# PROTECTION OF LYSOZYME WITH CHITOSAN USING RADIANT ENERGY VACUUM DEHYDRATION FOR CONTROL OF CLOSTRIDIAL NECROTIC ENTERITIS IN BROILER CHICKENS

by

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## Abstract

The aim of this study was to develop a heat-stable and slow-release lysozyme-based product using radiant energy under vacuum (REV) technology by formulating lysozyme in a chitosan matrix so that the product could be used as a feed additive for more effective control of clostridial necrotic enteritis (NE) in broiler chickens. Lysozyme/chitosan was made into pastes with water at 25-45% solids content. These were dried with REV technology (300 watts microwave power under 4 KPa absolute pressure). Lysozyme retained its full activity during this process. A REV-treated sample produced with a paste containing 25% solids had a $T_m$ of 124.6°C, which was substantially higher than that of spray-dried lysozyme (101.7°C). Thermal destruction curves constructed over a range of 100-130°C were used to determine D- and *z*-values of lysozyme and the REV-treated samples. REV products were consistently more heat-resistant than native lysozyme powder. A small scale feed pelleting study showed significantly better recovery of lysozyme in the chitosan protected REV product ($P<0.05$). The total porosity and pore size distribution of the REV products were analyzed with an Hg porosimeter. In the REV-treated samples, more than 80% of pore volume represented pore sizes ≥ 300 μm in radius, which were larger than those of native lysozyme powder. Surface structure analysis by scanning electron microscopy suggested the REV samples contained lysozyme evenly distributed inside the chitosan matrix. The REV samples released lysozyme more slowly in aqueous solution than lysozyme powder alone in water.

A cage study was conducted to demonstrate the effect of Entegard™REV, the REV-treated lysozyme antimicrobial mixture, on the performance of broiler chickens and necrotic enteritis disease reduction of birds that were challenged with *Eimeria maxima* and *Clostridium perfringens*. Entegard™REV included in feed at 200 g/metric ton (MT) was very effective in reducing the negative health effects in the birds following NE challenge, and its ability to control the disease was not statistically different from a commonly used antibiotic growth promotant, bacitracin methylene disalicylate (BMD), at 55 g/MT.

## Preface

Some of the work presented in this thesis has been a collaborative effort. Mr. John Jackson of Department of Pharmaceutical Sciences assisted with use of the differential scanning calorimeter. Mr. Derrick Horne of BioImaging Department helped me in preparation of scanning electron micrographs of lysozyme, chitosan and REV-treated samples. Ms. Kadek Okuda Andersen of Department of Chemistry helped in the attempts to differentiate lysozyme from chitosan in the REV matrix with Raman microspectroscopy. Mr. Zefferino Santucci of Neova Technologies Inc. helped in the analysis of the enzymatic activity of lysozyme in various samples with the Shugar turbidimetric method. For the broiler chicken study, Dr. Greg Mathis of Southern Poultry Research, Inc. helped in execution of the trial and Dr. Charles Hofacre of University of Georgia, Athens prepared the *Clostridium perfringens* challenge broth and conducted the necrotic enteritis lesion scoring. The study strictly followed the State of Georgia animal research ethical guidelines.

I designed and executed all of the experiments described in this thesis with the guidance from my supervisor Dr. Tim Durance unless stated otherwise. I also conducted all the statistical analyses of the data generated from the experiments.

Two publications arising from work presented in this dissertation are as follows:

1. Zhang, G., Yagmaee, P., Holley, R. A., Durance, T. D. Use of radiant energy under vacuum (REV) dehydration to increase the enzymatic stability of lysozyme during poultry feed pelleting. Submitted. (Chapter 2-5)
2. Zhang, G., Mathis, G. F., Hofacre, C. L., Yaghmaee, P., Holley, R. A., Durance, T. D., 2010. The effect of a radiant energy-treated lysozyme antimicrobial blend on the control of clostridial necrotic enteritis in broiler chickens. Avian Dis. 54: 1298-1300. (Chapter 6)

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## List of Abbreviations

ABS: absorbance

ANOVA: analysis of variance

AGP: antibiotic growth promotant

*Asp*: aspartic acid

Au: gold

$a_w$: water activity

BMD: bacitracin methylene disalicylate

CCD: charge-coupled device

*Cl.*: *Clostridium*

CPE: *Clostridium perfringens* enterotoxin

DSC: differential scanning calorimetry

EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

FCR: feed conversion ratio

GHz: gigahertz

*Glu*: glutamic acid

Hg: mercury

KDa: kilodalton

KPa: kilopascal

LB: Luria broth

LSD: least significant differences

MHz: megahertz

MIC: minimal inhibitory concentration

MT: metric ton

NE: necrotic enteritis

ppm: parts per million; μg/mL

REV: radiant energy under vacuum

rpm: rotation per minute

SEM: scanning electron micrography

SFP: Shahidi-Ferguson Perfringens

spp.: species

P: probability level

PBS: phosphate buffered saline

PBST: phosphate buffered saline with Tween® 20

Pd: palladium

$T_m$: melting temperature

## Acknowledgements

I must begin by thanking my supervisor, Dr. Tim Durance, for his invaluable guidance, immense patience and financial support. It was him that made this challenge a fun one.

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With great appreciation, I would like to acknowledge the ever prompt and unconditional technical assistance of Dr. Parastoo Yaghmaee.

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Last but most important, I would like to thank my family Yinghua and Aaron for being there for me all the time with your love and support!

## Dedication

I dedicate this thesis to my mom, Dr. Qiudong Wang.

# 1. Introduction

## *1.1 Necrotic enteritis*

### 1.1.1 *Clostridium perfringens* type A

*Clostridium perfringens* is a Gram positive, obligate anaerobic, spore-forming rod (Figure 1). Depending on the lethal toxins produced it has been classified into five different types (Table 1, Cato et al., 1986). *Cl. perfringens* type A, which produces primarily alpha toxin (phospholipase C) is the major group associated with necrotic enteritis (NE) in chickens although type C (which produces both alpha and beta toxins) has also been implicated in some cases (Wages and Opengart, 2003b). *Cl. perfringens* type A also causes cholangiohepatitis (Onderka et al., 1990) and gangrenous dermatitis (Wages and Opengart, 2003a) in chickens. For over 30 years the alpha toxin was thought to be the key virulence factor of NE but more recently, a gene knockout mutant of *Cl. perfringens* which was alpha toxin-negative was capable of causing NE, and this raised doubt concerning the essential role of alpha toxin in pathogenesis (Keyburn et al, 2006).

*Cl. perfringens* type A is also known as a common foodborne pathogen. *Clostridium perfringens* enterotoxin (CPE), a small single-chain polypeptide has been identified as the virulence factor for the symptoms of *Cl. perfringens* type A food poisoning (Brynestad and Granum, 2001). CPE is only produced by a small portion of the global

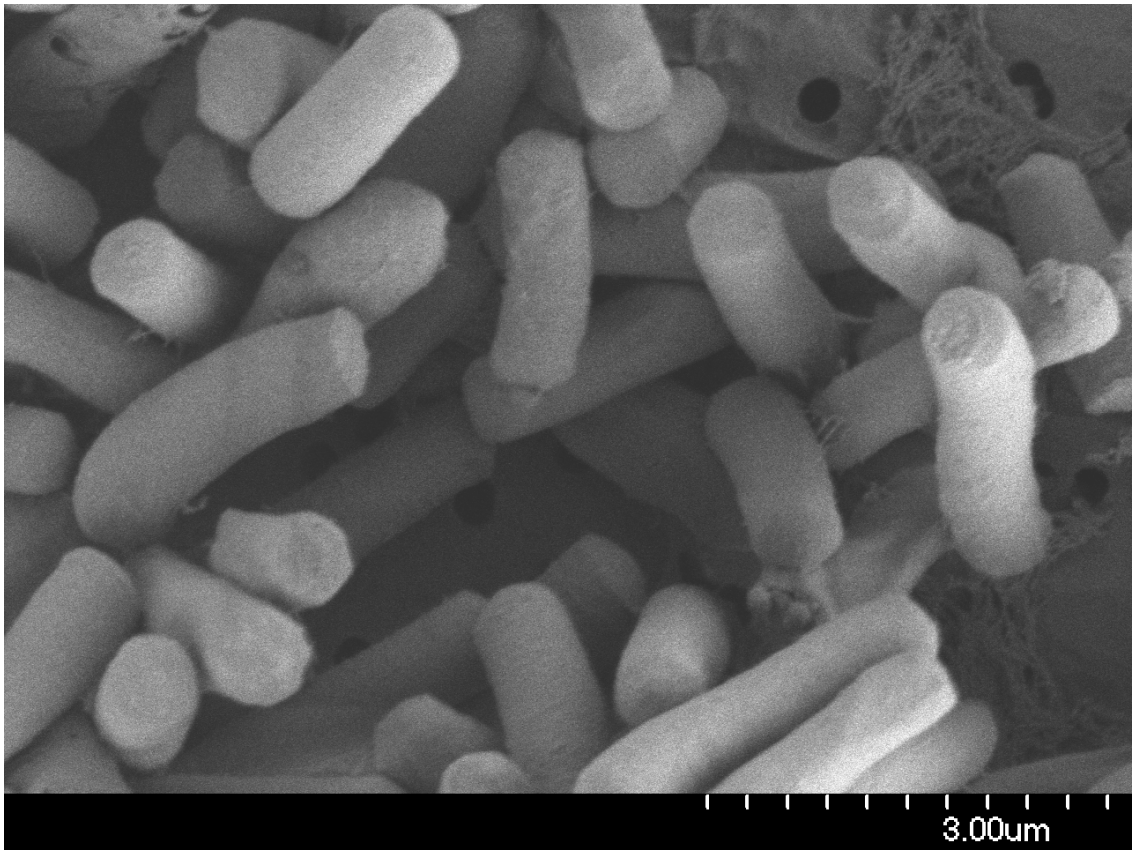


**Figure 1. Scanning electron micrograph of *Cl. perfringens* type A (IM248) vegetative cells.**

(Figure originally published by Zhang et al., 2006a. Copyright Society for Applied Microbiology)

| *Cl. perfringens* type | Toxin produced | | | |
|---|---|---|---|---|
| | *Alpha* | *Beta* | *Epsilon* | *Iota* |
| A | + | - | - | - |
| B | + | + | + | - |
| C | + | + | - | - |
| D | + | - | + | - |
| E | + | - | - | + |

**Table 1. Classification of *Cl. perfringens* by toxin type.**

(Table originally published by Cato et al., 1986. Copyright Lippincott Williams and Wilkins)

*Cl. perfringens* strains. Although *Cl. perfringens* type A often causes NE in avian species, severe food borne NE in humans is rare (Brynestad and Granum, 2001).

### 1.1.2 Avian necrotic enteritis, the disease and the concerns

Clostridial necrotic enteritis was first described by Parish in 1961 and reproduced experimentally with *Cl. perfringens*. Since then, this disease has been reported from most areas of the world where the broilers are produced under intensive management conditions, and it has had significant economic impact on the poultry industry (van der Sluis, 2000). Acute clinical forms of the disease include increased mortality and gross lesions consisting of large areas of necrosis of the lining of the lower small intestine, caeca and in some cases liver (Wages and Opengart, 2003b). A subclinical form has been associated with less efficient feed conversion ratio and retarded growth rate (Kaldhusdal and Hofshagen, 1992). Intestinal stresses caused by dietary risk factors and coccidiosis etc. are considered predisposing factors for the disease (Wages and Opengart, 2003b). In fact, experimentally induced NE is rather difficult to achieve in a controlled setting. Most research challenge models involve inoculating the birds with a combination of the protozoan *Eimeria* spp. and *Cl. perfringens* (George et al., 1984).

A variety of antibiotic growth enhancers, such as virginiamycin (a streptogramin) and bacitracin (a cyclic polypeptide) have been routinely used in feed to control the incidence of NE. However, prophylactic applications of antibiotics are thought to induce antibiotic-resistant strains of pathogens (Emborg et al., 2004). In January 2006, the

European Union officially banned the use of antibiotic growth promotants (AGP) in animal feed, including virginiamycin and bacitracin, which are still widely used in feed in North America. Similar actions may soon be seen in other parts of the world due to increasing public awareness about the possible negative impact of antibiotics on the environment and human health. It is believed that one of the most serious problems faced by poultry producers without access to antibiotics will be an increase in NE (Newman, 2000). In recent years, alternative approaches in addition to management strategies have been avidly pursued by poultry health personnel to reduce or eliminate the use of antibiotics in feed. Inclusion of the polyether ionophore group of coccidiostats in feed can reduce the incidence of NE. However, the development of commercial coccidial vaccines for broilers will phase out these chemical products in the future. Recent studies indicate that administration of natural gut microflora, also known as competitive exclusion, can effectively reduce the effects of clostridial NE (Hofacre et al., 1998; Kaldhusdal et al., 2001). Use of digestive enzymes in feed is becoming increasingly popular due to their ability to alleviate the effect of NE by reducing the viscosity of the gut content and the amount of nutrients available for pathogens (Jackson et al., 2003). Various plant extracts possessing anti-clostridial properties also hold promise as novel feed additives to fight NE (Garcia et al., 2002). More recently, short-chain fatty acids such as butyric acid and lauric acid, as well as essential oils such as thymol and cinnamaldehyde, microencapsulated in a poly-sugar matrix have been shown to contribute to the prevention of clostridial NE (Timbermont et al., 2010). New compounds from natural sources, may be an attractive alternative for use against *Cl.*

*perfringens* and some may replace antibiotics as growth promoters. Hen egg white lysozyme, a natural antimicrobial protein, could fill this need.

## *1.2 Lysozyme*

### 1.2.1 Introduction

Lysozyme (EC 3.2.1.17, muramidase) was first discovered and named by Alexander Fleming (1922). Lysozymes are ubiquitous in both the animal and plant kingdoms and play an important role in an organism's innate defense mechanism. For example, it occurs in several human secretions such as milk, saliva, and tears. Hen egg white lysozyme is the classic representative of the lysozyme family and this group of similar enzymes are called *c* type lysozyme (chicken- or conventional-type). There are also other types such as *v*, *b* and *h* types from viral and plant sources (Losso et al., 2000). Commercially, lysozyme is extracted from hen egg white due to its abundance in eggs. Each egg contains about 0.3-0.4 g of lysozyme. In this thesis, unless otherwise specified, the word "lysozyme" refers to hen egg white lysozyme.

### 1.2.2 Antimicrobial activity of lysozyme

Lysozyme is a 14.6 kDa single peptide that can result in cell lysis by cleaving the β-1, 4 glycosidic linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine in the peptidoglycan layer of the bacterial cell wall (Proctor and Cunningham, 1988). Lysozyme has an extremely high isoelectric point (>10) and consequently is highly cationic at neutral or acidic pH. The four disulfide bonds in lysozyme make the

molecule unusually compact so it has considerable heat stability. In solution, lysozyme is stable at low pH and is active over a range from 1°C to near 100°C (Charter and Lagarde, 1999). Proteolytic enzymes such as trypsin, chymotrypsin and papain do not hydrolyze native lysozyme but once lysozyme is denatured, it becomes susceptible to digestion by these enzymes. Pepsin, on the other hand, readily digests native lysozyme (Losso et al., 2000).

The lysozyme molecule consists of two lobes (α- and β-domains) linked with a long α-helix structure (Imoto, 1996). The unique tertiary structure maintained by the four disulfide bonds is key to lysozyme's catalytic activity. The active site of lysozyme contains 6 subsites that can bind 6 sugar units of the peptidoglycan substrate. Hydrolysis happens at the β-1, 4 glycosidic bond between the $4^{th}$ and $5^{th}$ sugar units, with the catalytic groups *Glu-35* and *Asp-52* of the lysozyme molecule (Imoto, 1996).

Gram positive bacteria are more susceptible to lysozyme because they have a thick cell wall of which about 90% is peptidoglycan. In Gram negative organisms, the peptidoglycan layer only makes up 5-10% of the cell wall and it is protected with an outer membrane containing lipopolysaccharide (Losso et al., 2000). This outer membrane acts as a barrier against macromolecules and antimicrobial agents such as lysozyme. Although lysozyme is most effective against certain Gram positive bacteria, its antimicrobial spectrum can be broadened towards other organisms and even some Gram negative bacteria when it is used in combination with other compounds/synergists. Gram negative bacteria become susceptible when the outer membrane has been

disrupted by compounds such as EDTA, aprotinin and organic acid (Pellegrini et al., 1992; Johnson, 1994; Gill and Holley, 2000). Lysozyme also possesses antiviral activities (Cisani et al., 1984), which was reported to be associated with its strong cationic properties in low to neutral pH ranges. Novel antibacterial activities of lysozyme, independent of its lytic mechanism, were reported recently by a number of researchers (Ibrahim et al., 1997; Masschalck et al., 2002).

### 1.2.3 Lysozyme and *Cl. perfringens* type A

Lysozyme has antimicrobial activity against many Gram positive bacteria including *Clostridium* species such as *Cl. tyrobutyricum*, *Cl. thermosaccharolyticum* and *Cl. botulinum* (Hughey and Johnson, 1987). It has been successfully used in the cheese industry as a bio-protectant for more than 30 years to control *Clostridium tyrobutyricum* which causes the "late blowing" of hard and semi-hard cheeses as a result of butyric fermentation (Carini et al., 1985). A study on the effect of *Eimeria tenella* and *Cl. perfringens* infection on the blood lysozyme and complement levels in broiler chickens showed a clear correlation between the lysozyme concentration and the severity of infection (Sotirov and Koinarski, 2003). Although the ability of lysozyme to inhibit several *Clostridium* species was documented, there were limited reports on the inhibitory effect of lysozyme against *Cl. perfringens*. Lysozyme does not kill bacterial endospores due to their unique capsular structure, but it works to stimulate spores to germinate into vegetative cells (Lewis, 1969). Lysozyme at <10 μg/mL has been successfully used in culture medium to stimulate the recovery of injured *Cl. perfringens* spores (Labbe and Chang, 1995). More recently, lysozyme at 156 μg/mL was found to

inhibit a number of *Cl. perfringens* strains isolated from chickens that had died of severe necrotic enteritis (Zhang et al, 2006a). The destructive effect of lysozyme on *Cl. perfringens* vegetative cells was illustrated in the scanning electron photomicrograph (Figure 2). Lysozyme at a sublethal concentration (50 μg/mL) was found to significantly inhibit alpha toxin production (Zhang et al., 2006a). A number of cage and floor pen trials on broiler chickens using a blend of lysozyme and other natural antimicrobials (Entegard™, Neova Technologies Inc. Abbotsford, Canada) significantly reduced NE mortality and enhanced broiler performance (Mathis et al., 2005). The other antimicrobials used included nisin, other egg proteins and sequestering agents such as citric acid, chitosan or EDTA. The antimicrobial blend, Entegard™, appeared to be nearly as effective as virginiamycin in preventing both clinical and subclinical effects of *Cl. perfringens* in a floor pen study that approximated U.S. poultry industry standards (Mathis et al., 2005).

