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Nisin Treatment for Inactivation of *Salmonella* Species and Other Gram-Negative Bacteria†

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Nisin, produced by *Lactococcus lactis* subsp. *lactis*, has a broad spectrum of activity against gram-positive bacteria and is generally recognized as safe in the United States for use in selected pasteurized cheese spreads to control the outgrowth and toxin production of *Clostridium botulinum*. This study evaluated the inhibitory activity of nisin in combination with a chelating agent, disodium EDTA, against several *Salmonella* species and other selected gram-negative bacteria. After a 1-h exposure to 50 μg of nisin per ml and 20 mM disodium EDTA at 37°C, a 3.2- to 6.9-log-cycle reduction in population was observed with the species tested. Treatment with disodium EDTA or nisin alone produced no significant inhibition (<1-log-cycle reduction) of the *Salmonella* and other gram-negative species tested. These results demonstrated that nisin is bactericidal to *Salmonella* species and that the observed inactivation can be demonstrated in other gram-negative bacteria. Applications involving the simultaneous treatment with nisin and chelating agents that alter the outer membrane may be of value in controlling food-borne salmonellae and other gram-negative bacteria.

Nisin is an antibacterial peptide produced by *Lactococcus lactis* subsp. *lactis* that exhibits a broad spectrum of inhibitory activity against gram-positive organisms including bacterial spores. In a recent international patent application by Blackburn et al. (2), it was reported that nisin used in combination with a chelating agent exhibited a bactericidal effect towards both gram-positive and gram-negative bacteria. Kordel and Sahl (5) showed that *Escherichia coli* exhibited nisin sensitivity when the outer membrane could be altered by treatments such as osmotic shock. In the United States, nisin has received GRAS (generally recognized as safe) status and is approved for use in some processed cheese spreads to prevent the outgrowth of clostridial spores and toxin production. In addition, nisin has been used to inactivate thermophilic spoilage organisms in canned goods and to extend the shelf life of milk and dairy products (3). The bactericidal action of nisin occurs in the cytoplasmic membrane of vegetative cells (6). Cellular damage can range from the loss of the proton motive force to the disruption of the cellular integrity of the membrane. Recently, Liu and Hansen (7) demonstrated that the dehydroalanine and dehydrobutyrine residues in active nisin play a direct role by acting as electrophilic Michael acceptors toward nucleophiles in the cytoplasmic membrane.

The outer membrane of gram-negative bacteria acts as a permeability barrier for the cell. It is responsible for preventing molecules such as antibiotics, detergents, and dyes from reaching the cytoplasmic membrane (8). Gram-negative bacteria are not generally sensitive to nisin (4, 9). Although the cytoplasmic membrane should be susceptible, the outer membrane protects the cell by excluding nisin (6). Magnesium ions serve to stabilize the lipopolysaccharide layer of the outer membrane. Chelating agents, such as EDTA, bind magnesium ions in the lipopolysaccharide layer and produce cells with increased susceptibility to antibiotics and detergents (8). In this study, we evaluated the effect of nisin against salmonellae and other gram-negative bacteria in which the outer membrane of the cell was compromised. Nisin treatments, applied in combination with EDTA, were effective against several gram-negative bacterial species, including environmental isolates and strains from the American Type Culture Collection.

MATERIALS AND METHODS

Strains. Test strains were obtained from environmental and type culture collections (Table 1). Cultures were maintained in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) stored at 4°C and were transferred to fresh BHI broth 24 h prior to experiments.

Nisin. Purified nisin (Aplin & Barrett Ltd, Dorset, England) was stored at 4°C in a desiccator. Stock solutions of nisin were prepared at a concentration of 1 mg/ml, suspended in 0.02 N HCl (pH 2), and stored at −10°C for up to 2 months. Nisin activity was verified by using *L. lactis* subsp. *cremoris* ATCC 14365 and *Listeria monocytogenes* Scott A (North Carolina State Culture Collection, Raleigh, N.C.) in an inhibition assay based on that of Barefoot and Klaenhammer (1).

