Nisin, an antimicrobial peptide used in food preservation was evaluated for thermal stability. Nisin retained antimicrobial activity after having been heated at 121°C for seven hours. Anhydrous nisin and concentrated nisin solutions (5-10 mg/ml) were still active after having been heated up to 190°C or 230°C. When exposed to high temperatures of 200 or 250°C for up to one hour, nisin lost all antimicrobial activity.

After 3.5 hours of heating at 121°C an inactive nisin degradation product, designated nisin L, was isolated. In comparison to native nisin, nisin L had reduced activity against *Pediococcus pentosaceus* FBB-61 and no activity against *Bacillus cereus* T vegetative cells or spores. Structural changes to nisin L were studied by high performance liquid chromatography (HPLC), ion spray mass spectroscopy (MS), $^1$H nuclear magnetic resonance
spectroscopy (NMR) and circular dichroism (CD) spectroscopy and compared to original nisin.

There was no difference in molecular weight between nisin and nisin L. Both MS spectra contained nisin, with average molecular weight (MW) of 3354 daltons (D), and hydrated nisin with average molecular weight of 3372 D. Nisin L had higher proportion of hydrated molecules, and it had molecules with more than one water addition. Proton NMR analysis of nisin L indicated that dehydrobutyrine 2 and dehydroalanine 5 residues had been altered, and that several new hydrogen resonances appeared. Water additions in nisin L are likely to have occurred at dehydroresidues, making them inactive. Nisin L was found to be more polar, as would be expected for a more hydrated peptide. Analysis of CD spectra indicated that nisin L had smaller content of α helix and therefore lesser membrane spanning capability. Tandem mass spectroscopy of original nisin revealed that it was hydrated at lysine 34 residue.
Structural Analysis of Thermally Inactivated Nisin

by

Tamara Musafija-Jeknic

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CONTRIBUTION OF AUTHORS

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Lantibiotics are small antimicrobial peptides produced by Gram positive bacteria from genera: *Lactococcus*, *Bacillus*, *Staphylococcus*, *Streptomyces* and *Streptococcus*. The unusual structure of lantibiotics which contain modified amino acids and thioether bridges is a unique biological phenomenon. Thioether bridges, built by lanthionine and β-methyllanthionine, confer higher thermal stability to lantibiotics. Modified amino acids, such as dehydroalanine and dehydrobutyrine are responsible for the biological activity of lantibiotics.

Nisin was the first recognized and most widely studied lantibiotic. We were interested in determining what are the upper limits of its thermal stability and what structural changes occur in the molecule upon heat treatment. Nisin is used as a natural food preservative and this information is important in its application in thermally processed foods. Additionally, nisin has an advantage over other food preservatives because it is a peptide that can be easily manipulated and improved by genetic engineering. The information obtained in this study will be useful in future protein engineering studies on nisin.

Our experimental approach was to compare activity and structure of heated and non heated nisin. After prolonged heating at 121°C and HPLC analysis we discovered an inactive nisin...
degradation product. Only in purified form did it have some activity against *Pedicoccus pentosaceus* FBB-61, but it had no activity against *Bacillus cereus* T bacterial cells or spores. This degradation product was designated nisin L.

The purpose of this study was to find structural differences that had led to the loss of antimicrobial activity in heat inactivated nisin. The structures of nisin and heat inactivated nisin were studied by high performance liquid chromatography (HPLC), ion spray mass spectroscopy (MS), $^1$H nuclear magnetic resonance (NMR) spectroscopy and circular dichroism (CD) spectroscopy.
2.1. Discovery of nisin

De Vuyst and Vandamme (1994) have given a historical account on the discovery of nisin. In 1928 Rogers discovered a diffusible substance in culture broths of *Lactococcus lactis* that was inhibitory to other lactic acid bacteria. Whitehead in 1933 noted the same phenomenon and suggested that this inhibitory substance was a protein. There was not much attention given to this inhibitory phenomenon until in 1944 Matick and Hirsch found that this substance was also active against several pathogenic Gram positive bacteria. In 1947 they called this substance nisin for group N\(^1\) Inhibitory Substance and IN for they considered it an antibiotic (De Vuyst and Vandamme, 1994). There are two natural variants of nisin - nisin A and nisin Z. Nisin Z differs from nisin A in having an asparagine residue instead of histidine at position 27 (Mulders et al., 1991). Nisin Z is widely distributed in populations of *Lactococcus lactis* (de Vos et al., 1993).

---

\(^1\) Dairy Lactococci used to be classified as Streptococci belonging to Lancefield group N.
2.2. Structure and properties

Nisin is the most important representative of lantibiotics-polycyclic bacteriocins that are naturally active against Gram positive and sporogenic bacteria. It is a small, cationic peptide of 34 amino acids. On the basis of its ring structures, it belongs to group A lantibiotics, together with subtilin, Pep 5, epidermin and gallidermin. These are long stretched, cationic, amphiphilic polypeptides (Jung, 1991). The structure of nisin was described by Gross and Morell in 1971 (Fig. 2.1) and its molecular weight was estimated to be 3510 D (Gross and Morell, 1967). Nisin was the first peptide in which it was recognized that lanthionine rings can be formed naturally by posttranslational modification. Prior to this, it was believed that lanthionine rings form as an artifact in hydrolyzates of wool or hair (Shiba et al., 1986). The structure of nisin was confirmed by chemical synthesis (Shiba et al., 1986; Wakamiya et al., 1991). Mass spectroscopy revealed that nisin's molecular weight is 3354 D (Barber et al., 1988). Nisin contains five thioether bridges in lanthionine and \( \beta \)-methylanthionine residues and has unusual residues dehydrobutiric acid (DHB) and dehydroalanine (DHA) (Fig. 2.2). These unusual amino acids are made from serine, threonine and cysteine in a pre-nisin molecule through posttranslational modifications (Jung, 1991). Nisin biosynthesis is inhibited by inhibitors of RNA synthesis (Hurst, 1966; Ingram, 1970).
Fig. 2.1 Structure of nisin. Abu: Aminobutyric acid, dha: dehydroalanine, dhb: dehydrobutyrine. (Shiba, 1986).

Fig. 2.2 Structure of unusual amino acids in nisin.
Ingram followed incorporation of radiolabeled threonine and cysteine into nisin and found that secreted nisin was not radiolabeled. He postulated a scheme by which unusual amino acids are made: serine, threonine and cysteine are first incorporated into a polypeptide chain, then serine and threonine are dehydrated to the dehydroamino acids which condense with cysteine residues to give β-methyllanthionine and lanthionine (Ingram, 1970). This scheme is still valid. Today, it is believed that synthesis occurs at the membrane, with the help of modification enzymes that take part in dehydration of serine and threonine (Hurst, 1978) and formation of thioether bridges (de Vos et al., 1995). Nisin is transported across the membrane after the cleavage of the leader sequence (Hansen, 1991).

Nisin is readily soluble at low pH (57 mg/ml at pH 2), but is sparingly soluble at pH 8 to 12 (0.25 mg/ml). At high pH nisin forms multimers via intermolecular reactions between nucleophilic groups and dehydro residues (Liu and Hansen, 1990). Addition of nucleophiles, thyoglycolate and 2-mercaptoethanesulfonate to nisin, led to the loss of antimicrobial activity and the loss of vinyl resonances of dehydro residues. Products of cyanogen bromide cleavage of nisin were 90% less active against spores of *B. cereus* T (Liu and Hansen, 1990). Nisin A is more soluble than nisin Z at low pH, at pH values greater then 7 solubility decreases by almost two orders of magnitude for both. Optimal stability for nisin A and Z is at pH 3 (Rollema et al., 1995). The solubility of nisin A in nonaqueous environments is not affected by pH, nisin is soluble at
both low and high pH (van den Hooven et al., 1993). Theoretically calculated isoelectric point of nisin A is 11.2.

2.3. Genetics

Nisin genes are organized as an operon (Engelke, 1994). Nisin Z genes are situated on 70 kb conjugative transposon Tn5267 together with genes for sucrose fermentation (Rauch et al., 1991). Genes for production of nisin A are located on the transposon Tn5301 (Dodd et al., 1991). There are eleven open reading frames (ORF) in the nisin operon: nisA coding for a structural gene for pre-nisin, a 57 residue long precursor peptide (Kaletta, 1989), nisB that codes for a membrane associated protein for which it was suggested that it dehydrates serine and threonine to dehydroalanine and dehydrobutyryl (de Vos, 1995). NisC codes for a protein that helps in stereospecific addition of sulfur to dehydroresidues, and in the process they change from L to D conformation (Jack and Sahl, 1995; Sahl et al. 1995). Genes lan B and lan C are unique to lantibiotics and common to all lantibiotic A operons sequenced so far. In Pep5 biosynthesis, Pep C was recognized as crucial to thioether bridge formation and by analogy this role is suggested for all Lan C proteins (Meyer et al., 1995). Nis T codes for ATP dependent transport protein (Engelke, 1992). Product of nisl is a lipoprotein that anchors itself in the membrane of a nisin producer and has a role in its immunity (Engelke, 1994). However, Nis I by itself can not
assure complete immunity (Qiao et al., 1995). Downstream from *nis I* are genes *nisP* involved in cleavage of prenisin, *nisR* important in regulation of nisin biosynthesis (Van der Meer, 1993) and *nisK* coding for a histidine kinase, important in nisin regulation (Engelke, 1994). Adjacent to *nisK* there are three additional open reading frames (ORF): *nisF, nisE* and *nisG* that seem to be involved in immunity to nisin. Downstream from these ORFs, three ORFs with opposite orientation were found, one of which belongs to *sacR* in the sucrose operon. This may indicate that all of the nisin cluster genes in *L. lactis* 6F3 have been identified. The nisin gene cluster in *L. lactis* 6F3 comprises of 15 kb of DNA (Siegers, 1995). The signal for continuing nisin production is mature nisin (Kuipers, as reported by de Vos et al., 1995).

