DEVELOPMENT OF PROTECTIVE FILMS FOR ENHANCING RUMINAL BYPASS OF MICRONUTRIENTS

by

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Abstract

A series of experiments were conducted in which wheat gluten film forming solutions were modified by various means to produce films that would improve resistance to ruminal degradation. There was an interaction between pH and temperature, whereby low pH (pH 3) and high temperature (75°C) of film forming solution resulted in films that had highest resistance to microbial degradation. Strategies that proved unsuccessful in improving resistance to ruminal degradation included use of the cross-linking enzyme, transglutaminase; induction of a Maillard reaction; and increasing the time of heating film forming solutions. Utilizing HCl versus acetic acid as the means to lower the pH of the film forming solution resulted in poor film formation, which can be attributed to glutenin's solubility in acetic acid. When acetic acid was replaced with 25% HCl, however, no compromise in film formation was observed and ruminal degradability was not affected. In order to develop an economical and effective means of encapsulating lysine for rumen bypass, wheat gluten and corn zein were evaluated as encapsulation materials. Coating soybean meal with either wheat gluten or zein resulted in superior protection against ruminal degradation when compared to unprotected soybean meal. Compared to wheat gluten, zein was observed to have greater resistance to ruminal degradation *in vitro*. Lysine was encapsulated by the spray dry method with wheat gluten or zein. Results from an *in vitro* ammonia release experiment were inconclusive with respect to effectiveness of the encapsulation process. Wheat gluten films were approximately 59% degraded in pepsin solution. Our results suggest that 72% of the wheat gluten film will bypass the rumen and 59% of the bypass fraction of the film will be degraded in the abomasum. Wheat gluten and zein are suitable candidates for development of encapsulated nutrients for ruminal protection.

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Dedication

This is dedicated to my beautiful mother, who passed away during my graduate career. She was my best friend and biggest support in my life. The gratitude that I have for her and all that she has done for me cannot be expressed with words. I love and miss you Mom.

CHAPTER 1 - Literature Review

Justification for Encapsulation

As a result of intensified production levels and increased genetic potential of ruminants the supply of available nutrients is inadequate to meet requirements. Amino acids are primarily supplied in the ruminant animal by digestion in the small intestine of microbially synthesized protein and rumen undegradable food protein (UDP). Chalupa (1974) demonstrated that rumen microbial protein production is sufficient to meet amino acid requirements for maintenance only. Even when high quality protein supplements are fed, because of extensive breakdown of protein within the rumen, protein supply may still be insufficient to meet animal requirements. Peptides and amino acids, either present in the diet or arising from proteolysis in the rumen, are utilized for the growth of ruminal microorganisms by incorporation into microbial protein (Wallace, 1996). In addition to this, a large quantity of amino acids resulting from proteolysis in the rumen are deaminated and used as a source of energy, rather than being incorporated into microbial protein (Tamminga, 1979). The degradation of protein in the rumen is a more rapid process than the rate at which energy is generated for microbial growth. Therefore, the value of protein supplements is lost because dietary protein-N is converted to ammonia-N, most of which diffuses from the rumen to be excreted as urea (Broderick, 1991).

Another limitation to nutrient availability is a result of the high grain, low roughage rations fed to high producing animals. These diets have the effect of reducing saliva secretions and subsequent ruminal pH. This is associated with a reduction in the flow of liquid and solid ruminal digesta, thereby reducing the efficiency of microbial protein synthesis in the rumen (Croom et al., 1990). High grain diets compromise the growth of certain species of microorganisms, such as protozoa, which in turn compromises the production of the nutrients those organisms are involved in synthesizing such as choline chloride (Drouillard et al., 1998). Choline is a B-vitamin which exists predominantly in phospholipids contained in the membranes of all cells in the body (as phosphatidyl choline). It functions in cell membrane integrity, lipid digestion and transport, and as a methyl donor and precursor to betaine.

Due to the high level of nutrient requirements and extensive breakdown of these nutrients in the rumen, as well as limitations to ruminal protein production and inadequate use of supplemental food protein, the need for ruminally protected nutrients is apparent. Providing a source of UDP in the feed such as fish meal, blood meal or soybean meal is an alternative source of available amino acids however these are generally lacking in at least one essential amino acid (Merchen and Titgemeyer, 1992) and are a waste of those amino acids that are supplied in excess (depending on the amino acid profile) of requirement.

Lysine and methionine usually are the first limiting amino acids for dairy and beef cattle (Richardson and Hatfield, 1978; Schwab et al., 1992). First and second limiting amino acids are determined mainly by the diet of an animal, but lysine and methionine are the usual culprits due to their high requirement. For high producing dairy cattle lysine is in high demand for milk protein synthesis, and methionine is related to production of milk fat because of its function as a methyl donor in transmethylation reactions in lipid biosynthesis (Lehninger, 1977). Dairy and feedlot diets are high in corn and corn byproducts such as distiller's grains. Corn and corn proteins are low in lysine content and therefore the need for supplementing a protected form of this amino acid may exist under certain conditions. As lysine and methionine are the first limiting amino acids they have been the main target for ruminal protection from degradation.

Performance of Ruminants Fed Ruminally Protected Nutrients

Increases in nitrogen retention and/or ruminant performance from post-ruminal infusion of limiting amino acids and other nutrients such as choline chloride demonstrate the potential for encapsulation of these nutrients (Burris et al., 1976; Hall et al., 1974; Sharma and Erdman, 1989). Actual results with supplemental ruminally protected nutrients have been variable though. Merchen and Titgemeyer (1992) suggested that source of supplemental CP can influence response of growing cattle to ruminally protected lysine (RPLys) and methionine (RPMet) supplementation. Oke et al. (1986) demonstrated an increase in feed conversion and gain in growing steers fed RPMet and RPLys in a diet supplemented with soybean meal and also an increase in nitrogen balance in lambs fed RPMet and RPLys. Wright and Loerch (1988) only

found increased gains in finishing steers fed at a level of 0.09% RPMet + 0.06% RPLys compared to those fed a urea control diet. Hussein and Berger (1995) observed improvements in DMI and gain:feed with supplementation of RPLys and RPMet, but reported no effect on gain or carcass characteristics. Rumen protected lysine and RPMet have been observed to modify milk fatty acid composition in ewes by increasing the ratio between long-chain and short chain fatty acids; however also increasing the ratio of unsaturated:saturated fatty acids (Sevi et al., 1998) which is desirable from a consumer health perspective. Donkin et al. (1989) found that addition of RPMet and RPLys to a corn based diet increased milk protein percentage and yield and plasma methionine and lysine concentrations. It has also been shown that supplementation of RPMet and RPLys to primiparous beef cattle increases milk production (Hess et al., 1998). There are increasing numbers of encapsulated lysine and methionine products available commercially. Some of these in the U.S. include Smartamine M (Aventis Animal Nutrition), AminoShure–L (Balchem), Mepron M85 (Degussa Corporation), and Met-Plus (Nisso America, Inc.). These products contain between 38 to 85% of D, L-methionine and/or D, L- lysine; however, the bioavailability of lysine/methionine in the small intestine differs greatly among products. Koenig and Rode (2001) investigated the efficacy of RPMet (Meprone M85) in lactating dairy cows and found that although the product contained 85% Met the intestinal bioavailability of Met was only 28% (on average) based on plasma methionine response. When blood plasma methionine concentration was compared among steers fed Smartamine M, Mepron M85, or METHIO-BY (Inter-Harz GmbH) it was concluded that Smartamine M was the most effective source of RPMet, as it resulted in the greatest elevation in blood plasma methionine (Sudekum et al., 2004). In recent literature, Swanepoel et al. (2010) tested a commercially available form of RPLys from Ajinomoto. This product increased intestinal absorption of lysine and utilization of other amino acids; however, there was no improvement in milk protein synthesis and a decrease in milk fat yield and milk fat percentage. Swanepoel et al. (2010) concluded that it was not advisable to supplement RPLys at this time due to the lack of predictability of intestinally absorbable lysine needs.

Due to the high sulfur amino acid content of wool and the fact that methionine is a sulfur containing amino acid, the supplementation of rumen protected methionine to increase wool production in sheep has been investigated. Robertson et al. (1999) found that RPMet

(Smartamine) increased wool growth in muskoxen. Feeding an encapsulated methionine supplement to growing/finishing lambs has also resulted in increased gains by 11% and feed efficiency by 9%, however only when the basal ration was supplemented with corn-urea or corn-blood meal-feather meal (Mowat and Deelstra, 1972).

Supplementation of ruminally protected choline (RPC) in high concentrate diets has shown to improve animal performance (Bindel et al., 2000; Bryant et al., 1999; Drouillard et al. 1998); however, Bryant et al. (1999) did not find consistent results among different levels of RPC in cattle and sheep. Like RPMet and RPLys, the response to RPC depends on the rumen undegradable protein content of the diet (Hartwell et al., 2000), and unlike RPMet and RPLys, on the level of supplemental fat in the diet. As ruminal protozoa are the principal suppliers of choline to the ruminant, excess fat in the diet will further limit the production of choline as fat can be toxic to rumen microorganisms. Drouillard et al. (1998) found that RPC increased ADG by 6.5%, decreased DMI, increased dressing percentage, and improved gain:feed by 12% in steers fed diets with no supplemental fat, but had no effect on efficiency or gain when fat was added to the diet. This is in contrast to a study done by Bindel et al. (2000), where effects on finishing performance did not show many interactions between RPC and supplemental tallow. Bindel et al. (2000) concluded that moderate levels (20 g/d) of supplemental RPC improved growth performance of finishing cattle without negatively affecting carcass characteristics.

Desirable Properties of Film for Coating/Encapsulating Nutrients

Films that are to be used for encapsulation of nutrients with the aim of protection against ruminal degradation should have these specific properties/characteristics, some of which have been mentioned by Drouillard (patent pending): 1) insoluble in the rumen of the animal (generally where the pH is greater than about 5); 2) soluble in acidic (pH 1.5-2) conditions of the abomasum (therefore altering the site of digestion to the duodenum); resistant to microbial attack; 3) prevent other interaction with components of a mixture; and 4) possess mechanical properties (flexibility and strength) such as to withstand breakage. The encapsulated product should have a high content of the core/active ingredient, a smooth surface and suitable specific

gravity (1.2 – 1.7g/cm^3) (Sykora et al., 2007). Specific gravity is important to take into consideration, as this affects the ruminal passage rate and avoids regurgitation (and thus damage to encapsulate) if the product is of an appropriate density (Vandamme and Ellis, 2004). Sykora et al., (2007) reported that the optimal size of an encapsulated product for cattle is up to 6 mm, but Yoshimaru et al. (1999) stated that feeds need a particle diameter of less than or equal to 1 mm to enable passage through the omasum. The film also should have organoleptic characteristics that encourage uptake by the animal (i.e. mainly pertaining to odor). Besides efficacy, the criteria for rumen stable delivery systems should also include safety and cost effectiveness (Wu and Papas, 1997). Safety issues pertain to the animal as well as the end product safety for human consumption because there should be no unfavorable residues in the meat. Ultimately a ruminally protected nutrient should be competitive in price with a similar or greater benefit in performance to other encapsulated products on the market and in comparison to feeding a protein supplement.

