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MECHANISMS AND KINETICS OF NISIN RELEASE FROM CALCIUM ALGINATE FILMS

A Dissertation in

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by

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ABSTRACT

Several studies have shown that calcium alginate matrices containing nisin effectively inhibits microbial growth in various foods such as beef, poultry, milk, and mushrooms, but they only present qualitative information on overall effectiveness of these systems on microbial inhibition. In order to design an effective antimicrobial packaging system for commercial applications, it is important to study the impact of various factors that govern the structure and properties of calcium alginate films and the diffusion of nisin from these films. It is known that nisin is a cationic polypeptide, whereas alginate is an anionic polymer. Ionic interactions between nisin and alginate might hamper the release of nisin from the alginate matrix, leading to reduced antimicrobial activity. Hence, it is essential to characterize interactions between nisin and alginate and to investigate the antimicrobial activity of nisin released from calcium alginate films. The overall goal of this research was to study the mechanisms and kinetics of nisin release from calcium alginate films. The objectives were to: (a) modify agar diffusion assay for better quantification of nisin; (b) determine factors affecting the release of nisin from calcium alginate films; (c) investigate the interaction between nisin and alginate; (d) determine the kinetics of release; (e) evaluate the physical, mechanical and optical properties of these films; and (f) investigate antimicrobial activity of the released nisin.

Agar diffusion bioassay, using *Micrococcus luteus*, is a commonly used method for quantification of nisin concentration. However, the correlation between inhibition zone and nisin concentration is not well established at higher nisin concentrations. The

objective of this study was to investigate the effect of different well size (small and large) and pre-diffusion times (0, 24, 48 and 72 h) at 4°C on the sensitivity, precision and accuracy of nisin quantification. Regression analysis indicate that small wells were better than large wells because of smaller standard deviation, higher predictive accuracy, and better discrimination between mean inhibition zones at neighboring nisin concentrations. Statistical analysis by Tukey's test showed that pre-diffusion resulted in significantly different inhibition zones at different nisin concentations. Pre-diffusion also improved sensitivity of the assay at different nisin concentrations. Different regression models were considered to explore the relationship between inhibition zone and nisin concentration for all pre-diffusion times. A spline model was the best fit model and 48 h was the best pre-diffusion time.

Factors affecting the release of nisin from calcium alginate films were studied by the disc agar diffusion bioassay. Calcium alginate films were formed by two methods – cross-linking before drying (CBD) and cross-linking after drying (CAD) – using various alginate compositions, cross-linking times and Ca^{2+}concentrations. The method of film formation, cross-linking time, and Ca^{2+}concentration had a significant effect ($p<0.05$) on nisin release. Alginate composition did not affect nisin release significantly ($p>0.05$). SEM images showed that film formation method influenced the microstructure of films considerably. Films made by the CBD method and cross-linked with high Ca^{2+} concentration had the highest nisin release.

The binding between nisin and alginate was investigated for various concentrations and compositions of sodium alginate and in the presence of Ca^{2+}. Six concentrations of sodium alginate (1.67-33.33 mg/ml) were added to nisin solution (0.5

mg/ml) and the free nisin remaining was measured by HPLC. Results indicated that nisin binds with alginate, but this binding was reversible in the presence of Ca^{2+}. The composition of alginate did not have a significant effect ($p>0.05$) on the nisin-sodium alginate binding. However, alginate with higher guluronic acid content binds less nisin in the presence of Ca^{2+}.

Diffusion of nisin from calcium alginate films cross-linked with various concentrations of Ca^{2+} (0.18-1.25 M) was investigated. The effect of cross-linking on the degree of swelling, mechanical properties, and optical properties of calcium alginate films was evaluated. The amount of nisin released from calcium alginate films increased significantly ($p<0.05$) with Ca^{2+} concentration. The mechanism involved in the diffusion process was investigated using the Power law model. Nisin diffusion from films cross-linked with 0.18M Ca^{2+} exhibited anomalous behavior, all other films exhibited near-Fickian behavior. Nisin release from these films fitted well to Fick's second law of diffusion ($R^2>0.90$). The effective diffusivity of nisin into water at 21°C was of the order 10^{-8} cm^2/s and did not change significantly ($p>0.05$) with Ca^{2+} concentration. This released nisin retained its antimicrobial activity. Films cross-linked with low Ca^{2+} concentrations underwent significant swelling. The transparency, tensile strength and elastic modulus of the films decreased, whereas elongation at break increased, with increasing Ca^{2+} concentration.

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Chapter 1

Introduction

Active packaging materials change the environment around the food product to extend its shelf-life, enhance microbial safety and/or improve organoleptic properties. Hence, active food packaging provides additional functions over traditional passive packaging, which is limited to protection of the food product against external influences (Rooney 1995). One promising type of active packaging is incorporation of antimicrobial substances in food packaging materials. Antimicrobial food packaging acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself (Appendini and Hotchkiss 2002). Antimicrobial packaging can improve product shelf-life by delivering antimicrobials on food surfaces, where microorganisms usually grow and cause spoilage (Greer 1982; Torres and others 1985).

Edible films and coatings in direct contact with foods have generated significant interest in the recent years. Alginate is one such polymer that can also be used as an edible film or coating to improve the shelf-life and quality of food products. Alginates are naturally occurring, high molecular weight polysaccharides extracted mainly from brown algae (Pheophyceae) and bacteria. Alginate is an anionic copolymer of β-D-mannuronic acid and α-L-guluronic acid. Alginates have the unique property of forming irreversible heat-stable gels, which can set at ambient temperatures in the presence of divalent ions such as Ca^{2+}, Ba^{2+}, Sr^{2+}, etc. Alginate-based edible coatings containing antimicrobials such as malic acid, glucose oxidase and nisin have been successfully applied onto various

plant, meat and dairy-based food products (Cutter and Siragusa 1997; Fang and Tsai 2003; Field and others 1986; Lu and others 2009, Luttmann 2011; Millette and others 2007; Natrajan and Sheldon 2000; Raybaudi-Massilia and others 2008; Wan and others 1997).

Nisin is a bacteriocin produced by lactic acid bacteria fermentation. It is a cationic polypeptide composed of 34-amino acids and is considered 'generally regarded as safe (GRAS)' by the Food and Drug Administration and the World Health Organization. Nisin is used in a variety of dairy, plant and meat-based food products. It shows antimicrobial activity against a wide range of gram-positive bacteria (Hurst and Hoover 1993). Various factors such as pH, salt, endogenous proteases, fat and protein in the food can adversely affect the antimicrobial activity of nisin, resulting in loss of activity over time (Abee and others 1994; DelvesBroughton and others 1996; Jung and others 1992; Rose and others 1999; Scott and Taylor 1981; Zapico and others 1999). The inhibitory activity of nisin can be extended by encapsulating it into the packaging material and designing the system so as to get the desired rate of nisin release on the food surface.

Several studies have reported that a calcium alginate matrix (films/coatings/beads) containing nisin effectively inhibits microbial growth in various foods such as beef, poultry, milk, and mushrooms (Cutter and Siragusa 1997, Fang and Tsai 2003, Lu and others 2009, Luttmann 2011, Millette and others 2007, Natrajan and Sheldon 2000, Wan and others 1997). The release of nisin from calcium alginate coatings in contact with the food product results in microbial inhibition, thereby increasing its shelf-life. However, all these release studies only present qualitative information on the effectiveness of nisin containing alginate matrices on microbial

inhibition. In order to design a desired release matrix using calcium alginate, it is important to study the impact of different factors that govern the structure and properties of calcium alginate films, and to model the diffusion of nisin from these films. Nisin is a cationic polypeptide, whereas alginate is an anionic polymer. Ionic interactions between nisin and alginate might hamper the release of nisin from the alginate matrix leading to reduced antimicrobial activity. Hence, it is essential to characterize any interactions between nisin and alginate and to investigate the antimicrobial activity of nisin released from calcium alginate films.

1.1 Research questions

1. How do factors such as alginate composition, method of film formation and cross-linking parameters affect the release of nisin from alginate films?
2. Is there any interaction between nisin and alginate? What is the effect of this interaction on nisin release from calcium alginate films?
3. What is the kinetics of nisin release from alginate films?
4. Is there any loss of antimicrobial activity of the nisin released from alginate films?

1.2 Hypotheses

1. The release of nisin from calcium alginate films into an aqueous medium is affected by the method of film formation, alginate composition, nisin concentration and cross-linking parameters such as cross-linking time and the

amount of cross-linker

2. Nisin binds to alginate due to ionic interactions
3. The release kinetics of nisin from calcium alginate films follows Fickian diffusion
4. Nisin released from calcium alginate films retains its antimicrobial activity

1.3 Objectives

The overall objective of this research was to study the mechanisms and kinetics of nisin release from calcium alginate films. The specific objectives were to: (a) modify agar diffusion assay for better quantification of nisin; (b) determine important factors affecting the release of nisin from calcium alginate films; (c) characterize the interaction between nisin and alginate; (d) determine the kinetics of release; (e) evaluate the physical, mechanical and optical properties of these films; and (f) investigate the antimicrobial activity of the released nisin.

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Chapter 2

Literature review

2.1 Active packaging

Active packaging implies an ‘active’ interaction of the package with its internal atmosphere or with the food. Active food packaging concepts provide some additional functions over traditional passive packaging, which is limited to protection of the food product against external influences. Active packaging materials change the environment around the food product to extend its shelf-life, enhance microbial safety and/or improve organoleptic properties. It is the application of specific packaging properties to specific situations. For instance, addition of ethylene releasing sachets in high moisture bakery products to suppress mold growth when low oxygen barrier packaging is used (Rooney, 1995); or use of moisture regulator packs for packaging Portuguese cheese (Pantaleao and others 2007). There are opportunities for reducing packaging cost by complementing active packaging with cheaper traditional passive packaging. Also, the consumers may have preference for active packaging systems since this type of packaged foods contain fewer additives, has better quality and safety (Han and Floros 2007). One promising type of active packaging is antimicrobial packaging, which involves incorporation of antimicrobial substances in food packaging materials.

2.2 Antimicrobial packaging

Antimicrobial food packaging acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself (Vermeiren and others 1999). Antimicrobials can be applied before or after packaging the food product; or they can be applied to the packaging material by direct addition or immobilization (Figure 2-1). Antimicrobial activity is achieved by release of antimicrobial from the packaging material on the food surface or package headspace; or inhibition of microbial growth by the immobilized antimicrobial at food-package contact surface.

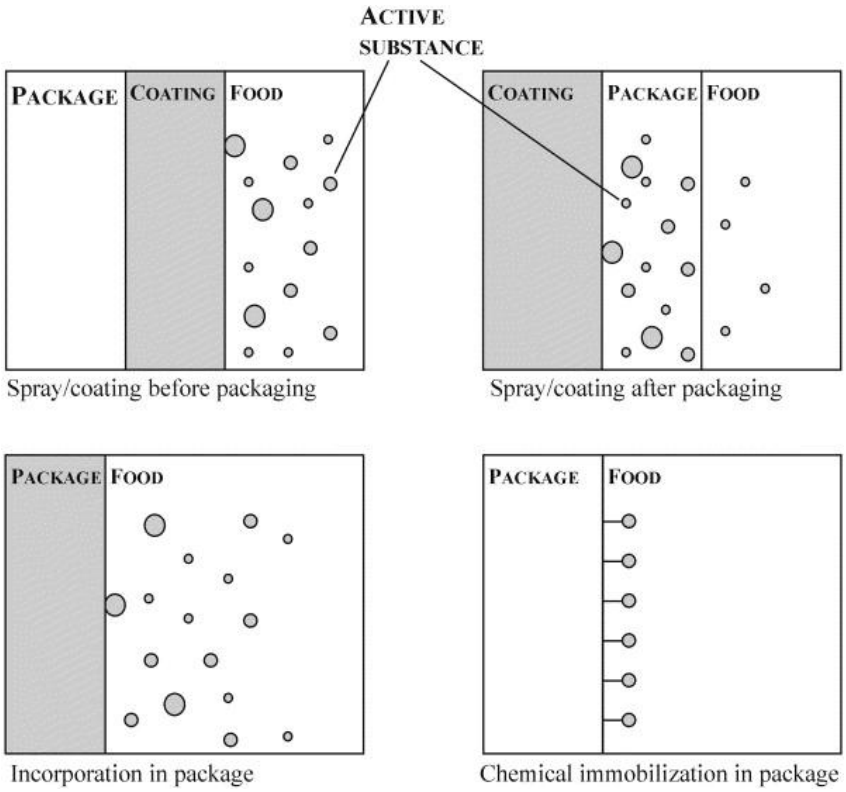


Figure 2-1. Migration of the antimicrobial in different applications of antimicrobial packaging systems (Han, 2000)

Antimicrobial packaging systems have characteristic antimicrobial activity and spectrum to different microorganisms. The following factors must be considered for designing an efficient antimicrobial system (a) antimicrobial agent, (b) target microorganisms, (c) internal package atmosphere, (d) packaging material and (e) food (Han and Floros 2007). Various compounds have been proposed and tested for incorporation in food packages including Ag-based compounds (Ishitani, 1995), organic acids (Han and Floros 1997), bacteriocins, such as nisin and pediocin (Dobias and others 1999, Ming and others 1997, Siragusa and others 1999), fungicides such as benomyl (Halek and Garg 1989) and imazalil (Weng and Hotchkiss 1992), organic compounds such as triclosan (Cutter 1999), etc. Table 2-1 shows studies applying antimicrobial packaging on various food systems.

Direct application of antimicrobials to the food may result in immediate destruction of the target microorganism, but would not prevent the recovery and growth of injured organisms (Hoffman and others 2001). Also, there may be activity loss due to its cross-reaction with food components such as lipids and proteins (Han and Floros 1997; Thomas and others 1998). According to Hoffman and others (2001), incorporation of antimicrobials in the food package could reduce the post-process growth of food pathogens. One of the major challenges of antimicrobial packaging is to obtain a continuous release of the antimicrobial for a longer duration and under product storage conditions. The increasing demand for fresh, safe and convenient food products will encourage the development and advances in antimicrobial packaging. A combination of natural antimicrobials in biodegradable packaging materials can be predicted to be a popular and preferred choice of the consumers as well as the manufacturers in the future.

Table 2-1. Studies on antimicrobial coatings on food products

Product	Antimicrobial Coating	Observation/Results	Reference
Vacuum packed bologna, cooked ham, pastrami	Acetic /propionic acid in chitosan	*Enterobacteriaceae* and *S. liquefaciens*	Ouattara and others 2000
Beef	Nisin and EDTA in polyethylene and polyethylene oxide films	*B. thermospacta*	Cutter, and others 2001
Turkey deli meat	Sakacin A in pullulan films	*L. monocytogenes*	Trinetta and others 2010
Chicken	Nisin and calcium propionate in corn zein film	*L. monocytogenes*	Janes and others 2002
Veal	Nisin in cellophane	Total aerobic bacteria	Guerra and others 2005
Cold-smoked salmon	Bacteriocin produced by *Lactobacillus curvatus* CWBI-B28 on plastic films	*L. monocytogenes*	Ghalfi and others 2006
Strawberries	Potassium sorbate in starch	Mesophilic aerobes, mold yeast	Garcia and others 2001
Apples	Lemongrass, oregano and vanillin in alginate	Native psychrophilic aerobes, moulds, yeast and *L. innocua*	Rojas-Grau and others 2007
Melon	Chitosan in methylcellulose	*E.coli, S. aureus, Salmonella spp*.	Krasaekoopt and Mabumrung 2008
Carrots	Chitosan in starch	Mesophilic aerobes, mold, yeast and psychrotrophic bacteria	Andrade and others 2004
Cheese	Chitosan	*L. monocytogenes*	Coma and others 2002

2.3 Nisin

Nisin is a bacteriocin that exhibits antimicrobial activity towards a wide range of Gram-positive bacteria including spore-formers, but shows little or no activity against Gram-negative bacteria, yeasts and molds (Hurst 1981). Nisin is the only bacteriocin widely used as a food preservative and is recognized 'generally regarded as safe (GRAS)' by the Food and Drug Administration (FDA) and the World Health Organization (WHO). It is permitted for use in over 50 countries as a food preservative and finds application in a variety of plant, dairy and meat-based food products. Nisin has no flavor, it is non-toxic, rapidly digestible by pancreatic proteases, and heat stable at low pH (Delves-Broughton and others 1996).

2.3.1 Structure

Nisin is a low molecular weight (3500 Daltons) polypeptide with 34-amino acids and belongs to a group of bacteriocins called 'lantibiotics' (small polypeptides < 4000 Daltons). Nisin is a member of the type A lantibiotics, which are elongated, amphiphilic, screw-shaped peptides with a net positive charge (Wiedemann and others 2004). It contains the rare thio-ether amino acids lanthionine and β-methyllanthionine and the unsaturated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb) formed in a post-translational modification process. In this process, the unsaturated amino acids dehydroalanine (Dha) and dehydrobutyrine (Dhb) are formed from serine and threonine residues, respectively. Subsequently, specific addition reactions occur between cysteine residues and some of the unsaturated amino acids, which results in the formation of

lanthionine and β-methyllanthionine residues (Rollema and others 1996). The thio-ether bridges of the lanthionines form intramolecular cross-links resulting in five cyclic ring structures in the molecule (Figure 2-2).

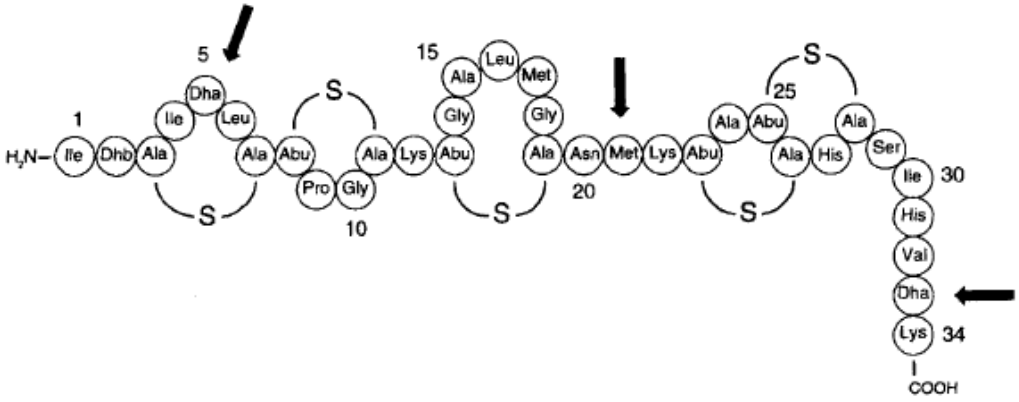


Figure 2-2. Schematic representation of primary structure of nisin A. Arrows indicate sites of modification (Rollema and others 1996)

The thio-ether bonds give nisin two rigid ring systems: 3-rings from the N-terminal (amino acids 1-19) and 2-rings from the C-terminal (amino acids 23-34) separated by a hinge region (amino acids 20-22), wherein considerable flexibility is observed. The ring systems give nisin a screw-like structure possessing amphipathic properties. The amphipathic property of nisin is characterized by the N-terminal region being more hydrophobic than the C-terminal region and also because the hydrophobic moieties are positioned opposite to the hydrophilic moieties throughout the length of the nisin molecule (Breukink and others 1999). This amphipathic character is imperative for its biological function (Van and others 1991; vandenHooven and others 1996). The

hydrophilic C-terminal part contains positively charged side chains of lysine and histidine residues. The *pKa* values of the side-chain groups in histidine and lysine residues are 6.5 and 10.0, respectively (Abee and Delves-Broughton, 2003). The net charge of nisin is therefore pH dependent.

The three known variants of nisin are nisin A, nisin Z and nisin Q. Nisin A and nisin Z have similar structures with 34 amino acids and five intramolecular sulphide rings. The only difference is that nisin Z has a substitution of Asn^{27} for His^{27}. They also have similar antimicrobial activities (Mulders and others 1991). Nisin Q, which was recently discovered, differs from nisin A in four amino acids and the detailed characterization and structural analysis of nisin Q are yet to be studied (Zendo and others 2003).

2.3.2 Mode of action

Nisin is cationic owing to the presence of three lysine residues plus one (in nisin Z*)* or two (in nisin A) histidine residues (Abee and Delves-Broughton 2003). The first report on the mode of action of nisin dates back to mid-1990's, when Ramseier (1960) observed leakage of UV-absorbing intracellular compounds from treated cells, suggesting a detergent effect. Subsequent studies led to believe that the primary mode of action of nisin is by formation of transient pores in the bacterial cytoplasmic membrane resulting in dissipation of membrane potential and rapid efflux of essential cytoplasmic substances, such as amino acids, potassium ions and nucleotides. This leakage of cellular materials abruptly stops all biosynthetic processes, causing cell death (Ruhr and others 1985; Sahl

and others 1987). The antimicrobial action of nisin can be described in 3 steps as follows 1) binding to the cytoplasmic membrane 2) insertion into the lipid phase of the membrane and 3) pore formation. Breukink and others (1997) reported that presence of the negatively charged phospholipids in the cellular membrane is essential for each of these steps. They varied the anionic lipid content in the bacterial membrane system and found that nisin binding remarkably increased as lipid content increased above 40%. The anionic lipid content in Gram-positive bacteria is much higher than that in Gram-negative bacteria (O'Leary and others 1988) and may be responsible for the higher activity of nisin against Gram- positive bacteria (Breukink and others 1997).

Studies by Brotz and others (1998) suggest that nisin uses bacterial cell well precursor, lipid II, as a docking molecule for specific binding to the bacterial membranes. Wiedemann and others (2001) proposed a model for lipid II mediated nisin pore formation (Figure 2-3). According to them, the N-terminal of nisin first binds to the carbohydrate moiety of lipid II in a 1:1 stoichiometry ratio. The C-terminal part of nisin then translocates across the bacterial cytoplasmic membrane. For this step the flexible hinge region between ring clusters A, B, C and D, E is important. Through this specific interaction with the cell wall precursor lipid II, nisin forms defined pores which are stable for seconds and have pore diameters of 2 to 2.5 nm (Wiedemann and others 2004). Several nisin-lipid II complexes are presumed to assemble for a functional pore resulting in rapid efflux of cytoplasmic constituents. The position of the lipid II hydrophobic tail in the cytoplasmic membrane is still unknown.

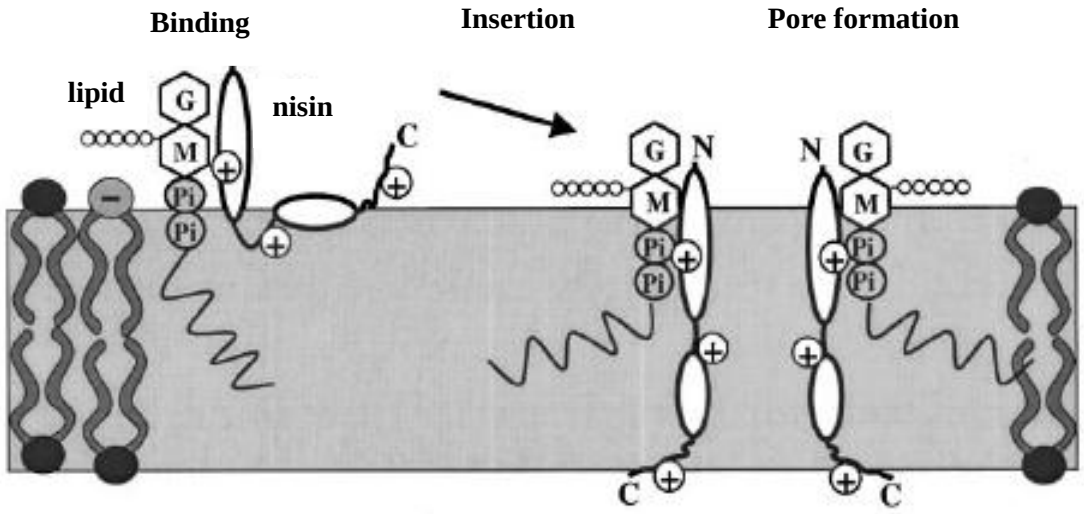


Figure 2-3. Model for the lipid II mediated nisin pore formation (Wiedemann and others 2001)

2.3.3 Stability and solubility

Nisin shows excellent stability when stored in dry and dark conditions below 25°C (Delves-Broughton 2005). In solution or aqueous suspension, nisin retains its maximum activity between pH 3 and 3.5. Davies and others (1998) found that autoclaving buffered nisin solutions at 121°C for 15 mins are very stable to autoclaving at pH 3 (<15% activity loss). However, nisin solutions of pH 1 and pH 5 had an activity loss of more than 90% and 50%, respectively. Even greater losses would be expected at higher pH (near neutral). This is probably due to modification of Dha and Dhb residues by the neucleophiles present at high pH, decreasing the stability and solubility of nisin (Liu and Hansen 1990). The retention of nisin activity in a food system depends on the storage temperature, pH, intrinsic factors of the food and period of storage. Nisin activity can be adversely affected by the presence of proteolytic enzymes (microbial, plant or

animal origin) or certain food additives such as titanium oxide and sodium metabisulphite (Delves-Broughton 2005). For food products intended for storage at temperatures >25^{0}C, higher levels of nisin needs to be incorporated to ensure its antimicrobial activity is retained for longer duration.

The solubility of nisin is strongly dependent on pH and ionic strength. The highest solubility is observed at low pH and low ionic strength (Rollema and others 1995). Liu and Hansen (1990) observed that the solubility of nisin dropped sharply from 57 mg/ml at pH 2, to 1.5 mg/ml at pH 6 and 0.25 mg/ml, at pH 8.5. However, in practical food applications the levels of nisin is unlikely to exceed 0.25 mg/ml and the poor solubility at non-acidic pH is not an issue of concern (DelvesBroughton and others 1996).

2.3.4 Commercial applications in foods

Commercially, nisin is available as Nisaplin®. It is an off-white/light tan colored powder containing about 2.5% nisin A and the balance material being salts and milk solids derived from the fermentation of a modified milk medium by nisin-producing strains of *L. lactis* subsp. *lactis* (Abee and Delves-Broughton 2003). The antimicrobial activity of nisin is expressed in International Units (IU). Pure nisin has an activity of 40 x 10^6 IU/g. Nisaplin® has an activity of 10^6 IU/g (Danisco, USA). Nisaplin® is widely used in food processing applications. It is effective over a range of pH (3.5-8.0) and is used in a variety of foods including processed cheese, cream products such as whipped or thickened sour cream, dairy and fat-based desserts, yogurt, fruit and vegetable preparations such as pasteurized juices and pulps, dips and snacks, pasteurized soup,

processed meats and beer. Nisaplin® can be added to the food directly as a dry powder or as a pre-suspension in water or milk. For heat processed foods, the manufacturers recommend thorough dispersion of nisin in the food substrate prior to the thermal process (Danisco, New Century, KS).

The recommended dosage of nisin is in the range 25-500 mg per kg or liter of food (Danisco, New Century, KS). Table 2-2 shows the major categories of food in which nisin is used, typical spoilage or pathogenic bacteria in these products controlled by nisin and the recommended nisin dose level.

Table 2-2. Typical levels of nisin used in food applications (Delves-Broughton 2005)

Food application	**Typical target organisms**	**Level of nisin (mg/kg or mg/L)**
Processed cheese	*Clostridium* spp. & *Bacillus* spp.	5 - 15
Pasteurized milk & milk products	*Clostridium* spp. & *Bacillus* spp.	0.25 - 10
Pasteurized chilled soups	*B.* cereus & *C.* pasteurianum	2.5 – 6.2
Crumpets	*B.* cereus	4 – 6.25
Canned foods (high acid)	*C. botulinum* & *C. thermosaccharolyticum*	2.5 – 5.0
Dipping sauce	Lactic acid bacteria	1.25 – 6.25
Salad dressings	Lactic acid bacteria	1.25 - 5
Beer: Pitching yeast wash Post fermentation	 Lactic acid bacteria *Lactobacillus, Pediococcus*	 25 – 37.5 0.25 – 1.25

2.3.5 Assay methods

Alhough nisin is ‘GRAS’, it is considered natural only when used in concentrations found in foods naturally fermented with a nisin-producing starter culture. Various countries have set limits for maximum levels of nisin in foods. The activity of nisin decreases during food processing and storage, due to the temperature, pH, and components of the food (Delves-Broughton 1990). Therefore, the ability to quantify nisin is essential for monitoring nisin quantities added into foods as well as its stability throughout the product's shelf-life. Several methods have been developed for detection and quantification of nisin as discussed below.

2.3.5.a Agar diffusion bioassay

Agar diffusion bioassay is most widely used for quantification of nisin activity in food samples as it directly measures the antimicrobial activity of nisin. It is based on measuring the inhibition zone produced by nisin sensitive microorganisms in agar plates, and correlating it to the antimicrobial activity of nisin. Inhibition zone is the result of diffusion of nisin into the agar medium and simultaneous growth of the indicator organism. Greater nisin concentrations result in larger inhibition zones. For quantification of nisin in an unknown sample, the inhibition zone is measured and nisin activity is predicted from a calibration curve plot of inhibition zone vs. logarithm of nisin concentration.

In this method, liquid agar containing nutrient broth is inoculated with an indicator organism (sensitive to nisin) and poured in a petri dish to form an agar bed.

