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Involvement of Dehydroalanine and Dehydrobutyrine in the Addition of Glutathione to Nisin

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Nisin variants and fragments were reacted with glutathione, and the products of the reactions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography/mass spectrometry (LC-MS). Reactions between glutathione and either [Ala5]nisin or [Ala33]nisin resulted in products with two glutathione molecules conjugated to one nisin variant molecule. Only one glutathione molecule was added to [Ala5,Ala33]nisin. Fragmentation of the nisin molecule resulted in nisin 1−12, nisin 1−20, and nisin 1−32 fragments. Each fragment retained two dehydro residues, which subsequently underwent reaction with glutathione. The data indicated that the dehydroalanine residues of nisin are sites of addition for glutathione. Such addition renders the nisin molecule inactive.

KEYWORDS: Nisin; glutathione; dehydroalanine; MALDI-TOF MS; LC-MS

INTRODUCTION

The lantibiotic nisin is an antibacterial peptide that is widely used as a natural preservative in the food industry. Lantibiotics, so-termed because of their characteristic sulfide ring formations, are small membrane active peptides containing posttranslationally modified amino acids (*1*). This involves the dehydration of serine and threonine to dehydroalanine and dehydrobutyrine, respectively. The dehydrated residues may then react with neighboring cysteine residues to form lanthionine or β-methyllanthionine residues (**Figure 1**).

Nisin exhibits antimicrobial activity against a broad spectrum of Gram-positive bacteria, including the spores of *Bacillus* and *Clostridium*. This activity is thought to be the result of two distinct killing mechanisms: (i) the N-terminal domain of nisin is able to interact with lipid II molecules and subsequently inhibit peptidoglycan synthesis, or (ii) the C-terminal domain can translocate across the cell membrane to form pores and dissipate the proton motive force (*2*). Furthermore, it has been proposed that nisin activity against spores is due to a reaction with sulfhydryl (−SH) groups of freshly germinated spores (*3*). The presence of the dehydrated amino acids and ring structures of nisin is essential for complete retention of antibacterial activity (*4*−*6*).

Nisin's high level of antibacterial activity, coupled with its heat and acid resistance, has resulted in its successful application in several dairy products, canned foods, and vegetables (*7*). However, it has had limited success as an antimicrobial agent in fresh meat products, and the use of nisin in this food matrix is restricted to heat-treated meat or in conjunction with other preservatives (*8*−*13*). This failure to inhibit target bacteria in fresh meat products was thought to be the result of nisin binding to meat components and surfaces, its sensitivity to food enzymes, interactions with phospholipids, poor solubility, or uneven distribution on the meat product (*14*−*18*).

Our laboratory hypothesized that nisin was inactivated in fresh meat by an enzymatic reaction with glutathione, a low molecular mass (307 Da) thiol compound found in meat tissues (*19*). The in vitro reaction between pure nisin and glutathione confirmed that under optimum conditions, glutathione adds to multiple sites on a nisin molecule (*20*). It has been proposed that the dehydro residues of nisin are able to act as Michael acceptors toward sulfhydryl groups (*21*). The aim of this study was to investigate the sites of addition of the glutathione molecules by employing engineered nisin variants in which the dehydroalanines were replaced with saturated alanine residues. This approach enables the role of the dehydrated residues to be assessed leading to a better understanding of the loss of activity of nisin in meat products. Nisin variants were reacted with glutathione, and the products of the reactions were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and liquid chromatography coupled with mass spectrometry (LC-MS) techniques.

MATERIALS AND METHODS

Materials. Pure nisin was obtained from Aplin & Barrett Ltd. (Langford, U.K.). Glutathione and sinapinic acid were obtained from Sigma Chemical Co. (St. Louis, MO).