2.4. Applications of nisin

The first applied study of nisin was its application to control butyric blowing in the manufacture of Swiss type cheeses (Hirsch, 1951). In the United Kingdom nisin was first marketed in 1953 (De Vuyst and Vandamme, 1994). In 1969 it was accepted as a legal and safe food additive by FAO/WHO. Today nisin is used as a natural food preservative in many countries around the world (Ray, 1992), with applications varying from baby foods, milk, dairy products and different canned foods (Delves - Broughton, 1990). Nisin can be used to control important food pathogens like: *Clostridium botulinum* in
pasteurized cheese spreads (Somers and Taylor, 1987), *Listeria monocytogenes* (Benkerroum and Sandine, 1988; Monticello and O'Connor, 1990), different *Bacillus* species (Oscroft et al., 1990), *Salmonella* species (Stevens et al., 1991); and it is also active against several mastitis causing pathogens (Broadbent et al., 1989). Nisin can be used in production of brandy (Henning et al., 1985), beer (Ogden, 1988) and wine (Radler, 1990; Daeschel et al., 1991). However, there are some limitations to nisin's usefulness in food systems. Nisin is not very efficient in controlling outgrowth of *C. botulinum* spores in cooked meat (Scott and Taylor, 1981), bacon (Taylor and Somers, 1985), or in model cured meat system (Rayman et al., 1983). Nisin's efficacy to control *L. monocytogenes* in fluid milk was lower in milks with higher fat content (Jung et al., 1992); and also nisin is not readily soluble at neutral pH values (Liu and Hansen, 1990). Sometimes nisin can inhibit dairy starter cultures (Hirsch, 1951; Lipinska, 1977). This problem can be overcome by using beneficial bacterial starters that are resistant to nisin (Lipinska, 1977). Bacterial strains naturally resistant to nisin occur spontaneously, and can be further selected for by exposure to higher nisin concentrations (Ming and Daeschel, 1993). *Bacillus cereus* produces an enzyme, nisinase, a dehydropeptidase that inactivates nisin (Jarvis and Farr, 1971).

In the USA, nisin is recognized as a GRAS substance (Federal register, 1988) and it is approved for use in processed cheese spreads. It is believed that humans have consumed milk and dairy products containing nisin for the last 8000 years (Ray, 1992).
Toxicological studies on nisin have shown that it has no adverse effects on rats (Frazer et al., 1962; Lipinska, 1977). It is not toxic to humans in quantities up to $3.3 \times 10^7$ U/kg body weight (Aplin and Barret, 1988). Additionally, it is degraded by chymotrypsin in the digestive tract and has no adverse effect on gastrointestinal flora (Heinemann and Williams, 1966).

The use of nisin can be extended to more products, as addition of chelating agents, such as EDTA, renders Gram negative bacteria susceptible to it (Blackburn et al., 1989; Stevens et al., 1991). Nisin can be used on food contact surfaces to prevent adhesion of \textit{L. monocytogenes} (Bower and Daeschel, 1995). Potential for use of nisin in new food products, cosmetics, veterinary and human medicine is ever growing (Applied Microbiology, 1993 Annual Report).

2.5. Thermal stability of nisin

It has long been known that nisin remains stable after heating at $121^\circ$C in acidic conditions. Tramer reports that there was no loss of nisin activity after 15 minutes at $121^\circ$C at pH 2, but that there was 40% loss of activity at pH 5 and 90% loss at pH 6.8 (Hurst, 1981). Time for 100 IU of Nisaplin (commercial grade preparation containing 1% nisin) to lose 50% of initial activity ($t_{1/2}$), when heated in an oil bath at $121^\circ$C at pH 2 is 40 minutes, at pH 4 it is 20 minutes and at pH 6 approximately 0.2 min (Dobias, 1983). Retention
of nisin activity after heating for 15 min at 121°C is highly dependable on pH. In buffer pH 3 100% activity was retained, pH 4 - 71%, pH 5 - 35%, pH 6 - 14.5% and at pH 7 only 0.5% of activity was retained. When applied in food systems, retention of nisin activity was higher in foods with lower pH values and lower fat content (Aplin and Barrett, 1988). Residual activity of nisin films adsorbed on silanized silica surfaces when subjected to convective heat at 120°C at pH 7 was 75 and 80% after 15 min for high and low hydrophobic surfaces, respectively. With nisin films treated at pH 3, residual activity was 25 and 20% after 15 min. Activity was completely lost after one hour in both treatments. Higher residual activity at pH 7 was explained in terms of greater adsorbed mass of nisin (Bower, 1994). Activity of nisin Z after one day at 37°C and 75°C was recovered in pH range 2 to 4, with more then 80% activity recovered at pH 3 (Rollema, 1995).

2.6. Structure and function relationships

There have been many studies on the activity of nisin fragments generated by aging, cleavage, digestion and chemical synthesis. The residues most important for activity are: DHA-5 (Hansen, 1991; Rollema et al. 1992; Chan et al. 1986, Kuipers et al. 1992, Wakamiya et al. 1991) and DHB - 2 (Wakamiya et al., 1991). Fragment 1-19 is the minimal structure required for activity (Wakamiya et al.,1991). Residues DHA-33 and Lys-34 are not important for activity (Chan et al.,1986; Lian,1992) or
conformational stability of nisin (Lian, 1992). Presence of dehydroalanine is directly related to nisin’s activity against cells of *Staphylococcus aureus* (Gross and Morrel, 1967).

In protein engineering studies on nisin Z DHA-5 was replaced by DHB, and the resulting molecule had lower activity against vegetative cells. This mutation increased nisin’s chemical stability when exposed to acid; and to 75°C at lower pH (1-4) for 24 hours (Rollema et al., 1995). When Met-17 and Gly-18 in pre-nisin molecule were changed to Gln and Thr, two mutant nisins were produced 90% of which contained DHB at position 18 (as in subtilin), and 10% had a Thr residue. The first nisin mutant had the same activity as the wild type. The second mutant had lower activity against *B. cereus* and *Streptococcus thermophilus* cells then original nisin, but was twice as active against *Micrococcus luteus* (Kuipers et al., 1992). Introduction of lysine residues instead of Asn-27 and His-31 considerably enhances nisin’s Z solubility at neutral pH and does not affect its antimicrobial activity (Rollema et al., 1995).

2.7. Nuclear magnetic resonance (NMR) spectroscopy studies

NMR studies on lantibiotics are numerous and employ an array of different experimental approaches. One dimensional proton NMR studies can be used for a quick assessment of vinyl protons of dehydro residues (Hansen, 1993; Mulders et al., 1991) while
assignment of crowded and complex spectra in the region from 0-5 ppm requires two dimensional analysis. Studies on nisin have been conducted in different environments: water (Slijper et al., 1989; van de Ven et al., 1991; Lian, 1992), sodium phosphate buffer (Chan et al., 1989), DMSO-d₆ (Palmer et al., 1989, Goodman et al., 1991) and in nonaqueous environments (van den Hooven et al., 1993).

Nisin in aqueous environments does not have defined secondary structure elements and its conformation is largely defined by thioether bridges (Slijper et al., 1989). The N-terminus is well constrained, Pro-9 points in the direction of ring A, and side chains of Met-17 and 21 face each other (Slijper et al., 1989). Similarly, Lian (1992) reports that nisin is a very flexible molecule, and that there is no preferred overall conformation for nisin in water. Nisin has two domains: 3-19 and 23-28 that are connected by a "hinge" region around Met-21. The molecule has an amphiphilic N-terminus. (van de Ven et al., 1991).

Study of synthetic nisin fragments and nisin in DMSO-d₆ revealed a kinked rod like conformation. Nisin's dimensions are given as 5 nm long and 2 nm wide. The molecule has a dipole moment bigger then 50 Debyes (Goodman et al., 1991). The study of synthetic fragments of rings A and B showed that these are rigid structures, that ring B has a type II β-turn, and that model of ring A with D alanines is more flexible then the model constructed with L alanines (Palmer et al., 1989).

