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Research Note

Interactions of Nisin with Glutathione in a Model Protein System and Meat

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ABSTRACT

Loss of nisin activity in meat has been ascribed, in part, to the formation of a nisin-glutathione adduct. Activity is lost more quickly in raw meat than in cooked meat, and this has been taken as evidence that the reaction is enzyme mediated. Formation of the nisin-glutathione adduct has been confirmed but is shown not to be enzyme mediated. Retention of activity in cooked meat is shown to be due to the loss of free sulfhydryl groups during cooking as a result of the reaction of glutathione with proteins and not a result of the inactivation of endogenous enzymes. Microbial enzymes do not appear to play a role, as similar losses are seen in raw and cooked meat extracts, both of which contained undetectable levels of microorganisms.

Nisin is a broad-spectrum bacteriocin produced by strains of the lactic acid bacterium *Lactococcus lactis* subsp. *lactis.* It belongs to the Ia subgroup of bacteriocins known as lantibiotics and consists of 34 amino acids with a molecular weight of 3,354 Da. *(5).* It exhibits antimicrobial activity towards a wide range of gram-positive vegetative bacteria and is particularly effective against bacterial spores *(5).*

Although used successfully as a preservative in numerous food systems, nisin demonstrates variable effectiveness in meat products, where organisms that show nisin sensitivity in vitro are found to be less sensitive when growing in meat. A number of possible reasons for this have been identified. These have included the nature of the spoilage microflora and preservation parameters *(7),* nisin binding to meat proteins *(1–4, 17),* degradation by meat proteases *(1, 16),* uneven mixing *(4),* adsorption by fat *(1–3, 5, 11, 13),* and pH conditions *(4, 17).*

Glutathione (GSH) (γ-glutamyl-cysteinyl-glycine) is a 307-Da thiol-containing tripeptide. It is found in mammalian cells, plants, and microorganisms and at levels of 156 to 627 nmol/g wet weight in beef, chicken, and pork *(22)* but not in dairy products. It is a strong reducing agent important for cellular defense, especially against free radicals and hydroperoxides. The free sulfhydryl group of GSH has been proposed as the binding site for nisin in fresh meat *(20).* Its reaction with the dehydro amino acid residues of nisin yields GHS S-conjugates and this has been found to be partially mediated by a GHS S-transferase (GST), which constitutes about 0.3% of the total soluble protein in bovine muscle *(18).*

Here we report an investigation which partially supports this observation but indicates that the reaction is nonenzymatic and provides an alternative explanation for the observed enhancement of nisin activity in cooked meats.

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MATERIALS AND METHODS

Bacterial strains and media. The organisms used for this study were *Micrococcus luteus* NCIB 8166 and *Listeria monocytogenes* NCTC5105. All bacteria were stored frozen on beads (Protect, Technical Service Consultants Ltd., Heywood, Lancashire, UK) at −80°C. For resuscitation, one bead was added 10 ml of brain heart infusion broth (*L. monocytogenes*) or nutrient broth (*M. luteus*) and incubated at 30°C for 24 h.

All microbiological media were supplied by Oxoid (Basingstoke, Hampshire, UK), unless otherwise stated. For serial decimal dilutions, maximum recovery diluent (MRD; 0.85% NaCl and 0.1% bacteriological peptone) was used.

Preparation of stock solutions. A stock solution of nisin was prepared by dissolving 0.01 g of purified nisin (50×10^6 IU of nisin per g, batch no. NP 148-149, Danisco, Beaminster, UK) in 50 ml of sterile 0.02 M hydrochloric acid to give an activity of 10^4 IU of nisin per ml. The solution was then sterilized by filtration through a 0.2-μm membrane (Minisart, Sartorius, Hannover, Germany).

A stock solution of GSH was prepared by dissolving 3.04 g of GSH lyophilized powder (G6529, Sigma, Gillingham, UK) into 20 ml of sterile 50 mM phosphate buffer (pH 6.5) to give a concentration of 500 mM. The pH of the solution was then adjusted to 6.5 by using NaOH and sterilized by filtration through a 0.2-μm membrane.