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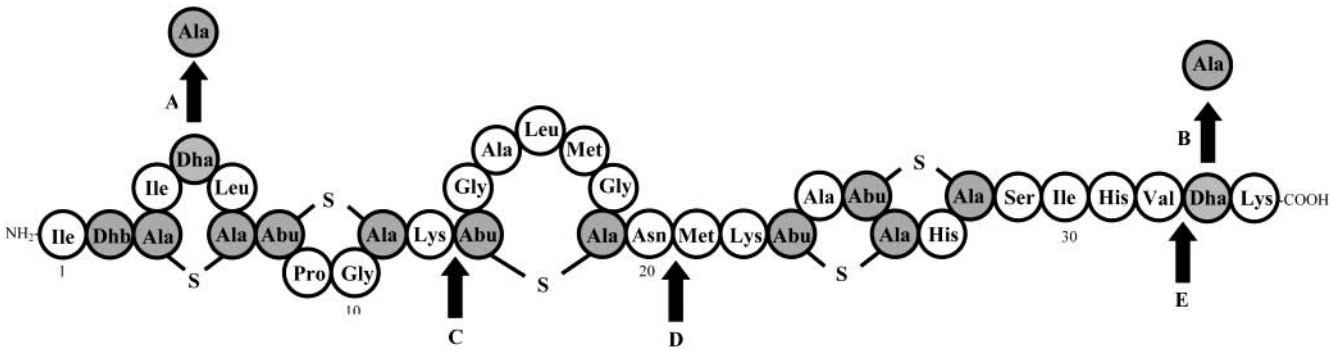

Figure 1. Structure of mature nisin molecule. Amino acids that have undergone posttranslational modification are shaded. Sites of engineered residues are indicated for nisin variants: (**A**) [Ala5]nisin A; (**B**) [Ala33]nisin A; (**AB**) [Ala5,Ala33]nisin A. Enzymatic cleavage sites (shown by arrows) generate fragments nisin 1–12 (**C**), nisin 1–20 (**D**), and nisin 1–32 (**E**).

Construction of Nisin Variants. Nisin variants and fragments were constructed and purified at the Institute of Food Research (Norwich, U.K.). [Ala5]-, [Ala33]-, and [Ala5,Ala33]nisin A variants were produced and purified by means of a protein engineering system as described by Dodd et al. (*22*). Briefly, a lactococcal expression system was developed using a gene replacement technique in which variant *nisA* genes were substituted for the chromosomal wild-type gene. Fragments of nisin were generated enzymatically by proteolytic digestion with α-chymotrypsin (nisin 1−20) or trypsin (nisin 1−12) and purified as described by Chan et al. (*4*). In addition, low levels of the natural degradation product nisin 1−32, generated by cleavage of the carboxy-terminal dipeptide (*23*), were identified in the nisin A and [Ala5]nisin A samples used in this study. The structure of nisin highlighting the variants and fragments is shown in **Figure 1**. The relative molecular masses of the nisin A variants and fragments were confirmed by MALDI-TOF MS as described below.

Activity Assay for Nisin. Glutathione S-transferase was purified from fresh beef, as previously described by Williamson and Ball (*24*). Stock solutions of nisin and the nisin variants (100 μg mL^{-1}) and purified glutathione S-transferase (1 mM) were dissolved in 50 mM sodium phosphate buffer (pH 6.0). Glutathione was dissolved in the sample buffer at a concentration of 50 mM, and the solution was adjusted to pH 6.0. Final working assays consisted of glutathione (25 mM), nisin (50 μg mL^{-1}), and glutathione S-transferase (1 μM). Assays were carried out at the optimum temperature of 37 °C. The reactions were stopped after 24 h by adding trifluoroacetic acid (TFA) to a final concentration of 0.3%. The products were analyzed by MALDI-TOF MS and LC-MS.

MALDI-TOF MS Analysis. All mass spectra data were acquired on a linear MALDI-TOF MS equipped with pulsed ion extraction technology (Bruker Proflex III, Billerica, MA) and a 125 cm flight tube, in a positive ion linear mode with a nitrogen laser (λ = 337 nm) for desorption/ionization of the samples and an acceleration voltage of 20 kV. Samples were prepared for MALDI-TOF analysis using the dried droplet method (*25*). A saturated solution of the sinapinic acid matrix was prepared in a solution containing two parts of 0.1% TFA and one part of acetonitrile. The sample was mixed with the matrix (1:1), spotted on a stainless steel probe, and allowed to air-dry.