### *1.3 Protection of lysozyme by encapsulation/entrapment*

One practical problem encountered in the administration of the lysozyme antimicrobial blend through feed is the inactivation of lysozyme during the pelleting process which involves high heat. Pelletization of mash feed before it is fed to the animals has been practiced for more than half a century (Cadet, 1965). Pelleted feed offers a number of advantages over the mash or grain forms, such as decreased wastage, reduced selective feeding and ingredient segregation, and improved digestibility and palatability (Thomas and van der Poel, 1996). The high temperature (>80°C) involved in the pelleting process can also reduce the incidence of pathogenic organisms. Increased bulk density of the

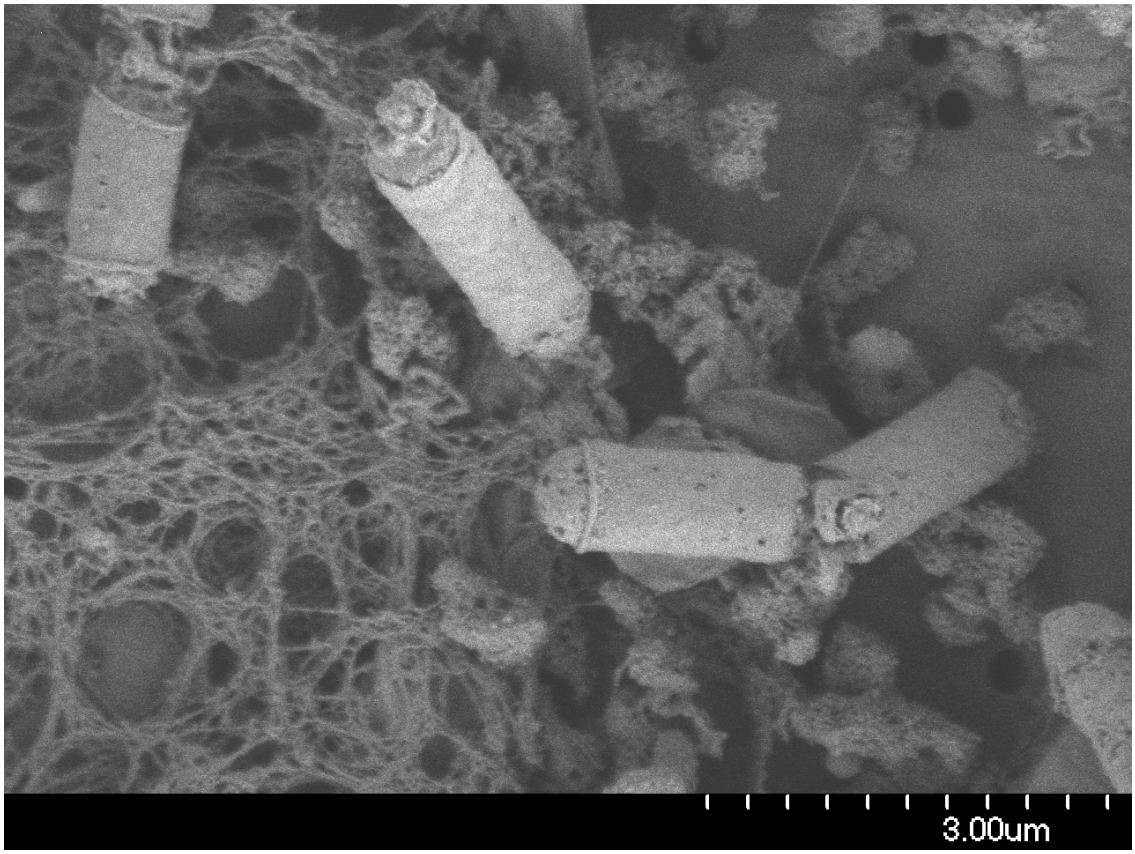


**Figure 2. Scanning electron micrograph of *Cl. perfringens* IM248 treated with 100 µg/mL lysozyme.**

(Figure originally published by Zhang et al., 2006a. Copyright Society for Applied Microbiology)

feed means increased feed intake and cheaper transportation. It was estimated that >80% of poultry feeds in developed countries are in pelleted forms (Thomas and van der Poel, 1996). In modern feed mills, the mash feed is pelleted in a “roller and die” pellet press (Thomas and van der Poel, 1996). Before entering the pellet press, the mash feed is often pretreated by methods such as conditioning with steam (Skoch et al., 1981) or is subjected to the use of an expander (Veenendaal, 1990) to increase temperature/moisture levels for better starch gelatinization and pellet integrity (reducing fines). The temperature of pellets after leaving the die is generally higher than that of the conditioned meal due to the frictional heat generated in the die. Finally, pellets are cooled with ambient air (Thomas and van der Poel, 1996). Post-pellet spray technology allows application of temperature sensitive materials such as feed enzymes to be incorporated in pelleted feed, bypassing the high temperature process. However, currently, only about 60% of the feed mills in North America are equipped with this technology (personal communication, Agri Stats, Inc. Fort Wayne, IN).

A pelleting study done at Neova Technologies Inc. (unpublished data) revealed that without protection, there could be > 50% loss of lysozyme after the pelleting process, especially when an expander was used prior to pelleting. Therefore, lysozyme within the product for NE control needs to be effectively protected during the pelleting process, preferably by the means of encapsulation. Encapsulation is a process where one material or a mixture of materials is coated with or entrapped in another material or system (Risch, 1993). The material being encapsulated is refereed to as the core, payload, actives, internal phase or fill, whereas the wall is sometimes called a shell, coating, wall

material, carrier or membrane. The current encapsulation techniques include spray drying, spray cooling/chilling, extrusion coating, centrifugal extrusion and inclusion complexing, etc. (Risch, 1993). Encapsulation provides protection from oxidation, heat, undesirable volatility, and sometimes also offers controlled release. Encapsulation has found applications in a number of fields: pharmaceutical, biomedical, agriculture, food, cosmetics, to mention a few. Encapsulation may protect lysozyme from heat denaturation during feed pelleting and also from gastric (pepsin) digestion in the bird's digestive tract. A recent study at Neova showed an increase in lysozyme protection (up to 25% loss) during pelleting when lysozyme was encapsulated with hydrogenated vegetable oil and calcium stearate.

## *1.4 Radiant energy under vacuum (REV)*

### 1.4.1 Introduction

REV, or vacuum microwave dehydration is a rapid and efficient drying method that can yield products with unique characteristics and also retain their biological functions with minimal damage, compared to conventional drying methods (Scaman and Durance, 2005). This dehydration method uses microwave radiation for heat generation and temperature is kept low by reducing the chamber pressure to below atmospheric pressure, but above the triple point pressure of water (0.61 kPa). The electromagnetic wave penetrates into the biomaterial and converts to thermal energy, providing even and rapid heating. Vacuum lowers the boiling point of water and creates a pressure gradient

that increases the rates of mass and heat transfer. *In situ* vaporization of the water also provides an expansive force to generate an open and porous structure in the product being dried, which further enhances the drying rate. Because the temperature can be kept at low levels (< 45°C) during the process and the drying occurs quickly at low oxygen pressure, damage to biological activities of the materials can be kept at minimum (Scaman and Durance, 2005). As early as in the 1940's, radio-frequency energy had been used in conjunction with vacuum for rapid dehydration of potatoes, cabbage, and penicillin (Rushton et al., 1945; Brown et al., 1947). Although the radiation applied was below the microwave frequency, these examples did convey the concept of converting electromagnetic energy to heat inside a material for dehydration. Commercially, microwave vacuum technology has been used to dry a variety of food products such as orange juice, grapes, blueberries, chicken broth and also agricultural crops such as grains, rice and peanuts (Scaman and Durance, 2005).

### 1.4.2 Microwave generation

Microwaves are electromagnetic radiation with wavelengths ranging from 1 mm to 1 m (frequencies from 300 MHz to 300 GHz). Microwaves in household ovens and commercial equipment are generated by a magnetron. A magnetron is a vacuum diode in which the cathode is surrounded by a coaxial anode (Love, 1995, Figure 3). The anode ring consists of even numbers of vanes that extend towards the cathode which is positioned in the centre of the magnetron. Alternating vanes are connected with conducting straps and share the same polarity. A constant magnetic field parallel to the

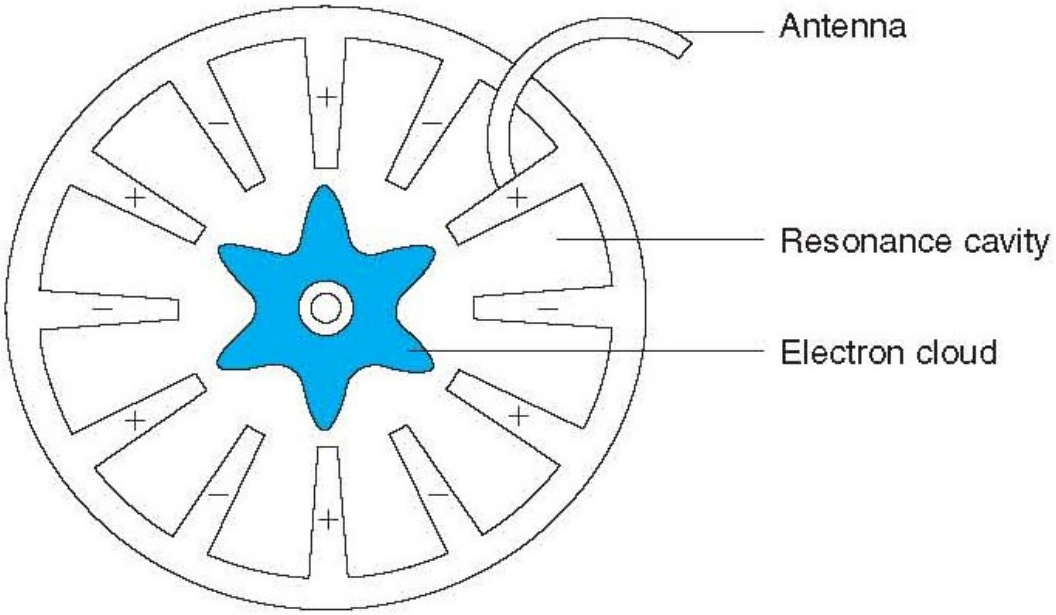


**Figure 3. Cross-section of a magnetron.**

Rotating spokes of the electron cloud induce an alternating current in the resonance cavities at the microwave frequencies. (Figure originally published by Love, 1995. Copyright Academic Press.)

axis of the cathode is provided with a pair of powerful permanent magnets. A direct current runs through the cathode and heats up the filament which emits electrons. Under a high potential difference (such as 4000 volts) between the cathode and anode vanes, the electrons travel at high speed towards the vanes with a deflected trajectory due to the strong magnetic field. This forms a cloud of electrons around the filament with rotating "spokes" that touch every alternating vane, which induces an alternating current with high frequencies inside the open area between the vanes (resonance cavities, Figure 3). An antenna transfers the microwaves generated in the resonance cavities through a waveguide into the dehydration chamber of the machine (Scaman and Durance 2005).

### 1.4.3 Energy transfer with microwave radiation

Microwaves do not have intrinsic heat. Electromagnetic energy is converted to thermal energy by interaction of the rapidly oscillating electric field with the molecules of the heated subject. The high frequency oscillation of the microwave field makes the charged ions or polar/dipolar molecules in a biomaterial migrate, vibrate or rotate in a random pattern, which generates heat. Water, due to its permanent dipole structure, and its relatively high dielectric constant (microwave permittivity) and high dielectric loss factor, is often the main component of a biomaterial that is affected by microwave radiation (Scaman and Durance, 2005). Microwaves can penetrate through a solid material and provide more even heating than conventional conductive or convective heat. Macromolecules such as lysozyme and chitosans have a much lower dielectric constant and do not absorb microwave energy well (Nakamura et al., 1988; Bonincontro et al., 2004; Seoudi and Nada, 2007). Since dehydration is a process of removing water,

the microwave can be a very useful tool to convert water to vapour with the heat generated. By reducing the boiling point of water under strong vacuum, this conversion can be achieved at reduced temperature, causing minimal or no thermal damage to the biological material being dried.

### 1.4.4 Characteristics of REV dehydration

For biological solids, the combination of vacuum and microwave offers the fastest dehydration rate among the different drying methods available (Scaman and Durance, 2005). A comparison of drying rates of fixed-sized fresh carrot slices by three different drying technologies demonstrated the superior dehydration rate of REV (Lin et al., 1998). Freeze-drying required approximately 72 h to reach the end point of drying (9% moisture content) and hot air-drying required 8 h. However, it only took 33 min for the carrot slices to reach the end point of drying with REV. In the same study, the authors also looked at the rehydration ratio of the dried carrot slices and it was found that the rehydration potential of the REV-dried samples lies in between freeze- and air-dried products (Lin et al., 1998). A comparison of the microstructures (SEM pictures) of air-, freeze- and REV-dried apple and potato slices indicated the resemblance of REV-dried products to freeze-dried products: both contained tissue networks with large pores (Durance and Liu, 1996; Sham et al., 2001). For less heat-sensitive materials that can be prepared in a homogenous liquid suspension, another rapid dehydration method is spray drying. In the spray drying process, the liquid being dried gets atomized into tiny droplets (10-500 μm in diameter) with a nozzle into a chamber filled with hot air (up to 300°C). The particles dry quickly and thermal damage is usually minimal. The dried

powder is usually gathered with a cyclone device (Masters, 1991). However, the feed materials for spray drying are limited to pumpable liquids and the cost of spray drying can be high relative to other hot air drying methods.

REV has been shown in numerous reports to allow dehydration of tissue-foods from plants with excellent retention of biological activity, flavors, and vitamins (Kwok et al., 2004; Purnama et al., 2010). In one study (Kim et al., 1997), survival of REV-dried lactic acid bacteria in plain yogurt was superior to spray-dried and freeze-dried samples. Similarly, higher levels of α-amylase activity were reported to be retained in microwave dried rice koji, compared to freeze-, vacuum-, and air-drying (Kim et al., 1999).

Natural polymers have been used to immobilize enzymes. An emulsion encapsulation method had been used to combine a number of hydrocolloids such as gelatine, starch, microcrystalline cellulose and algin, cross-linked with glutaraldehyde, to encapsulate/entrap peroxidases suitable for the production of hydrogen peroxide. The resulting matrix allowed long term antimicrobial activity by controlled release of hydrogen peroxide (King, 1995). In recent studies, REV techniques have been described for generation of dry porous materials from gels or solutions of hydrocolloids such as starch, methyl cellulose, and pectin (Durance et al., 2005). These reports indicate that dry porous solids may be produced without subjecting materials to destructive temperatures or to oxygen. Moreover, depending on the material used, the matrix may serve an encapsulation/entrapment type of function (Durance et al., 2005).

## *1.5 Hypotheses*

The following hypotheses are proposed in this thesis study:

1. REV process allows production of a porous material that contains lysozyme in a continuous matrix of chitosan, without negatively affecting the enzymatic activity of lysozyme.

2. The heat stability of REV-embedded lysozyme will be increased by the unique matrix structure, compared to native commercial lysozyme powder.

3. The release rate of lysozyme in water will be reduced when embedded in the chitosan matrix.

4. The REV-treated material will have increased efficacy in controlling NE in broiler chickens, compared to the product containing unprotected lysozyme.

## *1.6 Objectives*

### 1.6.1 Aim 1: to produce the lysozyme-chitosan matrix using REV processing and evaluate the effect of REV process on lysozyme activity

The process parameters were power (watts), vacuum level (mm Hg) and processing time (min). The initial solids content of the material mixture were also optimized. After processing, the final moisture content, and water activity were measured. Lysozyme

activities of the samples before and after REV were tested using the micrococci cell wall turbidimetric method (Shugar, 1952).

### 1.6.2 Aim 2: to evaluate the effect of REV process on the thermal stability of lysozyme antimicrobial mixture

Thermal stabilities of REV-treated samples were evaluated in 3 steps:

1. Denaturation temperatures of lysozyme powder and REV-treated powder samples were determined from thermograms obtained with a differential scanning calorimeter.

2. The D- and z-values of the commercial lysozyme powder and the REV processed samples were determined by constructing decimal destruction curves over temperatures from 100°C to 130°C.

3. A feed pelleting trial was conducted using a California pellet mill to verify the thermal protective effect of the REV-treated matrix. The residual lysozyme contents in the feed were quantified with an antigen competitive ELISA.

### 1.6.3 Aim 3: to investigate the microstructure of the REV-treatment samples

Total porosity, pore size and distribution of the samples were determined using a porosimeter. The surface structures of the samples were also visualized with scanning electron microscopy and Raman microspectroscopy.

### 1.6.4 Aim 4: to determine the release rate of lysozyme in the REV matrix

Lysozyme release rates of lysozyme, lysozyme/chitosan powder mixture and the REV-treated materials were measured as described by Park et al, 2004, with some modifications. The amounts of lysozyme released in solutions were determined with the Shugar method.

### 1.6.5 Aim 5: to evaluate the efficacy of REV-treated product in controlling experimentally-induced NE in broiler chickens

The efficacy of the REV-treated product (administered through the feed) was evaluated for the control of clostridial necrotic enteritis in a cage study. The experiment involved 30 cages starting with 10 male broiler chickens in each cage. The treatments were replicated in six blocks, randomized within blocks of five cages each. Coccidial and *Cl. perfringens* challenges were performed according to established protocols (Mathis et al., 2005). The study ended on day 28. Feed consumption, feed conversion ratio, live weight gain, mortality rate and lesion scores were monitored and analyzed.

# 2. Dehydration of Lysozyme-Chitosan Paste with the REV Process and the Effect of the REV Process on Lysozyme Activity

## *2.1 Introduction*

Hydrocolloids such as starch, pectin and methyl cellulose have been used successfully as carriers (coating materials) for encapsulating bioactive compounds such as penicillin G by forming a porous material after co-dehydration with REV technology (Durance et al., 2005; Yaghmaee and Durance, 2007). Water-soluble chitosan was chosen to be the hydrocolloid for this lysozyme REV study. Previous studies showed that chitosan also had an anti-clostridial effect, and when combined with lysozyme, produced synergistic effects against *Cl. perfringens.* The optimal ratio of lysozyme/chitosan mixture was determined to be 1:3 (Neova Technologies Inc., personal communications).

Chitosan is a linear polysaccharide composed of randomly distributed β-(1, 4)-D-glucosamine (deacetylated units) and N-acetyl D-glucosamine (acetylated units). It is produced commercially by deacetylation of chitin, the most abundant amino polysaccharide in nature (No et al., 2007). Most commercial chitosan products are sourced from the exoskeletons of crustaceans and the cell walls of fungi. Due to its non-toxic, biocompatible and biodegradable nature, it has found numerous applications in the biomedical, food and chemical industries (No et al., 2007). Research has demonstrated chitosan and its derivatives to be suitable pharmaceutical excipients and promising materials for drug delivery (Ilium, 1998; Prabaharan, 2008).