GST (G6511, Sigma) has an activity of 50 to 100 U/mg of protein and a molecular weight of approximately 40 kDa. A stock solution of 1 mM was prepared by dissolving 1 mg of the enzyme to 1 ml of sterile 50 mM phosphate buffer, pH 6.5. Its activity was confirmed by using the supplier's protocol.

Measurement of nisin activity. Nisin activity was measured by the horizontal agar diffusion method *(21).* In a sterile bottle, 5

ml of Tween 20 (P-7949, Sigma) and 5 ml of sterile MRD were mixed gently. The mixture was placed in an incubator at 55°C for 30 min. Five milliliters of the Tween-MRD suspension and the 3.8 ml of the *M. luteus* NCIB 8166 suspension were mixed gently with 250 ml of tempered Iso-sensitest agar (Oxoid). The mixture was aseptically poured over the surface of a glass bioassay plate of 3- to 4-mm depth, which had been previously sterilized with industrial methylated spirit. The agar was left to set, and the dried plate was placed in the refrigerator for approximately 45 min, as chilling facilitates the well-preparation stage. With a sterilized borer of 9-mm diameter and allowing 30 mm between adjacent wells and 20 mm between peripheral wells and the edge of the assay plate, 64 to 91 preassigned holes were made on the agar according to a random-number matrix board that was positioned underneath the assay plate. The agar plugs were then removed with a sterile spatula, and the wells were filled with equal volumes of the appropriate samples and five nisin reference standards (20 IU/ml, 10 IU/ml, 5 IU/ml, 2.5 IU/ml, and 1.25 IU/ml). Nisin standards were used for the preparation of a five-point standard curve. Sample extracts were diluted in order to fall in the range of the standard curve. The loaded plate was incubated at 30°C for 18 h. At the end of the incubation period, the diameter of inhibition zones formed around the wells was measured with digital calipers (CamLab Ltd., Cambridge, UK). All samples and standards were tested in quadruplicate, and the nisin concentration was calculated from the mean inhibition zone produced by each extract or standard by reference to the five-level standard curve.

Nisin activity in meat. Lean minced beef was purchased locally and was divided into two sachets of 500 g. Each was dosed with 500 IU of nisin per g by adding 25 ml of the nisin stock solution, and the mixture was blended manually to ensure that nisin was distributed as uniformly as possible. One was immediately transferred to a water bath of 72 to 73°C temperature. The temperature was monitored with a digital thermometer placed in the middle of a sachet. Once the temperature had reached 70°C, it was heated for another 2 min. Samples of 50 g of each portion were transferred to sterilized sachets, vacuum packed, and stored at 8°C. Initial extractions of both raw and heated samples were conducted immediately after their preparation. The remainder of two portions was then sampled periodically for nisin extraction. Sample extracts were stored at −18°C before their nisin content was measured.

Nisin extraction. Nisin was extracted from all the samples with water. Ten-gram portions of each sample were blended in a stomacher bag containing 90 ml of water until the sample was evenly suspended (Stomacher 400 laboratory blender, Seward, London, UK). Extracted samples were adjusted to a pH of 4 ± 0.05 with 5 M NaOH and filter sterilized. The extract was added to the wells of the bioassay plate.

Preparation of meat extract. Lean ground beef meat (320 g) was obtained from a local supermarket (fat content ca. 4.5%) and blended at maximum speed with 5× volume of phosphate buffer (100 mM, pH 7). The blended mixture was then centrifuged (model J2-21M/E, Beckman, Fullerton, Calif.) at 4°C for 20 min at 10,000 × *g*, and the supernatant was filtered through glass wool to remove floating lipids. Volumes of 150 ml of the meat extract were transferred aseptically into 250-ml Duran bottles, and 500 IU of nisin per ml was added to each as 7.5 ml of the previously prepared stock solution. After the addition of nisin, one of the bottles was transferred to a water bath at 72 to 73°C. The temperature was monitored with a digital thermometer placed in the middle of the Duran bottle. Once the temperature had reached 70°C, it was heated for another 2 min. Raw and heat-processed meat extracts were stored at 8°C.