Types of Films for Encapsulation of Nutrients and Desirable characteristics of rumen protected films

Protective films can be formed from a variety of different substances including proteins, polysaccharides, lipids, and synthetic polymers. Focus has been shifted away from synthetic film forming substances and towards environmentally friendly renewable polymers.

Protein

Proteins from several plant sources have gained considerable interest, and include corn zein, wheat gluten, and soy proteins because of their film-forming capabilities. Other proteins with film forming capabilities include gelatin, casein, whey protein, serum albumin, and ovalbumin. Wheat gluten is an attractive protein as it is readily available in sufficient quantities, of consistent quality and is relatively inexpensive (about $1/kg). Wheat gluten has been used to coat nuts (Noznick and Bundus, 1967) for the binding of salt as well as a replacement for

collagen in the manufacture of sausage casings (Turbak, 1972). Corn zein is also used in the formulation of coatings for nuts (Alikonis and Cosler, 1961) as well as confectionery (Alikonis, 1979) and pharmaceutical tablets (Andres, 1984). Both wheat gluten and zein have been investigated as a coating to increase egg shell strength and/or microbial resistance for eggshells (Tryhnew et al, 1973; Wong et al., 1996). Corn zein is readily available, being a byproduct of the corn-refining industry, but is more expensive than wheat gluten. In general, the main inadequacy of protein films is their poor water vapor barrier properties due to their hydrophilic nature (Gennadios et al., 1993). Film properties (water vapor and/or gas barrier, solubility in water or lipid, mechanical and rheological characteristics) are dependent on the type of material used, its formation, and method of application (Guilbert et al., 1995).

Wheat gluten has both elastic and cohesive properties, which can be attributed to the mixture of complex proteins therein. Wheat gluten is composed of 2 groups of protein molecules based on their extractability in aqueous ethanol (Osborne, 1907), specifically glutenins and gliadins. Gliadins are extractable/soluble in about 70% ethanol while glutenin is insoluble in aqueous ethanol (70%) due to its bigger size. Gliadins and glutenins are present in about equal amounts in wheat gluten and both are high in glutamine and proline (Hernandez-Munoz et al., 2003). They also have a fair amount of nonpolar amino acids which are responsible for their insolubility in water (Krull et al., 1971). Gliadins consist of single polypeptide chains, whereas glutenins represent the part of the protein fraction that in its quaternary structure has interchain disulfide bonds that connect different protein chains together (Lagrain et al., 2010; Shewry et al., 1986). It is these disulfide bonds that are reported to be responsible for the viscoelastic properties of wheat gluten (Roy et al., 1999), whereas the gliadin fraction is thought to be responsible for the viscous and extensible component of gluten (Payne et al., 1984). Changes in the number of cross-links (disulfide bonds), which is dependent on the number and distribution of cysteine residues available to form cross links, has a large effect on the physical properties of the HMW subunit polymers (Shewry et al., 1992). Glutenins are subdivided into 2 groups: high molecular weight (HMW) subunits with M_r of 95,000 to 14,0000 by SDS-PAGE (Payne et al., 1980), and low molecular weight (LMW) subunits with M_r of about 44,000 by SDS-PAGE (Bietz and Wall, 1972). Gliadins are further subdivided into 4 groups: α-, β-, and γ-gliadins with molecular weight between 30 000 to 50 000, and ω-gliadins with molecular weights between

44,000 to 74,000. The ω-gliadins consist of large proportions of glutamine, proline and phenylalanine residues, but have low concentrations of sulphur containing amino acids. The α-, β-, and γ-gliadins contain a higher molar percent of cysteine and methionine, however have less glutamine, proline and phenylalanine (Shewry et al., 1986). The LMW glutenin subunits have similar amino acid profiles to the α-, β-, and γ-gliadins, whereas the HMW subunits differ from the gliadins in their high content of glycine and low content of proline (Shewry et al., 1986).

Gennadios and Weller (1992) reported that films manufactured from wheat gluten which contained 82% protein had 120% higher tensile strength than films made from a wheat gluten product containing 75% protein content. Krochta et al. (1994) concluded that such observations indicate that other components present in commercial wheat gluten, such as starch, lipids and oligosaccharides disturb the homogeneity of protein film networks. Starch likely plays a main role in film weakening by retrograding when drying and forming crystalline regions in the film. In a study whereby films were made from the glutenin and gliadin fractions separately, the glutenin fraction was stronger, had a higher tensile strength and lower elongation at break and water vapor permeability value than gliadin films (Hernandez-Munoz, 2003).

Wheat gluten films are brittle, and addition of a plasticizer is required to impart flexibility or integrity to the film. Plasticizers reduce intermolecular forces and increase mobility of polymer chains, thereby imparting flexibility (Gontard et al., 1993; Irissin-Mangata et al., 2001). Water can act as a plasticizer as it alters the texture of polymers, but is very unstable due to evaporation during storage. Glycerol is a common plasticizer for both zein and wheat gluten films, although the more glycerol present in the film the more the water barrier properties of the film are compromised (Gontard et al., 1993). Another problem that arises with using glycerol is that during storage it will migrate to the surface of the film matrix due to binding limitations between itself and protein molecules (Park et al., 1994). This phenomenon is confirmed by lowered film elongation values during storage. Different molecular weight polyols and amines have been tested in comparison with glycerol as plasticizers and have found to be similar in properties such as solubility in water, transparency and water vapor permeability (WVP), however different in mechanical properties with an increase in elasticity and extensibility (Irissin-Mangata et al., 2001).

The mechanical properties of wheat gluten films also are dependent on temperature at which the film forming solution is prepared. The glass transition temperature (Tg) is a fundamental parameter in the study of film preparation as above this temperature the polymer changes from rigid and glassy to soft and pliable (Guilbert et al., 1995; Cherian et al., 1995). Glass transition temperature values are mainly influenced by chemical composition and plasticizer content, but can also be affected by cross-linking (Cherian et al., 1995). The glass transition temperature of wheat gluten is 50°C, and this temperature is lowered by about 10°C/1% of water content due to water being a plasticizer (Hoseney et al., 1986).

Zein (corn protein), avenin (oat protein) and kafirin (sorghum protein), like gliadin, are classified as prolamins (Osborne, 1924) as they contain a high content of the amino acid proline and are soluble in aqueous alcohol. Suitable films have been prepared from zein, avenin and kafirin, though zein has been more extensively studied (Gillgren and Stading, 2008; da Silva and Taylor, 2005; Zhang and Mittal, 2010). Avenin is more hydrophilic than zein, gluten or kafirin (Gillgren and Stading, 2008) as it is solubilized in a maximum of 45% ethanol (Kim, 1978). In contrast, kafirin and zein are more hydrophobic proteins, even more so than gluten (Taylor and Belton, 2002). Belton (1999) attributes this to their α-helical structure as opposed to gluten's β-sheet structure.

Polysaccharide

Polysaccharide films are similar to protein films in that they have low moisture barrier properties because of their hydrophilic nature. Polysaccharide films have been prepared from alginate, pectin, carrageenam, cellulose, starch, dextrin, vegetable and other gums (Guilbert et al, 1995 and Kester and Fennema, 1986).

Alginate and carrageenan are extracted from seaweeds and are mainly used as moisture barriers for coated foods (Krochta and De Mulder-Johnston, 1997). They are said to be used 'sacrificially' which means that moisture is first lost from the coating thereby acting as a

moisture barrier to the food. Alginate films are manufactured by evaporation of an aqueous alginate solution and ionic cross linking with a calcium salt (Krochta and De Mulder-Johnston, 1997).

Pectins are mainly made up of polymers composed of D-galacturonic acid. Gels are formed by de-esterification of pectin with chemicals and then solubilizing the products in water with calcium ions (Schultz et al., 1948). The resulting gel can then be formed into a film following evaporation of the water. Pectin films are like alginate and carrageenan films in that they are a sacrificing agent and reduce water loss (Kester and Fennema, 1986).

Production of cellulose films first requires the disruption of the crystalline structure of cellulose by initial treatment with base followed by reaction with various reagents, including chloroacetic acid, chloromethane, or propylene oxide (Krumel and Lindsay, 1976). This forms a cellulose chain with bulky substituents along the polymer connected via ether linkages. This reaction allows cellulose to be water soluble and to form a film.

Wolff et al. (1951) reported the procedure for producing films from amylose (starch). Amylose is solubilized in heated water saturated with n-butanol. The solution is then distilled to remove the butanol, filtered and cast onto a glass surface to dry at 21°C, producing a transparent film. Amylose films have been used as an oil barrier for potato chips (Murray et al., 1971) and to prevent clumping of raisins (Moore and Robinson, 1968). Yoshimaru et al. (1999) utilized porous starch to encapsulate a protease enzyme for protection against ruminal degradation. The enzyme was incorporated into the pores of the starch by mixing into solution, then spray drying to form microcapsules. The microcapsules were triple coated with Eudragit E100, AS-HF, and shellac. *In vitro* this product was 35% degraded by rumen microbes after 24 hours of incubation in rumen fluid. This was contrasted with 94% degradation of microcapsules prepared by the same method, except without shellac.

Lipid

The film forming process for lipids usually involves the melting and solidification of fats, waxes and resins (Debeaufort et al., 1998). Another common method involves applying a lipid-ethanol solution or lipid-water emulsion which after evaporation of the solvent leaves the lipid coating behind (Debeaufort et al., 1998; Kester and Fennema, 1986). Lipids that can be used for film formation include acetylated monoglycerides, natural waxes, and surfactants (Kester and Fennema, 1986). Lipid compounds that have been utilized as film forming substances include acetylated monoglycerides, natural waxes and fatty acids (Kester and Fennema, 1986). Lipid films in comparison to protein and polysaccharide films have better water vapor barrier properties due to their hydrophobic nature (Guilbert, 1995). The negative side to lipid films/coatings is that they may cause oganoleptic and textural problems due to oxidation/rancidity. (Guilbert et al., 1997). This may be more of a problem in edible coatings intended for human food rather than livestock. Fats have historically been used to coat fresh produce, especially fruits against moisture transfer and to increase shelf life. There are many commercial rumen protected products that have a lipid based coating. Ajinomoto developed an encapsulated product with hydrogenated fat as the coating for DL-methionine and lysine. The RPLys is reported to have an intestinal availability of 66.2% (Watanabe et al., 2006). Park et al. (2010) recently described a method of protecting an amino acid-enriched fatty acid from ruminal degradation and reported improved marbling and quality grade as a consequence. The method of protection involved combining calcium salts of fatty acids, amino acids methionine and lysine (1:2 ratio) and hydrogenated soybean oil. This mixture was then extruded and cut into 3 to 4 mm length pellets.

