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Multimethod assessment of commercial nisin preparations

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Nisin is a GRAS preservative effective against several Gram-positive organisms including *Listeria monocytogenes*. Commercial preparations are usually fermentation products containing 2.5% pure nisin along with insoluble material which, in this study, was found to influence the quantification and activity of nisin under different conditions. Commercially available samples of nisin were tested for efficacy using various methods, such as well diffusion, time to turbidity, and GUS (where a reporter compound is induced in response to nisin). SDS-PAGE detected a single peptide band, corresponding with the molecular weight of nisin. Protein quantified using the Bradford method indicated that the carrier of some samples was proteinaceous. Though the activity of commercially available nisin preparations is indicated on the label, end users should determine the effect of changing their source of nisin.
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Introduction

Nisin is a commercially available preservative that has GRAS status [9]. It is used in products such as pasteurized processed cheese, salad dressing, and liquid whole eggs to inhibit the growth of Gram-positive microorganisms including *Listeria monocytogenes*. Nisin, a bacteriocin produced by *Lactococcus lactis* ssp. lactis, has been purified and is a 34-amino-acid peptide with a molecular mass of 3.5 kDa. Two naturally occurring forms of nisin are available: nisin A and nisin Z. Nisin Z differs from nisin A by substitution of an asparagine residue at position 27 instead of histidine. The variants have identical minimum inhibitory concentrations against a range of organisms, though nisin Z shows better diffusion properties in agar [3]. Pure nisin has been assigned an activity of 40×10^6 international units (IU)/g. Its usage level in food products cannot exceed 10,000 IU/g in the US but has no limit in other countries.

Commercial preparations, which are fermentation products, contain 2.5% nisin (1×10^6 IU/g). The preparations are available as powders that are not completely soluble. To standardize the activity between lots, the activity against *Micrococcus luteus* is assessed. The assay, known as the "well diffusion assay," is rather unsophisticated. Wells are punched into agar containing *M. luteus*, and 10-fold dilutions of the preparation are pipetted into the wells. The reciprocal of the highest dilution showing a zone of inhibition is defined as one activity unit [5]. The assay cannot quantify the amount of nisin in the preparation, and is dependent on the strain of the target organism and assay conditions. The use of 10-fold dilutions also limits the discriminatory power of this assay. Previous research has compared methods to assay nisin in foods; however, published information comparing different commercial preparations to pure nisin is lacking [8,10]. Using the well diffusion assay, Scott and Taylor [9] showed differences in activity of the commercial preparation Nisaplin and pure nisin. They speculated that the milk proteins in the preparation bound nisin, limiting antimicrobial activity. In the work presented, the role of the carrier material of different commercial samples is examined.

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Materials and methods

Nisin preparations

All nisin samples were stored in powdered form at 4°C. Two samples of pure nisin, with activity of 40×10^6 IU/ml, were kindly provided by Applin and Barret (Ambicin, Dorset, UK) and Chr Hansen (CHN, Horsholm, Denmark). Commercially available nisin was provided by Rhodia (Madison, WI) (hereafter referred to as Novasin I and Novasin II to indicate changes in the commercial production and processing of Rhodia's nisin). Nisin was also purchased from Sigma (SN, St. Louis, MO). Both Novasin I and Novasin II are nisin Z. CHN and Ambicin are nisin A. Sigma does not specify if the preparation is nisin A or Z. All three commercially available samples (Novasin I, Novasin II, and SN) had activities of 1×10^6 IU/ml according to the label. Stock solutions with 1×10^5 IU/ml activity were made by dissolving nisin in nisin diluent (0.75% NaCl, 0.02 N HCl, pH 3.0) [1] and were stored at 4°C for no longer than 72 h. All subsequent dilutions of nisin were made in nisin diluent.