Microbial counts. Ten-milliliter samples of raw and heated meat extracts were aseptically removed and blended in a stomacher bag with 90 ml of MRD for 60 s in the stomacher lab blender. A decimal serial dilution prepared from this initial dilution was used to prepare replicate plate count agar (CM325, Oxoid) plates upon which 0.1 ml of dilutions was spread plated. Counts were enumerated after incubation at 25°C for 72 h.

Inactivation of nisin by GSH. Appropriate amounts of nisin, GSH, and GST stock solutions were added to 50 mM phosphate buffer (pH 6.5) in 50-ml sterile polypropylene tubes to produce final concentrations of 2,000 IU of nisin per ml, 1 μM of GST, and 50, 125, and 250 mM of GSH, respectively. Controls containing the same concentrations of nisin and GSH in the absence of the enzyme were also set up. The experiment was run at two temperatures, 20°C (close to the enzyme's optimum temperature of 25°C) and 4°C to represent the chill storage of meat. Samples were taken at time zero, after 12 and 24 h of storage at 4 and 20°C, respectively.

GSH heat stability. GSH was measured in sterile 50-ml polypropylene tubes containing 50 mM phosphate buffer of pH 6.5. Appropriate amounts of the stock solutions were added to produce final concentrations of 2,000 IU of nisin per ml and 50 and 250 mM of GSH. Samples were heated for 15, 30, and 60 min at 72°C and for 5 min in boiling water. Additional unheated samples containing the same nisin and GSH concentrations were prepared as controls. Heat-treated samples and controls were incubated at room temperature (20°C) for a period of 24 h. Sampling and water extractions of residual nisin were conducted at the beginning and at the end of incubation.

MALDI-TOF MS. All mass spectra were acquired on a linear matrix-assisted laser desorption/ionization–time of flight mass spectometry (MALDI-TOF MS) equipped with pulsed ion extraction technology (Proflex III, Bruker, Billerica, Mass.) and a 125-cm flight tube, in a positive ion linear mode with a nitrogen laser (λ = 337 nm) for desorption and ionization of the samples and an acceleration voltage of 20 kV. Samples were prepared for MALDI-TOF MS analysis according to the method described by Rose and others *(20)*. A volume of 0.5 μl of the extract was placed on a stainless steel MALDI-TOF probe and allowed to air dry. The probe was then dipped into MilliQ water for 30 s to remove water-soluble contaminants. Excess water was shaken off, and the sample was left to dry. Then 0.5 μl of a saturated solution sinapinic acid (Sigma Chemical, Basingstoke, UK) in a solution containing two parts 0.1% trifluoroacetic acid and one part acetonitrile was added to the sample spot and allowed to air dry. Bovine insulin (MH^+ = 5,734.557; $MH22^+$ = 2,867.782), obtained from Sigma Chemical, was used as a calibrant for external mass calibration. The spectra represent the result of 50 consecutive laser shots.

Inhibition of *L. monocytogenes* by nisin in the presence of BSA and GSH. The ability of nisin to inhibit the growth of *L. monocytogenes* NCTC5105 in 5 mM GSH solutions that had been heated at in the presence or absence of 10% (wt/vol) bovine serum albumin (BSA) was examined. Briefly, 5 mM of GSH was heated at 72°C for 2 min in the presence or absence of BSA (10% wt/vol). Nisin (from stock solution) diluted in double-strength brain heart infusion broth (Oxoid) was added after mixtures had cooled down to room temperature to give a final concentration of 50 IU of nisin per ml. Test and control samples were incubated