LC-MS Analysis. High-performance liquid chromatography (HPLC) chromatographic conditions were as follows: solvent A, 0.1% TFA in Milli-Q purified water; solvent B, 0.1% TFA in HPLC grade acetonitrile; flow rate, 1 mL·min^{-1}; gradient, 0.0−100% B in 45 min. A Phenomenex Jupiter C_4 column (250 mm × 4.60 mm packed with 5 μm beads; Torrence, CA) was used. The flow was split between the mass spectrometer and the diode array detector in the approximate ratio of 1:8. Diode array spectra were recorded simultaneously with mass spectra in the range of 190−600 nm. All nisin variants and their fragments and reaction products were analyzed using a Micromass Quattro II triple quadrupole mass spectrometer (Micromass, Manchester, U.K.), coupled to a Hewlett-Packard 1050 quaternary pump HPLC system equipped with a 1050 autoinjector and 1050 diode array detector (Agilent Technologies U.K. Ltd., Stockport, U.K.). Spectra were obtained in positive ion electrospray mode using a Micromass Z-spray ion source. The electrospray probe was operated at 3.5 kV, and the cone voltage was 28 V. The source and desolvation temperatures were 120 and 350 °C, respectively. The nitrogen nebulizing and drying gas flow rate were optimized at 15 and 350 L h^{-1}, respectively. Spectra were recorded scanned in continuum mode (i.e., raw data acquisition) by cyclic scanning of the mass range *m*/*z* 400−2200, with a scan duration of 5.0 s and an interscan time of 0.2 s. The mass spectrometer was set to unit mass resolution or better (LM and HM resolution parameters both at 15.0). Mass spectrometric data were recorded and processed using MassLynx version 3.4 software (Micromass, Manchester, U.K.).

Table 1. Molecular Masses of Nisin Variants and Fragments Determined by MALDI-TOF MS and LC-MS

nisin fragment/variant	experimental mass (MALDI-TOF MS)	experimental mass (LC-MS)
nisin	3362.0	3353.6
[Ala5]nisin	3360.3	3355.6
[Ala33]nisin	3359.3	3355.6
[Ala5,Ala33]nisin	3366.3	nd[a]
nisin 1–32	3184.5	3185.6
nisin 1–20	1880.7	nd
nisin 1–12	1150.6	1150.1

[a] nd, not determined.

RESULTS

MALDI-TOF MS and LC-MS Analyses. MALDI-TOF MS and LC-MS analyses were performed on the nisin variants and fragments prior to the reaction with glutathione to confirm their molecular weight and to determine whether any degradation fragments were present in the sample. The experimental masses are shown in **Table 1**. There is a discrepancy in the molecular masses as reported by MALDI-TOF MS and LC-MS. This is likely due to the low resolving capabilities of the MALDI-TOF MS instrument used to obtain the measurements (*26*). This particular instrument was unable to resolve the complex isotopic pattern exhibited by a molecule of this mass. More recent delayed extraction and/or reflectron MALDI-TOF MS instruments may better be able to resolve this isotopic cluster.

MALDI-TOF MS was also used to examine the products of the reaction between the different nisin molecules and the glutathione. A shift in mass of increments of ∼+307 Da was used to determine the number of glutathione molecules that were added to the nisin molecules. **Figures 2** and **3** show the mass spectra of the products of the reactions.

