COMPARISON OF THE ZONE OF INHIBITION AND ENUMERATION ASSAYS FOR EVALUATING THE EFFECTIVENESS OF ANTIMICROBIAL PACKAGING FILMS

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Keywords: nisin, packaging films, shelf-life extension, *L. monocytogenes*

BACKGROUND:

New approaches to reduce microbial growth during the storage of food have included the addition of antimicrobial compounds (AMC) such as nisin into the packaging material. Effectiveness of this method requires that (1) the AMC not interfere with film formation, (2) that the AMC retain it's antimicrobial properties, and (3) that the AM properties migrate from the film. Nisin and lysozyme have been incorporated into protein films and exhibit inhibition of both gram - and gram + strains of bacteria using the zone of inhibition assay. An alternative test to evaluate the level of inhibition of films with AMC incorporated was developed. Nisin is a bacteriocin produced by *Lactococcus lactis* which is classified as a lantibiotic due to the presence of the lanthionine amino acid (Tagg et al, 1976; Hansen, 1994). Because of it's nontoxicity, nisin is approved as GRAS in the U.S. and 57 other countries (Hurst and Hoover, 1993). Lysozyme is another GRAS antimicrobial and inactivates bacterial cells by hydrolyzing the B-1,4 glucosidic link between NAM and NAG in the peptidoglycan layer of gram positive cells (Proctor and Cunningham, 1988).

OBJECTIVE:

The objective of this study was to compare the sensitivity of two methods for analyzing antimicrobial films: (1) the zone of inhibition assay methods and (2) an enumeration method in which a liquid media is exposed to the film surface after which the bacterial population is enumerated.

EXPERIMENTAL METHODS:

Film formation

The film was formed in petri dishes which were weighed and placed on a level surface prior to pouring the film solution. A corn zein film solution was made by mixing 6.75g corn zein, 40.63 ml of 95% ethanol, and 1.89 glycerine. The corn zein and ethanol were mixed together and stirred until the mixture clarified. Glycerine was then added and slowly heated while stirring continued. Once the mixture started to boil, the heat was lowered and stirring discontinued and allowed to boil for 5 more minutes. Ten ml of the corn zein solution was transferred to individual test tubes and the desired levels of lysozyme or nisin was added to each test tube and evenly dispersed. Five ml of that mixture was poured into the level petri dishes and allowed to dry overnight. Film weights were determined after reweighing the dried films in the plates then by subtracting this from the weight of the petri dishes before addition of the film solution.

Bacterial cultures

Listeria monocytogenes ATCC 15313 was grown in BHI broth at 37 C with moderate shaking. The inoculum was allowed to grow 12-18 hours and was then centrifuged for 20 minutes. Then the supernatant was decanted and the pellet was washed by mixing the cells thoroughly with 10 ml of 0.1% peptone water. The centrifugation and washing procedure was repeated and the pellet resuspended in 0.1% peptone water.

Zone of inhibition assay

Soft agar was inoculated with 100 ul of the bacterial culture, then poured onto hard agar plates. Nine mm diameter dics were cut from the film then placed on the dried soft agar surface. The plates were incubated for 48 hours (37^{o}C) under optimal growth conditions. Antimicrobial activity was determined by measuring the zone of inhibition (diameters). The total zone area was calculated and subtracted from the area of the film itself. The concentration of the AMC was expressed in mg of AMC/ml of casting solution as well as in ug of AMC per disc.

Enumeration assay

Fifteen ml of cell solution was poured onto the film surface which had been cast in the petri dishes. The petri dishes were placed on an orbit shaker at 50 rpm at room temperature. At 0, 1 and 2 hours one ml of the cell solution exposed to the film surface was removed and used for enumeration. The colony forming units (CFU/ml) for each sample which had been exposed to film containing different nisin and lysozyme concentrations at the three time intervals was determined.

RESULTS AND DISCUSSION:

The results of this study demonstrated that the zone of inhibition assay sometimes fails to detect the effectiveness of antimicrobial films depending on the bacteria evaluated. The zone assay detected no inhibition of *Listeria monocytogenes* up to the 6.0 mg/ml level (Table 1). Previous research has indicated that nisin has no inhibitory affect on *Listeria momcytogenes* using well diffusion assays (Harris et al, 1989). The enumeration asssy detected a log reduction from 8.57 to 6.08 and from 8.38 to 5.38 of *L. monocytogenes* after one hour and two hours, respectively, of exposure to zein films containing 6.00 mg/ml of nisin (Table 2).

When lysozyme was tested with *Listeria monocytogenes*, inhibition zones appeared at the 3.00 mg/ml (202.8 ug/disc) concentration. The inhibition zone increased in size from 39.7 mm^2 to 200.57 mm^2 as the concentration of lysozyme increased from 3.00 mg/ml to 24.10 mg/ml (1652.9 ug/disc) (Table 3). In the enumeration assay the log CFU/ml decreased from 8.53 to 6.91 in two hours of exposure to the films as the lysozyme concentration was increased from 0 to 24.10 mg/ml (1205.0 mg/film) (Table 4). For both the zone and enumeration methods, lysozyme was effective against *Listeria monocytogenes*, however the enumeration method

appeared to be more sensitive at lower concentrations.

CONCLUSION:

The results suggest that the zone of inhibition assay is not always sensitive to the effect of antimicrobials incorporated into films. The enumeration assay produced more consistent results which reflected small changes in antimicrobial concentrations through the differences in bacterial populations.

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Table 1. Zone inhibition of *Listeria monocytogenes* by films with different levels of nisin added.

Nisin in film (mg/ml)	Nisin in disc (ug)	Inhibition zone area (mm)
0.00	0.0	0
0.10	6.7	0
0.25	18.2	0
0.50	34.8	0
0.75	69.5	0
1.50	94.1	0
3.00	200.9	0
6.00	525.4	0

Table 2. Enumeration assay of *Listeria monocytogenes* by films with different levels of nisin added.

Nisin in film	Nisin in disc	Log CFU/ml		
(mg/ml)	(mg)	0hr	1 hr	2 hr
0.00	0.0	8.72	8.57	8.38
0.10	0.5	8.72	8.28	8.00
0.25	1.3	8.72	7.70	7.28
0.50	7.5	8.72	6.71	6.40
0.75	9.0	8.72	6.59	6.15
1.50	12.0	8.72	6.30	6.15
3.00	15.0	8.72	6.28	6.04
6.00	30.0	8.72	6.08	5.38

Table 3. Zone inhibition of *Listeria monocytogenes* by films with different levels of lysozyme added.

Lysozyme in film (mg/ml)	Lysozyme in disc (ug)	Inhibition zone area (mm)
0.00	0.0	0
1.77	36.5	0
1.90	94.1	0
3.00	202.8	39.71
6.00	372.3	52.64
12.00	788.4	73.79
24.10	1652.9	200.57

Table 4. Enumeration assay of *Listeria monocytogenes* by films with different levels of lysozyme added.

Nisin in film	Nisin in disc	Log CFU/ml		
(mg/ml)	(mg)	0hr	1 hr	2 hr
0.00	0.0	8.11	7.97	8.53
0.77	3.9	8.11	7.51	7.00
1.50	7.5	8.11	7.23	7.23
3.00	15.0	8.11	7.11	7.04
6.00	30.0	8.11	7.18	6.86
12.00	60.0	8.11	7.04	6.87
24.10	120.5	8.11	6.95	6.91