After it solidifies, wells are made on the agar plates with a cork borer. The nisin samples are dispensed into these wells and inhibition zones formed after 24-48 h are measured with a vernier caliper. The formation of inhibition zone is the result of diffusion of nisin into the agar medium and simultaneous growth of the indicator organism. Greater the nisin concentration, larger will be the zone diameter. For quantification of nisin in an unknown sample, the diameters of the zones are measured and correlated with a standard curve plotted for diameter of zone vs. logarithm of nisin concentration.

Tramer and Fowler (1964) reported that addition of 1% Tween 20 to the agar aids in diffusion of nisin. In this assay *Micrococcus luteus* was used as an indicator strain, and the diameter of the inhibition zone was found to be directly related to the nisin concentration in the range of 0.5-10 IU/ml. Wolf and Gibbons (1996) improved the accuracy and precision of this method by reducing the concentration of agar from 1.5% to 0.75% and buffering the agar with phosphate salts. They found that the diameter of the inhibition zone is directly related to the logarithm of nisin concentration in the range of 0-1000 IU/ml. Pongtharangkul and Demirci (2004) tested three strains of nisin-sensitive microorganisms (*Micrococcus luteus, Lactobacillus sakei, Brochothrix thermosphacta*) to evaluate the effects of nisin-sensitive strains and pre-diffusion on the accuracy and precision of nisin quantification. They reported that pre-diffusion of nisin at 4°C with *L. sakei* as the indicator organism showed highest sensitivity, accuracy and precision for nisin quantification.

Agar diffusion method facilitates detection of very low levels of nisin activity (0.5 IU/ml or 0.0125 IU/µg), which may be encountered in food applications. However, it is time consuming, taking a minimum of two days to obtain the results.

2.3.5.b. High Performance Liquid Chromatography (HPLC)

Liu and Hansen (1990) developed a method for quantification of nisin using HPLC. Their protocol has been adopted by Rollema and others (1995) and Buonocore and others (2003) with some modifications. A reverse phase C-18 column of size 250 x 4 mm was used with water and acetonitrile (with 0.1% TFA) as solvents. Nisin was detected at wavelengths of 254 and 225 nm. HPLC method for nisin quantification is quick and reproducible. However, it has poor sensitivity compared to gar diffusion bioassay. It cannot detect samples below activity 500 IU/ml. Rollema and others (1995) heat treated nisin to 75°C and compared nisin peak patterns obtained from HPLC with the biological activity of same nisin sample as determined by agar diffusion assay with *M. flavus* (now *M. luteus*) over pH range 2-8. They found that at every pH value, the amount of nisin obtained by agar diffusion assay was higher than that obtained by HPLC. According to them, this may be because some degradation products of nisin (eg. Nisin 1-32) retain full or slightly reduced biological activity.

2.3.5.c. Colorimetric method

Ripoche and others (2006) used a colorimetric method, bicinchoninic acid protein assay (BCA), as described by Wiechelman and others (1988) for quantification of nisin. In this method, equal volumes of pure nisin sample and QBCA reagent were mixed and heated at 60°C for 1 hour and the absorbance was measured at 560 nm using a spectrophotometer. This method has been widely used for protein assay. However, it is sensitive to interference by other proteins or peptides that may be present in nisin or

Nisaplin® samples such as whey proteins, casein, peptides in yeast extract etc. used during production of nisin by fermentation. Thus using this assay for nisin quantification may not be accurate.

Fang and others (2007) have proposed MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay (MCA) for quantitative measurement of polypeptide bacteriocins in solutions with nisin as an example. A solution of nisin is incubated with an indicator bacterium, *Micrococcus luteus,* followed by addition of MTT and another incubation period. The viable bacteria are quantified by measuring the cleavage of the yellow tetrazolium salt MTT into purple formazan in the presence of metabolically active bacterial cells. They also compared MCA with standard agar diffusion assay (ADA) and found a high correlation coefficient (r^2=0.975±0.004) between optical density (OD) and the inhibitory effect of nisin on a bacterial strain *Micrococcus luteus* in the nisin activity range of 0.125~32 IU/ml. The MCA described in this study was very quick with quantification of nisin took only 7~8 hours and the detection limit was at the level of 0.125 IU/ml when compared to 12 IU/ml and 24~28 h for ADA. Though the MCA provides an accurate and rapid method for quantification of nisin in solutions, the plot of absorbance vs. nisin concentration is linear only in the range 0.125-32 IU/ml.

2.3.5.d. Other methods

i) Capillary zonal electrophoresis. Rossano and others (1998) described a method for nisin assay by using capillary zonal electrophoresis (CZE). This assay was used to

analyze nisin from milk and a linear response of peak area vs nisin concentration was achieved in the range of 10 - 100 µg of nisin per ml milk. They extracted the interfering substances such as lipid and casein from milk using acetonitrile followed by analysis of nisin by CZE.

ii) Photometry. In this method, 100 µl nisin solution is added to broth medium inoculated with an indicator strain and incubated at 30°C. The absorbance was measured at 600 nm after 4 hours. The response was calculated as the ratio between the absorbance of a sample and that of the blank (uninnoculated sample). When compared with agar diffusion bioassay, this method had better reproducibility and its less time consuming; however it is has poor sensitivity and shows a linear relationship when the response is in the range 0.1 – 0.9 only (Parente and others 1995).

iii) Bioluminescence. A bioluminescent method has been developed by Immonen and Karp (2007) for nisin quantification. In this assay the whole luciferase operon from *Photorhabdus luminescens* was cloned under the control of nisin inducible *nisA* promoter and transformed to *Lactococcus lactis* strain. The modified *L. lactis* strain exhibited a nisin-inducible bioluminescent phenotype. At certain inducer concentrations, the *nisRK* genes of these modified *L. lactis* strains allow them to sense nisin and relay the signal to initiate transcription from *nisA* promoter. This induction leads to detectable luminescence within ten minutes which can be directly measured. This method can detect nisin concentration as low as 0.1 pg/ml in 0.1% Tween 80 solution, and 3 pg/ml in low-fat milk. Time needed for the assay is only 3 hours. Similar technique has been applied for quantification of nisin in food products such as processed cheese, salad dressings, canned tomatoes, and liquid egg products (Hakovirta and others 2006).

2.3.6 Loss of activity in foods during storage

Various factors in food can adversely affect the action of nisin. In non-thermal or minimal heat processed foods, proteolytic enzymes from microbial, plant or animal origins can degrade nisin (Delves-Broughton and others 1996). The action of nisin Z on *Listeria monocytogenes* was found to be adversely affected by di-and trivalent cations such as Ca^{2+}, Mg^{2+} and Gd^{3+} (Abee 1994). Factors such as pH, storage duration, temperature of storage are keys factors in determining the shelf-life stability of nisin when incorporated in food products.

The activity of nisin in milk is strongly affected by the milk composition. Jung and others (1992) reported that milk fat had an adverse effect on the antimicrobial activity of nisin whereas addition of the emulsifier Tween 80 to nisin containing high fat milk enhanced its activity. Zapico and others (1998) studied the effect of homogenization of milk on nisin activity and found that the antimicrobial activity of nisin was considerably reduced by homogenization. This loss in activity was thought to be due to the adsorption of nisin on the fat-water interface, thus making it unavailable for interaction with bacterial cells.

Rose and others (1999) studied the inactivation of nisin in fresh meat. They concluded that nisin binds to sulfhydryl-containing enzyme, glutathione (GSH), present in meats and gets subsequently inactivated. Salt, fat, pH, curing agents and food particle size are some of the factors that may affect the activity of nisin in meat. Scott and Taylor (1981) reported that for some strains of *Clostridium botulinum*, the spore growth was prevented by < 50 IU/ml nisin in a brain heart infusion medium. However, nisin did not

prove effective in a cooked meat system, even at a concentration of 2000 IU/ml. They attributed this activity loss of nisin in meat system to interaction of nisin with proteins, fat and meat particles.

2.3.6.1 Mechanism of activity loss. The unsaturated amino acid residues in nisin molecule are susceptible to chemical modification due to the high reactivity of Cα–Cβ double bonds, thus limiting the chemical stability of the compound (Rollema and others 1996). Dehydroalanine (Dha) residues at position 5 (N-terminal) and position 33 (C-terminal) are prone to acid-catalyzed hydrolysis (Gross and Morell 1967; Rollema and others 1996). Addition of water molecule to the Dha residue leads to the formation of 2-hydroxyalanine residue, which then splits into an N-terminal peptide amide and a C-terminal pyruvyl peptide, as shown in Figure 2-4. The removal of C-terminal residues did not affect the biological activity. However, the modification of Dha5 at the N-terminal, which involved breaking open the first ring, significantly reduced the biological activity as compared to native nisin (Chan and others 1996, Rollema and others 1996). Thus, dehydroalanine (Dha) at position 5, or an intact first ring at the N-terminus is crucial for maintaining the functional properties of nisin. Chan and others (1996) also report that activity is lost due to cleavage of Dha5 to yield [des-Dha5]-nisin. Prolonged storage of the nisin-incorporated food or heating in acid leads to the formation of [des-Dha5]-nisin which could eventually cause loss of nisin activity.

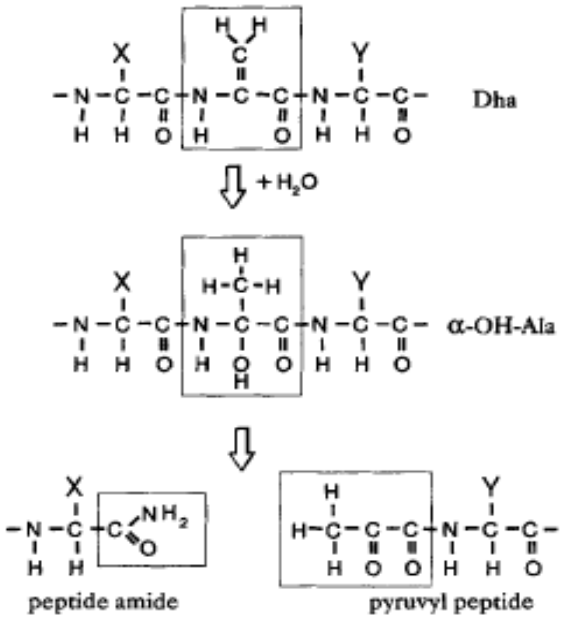


Figure 2-4. Schematic of acid-catalyzed hydrolysis of amino acid Dha causing cleavage of the polypeptide (Rollema and others 1996)

2.4 Alginate

Alginates are naturally occurring, high molecular weight polysaccharides extracted mainly from brown algae (*Pheophyceae*) and bacteria. Commercial alginate is extracted from algae, mainly *Laminaria hyperborea, Macrocystis pyrifera, Laminaria digitata, Ascophyllum nodosum, Laminaria japonica, Eclonia maxima, Lessonia nigrescens, Durvillea antartica* and *Sargassum* spp. Production of alginate from bacteria such as *Azotobacter vinelandii* and *Pseudomonas* species has been explored but it is not economically feasible yet (Draget 2009). It occurs in the cell wall and intercellular spaces of brown algae and provides both strength and flexibility to the plants. Alginic acid or

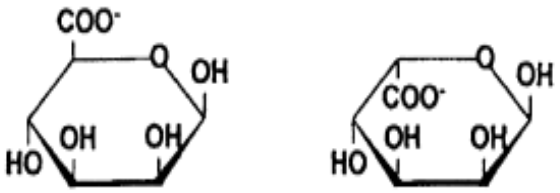
algin is the free acid form of alginate and it is also the intermediate product in commercial manufacture of alginates. It has limited stability and is modified to different salts (Na, K, Ca, Mg etc) of alginic acid to obtain stable alginates. The sodium and calcium salts of alginate are 'Generally Recognized as Safe (GRAS)' by the FDA according to 21 CFR 184.1724 and 184.1187, respectively.

2.4.1 Chemical composition

Alginate is an anionic copolymer of β-D-mannuronic acid (M) and its C-5 epimer, α-L-guluronic acid (G), linked together to form linear molecules with (1,4) glycosidic bonds (Figure 2-5). D-mannuronic acid is $^4C_1$ with diequatorial links while L-guluronic acid is $^1C_4$ with diaxial links. The composition and sequence of M and G blocks may vary widely depending on the algae source. The blocks are either similar or strictly alternating (MMMMMM, GGGGGG or MGMGMG). Biosynthesis of the polymer in seaweeds and its subsequent genetic and environmental control will determine the block structure distribution of alginates (Onsoyen 1997).

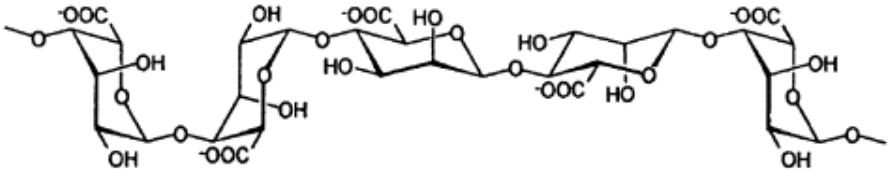
In the polymer chain the monomers will tend to be in their most energetically favorable configuration. The equatorial/equatorial glycosidic bond in M-M results in a flexible ribbon-like polymer in M-block regions and the axial/axial glycosidic bond in G-G results in a buckled and stiff polymer in the G-block regions. The MG block regions have intermediate flexibility (Onsoyen 1997).

(a)



β-D-mannuronate (M) **α-L-guluronate (G)**

(b)



G G M M G

(c)

Figure 2-5. Structure characteristics of alginate (a) monomers, (b) chain conformation and (c) block distribution (Draget and others 1996)

Alginates can have a wide range of molecular weights (MW 20–600 kDa) depending on the degree of polymerization (Onsoyen 1997). Aqueous solutions of alginate have non-Newtonian characteristics. It exhibits shear thinning i.e. viscosity decreases with increasing shear rate. The viscosity of an alginate solution depends on the concentration of the polymer and molecular weight distribution (Augst and others 2006).

2.4.2 Gelation and egg box model

Alginates have the unique property of forming irreversible heat-stable gels, which can set at ambient temperatures in the presence of divalent ions such as Ca^{2+}, Ba^{2+}, Sr^{2+}, etc. to name a few. Gelation has been demonstrated to result from specific and strong interactions between these divalent ions and blocks of guluronic acid residues. The gel forming capacity and the resulting gel strength thus depends on the amount of G-blocks and its length. High guluronic acid content and long block lengths results in strong calcium alginate gels (Smidsrød 1974). The divalent cation fits between four guluronic acid molecules like an egg in an egg-box as shown in Figure 2-6. Hence it is called an 'egg-box' model (Grant and others 1973; Morris and others 1978; Smidsrød 1974). In this model, calcium ions are packed in the cavity between two diaxially linked guluronic acid residues. Calcium induces chain-chain associations constituting the junction zones responsible for gelation of alginate (Braccini and Perez 2001).

The formation of the junction zones and eventually the hydrogel, can be described as a cooperative process with an unfavorable binding of the first ion and a more favorable binding of the following ones (zipper mechanism). In order to form gels, the alginate must contain a certain proportion of homopolymeric G-blocks. It has been reported that in the case of Ca^{2+}, a minimum of eight to 20 adjacent G-units are required for the formation of a stable junction (Donato and Paoletti 2009). Alginates can form gels with acid as well, by addition of protons to alginate solution. The extent of gel formation can be controlled by regulating crosslinking time and the concentration of calcium or acid. The kinetics of calcium alginate gel formation has been studied extensively and it has

been found to be influenced by alginate concentration, alginate composition, calcium salt particle size and concentration (Blandino and others 1999; Draget and others 1991).

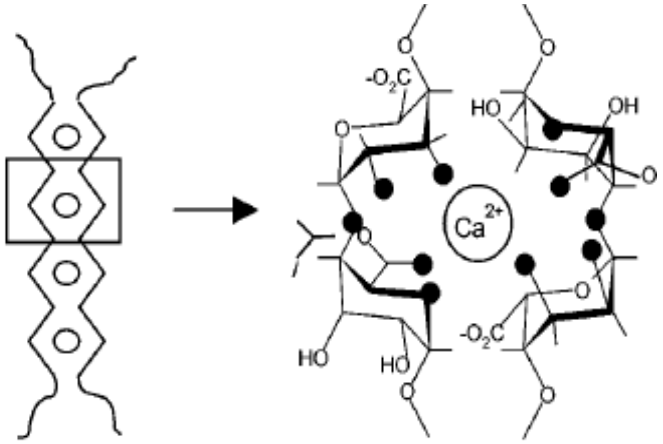


Figure 2-6. Calcium coordination of 'egg-box model' with guluronate chains (Braccini and Perez 2001)

Alginates can be cross-linked by diffusion setting or internal setting method (Draget 2009; Onsoyen 1997). *Diffusion setting* is characterized by rapid gelling kinetics and occurs when cross-linking ions such as Ca^{+2} diffuse into an alginate solution by dipping or spraying. It is commonly used as an immobilization technique. *Internal setting* is characterized by addition of cross-linking ions to an alginate solution in a controlled manner, usually achieved by controlling pH, using slowly soluble cross-linking ion or by using sequestrant such as phosphate or citrate. The diffusion and internal setting may be combined to produce a rapidly gelled outer membrane or coating.

2.4.3 Alginates as edible films and coatings

Alginates are used as food additives owing to its multi-functional properties such as thickening, gelation and stabilizing aqueous mixtures, dispersions and emulsions. Alginate offers controlled gelling at extreme temperatures, freeze-thaw stability and desired mechanical strength which makes it a popular choice for restructured food products, bakery industry and confectionary applications (Onsoyen 1997). Alginate has been used for commercial production of restructured foods such as onion rings, pimento olive fillings, apple pieces for pie fillings, meat chunks for pet food, fish patties etc. For application in confectionary products like jams, jellies, fruit fillings etc., the synergistic gelling between high guluronate alginate and pectin may be utilized. Alginate/pectin system gives thermo reversible gels independent of sugar content, in contrast to pectin gels, which may be used for low-calorie products (Draget and others 1996). An important application of alginates in bakery is to thicken cold-prepared creams, make them heat resistant and freeze-thaw stable. Sodium alginate in these cream fillings will interact with calcium in the milk powder to for heat stable calcium alginate network throughout the cream (Onsoyen 1997). Alginate films can also be used to improve the shelf-life and quality of food products. They can used in pastries to prevent migration of water from fillings to the remainder cake, edible coatings on meats and fresh produce to reduce water loss and improve microbial quality. Alginate-based edible coatings containing antimicrobials has been successfully applied on various fruits, meat and dairy-based food products (Cutter and Siragusa 1997; Fang and Tsai 2003; Field and others

1986; Lu and others 2009, Luttmann 2011; Millette and others 2007; Natrajan and Sheldon 2000; Raybaudi-Massilia and others 2008; Wan and others 1997).

2.5 Nisin containing alginate matrices

Alginate coatings have been shown to be an effective delivery system for nisin in various meat, poultry, seafood and dairy products. Cutter and Siragusa (1997) immobilized 100 μg/ml nisin in calcium alginate gels and incorporated in beef carcasses to study its effect on the inhibition of *Brochothrix thermospecta*, gram-positive bacteria responsible for spoilage in vacuum-packaged red meat. Beef tissue coated with calcium alginate containing nisin was shown to significantly ($p \leq 0.05$) suppress growth of *B. thermosphacta* for up to 7 days with growth of 6.56 log CFU/g, compared to samples coated with calcium alginate, samples treated with nisin solution and untreated samples, where the *B. thermosphacta* had grown to 7.18, 7.04, and 6.92 log CFU/g, respectively. They concluded that incorporation of nisin in calcium alginate gels is more effective in reducing the bacterial population on the carcass surface than direct application.

Fang and Tsai (2003) investigated the effect nisin, organic acids, chelators and their combinations immobilized in calcium alginate gels, on the growth of *Escherichia coli* O157:H7 in ground beef stored at 10°C and 30°C for 10 days. Nisin immobilized in calcium alginate provided significantly higher ($P<0.05$) inhibition of *E. coli* O157:H7, as compared to the non-immobilized control when stored at 10°C. The highest inhibition was achieved with the combination of nisin and acetic acid immobilized in calcium alginate. The authors found that the use of calcium alginate immobilization enhances the

effectiveness of the combination systems of nisin/EDTA, nisin/acetic acid and nisin/potassium sorbate on the growth of *E. coli* O157:H7 in ground beef at low temperature (10°C) but it was not effective at higher temperature (30°C).

Millette and others (2007) inoculated beef muscle slices with *Staphylococcus aureus* (10^4 CFU/g) were coated with palmitoylated alginate-based films containing 0, 500 or 1000 IU/ml of nisin. After 7 days of storage at 4°C, the concentration of *S. aureus* in beef samples covered with alginate films containing 500 and 1000 IU/ml of nisin was significantly reduced ($P<0.05$) by 0.91 and 1.86 logCFU/cm^2, respectively. No inhibition of *S. aureus* was observed on meat control.

Lu and others (2009) investigated the effect of nisin and EDTA treatment with and without calcium alginate coatings, on the microbial, chemical and sensory quality of northern snakehead (*Channa argus*) fillet stored at 4°C for 7 days. They found that nisin (1000 IU/ml) and EDTA (150 μg/ml) treatment without alginate significantly ($p<0.05$) inhibited the growth of total viable mesophilic bacteria and total psychrophilic bacteria than alginate coatings. Alginate coatings without antimicrobial significantly reduced chemical spoilage ($p<0.05$) and retarded water loss ($p<0.05$) while increasing the overall sensory scores ($p<0.05$) of fish fillets compared with those of without alginate. There was no significant difference ($p > 0.05$) between the coating treatments with and without nisin and EDTA.

Natrajan and Sheldon (2000) studied the effectiveness of calcium alginate films containing various nisin concentrations (0, 100, 300, 500 mg/ml), in inhibiting the growth of *Salmonella* Typhimurium on poultry skins. They inoculated drumstick skin samples with *S. Typhimurium* (10^5 log CFU/5.1 cm^2 of skin area) and measured the log reduction

of *S. Typhimurium* when stored at 4°C for 96 hours. No significant inhibition ($p > 0.05$) was observed for the 0 and 100 mg/ml nisin-containing alginate coating whereas 300 and 500 mg/ml nisin containing coating treatment yielded significantly larger ($p < 0.05$) population reductions during storage. The authors concluded that alginate based edible films could act as vehicles for delivering nisin to the surface of meats and other food products without a significant loss of inhibitory activity.

Wan and others (1997) studied the effect of free nisin and nisin incorporated in calcium alginate gel, on the inhibition of *Lactobacillus curvatus* in reconstituted skimmed milk stored at 30°C for 21 days. They tested for four concentrations of nisin 50, 125, 250 and 500 IU/ml. At nisin concentrations 250 and 500 IU/ml, both free and alginate incorporated nisin (1:4) was effective in inhibiting the growth of *L. curvatus* in skim milk for 21 days with viable count below the detection limit of 1 CFU/ml.

All the above mentioned studies only present qualitative information on the effectiveness of nisin containing alginate matrices on microbial inhibition. In order to design a desired release matrix using calcium alginate, it is important to study the impact of different factors that govern the structure and properties of calcium alginate films and the diffusion behavior of nisin from these films. Nisin is a cationic polypeptide, whereas alginate is an anionic polymer. Ionic interactions between nisin and alginate might hamper the release of nisin from the alginate matrix leading to reduced antimicrobial activity. Hence, it is essential to characterize any interactions between nisin and alginate and to investigate the antimicrobial activity of nisin released from calcium alginate films.

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Chapter 3

Theoretical considerations for modeling diffusion of nisin from calcium alginate films

3.1 Diffusion

Diffusion is a process by which matter (molecules, ions or other small particles) is transported from a region of higher concentration to a region of lower concentration, by random molecular motion also known as Brownian motion (Cussler, 1997). Thus, the driving force for diffusion is a concentration (chemical potential) gradient.

Diffusion can be described quantitatively using Fick's laws:

$$F = -D\frac{\partial c}{\partial x} \quad \text{(Eq. 3.1)}$$

$$\frac{\partial c}{\partial t} = D\frac{\partial^2 c}{\partial x^2} \quad \text{(Eq. 3.2)}$$

where, F = flux or rate of transfer per unit area (mol/m^2s)

c = nisin concentration in the film (mol/m^3)

D = diffusion coefficient or diffusivity (m^2/s)

x = position coordinate measured in the direction of diffusion (m)

t = time (s)

Equations 3.1 and 3.2 refer to Fick's first and second laws which account for steady and unsteady state diffusion, respectively. The negative sign indicates that diffusion occurs in the direction opposite to that of increasing concentration. These equations are valid only for an isotropic medium, in which the structure and diffusion properties in the

neighborhood of any point are the same relative to all directions. In dilute solutions, D is a constant. D value depends on the nature of the medium, diffusing components and temperature (Islam, 2004). The typical value of diffusion coefficient in gases is around 2 x 10^{-5} m^2/s, in liquids it is 8 x 10^{-8} m^2/s, and in solids it is around 2 x 10^{-11} m^2/s (Cussler 1997, Varzakas and others 2005).

3.2 Power law model

A standard procedure to investigate the diffusion mechanism in a thin sheet is by fitting early portion of the curve (where the fractional release $M_t/M_\infty < 0.6$) to the power law model. (Crank 1975; Han and Floros 2000; Ritger and Peppas 1987):

$$\frac{M_t}{M_\infty} = kt^n \qquad \text{(Eq. 3.3)}$$

where, k is a constant that characterizes the macromolecular network system and n is the diffusional exponent characteristic of the release mechanism. A value of n = 0.5 (Case I transport) indicates Fickian diffusion & release is proportional to $t^{0.5}$. A value of n = 1.0 (Case II transport) indicates non-Fickian diffusion and the release is directly proportional to t. Values between $0.5<n<1.0$ (Case III transport) indicate anomalous or non-Fickian diffusion. Values of $n<0.5$ shows a pseudo-Fickian behavior, where the sorption curves resemble Fickian diffusion curves. Values of $n>1$ define Super Case II transport in which a pronounced acceleration in solute release occurs toward the latter stages of release experiments, resulting in a more rapid relaxation controlled transport (Neogi 1996).

3.3 Analytical solutions

Depending on the geometry, initial and boundary conditions, there have been different analytical solutions proposed for Eq. 3.2. In our case, nisin was diffused through calcium alginate films into a well stirred solution (water). The following assumptions were made:

- the calcium alginate matrix is isotropic and homogeneous
- diffusion takes place from both sides of the film
- diffusion is one dimensional
- diffusion is Fickian
- surface concentrations on both the sides of the film are equal

If c is the concentration of nisin diffusing out of calcium alginate films, c_0 is the initial concentration of nisin uniformly distributed in the film and h is the film thickness, Eq. 3.2 can be solved for the following initial and boundary conditions:

$c= c_{0,}$ $t= 0$

$c= 0,$ $x= -h/2$

$c= 0,$ $x= -h/2$

The solution of Eq. 3.2 under these conditions is (Crank 1975)

$$\frac{C}{C_0} = \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^{\mathrm{n}}}{2\mathrm{n}+1}\exp\{-D_{eff}\,(2\mathrm{n}+1)^2\pi^2 t/h^2\}\left(\cos\frac{(2n+1)\pi x}{h}\right) \qquad \text{(Eq. 3.4)}$$

The fraction of nisin released from calcium alginate films can be calculated by integrating Eq. 3.4 over space and time:

$$\frac{M_t}{M_\infty} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\exp\{-(2n+1)^2\frac{D_{eff}\pi^2 t}{h^2}\} \quad \text{(Eq. 3.5)}$$

where, M_t is the amount of nisin released from the calcium alginate film at time t, M_∞ is the nisin released at infinite time and D_{eff} is the effective diffusivity of nisin, assumed constant.

For short times, when $M_t/M_\infty < 0.6$, the solution simplifies to (Crank 1975; Ritger and Peppas 1987; Han and Floros 2000)

$$\frac{M_t}{M_\infty} = \sqrt{\frac{16\,t\,D_{eff}}{\pi h^2}} \quad \text{(Eq. 3.6)}$$

3.4 References

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Chapter 4

Modified agar diffusion bioassay for better quantification of nisin*

Abstract

Agar diffusion bioassay, using *Micrococcus luteus*, is a widely used method for quantification of nisin concentration. The inhibition zone produced by incubating agar plates containing nisin wells is used as a measure of nisin concentration. The objective of this study was to investigate the effect of different well size (small and large) and pre-diffusion times (0, 24, 48, and 72 h) at 4°C on the sensitivity, precision and accuracy of nisin quantification. Regression analysis indicated that small wells were better than large wells because of smaller standard deviation, higher predictive accuracy, and better discrimination between mean inhibition zones at neighboring nisin concentrations. Statistical analysis by Tukey's test showed that pre-diffusion reslted in significantly different inhibition zone at different nisin concentrations. Pre-diffusion also improved sensitivity of the assay at different nisin concentrations. Different regression models were considered to explore the relationship between inhibition zone and nisin concentration for all pre-diffusion times. A spline model was the best fit model, and 48 h was the best pre-diffusion time.