Cell buffer. Cell buffer was composed of 50 mM Tris-HCl (pH 7.2), 1 mM $MgSO_4$, 4 mM $CaCl_2$, 0.1 M NaCl, and 0.1% gelatin (all chemicals supplied by Fisher Scientific Co., Dallas, Tex.). The pH was adjusted with 10 M HCl or 3 M NaOH.

Sequential treatment. *Salmonella* species were propagated in BHI broth at 37°C for 2 h to a population density of approximately 5×10^7 CFU/ml (optical density at 600 nm, ~0.1) and centrifuged in an Eppendorf microcentrifuge at 14,000 rpm for 5 min. Cells (~5×10^7 CFU/ml) were resuspended in either cell buffer (pH 6.5) (2 tubes prepared) or 20 mM disodium EDTA in cell buffer (pH 6.5) (2 tubes

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TABLE 1. The effect of nisin-EDTA treatment[a] on gram-negative species

Organism	Log reduction of bacterial population[b] after treatment at 37°C for:	
	30 min	60 min
Salmonella anatum 1920-1[c]	3.3	4.1
Salmonella binza 1042-2[c]	3.2	3.2
Salmonella broughton 1002-2[c]	3.8	4.7
Salmonella choleraesuis ATCC 10708[d]	4.2	4.7
Salmonella derby 2630-1[c]	4.2	4.1
Salmonella enteritidis Puerto Rico #1[c]	2.5	3.6
Salmonella hadar 3503-2[c]	3.9	3.7
Salmonella havana 843-2[c]	3.8	3.9
Salmonella heidelberg[e]	3.3	4.3
Salmonella indiana 56[c]	4.4	4.3
Salmonella infantis 310-2[c]	3.7	5.3
Salmonella montevideo 2864-1[c]	3.4	3.2
Salmonella muenster 2613-1[c]	3.3	3.2
Salmonella newington 1002-3[c]	3.1	3.9
Salmonella othmarschen 290-3[c]	3.3	3.3
Salmonella thomasville 967-2[c]	3.9	4.2
Salmonella thompson 292-2[c]	4.7	5.0
Salmonella typhimurium ATCC 14028[d]	4.0	3.8
Salmonella typhimurium 83[c]	3.0	5.2
Salmonella worthington 206-4[c]	4.2	4.3
Enterobacter aerogenes ATCC 13048[d]	—[g]	5.4
Shigella flexneri ATCC 12022[d]	—	6.7
Citrobacter freundii ATCC 13048[d]	—	5.2
Escherichia coli O157:H7 HC 19386[f]	—	6.9
Escherichia coli ATCC 25922[d]	—	6.6

[a] 50 μg of nisin per ml and 20 mM disodium EDTA.
[b] Untreated cell populations ranged from 3.5×10^7 to 2.4×10^8/ml; nisin-EDTA-treated cell populations ranged from 9.0×10^0 to 2.0×10^5/ml. Other controls (cells suspended in 20 mM disodium EDTA in cell buffer and cells suspended in 50 μg of nisin per ml in cell buffer) showed no significant reduction in population (≤1-log-cycle reduction) compared with the untreated control. Results represent the average of two trials.
[c] Poultry isolate obtained from Frank T. Jones, Poultry Science Extension, North Carolina State University, Raleigh, N.C.
[d] American Type Culture Collection, Rockville, Md.
[e] Chicken isolate obtained from Nelson Cox, USDA Agricultural Research Service, R. B. Russell Agricultural Research Center, Athens, Ga.
[f] Human isolate obtained from Bureau of Microbial Hazards, Health and Welfare Canada, Ottawa, Ontario, Canada.
[g] —, time period not tested.

prepared). All four tubes were incubated at 37°C for 30 min. Cells were harvested by centrifugation for 5 min (all centrifugation was performed in an Eppendorf microcentrifuge) and washed once with cell buffer. Cells contained in one tube from each treatment above were resuspended in cell buffer and immediately transferred to pour plates of BHI agar (Difco). Cells from the remaining tubes (EDTA in cell buffer and cell buffer alone) were resuspended in 50 μg of nisin per ml in cell buffer (pH 6.5) and incubated at 37°C for 30 min. Cells were harvested by centrifugation (5 min), washed twice in cell buffer, and pour plated in BHI agar. Dilutions were performed with cell buffer. All plates were incubated at 37°C for 48 h before colonies were counted. All trials were replicated twice.