Assignments for proton NMR spectra were confirmed with NMR resonances for nisin A that was labeled with ¹⁵N and ¹³C. In ¹⁵N and
two dimensional NMR studies chemical shifts for carbon and nitrogen atoms were identified (Sailer et al., 1993).

NMR studies of nisin in nonaqueous environments designed to mimic membrane conditions, i.e. dodecylphosphocholine (DPC), sodium dodecyl sulfate (SDS) micelles and trifluoroethanol (TFE) in water, revealed that the conformation differs from the one in water, and that the major conformational change occurs at the amino terminus (van den Hooven et al., 1993). However, this change is not dramatic and does not affect the overall folding of the molecule. The circular dichroism (CD) spectra of nisin in these environments also showed differences with respect to aqueous spectra, but no attempt was made to deduce common secondary structures from the spectra (van den Hooven et al., 1993). In CD and H NMR studies on Pep5, a group A lantibiotic of 34 amino acids and three sulfide rings, it was found that in water it has a flexible structure. Titration from water to TFE, revealed two state random-coil to helix transition. In TFE, an α helical stretch was observed for the linear region from Gln-14 to Phe-23 (Freund et al., 1991).

2.8. Mode of action of nisin

Nisin is active against bacterial vegetative cells and spores, apparently by two different mechanisms. The two mechanisms complement each other.
2.8.1. Action against bacterial vegetative cells

The primary target for nisin's antimicrobial action is the cytoplasmatic membrane. This has been demonstrated by studies on artificial liposomes (Ruhr and Sahl, 1985; Kordel et al., 1989; Gao et al., 1991; Garcia et al., 1993); on liposomes made from nisin sensitive cells (Ruhr and Sahl, 1985; Henning et al., 1986) and by studies on effects of nisin on cells of L. monocytogenes (Abee et al., 1994; Winkowski et al., 1994).

Nisin is naturally active against Gram positive bacteria. Gram negative bacteria are protected from nisin's action by the lipopolysaccharide membrane. When this outer membrane is disrupted by osmotic shock (Kordel and Sahl, 1986); chelating agents (Blackburn et al., 1989; Stevens et al., 1991) or as a result of a mutation (Stevens et al., 1992), the cell wall is exposed and permeable to nisin which lysis the inner membrane. Lysis is a result of pore formation, through which ions and small intracellular molecules are lost. It is proposed that the pore could be 1 nm in diameter for black lipid membranes\(^2\), with a lifetime of milliseconds to seconds (Benz et al., 1991). The loss of ions leads to dissipation of proton motive force (PMF) which in turn leads to greater intracellular ATP hydrolysis (in cell's futile attempt to regenerate the proton gradient (Winkowski et al., 1994)); this and

\(^2\) A solution of lipids is applied to the hole in Teflon foil; when this "membrane" turns black, it means that the bilayer has been formed.
the leakage of ATP lead to energy depletion which together with disrupted transport across the membrane disables all major cellular processes. The leakage of ATP lead to energy depletion which together with disrupted transport across the membrane disables all major cellular processes. The dissipation of PMF is a common mechanism of action for many bacteriocins and antimicrobial proteins (Montville and Bruno, 1994).

There is not much specificity in nisin action, and it is not likely that a specific receptor is involved. Phospholipid composition in artificial membranes is important (Abee et al., 1991); anionic liposomes are inhibitory to nisin action (Garcera et al., 1993); nisin makes pores in zwitterionic phosphatidylcholine (PC) liposomes, but not in anionic phosphatidylglycerol (PG) liposomes (Driessen et al., 1995). Negatively charged phospholipids in the membrane electrostatically attract cationic nisin and this association disturbs the lipid dynamics on lipid-water interface. Nisin immobilizes some of PG, which can be inferred from the signal of the $^{31}$P NMR. This does not happen with PC liposomes (Driessen et al., 1995). Membrane fluidity is important for nisin action. Spontaneous nisin resistant L. monocytogenes Scott A mutants had more linear fatty acids in the membrane than the wild type strain and consequently lower membrane fluidity (Ming and Daeschel, 1993; 1995).

Nisin is more active against energized cells (actively growing, or energized with glucose); it requires a membrane potential (inside negative) of 100 mV at neutral pH, and is more active at slightly
acetic pH (Sahl, 1991). In liposomes made from *E. coli* lipids or dioleoylglycerophosphocholine (DOPC), nisin induced efflux of carboxyfluorescein at low nisin/lipid ratios in the absence of membrane potential. However, the effect was enhanced in the presence of membrane potential. For *E. coli* liposomes, a saturation in efflux of carboxyfluorescein is reached at 5 μg of nisin/mg of lipid (Garcera et al., 1993). Nisin dissipated the membrane potential in liposomes made of *E. coli* PE and egg PC and inhibited oxygen consumption by Cyt c oxidase in proteoliposomes (Gao et al., 1991).

There are three models proposed on how nisin disrupts the cytoplasmatic membrane: 1.) Detergent-like action where the membrane would be disrupted at many sites simultaneously (Ramseier, 1960, as reported by Ruhr and Sahl, 1985). 2.) Pore formation by "barrel-stave" model, where after initial aggregation of nisin molecules at the membrane and membrane energization, the aggregates are drawn into the membrane in such a way that their hydrophobic surface is exposed to the core of the membrane while their hydrophilic side forms the aqueous channel. The repulsion of parallel oriented dipoles, with positive charges at the inner surface of the pore, would open the pores, but also account for their instability (Sahl, 1991). 3.) Pore formation by "wedge" model where electrostatic forces contribute to nisin’s association at the membrane, where the membrane potential plunges the molecules into the membrane, but in this model the polar head groups of PGs are following the molecules and take part in the formation of the pore (Driessen et al., 1995).
In all models, a common observation is that more than one molecule of nisin is needed to form a pore.

That nisin indeed inserts into the membrane has been demonstrated by fluorescence studies on genetically engineered mutant that had Ile-30 changed to Trp. In contact with liposomes, Trp-30 showed a blue shift in emission wavelength maximum, indicating that it had entered a more hydrophobic environment (Giffard et al., 1995).

Nisin Z has a similar mode of action to nisin A, but has faster diffusion properties in agar than nisin A (de Vos et al., 1993). In studies of mode of action of nisin Z against *L. monocytogenes* Scott A, grown at high and low temperatures, it was noted that when cells were grown at 30°C, the action of nisin Z was prevented below 7°C, but that cells grown at 4°C were still sensitive to nisin Z at low temperatures. This effect is probably due to decreased membrane fluidity at lower temperatures. The nature of the pores was the same at both temperatures (Abee et al., 1994).

In addition to forming pores in the membrane, nisin and Pep 5 induce autolysis in *Staphylococcus simulans* 22 (Bierbaum and Sahl, 1987). Nisin is not active against mycoplasma or eukaryotic cells (Sahl, 1991). Against fungi it is active only when applied in very high concentrations (Henning et al., 1986). In high concentrations (250 - 500 mg/kg body weight), nisin was found to be active against malarial parasites in mice (Gross and Morell, 1967).
2.8.2. Action against bacterial spores

Nisin is sporostatic. It inhibits outgrowth of the spore to a vegetative cell. (Campbell and Sniff, 1959). Heat shocked spores in presence of nisin go through all the stages of germination with accompanying swelling and loss of refractivity that can be followed by phase contrast microscopy, but the spore coat never ruptures and the vegetative cell never emerges (Hitchins et al., 1963).

It has been demonstrated that 0.1 μM nisin inhibits 0.25 mg/ml of germinating B. cereus T spores via interaction of dehydroresidues with sulfhydryl groups in the spore wall (Morris et al., 1984). The sulfhydryl groups could also interact with sulfhydryl containing enzymes, glutathione or coenzyme A (Gross and Morell, 1967). When dehydroalanine 5 was modified, the sporostatic activity of nisin was lost (Liu and Hansen, 1990). Further evidence to this point was provided when subtilin’s DHA 5 was mutated to alanine resulting in the loss of sporostatic activity. The activity of subtilin against vegetative cells was still intact, suggesting that different mechanisms of action are employed against cells and spores (Liu and Hansen, 1993). The presence of dehydroalanines was found important for activity against cells (Gross and Morell, 1967), but in this study other possible modifications to the molecule have not been excluded.

Nisin has been found to inhibit the murein synthesis by forming complexes with undecaprenylpyrophosphate activated intermediates
Interference with the formation of cell wall components contributes to the inhibition of spore outgrowth.

2.9. References


Barber, M., Elliot, G. J., Bordoli, R. S., Green, B. N. and Bycroft, B. W. 1988. Conformation of the structure of nisin and its major degradation product by FAB-MS and FAB-MS/MS. *Experientia* 44: 266-270.


CHAPTER 3.

