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Inhibition of *Listeria monocytogenes* on Pork Tissue by Immobilized Nisin

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ABSTRACT

Nisin was immobilized in 1% calcium alginate gels to test their ability to inhibit *Listeria monocytogenes* growth and their stability on cooked pork. Nisin added to calcium alginate gels and immobilized on cooked pork tenderloins inoculated with *L. monocytogenes* reduced the population more than did free nisin treatment alone. Nisin (10^4 IU/ml) immobilized in alginate reduced counts by 1.74 $\log_{10}$ units vs. 0.53 log units decrease from the free nisin treatment alone after 14 days at 4℃. Immobilized nisin at 5 x 10^3 IU/ml reduced 1.14 log units of *L. monocytogenes* compared to 0.49 log units with samples treated with free nisin at 4 ℃ after 14 days of incubation. The stability of nisin on cooked pork can be improved by alginate immobilization treatment which suggests this technique may have potential for meat decontamination.

Key words: Nisin, immobilization, *Listeria monocytogenes*, calcium alginate gels.

INTRODUCTION

The polypeptide nisin is a GRAS (generally recognized as safe) additive which shows antimicrobial activity against some Gram-positive bacteria[1]. It has been approved for use in pasteurized cheese spreads in the United States to prevent spore outgrowth and toxin production by *Clostridium botulinum*[2]. The action of nisin against *Listeria monocytogenes* have been reported. For instance, Fang and Lin[3-5] showed that growth of this pathogenic bacteria on raw and cooked pork was effectively prevented by nisin treatment. Although nisin is effective on the inhibition of *L. monocytogenes* growth, it is unstable on pork. Fang and Lin[3] reported that the activity of nisin remaining on cooked pork decreased rapidly after incubation at 20 °C. There also are problems of low solubility, uneven distribution, and lack of nisin stability on the meat surface.

Immobilization techniques in microbial processes have attracted increasing interest in the past decade. Several advantages of the immobilization technology have been reported[6-8]; for example, immobilized of whole microbial cells eliminates the use for isolating and purifying enzymes and provides more stable activity. Calcium alginate has been used as a food additive to reduce shrinkage loss of various foods such as lamb[9], chicken pieces[10] and beef[11]. Spettoli

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et al.[6] used immobilized whole cell of *Leuconostoc oenos* ML 34 with calcium alginate to carry out malolactic fermentation in wine, while Siragusa and Dickson[11] demonstrated the inhibition of *L. monocytogenes* by organic acids immobilized in a calcium alginate gel.

The objective of this investigation was to test the use of the commercial nisin immobilized on the surface of inoculated pork tissue. The antimicrobial activity of the immobilized nisin on the indicator strain *L. monocytogenes* and the stability of the nisin were examined in this study.

MATERIALS AND METHODS

I. *Cultures*

Listeria monocytogenes Scott A was obtained from the Culture Collection and Research Center, Food Industry Research and Development institute, Taiwan, Republic of China. *Lactobacillus sake* CCRC 14622 was obtained from the culture collection of Graduate Institute of Food Science and Technology, National Taiwan University, Taipei, Republic of China. *Listeria monocytogenes* and *Lactobacillus sake* were maintained on Tryptic soy agar (TSA, Difco) and on MRS (Difco) slants at 4 °C, respectively. Both strains of cultures were transferred monthly. For preparation of inoculum and standard curve, one loop of *L. monocytogenes* culture from TSA slant was streaked onto TSA plate and incubated for 24 h at 30 °C. A well isolated colony on the TSA plate was transferred into 100 ml of Tryptic soy broth (TSB, Difco) and incubated at 30 °C for 24 h. After the second incubation, *L. monocytogenes* was inoculated to 200 ml TSB at 1% inoculum (v/v). A standard curve of *L. monocytogenes* was prepared and the regression line determined as described by Fang and Lin[5]. The number of cells/ml of this pathogenic bacterium was adjusted by dilution.