SDS-PAGE

Tricine SDS-PAGE was performed based on the standard protocol recommended by Bio-Rad (Hercules, CA) for separation of proteins 1–1000 kDa with the following modifications. The acrylamide–bisacrylamide mixture was 30% acrylamide and 0.8% bisacrylamide, and was mixed with gel buffer, water, and glycerol in the ratio 4.9:5:3.5:2 for the running gel and 0.8:1.55:3.89 (no glycerol) for the stacking gel. Ten microliters of TEMED and 50 μl of ammonium persulfate (10% in H_2O) were added to each gel. A 50/50 mixture of 200 IU/ml nisin and Tricine SDS-PAGE buffer was boiled and 15 μl of each sample (approximately 2.5 μg/ml pure nisin) was added to each well in the gel. The gel was initially run at 25 V. When the dye reached the running gel, voltage was

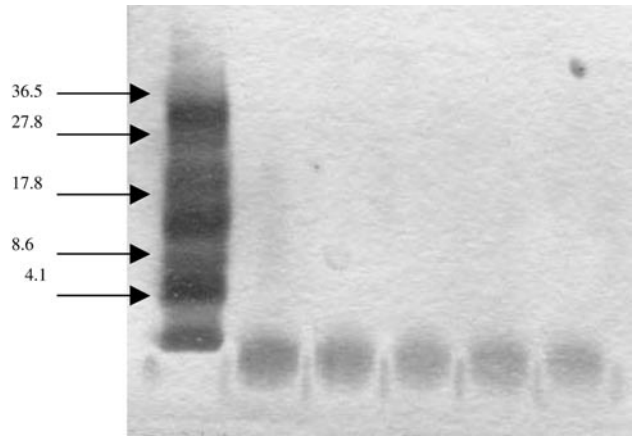

Figure 1 SDS-PAGE of nisin samples. From left to right: marker (kDa), SN, Ambicin, CHN, Novasin I, Novasin II.

increased to 150 V. The gel was stained according to the Bio-Rad Silver Stain protocol.

Bradford assay

The Bradford assay for protein determination was performed using Bradford Reagent (Bio-Rad) diluted five-fold in distilled water. Reagent (990 μl) was added to an Eppendorf tube containing 6 μl of distilled water and 4 μl of nisin sample, prepared to 5×10^4 IU/ml (1.25 mg/ml protein). The absorbance at 595 nm was monitored. Bovine serum albumin (Sigma) was used as the standard.

GUS assay

The assay is based on the protocol [7] described by de Ruyter *et al* [2], with some modifications. A plasmid (pNZ8008) [2] conferring resistance to chloramphenicol and containing a nisin-inducible promoter fused to the *gus*A gene was introduced to *La. lactis* by electroporation. Successful transformation was confirmed by growing cells on M-17 agar containing 0.5% glucose, 5 μg/ml chloramphenicol, 0.5 mM 5-bromo-4-chloro-3-indolyl glucuronide, and 20 IU/ml nisin. Colonies of transformed cells turned blue on the plates, were isolated, grown in CGM-17 broth (M-17 broth supplemented with 5 μg/ml chloramphenicol and 0.5% glucose), and stored with 30% glycerol at -70°C. Before each assay, frozen cells were revived in CGM-17 overnight at 30°C and diluted 1:10 in prewarmed fresh broth for 90 min. Nisin was added to fresh culture in a 9:1 ratio and incubated an additional 90 min. Cells were collected by centrifugation for 5 min, were resuspended in 200 μl of 1 M sodium phosphate buffer, pH 7, and 25 μl of a 9:1 acetone:toluene mixture and incubated for 20 min. Lysed cells (100 μl) were added to 2.85 ml of GUS buffer (50 mM sodium phosphate buffer, pH 7, 10 mM disodium EDTA, pH 8, 0.1% Sarcosyl, 0.1% Triton X-100, and 10 mM β-mercaptoethanol) and 189 μl of 2 mM 4-methylumbelliferyl β-D-glucuronide. The reaction was monitored using a Perkin Elmer LS 50B luminescence spectrometer under the following conditions: excitation slit 2.5 nm, excitation wavelength 325 nm, emission slit 2.5 nm, emission wavelength 435 nm. There was approximately 8 min between the addition of lysed cells to the substrate and the absorbance measurement.