STRUCTURAL ANALYSIS OF THERMALLY INACTIVATED NISIN.*

Tamara Musafija-Jeknic, Steven J. Gould and Mark A. Daeschel

*This chapter will be submitted to *Applied and Environmental Microbiology* for consideration as a research manuscript.
3.1. Abstract

Heat stability of the lantibiotic peptide nisin, a natural food preservative, was studied using differential scanning calorimetry and heating at 121°C. Temperature of the thermal transition for nisin solution at pH 4 was 63°C, and for solid nisin it was 150°C. It was found that nisin powder and concentrated nisin solutions (10 and 20 mg/ml) still retained antimicrobial activity after having been heated to 190°C and 230°C, respectively. However, when nisin solutions were held at high temperatures for 15, 30 and 60 minutes; no antimicrobial activity was found. Nisin still remained active after seven hours of heating at 121°C at pH 4. The extent of nisin degradation after prolonged heat treatment was evaluated by reverse phase HPLC and activity assays of isolated fractions. A nisin degradation product with greatly reduced antimicrobial activity was isolated after 3.5 hours of treatment at 121°C. The heat altered nisin, designated nisin L, had the same molecular mass as native nisin, but different ¹H NMR and CD spectra. In contrast to native nisin, nisin L was less active against *Pediococcus pentosaceus* FBB-61 in agar well diffusion assays and could not inhibit spore outgrowth or growth of vegetative cells of *Bacillus cereus* T. Nisin L had lost the dehydrobutyrine-2 vinyl resonance, and had reduced resonances for the dehydroalanine-5 residue. As judged by changes in one dimensional ¹H NMR spectra, most of the structural changes occurred in the region 3.5 - 4 ppm where hydrogens of the β-carbons of residues involved in the lanthionine bridges were. Analysis of
circular dichroism spectra for nisin in a hydrophobic environment showed 24% antiparallel, 8% parallel \( \beta \)-sheet, 25% turns, 14% \( \alpha \) helix and 30% other secondary structures. Nisin L had a smaller content of \( \alpha \) helix and more antiparallel \( \beta \)-sheet.

3.2. Introduction

Nisin is a bacteriocin produced by many strains of *Lactococcus lactis* ssp.*lactis*, a common dairy starter bacterium. Nisin, in its secreted form, is a peptide of 34 residues, containing the unusual amino acids dehydroalanine and dehydrobutyrine as well as five thioether bridges (13). It is the most studied and most prominent representative of lantibiotics and belongs to group A lantibiotics together with PEP 5, epidermin and subtilin (17). There are two naturally occurring nisin variants, nisin A that has a histidine at position 27 and nisin Z that instead of histidine has an asparagine at the same position (22).

Nisin has been used in food industry as a natural preservative several decades (7). It is active against many Gram positive bacteria and their spores; and it is active against Gram negative bacteria if their outer membranes are compromised (3, 25).

Heat stability of nisin is dependent on pH. Tramer reported that there was no loss of nisin activity after 15 minutes at 121°C at pH 2, but that there was 40% loss of activity at pH 5 and 90% loss at pH 6.8 (16). The time for 100 IU of Nisaplin (commercial preparation
containing 1% active nisin) to lose 50% of initial activity (t 1/2), when heated in an oil bath at 121°C, at pH 2 was 40 minutes, at pH 4 it was 20 minutes and at pH 6 was approximately 0.2 min. When heated at 121°C for two hours in buffer pH 2, nisin retained 23% initial activity, but only 8% in buffer pH 4 (8). When applied in food systems, retention of nisin activity was higher in foods with lower pH values and lower fat content (2). Nisin is a very heat stable protein, and as such very interesting in terms of application in thermally processed foods.

In this study, we exposed nisin to increasing temperatures to evaluate the upper limits of its heat stability. More importantly, we tried to elucidate structural and conformational changes to the molecule upon heating and to correlate these changes with its antimicrobial activity. Structural changes were assessed by comparing the structure of nisin that had lost a substantial amount of activity as a result of heating, designated nisin L, with the structure of native nisin. Four different methods of structural analysis were used: high performance liquid chromatography, ion spray mass spectroscopy, one dimensional proton nuclear magnetic resonance spectroscopy and circular dichroism spectroscopy. The antimicrobial activities of native and heat altered nisin against bacterial vegetative cells and spores were compared.

The premise behind this study was to find those portions of nisin structure that are affected most by heat and to evaluate what effect these changes have on antimicrobial activity, so that in the
future, by modes of protein engineering, these structures could be made more heat stable.

3.3. Materials and methods

Nisin with inhibitory activity of 50 X 10^6 IU/g, lot number NP 72, was obtained from Aplin and Barret, UK.

Heat treatment at 121°C Ten ml of solubilized nisin (2.6 mg/ml) in citrate phosphate buffer (CPH) pH 4 (0.01M C₆H₈O₇H₂O, 0.02M Na₂HPO₄, 0.045M NaCl) was aliquoted into vials and heated at 121°C from 0.5 to 7 hours. Duplicate samples were taken and analyzed for antimicrobial activity and for the extent of nisin degradation by reverse phase HPLC. In a separate experiment, under the same conditions nisin samples were removed after 2.25 and 3.5 h of heating and analyzed. Furthermore, nisin was heated in CPH buffer pH 6 for 3.5 h. Unheated nisin of the same concentration was used as a positive control, while heated buffer was a negative control.

Treatment of nisin at high temperatures Nisin solutions in CPH buffer - 60 μl of 10 mg/ml in pH 4 and 5 mg/ml at pH 6 were heated to 200° and 250°C using a pressurized differential scanning calorimetry (DSC) cell (Du Pont 910) with 400 psi pressure and held at these temperatures for 15, 30 and 60 minutes. The heating rate was 10°C/min. DSC was calibrated using water and indium. The runs were analyzed using thermal analysis program TA 4.0 (Du Pont). Experiments were done in duplicate. Large volume stainless steel capsules (Perkin Elmer) with 60 μl of buffer were used as a
reference. After heat treatment, capsules were opened and 25 µl of each sample was assayed for activity in agar wells; an additional 10 µl was applied to antibiotic discs and assayed.

Solid nisin was analyzed under atmospheric pressure in sealed aluminum sample pans (Du Pont), using an empty pan as a reference. Heating rate was 10°C/min and samples were heated up to 230°C. After heating, the pans were cooled and opened, the contents were dissolved in 10 µl 0.02N HCl, pH 2. Antibiotic paper disks were placed in the pan until the solution was absorbed, and then placed on MRS (Difco, Detroit, MI) agar plates with freshly spread culture of *Pediococcus pentosaceus* FBB-61.

In a dry heating oven, 1 ml of nisin samples concentration 5 mg/ml in 0.02 N HCl, 0.08M NaCl, pH2, were heated in glass vials up to 180, 190, 200, 210, 220 and 230°C and taken out as soon as their respective temperatures were reached. After cooling the residue was dissolved in the starting volume of the same solvent and 50 µl of each sample were assayed for activity.

**Antimicrobial activity** Antimicrobial activity of nisin was measured by agar well diffusion assay on MRS nutrient medium plates inoculated to a final volume of 0.1% with a freshly grown overnight culture of *Pediococcus pentosaceus* FBB-61. Fifty microliters of sample were applied per well. After overnight incubation at 37°C, the inhibition zones were measured and compared to inhibition zones of nisin standards (6). Activities of original nisin (O), heat inactivated nisin (L) and active nisin recovered after the heat treatment (N) were also compared in terms
of arbitrary units, where a series of twofold dilutions in water acidified with 1M HCl, pH 2, and water was assayed by agar well diffusion method against *Pediococcus pentosaceus* FBB-61. Minimal inhibitory concentration (MIC) were measured against vegetative cells of *Bacillus cereus* T in tryptic soy broth (TSB) (Difco, Detroit, MI) by measuring OD at 600 nm after overnight incubation at 37°C. Experiments were done in two replicates.

**Preparation of spores** Spores of *B. cereus* T were a generous gift of Dr. J. N. Hansen. They were grown in TSB broth, a single colony was isolated, propagated in TSB and then grown on fortified nutrient agar plates for two days at 30°C, and stored one day at 4°C. The spores were then harvested from the plates, centrifuged at 4°C several times until no vegetative cells were observed under microscope, diluted in sterile cold water and kept refrigerated until needed. This method was developed by K. Johnson from the University of Minnesota and communicated to us by P. M. Foegeding from the North Carolina State University.

**Measurement of sporostatic activity** Spores of *B. cereus* T were diluted in 5 ml of TSB to give final concentration of 1000 spores/ml and heat shocked for 15 min at 75°C. Original nisin was added at concentration of 100 and 1000 IU, and the same dilution (mg/ml) was used for nisin L. After an initial OD 600 nm measurement, absorbance was monitored over a 20 hour period. Two replicates were done for each treatment.