Lysozyme and chitosan powders were mixed together with water into pastes with various solids contents and dried in an experimental microwave vacuum drier. The original plan was to optimize the power level (watts), vacuum level (mm Hg) and process time in order to maximize the lysozyme recovery rate. A preliminary run with a pre-selected power and vacuum level with the equipment (300 watts and 4 KPa) yielded porous dry material that retained 100% of lysozyme activity. A processing time of 10 min was sufficient to produce sufficiently dry material ($a_w \leq 0.40$) and temperature of the material at the end of drying never exceeded 45°C. Therefore, these process parameters were chosen for the REV treatment for all of the samples. The objective of this study is to produce REV-treated samples with optimum initial solids levels and maximum lysozyme activity retention.

## *2.2 Materials and methods*

### 2.2.1 Moisture and water activity ($a_w$) measurements

The moisture contents of native lysozyme powder, chitosan powder, fresh lysozyme/chitosan pastes and REV-dried materials were measured using a vacuum oven method (100°C for 5 h, AOAC 17.006). The $a_w$ of fresh and dried pastes were measured with an Aqua Lab $a_w$ meter (Model series 3, Decagon Devices, Inc., Washington, USA).

### 2.2.2 Dehydration of lysozyme-chitosan paste with the REV process

Lysozyme (Neova Technologies Inc. Lot# LA5392) and chitosan (Shanghai Freemen Chemicals Co., Ltd., China. MW ca. 15 KDal) were tested for moisture contents before mixing. The lysozyme and chitosan mixture (1:3 solids ratio) was blended with distilled water using a Braun hand mixer at 10,000 rpm for 3-5 min to yield a paste with homogeneous consistency. Triplicate paste samples were made with the following solids content: 25%, 30%, 35%, 40% and 45% (w/w) and stored in sealed Whirlpak Retain® bags at room temperature. The pastes were tested for actual solids content, lysozyme enzymatic activity and $a_w$ before being subjected to the REV process. About 4 g of the paste were weighed into a clean, dry custom-made cylindrical quartz container (3 cm in diameter, Figure 4). The paste material was pressed to the bottom of the container with a spatula to avoid "boil-over" inside the dehydration chamber of the vacuum microwave dryer. Three samples of each paste were loaded to the Teflon sample holder and dried in a Microwave Vacuum Dryer (Model# VMD 900W, EnWave Corporation Vancouver BC Canada, Figure 5), at 300 watts power and pressure of 4 KPa for 10 min. The temperature profile during the process was monitored using an infrared thermometer. After drying, the samples were weighed immediately and the $a_w$ was measured (Figure 6). After $a_w$ testing, the samples were packaged in Whirlpak bags under nitrogen at a relative humidity of 18% in a glove box (containing desiccant) to minimize moisture change. Before the moisture and lysozyme activity testing, the samples were coarsely ground using a mortar and pestle to a particle size of 60 Mesh (screen) and the


**Figure 4. A lysozyme/chitosan paste with 25% initial solids in a quartz container, and the plastic holder that holds the quartz containers.**


**Figure 5. A research microwave vacuum drier: Model# VMD 990W (EnWave Corporation, Vancouver, BC, Canada).**


**Figure 6. REV-dried lysozyme/chitosan mixture from a 25% initial solids paste sample.**

remaining samples were stored at room temperature in sealed 10 mL glass serum vials, with headspace flushed with pure nitrogen gas before any further experiment.

### 2.2.3 Lysozyme enzymatic activity testing

Enzymatic activities of lysozyme samples were tested using the Shugar turbidimetric method (Shugar, 1952), with some modifications:

1. The substrate solution was prepared by adding 50-60 mg lyophilized *Micrococcus lysodeikticus* (Sigma M-3770) to 100 mL of sterile phosphate buffer (0.07 M, pH 6.2) and the substrate was gently suspend in buffer and incubated at 37°C for 30 min before use. An absorbance reading of 1.7 ± 0.1 at 450 nm was desired. The solution was adjusted by adding more substrate or buffer to obtain the target absorbance (ABS).

2. To exclude contamination of lysozyme (from airborne particles in the laboratory) in the prepared substrate suspension, the absorbance was read over a 3-min period. There should be minimal absorbance change (<0.1). When contamination took place, the substrate suspension was re-made.

3. About 28-30 mg of lysozyme standard (LOT# LF8134, Neova Technologies Inc. Abbotsford, BC) and samples (based on total weight) were weighed and dissolved in 30 mL of phosphate buffer. Three serial dilutions were made.

4. From the main screen of the spectrophotometer (ATI Unicam UV3, Cambridge, UK), "Library" was pressed to load the method and then select "Rate Lysozyme" (for continuous detection at 450 nm for 3 min).

5. An empty plastic cuvette was placed into the spectrophotometer and adjusted to zero. Then 2.9 mL of prepared *Micrococcus* substrate suspension was added (the initial reading should be 1.7 ± 0.1).

6. One hundred μL of the working (lysozyme) standard solution were added to the substrate suspension and mixed well by pipetting solution up and down. The absorbance (after 2-3 sec delay) was recorded for 3 min. The recommended rate value (absorbance change/min) should be between 0.028 and 0.038. If rate was out of this range, the dilution factor was adjusted.

7. Both standard and sample were tested in duplicate and the average was taken as the result.

8. Calculation:

Lysozyme activity (Shugar Units/mg) = $(ABS_{initial} - ABS_{final})$*DilutionFactor / (Constant*Time*Initial lysozyme concentration*Volume)

Constant = 0.001; Time = 3 (min); Volume = 0.10 (mL)

Example of lysozyme activity calculation for the freeze-dried standard:

(1.774-1.66) x (5/0.15) / (0.001 x 3 x (29.6 mg/50 mL) x 0.10 = 21,396 Shugar Units/mg

Example of lysozyme activity calculation for a powder sample

(1.779-1.666) x (5/0.15) / (0.001 x 3 x (29.4 mg/50 mL) x 0.10 = 21,353 Shugar Units/mg

Corrected lysozyme activity:

Lysozyme activity of sample x 24,000 / Lysozyme activity of standard = 21,353 x 24,000 / 21,396 = 23,951 Shugar Units/mg

24,000 Shugar Units/mg is the theoretical full lysozyme activity.

The unit definition: using *Micrococcus lysodeikticus*, one unit of enzyme will cause a decrease in absorbance at 450 nm of 0.001 per min at 25°C.

### 2.2.4 Minimal inhibitory concentration (MIC) of lysozyme/chitosan mixture before and after REV treatment

A micro-broth dilution assay (Zhang et al., 2006a) was used to evaluate the antimicrobial efficacy of lysozyme/chitosan against a *Cl. perfringens* strain (IM248) associated with severe NE disease (Animal Industry Branch, Ministry of Agriculture and Food, Province of British Columbia, Canada). Sterile 96-well polystyrene microtitre plates with well capacities of 300 µL were used (Falcon, Becton Dickinson and Company, Franklin Lakes, NJ) and 100 µL of fresh Luria broth (LB) was added to each well of the plate except for the first column. The medium pH was adjusted to 6.5 to simulate the intestinal environment (Sturkie, 1999). Lysozyme/chitosan mixtures at 10,000 µg/mL (based on total solids) were filter-sterilized by passage through a 0.45 µm membrane filter (Corning Incorporated, Corning, NY). Two hundred µL of the lysozyme stock solution were added to each well of the first column using a multi-channel pipettor (Eppendorf, Hamburg, Germany). Then 100 µL of the stock solution

was removed from the first column and mixed thoroughly with the broth in the corresponding wells of the second column 6 times. Subsequently a 100 μL aliquot of each well from this column were removed and mixed with the corresponding wells of the next column. This doubling dilution was done in rows across the plate except for the last column that was kept for use as controls. This dilution procedure resulted in a gradient of antimicrobial mixture concentrations from 0 to 10,000 μg/mL across the plate. Ten μL of fresh *Cl. perfringens* culture in brain heart infusion after overnight incubation at 41°C (approximately 2.0 x $10^8$ CFU/mL) were inoculated in each well of the plate to yield a final population density of $10^5$ CFU/mL. The microtitre plate was incubated in an anaerobic jar (BBL, Becton Dickinson and Company, Sparks, MD) at 41°C for 24h. Bacterial growth was measured by a change in absorbance at 560 nm using an automated microplate reader (Labsystems, Multiskan MS 3.0, Vantaa, Finland). The MIC was determined as the lowest antimicrobial concentration that resulted in inhibition of *Cl. perfringens* growth (lack of increase in absorbance reading). Five μL of the contents in those wells were also spotted on Shahidi-Ferguson Perfringens Agar (SFP, Oxoid, Basingstoke, UK) for confirmation of inhibition (absence of growth). The MIC assay was carried out in duplicate and the assay was also repeated three times on different occasions.

### 2.2.5 Statistical analysis

For comparison between two means, Student's *t*-test was used to determine the statistical differences using SigmaStat software (Version 2.0). Differences were deemed significant at the $P < 0.05$ probability level.

## *2.3 Results*

In general, the samples with lower starting solids levels had a visually more porous structure (also under a stereoviewer (Volpi Manufacturing, Auburn, NY)) which was confirmed by the porosity data. The 40% and 45% (solids) pastes were quite hard and did not produce a very porous structure after REV. Therefore, only the samples with 25%, 30% and 35% (initial solids) were subjected to further study. The tested solids levels of the paste at 24.47%, 29.35% and 34.67%, respectively, were very close to the target percentages. The initial $a_w$ was around 0.99 for all samples at room temperature and the dried samples had an $a_w \leq 0.40$. The calculated moisture contents of the dried samples (based on weight loss and tested solids contents) were consistently lower than the tested moisture levels of the finely ground powder, indicating rapid moisture ingress into the dried products. The final moisture contents of all samples stabilized at about 15%. No loss of lysozyme enzymatic activity was found for any of the samples after REV treatment (Figure 7). The temperatures of the samples during the REV drying are shown in Figure 8. In the current REV tests, the temperature of the vials never reached 45°C. The MIC values of lysozyme/chitosan mixture (25% solids samples) before and

after REV treatment is shown in Figure 9. The MIC of the mixture before and after REV treatment were both determined at 156 μg/mL (based on solids).

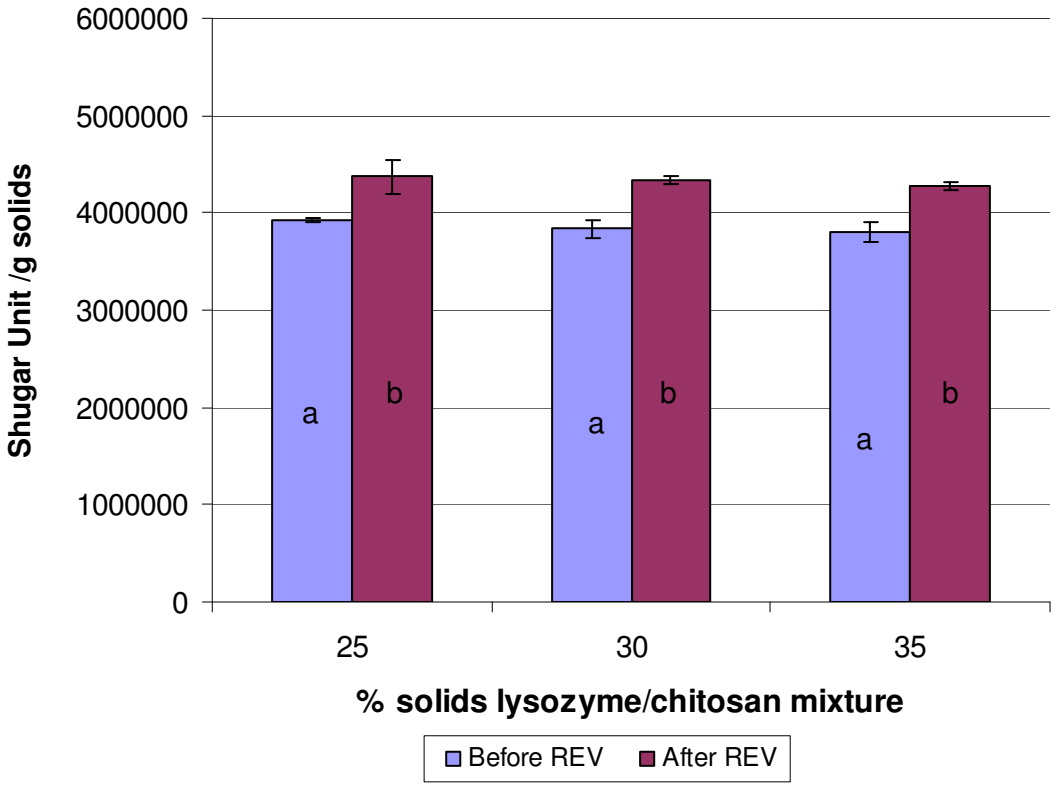


**Figure 7. Lysozyme enzymatic activity before and after REV.**

Bar graph shows the mean value and standard error measured from three independent experiments. Results labeled with different letters are significantly different from each other ($P < 0.05$).

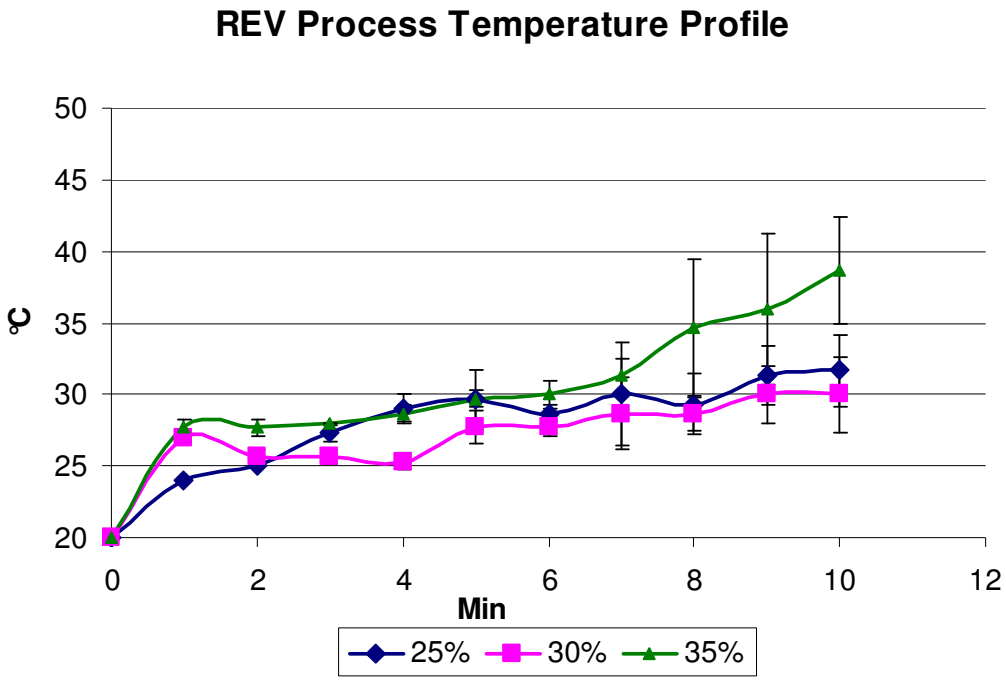


**Figure 8. The temperature of the samples with different initial solids during the REVdrying process.**

Line graph shows the mean value and standard error measured from three independent experiments.

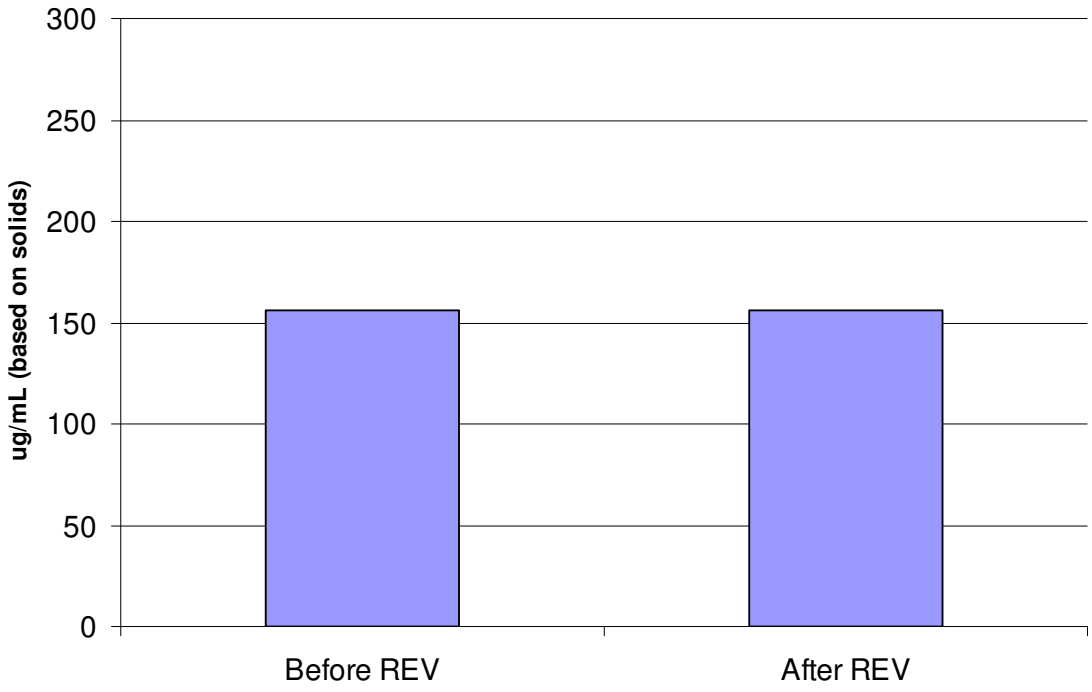


**Figure 9. The MIC values of the lysozyme/chitosan mixture against *Cl. perfringens* IM248 before and after REV treatment.**

## *2.4 Discussion*

Results showed that the water-soluble chitosan could serve as a suitable hydrocolloid carrier for lysozyme and a simple REV process (300 watts at 4 KPa pressure for 10 min) was sufficient to produce a porous material without significant loss of lysozyme activity. Indeed, REV treatment yielded a measurable, consistent increase (about 10%, $P<0.05$) in lysozyme activity. Hamaguchi et al. (1960) observed that when hen egg white lysozyme was heated at 50°C for 4 h, the activity measured was almost double that of the unheated enzyme. However, the reason for the activity increase by mild heating was not clear. In the current REV tests, the temperature of the vials was lower than 50°C. As expected, the antimicrobial activity of the mixture towards *Cl. perfringens* did not change after the REV treatment.

# 3. Effect of REV Process on the Thermal Stability of Lysozyme Antimicrobial Mixture

## *3.1 Introduction*

A REV process of 300 watts at 4 KPa for 10 min produced satisfactory porous chitosan-lysozyme materials with no loss of lysozyme enzymatic activity. Whether this product could withstand high temperature exposures and offer a protective effect on lysozyme activity needed to be evaluated. In the following experiments, the thermal stability of lysozyme in the REV-treated powders was tested by DSC analysis, D- and z-value determination and also during a small scale feed pelleting trial.