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4.1 Introduction

There are increasing food safety concerns among the governmental agencies, food industry and consumers alike. As consumer demands for 'natural', 'safe' and 'minimally processed' foods are also increasing, antimicrobial peptides from bacteria that target food borne pathogens without adverse effects to human health, have received considerable attention (Cleveland and others 2001). Nisin is one such bacteriocin produced by lactic acid bacteria (*Lactococcus lactis* subsp *lactis)* fermentation and is approved for use in over 50 countries. Nisin has no flavor, it is non-toxic, rapidly digestible by pancreatic proteases, and heat stable at low pH (DelvesBroughton and others 1996).

Nisin is a polypeptide composed of 34-amino acids and is classified as 'generally regarded as safe (GRAS)' by the Food and Drug Administration (FDA) and the World Health Organization (WHO). It is used in a variety of dairy, plant-based and meat-based food products. Nisin shows antimicrobial activity against a wide range of gram-positive bacteria such as *Bacillus*, *Clostridium*, *Listeria* and *Staphylococcus* species, but has little or no activity against gram-negative bacteria or fungi (Hurst and Hoover 1993). Nisin attacks the target microorganism by interacting with lipid molecules in the microbial cell and forming pores in the bacterial cytoplasmic membrane. This permeabilization of the cytoplasmic membrane results in rapid leakage of essential cellular constituents, causing cell death (Wiedemann and others 2001). The antimicrobial activity of nisin is expressed in International Units (IU), and it is available as Nisaplin®. One gram of pure nisin has an activity of 40 x 10^6 IU while Nisaplin® contains about 2.5 % nisin, and one gram of Nisaplin® has an activity of 10^6 IU (Danisco USA Inc., New Century, KS).

Nisin is considered ‘natural’ only when used in concentrations found in foods naturally fermented with a nisin-producing starter culture (Cleveland and others 2001). Various countries have set limits for maximum levels of nisin in foods. Therefore, several techniques have been developed for detection and quantification of nisin, such as *methylene blue and dilution assay* (Hirsch 1950), *turbidity assays* (Berridge and Barrett 1952), *agar diffusion bioassay* (Tramer and Fowler 1964; Rogers and Montville 1991; Pongtharangkul and Demirci 2004), *high performance liquid chromatography* (Liu and Hansen 1990; Rollema and others 1995; Buonocore 2003), *photometry* (Parente and others 1995), *capillary zonal electrophoresi* (Rossano and others 1998), *bioluminescence* (Immonen and Karp 2007), *colorimetric methods such as bicinchoninic acid protein assay* (Ripoche and others 2006) and *MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay* (Fang and others 2007).

The agar diffusion bioassay is most widely used method for quantifying nisin activity (Pongtharangkul and Demirci 2004). It is based on measuring the inhibition zone produced by nisin sensitive microorganisms in agar plates, and correlating it to the concentration of nisin. Inhibition zone is the result of diffusion of nisin into the agar medium and simultaneous growth of the indicator organism. Greater nisin concentrations result in larger inhibition zones. For quantification of nisin in an unknown sample, the inhibition zone is measured and the nisin concentration is predicted from a calibration plot of inhibition zone vs. nisin concentration.

Mocquot and Lefebvre (1956) developed the first agar diffusion assay for quantification of nisin. In their method, diffusion of nisin through agar is enhanced by addition of 0.3% Tween 80 to the agar and by overnight pre-diffusion of nisin at 4°C.

Tramer and Fowler (1964) improved this method by adding 1% Tween 20 to the agar, while eliminating the pre-diffusion step. In this latter assay, *Micrococcus luteus* was used as the indicator strain and the inhibition zone diameter was linearly related to nisin concentration in the range of 0.5-10 IU/ml. Wolf and Gibbons (1996) improved the accuracy and precision of this method by reducing the concentration of agar from 1.5% to 0.75%, and then buffering the agar with phosphate salts. They showed that the inhibition zone is directly related to the logarithm of nisin concentration in the range of 0-1000 IU/ml. However, Rogers and Montville (1991) showed that pre-diffusion of plates at 3 °C improved the sensitivity and reproducibility of the assay by resulting in larger inhibition zones and lower variability on different days. Pongtharangkul and Demirci (2004) also reported that 24-h pre-diffusion of the agar plates containing nisin wells at 4°C, produced larger inhibition zones and improved the accuracy and precision of nisin quantification as compared to the conventional bioassay. They concluded that nisin concentration should not exceed 300 IU/ml, because the predicted nisin concentration did not exhibit a good linear relationship with the actual nisin concentration above this limit. Therefore, it seems that there is a need to improve the pre-diffusion step and achieve better correlation between inhibition zone and nisin concentration, at higher nisin concentrations.

The objectives of this study were to investigate (a) the effect of agar well size and pre-diffusion time at 4°C, on the sensitivity, accuracy and precision of nisin quantification and (b) the relationship between inhibition zone and nisin concentration over a wide range of nisin concentrations.

4.2 Materials and Methods

4.2.1 Microorganism and media

The agar diffusion assay was performed according to the procedure described by Pongtharangkul and Demirci (2004). As in many previous studies, the nisin-sensitive microorganism used in this study was *Micrococcus luteus* (ATCC 10240). Stock culture was maintained at −80 °C in 20% glycerol. *M. luteus* was grown in Difco™ nutrient broth (Becton, Dickinson and Co., Sparks, MD) at 30°C on an orbital shaker (Model DS500E VWR International Inc., Radnor, PA) at 300 rpm for 24 h and refrigerated at 4°C before use. The media for agar bioassay consisted of 0.8% nutrient broth, 0.75% Bacto™ agar (Becton, Dickinson and Co., Sparks, MD) and 1% Tween 20 (BDH®, Solon, OH).

4.2.2 Nisin standard

Stock solution of Nisaplin® (Danisco USA Inc., New Century, KS) with nisin concentration of 125 µg/ml was prepared in sterile deionized water. Concentrations ranging from 0.625–125 µg/ml were prepared by dilutions in deionized water and utilized to construct the calibration plot.

4.2.3 Agar diffusion bioassay

The agar medium was autoclaved, cooled to 40°C in a water bath and inoculated with 1% v/v of 24 h culture of *M. luteus.* To ensure that an equivalent number of *M. luteus* cells were inoculated into the agar medium each time, the optical density of the inoculum was maintained at 1.7, when measured at 600 nm. The final population of

microorganisms was approximately 10^6 CFU/ml. Two different agar well sizes, small (3.5mm diameter and 2.6mm depth) and large (7mm diameter and 3.5mm depth) were used. Fifteen and twenty mililiter of liquid agar seeded with *M. luteus* was poured into the petri plate aseptically for small and large wells, respectively. This agar was allowed to solidify at 4°C for 30 min and 3-4 wells were made on each plate. Twenty and fifty µl of nisin solution in the concentration range 0.625–125 µg/ml was dispensed into the small and large wells, respectively. These agar plates containing nisin solution were stored at 4°C for 0, 24, 48 and 72 h (pre-diffusion) followed by incubation for 48 h in an environmental chamber maintained at 30°C and 75% RH. The inhibition zone was measured from the edge of the well with a digital vernier caliper (VWR International Inc., Radnor, PA). Four inhibition zone measurements were made for each well and averaged. Each treatment was replicated three times.

4.2.4 Data analysis

The mean inhibition zone was plotted against the logarithm of nisin concentration. From this data, a regression equation for each treatment was developed. The regression equation was of the type:

$$Z = A + B \log(C_N) \qquad \text{(Eq. 4.1)}$$

where, Z = Inhibition zone (mm), C_N = Concentration of nisin (µg/ml), A = constant and B = constant

The slopes of the regression equation indicated the sensitivity of the assay, the *precision* of the assay was inferred from the correlation coefficient (R^2) and the *accuracy* of the assay was inferred from the standard error. The *prediction accuracy* of the model

was inferred from the value of the Predicted Residual Sum of Squares (PRESS) (Kutner and others 2004a). PRESS values indicate how well a regression model predicts new observations. The smaller the PRESS value, the better the model's predictive ability. Results were analyzed using regression analysis and one-way ANOVA (α=0.05) in MINITAB® 14 (Minitab, State College, PA). Tukey's multiple range test (α=0.05) was used to determine any significant differences between mean inhibition zones.

To select the best pre-diffusion time, inverse prediction intervals of nisin were used (Kutner and others 2004b). An inverse prediction interval is the 95% confidence interval for the predicted value of nisin concentration and is computed from the regression of inhibition zone vs. nisin concentration. Smaller prediction intervals correspond to a higher probability of predicting an unknown nisin concentration from the observed inhibition zone correctly. The first step in this analysis was to find the best-fit calibration model. In addition to the log-linear model given in Eq. 4.1, quadratic models and spline models with knot points (K_N) at nisin concentrations 2.5, 6.25, 12.50, 18.75, 25 and 50 µg/ml were also fitted to the data to estimate the best fit model for each pre-diffusion time.

The quadratic model used was of the following type:

$$Z = A + BlogC_N \ + \ C(logC_N)^2 \qquad \text{(Eq. 4.2)}$$

while the spline model was as follows:

$$Z = A + BlogC_N \ + \ Cf(C_N) \qquad \text{(Eq. 4.3)}$$

where, C=constant and $f(\mathrm{C_N}) = \begin{cases} [\log(C_N) - \log(\mathrm{K_N})], & if\ \log(C_N) > \log(K_N) \\ 0, & otherwise \end{cases}$

The goodness of fit was measured by the Akaike information criterion (AIC), R^2 and lack of fit test. AIC is a goodness of fit measure that takes into account the value of the likelihood and the number of parameters in the model (Kutner and others 2004a). The lower the value of AIC, the better the model. The lack of fit test measures how well the model represents the data and it should not be satisfactorily significant. This analysis was done in R (R Development Core Team, 2011). After selecting the best model for each pre-diffusion time, the inverse prediction intervals of nisin as a function of the nisin concentration were computed (Kutner and others 2004b).

4.3 Results and Discussion

4.3.1 Effect of well dimensions

The plot of inhibition zones vs. nisin concentration for small and large wells using Eq. 4.1 is shown in Figure 4-1, and the results of regression analysis are presented in Table 4-1. The slopes for both wells were not significantly different ($p>0.05$) from each other, and their R^2 values were similar. Hence, their sensitivity and precision for nisin quantification is comparable. The standard error and PRESS value was lower for smaller wells. This indicates that the regression model for small wells had smaller standard deviation of the residuals and can predict new observations more accurately than the model with large well size.

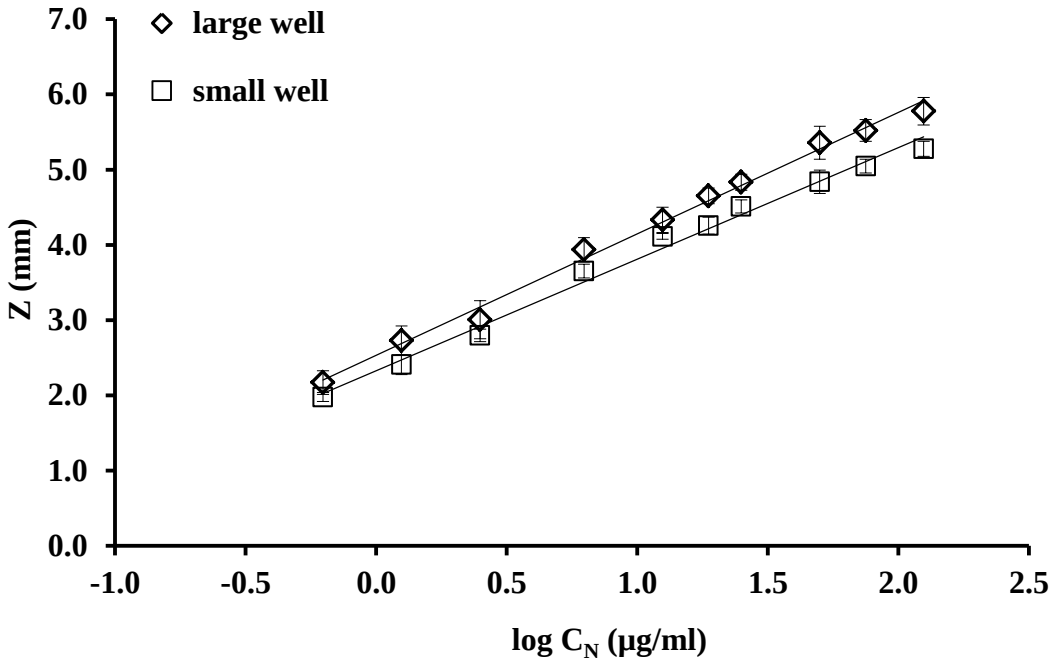

Figure 4-1. Inhibition zone vs. log nisin concentration for different well size. Data (Error bars: ±1 S.D) is an average of 3 replications.

Table 4-1. Regression analysis of inhibition zone vs. nisin concentration for different well size using Eq. 4.1

Well Size	Slope*	R^2	Standard error	PRESS value
Large	1.61[a]	0.98	0.18	0.98
Small	1.48[a]	0.99	0.10	0.61

*Values with a different superscript are significantly different ($p<0.05$)

For large wells, the inhibition zone at a nisin concentration of 1.25 µg/ml was not significantly different ($p>0.05$) from that of 2.50 µg/ml (Table 4-2). Similarly, no significant differences existed between nisin concentrations of 6.25 vs. 12.50, 12.50 vs. 18.75, 18.75 vs. 25, and 50 vs. 125 µg/ml. This indicates a poor separation of inhibition zones at neighboring nisin concentrations, for concentrations greater than 1.25 µg/ml. For small wells, the inhibition zones were significantly different from each other ($p<0.05$) for nisin concentration in the range 0.625-12.50 µg/ml. However, no significant differences existed between nisin concentrations of 12.50 vs. 18.75, 18.75 vs. 25, 50 vs. 75, and 75 vs. 125 µg/ml. Thus, there was a poor separation of inhibition zones at neighboring nisin concentrations for concentrations greater than 12.50 µg/ml. These Tukey's test results indicated that small wells distinguish better between inhibition zones for neighboring nisin concentrations in the range 0.625-12.50 µg/ml.

Comparing the results from regression analysis and Tukey's test, it appears that small wells are better than large wells, because of smaller standard deviation of residuals, higher prediction accuracy, and better distinction between inhibition zones at neighboring nisin concentrations. Hence, small well size was used in further studies comparing the effect of different pre-diffusion times on the sensitivity, accuracy and precision of the agar diffusion bioassay.

Table 4-2. Tukey's multiple test for inhibition zone with different well size

C_N (μg/ml)	Z (mm)* for well size	
	Large	Small
0.625	2.17 ± 0.16^{a}	1.98 ± 0.06^{a}
1.25	2.73 ± 0.2^{b}	2.41 ± 0.13^{b}
2.50	3.01 ± 0.25^{b}	2.80 ± 0.08^{c}
6.25	3.94 ± 0.16^{c}	3.65 ± 0.09^{d}
12.50	4.33 ± 0.17^{cd}	4.11 ± 0.04^{e}
18.75	4.65 ± 0.11^{d}	4.25 ± 0.11^{ef}
25	4.83 ± 0.11^{d}	4.51 ± 0.09^{f}
50	5.36 ± 0.22^{e}	4.84 ± 0.16^{g}
75	5.52 ± 0.15^{e}	5.05 ± 0.09^{gh}
125	5.78 ± 0.18^{e}	5.28 ± 0.10^{h}

*In a given column, values with different superscripts are significantly different ($p<0.05$)

4.3.2 Effect of pre-diffusion time

The plot of inhibition zone vs. nisin concentration for all pre-diffusion times is shown in Figure 4-2, and their regression analysis results are presented in Table 4-3. The slopes of the regression lines with pre-diffusion 0, 24, 48 and 72 h were all significantly different ($p<0.05$) from each other. This indicates that an increase in pre-diffusion time would increase assay sensitivity. This observation is in agreement with results from previous investigators (Pongtharangkul and Demirci 2004; Rogers and Montville 1991) who showed that pre-diffusion increased the inhibition zone and the slope of the regression line, enabling a more sensitive assay. During pre-diffusion, there is no microbial growth while nisin diffuses through the agar. A longer pre-diffusion allows a larger volume of nisin to diffuse into the agar before the onset of microbial growth, giving larger inhibition zones for the same nisin concentration. The R^2 values for all pre-

diffusion times were high, while the standard errors and PRESS values increased with increasing pre-diffusion time. Therefore, longer pre-diffusion times resulted in higher sensitivity, but lower accuracy of the assay.

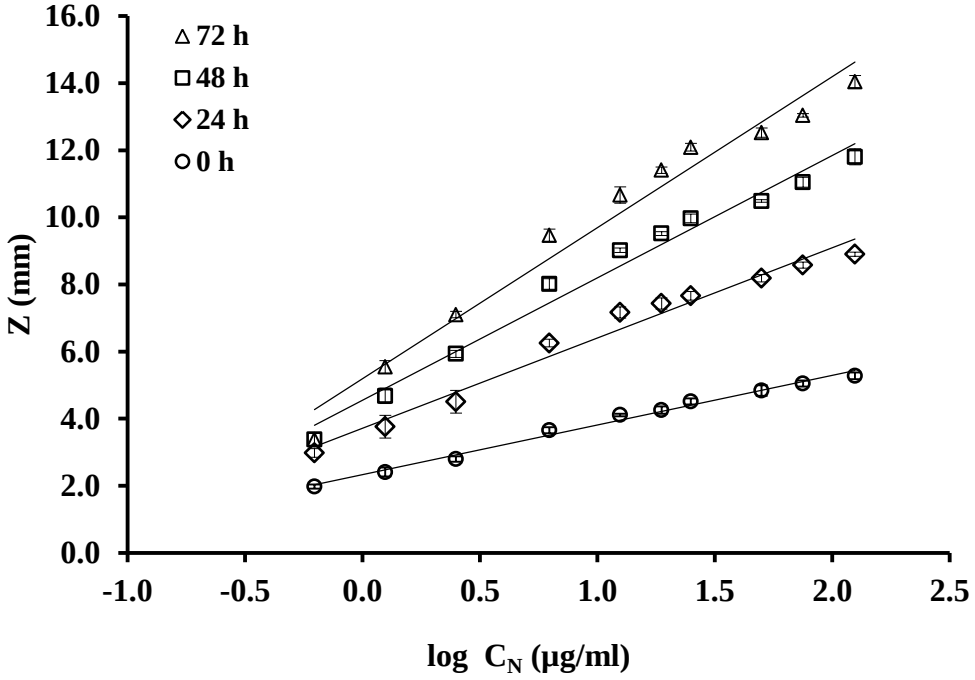

Figure 4-2. Inhibition zone vs. nisin concentration for different pre-diffusion times. Data (Error bars: $\pm$1 S.D) is an average of 3 replications.

Table 4-3. Regression analysis of inhibition zone vs. nisin concentration for different pre-diffusion times using Eq. 4.1

Pre-diffusion time (h)	Slope*	R^2	Standard error	PRESS value
0	1.48^a	0.99	0.10	0.61
24	2.69^b	0.98	0.35	4.00
48	3.64^c	0.98	0.40	5.07
72	4.50^d	0.97	0.58	11.13

*Values with a different superscript are significantly different ($p<0.05$)

As seen earlier for pre-diffusion time of 0 h, there was a poor separation of inhibition zones at neighboring nisin concentrations for concentrations greater than 12.50 µg/ml (Table 4-4). For 24 h pre-diffusion, inhibition zones were significantly different from each other ($p<0.05$) for nisin concentration in the range 0.625-25 µg/ml. However, there was no significant difference between inhibition zones at nisin concentrations 25 vs. 50, 50 vs. 75, and 75 vs. 125 µg/ml. For 48 h pre-diffusion, inhibition zones were significantly different from each other ($p<0.05$) for all nisin concentrations. For 72 h pre-diffusion, inhibition zones were significantly different from each other ($p<0.05$) for all nisin concentration, except for 25 vs. 50 µg/ml. Thus, pre-diffusion at 4°C improved the separation of inhibition zones at neighboring nisin concentrations up to 48 h, with 48 h giving the best separation.

Table 4-4. Tukey's multiple test for inhibition zones with different pre-diffusion times

C_N (µg/ml)	Z (mm)* for pre-diffusion time			
	0 h	24 h	48 h	72 h
0.625	1.98 ± 0.06^{a}	2.98 ± 0.13^{a}	3.38 ± 0.18^{a}	3.38 ± 0.19^{a}
1.25	2.41 ± 0.13^{b}	3.76 ± 0.34^{b}	4.68 ± 0.23^{b}	5.54 ± 0.19^{b}
2.50	2.80 ± 0.08^{c}	4.53 ± 0.34^{c}	5.93 ± 0.11^{c}	7.10 ± 0.10^{c}
6.25	3.65 ± 0.09^{d}	6.06 ± 0.14^{d}	8.02 ± 0.16^{d}	9.47 ± 0.18^{d}
12.50	4.11 ± 0.04^{e}	6.86 ± 0.03^{e}	9.02 ± 0.07^{e}	10.67 ± 0.24^{e}
18.75	4.25 ± 0.11^{ef}	7.43 ± 0.16^{f}	$9.520.06^{f}$	11.41 ± 0.09^{f}
25	4.51 ± 0.09^{f}	7.95 ± 0.01^{g}	9.97 ± 0.13^{g}	12.09 ± 0.11^{g}
50	4.84 ± 0.16^{g}	8.18 ± 0.11^{gh}	10.49 ± 0.04^{h}	12.52 ± 0.14^{g}
75	5.05 ± 0.09^{gh}	8.6 ± 0.09^{hi}	11.05 ± 0.15^{i}	13.04 ± 0.05^{h}
125	5.28 ± 0.10^{h}	8.91 ± 0.06^{i}	11.80 ± 0.24^{j}	14.05 ± 0.18^{i}

*In a given column, values with a different superscript are significantly different ($p<0.05$)

Based on the results from regression analysis and Tukey's test using the log-linear model (Eq. 4.1), it was found that longer pre-diffusion times resulted in higher sensitivity and better separation of inhibition zones at neighboring nisin concentrations, but lower accuracy of the assay. Hence, this model was not conclusive in selecting a best pre-diffusion time. Quadratic (Eq. 4.2) and spline (Eq. 4.3) models were fitted to the data and compared with the log-linear model. The AIC, R^2 and lack of fit test values for these models at all pre-diffusion times are presented in Table 4-5. The R^2 values for all models were high. For 0 h pre-diffusion time, the only models that passed the lack of fit test ($p>0.05$) were the quadratic and spline models with knot points at nisin concentrations of 6.25, 12.50, 18.75 and 25 µg/ml. For 24 h pre-diffusion time, only spline models with knot points at nisin concentrations of 18.75 and 25 µg/ml passed the lack of fit test ($p>0.05$). Similarly, for 48 h pre-diffusion time, only the spline model with knot point at

nisin concentration of 12.50 µg/ml passed the lack of fit test ($p>0.05$). None of the models passed the lack of fit test ($p<0.05$) at 72 h pre-diffusion time. Hence, 72 h pre-diffusion time was not considered for any further comparison. Based on the smallest AIC value for models that passed the lack of fit test ($p>0.05$), spline models with knot points at nisin concentration 12.50, 50 and 12.50 µg/ml were the best for pre-diffusion times 0, 24 and 48 h, respectively.

The plot of inhibition zone vs. nisin concentration using a spline model is shown in Figure 4-3. Spline model as the best-fit model in this study indicated a change in the driving force for diffusion of nisin through agar in the vicinity of the knot point concentration. The agar well has a limited volume of nisin solution. The progressive depletion of nisin solution from this well during pre-diffusion and incubation can reduce the driving force for diffusion of nisin through the agar. In addition, there was microbial growth during incubation at 30ºC. An increase in microbial cell density in the agar can reduce the free volume, slowing down nisin diffusion further. It should be pointed out that spline models are simplifications of a real situation where there is no real discontinuity. Although we have simplified the standard plot by using a spline model with one single knot point, the actual transition in the diffusivity of nisin would happen over a wide range of concentrations.

Table 4-5. R^2, AIC and lack of fit p-value of various regression models for all pre-diffusion times (computed using R)

Pre-diffusion time (h)	Model	R^2	AIC	Lack of fit p-value
0	Log-linear	0.99	-29.77	0.005
	Quadratic	0.99	-40.84	0.101
	Spline knot log(2.50)	0.99	-30.21	0.006
	Spline knot log(6.25)	0.99	-41.01	0.105
	Spline knot log(12.50)	0.99	-45.46	0.285
	Spline knot log(18.75)	0.99	-43.99	0.209
	Spline knot log(25)	0.99	-44.31	0.224
	Spline knot log(50)	0.99	-38.96	0.063
24	Log-linear	0.98	20.47	0.000
	Quadratic	0.99	4.04	0.009
	Spline knot log(2.50)	0.98	19.14	0.000
	Spline knot log(6.25)	0.99	7.55	0.003
	Spline knot log(12.50)	0.99	-0.40	0.030
	Spline knot log(18.75)	0.99	-5.52	0.112
	Spline knot log(25)	0.99	-9.64	0.283
	Spline knot log(50)	0.99	5.01	0.006
48	Log-linear	0.98	33.61	0.000
	Quadratic	0.99	-4.60	0.004
	Spline knot log(2.50)	0.99	19.25	0.000
	Spline knot log(6.25)	0.99	-11.87	0.030
	Spline knot log(12.50)	0.99	-14.07	0.053
	Spline knot log(18.75)	0.99	-4.37	0.003
	Spline knot log(25)	0.99	2.45	0.000
	Spline knot log(50)	0.99	24.00	0.000
72	Log-linear	0.97	56.56	0.000
	Quadratic	0.99	2.73	0.001
	Spline knot log(2.50)	0.99	30.86	0.000
	Spline knot log(6.25)	0.99	5.56	0.001
	Spline knot log(12.50)	0.99	7.84	0.000
	Spline knot log(18.75)	0.99	14.17	0.000
	Spline knot log(25)	0.99	19.52	0.000
	Spline knot log(50)	0.98	45.10	0.000

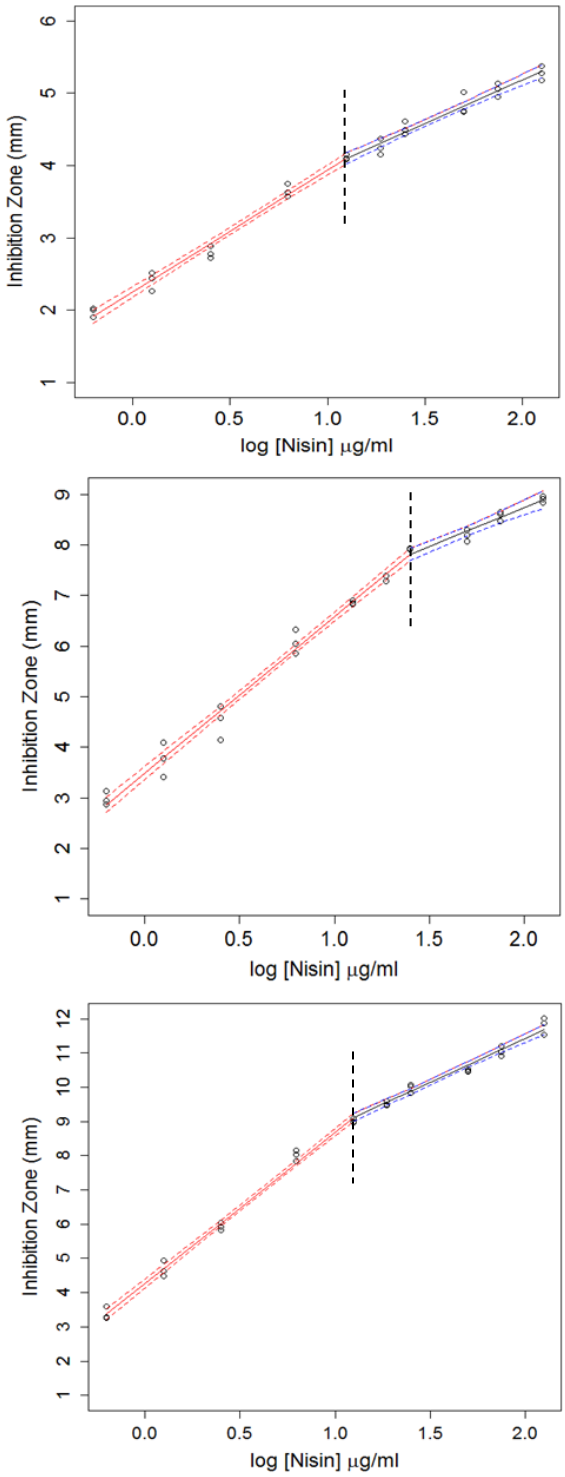

Figure 4-3. Inhibition zone vs. log nisin concentration using Eq. 4.3 for pre-diffusion times (a) 0 h, (b) 24 h and (c) 48 h generated using R. The dashed vertical line shows knot point. Dotted lines show 95% confidence interval.