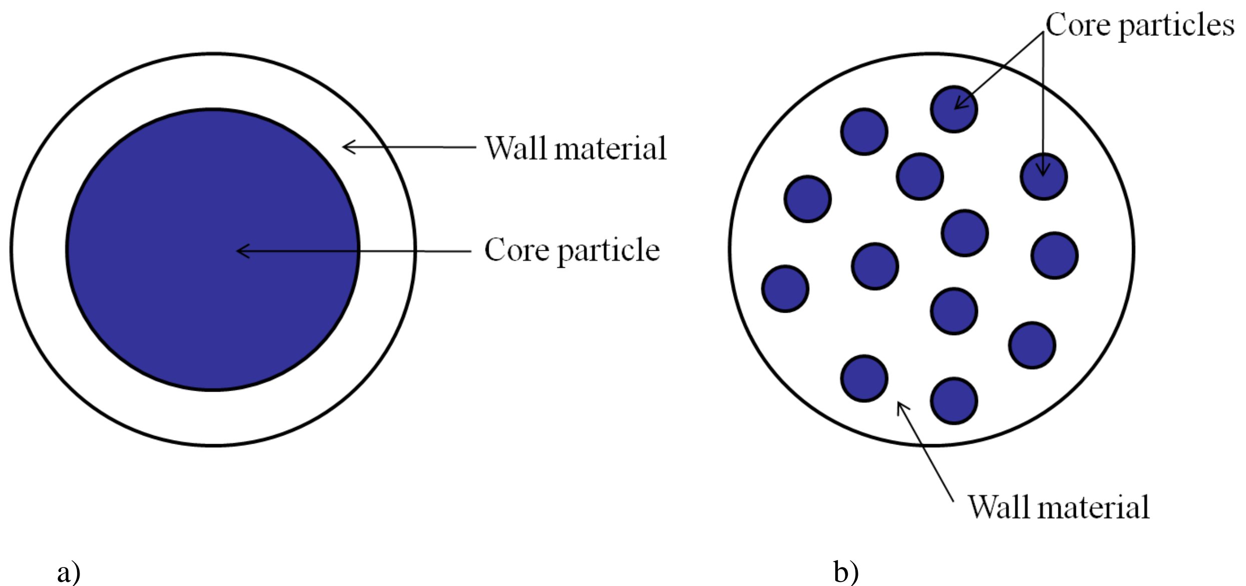
Composite

Composite films are films in which two or more film forming materials have been utilized to produce a product that has complementary functional properties and which address shortcomings of single compounds (Guilbert et al., 1997).

Encapsulation Strategies/Techniques

Encapsulation is defined by Augustin et al. (2010) as the "entrapment of a component (the core) within a secondary material (encapsulant matrix)". Desai and Park (2005) illustrated the two basic concepts in microcapsule formation (Figure 1.1). The first concept normally consists of a single particle (termed the core) enclosed by a wall of uniform thickness. It can be compared to a "hen's egg". The second concept consists of multiple cores that are embedded within a continuous matrix of wall material. Various encapsulation techniques exist and are most often used in the food industry, such as encapsulation of flavors, but also are used for drug delivery. Common encapsulation techniques include spray drying, spray freezing, extrusion coating, fluidized bed coating, and coacervation.

Figure 1.1 Conceptual diagram representing two types of microcapsules; a) Hen's egg encapsulation; b) Matrix type encapsulation



Spray Drying

Spray drying is a widely used method for encapsulation purposes as it is economical and effective. Spray drying involves dispersing the ingredient for encapsulation with the carrier/film forming solution usually at a ratio of 1:4 (Gibbs et al., 1999). The homogenized mixture is then “fed into a spray dryer and atomized with a nozzle or a spinning wheel” (Gibbs et al., 1999; Desai and Park, 2005). Water is evaporated from the atomized particles by hot air contacting the material, and the microcapsules collect at the bottom of the drier.

Spray Cooling/Chilling/Freezing

Spray chilling/freezing is similar to spray drying in that the core material and wall/encapsulating material are mixed and atomized; however, this is accomplished using cooled air as opposed to hot air (Desai and Park, 2005). This method causes the encapsulating/coating material to solidify around the core. Encapsulated products produced using this technique have typically used a lipid with a low melting point (such as hydrogenated vegetable oil) as the coating material. Spray cooling, chilling and freezing use different temperatures to achieve a spherical dry product. Spray cooling utilizes temperatures from 45-122°C, spray chilling from 32-42°C (Gibbs et al., 1999) and spray freezing uses very low temperatures in the negative °C range. MetiPEARL and LysiPEARL (Kemin Industries, Inc.), commercial rumen protected products have coined the term ‘Micropearls®’ to describe the process of producing their products by embedding them in prilled fat and then spray freezing this material at -60°C.

Fluidized Bed

Fluidized bed is a rather expensive technology whereby the core particles to be encapsulated are suspended in a temperature and humidity controlled chamber of high velocity air where the coating material is atomized (Desai and Park, 2005). The amount of coating that is applied to the core particles is dependent on the amount of time spent in the chamber. As the particles pass through the cycle multiple times, the random orientation of the particles at each

pass ensures uniform coating (Shahidi and Han, 1993). For hot-melt coatings, cool air is used to harden the encapsulate, and this usually applies to lipid based coatings. Solvent-based coatings use hot air to evaporate the solvent thus hardening the coating (DeZarn, 1995). Desai and Park (2005) listed the ways in which the fluidized bed method could be modified by changing the position of the spray nozzle. The spray nozzle for coating the core particles can be oriented as a top-spray, bottom-spray, and tangential-spray. The traditional top-spray method has its drawbacks as the distance the droplets travel before contacting the core particles cannot be controlled therefore coating imperfections can result due to premature droplet evaporation. Top spray does, however, produce a greater yield of encapsulated particles compared to bottom or tangential sprays (Jothi et al., 2010). The bottom spray method is a more controlled method as the path that the droplets travel is very short, thereby eliminating the chance of premature droplet evaporation. Tangential-spray method is used for very small particles and compensates for the low gravitational force of these fine particles. Fluidized bed is applied to various products for encapsulation, some of which include vitamins B and C, and minerals such as potassium chloride (Dezarn, 1995). Smartamine™ is produced by coating methionine with a stearic acid and pH-sensitive copolymer (poly-2-vinylpyridine-co-styrene) coating in a fluidized bed dryer using the solvent-free process (Prud'homme, 1996).

Extrusion

Extrusion is a technique that is commonly employed to form microcapsules that encapsulate flavors or colors; however, the product is soluble in water (Gibbs et al., 1999). The process consists of forcing the core material in a molten carbohydrate mass through a succession of dies into a bath of dehydrating liquid (e.g. isopropyl alcohol) (Desai and Park, 2005; Shahidi and Han, 1993). The mass solidifies upon contacting the liquid, thereby entrapping the core material. The extruded material is then cut to size, usually 1mm, and vacuum-dried (Gibbs et al., 1999; Shahidi and Han, 1993). One of the advantages of this method is that it achieves a true encapsulation as any core material that is exposed on the outside of the encapsulate is washed off in the alcohol bath (Gibbs et al., 1999). The extrusion process can be modified to create a spherical pellet such as in the case of extrusion spheronization. As effective controlled release of

a substance or drug is determined by a narrow size distribution and a smooth surface, this technique can be useful. Extrusion spheronization can produce pellets of uniform size with a high drug loading capacity (Ghai et al., 2009).

Coacervation

Coacervation is an expensive and complex, yet effective, method of encapsulation which frequently is regarded as the original method of encapsulation (Risch, 1995). Coacervation is often used to encapsulate flavor oils (Korus, 2001). In this process a solution is formed with 3 immiscible chemical phases, the core, coating and solvent phase (Jyothi et al., 2010). The coacervate phase is then deposited around the core ingredient. The coacervate wall is then hardened and the microcapsules isolated from the solution. The core material must be insoluble in the coacervation medium (Madene et al., 2006). Few proteins have been used as the 'wall' substance in the coacervation technique, although Mauguet et al. (2002) has successfully encapsulated hexadecane (an oil) with gliadin. Amino acids protected from ruminal degradation by entrapment within fat particles were further protected by the coating of chitosan by means of the simple coacervation method (Chiang et al., 2009).

Conclusions

Protection of nutrients, such as amino acids, from ruminal degradation by encapsulation has shown to be successful in improving animal performance (such as milk yield), although results have been inconsistent, and animal responses are small. There is a need for delivering nutrients to a targeted site for absorption, to increase efficiency of utilization and to meet increased requirement thereof. The reasons for inconsistent or poor responses from animals fed ruminally protected nutrients may be due to diet, body condition, stage of lactation (for dairy cattle), degree of ruminal protection, and efficiency of release and absorption of that nutrient (usually at the abomasum and small intestines respectively). In the case of ruminally protected amino acids, response will also be dictated by the extent and order of limitation of that amino

acid. In some cases more than one amino acid will be limiting which in turn limits response if only one or two of those amino acids are provided.

Protective films can be produced from proteins, lipids, polysaccharides or synthetic polymers, and the properties thereof can be modified by various processing methods to suit the application. A variety of encapsulation technologies exist, such as spray drying, fluidized bed drying, spray cooling, extrusion, and coacervation, to name a few, although the suitable method of creating an encapsulate will depend on the nature of the coating as well as the nutrient to be encapsulated.

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CHAPTER 2 - Processing Parameters for Wheat Gluten Films

Abstract

Various processing methods to produce wheat gluten films that are resistant to microbial degradation in the rumen were investigated, with the ultimate intent of using them to encapsulate nutrients for protection against ruminal degradation. Processing parameters that were applied to the film forming solution included modification of pH and temperature, addition of xylose to induce Maillard reactions, and addition of transglutaminase (Tgase) to promote cross linking of the wheat gluten proteins. Other treatments applied to film forming solutions consisted of 1) comparing acetic to hydrochloric acid (HCl) as the means for inducing pH change, and 2) comparing effects of the length of time for which thermal treatment is applied, either 10 or 50 min. Dry films were incubated in ruminal fluid *in vitro* for up to 8 h, and sometimes for 12 h. Response variables were either ammonia release or dry matter loss as indicators of degradability. An *in situ* experiment also was carried out. There were linear ($P < 0.01$) and quadratic ($P < 0.01$) effects of pH on degradability of films, with films prepared at pH 3 showing the greatest resistance to ruminal degradation. There also was a pH×temperature interaction, whereby low pH (pH 3) and high temperature (75°C) resulted in the lowest degradability compared to films manufactured at either pH 5 or 7.5 at 40 or 55°C. Low pH causes deamidation of proteins, while high temperature induces disulfide cross-links which may render films less digestible by ruminal bacteria. Xylose treatment did not confer any improvement in resistance to microbial degradation. Tgase treatment also did not improve resistance to degradation, as enzyme activity probably was hindered by the presence of ethanol. Glacial acetic acid was the preferred acid for pH change, as it resulted in a more homogeneous film, which can be attributed to glutenin's solubility in acetic acid. Replacing a limited amount of acetic acid with HCl did not affect degradability or film appearance. Prolonging thermal treatment of the film forming solution from 10 to 50 min did not affect ruminal degradability. There is potential to create protective films or coatings from wheat gluten for encapsulation of nutrients that will bypass the rumen.