## *3.2 Materials and methods*

### 3.2.1 Determination of lysozyme denaturation temperatures ($T_m$)

The denaturation temperatures for native lysozyme and the REV-processed samples were determined using a differential scanning calorimeter (TA Instruments Q100 DSC, New Castle, DE, USA), with refrigerated cooling. The purge gas was prepurified nitrogen at a pressure of 20 psi. Ten mg samples were placed in hermetically sealed aluminum pans using an empty pan as a reference. The samples were heated from ambient temperature to 220ºC at 10ºC/min and then cooled down to 30ºC at a rate of 20ºC/min. Tests were run in triplicate.

### 3.2.2 Determination of thermal kinetic parameters of lysozyme and REV samples (D-and *z*-values)

Lysozyme thermal stability was studied at: 100, 110, 120 and 130 °C. At 100°C, the sampling was done at 0, 10, 30 and 60 min. For the other temperatures, the sampling points were 0, 1, 5, 10 and 30 min. For each study, 5 mg of lysozyme or REV powder (25% starting solids) with moisture content adjusted to 14% (to mimic the typical feed moisture content) were placed in hermetically sealed aluminum pans. Triplicate samples were made for each sampling point. A preliminary experiment showed that under this setup, the DSC pans were heated to 130°C from ambient temperature in 60 s. The oil bath assembly included a glass beaker containing mineral oil and a Corning PC-420D laboratory stirrer and hot plate with temperature control probe (6795-420D, Corning, USA). This system was able to keep the temperature fluctuation within 1°C of the set point. DSC pans were lightly engraved with the letter "A" or "B" with a sharp needle to identify the lysozyme and REV samples, respectively. For the study, three lysozyme samples and three REV samples (dried from three separate runs) were grouped together in a small stainless steel mesh container (tea ball) with a chain and hook (for hanging on the rim of the beaker). Four such units were assembled to enable tests for four different heating durations. For each temperature study, four tea balls containing DSC pans were lowered in the oil at the same time. At each sampling point, one assembly was quickly removed from the oil and dipped into ice water for rapid cooling. After cooling for 5 min, the DSC pans were retrieved from the tea ball assembly and dried thoroughly with Kimwipe paper tissue. The DSC pans were carefully opened with a pair of small pliers and forceps. The opened aluminum pans with their contents were transferred to a pre-

labeled empty 1.5-ml microfuge tube. One ml of de-ionized water was added to each tube and they were vortexed vigorously to dissolve the soluble material. After settling, the supernatant was subjected to a lysozyme activity assay following the Shugar method (1952). The residual lysozyme activity was assessed as the percent activity recovered after heating, compared with the sample at time zero. Thermal destruction time curves were obtained by plotting the log (percentage activity recovered) over time to obtain D-values and the z-values were obtained by plotting the log D-values against the temperatures studied.

### 3.2.3 Stability of lysozyme during feed pelleting

The pelleting study was carried out using a California pellet mill (CPM, San Francisco, CA). Finely ground standard mash broiler feed (Purina Mills, Gainesville, GA) was used for this experiment (Appendix A). Lysozyme or REV-treated lysozyme/chitosan product, along with other ingredients of Entegard, was thoroughly blended with 75 kg mash feed to yield 20 ppm final lysozyme concentration. The feed and test samples were added to a Hobart mixer (Model M-802, Hobart Manufacturing Company Ltd., Troy, OH) and mixed for 15 min at speed level 1. About 300 g of mash sample was collected for each test product prior to the pelleting study. The mixed samples were then subjected to the pellet mill (85°C for 25 sec and 172.35 KPa steam pressure in the conditioner). Between sample runs, the mill was cleaned by pelleting starch containing canola oil that had a similar consistency of broiler mash feed. Pellets were collected mid-stream at the outlet of the mill, placed on a metal rack and allowed to air-dry for 15 min. For the ELISA assay of lysozyme content, 10 g of feed sample (mash or pellet) was placed in a

250 ml glass beaker and then mixed with 90 ml of standard phosphate buffered saline (PBS) supplemented with additional 2% NaCl. A Braun high speed hand mixer was used to homogenize the mixture for 5 min at 11,000 rpm. The lysozyme extraction was performed in triplicate for each feed sample. The homogenate was analyzed with an antigen competitive ELISA method developed by Neova Technologies Inc.

### 3.2.4 Detection of lysozyme content in feed

For the ELISA assay, a rabbit anti-lysozyme polyclonal antibody (Rockland Immunochemicals, Gilbertsville, PA) was diluted in PBS to 10 μg/mL and added (100 μL) to each well of a Nunc Maxisorp® 96-well ELISA plate (Nalge Nunc International, Rochester, NY). The plate was incubated at 4°C for 24 h and then washed 3 times with PBS supplemented with 0.05% Tween® 20 (Sigma P1379, PBST) contained in a plastic squirt bottle. The wells were then filled with 250 μL 3% fish gelatin (Sigma G7765) in PBS and incubated at 37°C for 2 h. The plate was emptied by striking up side down against a thin stack of paper towels and 100 μL feed samples and lysozyme standard (LOT# LF8134, Neova Technologies Inc., diluted to 1.0 μg/mL) were added to the first column of the plate. The samples were run in triplicate. The samples and the standard were serially diluted (2X) horizontally in PBS supplemented with 1% fish gelatin, with the wells of the last column kept as the negative control. Immediately after the dilution, 10 ng/mL of a custom made lysozyme-horseradish peroxidase conjugate (Antibodies Inc., Davis, CA) was added to each well of the plate (100 μL). The plate was incubated at 37°C for 1 h and then washed 3 times with PBST. Fifty μL of prepared 0.005% 3,3',5,5'-tetramethylbenzidine buffer (Sigma, T-5525) was added to each well and the

plate was further incubated at 37°C for 30 min. After the color development, the reaction was stopped by adding 50 μL 2N HCl and the plate was read at 450 nm using an automated microplate reader (Labsystems, Multiskan MS 3.0, Vantaa, Finland). The absorbance data were plotted and a standard curve of lysozyme was constructed. The lysozyme concentration in the feed was calculated with the plot of absorbance value and the dilution factor (10X).

### 3.2.5 Statistical analysis

All data collected were subjected to one-way ANOVA using SigmaStat software (Version 2.0). For comparison between two means, including the D- and z-values (slopes), Student's t-test was used to determine the statistical differences. For comparisons among many groups, statistical differences among the means were detected by Tukey's test. Differences were deemed significant at the $P < 0.05$ probability level.

## *3.3 Results*

### 3.3.1 Determination of lysozyme denaturation temperatures ($T_m$)

The thermograms of lysozyme, chitosan and the REV-treated sample obtained from the DSC analysis are shown in Figure 10. In this analysis, it was clear that the lysozyme/chitosan REV-processed blend had superior thermal stability when compared to the native lysozyme powder. Lysozyme had a melting (denaturation) temperature of 101.7°C. The REV sample generated from the paste with 25% solids had a $T_m$ of 124.6°C. Chitosan had a $T_m$ of 131.8°C, slightly higher than the REV-treated sample.

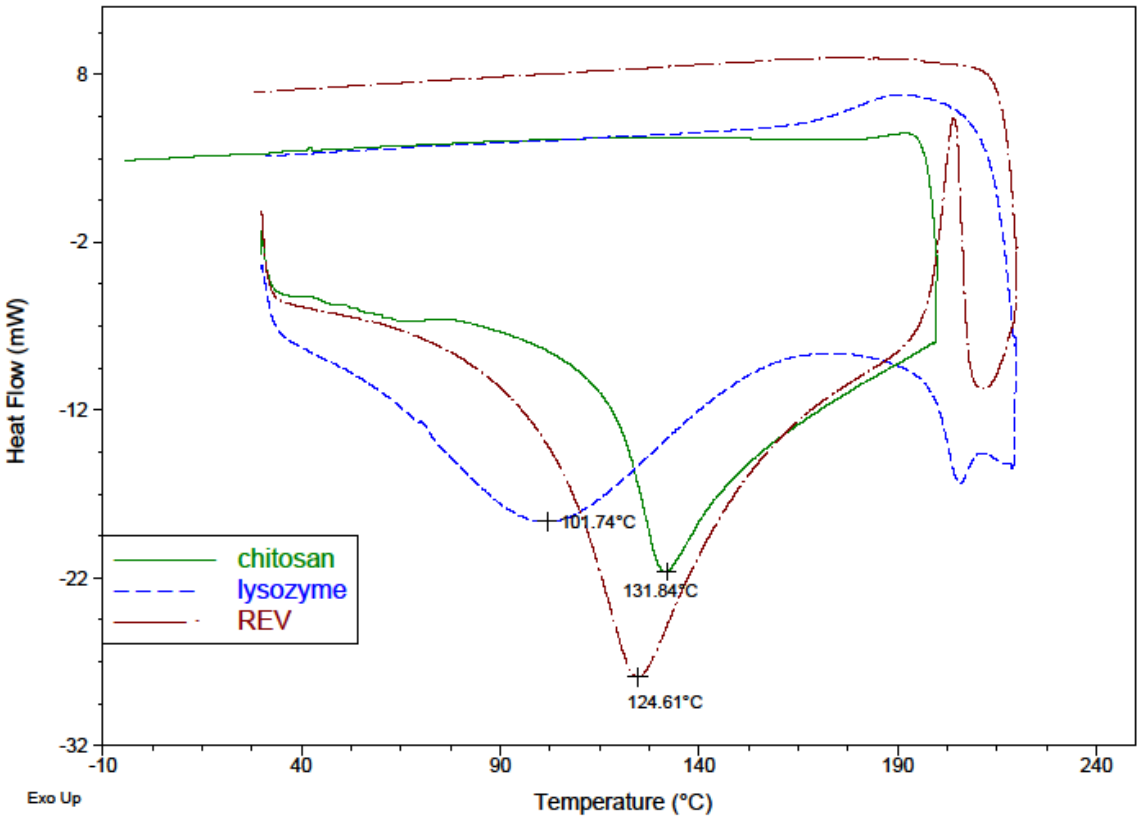


**Figure 10. Thermograms of lysozyme, chitosan and lysozyme-chitosan REV sample.**

The 30% and 35% (solids) samples also showed denaturation temperatures similar to the 25% solids samples (Figure 11).

### 3.3.2 Stability of enzymatic activity (D- and z-values) in lysozyme and REV-treated samples

The thermal destruction curves of lysozyme and the REV samples during treatment at 100-130°C are shown in Figures 12-15. The D-values were calculated for each temperature as the negative reciprocal of the regression slope of the curves. The D-values of lysozyme and REV-treated samples are listed in Table 2.

From the calculated D-values, it is evident that REV products had heat stability which was superior to native lysozyme in terms of retained enzymatic activity. For example, when heated at 110°C, it only took native lysozyme 74.63 min to lose 90% of activity while it took the REV-treated sample 166.7 min to lose 90% of enzymatic activity (Table 2). The incremental thermal stability as assessed by *z*-values of lysozyme and REV products was calculated based on the D-values using the same function (Figure 16). For lysozyme, the *z*-value was 12.82°C and for REV product, it was 15.46°C which was slightly higher than the native lysozyme, but not statistically different ($P > 0.05$).

### 3.3.3 Stability of lysozyme during feed pelleting

Lysozyme concentration in feed samples before and after the pelleting process is shown in Table 3. The percentage recovery rate, based on the residual lysozyme concentration (μg/g) in the mash feed samples (before pelleting) is graphed in Figure 17.