II. *Preparation and Inoculation of Pork Tenderloins*

Pork tenderloins were obtained from Shinung Co., Taiwan, and were transported in ice to the laboratory and stored at -20 °C for 48 h. Samples were thawed at room temperature for 2 h and cut into pieces of 5.0 by 4.0 by 0.5 cm (about 12 g per piece) with a sterile knife before the experiments were conducted. The pork samples were then cooked at 121 °C for 15 min and the fat was aseptically drained from the sample. After cooling at 4 °C for 2 h, the samples were subjected to their respective treatments.

III. *Reagent Preparation*

One percent (w/v) sodium alginate (Wako Pure Chemical Industries, Ltd., Japan) was prepared in distilled water and autoclaved at 121 °C for 15 min. Ninety mM $CaCl_2$ (Hayashi Pure Chemical Industries, Ltd., Japan) was also prepared in distilled water and autoclaved at the same condition. Purified nisin (Aplin & Barrett Ltd., Dorset, England) was stored at 4 °C in a desiccator. A nisin working solution was prepared by dissolving one gram of nisin in 100 ml of double distilled water, giving 10^4 IU/ml, and was sterilized by filtration (0.2 μm Nylon membrane filter). Fresh-prepared nisin solution was used for the nisin immobilization treatment.

IV. *Nisin Immobilization and Experimental Protocol*

After the pork tenderloins were autoclaved and fat-drained as described above, each cooled sample was surface-inoculated[3] with 1.0 ml diluted *L. monocytogenes* culture to give initial populations of approximately 10^3 CFU/g. Immediately following 20 min of room temperature incubation, each sample was immersed in different concentrations of nisin solution (0, 5 x 10^3 and 10^4 IU/ml) for 1 min and then removed and allowed to drain. The inoculated pork tissue was attached to a sterile clamp and immersed in the alginate solution for 30 sec, then transferred to the $CaCl_2$ solution for another 30 sec. The treated sample was transferred to a sterile petri

dish and stored at 4 °C until assayed. Unimmobilized samples were not treated with alginate and $CaCl_2$ solution. At sampling time, 10 g of the duplicated samples were transferred aseptically to a sterile stomacher bag (Seward Medical Co., England). Then 90 ml of sterile physiological saline containing 0.1 % peptone (SP solution) was added to the bag, and the contents were stomached for 2 min (Model 400 BA7221 Seward Medical Co., England). The resultant slurry was diluted with SP solution or spread plated directly as appropriate onto Palcam medium (Oxide)[5] for *L. monocytogenes* enumeration. The plate diffusion assay method was used to determine the remaining nisin concentration on meat[13]. Each sample was placed in 120 ml of 0.02 N HCl (pH 2.0) and boiled for 5 min to release nisin. After centrifugation (750 x g, 5 min), the supernatant was used for assay. *L. sake* CCRC 14622 and brain heart infusion agar (soft agar, Difco) were used as the test organism and the assay medium, respectively.