Introduction

Encapsulated amino acids, vitamins, and other nutrients are becoming increasingly common in the ruminant feed industry. The purpose of encapsulation is to provide protection from premature digestion in the rumen, making it possible to increase bioavailability of the core ingredient in the small intestine. Encapsulated amino acids are effective at delivering a targeted amount of limiting amino acids compared to traditional methods of heating or chemically treating protein, which results in an excess supply of non-limiting amino acids. The main limitation to feeding encapsulated products is cost. These products are expensive due to the cost of the film forming/encapsulating materials used. Wheat gluten is an inexpensive alternative and has natural film forming capabilities. Processing factors that influence the extent of degradation in the rumen and subsequent uptake in the post-ruminal digestive tract have not been fully elucidated. The objective of our research was to identify the initial processing conditions under which wheat gluten will provide sufficient protection from microbial degradation in the rumen. Temperature and pH are two processing parameters that have a large effect on the final properties of films due to their ability to alter protein structure. Maillard reactions have long been used as a method for reducing ruminal digestion of proteins (Can and Yilmaz, 2002), and cross-linking enzymes (such as Tgase) have been used successfully to cross-link films made from soy protein isolate (Tang et al., 2005) and wheat gluten (Wang et al., 2004).

Materials and Methods

Generalized Procedure

Film forming procedures differed in each experiment, as the formula for film production was altered in an attempt to improve functional properties; however, the general procedure is herein described with the differences for each experiment highlighted in the respective sections.

Films were prepared with ethanol and water as solvents and glycerol as a plasticizing agent. Vital wheat gluten (54 g, or 13% w/v, Manildra Group, Shawnee Mission, KS); and glycerol (3 g) were mixed into 200 mL of 95% ethanol. Distilled water was then added slowly (100 mL) to the solution whilst mixing on a stirrer plate. The film forming solutions were adjusted to their respective pH, usually with glacial acetic acid, to pH 3. Total volume of the resulting solution after pH change was approximately 416 mL. The solution was then sheared for 8 min using a Polysciences CAT homogenizer (model X 120) on setting 4. Thereafter, the sheared solution was stirred and heated to the appropriate temperature, normally 75ºC, on a hotplate under reflux. The temperature was monitored by a digital wire thermometer that was inserted into the top of the reflux unit and came into direct contact with the solution. The heated solution was held at the temperature for 10 min whilst stirring and then centrifuged at 959 x g for 5 min at room temperature (20°C) using a Beckman JA-14 rotor to remove any remaining insoluble gluten. Approximately 14% wheat gluten ended up in the precipitate from this centrifugation step. The resulting supernatant (150 mL) was poured onto a 33.7 x 23.5 cm Teflon coated cookie tray and dried in a 55°C oven overnight. Dry films were peeled off the cookie tray and ammonia and total amino acid (TAA) release assays were then carried out on the films after analysis for nitrogen content.

Procedures used in the following studies were approved by the Kansas State University Institutional Animal Care and Use Committee protocol no. 2535. The ruminal fluid inoculum was obtained from a cannulated steer fed a forage based diet. At collection, ruminal fluid was strained through 4 layers of cheesecloth. The strained rumen fluid (SRF) was then filtered through eight layers of cheesecloth, and purged with carbon dioxide. The SRF was transferred into a separating funnel and left for approximately 30 min to allow ruminal fluid to stratify. Once separated, the middle layer, devoid of fibrous matter and rich in microorganisms, was used to prepare the inoculum. The strained rumen *in vitro* inoculum was prepared by the method described by Craig et al. (1984). Chloramphenicol (CAP) and hydrazine sulfate (HS) were used as inhibitors of N assimilation, and maltose was used as an energy source. Reducing conditions were maintained *in vitro* using 2-mercaptoethanol. Samples of the films (approximately 4 mg N) were weighed into plastic incubation tubes, and wetted with 8 mL of McDougall's buffer for about 1 h prior to collection of ruminal fluid. Blank tubes, including inoculum and ruminal fluid,

but without substrate, also were included. The buffer was pre-warmed and added to SRF in a 1:1 ratio. The appropriate reagents were added to the inoculum 5 min before the start of incubation. After mixing, 20 mL of inoculum plus reagent were added to all tubes and incubated at different time points up to 8 h. Tubes were covered with stoppers and placed into a shaking water bath at 39°C. There were 2 replicates per sample and a blank per time point. Incubation was halted by adding 2 mL of 65% (w/v) trichloroacetic acid (TCA) to each tube, immediately placing onto ice for 10 min to cool, and then placing samples into the freezer. Each tube was thawed, vortexed and centrifuged at 15,300 x g at 4°C for 15 min. The supernatant was kept at 4°C until analysis for TAA and ammonia. Recoveries of ammonia and TAA-N after each time point were computed with the following equations:

Ammonia recovery (%) = (NH_3-N/ NH_3-N added) x 100,

and

N recovery (%) = (NH_3-N + TAA-N/added N) x 100

In this formula, TAA-N was calculated from the TAA content of acid hydrolysates of each film. The proportion degraded (PD) for each sample for each time period was computed using the following formula:

PD = {mg NH3-N (at t) + [(µmol TAA (at t))/(µmol TAA/mgN)]}/mg added N

Experiment 1: Effects of pH on the Resistance of Wheat Gluten Films to Ruminal Fermentation

This study consisted of a randomized complete block design with a 5 x 8 factorial arrangement of treatments. Factor 1 was fermentation time (0, 2, 4, 6 and 8 h) and factor 2 was pH level (pH 2, 3, 4, 5, 6, 7.5, 9 and 11) at which the films were made. An *in vitro* ammonia and total amino acid (TAA) release assay was conducted on the dry films with film as the experimental unit. Block consisted of the day of rumen contents collection. There were 3 replicates per fermentation time. Results were analyzed using the Mixed procedure of SAS

(version 9.2; SAS Inst., Cary, NC), with day as the random effect. Fixed effects were pH, time and pH×time. A P-value less than 0.05 was declared significant for this and subsequent experiments. The pH of the film forming solution was altered using glacial acetic acid or 6N ammonium hydroxide. The temperature under which the film forming solutions were heated was 40°C for all treatments. The pH 2 treatment was later excluded from this experiment due to the large quantity of acetic acid in this film, which resulted in poor film formation.

Experiment 2: Effects of Temperature and pH on the Resistance of Wheat Gluten Films to Ruminal Fermentation

The study was a randomized complete block design with a 3 x 3 x 5 factorial arrangement of treatments. Factors were pH level (3, 5, and 7.5), temperature (40°C, 55°C and 75°C) and fermentation time (0, 2, 4, 6 and 8 h). The pH levels chosen for this study were chosen based upon the results from the previous pH study. Films manufactured at various pH's that displayed the highest (pH 5), intermediate (pH 7.5) and lowest (pH 3) degradability were included in this study. Temperature treatments were selected based upon previous studies by Roy et al. (1999) and Herald et al. (1995) whereby wheat gluten films were prepared via different heating temperatures of the film forming solution. An *in vitro* ammonia and total amino acid (TAA) release assay was conducted on the dry films with film as the experimental unit. Block consisted of day of rumen contents collection, and there were 2 blocks for each fermentation time point. Results were analyzed using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with day as the random effect. Fixed effects included temperature, pH, time, and all interactions. The pH of the film forming solution was altered by addition of glacial acetic acid or 6N ammonium hydroxide.

Experiment 3: Effects of Maillard Reaction on the Resistance of Wheat Gluten Films to Ruminal Fermentation

A maillard reaction is a condensation reaction between a carbonyl group of a reducing sugar and an amino group of amino acid. The purpose of this experiment was to induce a

maillard reaction by addition of the reducing sugar xylose, with subsequent heating of the film forming solution. Maillard reactions have been used with success to protect proteins to some extent from ruminal degradation (Coetzer, 2000). An *in situ* trial was carried out to estimate ruminal degradability of films. All films were manufactured at pH 3 and 75°C. Xylose was added as one of the treatments at a ratio of 10 wheat gluten:1 xylose. Attempts were made at encapsulating lysine with these films by adding lysine directly onto the wet, poured film; however, the lysine was found to dissolve while the film was drying, and left air pockets in the dry film due to lysine's solubility in water. In response to this observation, 20 g of lysine were mixed with the water (100 mL) that was to be added to the solution prior to adjusting pH. The resulting dry film had lysine incorporated into the protein structure of the film. Once dry, approximately 1 g of film was weighed in duplicate into nylon Ankom (Macedon, NY) bags (30 µm pores) and sealed with a heat sealer. These bags were placed in a larger 33 x 38 cm weighted lingerie bag, and then suspended in the rumen of a cannulated steer. Incubation times were 0, 4, 8 and 12 h. After incubation, bags were rinsed by gentle hand agitation in cold water until the rinse water was clear. The bags were then dried for 24 h in a 105°C oven. The dried bags were weighed to determine residual DM.

The study was a randomized complete block design with a 2×4 factorial arrangement of treatments. Factors were xylose content (with and without) and incubation time (0, 4, 8 and 12 h). Experimental unit was film. Block was by animal, as 3 cannulated animals were utilized on one day, with 2 replicates per treatment. Results were analyzed using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with animal as the random effect. Fixed effects were xylose, time and xylose×time.

Experiment 4: Effect of a Cross-linking Enzyme (Transglutaminase) on the Resistance of Wheat Gluten Films to Ruminal Fermentation

The objective of this study was to evaluate the effect of addition of a cross-linking enzyme, transglutaminase (Tgase; Ajinomoto, Fort Lee, NJ), on wheat gluten film stability in the rumen. Tgase catalyzes a reaction between the γ carbonyl group of glutamine and the ε amino group of lysine to form a covalent ε-(γ-glutamyl) lysine cross-link. Cross-linking with the aid of

this enzyme has been used to improve strength and stability of films intended for food packaging (Wang et al., 2004), and we hypothesized that Tgase treatment could render films less digestible to ruminal bacteria. The study was a randomized complete block design and treatments were arranged factorially as a $(2 \times 2 + 1) \times 5$. Factor 1 was Tgase level (10 U/g and 20 U/g of wheat gluten) and factor 2 was Tgase type (Activa TI and Activa GS). The plus one treatment was a control film without Tgase. All films were subjected to ammonia release and total amino acid assays and incubated for 0, 2, 4, 6 and 8 h.