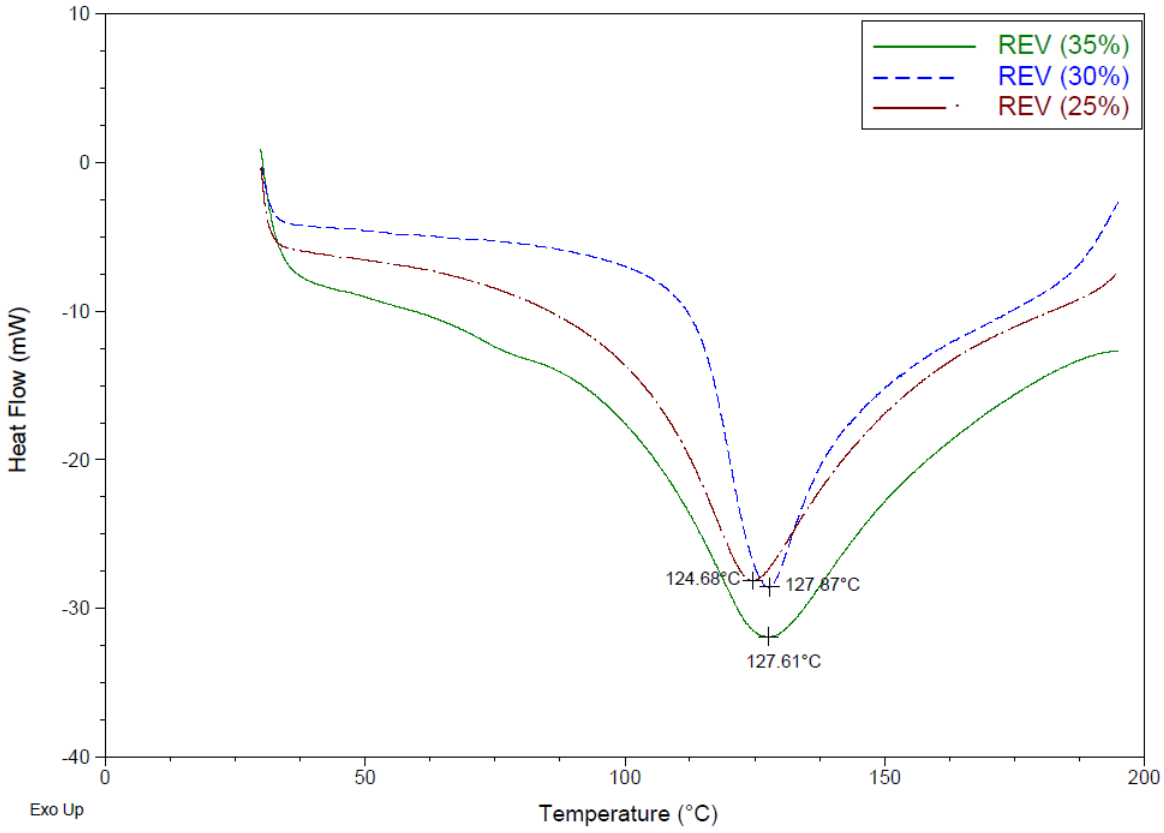


**Figure 11. Thermograms of REV samples with different starting solids content.**

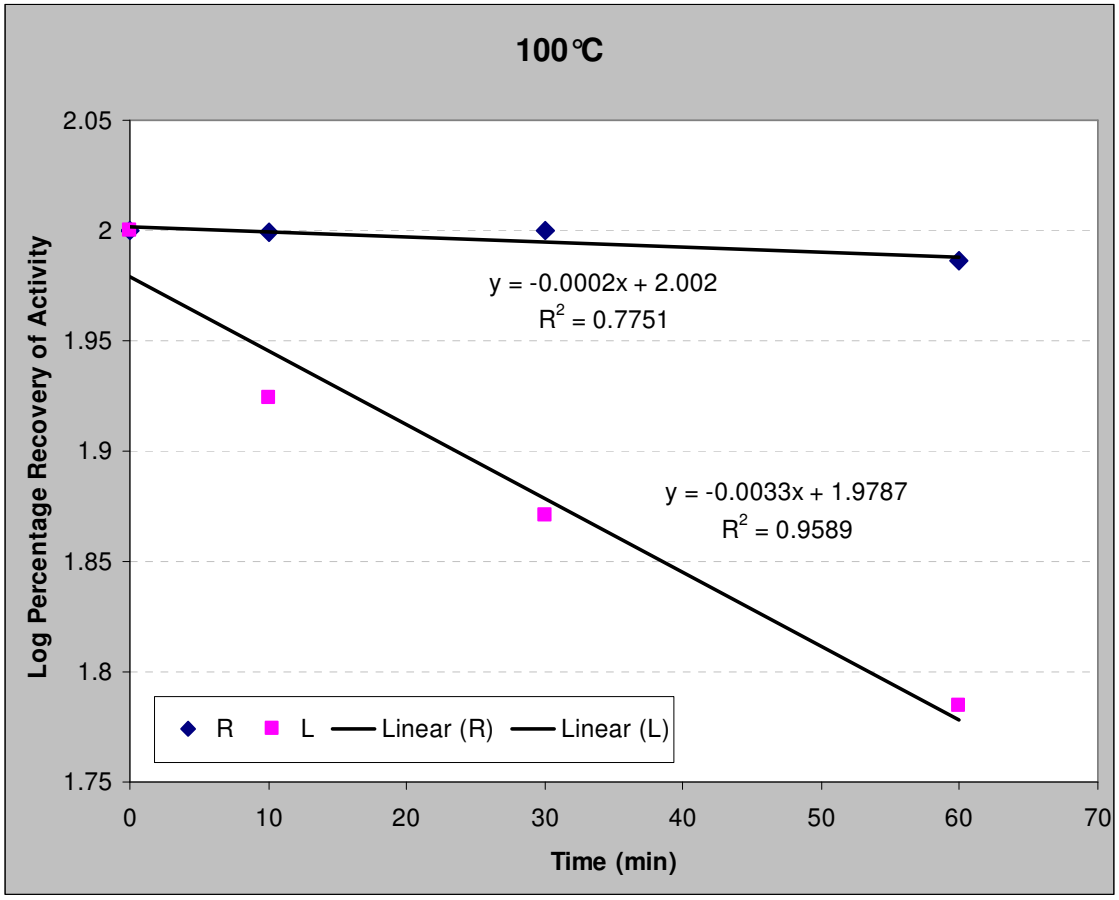


**Figure 12. Thermal destruction curve of lysozyme (L) and REV sample (R) at 100°C.**

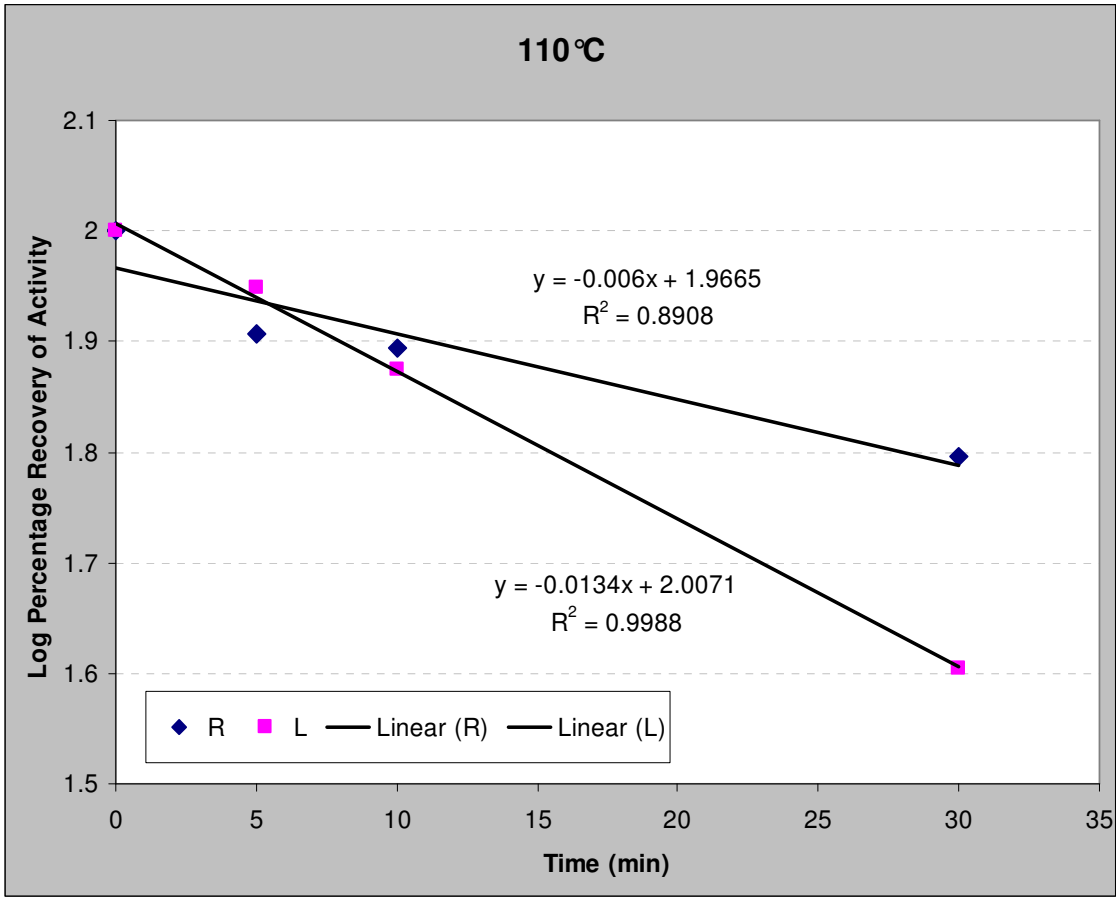


**Figure 13. Thermal destruction curve of lysozyme (L) and REV sample (R) at 110°C.**

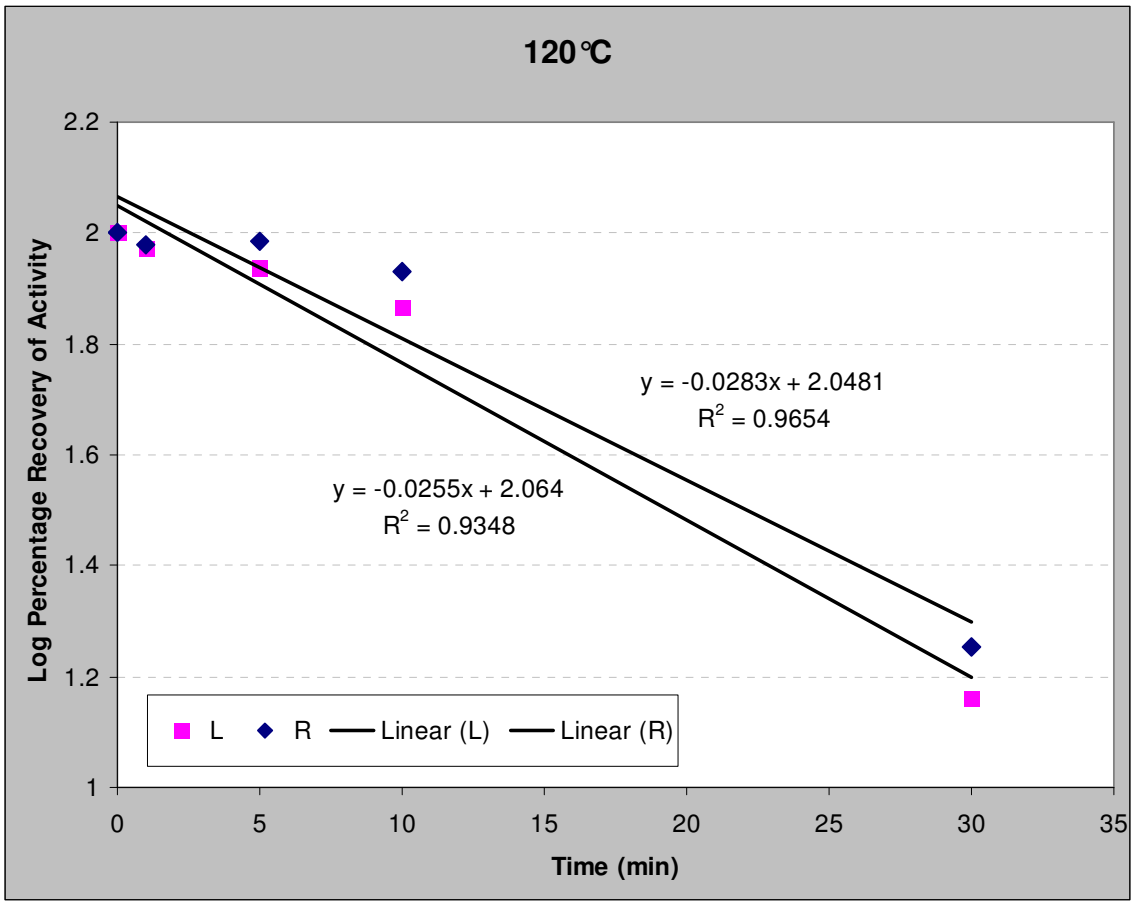


**Figure 14. Thermal destruction curve of lysozyme (L) and REV sample (R) at 120°C.**

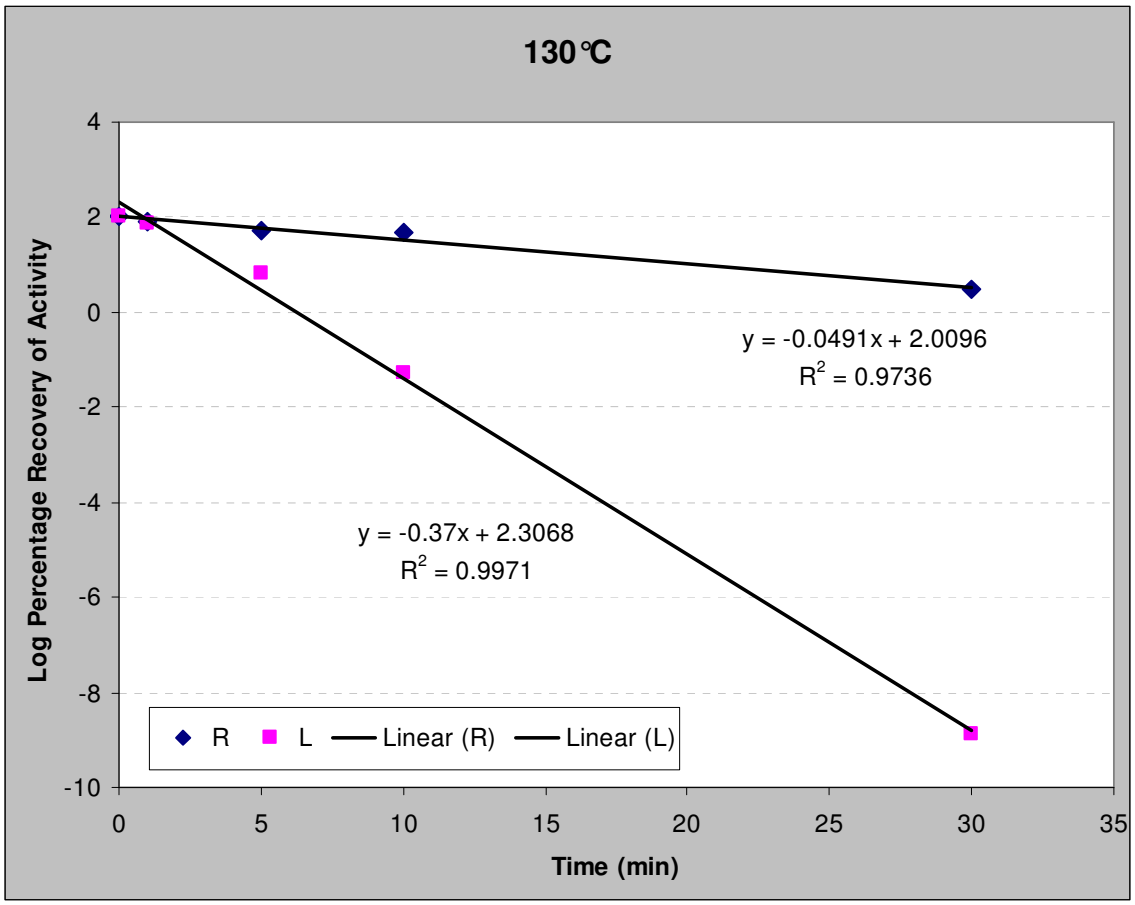


**Figure 15. Thermal destruction curve of lysozyme (L) and REV sample (R) at 130°C.**

| Temperature (°C) | Lysozyme D-value (min) | REV product D-value (min) |
|---|---|---|
| 100 ± 1 | 303.0[a] | 5000[b] |
| 110 ± 1 | 74.63[a] | 166.7[b] |
| 120 ± 1 | 35.34[a] | 39.22[a] |
| 130 ± 1 | 2.70[a] | 20.37[b] |

**Table 2. D-values of lysozyme and REV-treated product at different heating temperatures.**

Means within columns without common lowercase superscripts are significantly different ($P<0.05$).

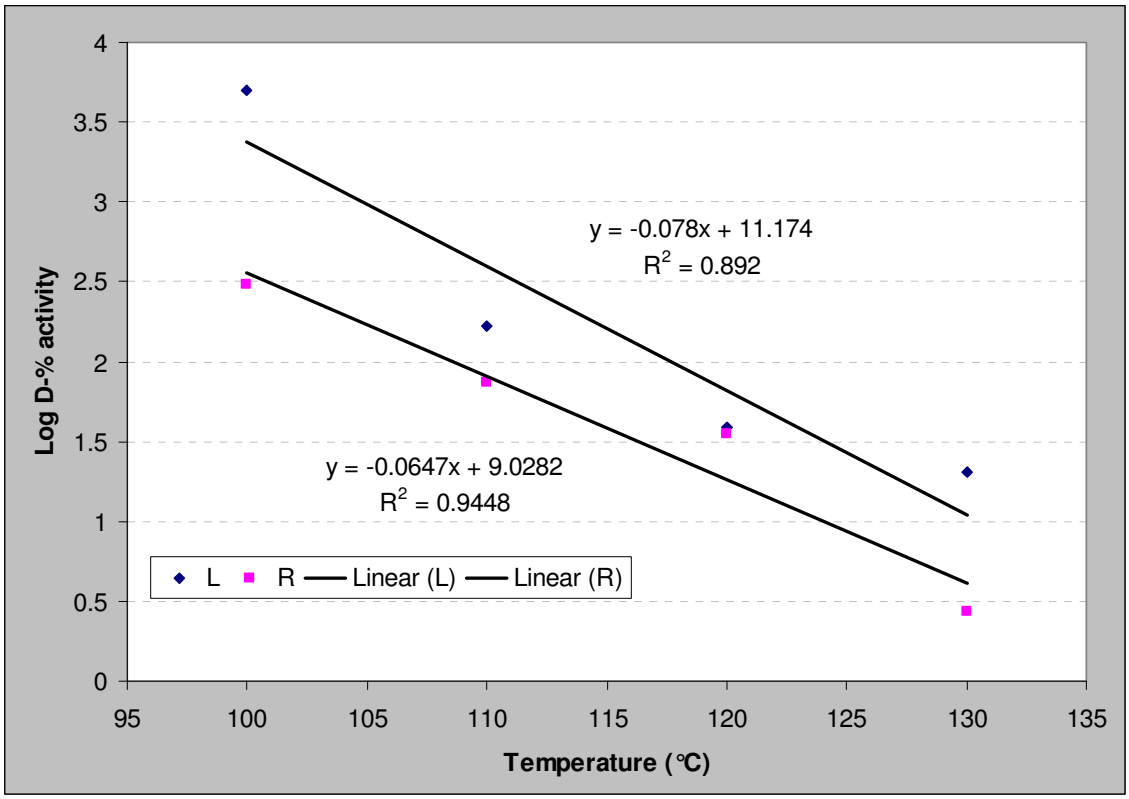


**Figure 16. Z-values of lysozyme (L) and REV sample (R).**

| Treatment | Lysozyme recovered (μg/g) |
|---|---|
| Mash feed supplemented with Entegard™ | 19.64±0.58[a] |
| Resulting pelleted feed | 15.44±1.28[b] |
| Mash feed supplemented with Entegard™REV | 19.45±0.95[a] |
| Resulting pelleted feed | 17.33±1.20[a] |

**Table 3. Lysozyme recovery from feed samples before and after the pelleting process.**

Means within columns without common lowercase superscripts are significantly different ($P<0.05$).

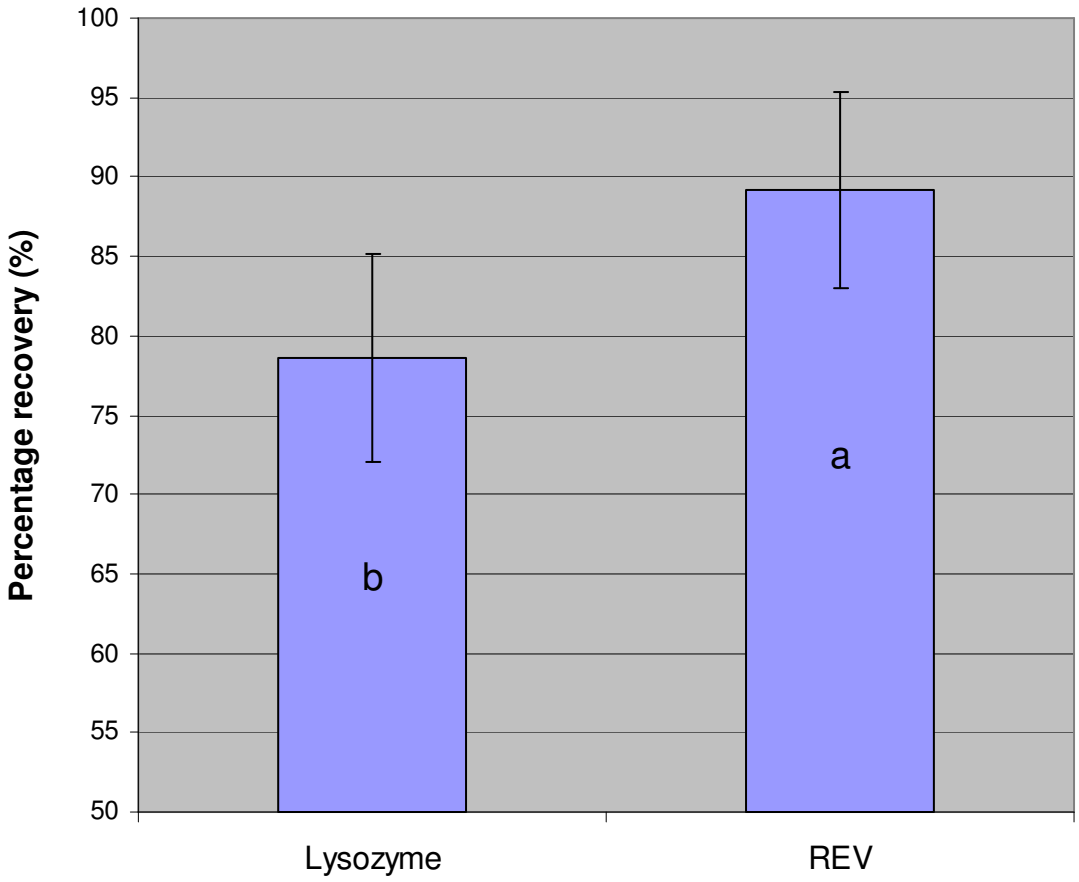


**Figure 17. Percentage of lysozyme recovery from pelleting of either lysozyme or REV-treated lysozyme/chitosan product.**

Bar graph shows the mean value and standard error measured from three independent measurements. Results labeled with a different letter are statistically different from each other ($P < 0.05$).

The lysozyme assays in both mash and pelleted feed supplemented with pure lysozyme and REV-treated product by ELISA showed on average that 78.60% lysozyme remained in the feed directly amended with lysozyme, and 89.12% lysozyme was recovered from the REV-treated samples. A significant difference was detected between the lysozyme concentrations in the "unprotected" feed samples before and after the pelleting process ($P<0.05$). This type of difference was not found with the feed samples to which REV-treated material was added (Table 3).

### *3.4 Discussion*

If there was no strong interaction between lysozyme and chitosan molecules in the REV-treated sample, the thermogram should have shown two separate endotherm peaks for lysozyme and chitosan. The DSC analysis revealed that the REV samples had only one endotherm peak that indicated a significantly higher denaturation temperature than native lysozyme. Kristiansen et al. (1998) studied the binding interactions between highly deacetylated chitosans and lysozyme using proton nuclear magnetic resonance spectroscopy. Electrostatic forces contributed to the strong binding between the two molecules and the complex was found to be strikingly stable. Therefore, the non-covalent interactions between lysozyme and chitosan molecules may have stabilized the secondary and tertiary structures of lysozyme and made the molecule more resistant to heat denaturation. Since there was not much difference among the samples produced with pastes having different solids levels, the 25% solids REV sample was used for further analysis because it easily formed a soft paste. The D-values obtained at different

temperatures also demonstrated better thermal stability of lysozyme when inside the REV product. These results were consistent with the observations from DSC analysis. The pelleting study confirmed that REV treatment yielded a product with better retention of the enzyme, which would be of practical value. The chitosan molecules surrounding the lysozyme might have protected lysozyme from severe heat denaturation during pelleting. Further, chitosan might also physically restrict lysozyme from binding with feed proteins, which might otherwise render lysozyme unavailable for antimicrobial action. The extent of difference between the recovery of lysozyme from unprotected enzyme and the REV-treated lysozyme/chitosan product in feed may been increased following more thermally severe pelleting conditions where an expander (steam conditioning and kneading prior to pelleting) is included. With such a process, the feed temperature could easily exceed 100°C.

# 4. Structural Analysis

## *4.1 Introduction*

The REV dehydration not only retained the full enzymatic activity of lysozyme, the resulting porous lysozyme/chitosan matrix was also found to have superior thermal stability for lysozyme activity over commercial spray-dried lysozyme powder. To fully understand the mechanism of the protective action of this REV matrix, a number of approaches were taken to look at the microstructure of the REV-treated material.

First compared were the total porosity and pore size distribution of native lysozyme powder and the REV-treated samples. In an attempt to understand the structural distribution of lysozyme and chitosan in the REV sample, the REV, pure lysozyme and pure chitosan samples were examined with Raman microspectoscopy (Renishaw Confocal Raman Microscope, System 1000, Gloucester, UK). Near-infrared (NIR) Raman spectra were acquired with the microscope equipped with a 785 nm diode laser source and a charge-coupled device (CCD) detector. A 50X objective (Leica Microsystems, Richmond Hill, ON, Canada) was employed to focus the laser to a 5-μm spot on the sample, which was irradiated with 40–50 milliwatts of laser power. However, due to the strong fluorescence generated from chitosan under the laser irradiation, the chitosan spectra totally covered the lysozyme spectra, making the differential localization of the two compounds in the REV sample impossible. In a second attempt, the samples were frozen under liquid nitrogen, sectioned, coated with gold and observed under a scanning electron microscope.