Simultaneous treatment. Bacterial test species were propagated in BHI broth at 37°C for 2 h to a population density of approximately 5×10^7 CFU/ml (optical density at 600 nm, ~0.1) and centrifuged for 5 min. Cells (~5×10^7 CFU/ml) were resuspended in one tube of each of the following: (i) cell buffer (pH 7.8) alone, (ii) 20 mM disodium EDTA in cell buffer (pH 8.0), (iii) 50 μg of nisin per ml in cell buffer (pH 7.0), and (iv) 50 μg of nisin per ml and 20 mM EDTA in cell buffer (pH 6.9). Cell suspensions were incubated for either 30 or 60 min at 37°C. The treated cells were harvested by centrifugation (5 min), washed twice with cell buffer, and immediately transferred to pour plates of BHI agar. Plates were incubated at 37°C for 48 h, and colonies were counted. All experiments were replicated twice.

RESULTS AND DISCUSSION

The inhibitory activity of treatments combining nisin and the chelating agent EDTA on *Salmonella* species was investigated. Inhibition was defined as greater than or equal to a 1-log-cycle reduction of the initial population. When EDTA treatment was followed sequentially by nisin treatment, only one of the *Salmonella* species tested exhibited sensitivity to nisin (data not shown). *Salmonella typhimurium* 83 showed a 1-log-cycle reduction in population. Cells treated with either EDTA or nisin alone were not inhibited (data not shown).

Upon simultaneous exposure to nisin and EDTA, inhibition was observed for all of the *Salmonella* species tested (Table 1). A 30-min exposure to 20 mM EDTA and 50 μg of nisin per ml in combination yielded a 2.5- to 4.7-log-cycle reduction for the species tested. When the exposure time was increased to 60 min, *Salmonella* species populations were reduced 3.2 to 5.3 log cycles. The control treatments (cell buffer alone, 20 mM EDTA in cell buffer, and 50 μg of nisin per ml in cell buffer) failed to inhibit cell populations for any of the species tested (data not shown). The log reductions of all replications were within 1 log cycle of each other.

Other gram-negative bacteria, including *Enterobacter aerogenes* ATCC 13048, *Shigella flexneri* ATCC 12022, *E. coli* O157:H7 HC 19386, *E. coli* ATCC 25922, and *Citrobacter freundii* ATCC 13048, were subjected to a 60-min exposure to the combination of 20 mM EDTA and 50 μg of nisin per ml. A 5.2- to 6.9-log-cycle decrease in population was observed for the species tested (Table 1). The control treatments (cell buffer alone, 20 mM EDTA in cell buffer, and 50 μg of nisin per ml in cell buffer) yielded no reduction in population for any of the species tested (data not shown). The log reductions of all replications were within 1 log cycle of each other.

These findings document the inhibitory activity of nisin, when used in combination with the chelating agent EDTA, against a wide variety of *Salmonella* species. In addition, this study extends the observations of Blackburn et al. (2). The data reported herein also suggest that inhibition of *Salmonella* species by a combination of nisin and EDTA is a time-dependent phenomenon, and the method of application (simultaneous versus sequential) is critical to achieving the desired effect. Increasing the nisin concentration above 50 μg/ml will, most likely, increase the magnitude of inactivation. Furthermore, the observed inactivation by nisin can be extended to other gram-negative bacteria. The observed population reductions by nisin are facilitated by the chelation of magnesium ions, present in the outer membrane, by EDTA. The removal of magnesium ions from the lipopolysaccharide layer of the outer membrane results in the loss of lipopolysaccharide and an increase in cell permeability (8). This increase in outer membrane permeability to nisin is proposed to facilitate inactivation of the cell via bactericidal action at the cytoplasmic membrane.

Applications involving simultaneous treatment with nisin and an outer membrane modifying-chelating agent such as

EDTA may be of value in controlling food-borne *Salmonella* species as well as other gram-negative pathogens in foods. The feasibility and practicality of this method for salmonella control are being investigated.

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