Films were prepared according to the methods previously described as the 'general procedure', with the exception that the film forming solution was heated to 50°C to facilitate optimum reaction conditions for the Tgase enzyme. The Activa TI preparation was added at 5.4 (10%) or 10.8 g (20%), while the Activa GS preparation was added at 9 (10%) or 18 g (20%) based upon their average activity of 100 and 60 U/g of powdered preparation. The Tgase was allowed to react with the wheat gluten protein for 8 min at 50°C. Due to the pH sensitive nature of the enzyme, pH of the film forming solution was only then adjusted with glacial acetic acid to pH 3. Thereafter, the film forming solution was treated the same as described in the generalized procedure, being subjected to homogenization, heating to 75°C under reflux, and centrifugation.

Results were analyzed using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with day as the random effect. Fixed effects included Tgase type, Tgase level, Tgase type×Tgase level and time. Experimental unit was film. Treatment means were compared among each other using F-protected ($P < 0.05$) orthogonal contrasts.

Experiment 5: Use of Acetic Acid versus Hydrochloric Acid to Control pH of Wheat Gluten Films and their Effects on Resistance of Films to Ruminal Fermentation

Acetic acid is a relatively weak acid, thus requiring its use in copious amounts to decrease pH of the film forming solution to 3. Due to cost considerations, a comparative study was carried out to elucidate differences between films formed with glacial acetic acid versus concentrated hydrochloric acid (HCl) as the means of decreasing pH of the film forming

solutions. Treatments consisted of various acetic acid levels (0, 25, 50, 75 and 100%). As 150 mL of acetic acid is required to lower the pH of our film forming solutions to 3, this served as our 100% acetic acid treatment level, therefore the 25, 50 and 75% treatments required using 25, 50 and 75% of 150 mL (37.5, 75 and 112.5 mL respectively) of acetic acid with concentrated HCl being used to make the final adjustments to obtain a pH of 3. An additional treatment was 100% of only 1N HCl. Thereafter, the film forming solution was treated the same as described in the generalized procedure, being subjected to homogenization, heating to 75°C under reflux, and centrifugation.

An *in vitro* ammonia and total amino acid (TAA) release assay was conducted (0 and 8 h incubation times) on the dry films with film as the experimental unit. Data were analyzed as a randomized complete block design using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with day as the random effect. Block consisted of day of ruminal contents collection. There were 2 replicates per fermentation time. The fixed effects were treatment, time and treatment×time.

Experiment 6: Effects of Prolonged Thermal Treatment on the Resistance of Wheat Gluten Films to Ruminal Fermentation

The objective of this experiment was to evaluate the difference between a short and extended period of heat treatment of the film forming solution on final film stability in the rumen. Film forming procedures were the same as stated in the generalized procedure with a few exceptions forthwith described. In this and subsequent experiments, the pH of the film forming solutions were adjusted (pH 3) with 112.5 mL glacial acetic acid and approximately 0.6 mL of HCl (75% acetic acid, 25% HCl). This study consisted of a randomized complete block design with 2 treatments, which consisted of heating the film forming solutions (80°C) under reflux and holding them at 80°C for either 10 min or 50 min on a hotplate. The method of analysis on these films was an *in vitro* ammonia release assay as previously described. Another method of evaluation, an *in vitro* digestibility assay using the Daisy incubator (Ankom, Macedon, NY), also was carried out. The method of incubation was slightly modified according to the "In Vitro True Digestibility" guidelines described by the manufacturer. The films were

weighed (0.25 g) into Ankom filter bags that were previously rinsed in acetone to remove a surfactant that may inhibit microbial digestion. Each bag, including blanks without substrate, was sealed with a heat sealer and placed into the digestion jar. Ruminal fluid (1000 mL) that was strained and separated was combined with McDougall's buffer (1000 mL) and added to each digestion jar. The digestion jars were purged with CO_2 for 30 seconds and placed into the Daisy incubator to incubate for 8 h. After 8 h the jars were drained of fluid and the bags rinsed with water until clear. The bags were then dried for 24 h in a 105°C oven. The dried bags were weighed to determine residual DM.

Results were analyzed using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with day as the random effect. Block consisted of day of ruminal contents collection. The fixed effect was treatment (2 replicates per treatment), and experimental unit was film.

Experiment 7: Effect of Analytical Grade Transglutaminase, With and Without Added Lysine, on the Resistance of Wheat Gluten Films to Ruminal Fermentation

The purpose of this experiment was to apply a more concentrated grade of Tgase enzyme to the film forming solution in an effort to improve the rumen microbial barrier properties of the wheat gluten film. A Tgase product with 10% active enzyme was obtained from Kinry Food Ingredients Co., Ltd. (Shanghai, China). Treatments were arranged as a $(2 \times 5 + 4) \times 2$ factorial arrangement of treatments. Factor 1 was level of Tgase (0 and 10 Units of enzyme activity/g of wheat gluten), factor 2 was level of lysine that was added to the film forming solution based upon the molar percent of glutamine (Gln) present in vital wheat gluten (1:1, 1:0.75, 1:0.5, 1:0.25, 1:0 Gln:Lys). Lysine was added to the reaction mixture, as it is the limiting substrate in wheat gluten (< 1%) for the cross-linking reaction. The molar percent Gln in wheat gluten was approximated to be 39% based on the glutamic acid analysis performed by the supplier of wheat gluten (Manildra Group, Shawnee Mission, KS); therefore, Lys was added in the following amounts for each respective Gln:Lys ratio: 26.7 g, 20.0 g, 13.3 g and 6.7 g. The 'plus 4' additional treatments in the model included: 1. Activa TI = addition of Activa TI (Ajinomoto, Fort Lee, NJ) without lysine; 2. Preheat = heating the film forming solution before adding Tgase

without lysine; 3. Double = doubling the amount of pure Tgase added to the film forming solution (with Gln:Lys in a 1:0.5 molar ratio); and finally, 4. SBM = soybean meal. Factor 3 was fermentation time (0 and 8 h). The 'preheat' treatment consisted of heating the film forming solution to 80°C prior to Tgase addition to increase the number of lysine and glutamine residues on the surface of the gluten molecule, thereby increasing its cross-linking reactivity. As the extent of reaction is determined by the number of exposed Gln and Lys residues on the surface of the protein, denaturing the protein by heat treatment, followed by cooling to 50°C before Tgase addition, is a method to increase the surface Gln and Lys residues available for reaction (Wang et al., 2007).

Following addition of Tgase, the wheat gluten solution was heated to 50°C in a closed conical flask in a water bath to facilitate optimum reaction conditions for the enzyme. The reaction took place in a closed conical flask to prevent evaporation of ethanol while the temperature of the solution was monitored with a digital thermometer wire probe inserted into the solution via a small hole in the lid. When the solution reached 50°C, the enzyme was allowed to react for 2 h at that temperature with occasional swirling of the mixture by hand. Films not requiring Tgase addition were subjected to the same procedure by incubating the film forming solution in the water bath for 2 h, but without Tgase. Ammonia released from the Tgase catalyzed reaction was measured immediately following the 2 h incubation period by squeezing approximately 5 mL of the film forming solution through cheese cloth, centrifuging at 959 x g for 5 min at 4°C using a Beckman JA-14 rotor, collecting the supernatant and analyzing for NH_3 on a plate reader (results not shown). The film forming solution was then treated similarly as described in the generalized procedure being subjected to pH change with acetic acid and HCl to pH 3, homogenization, heating to 75°C under reflux, and finally, centrifugation.

Once dry, the films were subjected to an ammonia release assay as previously described as well as an *in vitro* digestibility assay using the Daisy incubator. Incubation times for the ammonia release were 0 and 8 h. The incubation time for the Daisy incubator also was 8 h.

The study was a randomized complete block design with block consisting of the day of ruminal contents collection. There were 2 replicates per fermentation time. Results were

analyzed using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with day as the random effect. Fixed effects were treatment, time and treatment×time. Treatment means were compared using *F*-protected ($P < 0.05$) orthogonal contrasts.

Results

Experiment 1

Figure 2.1 displays the film degradability over time. There was a pH by hour interaction ($P = 0.0024$) as well as a quadratic effect ($P < 0.0001$) of pH on degradability, with pH 3 showing the lowest degradability values and pH 5 displaying the highest. There also was a linear effect of pH on degradability ($P < 0.001$).

Experiment 2

There was an interaction between pH and temperature ($P < 0.01$), whereby low pH (pH 3) and high temperature (75°C) films were most resistant to microbial degradation (Figure 2.2). There was no interaction between temperature and hour ($P > 0.05$) but there was a pH by hour interaction ($P < 0.01$), as well as a quadratic effect ($P < 0.01$) of pH on degradability, with pH 3 showing the lowest degradability values and pH 5 displaying the highest. Film degradability was decreased with increasing temperature ($P < 0.01$), with the lowest degradability occurring for films manufactured at 75°C.

Experiment 3

The percent of film that escaped the dacron bags after *in situ* incubation for 12 h is presented in Table 1. Film solutions with xylose added to induce Maillard reactions did not result in a higher percent of film escape from Dacron bags ($P > 0.05$) compared to films without xylose treatment. No treatment by time interactions occurred ($P > 0.05$).

Experiment 4

Figure 2.4 shows the *in vitro* degradability of films for this experiment. Activa GS treated films had greater degradability compared to films prepared with Activa TI at 10 or 20 U ($P < 0.0001$). Effectiveness of Activa TI was not different for the two levels ($P = 0.40$). Activa GS treated film solutions resulted in films that had greater degradation compared to control films without Tgase ($P < 0.001$). When comparing Activa TI to the control film without Tgase, it was evident that there were no differences ($P > 0.05$), therefore Tgase did not improve film barrier properties. There were no Tgase by time interactions ($P > 0.05$).

Experiment 5

Figure 2.5 displays the degradability of films that were manufactured using differing ratios of acetic acid and concentrated HCl. There were no effects of treatment or treatment by time interaction on degradability of films *in vitro* ($P > 0.05$). Films were, however, markedly different in appearance (Figure 2.6), with increasing amounts of HCl resulting in less homogeneous and more globular films.

Experiment 6

Figures 2.7 and 2.8 demonstrate the *in vitro* degradability of films with ammonia release assay and Daisy incubation procedures, respectively. There was no effect of prolonging thermal treatment on degradability of films with either the ammonia release assay or the Daisy incubation procedure ($P > 0.05$).

Experiment 7

Figure 2.9 displays the degradability of films when subjected to the Daisy incubation procedure. There was a linear effect of adding lysine to the film forming solution on degradability ($P < 0.0001$), whereby films with the highest lysine content resulted in the greatest degradability. Tgase treated films did not differ in degradability compared to films without Tgase ($P > 0.05$). Preheating the film forming solution did not improve reactivity of Tgase or reduce degradability compared to films that were not preheated ($P > 0.05$). Doubling the amount

of enzyme in the solution did not reduce degradability of films ($P > 0.05$). There also was no difference ($P > 0.05$) in degradability between the films that were treated with either 100 U Activa TI Tgase (Ajinomoto) or 1000 U Tgase (Kinry Food Ingredients). The control (SBM) was degraded significantly more than all treatments except those with a 1:1 ratio of Gln:Lys ($P < 0.0001$).