## *4.2 Materials and methods*

### 4.2.1 Porosity analysis

Total porosity and pore size distribution in lysozyme-chitosan REV products were evaluated using Hg injection capillary pressure measurements. These were performed in triplicate using an Hg porosimeter (Micromeritics AutoPore III, Folio Instruments Inc., ON, Canada). The penetrometer used was designed for powder, being equipped with a 3 mL bulb and having a 1.190 mL stem volume (Model# 0806 160580). Analysis was performed in two stages: low pressure analysis (0.69-206.82 KPa) corresponded to pore radius of 172.6-3.0 μm and high pressure analysis (206.82-227502.00 KPa) corresponded to pore radius of 3.0-0.0027 μm. The samples were placed in a dessicator along with phosphorous pentoxide for 7 days before analysis to remove any residual moisture.

Before the analysis, a thin layer of silicon-free vacuum grease (Apiezon, M&I Materials, Manchester, UK) was applied to the glass rim of the bulb. The penetrometer (including the lid) was weighed. About 0.4 g of sample was weighed and filled in the bulb of the penetrometer unit and the unit was then carefully closed. Samples with different starting solids contents, each from three separate REV dehydration runs were analyzed. For the low pressure analysis, the base screw covering the low pressure chamber was loosened and the stainless steel rod was removed from the chamber. The stem of the penetrometer was slowly loaded into the cavity until the plastic spacer contacted the rim of the cavity. The chamber cover was replaced and closed before the run started using the computer

interface provided with the AutoPore equipment. When the system was idle (after the low pressure analysis) and the pressure on the computer read 14.91 MPa, the Hg filled penetrometer was carefully removed from the chamber. With the stem pointing upward, the unit was weighed again and the weight was recorded. The steel rod was replaced in the cavity of the low pressure chamber and the port was closed with the cover. For the high pressure analysis, the high pressure chamber lid was unscrewed and gently lifted up all the way to expose the grip which was facing down. With the stem pointing up, the penetrometer was slowly inserted upward into the grip for certain depth and then the chamber lid was slowly lowered so that the end of the penetrometer bulb was just immersed in the mineral oil that was stored in the high pressure chamber. The stem of the penetrometer was slowly slid down by hand until the end of the bulb fit in the pin at the bottom of the oil chamber, with the upper portion of the stem still lodged inside the grip. When the penetrometer was confirmed to be properly aligned with both the pin at the bottom and the grip at the top, the chamber lid was gently lowered by pressing down the two handles on the lid. The air bubbles trapped in the oil were released by opening and closing the lid several times. Before the start of high pressure analysis, the system was calibrated by the computer with the lid open. After the calibration, the lid was tightly closed by rotating the handles and the “OK” button was pressed to commence the analysis.

### 4.2.2 Scanning electron microscopy analysis

The SEM sample preparation was performed according to the method of Kalab (1983) with some modifications. The samples were frozen in liquid nitrogen and fractured on a metal plate using a chilled scalpel. The pieces were mounted on aluminum SEM stubs using conductive tape, dried under vacuum and coated with a thin layer (5nm) of Au/Pd. Samples were viewed and imaged on a Hitachi S4700 field emission SEM (Hitachi, Tokyo, Japan) at the UBC BioImaging Facility.

### 4.2.3 Statistical analysis

All numerical data collected were subjected to one-way ANOVA using SigmaStat software (Version 2.0). For comparisons among many groups, statistical differences among the means were detected by Tukey's test. Differences were deemed significant at the $P < 0.05$ probability level.

## *4.3 Results*

### 4.3.1 Porosity analysis

The total porosity (Table 4) of the 30% solids samples was significantly higher than that of 35% solids samples, while the porosity of native lysozyme was significantly higher than that of 25% and 35% solids samples ($P < 0.05$).

The pore size distribution of the REV-treated samples and lysozyme are shown in Figures 18-21. In the REV samples, more than 90% of pore volume consisted of pore sizes ≥ 100 μm in radius. There was little Hg intrusion at pore size values ≤ 100 μm. All REV samples analysed (25 to 35% solids) showed similar pore size distribution. Although there was a significant difference in total porosity between the 30% solids and the other samples, this may not have any practical significance in explaining thermal stability of the product. In contrast, more than 90% of the pores in the pure lysozyme sample had a diameter ≤ 100 μm (Table 4).

| Sample | Total porosity (%)* | Pore size distribution (radius) | | |
|---|---|---|---|---|
| | | <10 μm | 10-100 μm | >100 μm |
| REV with 25% solids | 48.09±2.85[ab] | 0.35% | 4.50% | 95.15% |
| REV with 30% solids | 52.54±1.15[bc] | 0.21% | 2.53% | 97.26% |
| REV with 35% solids | 45.03±0.65[a] | 0.91% | 7.16% | 91.93% |
| Lysozyme | 54.33±3.68[c] | 18.49% | 75.59% | 5.92% |

**Table 4. Total porosity of lysozyme and REV-treated product obtained from the porosimeter analysis.**

*Means within columns without common lowercase superscripts are significantly different ($P<0.05$).

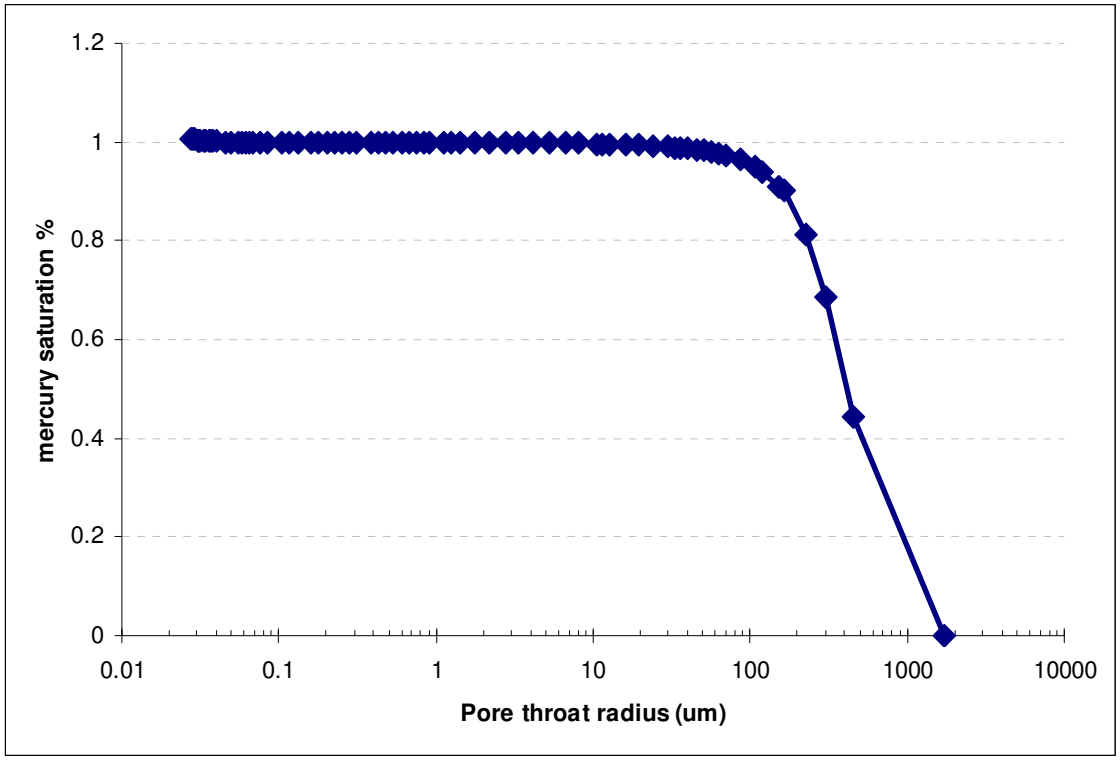


**Figure 18. Pore size distribution of REV sample dried from a paste with 25% initial solids.**

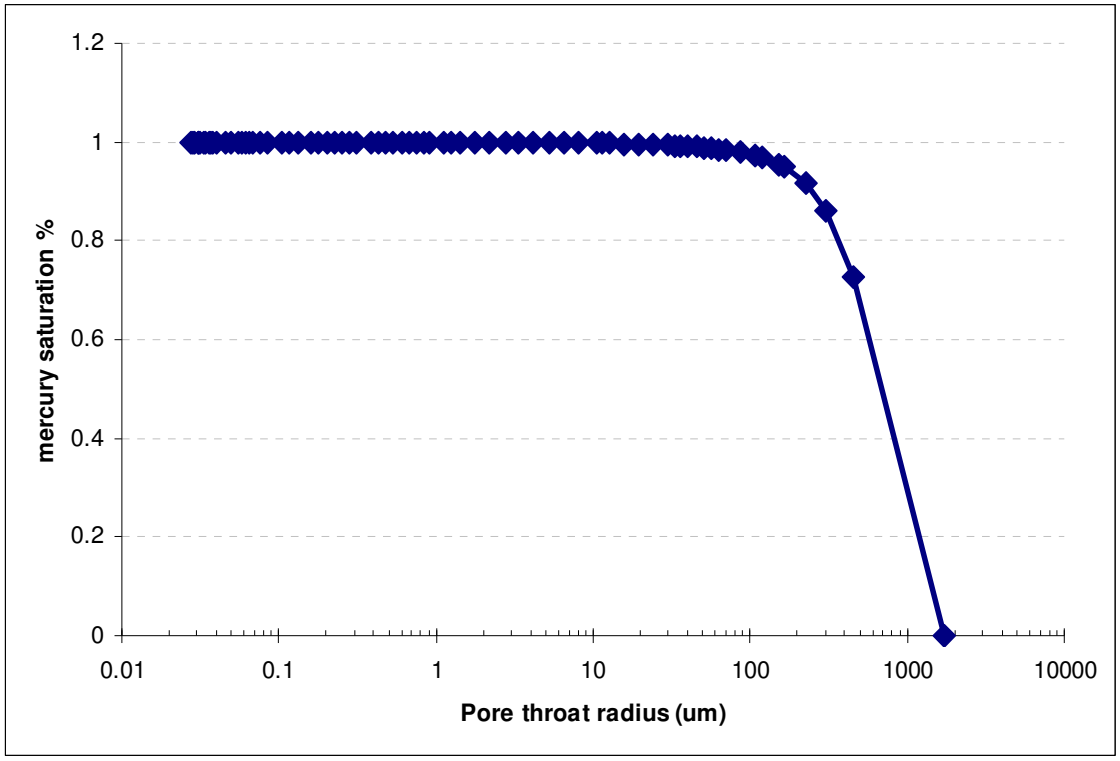


**Figure 19. Pore size distribution of REV sample dried from a paste with 30% initial solids.**

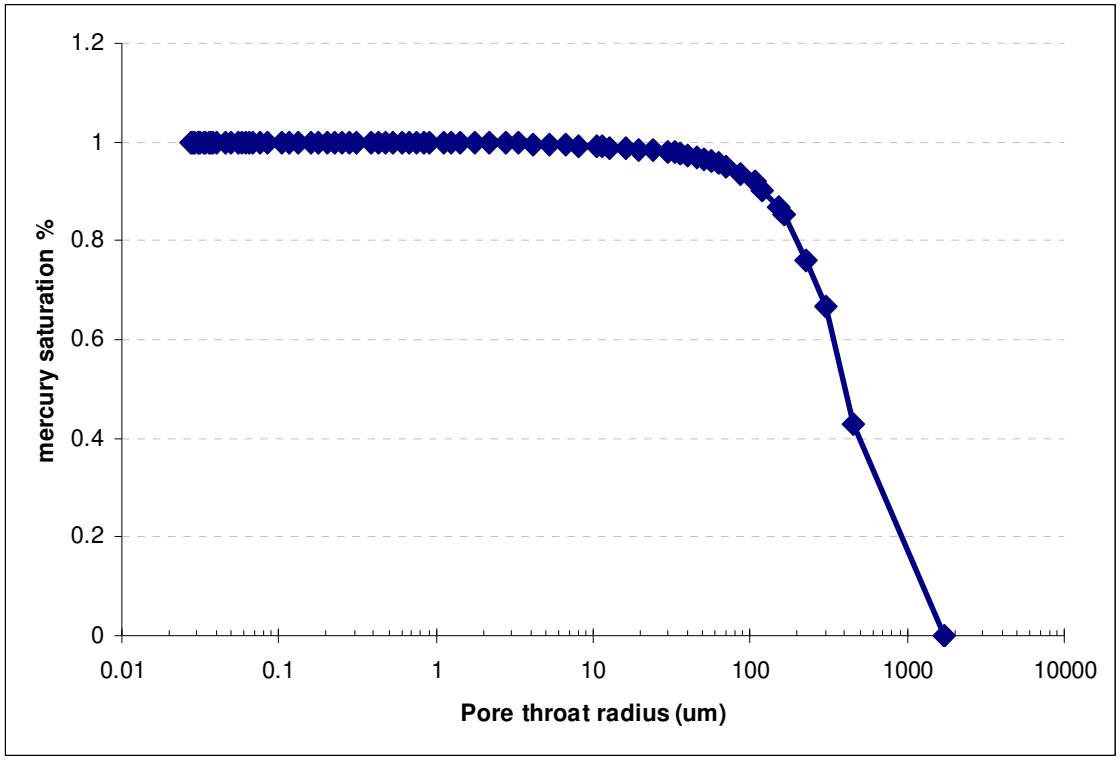


**Figure 20. Pore size distribution of REV sample dried from a paste with 35% initial solids.**

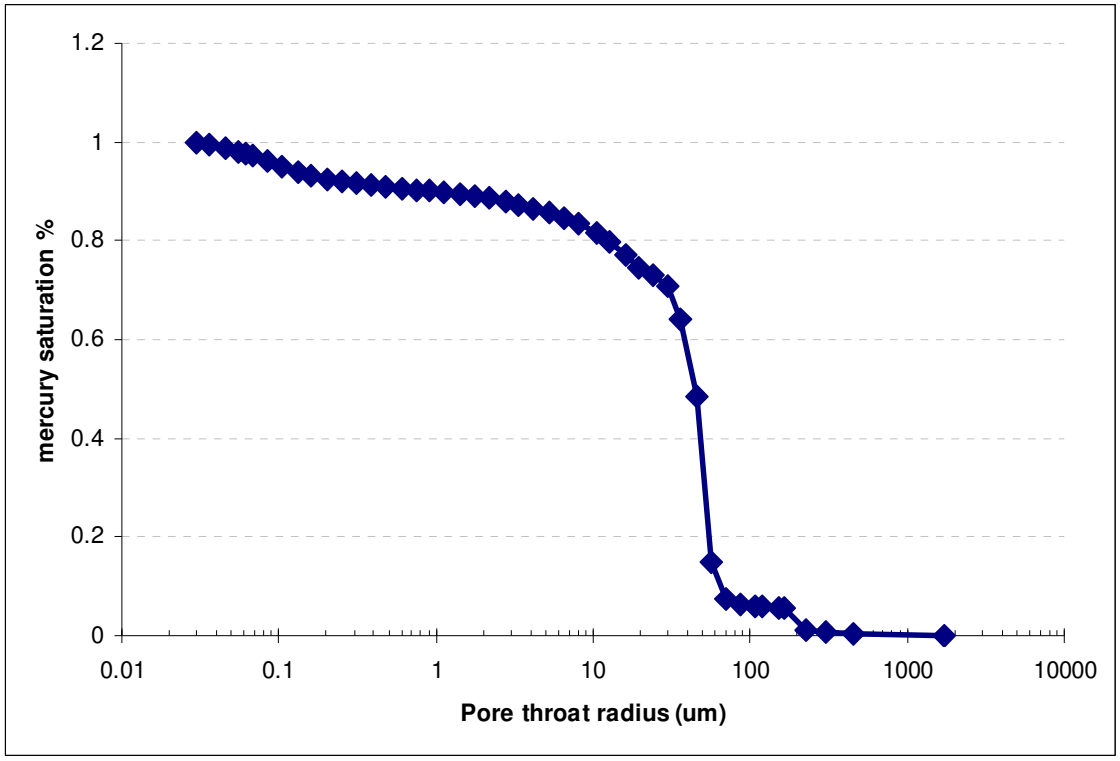


**Figure 21. Pore size distribution of a spray-dried commercial lysozyme powder sample.**

### 4.3.2 Scanning electron microscopy analysis

Scanning electron micrographs of lysozyme, chitosan and REV samples are shown in Figures 22-25, respectively. Unlike fully hydrated samples, the very low water content of the chitosan/lysozyme mix reduced the probability of displacement of the solid phase during freezing due to the lack of ice crystal formation. The use of liquid nitrogen increased the brittleness of the mixture. When compared at the same magnification, the general impression was that the sections of the REV-treated sample resembled the surface appearance of pure chitosan, which was coarse and fibrous-like (Figures 24 and 25). The lysozyme sample, no matter whether spray-dried or dried with REV, possessed a very smooth surface (Figures 22 and 23). The image in Figure 25 was interpreted to mean that lysozyme had been well mixed with chitosan on a molecular basis and was evenly distributed throughout the chitosan matrix.