The spline model equations for inhibition zone vs. nisin concentration for pre-diffusion times 24 and 48 h are:

For 24 h pre-diffusion:

$$Z = 3.49 + 3.09\log(C_N) - 1.54f(C_N) \quad \text{(Eq. 4.4)}$$

where, $f(\mathrm{C_N}) = \begin{cases} [\log(C_N) - \log(18.75)], & if\ \log(C_N) > \log(18.75) \\ 0\,, & otherwise \end{cases}$

For 48 hr pre-diffusion:

$$Z = 4.27 + 4.41\log(C_N) - 1.83f(C_N) \quad \text{(Eq. 4.5)}$$

where, $f(\mathrm{C_N}) = \begin{cases} [\log(C_N) - \log(12.50)], & if\ \log(C_N) > \log(12.50) \\ 0, & otherwise \end{cases}$

4.3.3 Selecting the best pre-diffusion time

After finding the best-fit model for each pre-diffusion time, the next step was to determine the best pre-diffusion time. Nisin concentration and its 95% inverse prediction interval were calculated using spline models for each pre-diffusion time. Pre-diffusion time with smaller prediction intervals was considered better.

The plot of inhibition zone vs. log nisin concentration with the observed and predicted values of nisin concentration and its 95% inverse prediction interval is shown in Fig 4-4. The inverse prediction interval for various nisin concentrations and pre-diffusion times is shown in Fig 4-5. It appears that longer pre-diffusion time produced shorter prediction intervals with 48 h pre-diffusion time having the shortest prediction interval. Hence, our results suggest that 48 h pre-diffusion time should be used to estimate nisin concentration in the range 0.625-125 µg/ml. When nisin concentration was

less than 18.75 µg/ml (log 1.3), the prediction interval for 24 h pre-diffusion time was similar to that of 48 h pre-diffusion time. Therefore, 24 h pre-diffusion time can be used to predict nisin concentrations upto 18.75 µg/ml.

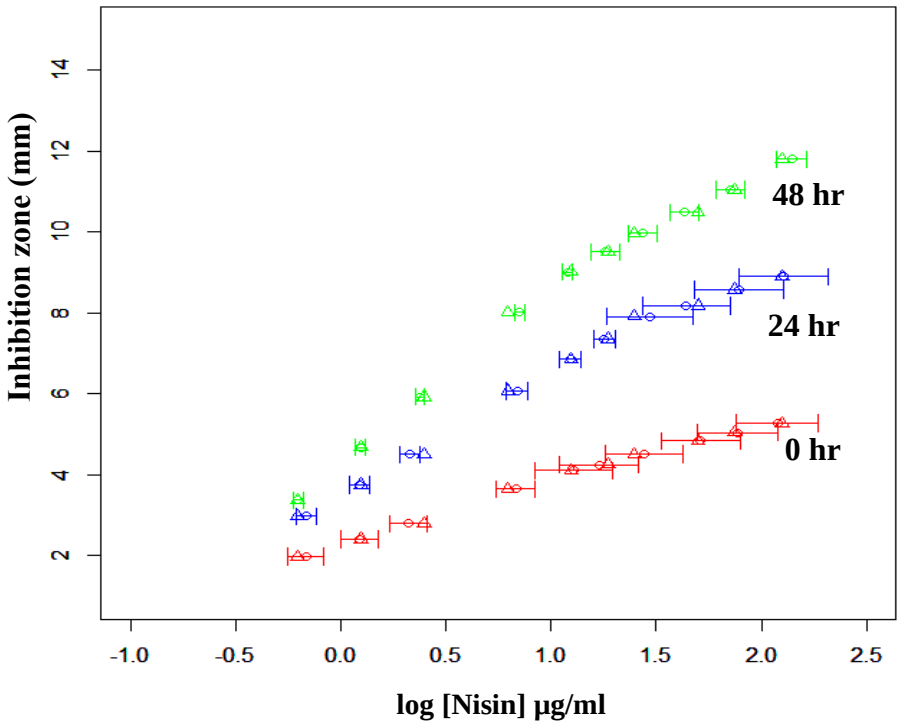

Figure 4-4. Inhibition zone vs. log nisin concentration with observed (○) and predicted (△) values (using Eq. 4.3) of nisin concentration generated using R and showing 95% inverse prediction intervals for different pre-diffusion times.

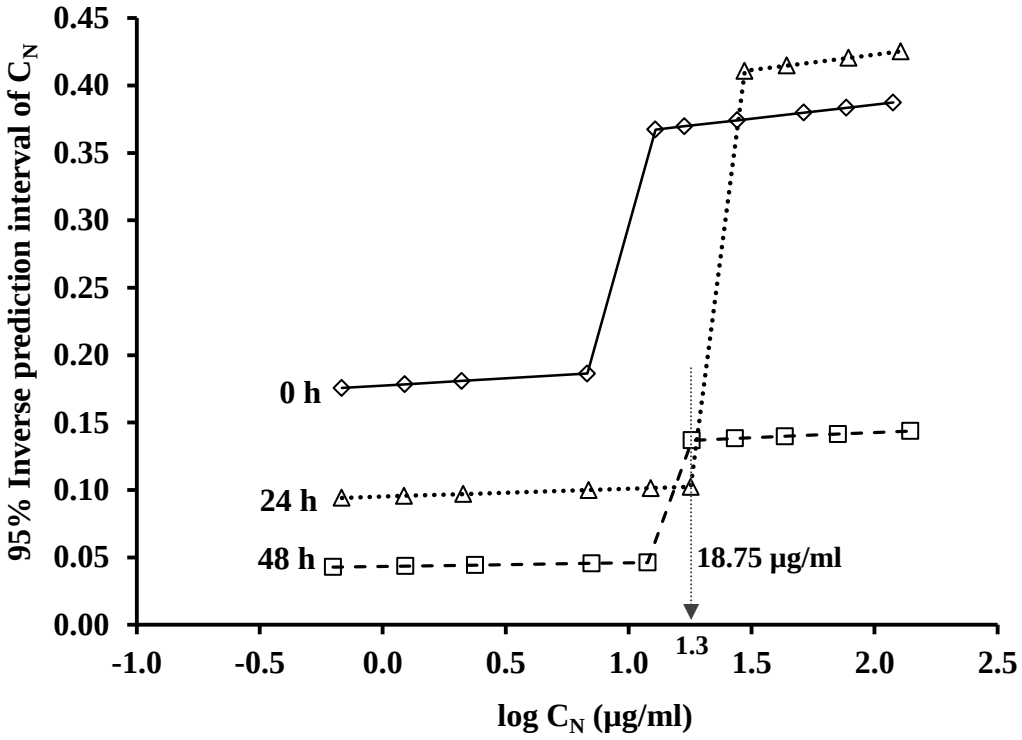

Figure 4-5. 95% inverse prediction interval of nisin concentration vs. log nisin concentration for spline models with different pre-diffusion times

4.4 Conclusions

In our agar diffusion bioassay, small wells demonstrated higher accuracy for quantification of nisin. Pre-diffusion improved separation of inhibition zones at various nisin concentrations. Pre-diffusion also improved the sensitivity of the assay. Spline models produced excellent correlation between nisin concentration and inhibition zone for 0, 24 and 48 h pre-diffusion times. The statistical models tested in this study had poor correlation between inhibition zone and nisin concentration at 72 h pre-diffusion time. 48 h was the best pre-diffusion time for nisin concentration in the range 0.625-125 µg/ml. 24

h pre-diffusion time can be used for nisin concentrations only upto 18.75 µg/ml, without compromising accuracy of prediction. This modified agar diffusion assay can be used for quantification of nisin in solutions and monitoring rate of nisin production during fermentation.

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Chapter 5
Release of nisin from calcium alginate films as affected by film formation method, cross-linking and alginate composition

Abstract

In order to design a desired release matrix for nisin using calcium alginate, it is important to study the impact of different factors that govern the structure and properties of calcium alginate films and hence, the diffusion of nisin from these films. The objective of this research was to investigate the effect of method of film formation, cross-linking parameters and alginate composition on the release of nisin from calcium alginate films using disc agar diffusion bioassay. Films were formed by two methods – cross-linking before drying (CBD) and cross-linking after drying (CAD) – using various alginate compositions, cross-linking times and Ca^{2+} concentrations. The method of film formation, cross-linking time and Ca^{2+} concentration had a significant effect ($p<0.05$) on nisin release. Alginate composition did not have any significant effect ($p>0.05$) on nisin release. Highest nisin release was obtained for films made by CBD method and cross-linked with high Ca^{2+}. SEM images showed that film formation method influenced the microstructure of the film.

5.1 Introduction

Nisin is a bacteriocin produced by lactic acid bacteria *Lactococcus lactis* subsp. *lactis.* Nisin shows antimicrobial activity against a wide range of gram-positive bacteria such as *Bacillus*, *Clostridium*, *Listeria* and *Staphylococcus* species, but has little or no activity against gram-negative bacteria or fungi (Hurst and Hoover 1993). Nisin is cationic owing to the presence of three lysine residues plus one (in nisin Z*)* or two (in nisin A) histidine residues (Abee and Delves-Broughton 2003). It is permitted for use in over 50 countries as a food preservative and it is used in a variety of plant, dairy and meat-based food products (Delves-Broughton 2005).

Alginates are naturally occurring, high molecular weight polysaccharides extracted mainly from brown algae (Pheophyceae) and bacteria. It is an anionic copolymer of β-D-mannuronic acid and α-L-guluronic acid. Alginates have the unique property of forming irreversible heat-stable gels, which can set at ambient temperatures in the presence of divalent ions such as Ca^{2+}, Ba^{2+}, Sr^{2+}, etc. Gelation results from specific and strong interactions between these divalent ions and blocks of guluronic acid residues. The divalent cation fits between four guluronic acid molecules like an egg in an egg-box as and it is called an ‘egg-box’ model (Gregor and Grant 1973; Morris and others 1978; Smidsrød 1974). The extent of gel formation can be controlled by regulating the cross-linking time and concentration of cross-linker (Onsoyen 1997).

Several studies have reported that calcium alginate matrix (films/coatings/beads) containing nisin is effective in inhibiting microbial growth in various foods such as beef, poultry, milk and mushrooms (Cutter and Siragusa 1997; Fang and Tsai 2003; Lu and

others 2009; Luttmann 2012; Millette and others 2007; Natrajan and Sheldon 2000; Wan and others 1997). The release of nisin from calcium alginate coatings in contact with the food product results in microbial inhibition, thereby increasing the product shelf-life. However, these studies present only qualitative information on the overall effectiveness of nisin containing alginate matrices on microbial inhibition. In order to design a desired release matrix using calcium alginate, it is important to study the impact of different factors that govern the structure and properties of calcium alginate films and in turn, the diffusion of nisin from these films.

The purpose of this study was to investigate the effect of method of film formation, cross-linking parameters and alginate composition on the release of nisin from calcium alginate films using disc agar diffusion bioassay.

5.2 Materials and methods

5.2.1 Materials

Sodium alginate with two different compositions was obtained from Sigma-Aldrich (Saint Louis, MO). Chemical composition and sequence analysis of sodium alginate was performed by Exova (Santa Fe Springs, CA) using ^{1}H nuclear magnetic resonance spectroscopy (ASTM F2259-10). The chemical composition of different sodium alginates is shown in Table 5-1. Nisaplin®, a commercial grade of nisin, was provided by Danisco Inc. (New Century, KS). Nisaplin® contains 2.5% nisin and 1g Nisaplin® has an activity of 10^6 International Units. Anhydrous calcium chloride was

obtained from J. T. Baker (Gibbstown, NJ). Square plastic plates (110mmx15mm) used for casting films were obtained from Electron Microscopy Science (Hatfield, PA).

Table 5-1. Chemical composition of different sodium alginate samples

Alginate sample	**Fraction of mannuronate (M)[a]**	**Fraction of guluronate (G)[a]**	**M/G ratio**	**$N^{b}_{G>1}$**
Low guluronate	0.62	0.38	1.63	14.2
High guluronate	0.33	0.67	0.49	3.0

[a] Standard deviation (SD) for M and G values is ± 0.01.
[b] $N_{G>1}$ is the number of consecutive guluronate moieties in guluronic acid block sequons. Relative standard deviation (RSD) for $N_{G>1}$ is 10%

5.2.2 Microorganism and media

The nisin-sensitive microorganism used for this study was *Micrococcus luteus* ATCC 10240 (Pongtharangkul and Demirci 2004; Tramer and Fowler 1964). Stock culture was maintained at −80°C in 20% glycerol. *M. luteus* was grown in Difco™ nutrient broth (Becton Dickinson and Co., Sparks, MD) at 30°C on an orbital shaker at 300 rpm for 24 h and refrigerated at 4°C before use. The media for agar bioassay consisted of 0.8% nutrient broth, 0.75% Bacto™ agar (Becton, Dickinson and Co., Sparks, MD) and 1% Tween 20 (BDH®, Solon, OH).

5.2.3 Film formation

Sodium alginate film solution was prepared by dissolving 0.5 g of sodium alginate in 50 ml of deionized water and heating to 70°C until the alginate dissolved. This alginate solution was allowed to cool at ambient conditions for 4 h and Nisaplin® was

added to give nisin concentration of 300 µg/ml. Calcium alginate films were formed by the following two methods:

i) Cross-linking after drying (CAD). In this method, the cooled sodium alginate solution containing nisin was poured into a casting plate followed by drying and conditioning within an environmental chamber maintained at 50±5% RH and 21±1°C for 2 days. The dried sodium alginate films were cross-linked by spraying with calcium chloride solution (20 sprays/film corresponding to about 8 ml of $CaCl_2$) on both surfaces. The cross-linking parameters were: Ca^{2+} concentration (0.125 and 2.5 M) and cross-linking time (0.5 and 30 min). After cross-linking, these films tested for nisin release by disc agar diffusion assay.

ii) Cross-linking before drying (CBD). In this method, the cooled alginate solution containing nisin was poured into a casting plate and cross-linked by spraying with calcium chloride solution (20 sprays/film corresponding to about 8 ml of $CaCl_2$) on the air-side of the solution. This resulted in an instantaneous calcium alginate gel formation. This cross-linked gel was dried and conditioned in the casting plate within an environmental chamber maintained at 50±5% RH and 21±1 °C for 2 days. The cross-linking parameters were: Ca^{2+} concentration (0.125 and 2.5 M) and cross-linking time was the same as drying time (2 days). After drying, these films were tested for nisin release by disc agar diffusion assay.

For both film formation methods, films were washed by spraying with deionized water and dried gently by blotting between two tissue papers, before conducting the agar diffusion assay. This was done to ensure that the observed nisin release was not only due to the nisin present on the surface of the film, and also to remove any excess Ca^{2+} from

the surface of the films. For films formed by CBD method, Ca^{2+} solution was sprayed on the air-side of the sodium alginate solution in the casting plate. Nisin released when the air-side of the film was in contact with agar was compared with nisin released when the plate-side of these films was in contact with agar. This was done to test whether Ca^{2+} diffused uniformly throughout the sodium alginate solution, forming a homogenous calcium alginate gel.

5.2.4 Disc agar diffusion bioassay

The agar disc diffusion bioassay was performed according to the procedure outlined by Pongtharangkul and Demirci (2004) and Sanla-Ead and others (2012) with modifications. Films were cut into 8.5 mm diameter discs using a cork borer. The agar medium was autoclaved, cooled to 40°C in a water bath and inoculated with 1% v/v of 24 h culture of *M. luteus.* To ensure that an equivalent number of *M. luteus* cells were inoculated into the agar medium each time, the optical density of the innoculum was maintained at 1.7 when measured at 600 nm. The final population of microorganisms was approximately 10^6 CFU/ml. Agar medium seeded with *M. luteus* was dispensed into petri plates and allowed to solidify at 4°C for 30 min. After this, three calcium alginate film discs were placed on these solidified agar plate and the plates were stored at 4°C (for pre-diffusion) for 24 h followed by incubation within an environmental chamber maintained at 30°C and 75% RH (to allow microbial growth and prevent desiccation). The inhibition zone diameter was measured with a digital vernier caliper (VWR International Inc. Radnor, PA). The mean inhibition zone for each film was evaluated as an average of four measurements.

Inhibition zone is the result of diffusion of nisin through calcium alginate film onto the film surface, diffusion of this surface nisin into the agar medium and simultaneous growth of *M.luteus*. Hence, inhibition zone is a measure of amount of nisin released from calcium alginate films. Greater nisin release from the film will result in larger inhibition zones. The inhibition distance was calculated as follows:

$$Inhibition\ distance(mm) = \frac{(Inhibition\ zone - alginate\ disc\ diameter)}{2}$$

5.2.5 Scanning electron microscopy (SEM)

SEM was performed according to the procedure described in Ouwerx and others (1993). The cross-section of the alginate films containing nisin prepared by the CAD and CBD method was observed in a Field Emission Scanning electron microscope (FEI Nova™ NanoSEM 630) under low vacuum and 5 kV. Calcium alginate samples were prepared by drying in liquid CO_2 in a critical point-dryer. Calcium alginate films were dehydrated in a series of gradually increasing ethanol concentrations: 10, 30, 50, 70, 90 and 100%. Each step was allowed to equilibrate for 10 or 15 min, followed by critical point drying. The dried samples were fractured using a razor blade. The sodium alginate film without any cross-linking disintegrated in ethanol. Hence, it was observed under the scanning electron microscope without any pre-treatment.

5.2.6 Experimental design and statistical analysis

A 2^n full factorial experimental design was used. The experimental design (coded values) for films formed by CAD and CBD method is shown in tables 5-2 and 5-3, respectively. The coded and actual values of the factors are shown in table 5-4. The data were analyzed by one-way ANOVA (α=0.05) using the software Statistica 6.0 (Statsoft

Inc., Tulsa, OK). The dependent variable for every treatment was the inhibition distance. Each treatment was done in triplicates.

Table 5-2. Experimental design (coded values) for nisin release from films formed by cross-linking after drying (CAD) method

Experiment number	Run order	M/G ratio	Ca^{2+} concentration	Cross-linking time
1	2	high	low	low
2	7	high	low	high
3	8	high	high	low
4	4	high	high	high
5	3	low	low	low
6	6	low	low	high
7	1	low	high	low
8	5	low	high	high

Table 5-3. Experimental design (coded values) for nisin release from films formed by cross-linking before drying (CBD) method

Experiment number	Run order	M/G ratio	Ca^{2+} concentration	Contact side
1	7	high	low	low
2	4	high	low	high
3	3	high	high	low
4	1	high	high	high
5	6	low	low	low
6	2	low	low	high
7	8	low	high	low
8	5	low	high	high

Table 5-4. Coded and actual values of factors

Coded value	M/G ratio	Ca^{2+} concentration (M)	Cross-linking time (min)	Contact side
low	0.49	0.125	0.5	air-side
high	1.63	2.5	30	plate-side

5.3 Results and Discussion

The amount of nisin in each film disc was about 100 µg and the area of the disc was 57 mm^2. Thus, final concentration of nisin in each calcium alginate disc was about 1.8 $\mu g/mm^2$.

5.3.1 Cross-linking after drying (CAD)

The mean inhibition distance for various treatments is shown in Table 5-5 and the ANOVA table for all the main effects and their two-way interactions is presented in Table 5-6. Different levels of Ca^{2+} and cross-linking time had a significant effect ($p=0.00$) on the inhibition distance. The effect of different M/G on the inhibition distance was not significantly different ($p=0.20$). There was significant interaction effect ($p=0.0004$) between Ca^{2+} concentration and cross-linking time. There was a significant lack of fit ($p<0.05$) on fitting a linear regression model with two-way interactions, whereas there was no significant lack of fit ($p>0.05$) on fitting a saturated linear regression model with 3-way interactions between M/G, Ca^{2+} concentration and cross-linking time (Table 5-7). In this model, the 3-way interaction between the factors was significant ($p=0.01$) indicating that M/G could have a significant effect on the nisin release for certain combination of Ca^{2+} concentration and cross-linking time.

The main effects and significant interaction effects plot is presented in Figure 5-1 and Figure 5-2, respectively. The inhibition distance decreased on increasing the cross-linking time and Ca^{2+} concentration (Figure 5-1). However, as seen from the interaction plot in Figure 5-2, the effect of cross-linking time is significant ($p=0.0$) only at high Ca^{2+} concentration and for low M/G alginate. This decrease in the inhibition distance with increasing cross-linking time and Ca^{2+} concentration was probably due to the formation of a more densely cross-linked calcium alginate gel which hinders nisin release. Similar observation has been reported by other researchers (Al-Musa and others 1999; Aslani and Kennedy 1996; Badwan and others 1985; Zactiti and Kieckbusch 2006). Alginate with low M/G has higher guluronnate content. Since Ca^{2+} binds strongly with guluronnate blocks of alginate, higher guluronnate content would result in a stronger gel. Hence the effect of interaction between cross-linking time and Ca^{2+} concentration was significant ($p<0.05$) for alginate with low M/G ratio.

Table 5-5. Mean inhibition zone of various treatments for calcium alginate films formed by cross-linking after drying (CAD) method

Experiment number	M/G ratio	Ca^{2+} conc. (M)	Cross-linking time (min)	Mean inhibition distance (mm)*	95% Confidence interval (±)
1	1.69	0.125	0.5	7.59 ± 0.50	1.24
2	1.69	0.125	30	6.68 ± 0.49	1.22
3	1.69	2.5	0.5	6.29 ± 0.12	0.30
4	1.69	2.5	30	4.45 ± 1.32	3.27
5	0.49	0.125	0.5	5.94 ± 0.65	1.61
6	0.49	0.125	30	6.99 ± 0.73	1.82
7	0.49	2.5	0.5	6.69 ± 0.56	1.39
8	0.49	2.5	30	3.59 ± 0.55	1.38

Table 5-6. ANOVA table of all the main effects and their two-way interactions for calcium alginate films formed by cross-linking after drying (CAD) method

Effect	SS	df	MS	F	p-value
M/G ratio	1.22	1.00	1.22	2.56	0.1292
Ca^{2+}	14.31	1.00	14.31	29.98	0.0001
Cross-linking time	8.69	1.00	8.69	18.21	0.0006
M/G * Ca^{2+}	0.29	1.00	0.29	0.62	0.4443
M/G * Cross-linking time	0.18	1.00	0.18	0.38	0.5466
Ca^{2+} * Cross-linking time	9.65	1.00	9.65	20.20	0.0004
Lack of fit (Model error)	3.88	1.00	3.88	8.12	0.0116
Pure Error (Data error)	7.64	16	0.48		
Total SS	45.87	23			

Table 5-7. ANOVA table of all the main effects and their three-way interactions for calcium alginate films formed by cross-linking after drying (CAD) method

Effect	SS	df	MS	F	p-value
M/G ratio	1.22	1.00	1.22	2.56	0.1292
Ca^{2+}	14.31	1.00	14.31	29.98	0.0001
Cross-linking time	8.69	1.00	8.69	18.21	0.0006
M/G * Ca^{2+}	0.29	1.00	0.29	0.62	0.4443
M/G * Cross-linking time	0.18	1.00	0.18	0.38	0.5466
Ca^{2+} * Cross-linking time	9.65	1.00	9.65	20.20	0.0004
M/G * Ca^{2+} *Cross-linking time	3.88	1.00	3.88	8.12	0.0116
Pure Error (Data error)	7.64	16	0.48		
Total SS	45.87	23			

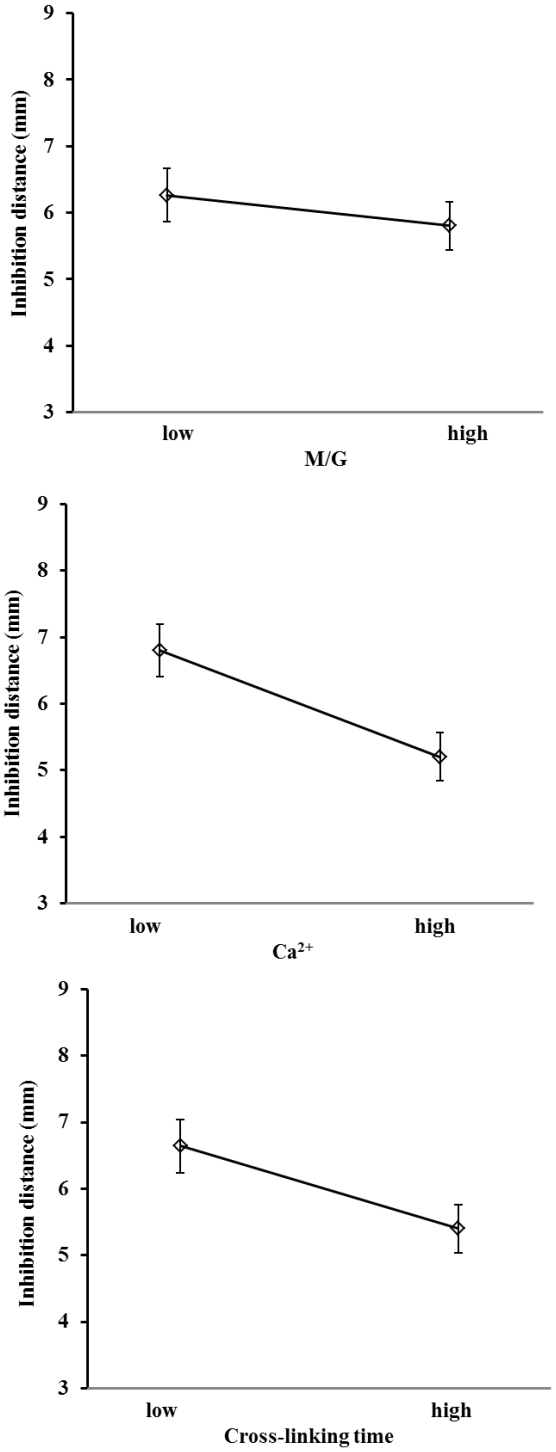

Figure 5-1. Inhibition distance vs. main effects for films formed by cross-linking after drying (CAD) method. The bars represent 95% confidence interval.

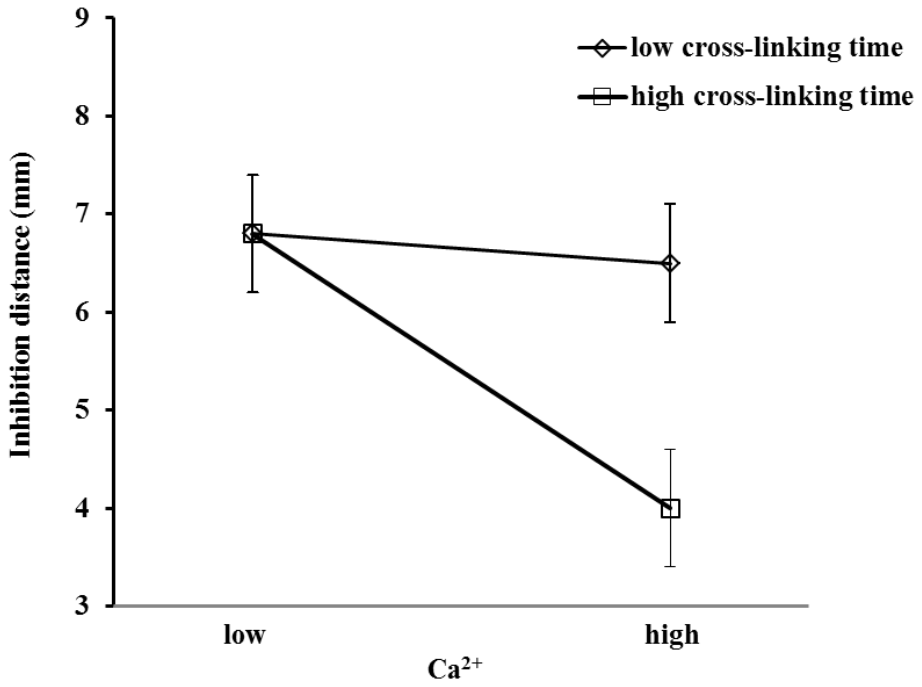

Figure 5-2. Inhibition distance vs. Ca^{2+} concentration at different cross-linking times for films formed by cross-linking after drying (CAD) method. The bars represent 95% confidence interval.

5.3.2 Cross-linking before drying (CBD)

The mean inhibition distance for various treatments is shown in Table 5-8 and the ANOVA table for all the main effects and their interactions is presented in Table 5-9. The main effects plot is presented in Figure 5-3. Among the main effects, only Ca^{2+} concentration was significant ($p = 0.00$). None of the interactions had any significant effect ($p>0.05$) on the inhibition distance. The side of the film in contact with agar did not have any significant effect ($p=0.56$) on the inhibition distance. This indicates that Ca^{2+} diffused uniformly through the sodium alginate solution giving homogeneous calcium alginate gels. The inhibition distance increased on increasing the Ca^{2+} concentration. This

behavior is opposite of that observed for nisin release from films made by CAD method. This could be due to ionic interaction between nisin and alginate. Nisin is cationic whereas, alginate is anionic in nature. When combined together in a matrix, ionic binding between them is likely to exist. It appears that this binding may be reversible in the presence of another cation such as Ca^{2+}. As Ca^{2+} is distributed uniformly throughout the calcium alginate film, higher concentration of Ca^{2+} can replace a higher amount of nisin bound to alginate. Hence, more nisin becomes available for release, resulting in larger inhibition distance.