A fully processed nisin molecule has three dehydro residues (Dhb2, Dha5, and Dha33), whereas [Ala5]nisin and [Ala33]-nisin have two dehydro residues (Dhb2 and Dha33 and Dhb2

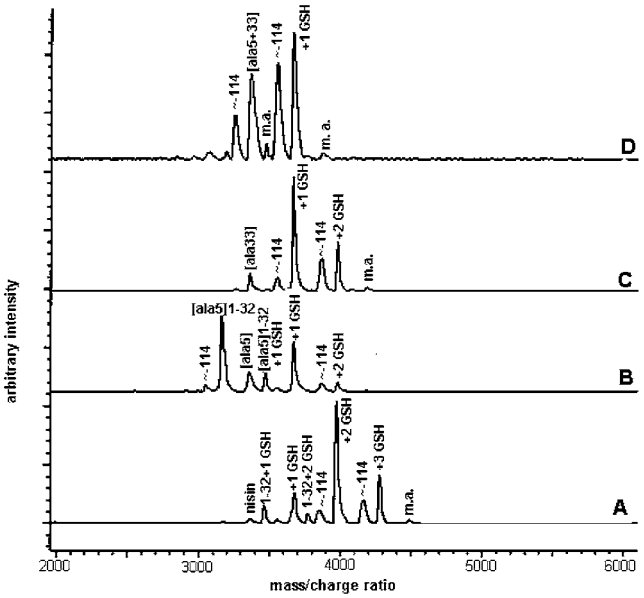

Figure 2. MALDI-TOF MS spectra of the products of the reaction between glutathione and (**A**) nisin; (**B**) [Ala5]nisin; (**C**) [Ala33]nisin; and (**D**) [Ala5,Ala33]nisin; m.a. refers to the presence of a matrix adduct.

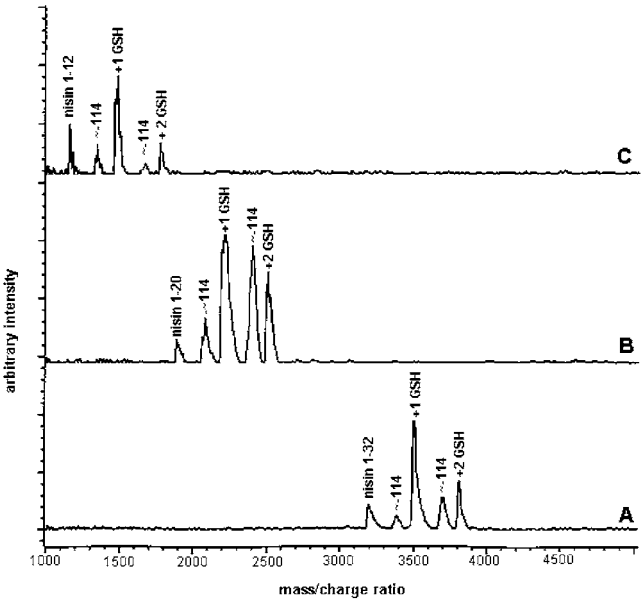

Figure 3. MALDI-TOF MS spectra of the products of the reaction between glutathione and (**A**) nisin 1–32; (**B**) nisin 1–20; and (**C**) nisin 1–12.

and Dha5, respectively) and [Ala5,Ala33]nisin has one dehydro residue (Dhb2). The products of the reaction between nisin and glutathione showed that up to three glutathione molecules can be added to one nisin molecule (**Figure 2A**). Reactions between the glutathione and the nisin variants showed that two glutathione molecules can be added to the single variants (**Figure 2B**,**C**) and that only one glutathione molecule can be conjugated to the double variant of nisin (**Figure 2D**). There was no apparent specificity for either the Dha5 or the Dha33 amino acid residues as the reaction proceeded with all nisin variants.

These data also showed an instability of the [Ala5]nisin molecule (**Figure 2B**). It appears that upon reaction of nisin with glutathione, a significant proportion of the sample had undergone cleavage of the two carboxy-terminal amino acids, whereas this is not apparent before the reaction with glutathione (**Table 1**). After cleavage, [Ala5]nisin 1−32 has only one dehydro residue (Dhb2) and undergoes the addition of a single

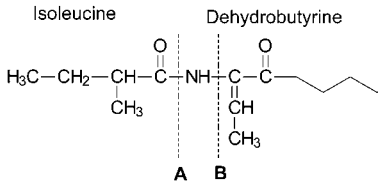

Figure 4. Structure of the first two amino acids in the N terminus region of nisin, indicating the potential sites of cleavage.