RESULTS AND DISCUSSION

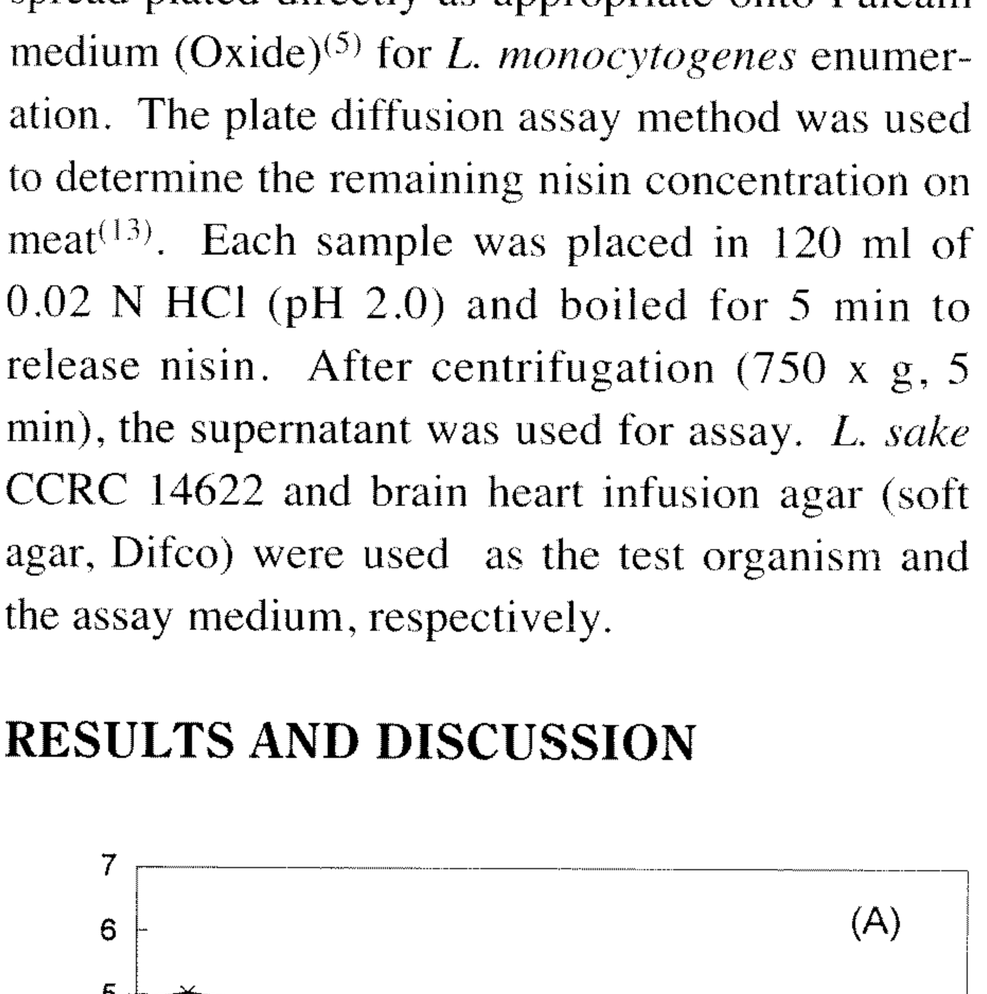

Figure 1. Effect of nisin on the growth of *L. monocytogenes* in TSB at 20 ℃. Nisin was added at 4.2 h after incubation.

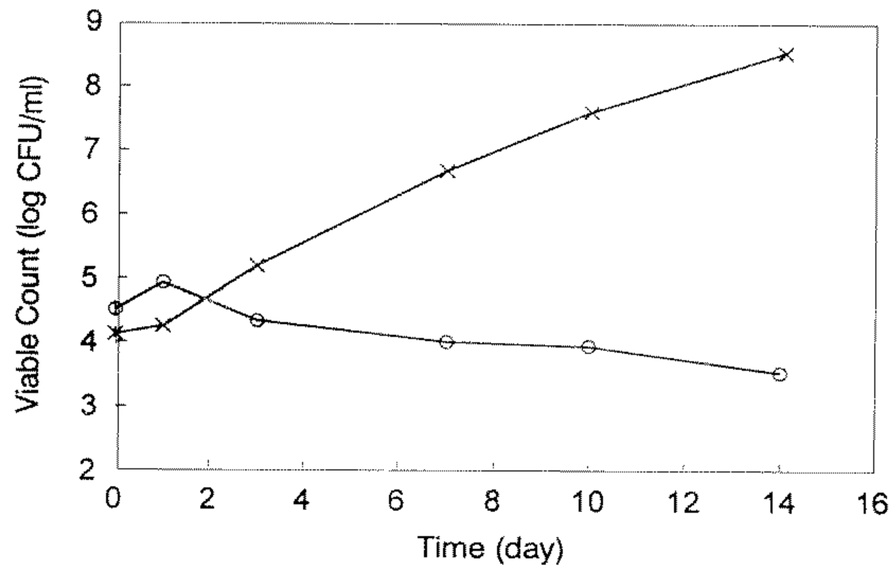

Figure 2. Bacteriocidal action of 500 IU nisin/ml on growth of *L. monocytogenes* in TSB at 20 ℃.