Table 5-8. Mean inhibition zone of various treatments for calcium alginate films formed by cross-linking before drying (CBD) method

Experiment number	M/G ratio	Ca^{2+} conc. (M)	Contact side	Mean inhibition distance (mm)*	95% Confidence interval (±)
1	1.63	0.125	up	6.53 ± 0.52	1.29
2	0.49	0.125	up	7.17 ± 1.72	4.27
3	1.63	2.5	up	13.48 ± 0.83	2.07
4	0.49	2.5	up	13.93 ± 0.74	1.84
5	1.63	0.125	down	6.88 ± 0.53	1.32
6	0.49	0.125	down	7.25 ± 1.54	3.82
7	1.63	2.5	down	13.47 ± 0.57	1.42
8	0.49	2.5	down	13.87 ± 0.68	1.68

Table 5-9. ANOVA table of all the main effects and their interaction on the inhibition distance for calcium alginate films formed by CBD method

Effect	SS	df	MS	F	p-value
M/G ratio	1.29	1.00	1.29	1.39	0.26
Ca^{2+}	271.70	1.00	271.70	291.91	0.00
Contact side	0.05	1.00	0.05	0.05	0.82
M/G * Ca^{2+}	0.01	1.00	0.01	0.01	0.92
M/G * Contact side	0.04	1.00	0.04	0.04	0.84
Ca^{2+} * Contact side	0.09	1.00	0.09	0.10	0.75
Pure Error (Data error)	15.82	17	0.93		
Total SS	289.00	23			

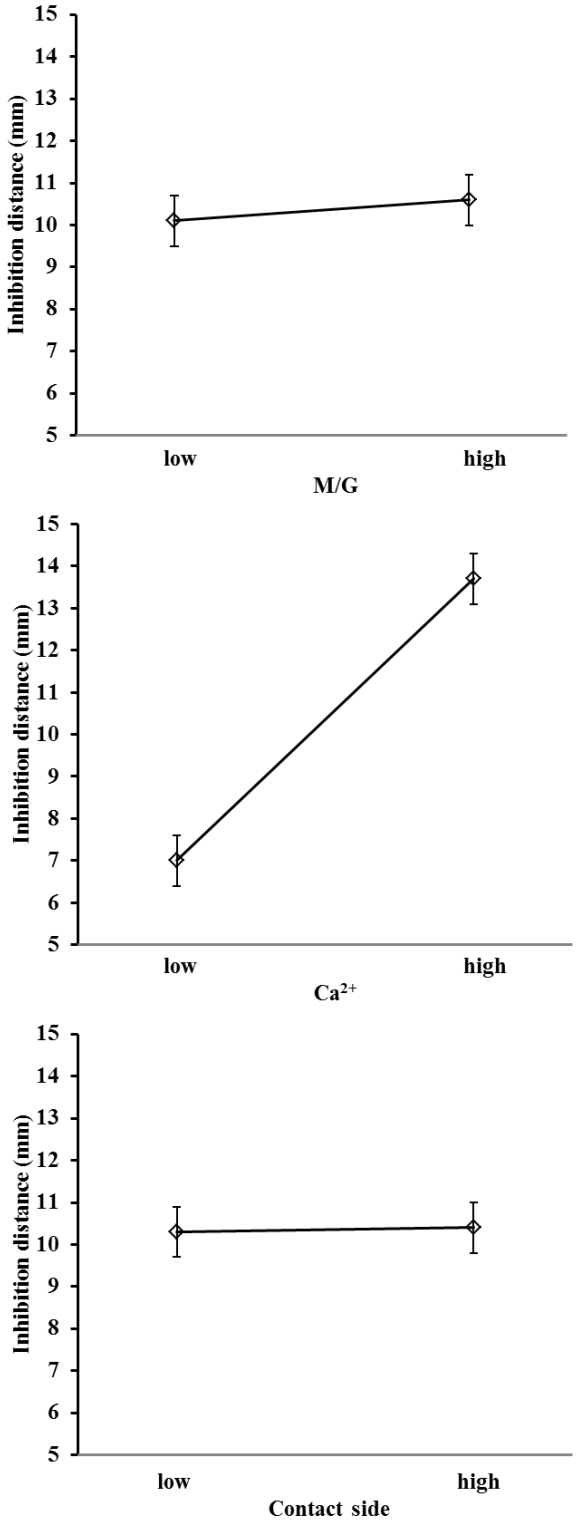

Figure 5-3. Inhibition distance vs. main effects for films formed by CBD method. The bars represent 95% confidence interval.

5.3.3 Method of film formation

The inhibition distance for nisin released from calcium alginate films formed by CAD method (cross-linked with 0.125 and 2.5M Ca^{2+} for 0.5 min) was compared with that released from calcium alginate films formed by CBD method (cross-linked with 0.125 and 2.5M Ca^{2+} and air-side in contact with agar) by Tukey's test (α=0.05). As seen in 5.3.1 and 5.3.2, the effect of alginate composition on the nisin release was similar for both film formation methods. Hence, films formed using alginate with M/G of 0.49 was used for this comparison.

When cross-linked with low Ca^{2+} concentration, the inhibition distance was not significantly different ($p<0.05$) for the different film formation methods (Figure 5-4). However, when cross-linked with high Ca^{2+} concentration, the inhibition distance was significantly higher ($p<0.05$) for films formed by CBD method those formed by CAD method. Overall, the film made by CBD method and cross-linked with high Ca^{2+} concentration resulted in the largest inhibition distance.

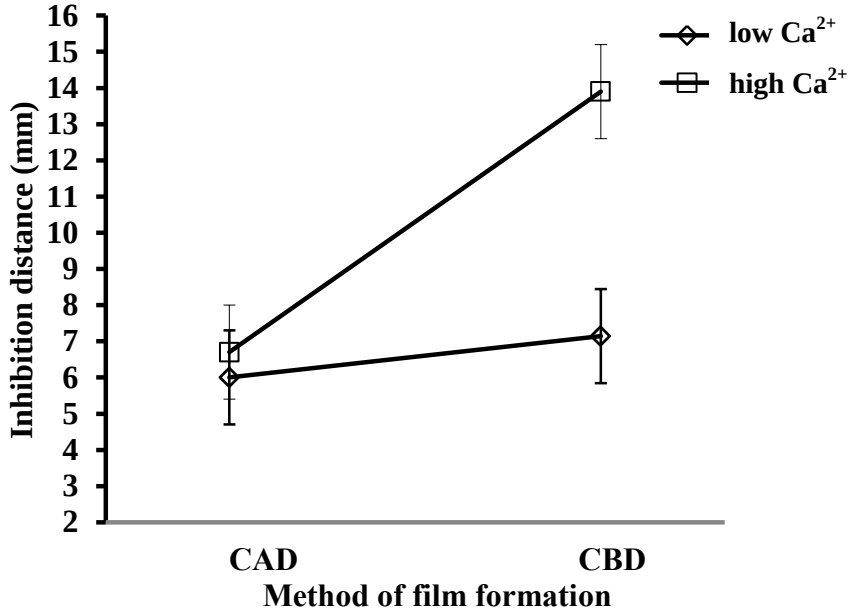

Figure 5-4. Inhibition distance vs. films formation method for different Ca^{2+} concentrations. The bars represent 95% confidence interval.

SEM images of the cross-section of alginate films prepared by the CAD and CBD method are shown in Figure 5-5. The cross-section of sodium alginate film appears smooth. Films formed by CAD method, different cross-linking times resulted in a different microstructure. Films cross-linked for 0.5 min show cross-linking mainly at the surface and have a smooth structure in the center. There was no such difference in structure between the surface and the center for films cross-linked for 30 min. However, these films had several layers. Films formed by CBD method had a different microstructure than those formed by CAD method. SEM images indicated that film formation method affects calcium alginate microstructure. In CAD method, Ca^{2+} is sprayed on dry sodium alginate film whereas in the CBD method, Ca^{2+} is sprayed on

sodium alginate film solution. Cross-linking alginate in solid vs. liquid state could have resulted in a different calcium alginate microstructure.

When Ca^{2+} is sprayed on sodium alginate, it reacts with the carboxylic groups of guluronic acid residues at the surface and then diffuses inward and reacts to form calcium alginate gel. Al-Musa and others (1999) studied the effect of different cross-linking procedures on the release of metoclopramide hydrochloride and cisapride from calcium alginate films formed by CAD method. They hypothesized cross-linking of alginate films to be an interfacial phenomenon. In case of our films formed by CAD method, it appears that cross-linking starts at the film surface, yielding an almost complete cross-linked surface. This highly cross-linked film interface can hinder the diffusion of Ca^{2+} into the adjacent alginate layers. The Ca^{2+} that successfully diffuses into the adjacent layers cross-links an additional number of guluronates forming calcium alginate gel, thereby obstructing the ionic movement further. This can slow down the Ca^{2+} diffusion considerably, resulting in a progressive reduction in the extent of cross-linking from the surface to the center of the film. However, in case of films formed by CBD method, Ca^{2+} diffuses throughout the sodium alginate solution forming a homogeneously cross-linked calcium alginate gel. This Ca^{2+} can replace higher amount of nisin that maybe bound to alginate resulting in higher free nisin and hence, larger inhibition distance.

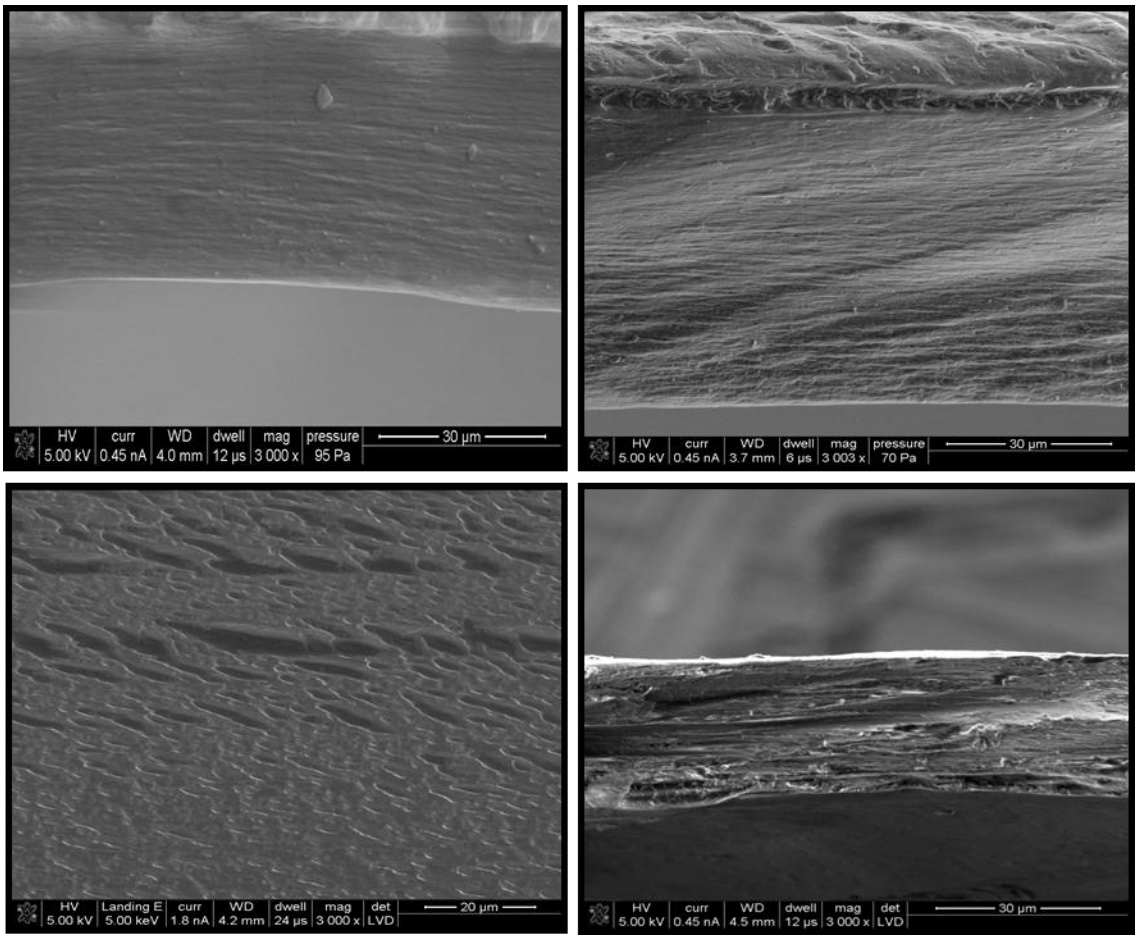

Figure 5-5. SEM images (3000x) of cross-section of sodium alginate film containing nisin (top left), calcium alginate film containing nisin formed by CAD method cross-linked with 2.5 M Ca^{2+} for 0.5 min (top right), calcium alginate film containing nisin formed by CAD method cross-linked with 2.5 M Ca^{2+} for 30 min (bottom right) and calcium alginate film formed by CBD method cross-linked with 2.5 M Ca^{2+} (bottom left).

5.4 Conclusion

Method of film formation, cross-linking time and Ca^{2+} concentration had a significant effect ($p<0.05$) on the nisin release. Alginate M/G ratio did not have any significant effect ($p<0.05$) on the nisin release. For films formed by CAD method, nisin release decreased with increasing cross-linking time and Ca^{2+} concentration. For films formed by CBD method, nisin release increased with increasing Ca^{2+} concentration. SEM images showed that film formation method influenced the microstructure of films considerably. Films made by the CBD method and cross-linked with high Ca^{2+} concentration had the highest nisin release.

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Chapter 6

Investigation of nisin binding with sodium and calcium alginate

Abstract

Ionic interactions between the cationic nisin and anionic alginate might hamper the release of nisin from the alginate matrix, leading to reduced antimicrobial activity. The objective of this study was to investigate the nature and reversibility of nisin alginate binding. The binding between nisin and alginate was investigated for various concentrations and compositions of sodium alginate (low and high guluronate) and in the presence of Ca^{2+}. Sodium alginate (1.67-33.33mg/ml) was added to nisin solution (0.5 mg/ml) and free nisin remaining in the solution was measured by HPLC. Results demonstrate that nisin binds with alginate, possibly through electrostatic interactions. The fraction of free nisin decreased with increasing alginate concentration. The composition of alginate did not have a significant effect ($p>0.05$) on the nisin-sodium alginate binding. This binding was reversible in the presence of Ca^{2+}. Higher Ca^{2+} concentration replaces a higher amount of nisin bound to alginate with high guluronate.

6.1 Introduction

Nisin is a polypeptide composed of 34-amino acids and is recognized 'generally regarded as safe (GRAS)' by the Food and Drug Administration (FDA) and the World Health Organization (WHO). It is used in a variety of dairy, plant and meat-based food products. Nisin shows antimicrobial activity against a wide range of gram-positive bacteria such as *Bacillus*, *Clostridium*, *Listeria* and *Staphylococcus* species (Hurst and Hoover 1993). Nisin contains three lysine residues plus one (in nisin Z*)* or two (in nisin A) histidine residues**.** The *pKa* values of the side-chain groups in histidine and lysine residues are 6.5 and 10.0, respectively (Abee and Delves-Broughton 2003). Nisin is cationic when the environmental pH is lower than the *pKa* values of its side chain groups, owing to the presence of primary amine groups ($-NH_3^+$) in these basic amino acid residues.

Alginates are naturally occurring, high molecular weight polysaccharides extracted mainly from brown algae (*Pheophyceae*) and bacteria. Alginate is an anionic copolymer of β-D-mannuronic acid (M) and α-L-guluronic acid (G), linked together to form linear molecules with (1,4) glycosidic bonds (Onsoyen 1997). At pH 2, alginate is uncharged due to complete protonation (Magnin and Dumitriu 2005). The negative charge increases with pH, and at pH 6.5 alginate is completely deprotonated. Alginates have the unique property of forming irreversible heat-stable gels, which can set at ambient temperatures in the presence of divalent ions such as $Ca^{2+,}$ $Ba^{2+,}$ Sr^{2+}, etc. The divalent cation fits between four guluronic acid molecules like an egg in an egg-box as and it is called an 'egg-box' model (Gregor and Grant 1973; Morris and others 1978;

Smidsrød 1974). Higher G content and longer block length results in stronger calcium alginate gels. Alginate is used as a food additive owing to its multi-functional properties such as thickening, gelation, and stabilizing aqueous mixtures, dispersions and emulsions.

When combined together into a matrix, ionic interactions between the cationic nisin and anionic alginate are likely to exist. Several studies have reported that calcium alginate matrix (films/coatings/beads) containing nisin is effective in inhibiting microbial growth in various foods such as beef, poultry, milk and mushrooms (Cutter and Siragusa 1997; Fang and Tsai 2003; Lu and others 2009; Luttmann 2011; Millette and others 2007; Natrajan and Sheldon 2000; Wan and others 1997). The release of nisin from calcium alginate matrix in contact with a food product results in microbial inhibition, thereby increasing product shelf-life. However, these studies provide only qualitative information on the overall effectiveness of nisin containing alginate matrices on the microbial inhibition. Ionic interactions between nisin and alginate might hamper the release of nisin from the alginate matrix, leading to reduced antimicrobial activity. In order to design effective antimicrobial systems, it is important to characterize the interactions between nisin and alginate.

The objectives of this research were to: a) investigate the binding between nisin and alginate for various concentrations and composition of sodium alginate; and b) study the nature (reversible/irreversible) of nisin-sodium alginate binding in the presence of Ca^{2+}.

6.2 Materials and methods

6.2.1 Materials

Sodium alginate with two different compositions of guluronate, low and high, was obtained from Sigma-Aldrich (Saint Louis, MO). Chemical composition and sequence analysis of sodium alginate was performed by Exova (Santa Fe Springs, CA) using ^{1}H nuclear magnetic resonance spectroscopy (ASTM F2259-10). Pure nisin powder (Danisco Inc. New Century, KS) was used for infrared spectroscopy studies. For binding studies, a commercial grade of nisin, Nisaplin® (Danisco Inc. New Century, KS), was used. Nisaplin® contains 2.5% nisin and one gram Nisaplin® has an activity of 10^{6} International Units. The elemental analysis of Nisaplin® was performed by Covance Laboratories (Philadelphia, PA) using Inductively Coupled Plasma (ICP) spectrometry as described in Official Methods of Analysis of AOAC INTERNATIONAL (2005). Anhydrous calcium chloride was obtained from J.T.Baker (Gibbstown, NJ). Square plastic plates (110mmx15mm) used for casting films were obtained from Electron Microscopy Science (Hatfield, PA).

6.2.2 Film preparation

Alginate films were prepared by dissolving 0.5 g of sodium alginate in 50 ml of deionized water and heating to 70°C until the alginate was dissolved. This solution was allowed to cool at ambient conditions for 4 h and poured in to a casting plate followed by drying and conditioning within an environmental chamber maintained at 50±5% RH and 21±1 °C for 2 days.

6.2.3 Measurement of free nisin

A stock solution of nisin with concentration 0.5 mg/ml was prepared by dissolving 20 mg of Nisaplin® in deionized water. Fifteen milliliter of this stock solution was dispensed into a petri dish. Different amounts (25-500 mg) and composition (high and low guluronate) of sodium alginate films were added to this solution. To determine the time taken for the binding reaction to reach equilibrium, 25 and 500 mg of sodium alginate films were used, giving alginate concentration of 1.67 and 33.33 mg/ml of nisin solution, respectively. To investigate nisin-alginate binding, six concentrations of sodium alginate films (1.67-33.33 mg/ml of nisin solution) were used. To study the nature (reversible/irreversible) of nisin-sodium alginate binding, 300 mg sodium alginate film was used, resulting in alginate concentration of 20 mg/ml in the nisin solution. The petri dishes containing nisin and sodium alginate solution were placed on an orbital shaker at 90 rpm within an environmental chamber maintained at 21±1°C. A 0.5 ml sample was drawn from this solution and the free nisin concentration was determined by HPLC. To determine the time taken for the binding reaction to reach equilibrium, free nisin concentration was determined at times 3, 6, 9, 12, 24 and 48 h. To investigate nisin-alginate binding, free nisin concentration was measured at the equilibrium time. To study the nature of binding, anhydrous calcium chloride (0.5, 1 and 1.5 M) was added to this solution after the nisin-alginate complex reached equilibrium and free nisin concentration was determined at 0, 3, 6, 9 and 12 h, after addition of Ca^{2+}. All experiments were done in triplicates.

The fraction of free nisin was calculated as follows:

$$Fraction\ of\ free\ nisin = \frac{[Nisin]_t}{[Nisin]_{t=0}}$$

where, $[Nisin]_t$ = concentration of nisin at time t (mg) and $[Nisin]_{t=0}$ = initial concentration of nisin (mg)

6.2.4 Quantification of nisin by High Performance Liquid Chromatography (HPLC)

The HPLC method was adopted with modifications from Liu and Hansen (1990) and Buonocore and others (2003). A C-18 reverse phase column with silica packing (size 5 µm and pore 100A°) and dimensions 250 mm x 4.6 mm was used (Restek Corporation, Bellefonte, PA, USA). A Shimadzu HPLC system (LC-20AD with LCSolution software; autosampler SIL-20AC HT; UV-VIS detector SPD-20AV) was used with the following operating parameters: injection volume 50 µl, UV absorption at 220 nm and temperature 40°C. Mobile phase was a mixture of acetonitrile containing 0.1% trifluroacetic acid (EMD Gibbstown, NJ, USA) and ultrapure water, flowing at 1ml/min. The mobile phase was run in gradient mode, wherein the concentration of acetonitrile was varied from 25-75% within 15 min.

The solutions were filtered with a 0.45 µm syringe filter (Iso-disc Nylon 13 mm x 0.45 µm, Restek Corporation, Bellefonte, PA, USA) before injecting into HPLC. To prevent column damage, the sodium alginate present in alginate-nisin solution was precipitated with the organic mobile phase (acetonitrile containing 0.1% trifluoroacetic acid). 0.5ml organic mobile phase was added to 0.5ml of alginate-nisin sample and centrifuged at 6000 rpm for 10 min. The free nisin concentration was measured in the

supernatant. A calibration plot was constructed for nisin peak area against nisin concentration of standard solutions ranging from 0.0025-0.5 mg/ml (Appendix A).

6.2.5 Fourier Transform Infrared (FT-IR) spectroscopy

FT-IR was conducted to investigate any changes in nisin conformation due to binding with alginate. A Bruker IFS 66/s equipped with a single reflection diamond ATR accessory (Harrick MVP-Pro™ Star, Bruker Optics Billerica, MA, USA) was used to acquire FT-IR spectra at ambient conditions. The sample compartment was flushed with N_2 gas to minimize the effects of water vapor and CO_2. Spectra were recorded from 400–4000 cm^{-1} at a resolution of 6 $cm^{-1.}$ Each spectrum represents an average of 100 scans. Background spectra consisted of the bare diamond crystal under the same experimental conditions. All spectra were plotted in absorbance units, –log (S/R), where S and R were the single channel spectra of the sample and the bare diamond crystal, respectively. All spectral manipulations were performed by the software OPUS 6.0 (Bruker Optics Billerica, MA, USA).

Spectra were collected for pure nisin powder and sodium alginate films with nisin and without nisin. A control sample of nisin was prepared to investigate the effect of dissolution in deinonized water on the conformation of nisin. Nisin solution with concentration 0.5 mg/ml in deionized water was poured on the casting plate used for making sodium alginate films. The water was evaporated by placing these plates within an environmental chamber maintained at 50±5% RH and 21±1 °C for 2 days. Spectra collected for the nisin powder scraped off from this plate served as the nisin control. Measurements were made for two replicates, with three samples for every replication.

6.2.6 Statistical analysis

The data was analyzed by one-way ANOVA (α=0.05) using MINITAB® 14 (State College, PA). Significant differences between the means were tested by Tukey's test (α=0.05).

6.3 Results and Discussion

Chemical composition of different sodium alginates is shown in Table 6-1. The elemental analysis of Nisaplin® is shown in Table 6-2. In addition to nisin, Nisaplin® was composed of Na and Ca.

Table 6-1. Chemical composition of sodium alginate samples

Alginate sample	Avg. molecular weight (kDa)	Fraction of mannuronate[a] (M)	Fraction of guluronate[a] (G)	M/G ratio	$N^b_{G>1}$
Low guluronate	46	0.62	0.38	1.63	3.0
High guluronate	150	0.33	0.67	0.49	14.2

[a] Standard deviation for M and G values is ± 0.01.
[b] $N_{G>1}$ is the number of consecutive guluronate moieties in guluronic acid block sequons. Relative standard deviation for $N_{G>1}$ is 10% (Exova, Santa Fe Springs, CA)

Table 6-2. Elemental analysis of Nisaplin®

Element	Amount (mg/g Nisaplin®)
Calcium	5.45
Sodium	335
Iron, Magnesium, Manganese, Phosphorous, Potassium, Zinc, Copper	ND*

*Not determinable (ND) as they were below the detection limits

6.3.1 Determination of equilibrium binding time for nisin-alginate complex

Fraction of free nisin measured in nisin solution containing different concentrations (low= 1.67 mg/ml and high= 33.33 mg/ml) and M/G of sodium alginate is shown in Figure 6-1 and the analysis of means by Tukey's test is shown in Table 6-3. Alginate film solutions with an M/G of 0.49 and alginate concentration higher than 33.33 mg/ml were too viscous too handle. Hence, alginate concentrations only up to 33.33 mg/ml were used for this study. Based on the results from Tukey's test, equilibrium was reached at different times for different film solutions. For alginate with an M/G of 1.69, equilibrium was reached between 0-3 h and 12-24 h for low and high sodium alginate concentrations, respectively. For alginate with an M/G of 0.49, equilibrium was reached between 3-6 h and 9-12 h for low and high sodium alginate concentrations, respectively. Equilibrium was reached within 24 hr for all solutions. Hence, 24 h was selected as the equilibrium binding time for further studies.

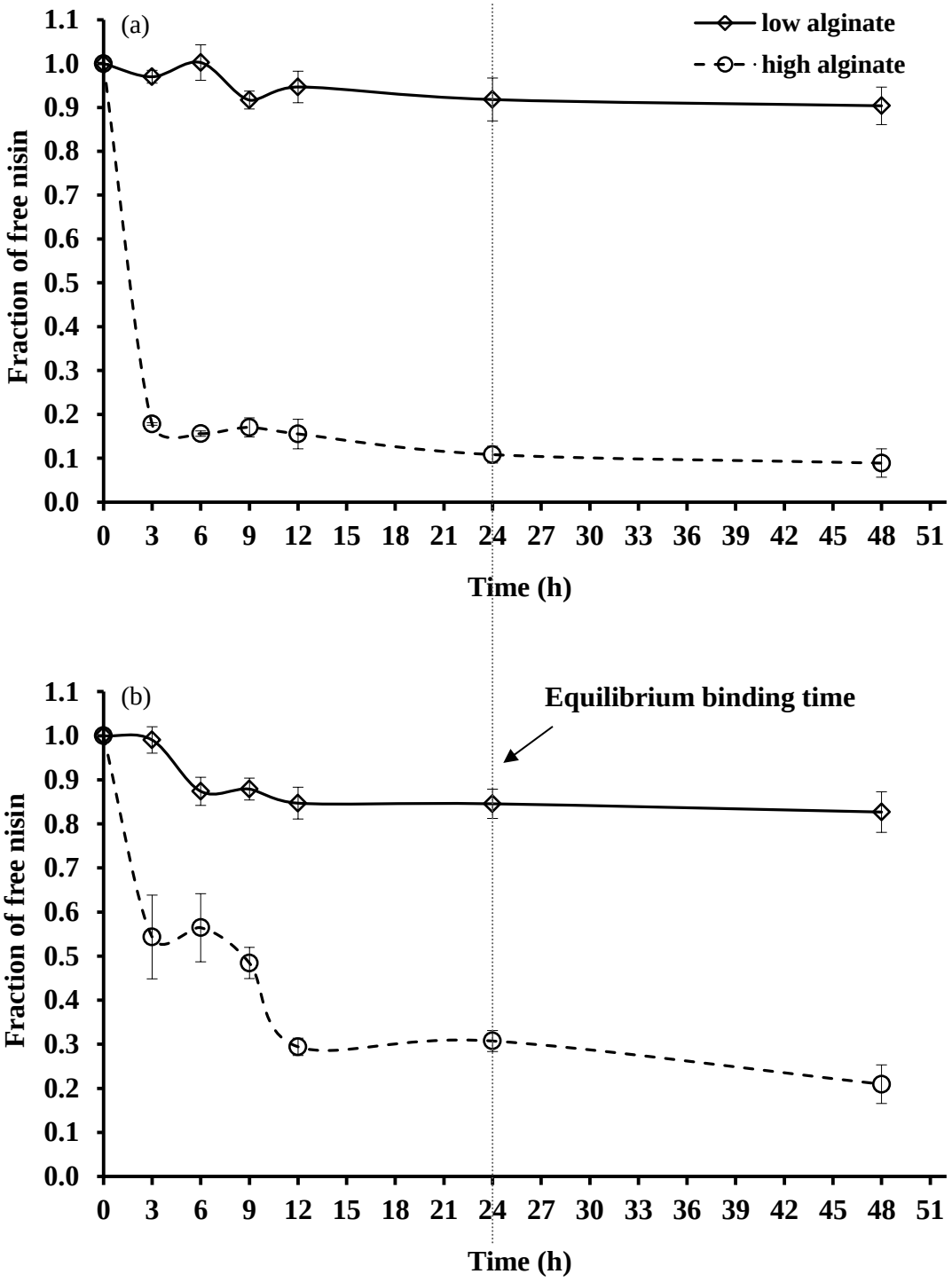

Figure 6-1. Fraction of free nisin measured in nisin solution containing different concentrations of sodium alginate with M/G ratio of (a) 1.63 and (b) 0.49. Each data point (Error bars: ±1S.D) is an average of 3 replications.