Figure 2.10 displays the degradability of films when subjected to the ammonia release assay. There was no effect of adding lysine to the film forming solution on degradability of films ($P > 0.05$). There were no differences in degradability of films treated with or without Tgase ($P > 0.05$). All other results from ammonia release were consistent with the Daisy incubation procedure.

Discussion

Experiment 1

The films made at pH 5 may be the most degradable due to this being closest to pH of the rumen. Ruminal bacteria thrive at pH 5 to 7, therefore the films made at pH 5 would be more susceptible to microbial attack. Film formation at pH 7.5 was hindered by poor protein dispersion due to this being the isoelectric region of wheat gluten. Poor film formation at the isoelectric region will compromise integrity of the film, making it more easily degraded by ruminal bacteria. Herald et al. (1995) reported an improved tensile strength of wheat gluten films prepared at pH 3.3 compared to pH 10, which was attributed to an enhanced dispersion of gluten proteins (mainly glutenin) in solution. The solubility of glutenin proteins in acetic acid may explain why pH 3 films, with the highest content of acetic acid, resulted in improved barrier properties to ruminal bacteria. Conflicting results have been reported for various pH studies using wheat gluten, although most research agrees that film formation is best under highly alkaline (9-13) or highly acid (2-4) conditions (Gennadios et al., 1993). In a study by Gontard et al. (1992), wheat gluten films prepared at pH 5 or 6 with high ethanol concentration (70%) resulted in films that had high solubility in water. Generally, a high solubility in water correlates with high solubility in ruminal fluid, as ruminal fluid contains a high concentration of water. As the concentration of ethanol was high in our treatments at pH 5 and 7, this is consistent with our results. Concentrations of ethanol and pH, therefore play an important role in film properties.

Experiment 2

The linear decrease in degradability with increased temperature of the film forming solution may indicate increased cross-linking through covalent S-S bond formation. The mechanism behind this may be that heated film forming solutions denature wheat gluten proteins, thereby reducing the S-S bonds already inherent in the protein, and exposing SH groups. During drying, disulfide bonds are formed by air oxidation, thereby cross-linking the protein molecules (Roy et al., 1999). This would contribute to the films being stronger and more resistant to microbial degradation. Roy et al (1999) found that solubility of wheat gluten films in water decreased when films were prepared at 75 or 95°C, which they credited to protein denaturation, disulfide bond formation, and the resulting increased molecular weight of the protein fractions. At moisture levels above 20%, which is the case for our experiment, the wheat gluten is already past its glass transition state at a temperature above 0°C. According to Lagrain et al. (2010), cross-linking reactions take place at temperatures above 70°C.

Experiment 3

Maillard reactions frequently have been used to reduce protein degradation in the rumen (Can and Yilmaz, 2002; Cleale et al., 1987). This is due to the ability of Maillard reaction products (MRP) to chelate minerals (McBurney et al., 1983; Friedman, 1996), which inhibits proteolytic bacteria in the rumen from degrading protein. Chelation may remove minerals that function as cofactors for proteolytic enzymes, thereby inhibiting proteolysis (Wallace, 1996). Another possible mechanism for reduced ruminal degradation of MRP may be due to stearic hinderance by the bulky sugar group, when bound to amino acids in the condensation reaction, could limit enzymatic ability to cleave this bond. Although films with added xylose did not improve barrier characteristics, they were dark brown in appearance (Figure 2.3), suggesting that a non-enzymatic browning reaction did take place. Degradability of films were approximately 45%, which is considerably high, even for 12 h incubation, as our previous *in situ* experiments (data not shown) have shown films to be only 28% degraded. This can be credited to lysine incorporation into the films, as lysine is water soluble, therefore soluble also in the rumen. It's probable that addition of free lysine to the film forming solution reacted with the xylose thereby

inhibiting formation of lysine-xylose condensation polymers for lysine inherent in the wheat gluten. This could explain why xylose treated films did not improve resistance to ruminal degradation.

Experiment 4 and 7

Tgase catalyzes a reaction between the γ carbonyl group of glutamine and the ε amino group of lysine to form a covalent ε-(γ-glutamyl) lysine cross-link. Wang et al. (2004) reported an overall improvement in the "physical and barrier properties" of Tgase treated wheat gluten films. A lower elongation at break, water vapor permeability, and an elevated tensile strength was found with Tgase treated films as well as a higher water resistance. Wang's method for film preparation consisted of cross-linking wheat gluten with Tgase in a jacketed bioreactor, dialyzing, then freeze drying the cross-linked gluten, and only then creating a film forming solution from this product. In contrast, Lai and Chiang (2006) found that addition of increasing amounts of Tgase increased water vapor permeability and decreased tensile strength. This study however, utilized a similar film preparation technique to ours whereby Tgase is added to a film forming solution consisting of wheat gluten, ethanol and glycerol.

Activa GS has been used in food applications to bond large pieces of meat together for production of restructured meat products. To explain the poorer barrier properties of GS compared to TI, or the higher degradability of GS in experiment 4, it seems likely that the high content of other ingredients in the enzyme preparation of Activa GS (such as sodium chloride, gelatin and phosphates) may have interacted with the protein network of the film to decrease efficacy of the Tgase. The higher nitrogen degradation in the Activa GS tubes may be attributed to microbial fermentation of the gelatin, an additional protein source, which is not present in Activa TI. Activa TI contains approximately 99% maltodextrin as filler ingredient that may have interacted with wheat gluten to negate any positive effects of the Tgase reaction. Attempts were made at purifying the Tgase enzyme from the Activa preparation using dialysis and subsequent cation-exchange column, but these attempts were unsuccessful and led us to use a more concentrated Tgase (1000 U) containing less filler material. It also is noteworthy that the reaction time for experiment 4 was only 8 min at 50°C, which may have limited the cross-linking reaction. One unit of enzyme activity is defined as the formation of 1μmol of hydroxamic acid/min at pH 6.0 and 37ºC assayed by the method of Folk and Cole (1966). Based on a

conservative estimate of 20% glutamine present in wheat gluten, we increased the reaction time to 2 h in experiment 7. A lack of improvement in the film's resistance to microbial degradation may indicate that Tgase induced cross-links are not an effective deterrent to microbial digestion, or that cross-linking bonds were not formed. Lai and Chiang (2005) concluded that the activity of Tgase was highly influenced by the solvent and pH of the system. The formation of hydroxyamate under the conditions of an ethanol:water film forming solution of 1:1 (pH 8) was very low compared to a wheat gluten tri-acetate buffer (pH 6) solution, indicating low reactivity under film forming conditions. Gan et al. (2008) found that cross-linking can be excessive, as this prevented release of the core product to a certain extent. Although they attributed this excessive cross-linking to the Maillard reaction, as the Tgase did not provide sufficient protection of the core ingredient, even where ε-(γ-glutamyl) lysine bonds were confirmed. There were no differences between Tgase sources (100 U Activa TI vs. 1000 U Tgase) in experiment 7, leading us to conclude that absence of improvement in resistance to degradability was probably not due to excess maltodextrin in the 100 U Activa TI preparation.

Experiment 5

Although no major differences were found between the treatments in terms of degradability, there were some notable differences in viscosity of the film forming solutions and appearance of dried films. The addition of increasing amounts of HCl contributed to the film solution having a thicker and more viscous consistency (Figure 2.6). Concentrated HCl without acetic acid resulted in a dry film that was globular and inconsistent in form. This observation is probably due to the glutenin portion of gluten being soluble in acetic acid (Osborne, 1907) but not HCl; therefore, a superior dispersion of wheat gluten in solution is achieved when acetic acid is used. Liao et al. (2009) found that acetic acid mainly modified wheat gluten by hydrolyzing the amide groups from side chains (especially Gln and Asn), but had minimal effect on peptide bonds. This resulted in less change in peptide molecular size and secondary structure of wheat gluten compared with HCl deamidation. In contrast, HCl denatured the wheat gluten by proteolysis. Acetic acid deamidated gluten, but had less molecular flexibility than HCl deamidated gluten, mainly because the peptides in HCl deamidated gluten were shorter in length. They also noted that acetic acid deamidated wheat gluten has more β-turns because more Glu

and Asp are exposed. This was said to increase the charge density and electrostatic repulsion of protein, thereby improving protein-water interaction and protein surface properties. An additional finding by Liao et al. (2009) was that the HCl wheat gluten solution had a higher disulfide bond content. These findings provide evidence that acetic acid and HCl have a role in final film properties and protein structure.

Experiment 6

In recent research by Wang et al. (2009), heating wheat gluten solutions to 70ºC for 30 min significantly reduced sulfhydryl (SH) groups compared to 10 min, with higher temperatures and longer treatment time resulting in a greater decrease in free SH content. The decrease in SH groups was proposed to be indicative of formation of disulfide cross-linking bonds. From these results, if there were indeed increased disulfide bonds present in the 80ºC 50-min treatment, it did not confer improved barrier properties against microbial degradation.

Conclusions

Processing parameters (of the film forming solution) that optimized resistance to microbial degradation included lowering of the pH to 3 (using mainly acetic acid) and increasing the temperature to 75°C for 10 min. Strategies that proved unsuccessful in improving resistance to ruminal degradation included use of the cross-linking enzyme, transglutaminase; induction of a Maillard reaction; and increasing the time of heating the film forming solution. Wheat gluten demonstrates potential for development of an economical means for encapsulation of micronutrients and amino acids. Wheat gluten films exhibited between 65 and 92% resistance to ruminal digestion (after 8 h) under *in vitro* conditions.