Immobilized nisin resulted in a greater reduction rate in plate count of *L. monocytogenes* attached to cooked pork surfaces after 14 days of storage at 4 °C when compared to nisin treatments without alginate (Fig. 1). The reduction in plate numbers of *L. monocytogenes* with immobilized nisin (10^4 IU/ml) and free nisin treatments after 14 days at 4 °C was 1.74 and 0.53 $\log_{10}$ units, respectively. When 5 x 10^3 IU nisin/ml was applied, the reduction in plate number of immobilized nisin and free nisin treated samples was 1.14 and 0.49 $\log_{10}$ units, respectively (Fig. 1). After day 0, *L. monocytogenes* counts on the inoculated control treatments, i.e., immobilization treatment without nisin addition, began to decrease throughout the incubation period (Fig. 2). These results indicated that immobilization alone had inhibitory effect on *L. monocytogenes*. Slight decrease in surface counts on meats treated with alginate have previously been reported[10]. Siragusa and Dickson[11] also reported that the lactic acid or acetic acid added to calcium alginate gels and immobilized on lean beef tissue reduced the population of *L. monocytogenes* significantly more than acid treatment alone. They found that lactic acid (1.7 % v/v) immobilized in alginate reduced counts by 1.3 $\log_{10}$ units vs. 0.03 log unit decrease for the acid treatment alone. Acetic acid (2 % v/v) reduced counts 1.5 and 0.25 log units, respectively[12]. The enhanced bactericidal effect on cooked pork by the alginate immobilization might be due to the alginate coating increasing the contact time of the nisin with the

Table 1. Residual nisin activity (IU/ml) on cooked pork treated with or without alginate at 4℃

Initial nisin activity (IU/ml)	Treatment	Nisin activity (IU/ml) during storage (day)				
		1	3	7	10	14
$5x10^3$	Immobilized	52.48	29.51	18.20	12.88	10.23
	Un-immobilized	22.91	10.96	6.76	3.47	2.34
$1x10^4$	Immobilized	269.15	117.49	61.66	40.74	29.51
	Un-immobilized	117.49	22.91	12.88	9.33	5.75

meat surface. In addition, the decreased rate of moisture loss due to alginate coating could have maintained a moisture surface environment for the antimicrobial agent to act on the tissue surface.

The effect of immobilization of nisin at 4 °C on the activity of this antimicrobial agent is indicated in Table 1. The activity of nisin remaining on the meat decreased rapidly after incubation at 4 °C without immobilization treatment. However, the activity of nisin did not decrease as rapidly when the tenderloin was treated with alginate. Fang and Lin[3] reported that nisin is not stable on pork. The activity of nisin on pork decreased with time, possibly as the result of low solubility and uneven distribution of nisin in meat[12], or by binding of nisin with meat particles[14]. The results of this investigation show that the stability of nisin on pork could be improved by the application of calcium alginate gel. Since the gel system fluidity of calcium alginate is not temperature dependent, it offers many advantages for immobilizing antimicrobial agents. For instance, living cells can be immobilized in alginate gel[15] which includes the prospect of using bacteriocin producing microorganism to inhibit the growth of specific pathogens on food surface. There is potential for the application of immobilized nisin to meat industry. Alginate with nisin can be sprayed on the surface of meat products to inhibit the growth of pathogen and to extend the shelf life of the products. In addition, since alginate has GRAS status so it could be applied to the meat processing operation without further food safety concern.

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以固定化乳酸鏈球菌素抑制豬肉中李斯特單胞菌之生長

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摘　要

本研究探討利用1%褐藻膠鈣固定乳酸鏈球菌素對李斯特單胞菌生長之影響及其於熟豬肉中之穩定性。以褐藻膠鈣固定乳酸鏈球菌素較未經固定處理之乳酸鏈球菌素能有效抑制李斯特單胞菌之生長。熟豬肉經4℃、14天貯存後，經固定化乳酸鏈球菌素(10^4IU/ml)處理者能降低該病原菌達1.74對數單位(log CFU/ml)，而未經固定化處理之乳酸鏈球菌素則只能降低0.53對數單位。若使用乳酸鏈球菌之濃度為$5x10^3$IU/ml，則固定化處理及未經固定化處理所能降低李斯特單胞菌之菌數分別為1.14及0.49對數單位。乳酸鏈球菌素於熟豬肉中之穩定性能因褐藻膠鈣固定處理而改善，顯示該固定化處理技術有應用於肉品以降低微生物污染之潛力。