glutathione molecule (**Figure 2B**). To a lesser extent, this phenomenon was also observed with the full-length molecule. In this case, the truncated peptide retains two dehydro residues and peaks are evident corresponding to the addition of both one and two glutathione molecules (**Figure 2A**). No such degradation was detected with the nisin variants that have had Dha33 replaced with an Ala residue (**Figure 2C**,**D**). The MALDI-TOF MS data showed that the number of glutathione molecules that can add to either the nisin or the nisin variants is consistent with the number of dehydro residues in the molecule. The MALDI-TOF MS analysis also showed the presence of matrix adduct peaks of ∼+215 Da, which is typical of the use of sinapinic acid as the matrix (personal communication, Randy Whittal).

Fragmentation of the nisin molecule involved cleavage of the C terminus region at the positions after 12, 20, and 32 amino acid residues, resulting in three fragments, each with two dehydro residues (Dhb2 and Dha5). Consistent with the number of dehydro residues remaining, the MALDI-TOF MS data showed that two glutathione molecules were added to each of the fragments (**Figure 3**). This suggested that the N terminus of the nisin molecule is highly susceptible to nucleophilic attack by glutathione and that Dha33 in the C terminus region is the site of the third glutathione addition.

In addition to the peaks corresponding to the addition of the glutathione molecule(s), a number of other fragmentation peaks were evident from the MALDI-TOF MS and the LC-MS data. The dominant fragment peaks (**Table 2**) were consistent with loss of either ∼114 Da, as detected by MALDI-TOF MS and LC-MS, or ∼129 Da, as detected by LC-MS. Screening of the reaction products by a reflectron MALDI-TOF mass spectrometer also showed the fragment peaks of less than ∼129 Da (data not shown). Because the peaks were seen in each of the product samples, it is likely that the fragmentation occurs at the N-terminal region of the molecule. This could be the result of a common phenomenon with mass spectrometry techniques, called postsource decay, which results in the fragmentation of the analyte molecule (*27*). This phenomenon is not typically seen with linear MALDI-TOF mass spectrometry but may be seen with other mass spectrometry instrumentation. The loss of ∼114 Da was seen with the linear mass spectrometer (**Figures 2** and **3**) and with the parent molecule of [Ala5,Ala33]nisin (**Figure 3D**) after the reaction with glutathione and not prior to the reaction (data not shown). It is possible that the resulting peptide fragments occur in nature when glutathione attaches to a nisin molecule. However, this loss of ∼114 Da could also be explained as fragmentation due to the conditions used for the MALDI-TOF MS analysis.

To determine the sites of cleavage, the monoisotopic mass of nisin after the addition of two GSH molecules was compared with the monoisotopic masses after cleavage at two different sites as shown in **Figure 4**. The monoisotopic mass of nisin with two GSH molecules added ($C_{163}H_{265}N_{48}O_{49}S_9$), as determined by MS-Isotope 1.3.1, Protein Prospector 3.4.1 (The Regents of the University of California), is 3966.7206. Cleavage

Table 2. Summary of the Dominant Peaks of the Products of the Reaction between the Nisin Variants and the Fragments with GSH after Analysis by MALDI-TOF MS and LC-MS