Table 6-3. Tukey's test for fraction of free nisin measured at various times in nisin solution containing different concentration and M/G ratio of sodium alginate

Alginate M/G ratio	Alginate conc.	Time (h)	Fraction of free nisin (mg/mg)*
1.63	low	0	1.00 ± 0.00^{a}
		3	0.97 ± 0.01^{ab}
		6	1.00 ± 0.04^{ab}
		9	0.92 ± 0.02^{ab}
		12	0.95 ± 0.04^{ab}
		24	0.92 ± 0.05^{ab}
		48	0.90 ± 0.04^{b}
1.63	high	0	1.00 ± 0.00^{a}
		3	0.18 ± 0.00^{b}
		6	0.16 ± 0.01^{bc}
		9	0.17 ± 0.02^{b}
		12	0.16 ± 0.03^{bc}
		24	0.11 ± 0.02^{cd}
		48	0.09 ± 0.03^{d}
0.49	low	0	1.00 ± 0.00^{a}
		3	0.99 ± 0.03^{a}
		6	0.87 ± 0.03^{b}
		9	0.88 ± 0.03^{b}
		12	0.85 ± 0.04^{b}
		24	0.85 ± 0.03^{b}
		48	0.83 ± 0.05^{b}
0.49	high	0	1.00 ± 0.00^{a}
		3	0.54 ± 0.09^{b}
		6	0.56 ± 0.08^{b}
		9	0.48 ± 0.04^{b}
		12	0.29 ± 0.02^{c}
		24	0.31 ± 0.02^{c}
		48	0.21 ± 0.04^{c}

*For a given alginate M/G ratio and concentration, values (Mean±1SD) with a different superscript are significantly different from each other ($p<0.05$)

6.3.2 Determination of free nisin

Figure 6-2 shows fraction of free nisin measured after 24 h (equilibrium binding time) in nisin solution containing different concentrations (1.67, 3.33, 6.67, 13.33, 20 and 33.33 mg/ml) and M/G ratio of sodium alginate. The analysis of means by Tukey's test is shown in Table 6-4.

Fraction of free nisin decreased significantly ($p<0.05$) with increasing alginate concentration from 1.67 to 13.33 mg/ml, and did not change significantly ($p>0.05$) beyond alginate concentration of 13.33 mg/ml. For sodium alginate with an M/G ratio of 1.63, free nisin fraction decreased from 0.94 to 0.09 as alginate concentration increased from 1.67 to 33.33 mg/ml, respectively. For sodium alginate with an M/G ratio of 0.49, free nisin fraction decreased from 0.95 to 0.25 as alginate concentration increased from 1.67 to 33.33 mg/ml, respectively. This progressive decrease in the availability of free nisin with increasing alginate concentration is an indication of binding between alginate and nisin. Overall, sodium alginate with different M/G ratio did not change the fraction of free nisin significantly ($p>0.05$).

Ideally, all of nisin should be bound to alginate, shown as dashed line in Figure 6-2. This was not observed in our case due to high viscoity of the alginate solution above 13.33 mg/ml, which could have prevented from proper mixing of the solution. The binding ratio at point A (19mg/ml sodium alginate), defined as moles of nisin bound per mole alginate, was found to be 0.36 and 11.28 for sodium alginate with M/G ratio of 1.63 and 0.49, respectively.

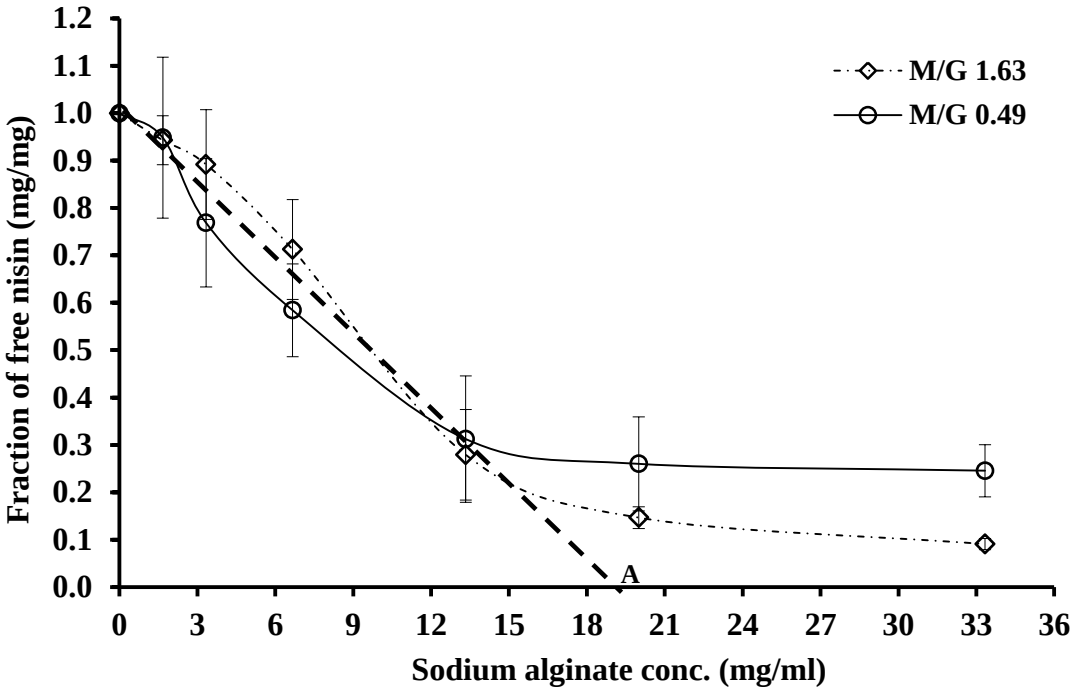

Figure 6-2. Fraction of free nisin measured after 24 h in nisin solution containing different concentrations and M/G ratio of sodium alginate. Each data point (Error bars: ±1S.D) is an average of 3 replications.

Table 6-4. Tukey's test for fraction of free nisin measured after 24 h in nisin solution containing different M/G ratio and concentrations of sodium alginate

Alginate M/G ratio	Sodium alginate conc. (mg/ml)	Free nisin fraction (mg/mg)*
1.63	0	1.00 ± 0.02^{a}
	1.67	0.94 ± 0.05^{ab}
	3.33	0.89 ± 0.12^{ab}
	6.67	0.71 ± 0.10^{b}
	13.33	0.28 ± 0.10^{c}
	20	0.15 ± 0.02^{c}
	33.33	0.09 ± 0.01^{c}
0.49	0	1.00 ± 0.02^{a}
	1.67	0.95 ± 0.18^{a}
	3.33	0.77 ± 0.14^{ab}
	6.67	0.58 ± 0.10^{bc}
	13.33	0.31 ± 0.21^{cd}
	20	0.26 ± 0.11^{d}
	33.33	0.25 ± 0.06^{d}

*For a given alginate M/G ratio, values (Mean±1SD) with different superscripts are significantly different from each other ($p<0.05$)

Electrostatic interactions between cationic active compounds and anionic polysaccharides such as carrageenan, pectin and xanthan has been reported (Asker 2009; Bonnaud and others 2010, Chacko 2008; Chang and others 2011). Alginate beads have been used as a potential delivery system for many cationic drugs such as propanolol (Segi and others 1989), chlorpheniramine maleate (Stockwell and Davis 1986) transforming growth factor- β_1 (Mumper and others 1994) and vascular endothelial growth factor (Gu and others 2004; Gu 2004). In most cases, complex formation between alginate and the cationic drug led to increased drug loadings into the alginate beads but decreased diffusion rates out of the beads. Stockwell and Davis (1986) found that release rate of

drugs from calcium alginate gels was dependent on the ionic nature of the drug, with the cationic drug chlorpheniramine maleate having a slower release rate than the anionic drug sodium salicylate. This was attributed to electrostatic interaction between the cationic drug and negatively charged alginate. Mumper and others (1994) investigated the efficacy of calcium alginate beads as a controlled release matrix for delivery of growth regulator protein, transforming growth factor-β_1 (TGF-β_1). They found that all the TGF-β_1 lost its bioactivity on incorporation in alginate beads, possibly due to a very high affinity of the alginate for the cationic TGF-β_1. Gu and others (2004) studied release of positively charged vascular endothelial growth factor (VEGF) encapsulated in calcium alginate gel beads. They observed high VEGF retention in the beads, probably due to an electrostatic interaction with the carboxylate groups of alginate.

In our study, nisin solution containing sodium alginate was turbid, indicating either poor solubility of the solutes or interaction of alginate with nisin and/or with the Ca^{2+} present in Nisaplin®. In the present experiment, pH of alginate-nisin (pH 4.4-6.1) mixture was below the pK_a of nisin side chains (histidine=6.5 and lysine=10.0). Hence, the amino group of nisin will be positively charged. There could be ionic interaction between the negatively charged carboxyl ($-COO^-$) groups of the uronic acids in alginate with positively charged amine ($-NH3^+$) groups of the basic amino acids, lysine and histidine, in nisin. This ionic interaction can lead to the formation of an insoluble precipitate, causing turbidity and unavailability of free nisin.

6.3.3 FT-IR

The spectra for pure nisin powder, nisin control, sodium alginate films and sodium alginate films containing nisin are shown in Figure 6-3 along with their peak assignments in Table 6-5. Sodium alginate with an M/G of 0.49 was used for the FT-IR study, since the alginate M/G did not have any significant effect ($p>0.05$) on nisin binding, as inferred from the binding studies using HPLC (6.3.2).

The conformation state of a peptide can be assessed by investigating its amide I, II, and III bands (Griebenow and others 1993). Amide I is primarily a C=O stretching vibration with some contributions from C-N. Amide II and III are heavily mixed vibrational modes. Among these amide bands, amide I is comparatively well understood and has been most commonly employed for IR investigations of protein secondary structure (Arrando and others 1993). Therefore, any change in nisin conformation in the presence of alginate, was restricted to comparing the peak shifts in amide I region only.

As seen in Figure 6-3, the spectrum for nisin powder and nisin control were identical in their amide I region. The dissolution of nisin in deionized water did not induce any discernable change in the amide I region and hence, in the secondary structure of nisin. The spectrum of sodium alginate containing nisin showed peak shifts from 1407 to 1409 cm^{-1} and 1592 to 1597cm^{-1}. In addition, new peaks were observed in the region 1750-1400 cm^{-1} due to the amide I and amide II absorbance bands of nisin.

In order to investigate changes in the amide I band of nisin in the presence of alginate, first-order derivative of nisin spectra with alginate and without alginate were compared (Figure 6-4). In first-order derivatives of spectra, a positive slope indicates presence of a peak. For the spectrum of nisin with alginate, a new peak was observed in

the amide I band of nisin at 1665 cm^{-1} as shown in Figure 6-4. Similar observation was made by El-El-Jastami and Lafleur (1997), who studied the structure of nisin bound to membrane lipids using infrared spectroscopy. They reported a shift in the amide I band to 1660 cm^{-1}, when bound to negatively charged membrane lipids.

The frequency of the C=O vibrations in the amide band region is related to the strength of the hydrogen bonds formed within the protein structure. The stronger the hydrogen bond, the lower the electron density of the C=O group and lower the frequency of the amide I absorption (Griebenow and others 1993). Peak shifts or appearance of new peaks at a higher wavenumber in the presence of alginate indicates less self-association of nisin. The appearance of a new peak in the higher energy region of amide I indicates that alginate induces a change in the secondary structure of nisin. Studies suggest that the amino acid composition, positive charge and conformation of nisin play an important role in its antimicrobial activity (El-Jastami and Lafleur 1997; Breukink and others 1997; Chan and others 1996; Giffard and other 1997; Moll and others 1997).The change in the secondary structure of nisin due to binding with alginate might affect its antimicrobial activity and needs further investigation.

Table 6-5. Peak assignment of sodium alginate and nisin (Griebenow and others 1993; Sartori and others 1996; vanHoogmoed and others 2003)

Sodium alginate (cm^{-1})	Nisin (cm^{-1})	Peak Assignment
3700-3000 (broad)		OH stretch
3000 - 2800		CH stretch
1592 (very strong, sharp)		COO^- stretching (asymmetric)
	1637	Amide I
	1513	Amide II
	1200-1367	Amide III
1407		COO^- stretching (symmetric)
1024		Antisymmetric stretch C-O-C

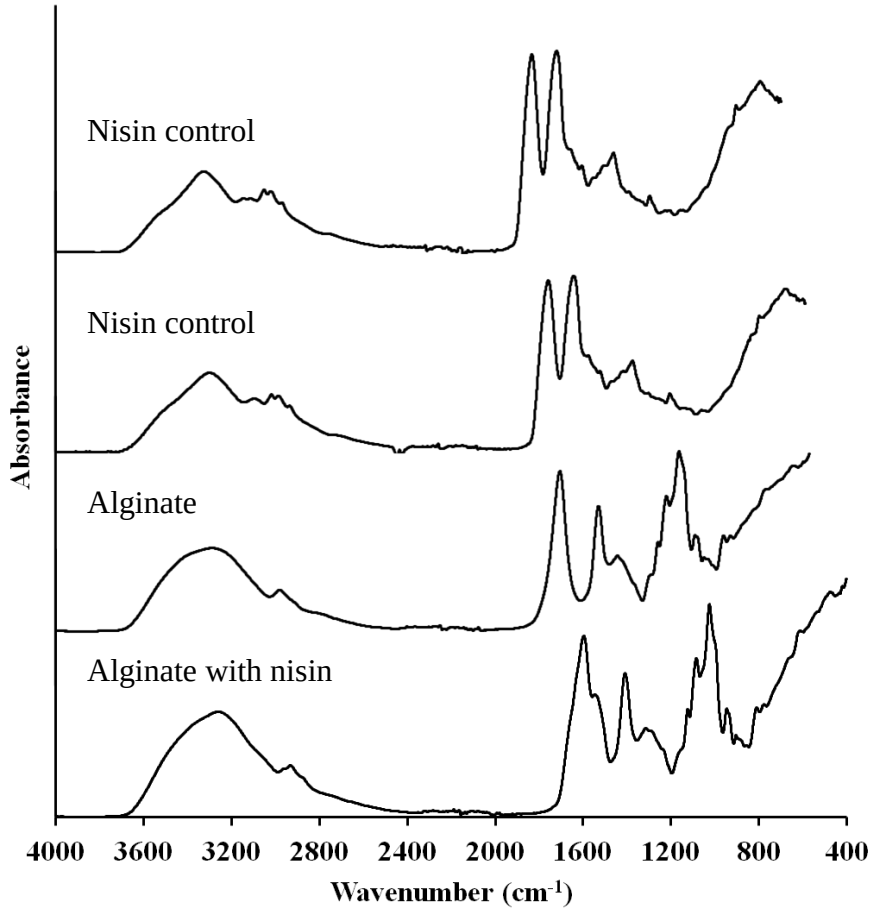

Figure 6-3. FT-IR spectra of nisin powder, nisin control, alginate films and alginate films containing nisin

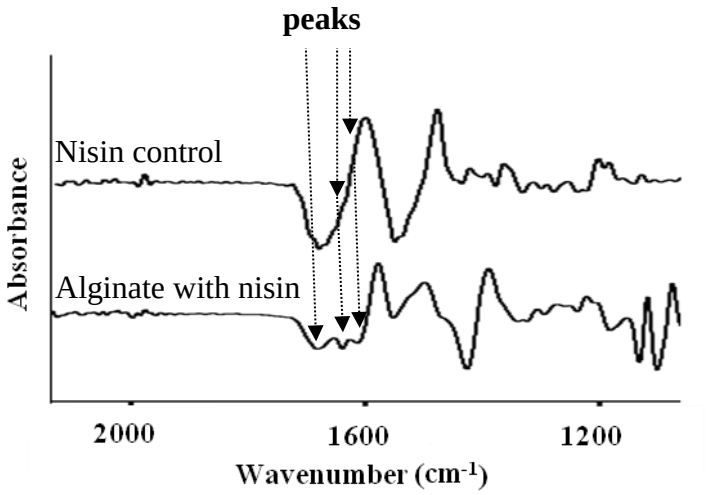

Figure 6-4. First order derivative of FT-IR spectra

6.3.4 Reversibility of nisin-alginate binding

In order to evaluate the reversible/irreversible nature of nisin-sodium alginate binding, different concentrations of Ca^{2+} (0.5, 1, 1.5 M) was added to nisin-alginate mixture, after 24 hr equilibration. On addition of Ca^{2+}, the nisin-sodium alginate-Ca^{2+} mixture separated into a liquid and a solid phase. The weights of this solid and liquid phase were measured at 12 h after addition of Ca^{2+} and are shown in Figure 6-5. For both compositions of alginate, the weight of solid gel was significantly higher ($p<0.05$) than the liquid, at every Ca^{2+}concentration. During sample preparation for quantification of free nisin by HPLC, no sodium alginate precipitate was obtained for any Ca^{2+} concentration, indicating that all of sodium alginate was involved in the gel formation. Hence, it appears that the liquid phase was mainly water with free nisin and the solid phase was mainly calcium alginate gel containing the remaining nisin.

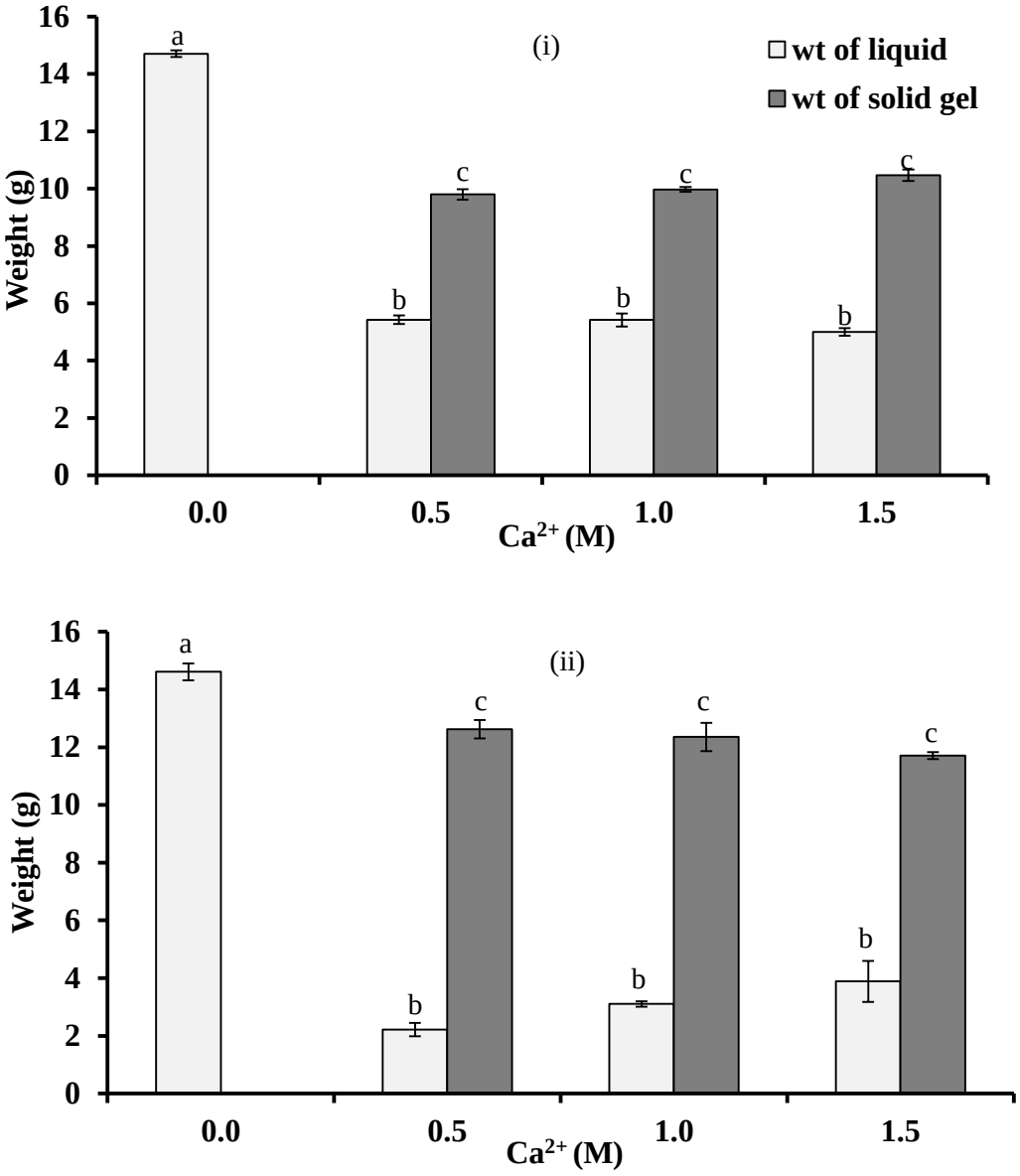

Figure 6-5. Weight of solid and liquid portion of nisin-sodium alginate-Ca^{2+} mixture measured at 12 hr after the addition of different concentrations of Ca^{2+} to nisin solution containing sodium alginate with M/G ratio of (i) 1.63 and (ii) 0.49. Each data point (Error bars: $\pm$1S.D) is an average of 3 replications. For a given M/G of alginate, values with different letters are significantly different from each other ($p<0.05$)

The free nisin concentration in the liquid portion was measured by HPLC. Fraction of free nisin present in the liquid portion of nisin-sodium alginate-Ca^{2+} mixture measured at different times is presented in Figure 6-6. The analysis of means by Tukey's test is shown in Table 6-6.

Fraction of free nisin increased significantly ($p<0.05$) on addition of 1.0 and 1.5M Ca^{2+}. No significant difference ($p>0.05$) was observed in the fraction of free nisin before and after addition of 0.5M Ca^{2+}. For all solutions, fraction of free nisin reached equilibrium within 9 h after addition of Ca^{2+} (Table 6-6). Fraction of free nisin increased significantly ($p<0.05$) with increasing Ca^{2+} concentration. For sodium alginate with an M/G ratio of 1.63, free nisin fraction increased from 0.14 to 0.36 with increase in Ca^{2+} concentration from 0.5 to 1.5M respectively, as seen at 12 h. For sodium alginate with an M/G ratio of 0.49, free nisin fraction increased from 0.18 to 0.56 with increase in Ca^{2+} concentration from 0.5 to 1.5M respectively, as seen at 12 h. Thus, the binding of nisin with alginate was reversible in the presence of Ca^{2+}.

Electrostatic binding between alginate and cationic compounds has been reported to be reversible in the presence of Ca^{2+} by other researchers as well. Gu and others (2004) added $CaCl_2$ in the release medium to mask electrostatic interaction between alginate and vascular endothelial growth factor (VEGF) encapsulated in calcium alginate beads. They observed an increased release of VEGF from calcium alginate beads with increase in the $CaCl_2$ concentration in the release medium and concluded that the presence of calcium can shield the ionic interaction between alginate and the encapsulated VEGF. Thu and others (1996) observed a marked increase in the release of poly-L-lysine from calcium alginate beads in the presence of calcium and strontium ions in the release medium.

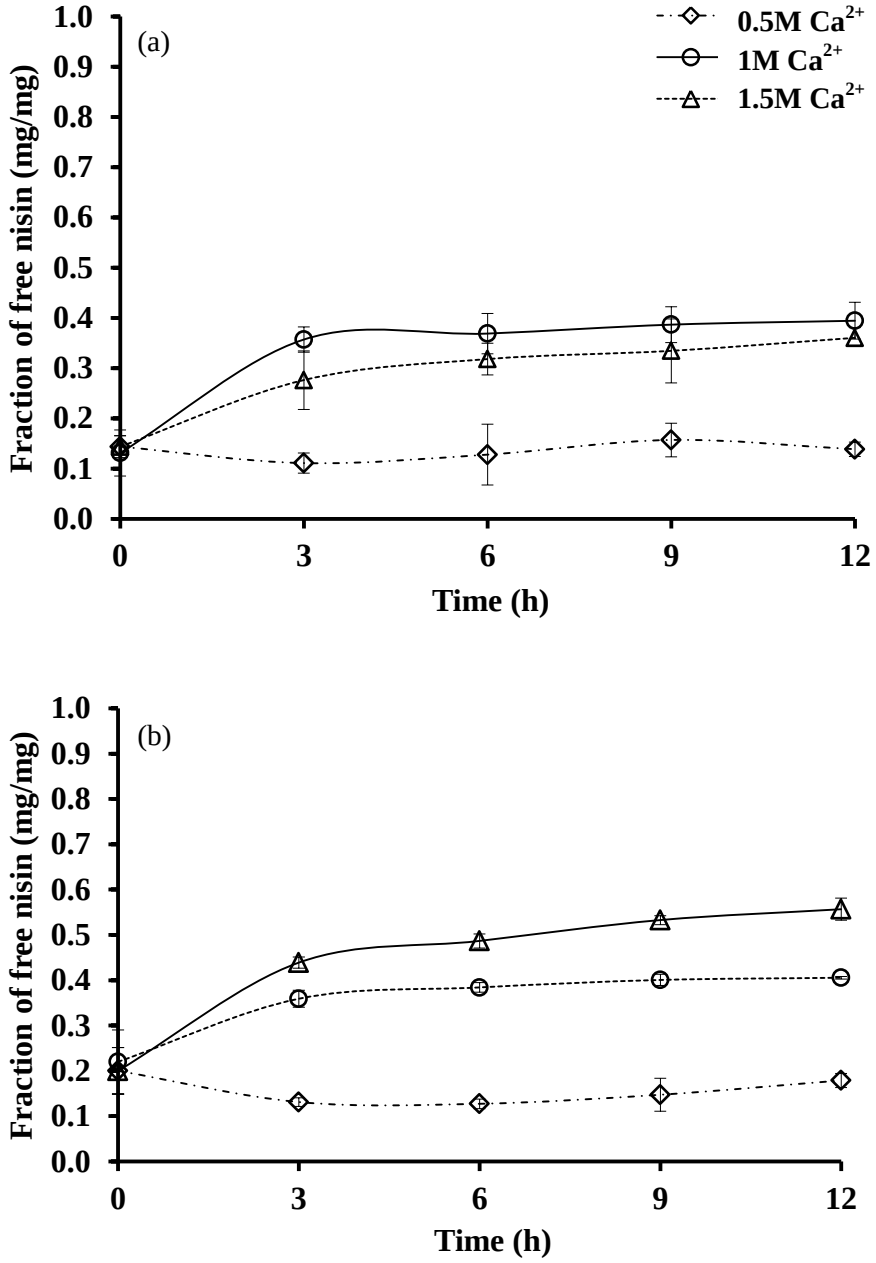

Figure 6-6. Fraction of free nisin measured after addition of different conc. of Ca^{2+} to nisin solution containing alginate with M/G ratio of (a) 1.63 and (b) 0.49. Each data point (Error bars: $\pm$1S.D) is an average of 3 replications.

Table 6-6. Tukey's test for fraction of free nisin measured at 12 h after addition of Ca^{2+} to nisin solution containing sodium alginate

Alginate M/G ratio	Ca^{2+} (M)	Time after addition of Ca^{2+} (h)	Mean free nisin fraction (mg/mg)*
1.63	0.5	0	0.14 ± 0.02^{a}
		3	0.11 ± 0.02^{a}
		6	0.13 ± 0.06^{a}
		9	0.16 ± 0.03^{a}
		12	0.14 ± 0.01^{a}
1.63	1	0	0.13 ± 0.05^{a}
		3	0.36 ± 0.02^{b}
		6	0.37 ± 0.04^{b}
		9	0.39 ± 0.04^{b}
		12	0.39 ± 0.04^{b}
1.63	1.5	0	0.14 ± 0.02^{a}
		3	0.28 ± 0.06^{b}
		6	0.32 ± 0.03^{b}
		9	0.33 ± 0.06^{b}
		12	0.36 ± 0.02^{b}
0.49	0.5	0	0.24 ± 0.05^{a}
		3	0.17 ± 0.03^{a}
		6	0.17 ± 0.04^{a}
		9	0.16 ± 0.04^{a}
		12	0.18 ± 0.02^{a}
0.49	1	0	0.29 ± 0.08^{a}
		3	0.36 ± 0.02^{b}
		6	0.38 ± 0.01^{b}
		9	0.40 ± 0.01^{b}
		12	0.41 ± 0.00^{b}
0.49	1.5	0	0.24 ± 0.05^{a}
		3	0.44 ± 0.01^{b}
		6	0.49 ± 0.02^{bc}
		9	0.53 ± 0.01^{c}
		12	0.56 ± 0.02^{c}

*For a given alginate M/G ratio, values (Mean±1SD) with different superscripts are significantly different from each other ($p<0.05$)

Fraction of free nisin measured at 12 h after addition of 1.5M Ca^{2+} to nisin solution containing sodium alginate was significantly higher ($p<0.05$) for sodium alginate with an M/G of 0.49 than that with an M/G of 1.63 (Figure 6-7). Similar observation has been reported by Thu and others (1996), who found that the binding between poly-L-lysine and calcium alginate decreased with increasing guluronic acid content of sodium alginate. This can be attributed to the high guluronate content of the alginate. The complex between alginate and nisin might be governed by electrostatic interactions between the negatively charged carboxyl groups of uronic acids in alginate and the positively charged amine side groups in nisin. Since guluronic acid blocks bind strongly with Ca^{2+}, it is relatively easy for Ca^{2+} to replace the nisin residues bound to the guluronate than those bound to mannuronate. Hence, higher amount of free nisin was available after addition of Ca^{2+} to alginate with high guluronic acid content.