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Figure 2.1 Effect of pH on film degradability *in vitro*

[SEM 1.40; pH linear and quadratic effect, $P < 0.001$; pH effect, $P < 0.0001$; time effect, $P < 0.0001$; pH × time effect, $P < 0.0001$]

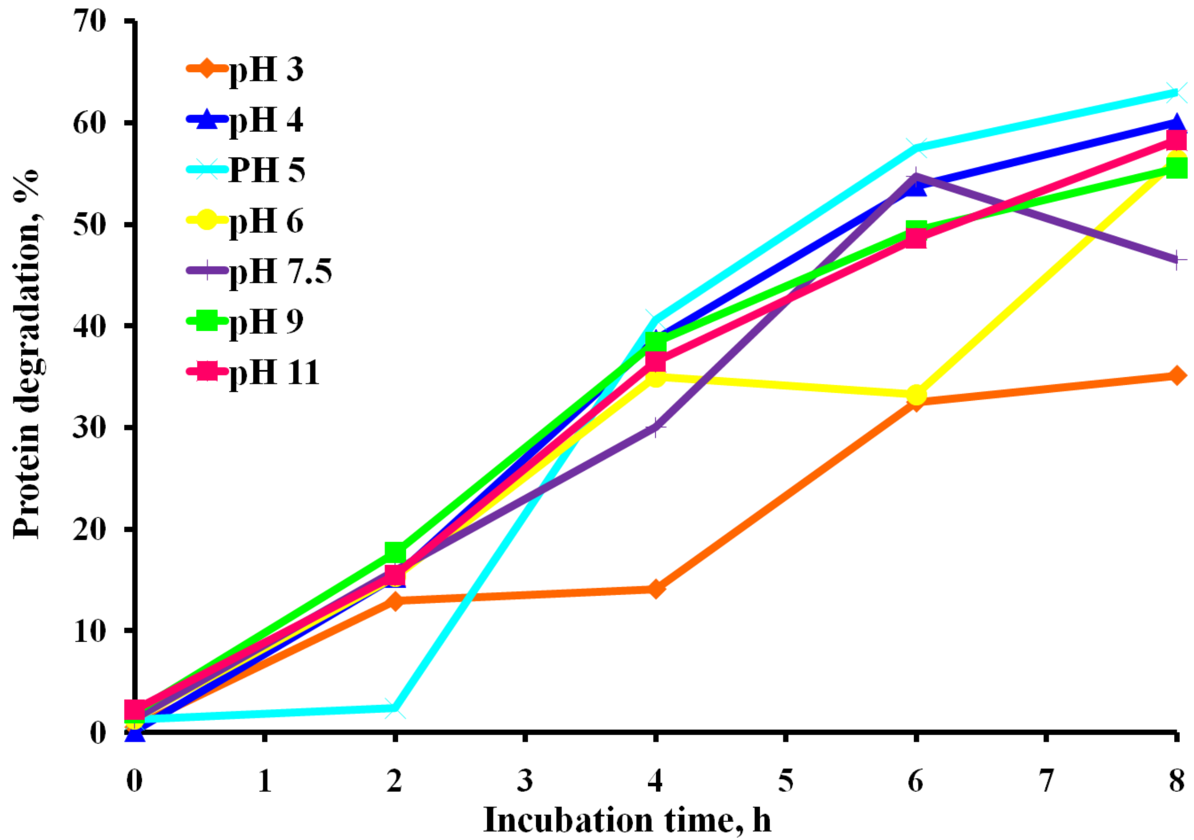


Figure 2.2 ***In vitro*** **protein degradation after 8 hours ruminal fermentation [SEM ranged from 10.34 to 11.43; pH × temperature interaction, $P < 0.01$; quadratic effect of pH, $P < 0.001$; linear effect of temperature, $P < 0.01$]**

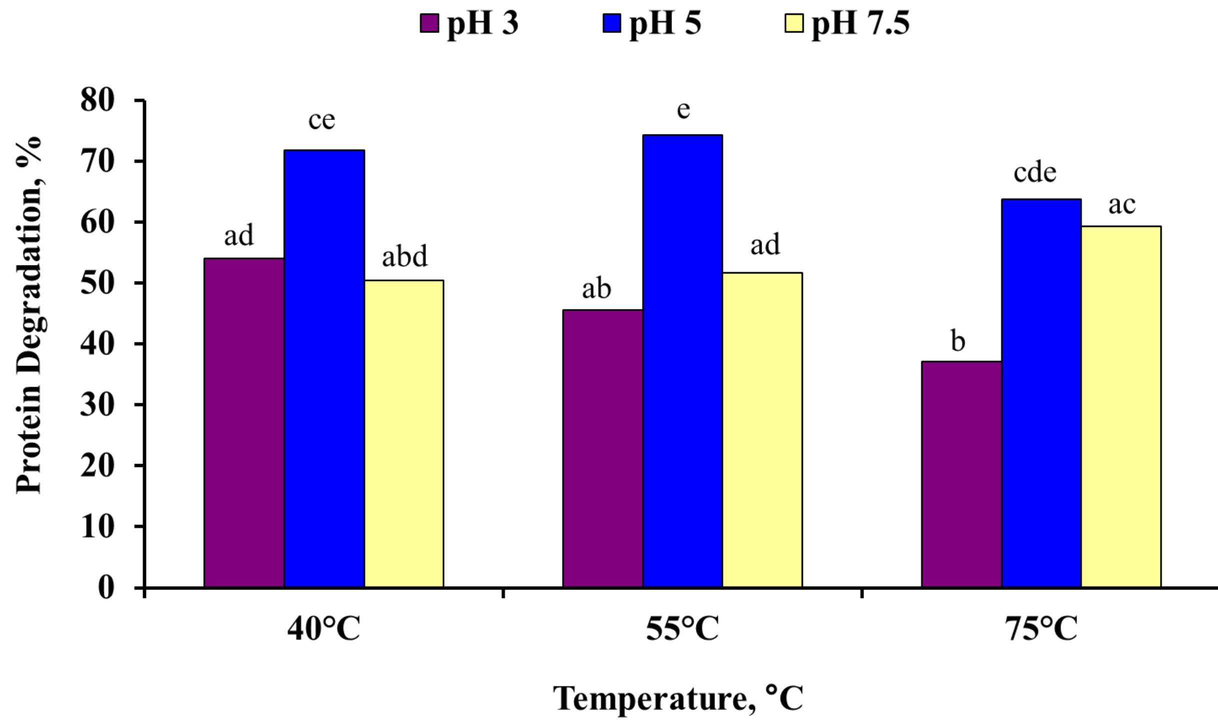


Table 1 *In situ* degradability of films with dissolved lysine with and without xylose [SEM ranged from 0.39 to 0.47; no xylose effect, P > 0.05]

Item	None	Xylose	SEM
Film digested after 12-h incubation,%	46.2	45.6	0.39

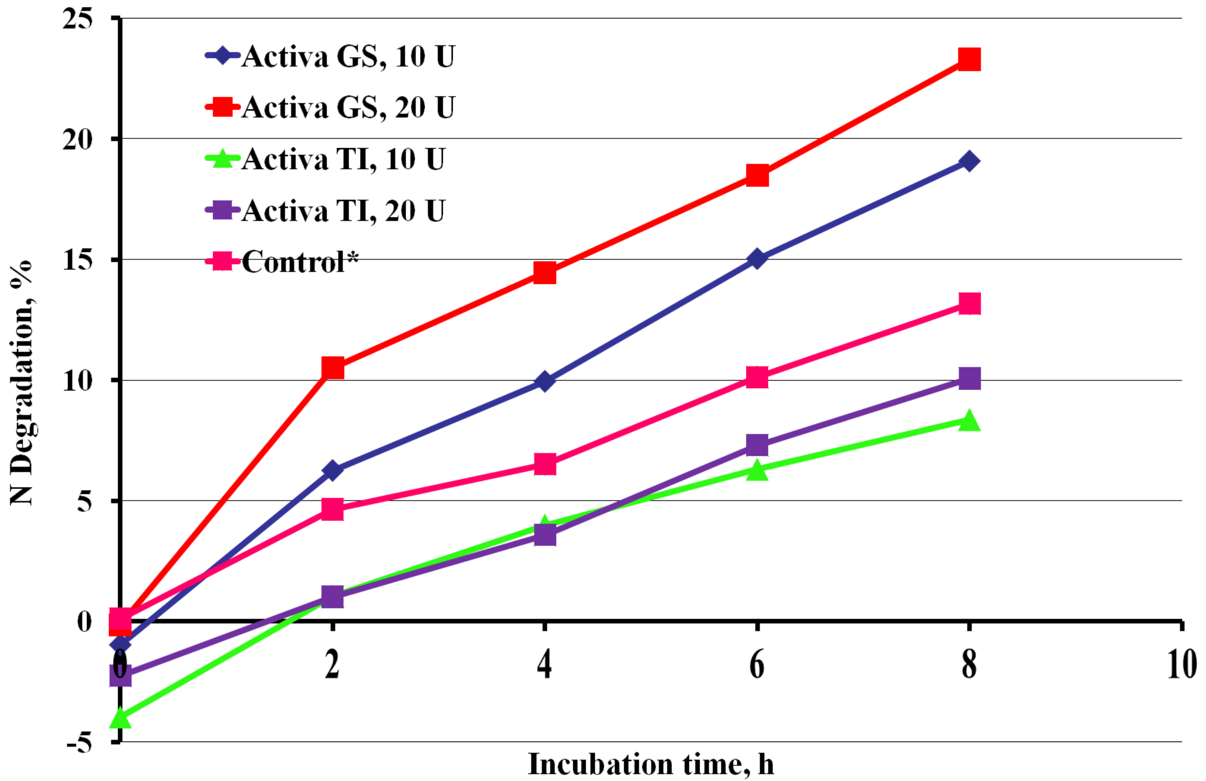
Figure 2.3 a. Film appearance without xylose treatment b. Film appearance with xylose treatment indicative of a non-enzymatic browning reaction

a.

b.

Figure 2.4 Effect of type and amount of commercial transglutaminase (Tgase) preparations (Activa GS and Activa TI) on degradability of films *in vitro*

[SEM ranged from 0.98 to 1.63; effect of Activa GS, ($P < 0.001$); effect of Activa TI ($P > 0.05$)]



*Control films prepared without Tgase

Figure 2.5 Effect of acetic acid and HCl, at pH 3, on degradability of films after 8 h *in vitro*
[SEM 0.79; no treatment effect, P > 0.05]

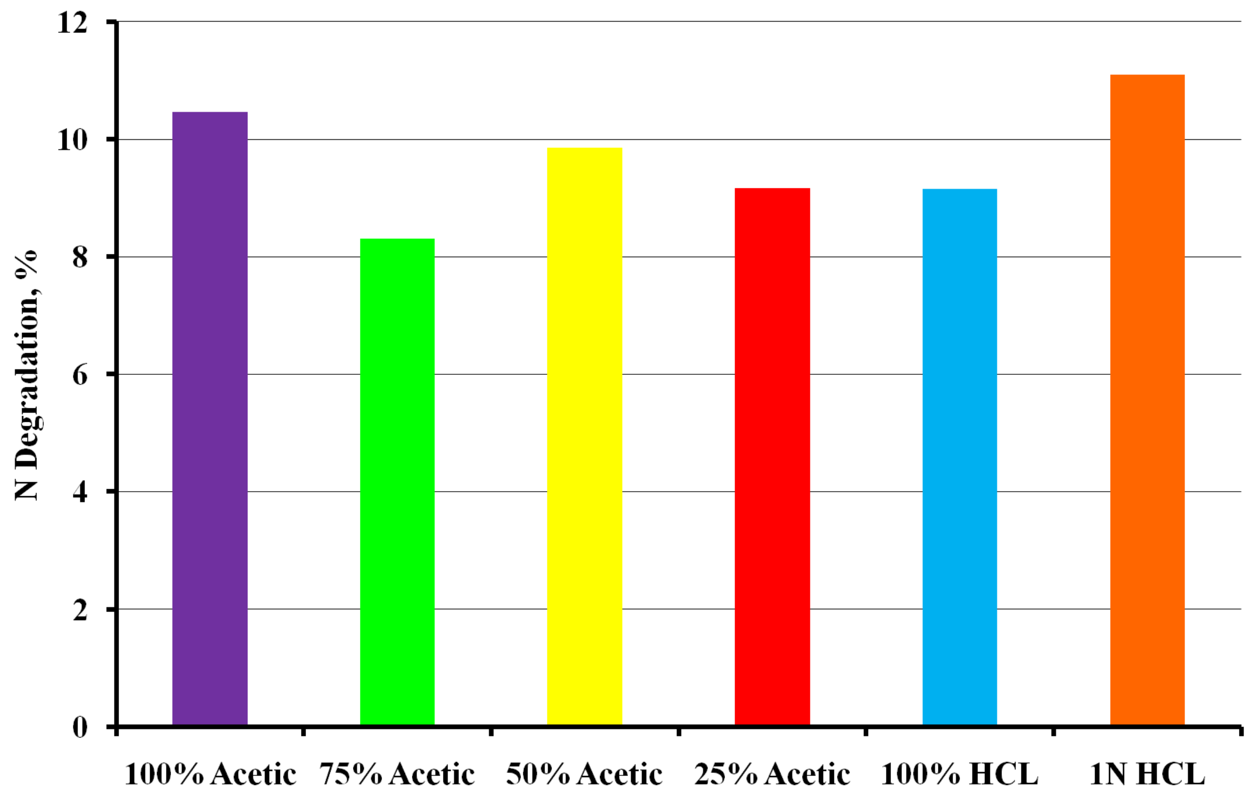


Figure 2.6 a. Appearance of films manufactured with 100% concentrated HCl. b. Appearance of films manufactured with 75% Acetic acid and 25% HCl

a.

b.