**HPLC separation** Analytical HPLC was performed on 20 μl samples using a Zorbax (Du Pont) reverse phase C-18 protein plus column (4.6
x 250 mm; pore size 80 Å) and isocratic elution with 35% acetonitrile, 0.1% TFA. The flow rate was 1 ml/min, supplied by an LC-6A pump (Shimadzu). Absorbance was monitored at 222 nm using a Beckman 163 variable wavelength detector. The chromatograms were recorded on a Beckman 427 integrator.

**Semipreparative liquid chromatography** In order to purify large quantities of heat inactivated nisin, nisin preparations of 2.6 mg/ml (0.01M citrate, 0.01M Na citrate and 0.075M NaCl) and 20 mg/ml in citrate buffer pH 4, heated for 3.5 h at 121°C, were purified by semipreparative LC. Repeated injections of 500 μl of heated nisin samples were applied on a Lobar LiChroprep RP -18 column (Merck) dimensions 10 x 240 mm; particle size 40-63 μm; and eluted isocratically at 34% aqueous acetonitrile, 0.1% TFA; or isocratically with gradually increasing acetonitrile concentration from 30 to 55%. The flow rate was 1 and 2 ml/min, respectively. The system used for HPLC analysis was used in this purification.

**Preparation of samples for MS analysis** Fractions (1 ml) were collected after HPLC separation of 200 μl of heated sample and repurified on HPLC. Fractions corresponding to pure peaks (1-1.5 ml) were concentrated in Centricon microconcentrators with 3000 MW cut off membrane (Amicon) to a volume of 130-150 μl, and further concentrated in a Speed Vac concentrator to 10-15 μl. Fractions collected after semipreparative LC separation (20 ml) were dried in a rotoevaporator, taken up in H₂O, lyophilized and resuspended in 200 μl H₂O. Five μl of each fraction were analyzed on a PE SCIEX API III Biomolecular Mass Analyzer (Perkin Elmer) by ion spray mass
spectroscopy. Samples were scanned in positive ion mode every three seconds in the mass to charge ratio range from 200 to 1800. Peaks with sufficient intensity were reinjected and analyzed by tandem MS; peaks with m/z 1119 and 1125 served as parent ions. The collision gas used was argon with 10% nitrogen. Water containing 0.1% TFA was mobile phase at a flow rate of 0.3 ml/h, supplied by Harvard syringe pump (Harvard Aparatus).

**Preparation of samples for NMR spectroscopy.** Nisin at 20 mg/ml was dissolved in citrate buffer (0.01M citrate and 0.01M Na citrate, pH 4) and heated at 121°C for 3.5 hours. Individual compounds were separated by semipreparative LC and fractions assayed for activity. Fractions of interest (15 - 20 ml) were completely dried under vacuum evaporation and taken up in D$_2$O, pH 7. Fractions from several runs were combined and lyophilized overnight, which yielded 23.3 mg of nisin L (reduced activity peak) and 29.1 mg of nisin N (recovered nisin peak). A sample of original nisin (20 mg) in D$_2$O pH 7, was prepared and designated nisin O.

**NMR spectroscopy** Spectra were obtained on a Bruker AM 400 spectrometer at 298 K. Samples (10-13 mg) were dissolved in D$_2$O pH 7. The data were obtained in 128 scans, with an acquisition time of 1.36 sec and a 2 sec relaxation delay time. The sweep width was 6024 Hz per scan, the pulse angle was 33°, and there were 16 K data points per spectrum.

**Trypsin digestion of nisin and nisin L** Nisin samples were dissolved in 30 mM sodium acetate, 5 mM TRIS, 5 mM CaCl$_2$, pH 7.55 (adjusted with HCl), at a concentration of 1 mg/ml. Trypsin (SIGMA)
was added in the same buffer at a final concentration of 0.08 mg/ml, and the reaction was carried on at 37°C for 17 hours, and then stopped by addition of 1-2 µl of 1 M HCl. Five µl of each digestion and of controls (without trypsin) were analyzed by RP liquid chromatography interfaced with mass spectroscopy (LCMS) and two digested fragments were analysed by LCMS followed by MS of m/z 748 and 596 parent ions. Conditions for LC were: RP C-18 HPLC column, dimensions 1 mm by 250 mm, with 5 µm particle size (Vydac, Hesperia, California). Gradient of 10 to 100% acetonitrile, 0.1% TFA in water, in 30 min followed by 5 min at 100% acetonitrile and from 40% to 100% acetonitrile, 0.1% TFA in water, in 20 min, with flow rate of 40 µl/min, was supplied by dual syringe solvent delivery system, Applied Biosystems 140B.

**CD spectroscopy** Native nisin was dissolved at 0.1 mg/ml in water, 50% and 90% trifluorethanol (TFE) (SIGMA) in water. The CD spectra were recorded from 184 to 260 nm in 0.1 and 0.02 cm cells with a Jasco J-720 spectropolarimeter. Samples of nisins O, L and N were recorded in 90% TFE. Each spectrum was an average of four runs from which the background spectrum of buffer was subtracted. Before running the CD spectra, an absorbance spectrum from 300 to 180 nm was recorded on a Carry spectrometer, to confirm that protein concentration was in the optimal range for use on the spectropolarimeter. The CD spectra were analyzed for five common secondary structures by variable selection program that uses singular value decomposition analysis to deduce secondary structures using a database of 33 reference proteins with known
secondary structures (5, 21). This program was developed in the laboratory of Dr C. W. Johnson, Department of Biochemistry and Biophysics, Oregon State University. Only the spectra obtained in 0.02 cm cell were used in this analysis.

3.4. Results

**Differential scanning calorimetry** The average denaturation temperature for solid nisin was 150°C, with an enthalpy value of 22 J/g, and for nisin in solution it was 63°C. Dilute solutions (0.1 mg/ml) and larger volumes gave more reproducible results than very concentrated solutions (5 to 20 mg/ml) (data not shown). Nisin was found to be readily soluble at 5 mg/ml in buffer pH 6.

**Nisin treatment at high temperatures** Nisin at high concentrations (5 mg/ml) was found to retain some antimicrobial activity after having been heated to 230°C. However, when held at either 200 or 250°C for 15 - 60 minutes at either pH 4 or 6, it was no longer active. Solid nisin retained activity up to 190°C.

**Isolation of a novel nisin degradation product** After heating at 121°C, samples of nisin were analyzed by analytical HPLC. Two distinct peaks were seen, one with retention time (RT) around 5 to 6 min and second one at RT of 9 to 10 min. Fractions collected from the first peak, after HPLC separation, did not have antimicrobial activity, while the fractions from the second peak were still active. Over time, the area of the first peak increased, suggesting formation
of a stable degradation product. (Fig. 3.2). The area of the second peak decreased with concurrent loss of antimicrobial activity (Table 3.1, Fig. 3.1). A mixture of equal amounts of heated and native nisin was analyzed by HPLC. Only the area of the second peak increased indicating that nisin coeluted with the second peak. Further conformation that peak 2 represented recovered nisin was provided by \(^1\)H NMR and MS. We prepared large quantities of the two peaks by semipreparative RP LC. Peak 1 represented nisin's major thermal degradation product and was designated nisin L (Fig. 3.3). When heated at pH 6 for 3.5 hours nisin lost all the activity. HPLC analysis of this preparation did not reveal any distinct peaks (Fig. 3.2)

Table 3.1. Activity of nisin after prolonged heat treatment at 121°C and areas of HPLC chromatographic peaks. Areas of peaks are given as percentages of the total area of respective chromatograms.

<table>
<thead>
<tr>
<th>hours</th>
<th>activity (IU)</th>
<th>% peak 1</th>
<th>% peak 2</th>
<th>ratio 2/1</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>800</td>
<td>0</td>
<td>87</td>
<td>87*</td>
</tr>
<tr>
<td>0.5</td>
<td>266</td>
<td>10</td>
<td>64</td>
<td>6.4</td>
</tr>
<tr>
<td>2.25</td>
<td>100</td>
<td>20.5</td>
<td>34</td>
<td>1.66</td>
</tr>
<tr>
<td>3.5</td>
<td>38.5</td>
<td>34</td>
<td>25</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>17.5</td>
<td>31.5</td>
<td>20</td>
<td>0.64</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>39</td>
<td>18</td>
<td>0.46</td>
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<td>7</td>
<td>5</td>
<td>43</td>
<td>5</td>
<td>0.12</td>
</tr>
</tbody>
</table>
Fig. 3.1. Activity of nisin after prolonged heat treatment at 121°C. Note that activity decreases as the ratio of areas of active over nonactive peak decreases.
Fig. 3.2 HPLC of nisin after heat treatment at 121°C. Note the increase in the area of the first peak with longer heating. Active peak marked with an arrow. a.) not treated; b.) 30 min; c.) 3.5 hours; d.) 6 hours; e.) 3.5 hours in buffer pH 6. Samples were prepared in CPH buffer, pH 4 at concentration 2.6 mg/ml. Analyzed 20 ul of sample, isocratic elution with 35% CH₃CN, 0.1% TFA in water.
Fig. 3.2 HPLC of nisin after heat treatment at 121°C.
Fig. 3.3. Activity of fractions collected after semipreparative LC of nisin (10 mg/ml, pH 4) heated at 121°C for 3.5 hours. Note the difference in the sizes of inhibition zones of fractions 3 and 4, corresponding to the first peak (nisin L), when compared to later eluting fractions of active peak (nisin N). Applied 50 μl of fractions per well.