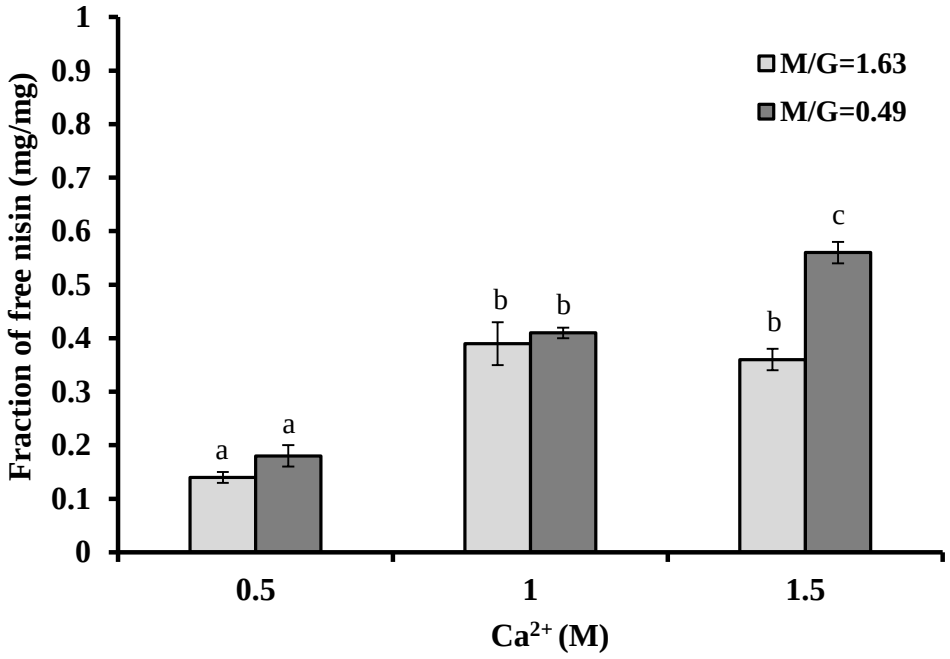

Figure 6-7. Fraction of free nisin measured at 12 h after addition of different concentrations of Ca^{2+} to nisin solution containing sodium alginate. Each data point (Error bars: $\pm$1S.D) is an average of 3 replications. Values with a different letter are significantly different from each other ($p<0.05$)

6.4 Conclusions

Nisin binds with alginate and the fraction of free nisin decreased progressively with increase in alginate concentration. Sodium alginate composition did not have any significant effect ($p>0.05$) on the binding between alginate and nisin. Binding with alginate induced a change in the secondary structure of nisin, as inferred from the FT-IR study. The binding between nisin and sodium alginate was reversible in the presence of Ca^{2+}, possibly due to higher Ca^{2+} concentration replacing a higher amount of nisin bound to high guluronic acid alginate.

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Chapter 7

Release kinetics of nisin from calcium alginate films

Abstract

In order to design a effective nisin-alginate antimicrobial packaging system, it is important to study the kinetics of nisin release from these films. In this research, diffusion of nisin from calcium alginate films cross-linked with various concentrations of Ca^{2+} (0.18-1.25M) was investigated. The effect of cross-linking on the degree of swelling, mechanical properties, and optical properties of calcium alginate films was evaluated. The amount of nisin released from calcium alginate films increased significantly ($p<0.05$) with Ca^{2+} concentration. The mechanism involved in the diffusion process was investigated using the Power law model. Nisin diffusion from films cross-linked with 0.18M Ca^{2+} exhibited anomalous behavior, all other films exhibited near-Fickian behavior. Nisin release from these films fitted well to Fick's second law of diffusion ($R^2>0.90$). The effective diffusivity of nisin into water at 21°C was of the order 10^{-8} cm^2/s and did not change significantly ($p>0.05$) with Ca^{2+} concentration. Films cross-linked with low Ca^{2+} concentrations underwent significant swelling. The transparency, tensile strength and elastic modulus of the films decreased, whereas elongation at break increased, with increasing Ca^{2+} concentration.

7.1 Introduction

Active food packaging provides additional functions over traditional passive packaging, which is limited to protecting food products against external influences (Rooney, 1995). Active packaging materials change the environment around the food product to extend its shelf-life, enhance microbial safety and/or improve organoleptic properties (Ozdemir and Floros 2004). One promising type of active packaging is incorporation of antimicrobial substances in food packaging materials. Antimicrobial food packaging acts to reduce, inhibit or retard the growth of microorganisms that may be present in the packed food or packaging material itself (Vermeiren and others 1999). Antimicrobial packaging can improve product shelf-life by delivering antimicrobials on food surfaces, where microorganisms usually grow and cause spoilage (Greer 1982; Torres and others 1985; Guilbert 1988)

Edible films and coatings in direct contact with foods have generated significant interest in recent years. Alginate is one such polymer that can also be used as an edible film or coating to improve the shelf-life and quality of food products. Alginates are naturally occurring, high molecular weight polysaccharides extracted mainly from brown algae (Pheophyceae) and bacteria. It is an anionic copolymer of β-D-mannuronic acid and its α-L-guluronic acid (Onsoyen 1997). Alginates have the unique property of forming irreversible heat-stable gels, which can set at ambient temperatures in the presence of divalent ions such as $Ca^{2+,}$ $Ba^{2+,}$ Sr^{2+}, etc. Gelation results from specific and strong interactions between these divalent ions and blocks of guluronic acid residue. The extent

of gel formation can be controlled by regulating the cross-linking time and concentration of cross-linker.

Studies have reported that a calcium alginate matrix (films/coatings/beads) containing nisin effectively inhibits microbial growth in various foods such as beef, poultry, milk and mushrooms (Cutter and Siragusa 1997; Fang and Tsai 2003; Lu and others 2009; Luttmann 2011; Millette and others 2007; Natrajan and Sheldon 2000; Wan and others 1997). The release of nisin from calcium alginate coatings in contact with the food product results in microbial inhibition, thereby increasing its shelf-life. However, the above mentioned release studies only present qualitative information on the overall effectiveness of nisin containing alginate matrices on microbial inhibition. In order to design a desired release matrix, more quantitative information is needed. For example, it is important to know the diffusivity of an antimicrobial compound from the film or coating. Diffusivity can be affected by various factors such as size and shape of the active substance molecules (Franssen and others 2004), polymer concentration (Ripoche and others 2006; Sebti and others 2004; Ozdemir and Floros 2003), degree of cross-linking (Zactiti and Kieckbusch, 2009), nature of plasticizer (Franssen and others 2004), environmental conditions such as pH (Mauriello and others 2005) and temperature (Teerakarn and others 2002; Sebti and others 2004). It is also important to define the physical, mechanical and optical properties of edible films when used in direct with food.

The objectives of this study were to: (a) study the knietics of nisin diffusion through calcium alginate films; (b) determine nisin diffusion coefficients; (c) investigate antimicrobial activity of released nisin; and (d) investigate the effect of cross-linking on the swelling, mechanical and optical properties of calcium alginate films.

7.2 Materials and methods

7.2.1 Materials

Sodium alginate with composition 33% mannuronate and 67% guluronate was obtained from Sigma-Aldrich (Saint Louis, MO, USA). Nisaplin®, a commercial grade of nisin, was provided by Danisco Inc. (New Century, KS, USA). Nisaplin® contains 2.5% nisin and one gram Nisaplin® has an activity of 10^6 International Units. Anhydrous calcium chloride was obtained from J.T.Baker (Gibbstown, NJ, USA). Square plastic plates (110mm x15mm) used for casting films were obtained from Electron Microscopy Science, (Hatfield, PA, USA). The petri plates (60 mm x 15 mm) used for the release kinetics study were obtained from VWR International (Radnor, PA, USA). Glass beads (diameter of 3 mm) were obtained from Fischer Scientific (Pittsburg, PA, USA).

7.2.2 Film formation

Sodium alginate solution was prepared by dissolving 0.5 g sodium alginate in 50 ml deionized water and heating to 70°C until the alginate was dissolved. This alginate solution was allowed to cool at ambient conditions for 4 h and Nisaplin® was added to give a nisin concentration of 300 µg/ml. Fifty milliliter of this solution was poured into casting plates and cross-linked by spraying (20 sprays/plate corresponding to about 8 ml of $CaCl_2$ solution) $CaCl_2$ solution with low (0.18, 0.25 M) and high (0.63, 1.25 M) Ca^{2+} concentration. These calcium alginate films were dried and conditioned within an environmental chamber maintained at 50±5% RH and 21±1 °C for 3 days. After drying, the films were washed thrice with deionized water to remove any excess Ca^{2+}.

7.2.3 Film thickness

The film thickness was controlled by always pouring 50 ml sodium alginate into the casting plate. Thickness of the conditioned films was measured using a digital micrometer (Max-Cal Inc, Japan). Measurements were taken at 5 random locations and the mean value was used in the evaluation of diffusivity, mechanical properties and transparency.

7.2.4 Nisin release from calcium alginate films

Measurements of nisin release from calcium alginate films were performed according to the method described in Chacko (2008). Calcium alginate films (3.5 x 3.5 cm^2) containing nisin were placed in petri dishes. To ensure double-sided diffusion, each petri dish was modified by sticking glass beads inside them (Figure 7-1) so as to elevate the film and prevent it from resting on the base of the dish. Ten milliliter of deionized water (release medium) was dispensed in each petri dish to completely submerge the film. The volume/surface area ratio for double sided diffusion experiments from polymeric films was 0.82 ml/cm^2, thus it was maintained between 0.31-155 ml/cm^2 (ASTM D4754). To minimize boundary layer formation around the film, petri dishes were placed on an orbital shaker at 90 rpm in an environmental chamber maintained at 21±1°C (Figure 7-2). 0.5 ml of the release solution was withdrawn at various times from 15-240 min and the amount of nisin released was measured by HPLC. A different petri dish was used for every time point. The experiment was done in triplicates for each treatment. The amount of nisin released at time t (M_t) and the fractional nisin release (M_t/M_∞) were calculated and plotted as a function of time. The amount of nisin released

at infinite time (M_∞) was taken to be the value at which the equilibrium was attained. Film thickness was measured at the end of each experiment after blotting the films dry with a tissue paper, and this thickness was used to calculate the effective diffusivity.

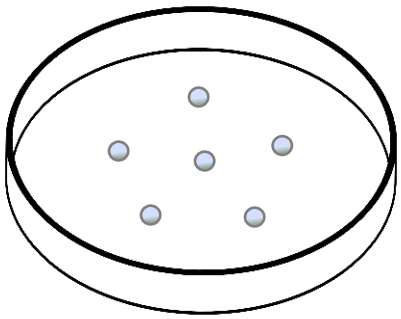

Figure 7-1. A schematic representation of a petri dish with glass beads

Figure 7-2. A pictorial representation of the nisin release experiments

The release mechanism was investigated by using the early portion of the release curve ($M_t/M_\infty<0.6$) to fit the following power law model (Crank, 1975; Langer and Peppas 1983; Ritger and Peppas 1987):

$$\frac{M_t}{M_\infty} = kt^n \qquad \text{(Eq.7.1)}$$

where, k is a constant that characterizes the macromolecular network system, and n is the diffusional exponent characteristic of the release mechanism. A value of n = 0.5 (Case I transport) indicates true Fickian diffusion and the release is proportional to $t^{0.5}$. A value of n = 1.0 (Case II transport) indicates non-Fickian diffusion and the release is directly proportional to t. Values between 0.5<n<1.0 (Case III transport) indicate anomalous diffusion. Values of n>1 define the Super Case II transport in which a pronounced solute release occurs toward the latter stages of diffusion, resulting in a more rapid relaxation controlled transport. Values of n<0.5 indicate pseudo-Fickian diffusion, where the sorption curves resemble Fickian diffusion curves but the approach to final equilibrium is slow (Neogi 1996).

7.2.5 Quantification of nisin by High Performance Liquid Chromatography (HPLC)

The HPLC method was adopted with modifications from Liu and Hansen (1990) and Buonocore and others (2003). A C-18 reverse phase column with silica packing (size 5 μm and pore 100A°) and dimensions 250 mmx4.6 mm was used (Restek Corporation Bellefonte, PA, USA). A Shimadzu HPLC system (LC-20AD with LCSolution software; autosampler SIL-20AC HT; UV-VIS detector SPD-20AV) was used with the following operating parameters: injection volume 50 μl, UV absorption at 220 nm and temperature

40°C. Mobile phase was a mixture of acetonitrile containing 0.1% trifluroacetic acid (EMD Gibbstown, NJ) and ultrapure water, flowing at 1ml/min. The mobile phase was run in gradient mode, wherein the concentration of acetonitrile was varied from 25-75% within 15 min.

The release solution was filtered with a 0.45 µm syringe filter (Iso-disc Nylon 13 mm x 0.45 µm, Restek Corporation, Bellefonte, PA, USA) before injecting into HPLC. To prevent column damage, the sodium alginate fractions present in alginate-nisin solutions were precipitated with organic mobile phase (acetonitrile containing 0.1% trifluoroacetic acid). 0.5 ml aliquot of alginate-nisin solution was diluted with 0.5 ml organic mobile phase and centrifuged at 6000 rpm for 10 min. The supernatant was used to measure the free nisin concentration. A calibration curve was constructed for nisin peak area against nisin concentration of standard solutions ranging from 0.0025-0.5 mg/ml (shown in Appendix).

7.2.6 Determination of effective diffusivity of nisin in calcium alginate

A mathematical model that describes nisin diffusion out of calcium alginate films can be derived from Fick's second law expressed in one dimension with a time-dependent diffusion coefficient (Crank, 1975):

$$\frac{\partial c}{\partial t} = D\,\frac{\partial^2 c}{\partial x^2} \qquad \text{(Eq.7.2)}$$

where, c is the nisin concentration in the film, D is the diffusion coefficient of nisin, x the coordinate measured in the direction of diffusion and t is time.

If c is the concentration of nisin diffusing out of calcium alginate films, c_0 is the initial concentration of nisin uniformly distributed in the film, and h is the film thickness, Eq. (7.2) can be solved for the following initial and boundary conditions:

$c = c_0$, $t = 0$

$c = 0$, $x = \pm h/2$

The solution of Eq. (7.2) under these conditions is (Crank 1975):

$$\frac{C}{C_0} = \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^{\mathrm{n}}}{2\mathrm{n}+1}\exp\{-D_{eff}\,(2\mathrm{n}+1)^2\pi^2 t/h^2\}\left(\cos\frac{(2n+1)\pi x}{h}\right) \qquad \text{(Eq. 7.3)}$$

The fraction of nisin released from calcium alginate films can be calculated by integrating Eq. (7.3) over space and time:

$$\frac{\mathrm{M}_t}{\mathrm{M}_\infty} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2\mathrm{n}+1)^2}\exp\{-(2\mathrm{n}+1)^2\frac{D_{eff}\pi^2 t}{h^2}\} \qquad \text{(Eq.7.4)}$$

where, M_t is the amount of nisin released from the calcium alginate film at time t, M_∞ is the nisin released at infinite time, and D_{eff} is the effective diffusivity of nisin, assumed constant.

For short times, when $M_t/M_\infty < 0.6$, the solution simplifies to (Crank 1975; Ritger and Peppas 1987; Han and Floros 2000)

$$\frac{\mathrm{M}_t}{\mathrm{M}_\infty} = \sqrt{\frac{16\,t\,D_{eff}}{\pi h^2}} \qquad \text{(Eq.7.5)}$$

7.2.7 Antimicrobial activity of released nisin

A verification study was conducted to measure the antimicrobial activity of released nisin from calcium alginate films containing different initial nisin loading, and compare this biologicaly active nisin with the total amount of released nisin. Calcium alginate films cross-linked with 1.25 M Ca^{2+} were formed as described in section 7.2.2. Three concentrations of Nisaplin® was added to sodium alginate solution to obtain nisin concentration of 150, 225 and 300 µg/ml giving total nisin load per film as 1225, 1837.5 and 2450 µg, respectively. Nisin release study was conducted as described in section 7.2.4. The release solution was withdrawn at 240 min. The amount of biologically active nisin was determined by agar diffusion bioassay (as described in section 4.2.3 with 48 h pre-diffusion time) and total nisin was determined by HPLC (as described in section 7.2.5). The experiment was replicated three times for every nisin concentration.

7.2.8 Degree of swelling (DS)

Calcium alginate films (3.5x3.5cm^2) containing nisin were weighed and immersed in 100 ml deionized water. The films were removed from water, blotted between filter papers to remove excess of surface liquid, and reweighed at times 2, 5, 10, 20, 30, 40, 60 and 90 min. The degree of swelling was measured as follows:

$$DS = \frac{w_s - w_i}{w_i} \qquad \text{(Eq.7.6)}$$

where, w_s is the weight of swollen film (mg), and w_i is the initial weight of the dry film (mg). Measurements were done in triplicates for each treatment.

7.2.9 Moisture content of films

For moisture content measurements, calcium alginate films (3.5 x 3.5cm^2) containing nisin were dried in an oven at 105ºC until constant weight. The percent moisture content, MC, was determined as follows:

$$MC = \frac{w_i - w_f}{w_i} \text{x } 100 \qquad \text{(Eq.7.7)}$$

where, w_i is the initial weight of the film and w_f is the final weight of the dried film. The measurement was done in triplicates for each treatment.

7.2.10 Mechanical properties

A Texture Analyzer TA.XT2i (Texture Technologies Corporation, Scarsdale, NY) was used to determine the ultimate tensile strength (UTS), elastic modulus (EM) and elongation at the break (EB) of calcium alginate films containing nisin. UTS is the maximum stress a film can sustain and was calculated in MPa as the ratio of maximum load and the original minimum cross sectional area of the specimen. EM is the ratio of stress in MPa to strain over the linear part of the stress-strain curve and it is a measure of film stiffness (Ozdemir and Floros 2008). EB is the percent change of the initial length of the film vs. that at the time of rupture, and it indicates the ability of the film to stretch (Gennadios and others 1996). All tests were performed on rectangular film strips of 1 cm width and 5 cm length according to the ASTM Standard Test Method D882-10 (ASTM, 2010). Initial grip separation and crosshead speed were set at 2 cm and 0.1 mm/s, respectively. All measurements were replicated thrice with three strips per treatment.

7.2.11 Optical properties

A Minolta spectrophotometer type CM 3500d (Konica Minolta Sensing, Inc, Ramsey, NJ, USA) with software SpectraQC 3.30 was used to determine the color and transparency of calcium alginate films containing nisin. For each treatment, measurements were made at three different locations on the film, and each treatment was replicated thrice. Hunter-Lab color scale (L= 0 [black] to 100 [white]; a= –80 [green] to 100 [red]; b= –80 [blue] to 70 [yellow]) was used to measure the L, a and b values of the films with reference to a white standard plate (L=96.87, a=0.19, and b=2.00). The yellowness index (YI), and whiteness index (WI) were calculated from the following equations (Rhim and others 1999):

$$YI = \frac{142.86b}{L} \quad \text{(Eq.7.8)}$$

$$WI = 100 - ((100 - L)^2 + \Delta a^2 + \Delta b^2)^{0.5} \quad \text{(Eq.7.9)}$$

where, Δa= $a_{standard} - a_{sample}$ and Δb = $b_{standard} - b_{sample.}$ YI and WI indicate the degree of yellowness and whiteness, respectively.

The transparency of calcium alginate films at 600nm (T_{600}) was calculated from the following equation (Han and Floros 1997):

$$T_{600} = \frac{\log(\%T_r)}{x} \quad \text{(Eq.7.10)}$$

where, $\%T_r$ = percent transmittance measured at 600 nm and x = film thickness (mm).

7.2.12 Statistical analysis

One-way ANOVA (α=0.05) using MINITAB® 14 (State College, PA) were applied for data analysis. Significant differences between means were tested by Tukey's test (α=0.05).

7.3 Results and Discussion

7.3.1 Release of nisin

The total amount of nisin in each film (3.5x3.5 cm^2) was 2450 µg, giving a nisin concentration of 200 µg/cm^2. Figure 7-3 shows release of nisin from calcium alginate films cross-linked with different concentrations of Ca^{2+}. The amount of nisin released, increased significantly ($p<0.05$) as Ca^{2+} concentration increased. The nisin release leveled off after about 180 min. The mean amount of released nisin at equilibrium was 160, 260, 490 and 900 µg for alginate films cross-linked with 0.18, 0.25, 0.63 and 1.25M of Ca^{2+}, respectively. This progressive increase in the amount of nisin released with increased Ca^{2+} concentration was due to the reversible binding of nisin with alginate in the presence of Ca^{2+}. Higher Ca^{2+} concentration replaces a higher amount of nisin bound to alginate, hence, more nisin becomes available for release. Additional studies indicated that no further increase in the amount of nisin released was possible for Ca^{2+} concentration higher than 1.25M (data not shown).

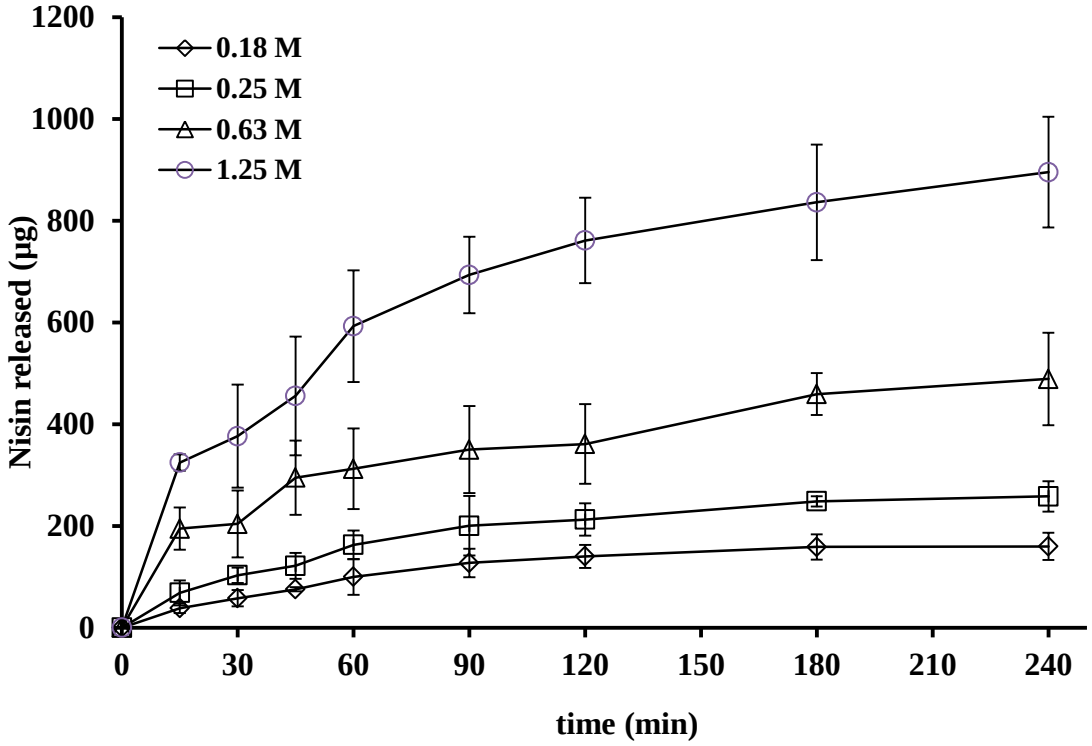

Figure 7-3. Amount of nisin released from calcium alginate films cross-linked with different concentrations of Ca^{2+}. Data (Error bar ±1SD) is an average of three replications.

Fractional release of nisin from calcium alginate films is shown in Figure 7-4. Several parameters, diffusional exponent (*n*), diffusional constant (*k*) and correlation coefficient (R^2) for the Power law model (Eq. 7.1), were calculated from the ln (M_t/M_∞) vs. ln t plot (Figure 7-5), and their values are presented in Table 7-1. The kinetics of nisin release from films for all concentrations of Ca^{2+} deviated from the ideal Fickian diffusion. For films cross-linked with 0.18M Ca^{2+}, the value of *n* was 0.81±0.09, indicating an anomalous release mechanism. The kinetics of nisin release from films cross-linked with 0.25, 0.62 and 1.25 M Ca^{2+}, exhibited near-Fickian mechanism with *n* values of 0.60±0.13, 0.42±0.02 and 0.41±0.03, respectively.

Deviations from the ideal Fickian behavior (n=0.5) for diffusion in glassy polymers has been reported by other researchers as well (Berens and Hopfenberg 1977; Chacko 2007; daSilva and others 2012; Korsmeyer and Peppas 1981; Ozdemir and Floros 2001; Wind and Lenderink, 1996; Zactiti and Kieckbusch 2009). This deviation has been attributed to polymer relaxation or rearrangement in the polymer structure due to stresses exerted by the penetrant (Rogers 1985; Frisch 1980). Korsmeyer and Peppas (1981) studied the diffusion of theophylline in cross-linked and dried poly(vinyl)alcohol gels. They hypothesized that the glass-to-rubber transition in the polymer due to counter diffusion of water into the originally dried (glassy) polymer resulted in the anomalous diffusion behavior. A similar phenomenon could be responsible for the deviation from ideal Fickian release mechanism of nisin in this study. A noticeable degree of swelling was observed in alginate films cross-linked with low Ca^{2+} concentrations (0.18 and 0.25M) due to absorption of water. This counter-diffusion of water into the dried calcium alginate films could have resulted in significant rearrangement of the alginate molecules due to the stresses exerted by water.

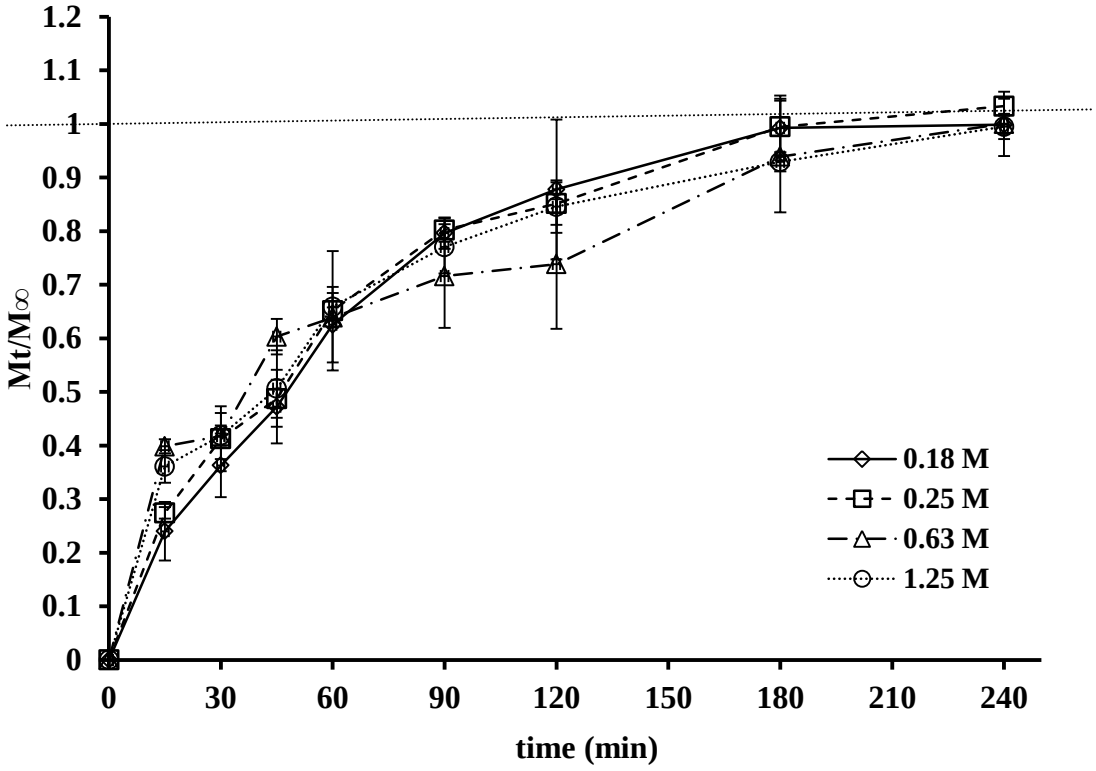

Figure 7-4. Fractional release of nisin from calcium alginate films cross-linked with different concentrations of Ca^{2+}. Each data point (Error bar ±1SD) is an average of three replications.

