</sup> can be envisaged. It is important to note first that, although we have shown that nisin<sup>1–12</sup> can induce nisin immunity [21], this can not be responsible for the antagonism described here, since the nisin-sensitive *L. lactis* strain MG1614 used for growth-inhibition assays lacks the transposon carrying the *nis* operon, including the genes conferring immunity (see [21]). The simplest explanation of the effects of nisin<sup>1–12</sup> is that it competes with nisin for some site which is essential for pore formation. The observation that nisin is able to form pores in protein-free liposomes [26] makes it unlikely that this essential site is a protein, but two other possibilities remain. First, nisin<sup>1–12</sup> could compete for a site on the cell membrane to which nisin must bind as a preliminary to pore formation – probably phosphatidyl glycerol [10,26]. Secondly, the competition could be at the level of the oligomerisation of nisin required for pore formation, if one postulates that an oligomer containing some nisin and some nisin<sup>1–12</sup> molecules is inactive. These two possibilities cannot be distinguished on the basis of the information presently available. The observation that nisin<sup>1–12</sup> does not antagonise the anti-bacterial activity of subtilin would imply either that subtilin binds to a different molecule in the membrane or that mixed nisin-subtilin oligomers cannot form. In either case,

there must be a clear discrimination in binding between the two structurally similar lantibiotics. Although much remains to be learned of the mechanism of action of nisin and the mechanism of nisin immunity [21], the present results show that nisin<sup>1–12</sup> will be a valuable tool in these further investigations.

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