**Mass spectroscopy analysis** Nisin L, nisin N and original nisin had similar distribution of ions. All had peaks of average mass of 3354 D plus a peak 18 amu larger that was interpreted as a water molecule addition. Nisin L had three more peaks with additions of 18 amu. In spectra of nisin O and N, hydrated peaks were of lower intensity then in nisin L. (Fig. 3.4). Tandem mass spectroscopy
of triply charged ions, m/z 1118 and m/z 1124, showed similar fragmentation patterns for nisin L and nisin. The ion with m/z 1030 of nisin L was used in tandem mass spectroscopy, but gave spectra of low intensity and its fragmentation did not reveal any additional structural information (data not shown).

Molecular weights of fragments of nisin L and nisin, after trypsin digestion, and structural assignments are given in table 2.

Table 2. Masses deduced from LCMS of tryptic digests of nisin and nisin L.

<table>
<thead>
<tr>
<th>expected fragments</th>
<th>nisin O digest</th>
<th>nisin L digest</th>
<th>structural assignment</th>
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<tr>
<td>935.49</td>
<td>1007.45</td>
<td>1036.59</td>
<td>Ala 3-Lys 12</td>
</tr>
<tr>
<td>1150.58</td>
<td>1150.7</td>
<td>1150.09</td>
<td>Lys 13-Lys 22</td>
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<tr>
<td>1232.57</td>
<td>1232.28</td>
<td>1194.65</td>
<td>Dhb 2- Lys 12</td>
</tr>
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<td>1491.5</td>
<td>1492.18</td>
<td>1495.69</td>
<td>not assigned</td>
</tr>
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<td>1495.69</td>
<td>1495</td>
<td>1495</td>
<td>not assigned</td>
</tr>
<tr>
<td>1510.08</td>
<td></td>
<td>1510.08</td>
<td>not assigned</td>
</tr>
<tr>
<td>1925</td>
<td></td>
<td>1925</td>
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</tr>
<tr>
<td>2139</td>
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<td>2221.78</td>
<td>2221.78</td>
<td>Abu 13-Lys 34</td>
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<td>nisin (1-31)</td>
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<tr>
<td>3354.47</td>
<td></td>
<td>3354.47</td>
<td>nisin</td>
</tr>
</tbody>
</table>
Fig. 3.4. Detail of mass spectra of fractions eluted from nisin L peak (a.) and nisin N peak (b.). Ion with m/z of 839.6 is four times charged molecular ion, [M+4H]^{4+}, of nisin (3354 D), followed by ions with m/z of 844, 848, etc, representing hydrated forms of nisin. Analyzed 5 µl of each fraction.
One dimensional 1H NMR spectra

The 400 MHz 1H NMR spectra of nisin L and nisin O were obtained at pH 7 and 25°C. The spectra were compared and a number of differences were found:

The resonance for the vinyl hydrogen of dehydrobutyryne-2 resonance was missing (1). The intensities of the resonance for the hydrogens of dehydroalanine-5 were much smaller (2). Furthermore, new resonances were observed in the 3.55 to 3.85 ppm region, indicating new aliphatic hydrogens on carbons directly attached to heteroatoms (3). In the case of nisin, this is the range where hydrogen resonances of beta carbon atoms of lanthionine residues are located. A new resonance appeared at 2.5 ppm (4), and a new doublet appeared at 1.55 ppm (6). The doublet resonance at 1.9 ppm has been changed (5), indicating different magnetic environment for Ile1. The doublet resonance at 1.13 ppm has been altered (7) (Fig 3.5 and Fig 3.6).
Fig. 3.5. One dimensional $^1$H NMR spectra of nisin. The peak at 4.85 ppm is water.
Fig. 3.6. One dimensional $^1$H NMR spectra of nisin L. Important differences with respect to nisin spectrum are marked. The small new peak at 2 ppm is acetonitrile impurity. The peak at 4.85 ppm is water.
Activity against vegetative cells and bacterial spores
Nisin L had lower activity than nisin in agar well diffusion assays (Fig 3.7). Its activity in arbitrary units against *Pediococcus pentosaceus* FBB 61 was $16 \times 10^6$ AU/g and original nisin had $36 \times 10^6$ AU/g. in deionized water, pH 7 (results are average from three separate experiments).

![Zone sizes in agar well diffusion assay](image)

Fig. 3.7. Comparison of zone sizes in agar well diffusion assay. Dilutions were prepared in acidified water, pH 2, applied 50 µl of sample per well.

Nisin L was not able to inhibit growth of vegetative cells of *Bacillus cereus* T when applied at the very high concentration of 500 IU/ml, while original nisin reduced the growth of cells at a concentration of 50 IU/ml and completely inhibited the growth at a concentration of 75 IU/ml (Fig 3.9).
Fig. 3.8. Overnight growth of *B. cereus* T vegetative cells with different concentrations of original nisin and nisin L.
Nisin L could not inhibit the outgrowth of *B. cereus* T spores at concentration of 100 IU/ml. However, when applied at 1000 IU/ml it delayed their outgrowth for 4 to 5 hours but was not able to inhibit it. Original nisin was active at both concentrations (Fig 3.9).

Fig. 3.9. Outgrowth of *B. cereus* T spores in presence of nisin and nisin L.
**CD spectroscopy** CD spectra of nisin were obtained in solvents of different polarities: 0, 50 and 90% TFE in water. The CD spectrum of nisin L was obtained in 90% TFE and compared with the spectrum of nisin in the same solvent. Nisin in water has more of a random coil structure than nisin in hydrophobic environment (Fig. 3.10). The spectra were analyzed by program Varslc1 that uses singular value decomposition analysis to deduce secondary structures using a database of 33 proteins with known secondary structures. The results are given in Table 3. and Fig. 3.11.

Table 3.3. Percentages of common secondary structures of nisin in solvents of different hydrophobicity and structure of nisin L.

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<td>8</td>
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<td>30</td>
<td>24</td>
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<td>25</td>
<td>20</td>
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<td>31</td>
<td>30</td>
</tr>
<tr>
<td>total</td>
<td>100</td>
<td>100</td>
<td>102</td>
<td>101</td>
</tr>
</tbody>
</table>
Fig. 3.10. Secondary structures of nisin in solvents of increasing hydrophobicity.

Fig. 3.11. Secondary structures of nisin, nisin L and nisin N in 90% TFE.
3.5 Discussion

For a protein produced by a mesophilic bacterium, nisin is unusually heat stable. After seven hours of heat treatment at 121°C, it was still active. However, the denaturation temperature of nisin in solution at pH 4 was 63°C, while for solid nisin it was 150°C. The difference in denaturation temperature for aqueous and solid forms is common for many biological molecules. In general, hydrophobic proteins have been found to be more thermally stable (1), and in the case of nisin, the thioether bridges further contribute to its thermal stability (14).

Our results confirm and stretch the boundaries of nisin’s remarkable heat stability. HPLC analysis revealed a new peak that accumulated with prolonged heat treatment at 121°C, and this suggested a formation of a specific stable degradation product. This peak was designated nisin L and isolated by preparative LC. In its purified form, nisin L, does not have antimicrobial activity against vegetative cells or spores of *Bacillus cereus* T, although it was active at reduced potency against *Pediococcus pentosaceus* FBB-61. Different sensitivity of indicator strains is an established phenomenon. We assayed activity against spores and cells of *B. cereus* T, as it is believed that different mechanisms are responsible for action against spores and vegetative cells. In nisin L, both activities were affected.
Initially, nisin solutions were prepared in buffer containing sodium phosphate and NaCl. Later, when using MS techniques that were sensitive to these ions, only citrate buffer was used. The formation of the product was not affected by lowering the ionic strength of the buffer. The fact that nisin L eluted earlier than nisin from a reverse phase column suggested that it is a more polar compound, consistent with the finding that nisin L was more hydrated. Trypsin digestion of nisin O and nisin L for 17 hours, yielded a variety of HPLC fragments. Digestion of nisin O had yielded fragments of nisin (3354 D) and nisin 1-31 (3058 D), but was not complete, as there were still some undigested peptides left. Nisin L was completely digested and yielded four fragments in common with nisin O. Trypsin cuts at carboxyl side of lysine or arginine residues and in case of nisin it should cut at two sites - lysine 12 and lysine 22. Analysis software for LC/MS systems, Bio ToolBox (PE Sciex), was used to estimate average molecular weight of fragments after trypsin digestion. Digestion pattern of nisin O has all the predicted fragments except for a fragment 13 - 22 (β methyllanthione’s aminobutyric acid 13 to lysine 22). This fragment lies between lanthionine bridges and is not easily accessible by trypsin. Nisin L had only one fragment that would be “theoretically” expected. Both nisin O and L had fragments of molecular weight 1195, 1492, 1495 that were not assigned. The fact that there was no undigested nisin L suggests that it didn’t have the conformational restraints of native nisin; and as such was a more accessible substrate. Absence
of expected fragments in nisin L could indicate a change in vicinity of or at lysine residues.