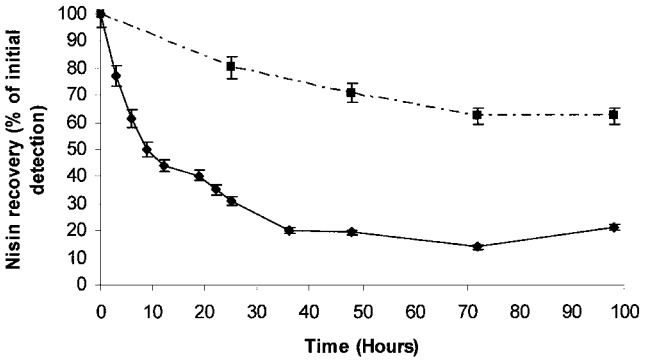

FIGURE 1. *Loss of water-extractable nisin content in lean vacuum-packed minced beef stored at 8°C. ♦, Raw meat; ■, heated meat (70°C, 2 min). Each point represents the mean ± standard deviation of three replicate samples assayed in quadruplicate.*

at 20°C for 24 h, and then an overnight culture of *L. monocytogenes* was diluted properly in MRD to give a final inoculum of approximately 10^3 CFU/ml. Changes in the population of *L. monocytogenes* were monitored over a period of 40 h at 30°C.

Measurement of free thiol content in BSA-GSH solution. The content of free thiol groups was measured in heated (72°C for 2 min) and nonheated BSA (10%, wt/vol) solutions with 2.5, 1.25, and 0.625 mM GSH. Solutions containing the same concentrations of GSH only and BSA only were also prepared and treated similarly. Standards of known GSH concentrations were prepared and used as reference for the calculation of the thiol contents of the test solutions. The thiol content of test and standard samples was determined by using Ellman's method *(6).* Briefly, 3 ml of sample extracts prepared in 80% ethanol were mixed with 2 ml of 100 mM phosphate buffer (pH 8) and 5 ml of deionized water. Then, 0.2 ml of 5,5′-dithiobis(2-nitrobenzoic acid) (Sigma) dissolved in 100 mM phosphate buffer (pH 7) was added to 3 ml of the previously prepared mixture in a photometer cuvette. Color developed within 2 min, and the absorbance was measured at 412 nm.

Statistical evaluation. For all experiments, three independent trials were conducted and measurements were performed in duplicate, unless otherwise stated. Statistical evaluation of results was performed with SPPS package v.11.0 for Windows using analysis of variance and Student's *t* test.

RESULTS AND DISCUSSION

Fate of nisin in pasteurized and nonpasteurized minced meat. The free (water extractable) nisin contents in raw and pasteurized minced beef were determined during storage in vacuum packs at 8°C. Initial levels of free nisin in the raw and the pasteurized meat were 23 and 11%, respectively, of the nisin added. This initial loss was taken to represent the binding of nisin to meat proteins and heating *(1–4, 8, 16).* The subsequent rate of loss of free nisin in pasteurized minced beef meat was significantly slower than in raw meat. In raw meat samples, more than 50% of the free nisin present initially was lost within the first 10 h of storage (Fig. 1). After 24 h, nisin levels declined more slowly, levelling out at around 20% of the initial concentration. In contrast, the heat-processed meat retained almost 80% of the remaining nisin activity after 24 h of storage,