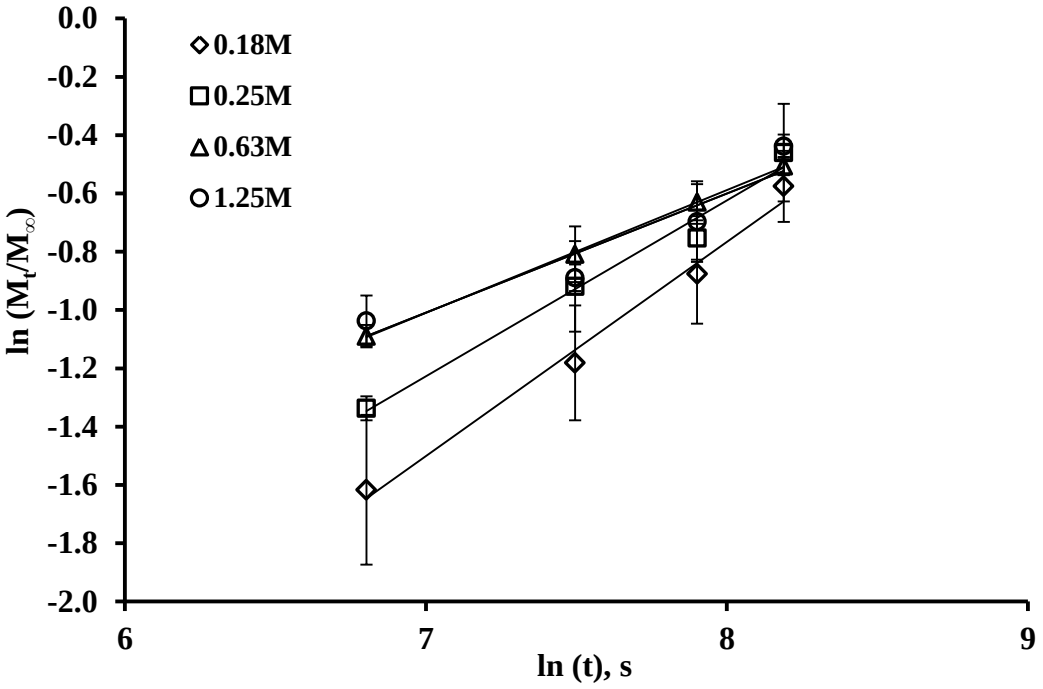

Figure 7-5. Fractional release of nisin from calcium alginate films cross-linked with different concentrations of Ca^{2+} modeled using Eq. 7.1. Each data point (Error bar ±1SD) is an average of three replications.

Table 7-1. Diffusional exponent (*n*), diffusional constant (*k*) and correlation coefficient (R^2) of nisin release calculated from data in Figure 7-6

Ca^{2+} conc. (M)	**Slope, *n***	**Intercept, $k \times 10^2$ (s^{-n})**	**R^2**	**Release mechanism**
0.18	0.81 ± 0.09	0.48	0.99	Anomalous
0.25	0.60 ± 0.13	0.53	0.98	near-Fickian
0.63	0.42± 0.02	2.37	0.99	near-Fickian
1.25	0.41 ± 0.03	13.85	0.90	near-Fickian

The data were adjusted to the diffusion model (Eq. 7.5), through a linear plot of M_t/M_∞ vs. $t^{0.5}$ (Figure 7-6). The initial and final film thickness, and the R^2, slope and calculated effective diffusivity values (calculated using the final thickness) are shown in Table 7-2.

Nisin release kinetics from all films fitted well to Fick's second law of diffusion (R^2>0.90). Nisin diffusion coefficients in the films had values in the order of 10^{-8} cm^2/s. The D_{eff} of nisin did not change significantly ($p>0.05$) with Ca^{2+} concentration. This shows that nisin release is independent of Ca^{2+} concentration in the film. It appears that effect of Ca^{2+} concentration on the hydrophilicity and structural properties of alginate films does not affect nisin diffusivity through these films significantly, and the primary mechanism for nisin release from these films was replacement of the bound nisin by Ca^{2+}.

The D_{eff} values of nisin obtained in this study are 10-fold lower than that reported in agarose gels (Ripoche and others 2006). Our values are 10-fold higher than nisin diffusivity values in cross-linked poly(vinyl)alcohol films at 25°C (Buonocore and others 2003) and 100-fold higher than that reported in corn zein and wheat gluten films at 25°C (Teerakarn and others 2002), and in whey protein isolate films at 10°C (Rossi-Márquez and others 2009).

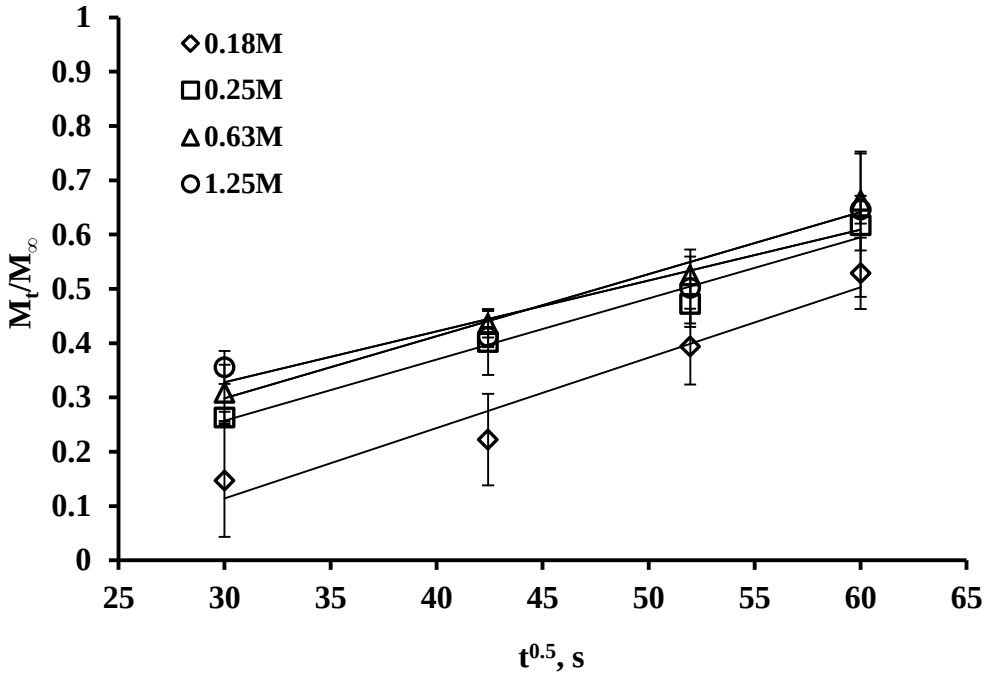

Figure 7-6. Fractional release of nisin from calcium alginate films cross-linked with various concentrations of Ca^{2+} modeled using Eq. 7.5. Each data point (Error bar = ±1SD) is an average of three replications.

Table 7-2. Initial and final film thickness and R^2, slope and effective diffusion coefficient of nisin calculated from data in Figure 7-6

Ca^{2+} conc. (M)	**Thickness (mm)***		**R^2**	**Slope**	**Effective diffusion coefficient*, D_{eff} x 10^{-8} (cm^2/s)**
	Initial	**Final**			
0.18	0.11 ± 0.03^{a}	0.58 ± 0.06^{c}	0.95	0.014 ± 0.001	14.63 ± 10.22^{a}
0.25	0.13 ± 0.03^{a}	0.44 ± 0.04^{d}	0.98	0.011 ± 0.004	5.18 ± 3.81^{a}
0.63	0.21 ± 0.04^{ab}	0.18 ± 0.04^{ab}	0.98	0.011 ± 0.003	0.86 ± 0.490^{a}
1.25	0.27 ± 0.05^{b}	0.30 ± 0.02^{b}	0.91	0.009 ± 0.001	1.54 ± 0.19^{a}

*Values with different superscripts are significantly different ($p<0.05$) from each other.

Films cross-linked with low Ca^{2+} concentrations (0.18 and 0.25 M) underwent significant increase ($p<0.05$) in thickness during the release study due to swelling whereas, films cross-linked with high Ca^{2+} concentrations (0.63 and 1.25 M) did not undergo any significant change ($p>0.05$) in thickness. Modeling of release from swelling polymeric systems is known as moving boundary or Stefan-Neumann problems. The constitutive equation for transport of solute in the presence of both diffusional and relaxational phenomena is highly non-linear and numerical solutions must be used due to the absence of exact analytical solutions (Ritger and Peppas, 1987). If alginate films cross-linked with low Ca^{2+} concentrations underwent significant swelling during the time periods used for calculating D_{eff}, then these values of D_{eff} are not representative of the actual value. Hence, it is important to know the kinetics of swelling in these films.

7.3.2 Antimicrobial activity of released nisin

Figure 7-7 shows the amount of biologically active and total nisin released from calcium alginate films with different initial nisin load. The amount of biologically active nisin released from calcium alginate films with initial nisin load of 1225, 1838 and 2450, was found to be 418, 657 and 1039 µg, respectively. The total amount of nisin released from calcium alginate films with initial nisin load of 1225, 1838 and 2450 µg, was found to be 363, 594 and 875 µg, respectively. The amount of nisin released increased significantly ($p<0.05$) with increase in initial nisin load from 1225 to 2450 µg. For a given initial nisin load, the amount of nisin measured by agar diffusion assay was not significantly different ($p>0.05$) from the total nisin measured using HPLC. Hence, we

conclude that nisin released from calcium alginate films retained its antimicrobial activity.

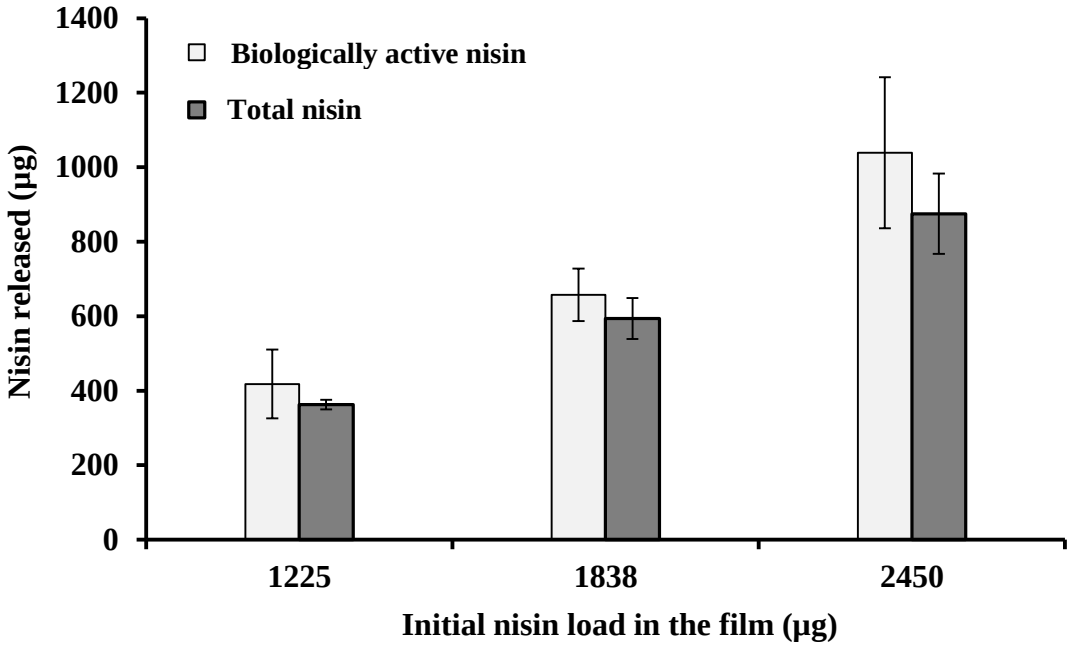

Figure 7-7. Amount of nisin released from calcium alginate films with different initial nisin load as measured by agar diffusion bioassay and HPLC. Values (Error bar = ±1SD) with a different letter are significantly different ($p<0.05$) from each other.

7.3.3 Degree of swelling (DS)

The degree of swelling of calcium alginate films in water is shown in Figure 7-8. The degree of swelling reduced significantly ($p<0.05$) with increasing Ca^{2+} concentration. Films cross-linked with low Ca^{2+} concentrations underwent significant ($p<0.05$) swelling. Films cross-linked with 0.18M Ca^{2+} reached equilibrium between 10 and 20 min after immersion in water, and their DS at equilibrium was about 11.5. Films cross-linked with

0.25M Ca^{2+} reached equilibrium between 2 and 5 min after immersion in water, and their DS at equilibrium was about 5.5. Films cross-linked with high Ca^{2+} concentrations did not exhibit any significant ($p>0.05$) swelling, and lost <5% of their initial weight (probably due to leaching out of solutes such as nisin and other components present in Nisaplin®). Higher Ca^{2+} concentrations probably increased chain-chain associations between guluronic acid blocks, resulting in a stronger network that hinders water absorption. Lower swelling of calcium alginate films with higher Ca^{2+} concentration has also been reported by Zactiti and Kieckbusch (2004).

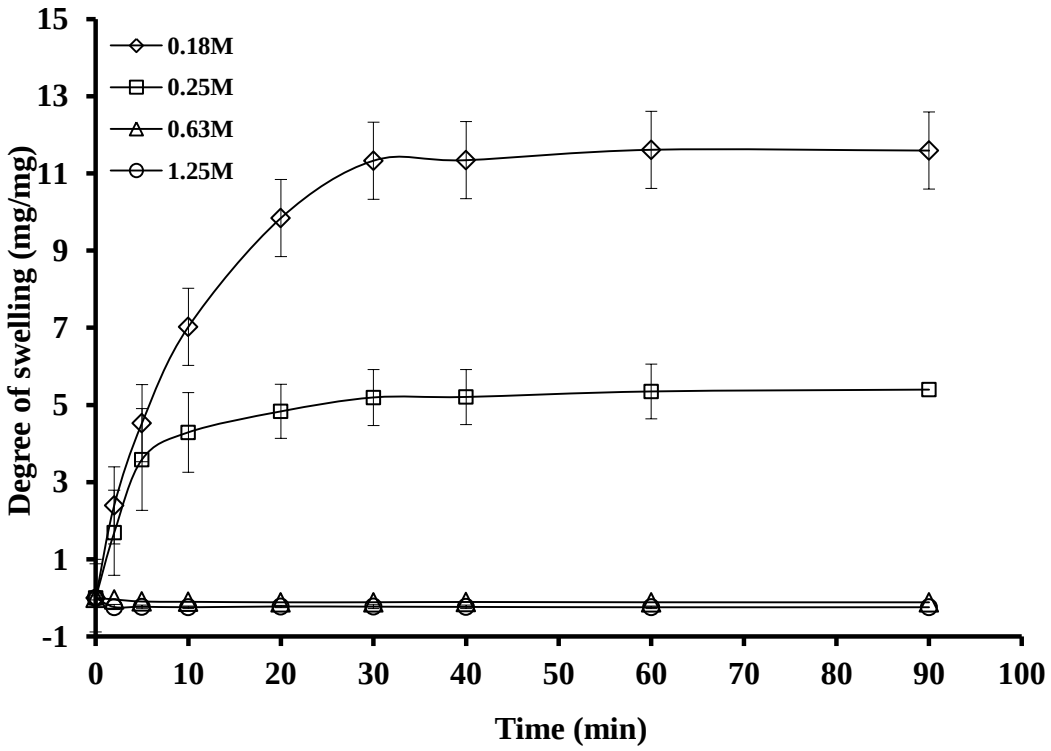

Figure 7-8. Degree of swelling for calcium alginate films with different Ca^{2+} concentrations. Each data (Error bar ±1SD) is an average of three replications. Symbols represent experimental values.

The D_{eff} of nisin from calcium alginate films was calculated for release kinetics between 15-60 min. Thus, the films cross-linked with 0.18 M Ca^{2+} underwent swelling during the time period used for calculating D_{eff}. This explains the anomalous behavior of nisin diffusion from these films, which resulted from a coupling effect of diffusion and relaxation-controlled release mechanism. For films cross-linked with 0.25 M Ca^{2+}, the swelling was significant up to 5 min of the immersion time in water. Although these films underwent swelling, this swelling was in the initial time period of release and hence did not have a significant effect on the diffusion mechanism of nisin from these films, as indicated by the near-Fickian diffusion behavior of nisin from these films.

7.3.4. Moisture content of films

The moisture content of calcium alginate films cross-linked with different concentrations of Ca^{2+} is shown in Table 7-3. Moisture content of the films increased significantly ($p<0.05$) with increasing Ca^{2+} concentration. This can be attributed to the hygroscopic nature of $CaCl_2$. Higher amount of $CaCl_2$ retains higher moisture in the film. This high moisture retention in the films with was likely the reason for significant ($p<0.05$) increase in film thickness with increasing Ca^{2+} concentration (Table 7-2).

7.3.5 Mechanical properties

The values of UTS, EM and EB for different calcium alginate films are given in Table 7-2. With increasing Ca^{2+} concentration, UTS and ME decreased significantly ($p<0.05$), while, EB increased significantly ($p<0.05$). This can be attributed to the higher water retention of calcium alginate films with increasing Ca^{2+} concentration. Water acts as a plasticizer and modifies the film's mechanical properties. A plasticizer increases film flexibility by reducing the intermolecular H-bonding along polymer chains, and by

increasing intermolecular spacing. The resulting increase in molecular mobility lowers the glass transition temperature and decreases the ratio of crystalline to amorphous region (Guilbert and others 1997). Increasing plasticizer concentration in a film results in lower TS and EM, and higher film elongation (Janjarasskul and Krochta 2010). Similar trends of decreasing tensile strength and increasing elongation of alginate films due to higher plasticizer effect have been reported by other researchers (Olivas and Barbosa 2008; Parris and others 1995).

Table 7-3. Moisture content and mechanical properties of calcium alginate films

Ca^{2+} conc. (M)	**Moisture content* (%)**	**UTS* (MPa)**	**EM* (MPa)**	**EB* (%)**
0.18	35 ± 8.67^{a}	6.17 ± 0.56^{a}	2.67 ± 0.69^{a}	13.08 ± 3.64^{a}
0.25	38 ± 3.84^{a}	5.42 ± 1.17^{a}	1.6 ± 0.45^{ab}	18.14 ± 1.59^{a}
0.63	54 ± 2.56^{b}	5.20 ± 0.29^{ab}	1.75 ± 0.54^{ab}	32.85 ± 3.13^{b}
1.25	56 ± 10.94^{b}	3.80 ± 0.40^{b}	1.27 ± 0.40^{b}	47.13 ± 5.43^{c}

*In a given column, values with a different superscript are significantly different ($p<0.05$) from each other.

7.3.6 Optical properties

The values of whiteness index (WI), yellowness index (YI), and transparency (T_{600}) for different calcium alginate films are shown in Table 7-.4. Sodium alginate solution is brown in color and becomes whitish on cross-linking with Ca^{2+}. The color differences between different films can be better described using color functions such as WI and YI. Ca^{2+} concentration did not significantly ($p>0.05$) change the WI and YI of the films. Higher Ca^{2+} concentrations significantly reduced the transparency of calcium alginate films. This indicates that higher Ca^{2+} concentration, and consequently higher

degree of cross-linking, made the films less transparent or more opaque. Polymers with higher cross-linking tend to exhibit an increased opacity due to smaller inter-chain spacing, allowing less light to pass through the film (Yang and others 2010; da Silva and others 2012). High concentration of Ca^{2+} also increased film thickness (Table 7-1), which may also be the reason for the reduced transparency. Other researchers have also reported that an increase in thickness decreases a film's transparency (Mali and others 2004; Trinetta and others 2011).

Table 7-4. Color functions and transparency of calcium alginate films

Ca^{2+} conc. (M)	**WI***	**YI***	**T_{600}***
0.18	89.64 ± 5.33^{a}	5.45 ± 1.37^{a}	18.49 ± 2.75^{a}
0.25	87.74 ± 4.37^{a}	6.59 ± 2.29^{a}	13.83 ± 0.90^{ab}
0.63	85.77 ± 6.55^{a}	8.51 ± 2.11^{a}	12.12 ± 3.01^{ab}
1.25	86.63 ± 3.41^{a}	10.37 ± 2.24^{a}	12.04 ± 3.10^{b}

*In a given column, values with a different superscript are significantly different ($p<0.05$) from each other.

7.4 Conclusions

The amount of nisin released from calcium alginate films increased significantly ($p<0.05$) with the Ca^{2+} concentration. Diffusion of nisin from films cross-linked with 0.18M Ca^{2+} exhibited anomalous or non-Fickian behavior, while those cross-linked with 0.25, 0.63 and 1.25M Ca^{2+} exhibited near-Fickian behavior, as inferred from the Power law model. Nisin release from films followed Fick's second law of diffusion ($R^2>0.90$). The effective diffusivity of nisin into water at 21°C was of the order 10^{-8} cm^2/s, and did not change significantly ($p>0.05$) with Ca^{2+} concentration. Films cross-linked with low

Ca^{2+} underwent significant ($p<0.05$) swelling, whereas films cross-linked with high Ca^{2+} did not show any significant ($p>0.05$) swelling. The transparency, tensile strength and elastic modulus of the films decreased, whereas elongation at break increased, with increasing Ca^{2+} concentration.

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Chapter 8

Conclusions and suggestions for future studies

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8.1 Conclusions

This research was undertaken to understand the interaction between nisin and alginate and to study the release kinetics of nisin from calcium alginate films, in order to develop an efficient antimicrobial packaging system for commercial applications on high moisture foods.

It was imperative to have a reliable and accurate technique to measure the amount and antimicrobial activity of the released nisin, in order to study kinetics and mechanisms of nisin release from calcium alginate films. In chapter 4, we improved the agar diffusion bioassay to determine best pre-diffusion time and achieved better correlation between inhibition zone and nisin concentration, at higher nisin concentrations. It was found that small wells were better than large wells because of smaller standard deviation, higher predictive accuracy, and better discrimination between mean inhibition zones at neighboring nisin concentrations. Among the statistical models tested, spline models produced excellent correlation between nisin concentration and inhibition zone and 48 h was found to be the best pre-diffusion time.

In Chapter 5, we studied the impact of different factors that govern the structure and properties of calcium alginate films such as alginate M/G ratio, method of film formation (cross-linking after drying (CAD) vs. cross-linking before drying (CBD)) and cross-linking parameters (time of cross-linking and concentration of Ca^{2+}). The key

finding from this study was that nisin release was significantly affected by the method of film formation and cross-linking parameters. Alginate M/G ratio did not have any significant effect ($p<0.05$) on the nisin release. For films formed by CAD method, nisin release decreased with increasing cross-linking time and Ca^{2+} concentration. For films formed by CBD method, nisin release increased with increasing Ca^{2+} concentration. SEM images showed that film formation method influenced the microstructure of films. Films made by the CBD method and cross-linked with high Ca^{2+} concentration had the highest nisin release, probably due to reversible binding between nisin and alginate in the presence of Ca^{2+}. This hypothesis of reversible binding between nisin and alginate in the presence of Ca^{2+} was tested in chapter 6.

In chapter 6, the stoichiometry and nature of binding between nisin and alginate was investigated. The key findings from this study were that nisin binds with sodium alginate possibly through electrostatic interactions and this binding between nisin and alginate was shown to be reversible in the presence of Ca^{2+}. This finding of reversible binding between nisin and alginate in the presence of Ca^{2+} was helpful in understanding the contrasting release behavior exhibited by the different film formation methods, as seen in chapter 5. It appears that in case of films formed by CAD method, cross-linking of alginate films was mainly an interfacial phenomenon. Cross-linking started at the film surface to yield an almost completely cross-linked surface which hampered the release of free nisin into the surrounding medium. In case of films formed by CBD method, Ca^{2+} diffused throughout the sodium alginate solution forming a homogeneously cross-linked calcium alginate gel. Higher amount of Ca^{2+} in the alginate matrix could replace higher

amount of nisin bound to alginate, thus resulting in higher free nisin and consequently higher nisin release.

After studying the interaction between nisin and alginate, and how this interaction affected nisin release, the next step was to study the release kinetics of nisin from calcium alginate films. In chapter 7, we investigated the kinetics of nisin diffusion through calcium alginate films. It was found that nisin release increased significantly ($p<0.05$) with Ca^{2+} concentration, validating the hypothesis of reversible binding between nisin and alginate in the presence of Ca^{2+}. One of the key findings from this study was that the release of nisin from calcium alginate films deviated from the ideal Fickian behavior. Diffusion of nisin from films cross-linked with low Ca^{2+} exhibited anomalous or non-Fickian behavior, while those cross-linked with high Ca^{2+} exhibited near-Fickian behavior. The effective diffusivity of nisin from the films into water at 21°C was of the order 10^{-8} cm^2/s, and it did not change significantly ($p>0.05$) with Ca^{2+} concentration. This released nisin retained its antimicrobial activity fully.

The overall findings in this dissertation provide insightful information on the mechanism and kinetics of nisin release from calcium alginate films. The primary mechanism of nisin release from calcium alginate films was due to replacement of bound nisin by Ca^{2+}. Nisin release from calcium alginate films increased with increasing Ca^{2+} concentration. The effective diffusivity of nisin from the films into water did not change significantly ($p>0.05$) with Ca^{2+} concentration, indicating that calcium alginate matrix does not bind nisin physically. However, some amount of nisin remained unavailable for release due to ionic interaction with sodium alginate. All of these finding will be helpful

in designing an efficient antimicrobial packaging system with desired release characteristics.

8.2 Suggestions for future work

1. Identify compounds and/or techniques that can "shield" nisin from binding with alginate. As discussed in section 2.5, nisin containing alginate coatings have been shown to be effective on microbial inhibition in several different foods such as beef, poultry, milk and mushrooms. Although the system of nisin and alginate is an effective antimicrobial coating, excess nisin needs to be added in the coating formulation to account for the loss of chemically unavailable nisin. Thus, there is a need to modify the calcium alginate matrix so as to 'shield' nisin from interacting with alginate. One approach will be addition of cations that have a stronger affinity for alginate than nisin, but do not interfere with calcium alginate gel formation.

2. Investigate the reversibility of nisin-alginate binding in the presence of Ca^{2+} using alginate with high guluronic acid content: Another approach to minimize loss of nisin bound with alginate can be to use alginate with high guluronnate (>67%). As seen in chapter 6, higher amount of free nisin was available after addition of Ca^{2+} to sodium alginate with high guluronic acid content. Since guluronic acid blocks bind strongly with Ca^{2+}, it is relatively easy for Ca^{2+} to replace the nisin residues bound to the guluronate than those bound to mannuronate. This study will be helpful in selecting the best

composition of alginate and cross-linking parameters for designing an efficient antimicrobial system.

3. Study the effect of pH, water activity and temperature of the release medium on diffusion of nisin from calcium alginate films: In this dissertation, we investigated diffusion of nisin through calcium alginate films in to water at 21°C. However, real food systems are complex and factors such pH amd water activity of the food can affect water sorption kinetics of calcium alginate films and in turn, affect diffusivity of nisin through these films. Similarly, different storage temperatures can have a significant effect on diffusivity of nisin through calcium alginate films. In order to better understand and predict diffusion of nisin from calcium alginate packaging films in to a food matrix, it is important to study the effect of various characteristics of the food matrix such as pH and water activity and the effect of storage temperature on nisin release kinetics.

4. Develop a mathematical model to describe release kinetics of nisin from calcium alginate films: As seen in chapter 7, diffusion of nisin from calcium alginate films deviated from ideal Fickian behavior. Hence, further work needs to be done to fully understand the release mechanism of nisin from calcium alginate films. A mathematical model will provide a tool to understand this diffusion process and also to predict the release kinetics of nisin under various conditions for which the model will be developed.

APPENDIX

Calibration plot of nisin by High Performance Liquid Chromatography (HPLC)

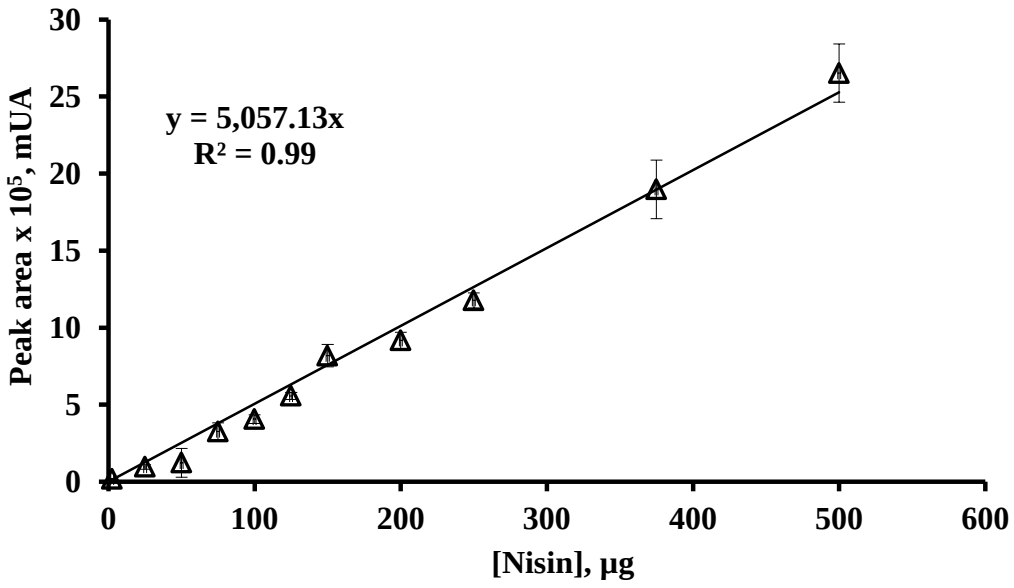

Figure A. Nisin calibration plot developed by HPLC technique. Each data point (Error bars: ±1 S.D) is an average of 3 replications. Symbol represents the experimental value.

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