## *4.4 Discussion*

The substantially larger pore sizes in the REV product may explain why lysozyme was more heat-resistant in this form. The REV sample contained larger pores that trapped larger volume of air than the untreated lysozyme powder and thus became less heat-conductive. A matrix with larger pores has a relatively smaller surface area than a structure with large number of smaller pores. A smaller surface area implies less direct heat exposure and less heat damage is likely to occur following sudden exposure to high temperatures. Alternatively, non-covalent interactions between lysozyme and chitosan

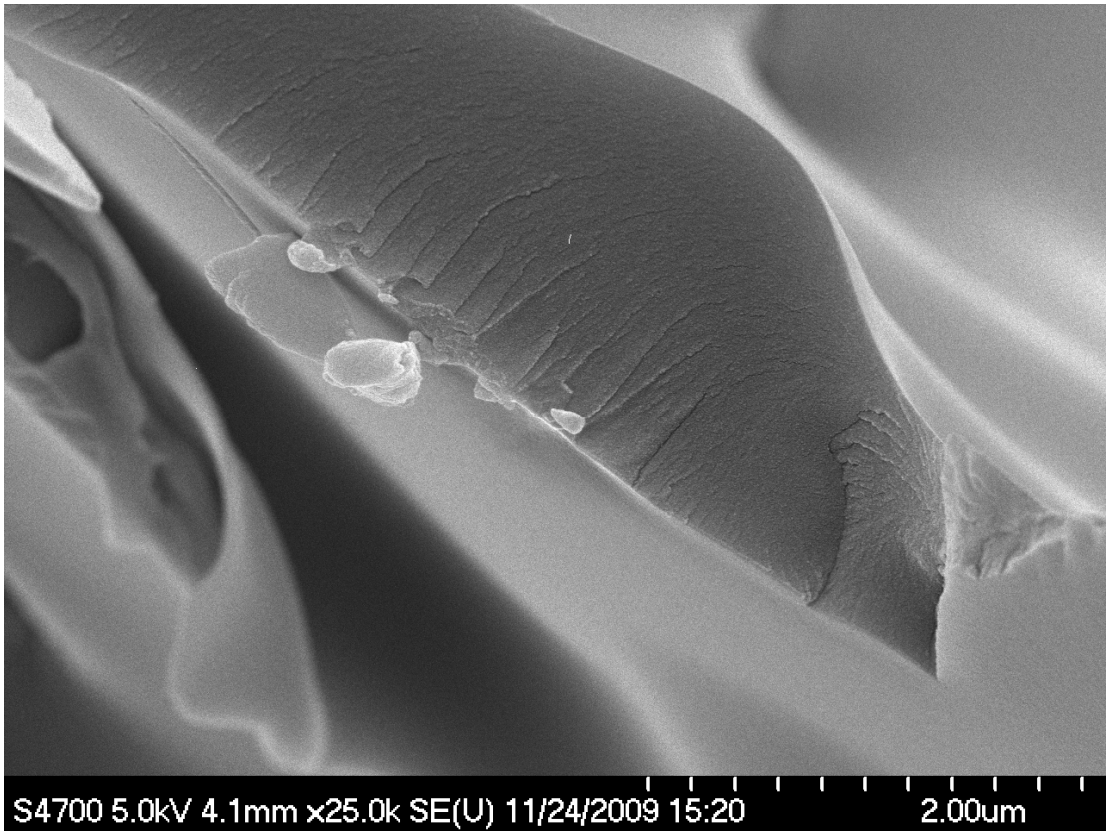


**Figure 22. Scanning electron micrograph of lysozyme sample (dried with REV).**

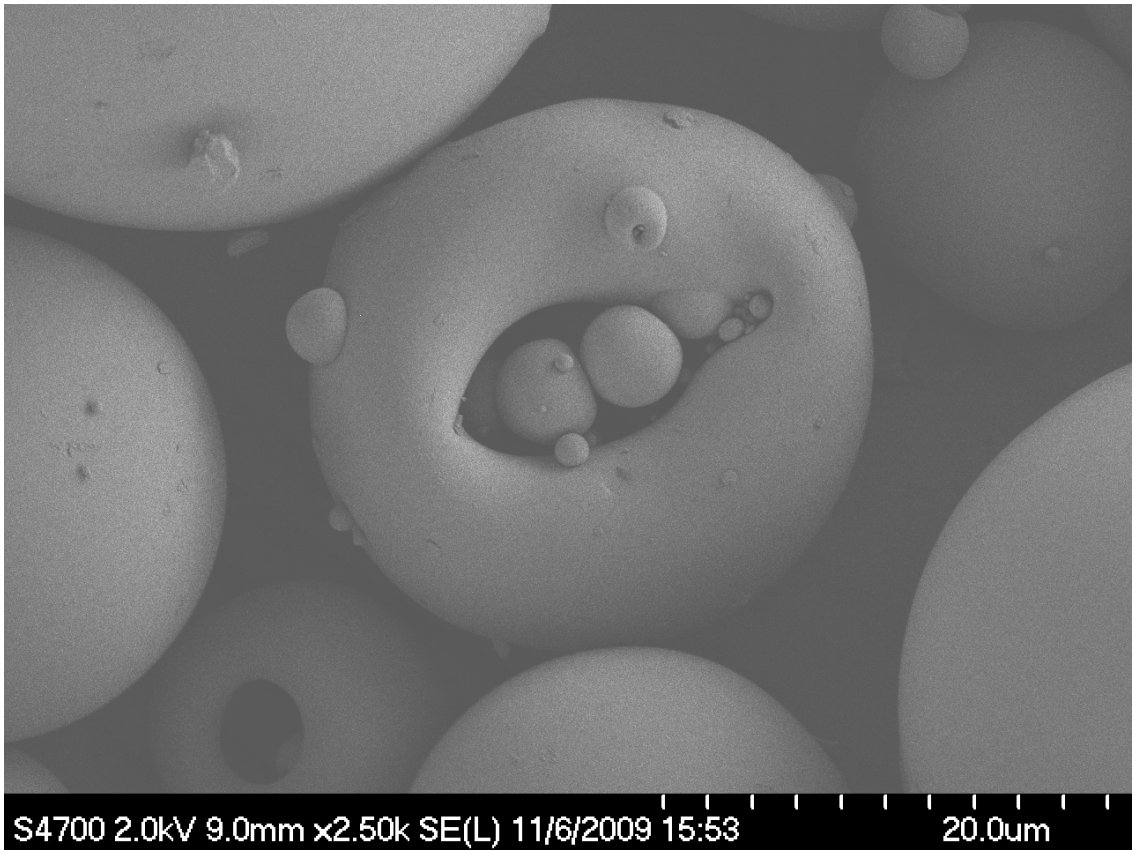


**Figure 23. Scanning electron micrograph of lysozyme sample (spray-dried).**

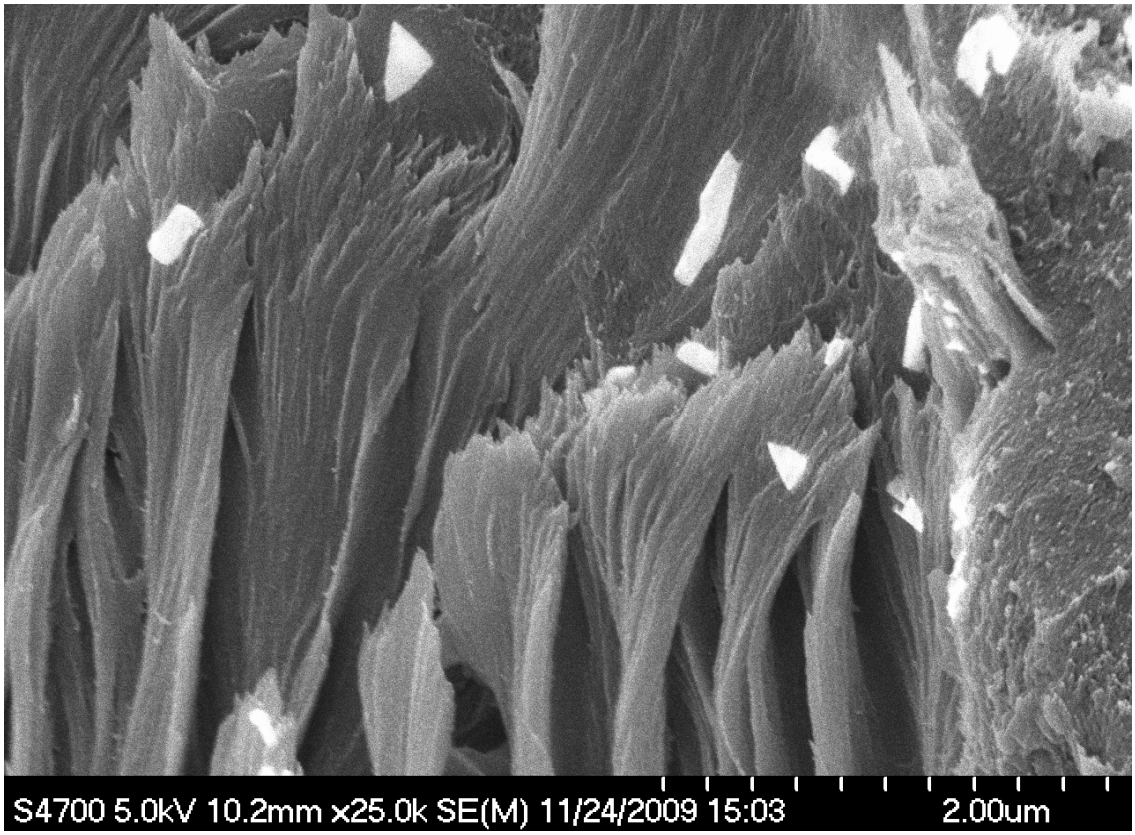


**Figure 24. Scanning electron micrograph of chitosan sample (dried with REV).**

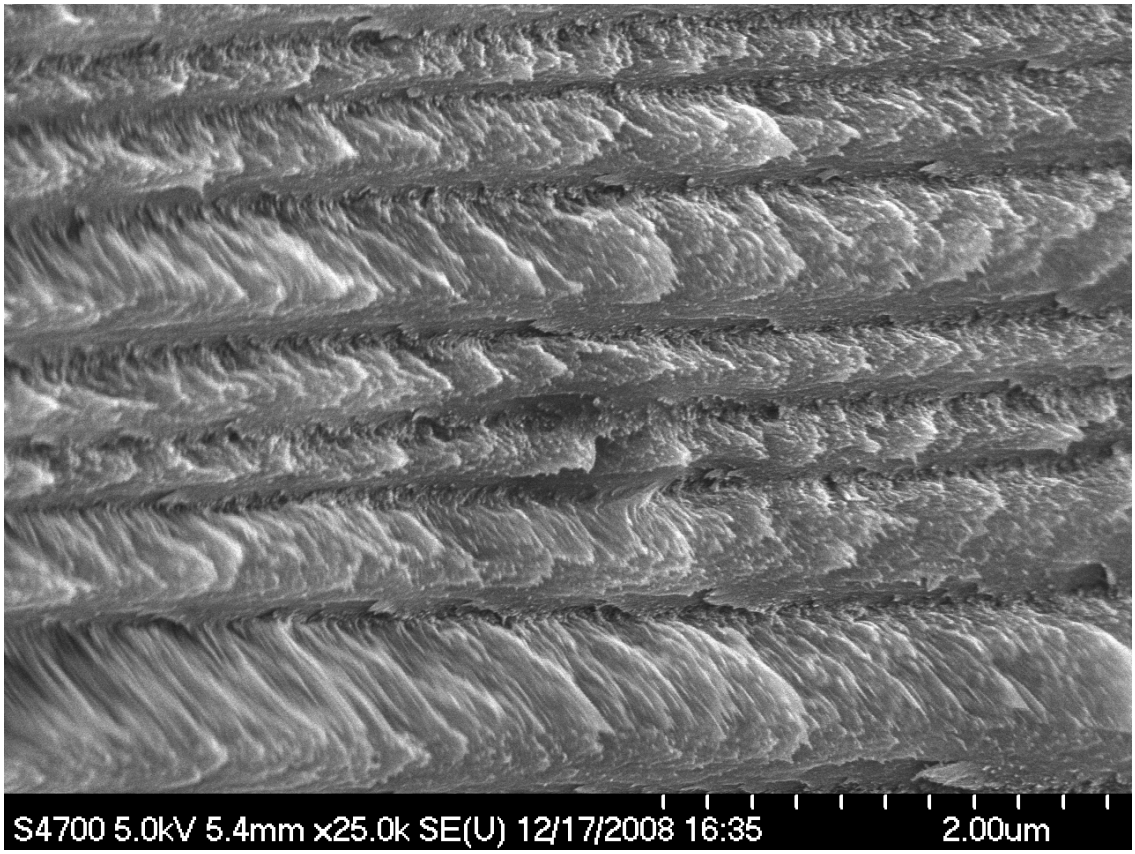


**Figure 25. Scanning electron micrograph of REV-treated lysozyme/chitosan mixture.**

molecules (Kristiansen et al, 1998) may have stabilized the secondary and tertiary structures of lysozyme. The fiber-like appearance of surfaces from sections of REV-treated mixture and chitosan suggested that large aggregations of lysozyme molecules in the REV matrix did not occur, following the observations that pure lysozyme had a uniquely smooth surface, no matter whether spray- or REV-dried. Therefore, lysozyme appeared to be evenly distributed within the chitosan carrier.

# 5. Lysozyme Release Rate

## *5.1 Introduction*

Lysozyme can be readily hydrolyzed by pepsin. Therefore, as a feed additive, it needs to be protected during its passage through the gastric section of the bird's digestive tract. Once lysozyme reaches the duodenum, pepsin is no longer active (at higher pH). Therefore, if the majority of lysozyme ingested can be released after 1 h, it can be effectively protected from gastric digestion (Duke, 1999).

Park et al. (2004) had demonstrated the slow release of lysozyme in water when lysozyme was incorporated into a dry chitosan film. If lysozyme was indeed evenly distributed (encapsulated) in the chitosan matrix in the REV product, it should also display a similar release pattern when suspended in water. This study looked at the release rate of lysozyme from the REV material in water.

## *5.2 Materials and methods*

The samples used in these experiments were: lysozyme/chitosan REV product, 1:3 lysozyme/chitosan powder mixture, and lysozyme powder by itself. Lysozyme release was measured using a spectrophotometric turbidity assay described by Park et al. (2004) with some modifications. One gram of each sample was weighed out in a 100 mL glass beaker and then suspended in 50 mL of sterile de-ionized water. The solutions were then stirred slowly (50 rpm) with magnetic stir bars using a Lab-line Mistral multi-

stirrer (Barnstead International, Dubuque, IA, USA) at room temperature. Aliquots of 6 mL were removed from each sample and sterilized by passing through a 0.45μm nitrocellulose membrane filter after periods of 5, 15, 30, 60 min, 4 and 24 h. Lysozyme activity of the aliquots was then analyzed using the Shugar method. Triplicate measurements were performed.

### *5.3 Results*

The amount of lysozyme released from the samples upon hydration is illustrated in Figure 26. During sampling, sterile filtration of the aliquots removed undissolved particles from the solution, resulting in the detection of only solublized lysozyme. Lysozyme release was substantially delayed in the REV samples compared to the others. The REV sample took almost 4 h to dissolve and release 95% of its lysozyme while this was achieved in the untreated lysozyme sample in < 30 min. By the time the lysozyme sample had reached its maximum activity (at 60 min), the lysozyme/chitosan powder blend sample reached 91% of maximum activity, whereas the REV sample had only reached 58.4%. In comparison, the lysozyme sample reached its peak activity at 60 min and the 1:3 lysozyme/chitosan powder blend reached its peak activity at 4 h.

### *5.4 Discussion*

The slower dissolution of lysozyme from the REV sample in water also served as indirect evidence of embedding or encapsulation of lysozyme in the REV matrix. The

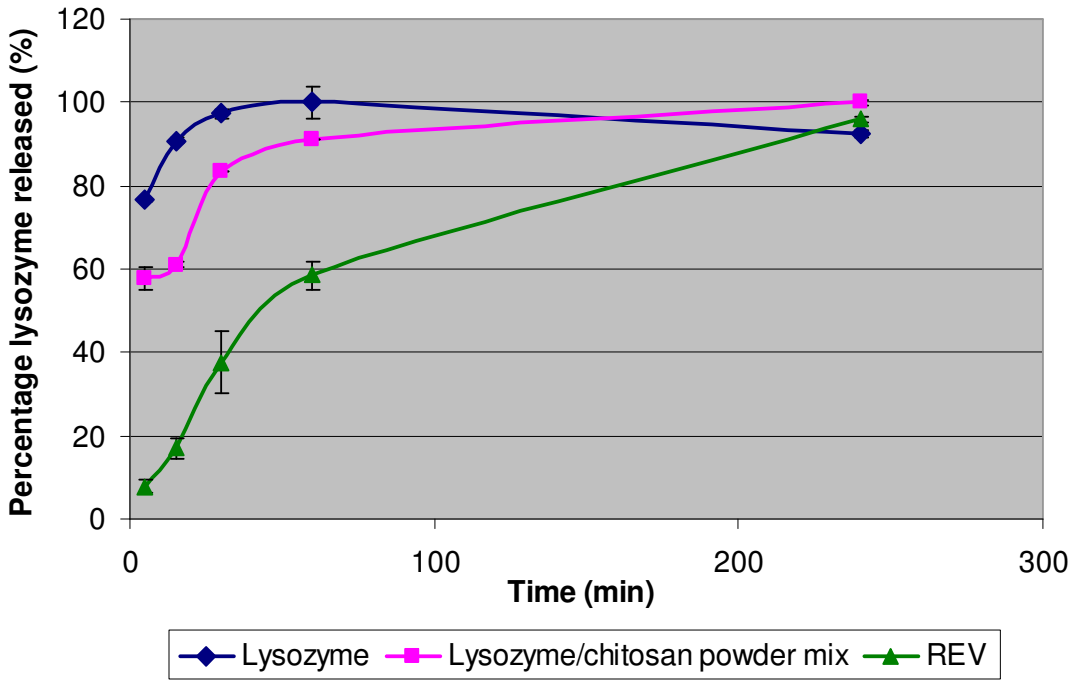


**Figure 26. The amount of released lysozyme from the samples as a function of time.**

REV = 25% solids REV-treated lysozyme/chitosan product

difference in time observed before the maximum activity plateau of enzyme release was reached can be attributed to the difference in solubility between lysozyme and chitosan, where chitosan is less soluble in water than lysozyme, from our previous observations. The different lysozyme release rate shown by lysozyme alone and the lysozyme/chitosan powder blend may have been due to the solution viscosity increase caused by the dissolving chitosan. In other work, for the lysozyme-chitosan plastic films produced by Park et al. (2004) it took more than two days to reach an activity plateau, or complete lysozyme release with a similar experimental setup. This may have been due to the absence of a porous structure in the films and the addition of the plasticizer glycerol which increased the polymer crosslinking and decreased the solubility of chitosan. Furthermore, the chitosan used for film manufacture might have had lower solubility.

The relatively slow release of lysozyme from the REV sample indicated its potential to be used in the poultry feed for targeted intestinal release in birds fed with this material. In chickens and turkeys, the digestive passage of feed can take between 2-5 h (Duke, 1999). Given the greater complexity and consistency of feed/digesta, it is very likely that the majority of the lysozyme in the REV product would be released after it passed the gastric portion of the digestive tract, and be able to target the intestine where NE develops.

# 6. Effect of REV-Treated Product on the Control of Clostridial Necrotic Enteritis in Broiler Chickens

## *6.1 Introduction*

A lysozyme/chitosan mixture was produced by mixing lysozyme, water-soluble chitosan and water into a paste and dried by the REV process into a porous material (Chapter 2). The REV process did not negatively affect the enzymatic activity of lysozyme. On the contrary, the thermal stability of lysozyme was significantly improved with the protection of chitosan in this unique structure generated by REV (Chapter 3). The REV-treated mixture also showed a slow-release feature for lysozyme in water (Chapter 5). Therefore, the REV-treated lysozyme/chitosan mixture holds great potential for greater efficacy to control NE in broiler birds, by improved lysozyme stability during the feed pelleting process and protection from peptic digestion in the chicken's digestive tract. To test the final hypothesis set for this thesis work, a cage study was conducted to demonstrate the effect of Entegard$^{TM}$REV, the REV-treated lysozyme/chitosan-based antimicrobial blend, on the performance of broiler chickens and necrotic enteritis (NE) disease reduction of birds that were challenged with *Eimeria maxima* and *Clostridium perfringens* at Southern Poultry Research (SPR), Inc., Athens, Georgia.