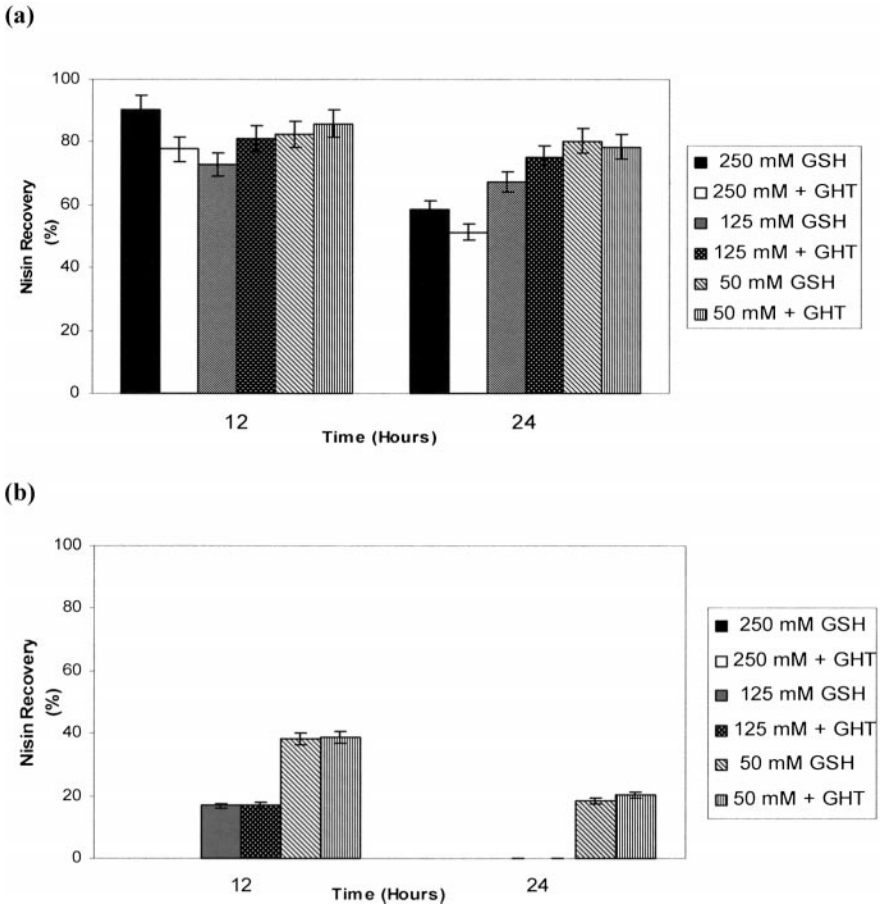

FIGURE 2. *Nisin recovery (%) following reaction with GSH in the presence or absence of GST at 4°C (a) and 20°C (b) for 12 and 24 h in phosphate buffer. Each point represents the mean of three replicate samples assayed in quadruplicate.*