	MALDI-TOF MS		LC-MS	
	experimental mass	mass difference	experimental mass	mass difference
nisin	3362.0		3353.6	
addition of one GSH molecule	3669.5	+307.5		
addition of two GSH molecules	3976.2	+614.2	3968.0	+614.4
fragment peaks	3860.2	−116.0	3839.0	−129
addition of three GSH molecules	4283.3	+921.3	4275.2	+921.6
fragment peaks	4169.0	−114.3	4161.3	−113.9
			4146.2	−129.0
[Ala5]nisin	3360.3		3355.6	
addition of one GSH molecule	3667.8	+307.5	3662.9	+307.3
fragment peaks	3554.6	−113.2		
addition of two GSH molecules	3976.0	+615.7	3970.2	+614.6
fragment peaks	3862.1	−113.9	3856.1	−114.1
			3840.7	−129.5
[Ala33]nisin	3359.3		3355.6	
addition of one GSH molecule	3666.5	+307.2	3662.7	+307.1
fragment peaks	3552.4	−114.1		
addition of two GSH molecules	3973.1	+613.8	3969.7	+614.1
fragment peaks	3859.1	−114.0	3855.9	−113.8
			3840.6	−129.1
[Ala5,Ala33]nisin	3366.3		nd	
addition of one GSH molecule	3673.5	+307.2		
fragment peaks	3560.4	−113.1		
nisin 1–32	3184.5		3185.6	
addition of one GSH molecule	3492.0	+307.5	3492.7	+307.1
fragment peaks	3377.0	−115.0		
addition of two GSH molecules	3799.0	+614.5	3800.2	+614.6
fragment peaks	3687.1	−111.9	3685.9	−114.3
			3671.0	−129.2
nisin 1–20	1880.7		nd	
addition of one GSH molecule	2187.6	+306.9		
fragment peaks	2073.3	−114.3		
addition of two GSH molecules	2494.0	+306.4		
fragment peaks	2382.7	−111.3		
nisin 1–12	1150.6		1150.1	
addition of one GSH molecule	1457.7	+307.1		+307.3
fragment peaks	1343.6	−114.1		
addition of two GSH molecules	1763.8	+613.2	1764.7	+614.6
fragment peaks	1648.1	−115.7	1650.5	−114.2
			1635.5	−129.2

[a] nd, not determined.

at the peptide bond between isoleucine and dehydrobutyrine results in a monoisotopic mass of 3852.6287 ($C_{157}H_{253}N_{47}O_{48}S_9$), and cleavage after the amine group results in a monoisotopic mass of 3837.6179 ($C_{157}H_{252}N_{46}O_{48}S_9$). The mass differences as compared with the major nisin molecule with two GSH added are 114.0919 and 129.1027, respectively.

DISCUSSION

The nisin molecule is a complex antibacterial peptide with 13 out of 34 residues posttranslationally modified to dehydro residues and thioether cross-linkages. Several studies have demonstrated the importance of such modifications to the antibacterial efficacy of nisin. In particular, the acid-catalyzed addition of a water molecule to Dha5 results in a loss of integrity of ring A of nisin and, subsequently, a decrease in its antibacterial activity (*6*). Similarly, cleavage of ring C in nisin results in the complete loss of activity (*4*), although the presence of dehydro residues is not essential. The engineered variants [Ala5]nisin, [Ala33]nisin, and [Ala5,Ala33]nisin retained a significant level of activity against vegetatively growing cells

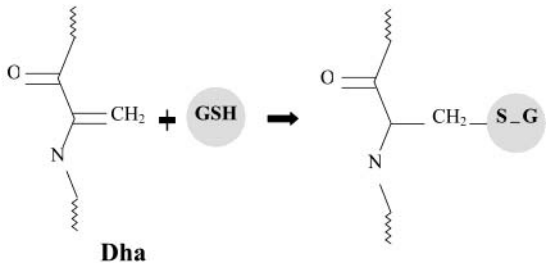

Figure 5. Proposed reaction of addition of glutathione to dehydroalanine.

(*28*). However, [Ala5]nisin displayed a much lower activity against the outgrowth of spores of *Bacillus subtilis* (*4*), supporting the proposal that the dehydroalanine residues play a vital role in the killing action against bacterial spores (*3*).

This work has provided an insight into the potential use of nisin as a natural preservative in meat products. It appears that the addition of glutathione to nisin is one reason for its poor activity in fresh meats (*19*, *20*). However, our studies have indicated that the successful application of nisin to this food matrix may be achieved by either (i) the addition of nisin to heat-treated products, as demonstrated by Davies et al. (*11*); (ii) the use of a food grade glutathione S-transferase inhibitor in fresh meat products; or (iii) employing an active nisin variant in which the dehydro residues are absent or unavailable for reaction with glutathione.

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