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Inactivation of Nisin by Glutathione in Fresh Meat

N.L. Rose, P. Sporns, M.E. Stiles, and L.M. McMullen

ABSTRACT

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to determine the fate of nisin in meat products. Nisin in 0.02N HCl was added to fresh and cooked meat and meat juice and samples were stored at 4 °C overnight. Residual nisin solutions and meat juice and meat extract supernatants were analyzed for antimicrobial activity and for nisin. Nisin was recovered from cooked meat extract and cooked meat juice; however, only nisin bound to a food component was detected in fresh meat extract. Mass spectra for raw meat and juice showed a signal 307 Da greater than the mass of nisin. Results indicated that nisin was likely inactivated in raw meat by an enzymatic reaction with glutathione.

Key Words: nisin, inactivation, MALDI-TOF MS, meat, enzymatic reaction

INTRODUCTION

Nisin is the most studied and highly characterized bacteriocin produced by lactic acid bacteria. Nisin is in a class of bacteriocins known as lantibiotics which are small peptides defined by antimicrobial activity and the presence of lanthionines and uncommon amino acids (Nes and Tagg, 1996; van De Ven and Jung, 1996). The structures of nisin and its uncommon amino acids are shown in Fig. 1. Nisin contains dehydro residues [dehydroalanine (DHA) and dehydrobutyrine (DHB)] and thioether crosslinkages [lanthionine and β-methylthionine] that arise from extensive posttranslational modification of serine, threonine and cysteine (Liu and Hansen, 1990; Kuipers et al., 1995; Stringer et al., 1995; van De Ven and Jung, 1996).

Nisin exhibits antimicrobial activity against many gram-positive bacteria, including the spores of Bacillus and Clostridium spp.; however, it has no effect against gram-negative bacteria, yeast or molds (Delves-Broughton et al., 1996). The phospholipid component of the cytoplasmic membrane of vegetative cells has been hypothesized to be the major target for nisin activity (Abee et al., 1995), whereas nisin acts on the sulfhydryl membrane groups for inactivation of germinated spores (Liu and Hansen, 1990; Morris et al., 1984). Nisin has been approved as a food additive in over 46 countries for use in processed cheeses, dairy products and canned foods (Delves-Broughton et al., 1996). The use of nisin in meats is limited because some meat components interfere with its activity (Henning et al., 1986).

Several researchers have examined potential methods for determination of the concentration of nisin in food products. Extraction and bioassays are typically used for the detection of bacteriocins in meats (Leisner et al., 1995; McMullen and Stiles, 1996). Bouksaim et al. (1998) used enhanced chemiluminescence for the detection of nisin in milk and whey. Each method has been effective; however, both are time-consuming and neither enables study of the interaction of the bacteriocin with the food components.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been an effective method for detection of bacteriocins in the culture supernatant of producer organisms and has potential for determining the fate of bacteriocins in foods (Rose et al., 1999). MALDI-TOF MS uses a pulsed laser beam to transfer energy to a spot of co-crystallized sample and matrix, which are then ejected into a vacuum for analysis. Ions formed are accelerated by an electric field and the time-of-flight of the ion through the drift tube is used to determine the mass of the ion. This method is valuable for molecular weight measurements (Costello, 1997; Siudak, 1994). Our objective was to use MALDI-TOF MS to study the fate of nisin in meats.

MATERIALS & METHODS

Bacterial strains and media

Carnobacterium diversum NCFB 2855 was used as a sensitive indicator for detection of nisin activity. A frozen stock culture was maintained at –70 °C in Bacto® APT (All-Purpose Tween) broth (Difco Laboratories, Detroit, MI) supplement-

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Nisin solutions and meat preparation

Pure nisin (Aplin & Barrett Ltd., Langford, England) was stored at 4 °C in a desiccator. Working solutions were prepared by dissolving 50, 125, and 250 µg of nisin in 1 mL 0.02N HCl (pH 2). A solution of 0.02N HCl was used as a control.

Fresh eye-of-round beef was purchased from a local supermarket. Pork was obtained from a federally-inspected processing plant, stored at −30 °C and thawed at room temperature before use in experiments. Meat samples (1.0 × 1.0 × 0.5 cm) were soaked in each nisin solution and the samples of raw pork and beef samples were prepared (Fig. 2). Similar spectra were obtained for the residual solution of the cooked meat.

MALDI-TOF MS

All mass spectra 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. The spectra are representative of 10 consecutive laser shots. Angiotensin II (MH⁺ = 1046.542) and bovine insulin (MH⁺ = 5734.557), from Sigma Chemical Co. (St. Louis, MO), were used for external mass calibration.

MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed on all residual nisin solutions and on the supernatant from the juice and extracts of the meat samples. The mass spectra of the residual nisin solutions (250 µg/mL) of cooked and raw meat were compared. A single peak corresponding to the mass of nisin was observed for the residual solution of the cooked meat. The nisin signal and an additional peak ≈307 Da greater than the nisin peak was detected in the supernatant of the fresh meat extract. Similar results were obtained for the supernatants from the raw meat (Fig. 3c) and cooked meat (Fig. 3d) juice samples.

RESULTS

Nisin assays

Prior to soaking the meat, the control (0.02N HCl) and nisin preparations were assayed by the “spot-on-lawn” method (Ahn and Stiles, 1990) for the number of arbitrary units of bacteriocin using C. divergens NCFB 2855 as the indicator strain (Table 1). Each solution was also analyzed by MALDI-TOF MS TOF analysis by the method described by Rose et al. (1999). Samples for analysis included initial and residual nisin solutions, extracts of raw and cooked meat soaked in nisin, and raw and cooked juice with nisin added. The supernatant (0.5 µL) of meat extract and juice samples was placed on a stainless steel MALDI probe and allowed to air dry. The probe was dipped into Milli-Q water and held static for 30 s to remove water-soluble contaminants from the sample. Excess water was shaken off the probe, and the sample was air-dried. When dry, 0.5 µL saturated solution of sinapinic acid (Sigma) in a solution containing two parts of 0.1% trifluoroacetic acid and one part of acetonitrile was added to the sample spot. MALDI-TOF MS samples for the initial nisin solutions were prepared without the washing step because of the pure nature of the samples.

Table 1—Activity (AU/mL) of control and nisin solutions

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<tr>
<th>Nisin conc (µg/mL)</th>
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<tbody>
<tr>
<td>0 (Control)*</td>
<td>0</td>
</tr>
<tr>
<td>50</td>
<td>1600</td>
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*0.02N HCl

Antimicrobial activity was almost half that of the initial nisin solutions. Antimicrobial activity was detected in the extract from the cooked meat sample; however, no activity was detected in any extracts from the raw meat sample. Antimicrobial activity was found in the juice from the raw and cooked meat, although the juice from the cooked meat had four times more activity than the juice from the raw meat.

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ed with 20% glycerol (vol/vol). Prior to experimental use, C. divergens NCFB 2855 was subcultured twice and grown overnight in APT broth. Solid agar medium was prepared by adding 1.5% (wt/vol) granulated agar (Difco) to APT broth. Soft APT agar was prepared with 0.75% agar (wt/vol).

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Fig. 2—Mass spectra of (a) control solution of 0.02N HCl, and (b) 250 µg/mL solution of nisin in 0.02N HCl. Average mass of nisin solution was 3365.77 ± 1.8 Da (n = 4).
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typically enzyme-mediated; therefore, the extract of cooked meat was analyzed to determine whether this reaction would occur after the enzymes were inactivated. The mass spectra of the cooked beef extract (Fig. 4a) showed a typical nisin ion peak; whereas no comparable signal was observed in either the raw pork (Fig. 4b) or raw beef (Fig. 4c) extracts. However, a signal >307 Da greater than the mass of nisin was observed for the raw pork and raw beef extracts.

**DISCUSSION**

With the use of MALDI-TOF MS and activity assays, we could determine the fate of nisin on raw meat. Extracts of raw meat samples that had been soaked in a nisin solution were lacking in nisin activity; whereas, those from cooked meat retained activity. Results also showed that the mass of nisin increased by >307 Da when it was applied to fresh meat and stored. In contrast, when nisin was applied to cooked meat, there was no shift in mass. This indicated that nisin reacted with a component of fresh meat and that the reaction was likely enzymatic because it did not occur in cooked meat.

Although it has activity against the outgrowth of spores and gram-positive foodborne pathogens, the use of nisin in meat products has been ineffective except when used in high concentrations (Scott and Taylor, 1981a; Rayman et al., 1983; Bell and De Lacy, 1986; Chung et al., 1989; Stevens et al., 1991; Mahadeo and Tatini, 1994). There have been several hypotheses about why nisin is inactive in meat; however, there is no scientific evidence to substantiate them. Its ineffectiveness in meat products has been attributed to reactions with meat components and surfaces, poor solubility, sensitivity to food enzymes, high bacterial loads, interaction with phospholipids and poor distribution throughout the meat product (Scott and Taylor, 1981a; Bell and De Lacy, 1986; Henning et al., 1986; Cutter and Siragusa, 1994; Stringer et al., 1995; Delves-Broughton et al., 1996).

These problems have been reportedly due primarily to the hydrophobic nature of nisin and its instability at neutral pH (Scott and Taylor, 1981b; Henning et al., 1986; Stringer et al., 1995; Delves-Broughton et al., 1996).