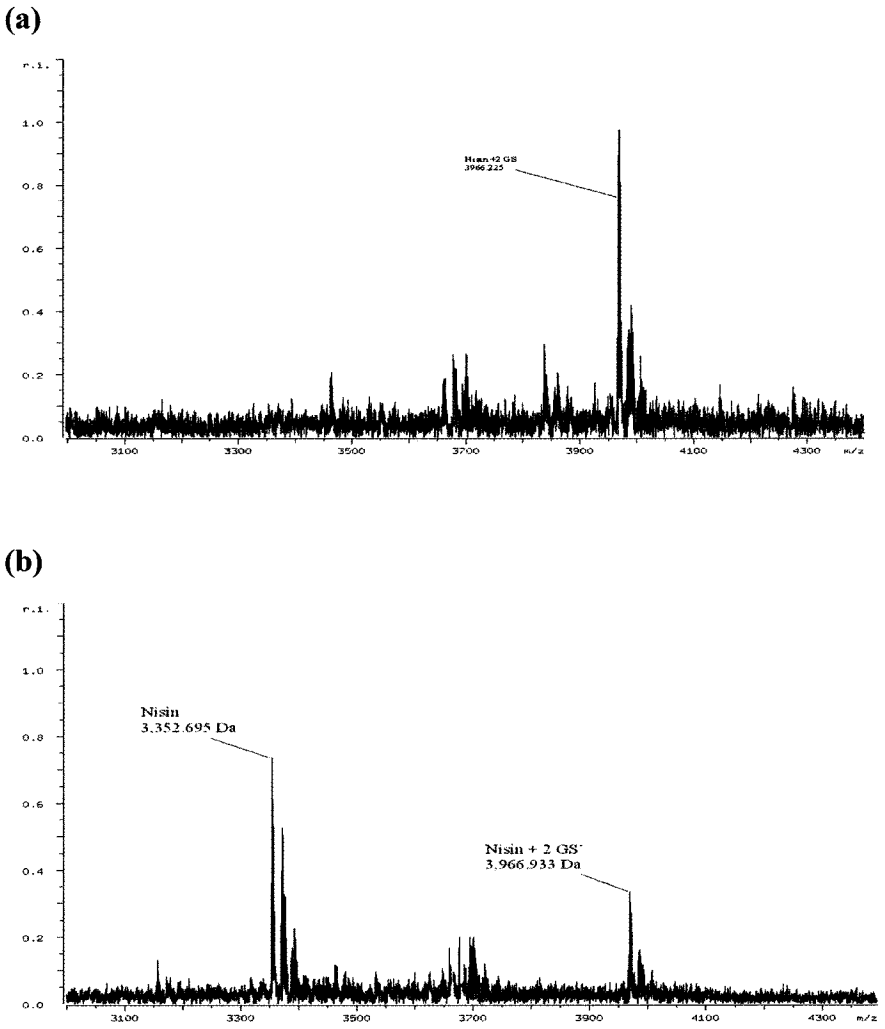

FIGURE 3. *Formation of the GSH-nisin adduct using MALD-TOF MS. Nisin 2000 IU/ml with (a) 250 mM GSH and (b) 50 mM GSH after 24 h in phosphate buffer at 20°C.*

and at the end of the fourth day, the same samples contained approximately 50% of the initial level (Fig. 1). A similar pattern of nisin loss was seen when a liquid meat juice was used instead of ground meat. The microbial count in the meat juice was undetectable ($<10^2$ CFU ml^{-1}) in both the heated and unheated samples over the first 24 h of storage at 8°C. Hence, the observed differences in nisin loss between heated and raw samples were not due to microbial action.

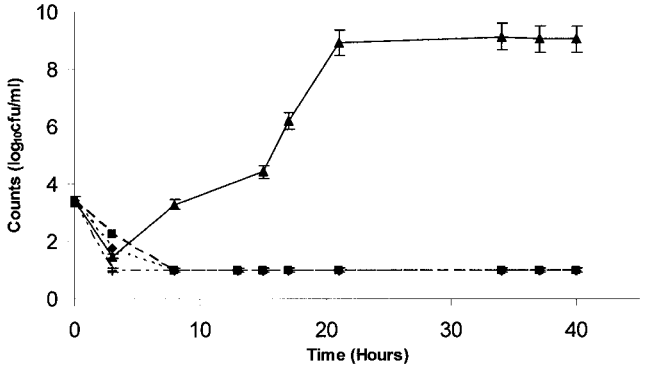

FIGURE 4. *Ability of nisin to inhibit growth of* L. monocytogenes *in brain heart infusion broth at 30°C containing GSH heated in the presence or absence of BSA.* ◆, *GSH plus BSA plus nisin;* ▲, *GSH plus nisin;* ■, *BSA plus nisin;* +, *nisin alone. Each point represents the mean ± standard deviation of two replicate samples assayed in triplicate.*

Nisin inactivation by GSH. Nisin inactivation by GSH in the presence or absence of the GST was evaluated at 20°C, close to the enzyme optimum *(18),* and at 4°C, representative of the storage temperature of chilled meat. The results shown in Figure 2 clearly confirm a loss of nisin activity in the presence of GSH, regardless of the presence of enzyme.