Well diffusion assay

An overnight culture of *M. luteus* ATCC 10240 (30°C, buffered brain heart infusion broth, pH 6.2) was used to inoculate Nutrient Agar (final cell concentration $\sim10^6$ cfu/ml) tempered to 45°C. Agar plates were allowed to solidify and 7-mm wells were punched. Nisin samples (45 μl) were added to the wells and the plates were incubated at 30°C. Duplicate plates were used for each sample. After 24 h, zones of inhibition were measured from the center of the well to the edge of cell growth using a ruler. Differences between plates were never greater than 0.5 mm, and the experiment was independently repeated.

Ninety-six-well liquid assay

Stationary phase *L. monocytogenes* Scott A (30°C; BHI) was diluted 1:100 with fresh medium to approximately 5×10^6 cfu/ml. Cells (180 μl) were added to a 96-well microtiter plate containing 20 μl of a 10× nisin solution in nisin diluent. The plates were covered and incubated at 30°C. Absorbance at 630 nm was read every 30 min for 24 h by a temperature-controlled Dynex plate reader with Revelation software.

Statistical analysis

Differences between nisin samples determined by the GUS assay were difficult to quantify since the assay is time-dependent and the complete set of samples could not be tested in each experiment. To overcome this, CHN (arbitrarily chosen) was tested in each assay and the values were treated as the baseline. The intensity value of CHN at each specific nisin concentration was assigned a value of one. For that concentration in that trial, other samples were expressed as a percentage of the CHN value. SN, Ambicin, Novasin I, and Novasin II were then compared using a one-way ANOVA (Excel, Silicon Valley, CA) for each concentration.

Results

SDS-PAGE

SDS-PAGE was used to determine if any contaminating proteins were present in the samples. Although the commercially available preparations are fermentation products, no bands were observed between the 36.5- and 4.1-kDa markers. There was only one broad band below the 4.1-kDa marker in each sample (Figure 1). This is consistent with the molecular weight of bacteriocins.

Bradford assay

Ambicin and CHN, the pure nisin samples, as well as SN, had similar protein concentrations before centrifugation. Preparations of Novasin I and Novasin II had more than quadruple the protein concentration of the other samples, but centrifugation and removal of the insoluble matter caused a drastic decrease in protein content (Table 1). Samples with precipitates had decreased protein concentration after centrifugation and removal of the

Table 1 Bradford determination of protein concentration of nisin samples

Sample	Protein concentration (μg/ml)	
	Not centrifuged	Centrifuged
Ambicin	144.0	137.8
CHN	155.5	99.0
SN	130.6	68.8
Novasin I	671.8	84.9
Novasin II	641.1	97.4

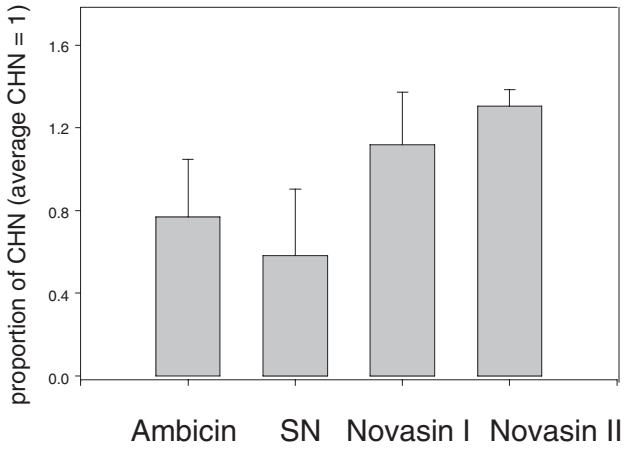

Figure 2 GUS assay response of samples relative to CHN at a concentration of 0.909 IU/ml nisin. Error bars represent 1 SD.

precipitate. The protein content in the CHN sample was decreased by about one third, and about half of the protein in the SN preparation was lost due to centrifugation. Ambicin changed the least after centrifugation and as also the only one that did not have a precipitate.