Nisin has been shown to be an effective inhibitor of *B. cereus* and *C. botulinum* spore outgrowth by inactivating the membrane sulfhydryl groups in the newly germinated spores (Scott and Taylor, 1981a; Morris et al., 1984; Liu and Hansen, 1990). The sulfhydryl groups are involved in many enzymatic reactions of the cell and, therefore, serve as potential sites of inhibition by sulfhydryl agents. A good sulfhydryl agent has been defined as a compound of moderate reactivity in a relatively bulky, charged and nontransportable molecule (Morris et al., 1984). Nisin meets the criteria of a good sulfhydryl agent because it is a 34-residue peptide that is relatively bulky, and its activity is associated with the unusual dehydro residues that could act as electrophilic Michael acceptors and readily react with mercaptans and other effective nucleophiles (Morris et al., 1984; Liu and Hansen, 1990). The reaction of nisin with mercap-

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**Table 2—Nisin recovered from supernatant of residual nisin solutions, meat juice and meat extracts**

<table>
<thead>
<tr>
<th>Meat Preparation</th>
<th>Nisin conc (µg/mL)</th>
<th>Raw Activity (AU/mL)</th>
<th>Cooked Activity (AU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>Residual nisin soln&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
<td>800</td>
</tr>
<tr>
<td></td>
<td></td>
<td>125</td>
<td>1600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>250</td>
<td>3200</td>
</tr>
<tr>
<td>Meat extract&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50</td>
<td>0</td>
<td>n.d.</td>
</tr>
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<td></td>
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<td>n.d.</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0</td>
<td>3200</td>
</tr>
<tr>
<td>Meat juice&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250</td>
<td>400</td>
<td>1600</td>
</tr>
<tr>
<td>Pork Meat extract&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250</td>
<td>0</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

<sup>a</sup>n = 4  
<sup>b</sup>n.d. not determined
Inactivation of Nisin by Glutathione . . .

Glutathione \([\text{N-(N-L-\gamma\text{-glutamyl-L-cysteinyl)glycine}}]\) is a major low molecular weight (307 Da) thiol compound found in cells (Fig. 5). GSH is widespread in nature and is found in both plant and animal tissues. Wierzchicka et al. (1989) determined the amounts of GSH in foods consumed by humans. They found that the concentration of GSH in beef, chicken, and pork was generally high with 156 to 627 nmol/g wet weight. The concentration of GSH is less in raw fish (21 nmol/g wet weight), and there is essentially no GSH in dairy products. GSH functions in the body to detoxify carcinogenic electrophiles and to protect cells against oxidative damage, and GSH reacts with electrophiles to yield GSH S-conjugates (Wierzchicka et al., 1989). These reactions are partially mediated by glutathione S-transferase and by glutathione peroxidase. Lee et al. (1996) reported that glutathione peroxidase was present in high amounts in fresh turkey thigh muscle (0.73 ± 0.04 U/g), and it was recovered in very low amounts (0.03 ± 0.01 U/g) after cooking. The amount of GSH in poultry meat is unaffected by heat. Williamson and Ball (1988) postulated that glutathione S-trans ferase may protect against oxidative damage in meat and they isolated the enzyme from fresh pork. Their results showed that the enzyme was most stable and active at pH 5.5 to 6.0, which is consistent with the pH of fresh meat products.

The enzyme responsible for catalyzing the reaction between nisin and GSH is glutathione S-transferase. Nisin exhibits some features common to known substrates of glutathione S-transferase, including its hydrophobicity and its electrophilic carbon atom (Sipes and Gandolf, 1991). Glutathione S-transferase catalyzes the reaction of GSH with compounds containing electrophilic carbon atoms, forming a thioether bond (Fig. 5) between the carbon atom and the sulphydryl group of GSH (Sipes and Gandolf, 1991). In the presence of an electrophile, mercaptans can add to a double bond; such addition could be done with GSH and glutathione S-transferase.

Our studies have illustrated the utility of MALDI-TOF MS in determining the fate of peptides, such as bacteriocins, in complex food matrices such as meat. Our data support the hypothesis that nisin is inactivated in raw meat due to a specific reaction with a compound in raw meat. Due to the noted mass increase of nisin, that compound is apparently GSH. From the inhibition studies, it is clear that nisin was inactivated when bound to GSH. GSH is not found in dairy products, which would explain why nisin is an effective antiin crobial agent in dairy products. Future work is needed on determining the rate of reaction between GSH and nisin, inhibition studies on glutathione S-transferase and the potential for use of nisin in cooked meat products is recommended.

**REFERENCES**


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