Formation of a GSH-nisin adduct after incubation for 24 h at 20°C in the absence of enzyme was confirmed by MALDI-TOF analysis, similar to the work of Rose and coworkers *(18).* With high levels of GSH (250 mM), the mass spectrum revealed a single peak of approximately 3,966 Da (≈3,353 + 614 Da) which corresponds to the addition of two molecules of GSH to one of nisin. The original nisin peak (3,352 Da) was not detected (Fig. 3a). With 50 mM GSH, two peaks of approximately 3,352 Da and 3,966.933 Da were detected corresponding to nisin and nisin plus two molecules of GSH (≈3,352 + 614 Da) (Fig. 3b).

The results show that formation of the nisin-GSH adduct occurs largely through a nonenzyme mediated reaction. At the higher temperature 20°C, in the presence of

TABLE 1. *Reduction of free thiol content of GSH plus BSA solutions immediately after heat treatment*[a]

Sample composition	Reduction of free thiol content after heat treatment (%)
2.5 mM GSH + 10% BSA	35.22 ± 0.02
1.25 mM GSH + 10% BSA	39.67 ± 0.01
0.625 mM GSH + 10% BSA	40.20 ± 0.03
2.5 mM GSH	5.84 ± 0.04
1.25 mM GSH	2.64 ± 0.03
0.625 mM GSH	6.6 ± 0.03
10% BSA	1.30 ± 0.02

[a] Values represent the mean ± standard deviation of three replicate samples measured in triplicate.

250 and 125 mM of GSH, formation of the adduct resulted in a complete loss of detectable nisin activity, with less than 1% of the initial nisin activity recovered from both enzymatic and control reactions after 24 h. Under conditions where residual nisin was detectable, there was no significant difference ($P > 0.05$) between levels in the enzyme treated samples and controls. At 4°C, using three concentrations of GSH and two sampling times, there was significantly greater loss of nisin in the presence of enzyme ($P < 0.05$) on only one occasion (250 mM GSH after 12 h); on all other occasions, there was no significant difference between the nisin loss with or without enzyme ($P > 0.05$).

GSH heat stability. Reduced loss of nisin in the cooked meat was not a result of degradation of GSH by heat. This was indicated by the fact that GSH solutions that had been heated at 72°C for 1 h or 100°C for 5 min retained the ability to inactivate nisin as measured by the nisin bioassay. With 250-mM GSH solutions, there was no significant difference in the loss of nisin when incubated with heated or unheated GSH solutions for 24 h ($P > 0.05$). The mean loss in nisin activity was 97%. At 50 mM GSH, the difference between heated and unheated solutions was again not significant with a mean loss of nisin activity loss of 74%.

Interaction of GSH with BSA during heating. Heating GSH with BSA removed the ability of GSH to reduce nisin activity. BSA is not a typical meat protein but was used as a simple experimental model because of its high solubility. Nisin (50 IU/ml) was able to inhibit *L. monocytogenes* when it was left for 24 h in a solution of GSH (5 mM) and BSA (10%) that had been heated together at 72°C for 2 min. No growth of *L. monocytogenes* was observed over 40 h of incubation at 30°C. However, when the same level of nisin was left with heated GSH for 24 h, it lost activity and *L. monocytogenes* was able to grow to levels in excess of 10^8 within 20 h (Fig. 4).

Heating GSH with proteins is known to reduce intramolecular disulfide bonds to sulfhydryl groups *(9, 12).* Reduction of disulfide bonds leads to conformational changes and subsequent exposure of hydrophobic regions, which promotes the interaction of proteins and leads to gel formation through new disulfide linkages *(10, 14, 15).* The consequent loss of free GSH in this way will reduce GSH-nisin adduct formation.

In our case, heating of GSH was shown to reduce significantly the levels of free sulfhydryl groups, indicating that GSH interacts with protein molecules during heating ($P < 0.05$) (Table 1). Controls containing GSH and BSA alone showed no significant change in thiol content after heat treatment ($P > 0.05$).

These results indicate that reduced nisin loss in heat-treated meat is due to lower levels of GSH in meat as a result of its binding to proteins during heat processing and not the result of heat inactivation of GST.

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