GUS assay

There was a linear relationship between the intensity signal and nisin concentration in the range 0.01–1 IU/ml for all samples. Denatured nisin (pH 8, boiled 15 min) did not give a signal in the same concentration range. When data were expressed as a percentage of CHN (used as the baseline in each assay), ANOVA indicated differences between the remaining four samples at concentrations of 0.196, 0.467, and 0.909 IU/ml (P=0.026, 0.024, and 0.037, respectively). Figure 2 shows the mean intensity and standard deviation of each sample compared to CHN. The 95% confidence intervals for the mean intensity of SN (0.332–0.944) and Novasin II (1.229–1.384) relative to CHN do not include 1.00, showing that they were significantly different from CHN. When

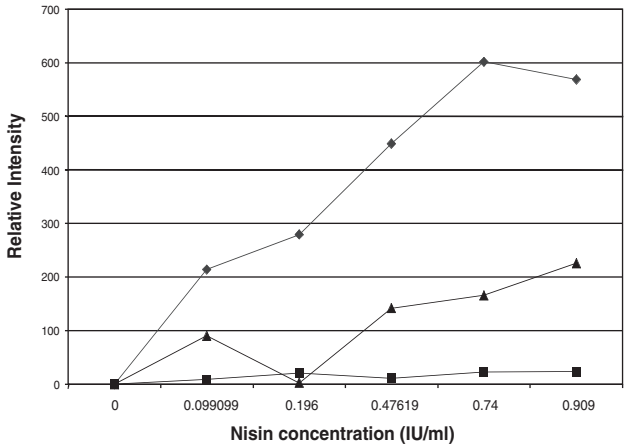

Figure 3 Nisin activity in the components of Novasin II as detected by the GUS assay. (◆) Novasin II; (■) supernatant; (▲) insoluble component (resuspended).

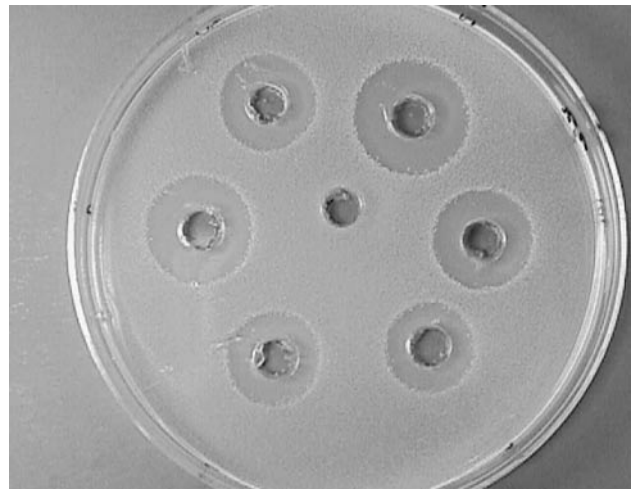

Figure 4 Well diffusion assay using *M. luteus* as an indicator to show the effect of centrifugation and washing of 1000 IU/ml Novasin II. Numbers in parenthesis are average size in millimeters ±SD of duplicate plates in replicate experiments. Clockwise from top left: supernatant (4.0±0.9), Novasin II (6.6±1.0), solid (4.3±0.7), wash 3 (4.2±0.6), wash 2 (4.2±0.6), wash 1 (5.9±0.6) (diluent control in center).

Novasin II was centrifuged and the supernatant and the resuspended solid were independently assayed, the solid portion gave a much stronger signal than the supernatant indicating that activity was associated with the insoluble matter (Figure 3).