## *6.2 Materials and methods*

### 6.2.1 Experimental rations

An unmedicated commercial type chicken starter ration compounded with feedstuffs commonly used in the United States was formulated. This ration (in pellet form) was fed *ad libitum* from the date of chick arrival until Day 28 of the study. The diet formulation details are included in Appendices A-D. Experimental treatment feeds were prepared from this basal starter feed. Quantities of all basal feed and test articles used to prepare treatment batches were documented. Treatment feeds were mixed at SPR to assure a uniform distribution of respective test articles. Regular Entegard$^{TM}$ (spray-dried commercial lysozyme based antimicrobial mixture) and Entegard$^{TM}$REV (REV-treated lysozyme/chitosan antimicrobial mixture) were added at 200 g/ton of feed (containing equal amounts of lysozyme). The antibiotic bacitracin methylene disalicylate (BMD) was added to a separate treatment at a rate of 55 g/ton of feed. All treatment feeds were pelleted using a California Pellet Mill (San Francisco, CA) and then crumbled before use. The pelleting condition was 80°C for 25 sec with a steam pressure of 172.35 KPa. Three pellet samples were collected mid-stream at the outlet of the pellet mill for each treatment group. Feed samples for all treatments were analyzed and the intended medication levels confirmed (Neova Technologies, Inc. Abbotsford, BC; Eurofins Scientific Inc., Memphis, TN). The feed was transferred to the building housing the chickens on trial and distributed among cages of the same treatment.

### 6.2.2 Test animals

One-day-old Cobb X Cobb strain male broiler chicks were purchased from Cobb-Vantress Hatchery (Cleveland, GA). At the hatchery, the birds were sexed and received routine vaccination. Only healthy appearing chicks were used in the study. Number and disposition of all birds not used for allocation were documented.

### 6.2.3 Housing

Upon arrival, chicks were raised in Petersime battery cages. At placement, the birds were fed the treatment feeds. The building housing the birds on trial was an insulated, concrete floored, metal structure that measured 40 feet by 100 feet in a north-south direction. The floor space per animal was 588 $cm^2$/bird. The feeder space was 8 birds/24 X 3.5 inch feeder. A thermostatically controlled gas furnace/air conditioner maintained uniform temperature. Even illumination was provided.

### 6.2.4 Experimental design

Eight day-old male broiler chicks were assigned to each of 6 replicate groups per treatment in a randomized complete block design. Five experimental treatments were included in this study: 1) non-*Cl. perfringens*-infected, non-medicated; 2) *Cl. perfringens*-infected, non-medicated; 3) *Cl. perfringens*-infected, Entegard™-medicated; 4) *Cl. perfringens*-infected, Entegard™REV-medicated; 5) *Cl. perfringens*-infected, BMD-medicated (Table 5).

| Treatment | Coccidial Challenge | *Clostridium perfringens* Challenge | Cages per treatment |
|---|---|---|---|
| 1. Non-infected, non-medicated | Day 14 | No | 6 |
| 2. Infected, non-medicated | Day 14 | Day 18, 19, and 20 | 6 |
| 3. Infected, Entegard™, 200 g/ton | Day 14 | Day 18, 19, and 20 | 6 |
| 4. Infected, Entegard™REV , 200 g/ton | Day 14 | Day 18, 19, and 20 | 6 |
| 5. Infected, BMD, 55 g/ton | Day 14 | Day 18, 19, and 20 | 6 |

**Table 5. Experimental design and the timing of coccidial and *Cl. perfringens* challenges.**

### 6.2.5 Experimental procedures

On day 14, all birds were orally inoculated with approximately 5000 live oocysts of *Eimeria maxima* per bird. Starting on day 18 and for 3 consecutive days, all birds, except the non-*Cl. perfringens* challenged and non-medicated control group, were given a fresh over-night culture (in Thioglycollate Broth) of *Cl. perfringens* through feed at approximately 1.0 x $10^8$ CFU/bird. The *Cl. perfringens* strain (CP-6) used was a field isolate known to cause NE and originated from a commercial broiler operation in Georgia.

All birds were weighed by cage on days 0, 14, 21 and 28. Feed was weighed in on day 0 and the remaining feed was weighed on days 14, 21 and 28. On day 21, three birds from each cage were randomly selected, weighed, euthanized by cervical dislocation, and the intestines were examined for the presence of necrotic enteritis (NE) lesions (George et al., 1984). The NE lesion scoring system used categories from 0 to 3, with 0 being normal and 3 being the most severe (0 = no gross lesion, 1 = thin-walled or friable, 2 = focal necrosis or ulceration, 3 = large patches of necrosis). Each bird was scored and then a mean group score was calculated. Data collected included live body weight (g), corrected feed conversion ratio, feed consumption (kg), lesion scores and NE mortality rate (%).

The facility was checked twice daily to ensure that all cages had water and that feed was available in every cage. The building temperature's range was maintained at an

appropriate temperature for the age of the birds. Even, continuous illumination was provided by fluorescent lamps hung vertically along the wall. Feed and water were given *ad libitum*. Observations included were the availability of feed and water, temperature control, and any unusual conditions of the facility. The birds were watched closely for any abnormal reactions. No abnormal reactions or conditions occurred during the course of this study. When dead birds were removed from cages, the cage number, date, weight of the bird, and probable cause of death were recorded.

### 6.2.6 Statistical analysis

All of the collected data were subjected to a two way ANOVA for a randomized complete block design where the cages were blocked by location using SigmaStat software (Version 2.0). The NE mortality rate per cage was transformed to the arc sin square root and analyzed as continuous data. Data were back-transformed and geometric means for the mortality proportions are presented. Statistical differences among treatment groups were detected by the least significant differences (LSD) test and the differences were deemed significant at a $P < 0.05$ probability level.

## *6.3 Results*

Performance, lesion scores and the NE mortality rates of the chickens receiving each of the five treatments are presented in Table 6.

| Treatment | Gain (Kg) | FCR | NE lesion score | NE mortality (%) |
|---|---|---|---|---|
| 1. Non-infected, non-medicated | 0.975[a] | 1.658[a] | 0.0[a] | 0.09[a] |
| 2. Infected, non-medicated | 0.685[c] | 2.173[c] | 1.4[c] | 32.06[c] |
| 3. Infected, Entegard™, 200 g/ton | 0.872[b] | 1.852[b] | 0.6[ab] | 15.89[bc] |
| 4. Infected, Entegard™REV , 200 g/ton | 0.892[ab] | 1.859[b] | 0.8[b] | 9.04[b] |
| 5. Infected, BMD, 55 g/ton | 0.898[ab] | 1.806[ab] | 0.4[ab] | 9.04[b] |

**Table 6. Effect of infection and medications on the broiler performance, lesion scores and NE mortality rate.**

Means within columns without common lowercase superscripts are significantly different ($P<0.05$).

FCR = Kg feed consumption /Kg weight gain

The infected and non-medicated (positive control) group gained significantly less weight and had a significantly higher feed conversion ratio (FCR) than the other treatment groups. This group of birds also had significantly more severe NE lesions and NE-related mortalities than the other groups. Both Entegard™ and Entegard™REV-treated groups performed significantly better than the positive control in weight gain and FCR. They also sustained fewer NE lesions and NE-related mortality. There was no significant difference in all of the four categories between the Entegard™REV and BMD treatment groups. For NE mortality, while the Entegard™ group was not statistically different from the positive control group the REV group was significantly better than the positive control and was not different from the BMD treated group.

### *6.4 Discussion*

A number of non-antibiotic products have been shown to have various degrees of efficacy in controlling NE. Recent studies indicate that administration of natural gut microbiotia, also known as competitive exclusion, can effectively reduce the incidence of clostridial necrotic enteritis (Hofacre et al., 1998; Kaldhusdal et al., 2001). The use of digestive enzymes in feed has become increasingly popular due to their ability to alleviate the effect of NE by reducing the viscosity of the gut content and the amount of nutrients available for pathogens (Jackson et al., 2003). Various plant extracts possessing anti-clostridial properties also hold promise as novel feed additives to combat necrotic enteritis (Garcia et al., 2002). Short-chain fatty acids and essential oils microencapsulated in poly-sugar matrix have also shown efficacy in the prevention of

clostridial NE (Timbermont, et al., 2010). The lysozyme-based natural antimicrobial blend used here in two formulations yielded consistent performance in controlling NE, and their efficacy was robust and in some cases comparable to BMD and virginiamycin (Mathis et al., 2005).

When birds were unprotected in the NE disease challenge model followed here, there was a significant reduction in broiler performance with increased lesion scores and mortalities (comparison of treatment groups 1 and 2). As anticipated, inclusion of the commercial antibiotic growth promotant BMD in feed successfully reduced these damaging effects.

The lysozyme-based natural antimicrobial blend Entegard$^{TM}$ was shown in preliminary work to be effective in controlling NE in broilers (Mathis et al., 2005). However, without protection of lysozyme in the formulation, there was considerable lysozyme activity loss due to pelleting and gastric digestion. It was possible that the Entegard$^{TM}$REV performed better than the unprotected product, Entegard$^{TM}$, because REV provided lysozyme better thermal stability, allowed less lysozyme binding with feed proteins due to obstruction by the chitosan and allowed less damage from pepsin digestion in the gizzard of the birds due to the slower release of lysozyme from the mixture. If the pelleting conditions used in this trial had involved higher temperature for a longer time, a more pronounced difference might have been detected between the regular Entegard$^{TM}$ and Entegard$^{TM}$REV treatments.

In summary, this study demonstrated that Entegard$^{TM}$REV, a chitosan-protected lysozyme antimicrobial formulation can be used to control NE in broiler chickens under circumstances where the use of antibiotics is not desired.

# 7. General Conclusion and Future Directions

Antibiotic growth promotants have been completely banned in the European Union and due to their perceived negative effects on human health, and with increasing public concern and awareness this trend may soon be followed in other parts of the world. After the banning of AGP, clostridial necrotic enteritis will become the No.1 disease faced by the poultry industry. Among a number of proposed alternative NE preventative remedies and strategies, a hen egg white lysozyme-based antimicrobial mixture, Entegard$^{TM}$, has demonstrated its efficacy in reducing the negative effects of NE in numerous broiler chicken cage and floor pen trials and its performance is nearly as effective as BMD and virginiamycin, two most commonly used AGP in Canada and the US (Mathis et al., 2005). Indeed, Entegard$^{TM}$ is one of the most promising AGP substitutes on the horizon.

This study investigated the possibility of protection of lysozyme with a novel dehydration technology in order to improve the efficacy of Entegard$^{TM}$ for the control of NE by increasing its thermal stability against pelleting temperatures and offering delayed release to bypass pepsin digestion in the chicken gut. The thesis has addressed the hypotheses and objectives laid out in Chapter 1. These included developing the REV process and producing a REV-dried lysozyme/chitosan matrix without losing the enzymatic and antimicrobial activity of lysozyme (Chapter 2); Characterizing and comparing the thermal stability of REV-treated products and native lysozyme (Chapter 3); Structural analysis of the REV-treated samples (Chapter 4); adjusting the release rate of lysozyme and REV-treated product (Chapter 5); and conducting a cage study to look

at the efficacy of the REV-treated product for the control of experimentally induced NE in broiler birds (Chapter 6). Each of these objectives is addressed below.

Although lysozyme was not the first enzyme to be dried with REV technology, this is the first time that lysozyme was dried with this novel dehydration method and furthermore, was protected by encapsulation with REV. Water-soluble chitosan was chosen to serve as a hydrocolloid carrier for lysozyme due to its synergistic action with lysozyme against *Cl. perfringens*. A simple REV dehydration process (300 watts at 4 KPa pressure for 10 min) produced a desirable porous material without negatively affecting the enzymatic and antimicrobial activities of lysozyme. The temperature of the material being dried was kept below 45°C during the REV process.

DSC analysis revealed that when lysozyme was encapsulated in chitosan and dried under REV it had a substantially higher denaturation temperature than the spray-dried commercial lysozyme powder. The subsequent thermal decimal destruction curves obtained from heating experiments confirmed the superior stability of lysozyme in REV powder to native lysozyme. Furthermore, the lysozyme protected by REV withstood pelleting better in feed and thus had increased availability in the finished feed.

Porosity analysis confirmed the substantially larger pore sizes of the REV matrix, which helped to explain the better heat-resistance. Non-covalent interactions between lysozyme and chitosan molecules may have stabilized the secondary and tertiary structures of lysozyme. Comparison of the SEM pictures of the REV-dried pure

lysozyme, pure chitosan and mixed and then dried product suggested that large aggregations of lysozyme molecules in the REV matrix did not occur, and further, it was likely that there was even distribution of lysozyme in the chitosan carrier. The slow release of lysozyme from the REV matrix in water also supported this conclusion. Although attempts to visualize the distribution of lysozyme molecules or micro-particles in the REV matrix by Raman microspectroscopy were not successful, it would be worthwhile to further investigate the distribution of lysozyme within the chitosan matrix with other techniques such as radio-isotopes, gold or antibody labelling of lysozyme or chitosan, provided that such modifications do not affect the REV process.

When suspended in water, the REV sample took almost 4 h to release 95% of its lysozyme activity, which was more than 8 times slower than untreated lysozyme. The relatively slow release of lysozyme from the REV sample indicated its potential to be used in poultry feed for targeted intestinal release in birds fed with this material. Given the greater complexity and consistency of feed/digesta, it is very likely that the majority of the lysozyme in the REV product would be released after it passed the gastric portion of the digestive tract, and be able to target the intestine where NE develops. It would be interesting to look at the fate of lysozyme in different parts of the chicken's digestive tract to fully understand the percentage loss of lysozyme in both REV-treated and untreated products. This could be achieved in a feeding trial with a standard marker such as chromic oxide. By analyzing the change in ratio of lysozyme and the inert marker concentration in digesta samples taken in different parts of the digestive tract, the percentage loss by pepsin digestion can be calculated. Certainly the indigenous

lysozyme content in different parts of the gut needs to be accounted for, too. This study demonstrated the suitability of REV technology as a novel method for encapsulation/entrapment of enzymes with biopolymers to offer protection and controlled release.

Entegard™ formulated with REV-treated lysozyme (Entegard™REV) performed better than the unprotected product, and its ability to reduce the negative effect caused by NE was not statistically different from BMD. Therefore, this study demonstrated that Entegard™REV, a chitosan-protected lysozyme antimicrobial formulation may be able to offer enhanced efficacy for control of NE in broiler chickens, compared to the original formulation, under circumstances where the use of antibiotics is not desired.

It is known that REV technology can offer a rapid and cost effective way to dry biological materials including live bacterial cultures. Recently, a *Lactobacillus salivarius* strain isolated from healthy chicken intestine had a synergistic inhibitory effect with Entegard™ against *Cl. perfringens*, and a floor pen trial using a combination of Entegard™ through feed and *L. salivarius* through drinking water showed significantly increased efficacy in controlling experimentally induced necrotic enteritis (Zhang et al., 2006b). This probiotic culture had moderate tolerance to Entegard™ (up to 200 μg/mL). There is an interest to formulate a powder blend of Entegard™ with *L. salivarius* for use in feed or drinking water. Because recent studies showed that the commonly freeze-dried *L. salivarius* culture died quickly when blended in feed or mixed with high concentrations of Entegard™, there is also a need to protect *L. salivarius*. It

will be interesting to explore the possibility of a REV dehydration process, with the assistance of a suitable protecting carrier, which may offer a rapid, non-destructive and economical method for large scale production of bacterial cultures.

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# Appendices

*Appendix A. Broiler starter feed formulation.*

| Ingredient name | lbs/ton | Percentage |
|---|---|---|
| Corn | 1192.4 | 59.62 |
| Soybean meal | 616.6 | 30.83 |
| Fat, animal | 56.4 | 2.82 |
| Dicalcium phosphate | 21.6 | 1.08 |
| Calcium carbonate | 14.0 | 0.70 |
| Salt | 8.8 | 0.44 |
| Standard micro | 9.0 | 0.45 |
| Choline 70 | 1.2 | 0.06 |
| Total | 2000 | 100 |

**Table 7. Broiler starter feed formulation.**

(Information from Southern Poultry Research, Inc. Athens, GA)

*Appendix B. Standard microingredient formulation.*

| **Ingredient name** | **lbs/ton** | **Percentage** |
|---|---|---|
| DL methionine | 4.58 | 0.2292 |
| Menadione 16 | 0.22 | 0.0110 |
| B12 Concentrate 300 | 0.03 | 0.0017 |
| Magnesium and potassium sulfate | 0.30 | 0.0150 |
| Thiamine 1 | 0.22 | 0.0110 |
| Niacin 98.0 | 0.08 | 0.0039 |
| Pyridoxine 1 | 0.38 | 0.0192 |
| Biotin 1 | 0.11 | 0.0055 |
| Ferrous monosulfate | 0.10 | 0.0050 |
| Vitamin D TRCR (7500) PX | 0.59 | 0.0294 |
| Selenium 0.06% | 0.80 | 0.0400 |
| Riboflavin Supplement 60 | 0.10 | 0.0050 |
| L-lysine | 0.55 | 0.0273 |
| Folic acid 2% | 0.07 | 0.0036 |
| Trace minerial A | 0.40 | 0.0200 |
| Vitamin E Supplement 227000 | 0.05 | 0.0026 |
| Calcium pantothenate 80 | 0.14 | 0.0068 |
| Zinc oxide 72 | 0.11 | 0.0053 |
| Manganese oxide 60 | 0.15 | 0.0073 |
| 650 A concentrate | 0.02 | 0.0010 |
| Total | 9.00 | 0.4497 |

**Table 8. Standard microungredient formulation.**

(Information from Southern Poultry Research, Inc. Athens, GA)

*Appendix C. Minimum added amounts of vitamins and minerals.*

| **Ingredient name** | **Units** | **Amount added** |
|---|---|---|
| Vitamin A | IU/lb | 3000 |
| Vitamin D3 | IU/lb | 1000 |
| Vitamin E | IU/lb | 6.0 |
| Vitamin B12 | µg/lb | 5.0 |
| Riboflavin | ppm | 6.6 |
| Niacin | ppm | 38.5 |
| D pantothenic acid | ppm | 11 |
| Menadione sodium bisulfite complex | ppm | 3.85 |
| Folic acid | ppm | 0.715 |
| Pyridoxine | ppm | 1.920 |
| Thiamin | ppm | 1.1 |
| Biotin | ppm | 0.055 |
| Manganese | ppm | 80.4 |
| Zinc | ppm | 64.2 |
| Iron | ppm | 15.78 |
| Copper | ppm | 2.4 |
| Iodine | ppm | 0.6 |
| Magnesium | ppm | 16.08 |
| Selenium | ppm | 0.24 |

**Table 9. Minimum added amounts of vitamins and minerals.**

(Information from Southern Poultry Research, Inc. Athens, GA)

*Appendix D. Calculated analysis of the starter feed.*

| **Ingredient/item name** | **Amount** |
|---|---|
| Metabolizable energy (Kcal/lb) | 1406 |
| Crude protein (%) | 21.86 |
| Lysine (%) | 1.22 |
| Methionine (%) | 0.58 |
| Methionine + cystine (%) | 0.95 |
| Crude fat (%) | 6.00 |
| Crude fiber (%) | 3.00 |
| Calcium (%) | 0.95 |
| Phosphorous total (%) | 0.69 |
| Sodium (%) | 0.22 |

**Table 10. Calculated analysis of the starter feed.**

(Information from Southern Poultry Research, Inc. Athens, GA)