Ion spray mass spectroscopy indicated that there was no change in molecular weight, but that nisin L had a higher percentage of hydrated nisin forms, with more then one water addition (Fig 3.4). Tandem MS revealed similar fragmentation patterns for triply charged parent ions, [M+3H]^{3+}, 1119 and 1125. The molecular weight was averaged at 3354 D, and all samples had an additional smaller peak of 3372 D that was interpreted as the hydrate. In nisin it was noted earlier that molecular ion 3354, is accompanied by ion 3372, and the authors interpreted this as having one of the dehydro residues in hydrated form (23). In our case, the water molecule has not been added to any of the dehydro residues in the nisin molecule, since $^1$H NMR of original nisin does not show the changes in their respective spectra. Hydrated nisin forms in nisin L, could have had water added to double bonds of dehydro residues, consistent with the change in their $^1$H NMR spectra.

It is remarkable how many resonances in $^1$H NMR spectra of nisin and nisin L had exactly the same chemical shifts. The most important changes in $^1$H NMR spectra of nisin L with respect to original nisin are the loss of the dehydrobutyrine (DHB) 2 vinyl resonance and much smaller resonances of DHA 5. These changes alone would account for the loss of antimicrobial activity, as documented in many reports (4,12,18,20, 28). Consistent with changes in DHB 2 and DHA 5, we see changes in resonances of residues adjacent to them, ie, Ile 1 and Ile 4. Previous authors have
assigned different resonances for γ methyls of Ile 1 and Ile 4. We clearly observed disappearance of the doublet resonance at δ 1.85 that was assigned to Ile 1 by Kuipers et al. (18). The doublet at δ 1.15, due to Ile 4, has been altered in nisin L, as a result of a change in adjacent DHA 5. In nisin L, a new doublet appeared at δ 1.6 that may be shifted Ile 1 or Ile 4 resonance.

Subtilin in aqueous solution undergoes spontaneous inactivation, concurrent with the loss of the dehydroalanine 5 vinyl resonance in NMR spectrum (14). In nisin, DHA 5 is more stable, but still we were surprised that DHB resonance was completely lost before DHA 5 resonance. In nisin Z, it was found that when DHA5 was changed to dehydrobutyrine, the mutant was more resistant to acid-catalyzed chemical degradation, but also less active (25).

New peak at 2.5 ppm could be interpreted as resonance of methyl hydrogens of γ carbon of hydrated form of DHB 2. In epilacin K7-(3-31) peptide, a lower molecular weight form of epilacin K7, a new type A lantibiotic, a methyl group of oxopropionyl 3 gave a signal at 2.4 ppm (29). In oxopropionyl residue, the methyl group was adjacent to carbonyl (C=O). If DHB 2 was hydrated to threonine, new resonances for γ carbon should be expected around 1.3 ppm, and for β carbon around 4.3 ppm. When a threonine residue was incorporated at position 18 in nisin Z, this is where new resonances appeared (18). In des-DHA 5 nisin a singlet at 2.45 ppm was interpreted as arising from the methyl group of the pyruvyl residue (24). The observed resonances of DHA 5 in nisin L, were of lower intensity, but still
present. The low intensity of this new peak warrants only tentative assignments.

Additional peaks of high intensity appeared in ppm range from 3.55 to 3.85 in nisin L spectra, and this is the range where CβH resonances of dehydroresides are. A β carbon resonance of changed DHB 2 could be one of these peaks.

Nisin assumes a more defined secondary structure in membrane-like environments. A conformational transition, as seen by NMR and CD spectroscopy was complete at about 70% TFE in water (29). In water nisin has a flexible, random coil conformation (11,19, 24). Pep5, a group A lantibiotic, when titrated from water to 90% TFE, showed a two state random coil to helix transition (9). In these studies, no attempt was made to deduce secondary structures from CD spectra.

We recorded nisin's CD spectra at 0, 50 and 90% TFE and found that 90% TFE in water gave us the best defined spectra, and consequently compared nisin L and nisin spectra at this TFE concentration. We found that antiparallel beta sheet is the major defined secondary structure, followed by "other" structures and turns. The high fraction of "other" structures is not surprising, since nisin contains lanthionine rings that contribute to rigidity in the molecule. The main change in the nisin molecule, when in an hydrophobic environment, was an increase in helical at the expense of antiparallel structures. The carboxyl terminus is probably the part of the molecule that assumes helical conformation. It was reported that this part of the molecule enters the membrane (10).
Inactive nisin L had a smaller percentage of alpha helix, suggesting that its membrane spanning properties have been affected.

The CD analysis program, Varslc1, randomly takes out the first three proteins from the combination. Then, the combinations that gave higher percentages for total structure are examined to see what proteins are missing in the combination, and these proteins are eliminated from the data set. It took a longer time to analyze CD spectra for nisin L and nisin N then original nisin. With nisin L we came to a total structure of 101%, and had 27 proteins left in combination out of a data set of 33. As a comparison, total structure of 102% for original nisin was reached with 30 proteins still left in combination. For recovered nisin, we could not get a structure better then 70% total, even with only 15 proteins left in combination. Recovered nisin was just as active as original nisin, but definitely did not have the same conformation as nisin. This recovered nisin represents the portion of the molecule that underwent reversible thermal transition but it seems it did not assume the correct conformation upon cooling. This suggests that there are more then just one "active" configuration for the molecule. This is to be expected, as nisin remains active after trypsin digestion (16) and as synthetic fragments of nisin still have activity (4, 30).

Generally, main mechanisms that are responsible for irreversible thermal inactivation of proteins are: deamidation of amide side chains, peptide bond hydrolysis, disulfide bond exchange, racemization of amino acids and destruction of cysteine (1). In our case, major recognizable detrimental effect of heat was the
destruction of dehydrobutyrine 2 and partial destruction of dehydroalanine 5.

3.6 Acknowledgments

We would like to thank Mr. Don Griffin for MS analysis and Mr. Rodger Konhart for NMR analysis. Appreciation is expressed to Dr. Curtis W. Johnson for advice on interpretation of CD data and Jeaninne Lawrence for technical help and advice. We would like to thank Dr. Edvard Kolbe who gave valuable advice on DSC methodology and interpretation. The help of Dr. Cindy K. Bower and Dr. Scott A. Rankin is appreciated. This work was supported by USDA National Research Initiative Competitive Grants Program, Food Safety Division.

3.7 References


International patent application number: PCT/US 89/02625.


CHAPTER 4.

ION SPRAY MASS SPECTROSCOPY OF NISIN*

T. Musafija-Jeknic, D. A. Griffin and M. A. Daeschel

*This chapter will be submitted to Biomedical and Environmental Mass Spectrometry for consideration as a short communication.
4.1 Abstract

Nisin is an antimicrobial peptide that contains the unusual amino acids dehydroalanine, dehydrobutyryne, lanthionine and β-methyllanthionine. Lanthionine and β-methyllanthionine make up the substructures of five thioether bridges. Ion spray mass spectroscopy revealed the presence of two to six time charged molecular ions that gave average molecular mass of 3353.73, which is in agreement with previously reported MW values for nisin. The spectrum had an additional peak of 3371.33 D representing hydrated nisin. Tandem mass spectroscopy of triply charged parent ions m/z 1119 and m/z 1125 was used to identify where this addition of 18 amu occurs. We propose that the water addition occurs at lysine 34. Tandem mass spectroscopy did not reveal significant fragmentation within lanthionine bridges.

4.2 Introduction

Nisin is a bacteriocin produced by Lactococcus lactis ssp. lactis that has many applications in food industry as a natural food preservative (4). The structure of nisin was described by Gross and Morrel, 1971 (5). Five thioether bridges, as well as the presence of dehydroalanine (DHA) and dehydrobutyryne (DHB), make nisin an intriguing molecule for mass spectroscopy analysis. Mass spectroscopy (MS) studies on nisin have been done using $^{252}$Cf
plasma desorption MS (PDMS), liquid secondary ionization MS (LSIMS) (1, 6, 8) and fast atom bombardment (FAB) mass spectroscopy (2). Molecular mass of nisin is 3354 (1, 2, 6, 8). Previous studies have revealed the presence of hydrated nisin with molecular weight of 3372, and the presence of a degradation product with molecular weight of 3157 that was interpreted as nisin lacking the two C terminal residues (2,8).