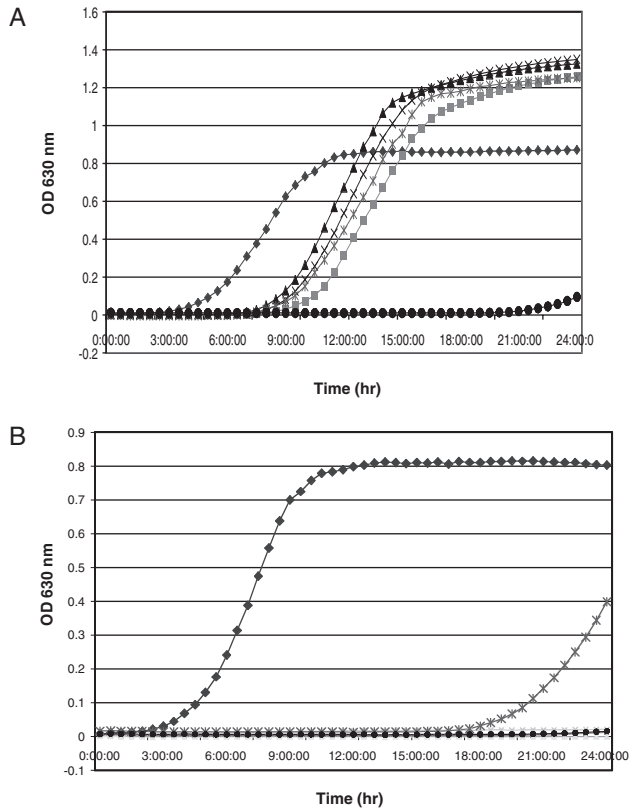

Figure 5 Response of *L. monocytogenes* Scott A to 25 IU/ml nisin (Panel A) or 50 IU/ml nisin (Panel B) at 30°C. (◆) Control; (∗) Novasin I; (●) Novasin II; (×) SN; (■) Ambicin; (▲) CHN.

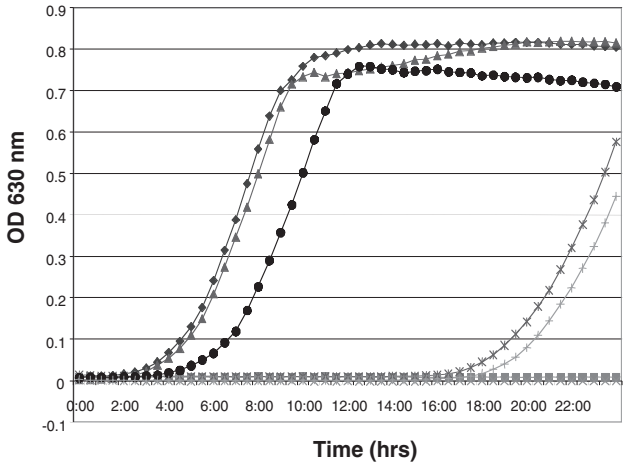

Figure 6 Response of *L. monocytogenes* Scott A to components of Novasin II, 100 IU/ml. (◆) Control; (■) Novasin II; (▲) supernatant; (×) wash; (∗) wash 2; (●) wash 3; (+) solid.

Well diffusion

Neither sodium dodecyl sulfate nor 5 M urea was able to solubilize the insoluble matter in the preparations (data not shown). Since nisin, but not the insoluble matter, can diffuse through the agar, the zones of inhibition represent only the activity of freely soluble nisin molecules. Initially, *L. monocytogenes* was used since it is the target pathogen for nisin activity. When *L. monocytogenes* was the target organism in the well diffusion assay, zones were very small and difficult to measure, even when up to 5000 IU/ml was used. Therefore, the sensitive indicator *M. luteus* was used. Irreproducible zones were obtained using *M. luteus*, except that Novasin II always gave the largest zone. This assay was better able to detect more drastic differences in nisin concentration. Since the GUS assay showed that nisin associated with the insoluble material caused a significant signal, the components were also used in the well diffusion assay to ensure that the nisin was still biologically active (Figure 4). Application of the supernatant caused a barely detectable zone, whereas the insoluble portion (resuspended to the original volume) caused a large zone. The resuspended component was centrifuged again, and the decanted liquid collected is referred to as "Wash." In a separate experiment, the solid portion was washed with nisin diluent multiple times (washes 1, 2, and 3). The final insoluble pellet was resuspended and is referred to as "Solid." Each experiment was repeated at least twice, using duplicate plates, and the average and standard deviations of the well sizes are given in the legend of Figure 4. Assays of the fractions consistently demonstrated that more nisin was released during the first wash than during the subsequent washes. Less nisin was present in the supernatant than was released during the first wash. After three washes, nisin still remained associated with the solid material.

Ninety-six-well assay

Growth curves of *L. monocytogenes* in the presence of nisin were generated by recording the increase in optical density over time. At 25 IU/ml, only Novasin II inhibited *L. monocytogenes* for more than 24 h (Figure 5A). Novasin II, CHN, SN, and Ambicin inhibited cells at a concentration of 50 IU/ml (Figure 5B). Novasin I increased lag time; however, after 18 h, the optical density began to increase, indicating the commencement of exponential phase.

The activity of the supernatant and washes of Novasin II were also assessed, and as shown in Figure 6, the supernatant caused almost no inhibition, whereas the first wash completely inhibited *L. monocytogenes*. The second wash solution was inhibitory to a lesser degree, as was the solid resuspended after three washes. The solution collected from the third wash had virtually no activity.

Discussion

All samples contained varying amounts of pure nisin. SDS-PAGE confirmed the presence of a protein in the molecular weight range expected for nisin. The Bradford assay gave much lower protein concentrations than the labeled concentration of nisin in each preparation (1.25 mg/ml) (Table 1). This is because bovine serum albumin, a large globular protein, binds the dye of the reagent differently than nisin. The loss of measurable protein after centrifugation indicates that the insoluble matter in Novasin I and Novasin II is proteinaceous or has a protein component. These proteins may have been too large to be detected in SDS-PAGE, since the highest molecular mass marker is 36.5 kDa. Additionally, large insoluble proteins may have been retained in the stacking gel. Nisin, which is known to undergo electrostatic or hydrophobic interactions with other proteins, may have been lost during centrifugation due to interaction with the insoluble material. Therefore, the protein content of Novasin I and Novasin II after centrifugation as determined by the Bradford assay may not accurately quantify the amount of nisin in the whole sample. The presence of protein in the carrier prevents a direct relationship between protein content and nisin to be established using the Bradford assay. Before centrifugation, the estimated protein concentration is the sum of nisin and carrier proteins. After centrifugation, protein concentration should correspond only to nisin (according to the single band obtained with SDS-PAGE), but will be underestimated if nisin bound to the carrier was removed during centrifugation.

Other studies have shown that the C and N termini of the nisin molecule play a role in pore formation [6]. If nisin is bound to solid carrier in Novasin II, it may be able to induce a response in the GUS assay but may not be able to interact with cell membranes to form pores. To investigate this, the Novasin II pellet was washed several times to release noncovalently bound nisin. Results of the well diffusion assay (Figure 4) show that nisin can dissociate from the solid and is active against target cells.

The well diffusion assay was not useful for determining differences among different nisin preparations, except that Novasin II always gave the largest zone. Results from the GUS and Bradford assays suggest that the differences among the preparations may have been below the sensitivity level of the well diffusion assay. Gross differences, however, could be observed using this technique. The activity of the soluble and insoluble components of Novasin II could be detected using the well diffusion assay. When nisin diluent was added to Novasin II powder, most of the nisin remained associated with the insoluble material. The association was not permanent, as is seen by activity in subsequent washes. Other components of Novasin II, such as salt, may be dissolved during the initial addition of nisin diluent to the powder, resulting in an environment that favors the reversible association of nisin with the insoluble protein. When the supernatant containing the substance inhibitory to nisin release is removed, and the sample is resuspended in fresh diluent, the equilibrium may shift and allow nisin to dissociate from the solid.

The advantage of the 96-well turbidity assay is that any problems with diffusion of insoluble matter are eliminated.

However, at high concentrations of SN, Novasin I, or Novasin II, the turbidity introduced by the preparations can be problematic. This assay also showed the greatest differences between preparations. However, since the assay measures turbidity, bactericidal and bacteriostatic effects cannot be distinguished. At 25 IU/ml nisin, a relatively low concentration, Novasin II almost completely inhibited growth while the nisin preparations caused similar increases in lag time (Figure 5A). Ambicin caused a greater lag time than CH, SN, and Novasin I; cells treated with Ambicin grew at a slightly slower rate. Since the GUS assay showed that Novasin II contained more nisin than the other preparations, it is possible that the Novasin II contained more active nisin than the 25 IU/ml calculated according to the label.

The methods employed showed that there was little difference among nisin preparations, except that Novasin II consistently showed greater activity. The GUS assay was the only method used that could quantify nisin concentration. It showed that Novasin II contained more nisin than the other samples, which may explain the apparent increased activity. While the interference of food components on the availability of nisin has been documented [4], this research is the first to conclusively show, using the GUS assay, that nisin may bind not only to food, but also to the carrier. Because of the carrier effect, food processors need to validate efficacy before changing sources of nisin.

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