Ion spray MS is a soft ionization technique that produces multiple charged molecular ions and is a method of choice in analyzing large proteins. It is also very useful because it allows interfacing of liquid chromatography to a mass analyzer. In this study on nisin, the method proved useful, as it allowed us to confirm previous structural assignments and to suggest a site at which hydroxylation in nisin occurs. It was proposed previously (2,8) that water addition most likely occurs at one of the dehydroresidues. We propose that hydroxylation occurs at lysine 34, based on tandem MS data for nisin and hydrated nisin and on $^1$H NMR spectrum of nisin, which was discussed in previous chapter.

4.3 Material and methods

Nisin, lot number NP 72, with activity of $50 \times 10^6$ IU/g was obtained from Aplin and Barrett Ltd. (Dorset, UK). Samples were prepared by dissolving nisin in water, and applying five μl for MS analysis.
Ion spray MS was performed on PE SCIEX API III Biomolecular Mass Analyzer (Perkin Elmer). Samples were scanned in positive ion mode every three seconds in the mass to charge ratio (m/z) range from 200 to 1800. After MS analysis, samples were reinjected and analyzed by tandem MS; triply charged peaks with m/z 1118 (nisin) or 1124 (hydrated nisin) served as parent ions. The collision gas used was argon with 10% nitrogen. Water containing 0.1% TFA was mobile phase at a flow rate of 0.3 ml/h, supplied by a Harvard syringe pump (Harvard Apparatus).

Data were analyzed with analysis software Bio ToolBox (Perkin Elmer SCIEX).

4.4 Results

Mass spectrum of nisin contained peaks with m/z ratio 560, 672, 839.6, 1118.8 and 1677 corresponding to molecular ion with six ([M+6H]^{6+}), five ([M+5H]^{5+}), four ([M+4H]^{4+}), three ([M+3H]^{3+}) and two ([M+2H]^{2+}) charges. The average molecular mass for these ions was 3353.87 D and in agreement with previously reported molecular mass for nisin. The peaks with m/z 675.2, 844 and 1124.8 correspond to five, four and three times charged ions of hydrated nisin, with molecular weight of 3371.67 D (Fig 4.1.). Nisin degradation product with molecular weight of 3157 D was also present. This degradation product was assigned as nisin 1-32 (2,8); alternatively, it can be interpreted as nisin 3-34.
Tandem mass spectroscopy on triply charged molecular ions m/z 1118.8 and m/z 1124.8 is given in figure 4.2. Structural assignments for fragments that were found in tandem mass spectroscopy are given in table 4.1 and figure 4.3.
Figure 4.1. Ion mass spectroscopy spectrum of nisin.
Figure 4.2. Tandem MS spectra of nisin. a.) daughters of m/z 1119, b.) daughters of m/z 1125.
Table 4.1. Fragments observed in tandem MS of nisin and their structural assignments.

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Figure 4.3. Structural assignments of fragments observed in mass spectroscopy of nisin. Fragments marked with an asterisk have an addition of 18 D.
4.5 Discussion

Ion spray mass spectroscopy proved to be a useful method in studying nisin. Some of the fragments we observed, were observed before, but were not assigned, such as m/z 1047 (2), that we assigned as triply charged ion B 32. As expected, we did not have much fragmentation within lanthionine bridges, with the exception of fragment m/z 300 that represents the cleavage of first lanthionine, with the retention of sulphur on Ala 3. The cleavage of lanthionine bridges in LSIMS and PDMS was reported earlier (2), and this particular fragment has been observed previously in FAB MS of nisin (1). In FAB MS of other lantibiotics no fragmentation was observed within their cyclic portions (7).

In comparison with previously reported data, our spectra contained more information on the carboxy terminus of the molecule. In nisin studies by other MS methods nisin's hydrated form (3372 D) was observed, but tandem MS analysis on it was never attempted. The daughter spectrum of hydrated nisin ion m/z 1125 was of low intensity, since the parent ion abundance is 17% with respect to nisin. Even though our spectra were of low intensity we were able to see this important difference in daughter spectra of nisin and hydrated nisin. Tandem MS of nisin has a series of fragments Y'' 32, Y'' 31 and Y'' 30 with molecular weights of 216, 315 and 452 D, respectively. In hydrated nisin we find this series with an addition of 18 D: 234, 333 and 470 D. This suggests that hydration occurs at DHA 33 or at Lys 34. We have recorded 1H NMR spectrum of nisin and
saw no changes in DHA 33 resonance (Fig 3.5), previous chapter. The doublet resonance at 5.8 ppm represents C^8H of DHA 33. It is removed downfield in the spectrum because of the deshielding effect of the double bond (6). If water was added to the double bond of DHA 33, this resonance would be lost.

We suggest that lysine 34 has been hydrated. Hydroxylation is a common modification of lysine and usually occurs on \( \partial \) C atom (3). Hydroxylysine has been found in collagen (7). We expect that lysine 34 has been hydroxylated at \( \partial \) C atom, as depicted in fig. 4.4.

Figure 4.4. Structure of hydroxylysine, adjacent to DHA 33 residue, as it would appear in hydrated nisin.
4.6 References


CHAPTER 5. 
SUMMARY

Novel nisin degradation product, designated nisin L was isolated after 3.5 hours of heat treatment at 121°C and characterized. Nisin L had lost antimicrobial activity against spores and vegetative cells of *B. cereus* T and had around 40% of original nisin's activity in terms of arbitrary units against *P. pentocaseus* FBB-61.

The most important structural changes in nisin L with respect to original nisin are:
- Higher proportion of hydrated nisin forms.
- Loss of DHB 2 and alteration of DHA 5 vinyl resonances that are crucial to nisin's antimicrobial activity.
- Appearance of new hydrogen resonances in the region 3.6 to 3.8 ppm in 1H NMR spectrum. These new resonances have not yet been assigned to a definite structural change.
- Nisin L is more polar then nisin.
- Smaller proportion of α helix, and slightly higher percentage of antiparallel β sheet.

We suggest that dehydroresidues have been altered by hydration and this explains higher polarity and higher percentage of hydrated nisin in nisin L. The loss of dehydroresidues and smaller percentage of α helix are responsible for the loss of antimicrobial activity.

New findings with respect to original nisin are that this is the first time that relative proportions of its secondary structures have
been reported. Antiparallel β sheet was recognized as the major defined secondary structure; before it was believed that nisin had mostly α helical structure. We suggest that lysine 34 is hydroxylated in hydrated nisin (3372 D). Extraordinary heat stability of nisin has been confirmed. It was seen that even after seven hours of heating at 121°C or after heating to 190°C or 230°C, nisin still retained detectable antimicrobial activity.

For a future researcher in this field these recommendations are put forth:
1. To determine which amino acid on carboxyl terminus of hydrated nisin have been hydroxylated:
   - Carboxypeptidase Y digestion of nisin and hydrated nisin up to the fourth terminal residue and comparison of amino acid products.
   - Total amino acid hydrolysis and analysis including hydroxylysine and threonine standards.
   - Tandem mass spectroscopy of ions m/z 452 and 470.
2. To evaluate structural changes in nisin L:
   - Recording and comparison of 1H NMR spectra of nisin and nisin L on a more powerful spectrometer (600 or 750 MHz).
   - More 2D NMR experiments, such as NOESY and ROESY, in different pH and temperature environments.
3. To determine which residues in nisin L have been hydrated, nisin should be heated in buffer made with 18O water. Then tandem mass spectroscopy of selected ions would give a definite answer where 18O has been incorporated.


Barber, M., Elliot, G. J., Bordoli, R. S., Green, B. N. and Bycroft, B. W. 1988. Conformation of the structure of nisin and its major degradation product by FAB-MS and FAB-MS/MS. *Experientia* 44:266-270.


APPENDIX

TWO DIMENSIONAL NMR COSY EXPERIMENT
INTRODUCTION

Double filtered J correlated spectroscopy (COSY) experiment was performed to confirm the findings from one dimensional NMR and to get a better resolution of peaks.

EXPERIMENTAL

Spectra were recorded on a Bruker 400 MHz NMR spectrometer, on 11-13 mg of sample in D$_2$O, at room temperature. Following parameters were used: D$_0$, D$_2$ and D$_3$ were 3 $\mu$s, and delay time D$_1$ was 1 second. There were 256 experiments with increments of 2.6 $\mu$s and 128 scans. Sweep width (SW) 1 was 1938 Hz, SW$_2$ was 3876 Hz, SI$_1$ was 512 word and SI$_2$ was 1024 word.

RESULTS

Results are given in figures A.1., A. 2., A. 3. and A.4.
Fig. A.1. Two dimensional COSY spectrum of nisin O. Peaks marked with arrows are unique to nisin O.
Fig. A.2. Two dimensional COSY spectrum of nisin L. Peak marked with an arrow is unique to nisin L.
Fig. A.3. A detail of two dimensional COSY spectrum of nisin O. Peaks marked with arrows are unique to nisin O.
Fig. A.4. A detail of two dimensional COSY spectrum of nisin L. Peaks marked with arrows are unique to nisin L.