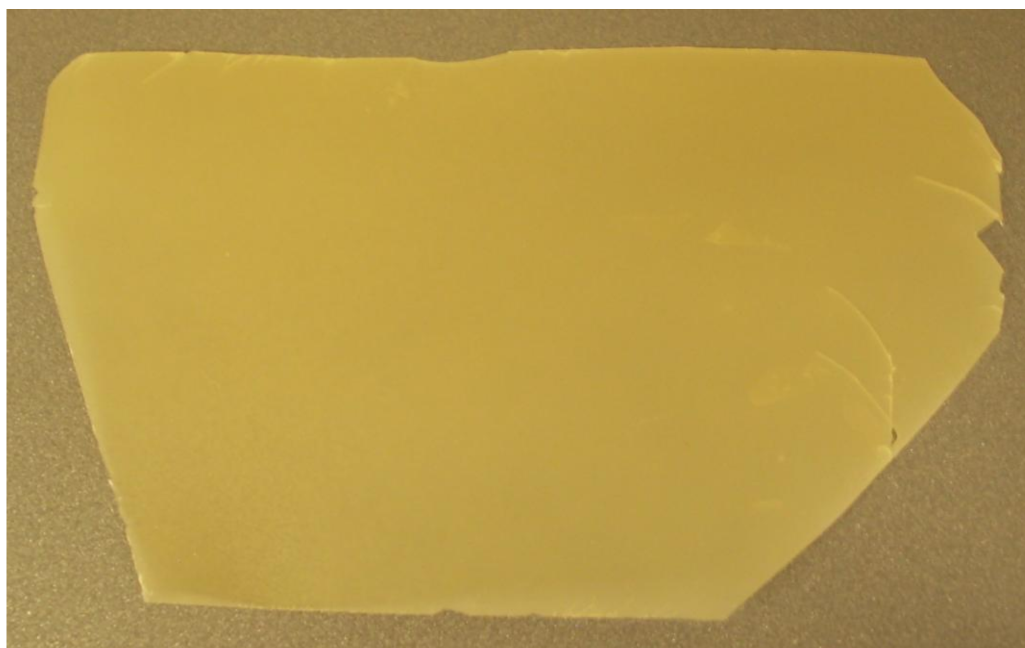


Figure 2.7 Effect of prolonged thermal treatment on degradability of films after 8 h *in vitro* [SEM 0.65; no treatment effect, P > 0.05]

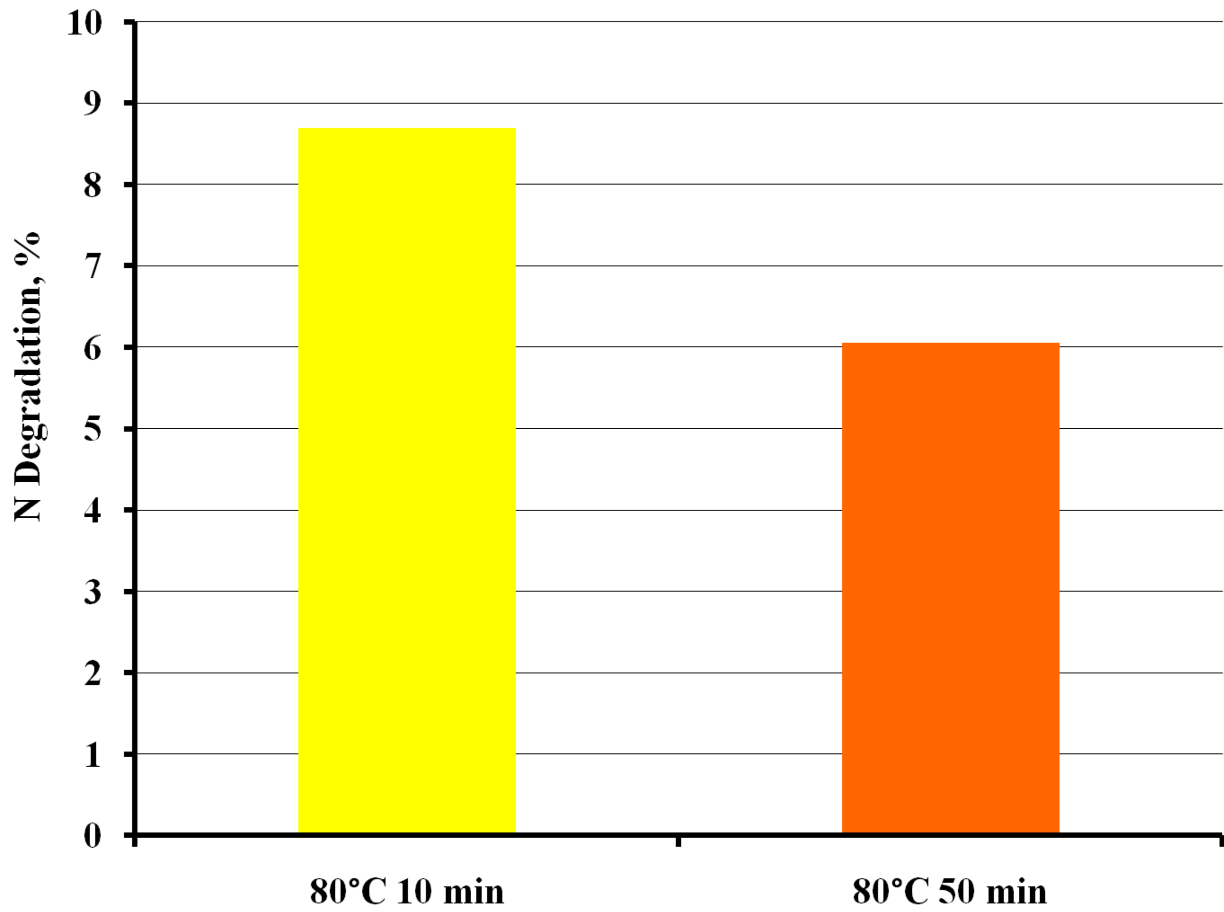


Figure 2.8 Effect of prolonged thermal treatment on degradability of films after 8 h in the Daisy incubator system

[SEM 0.38; no effect of prolonged thermal treatment P > 0.05]

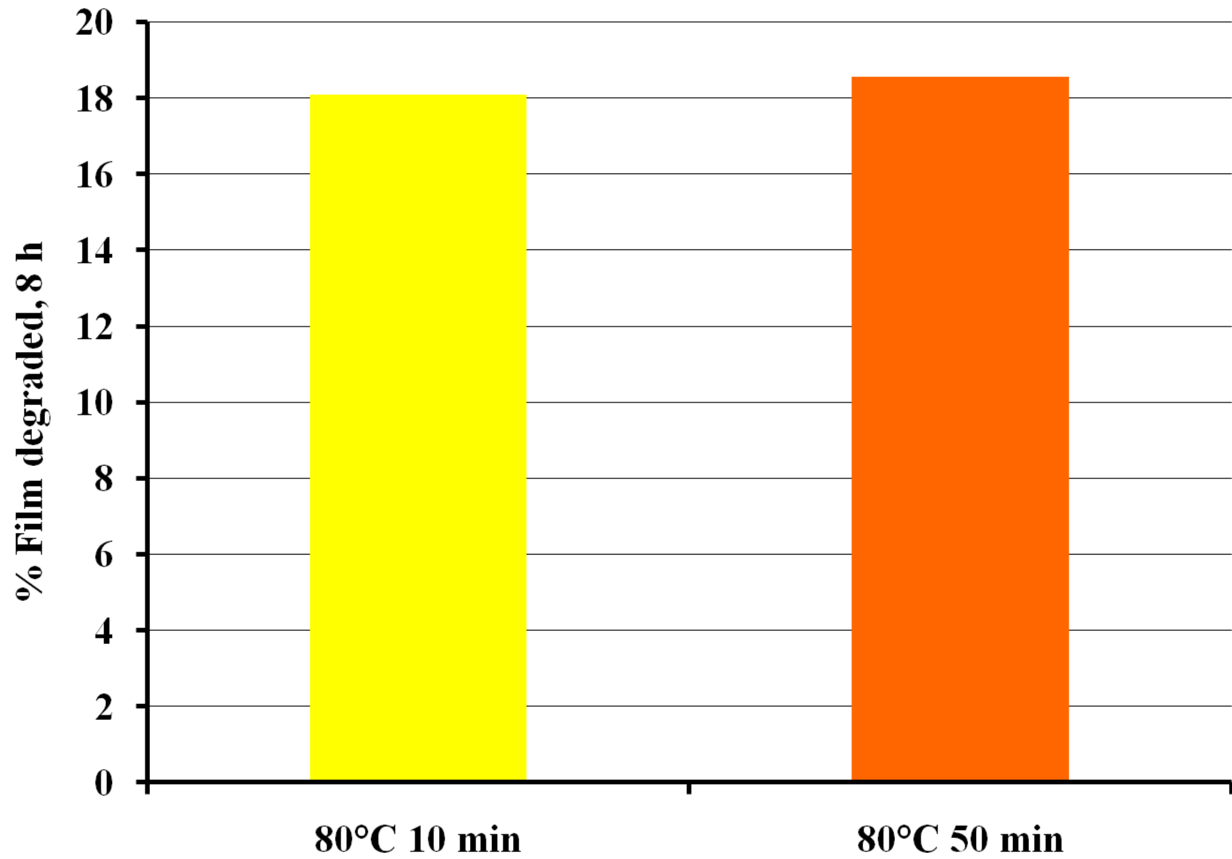
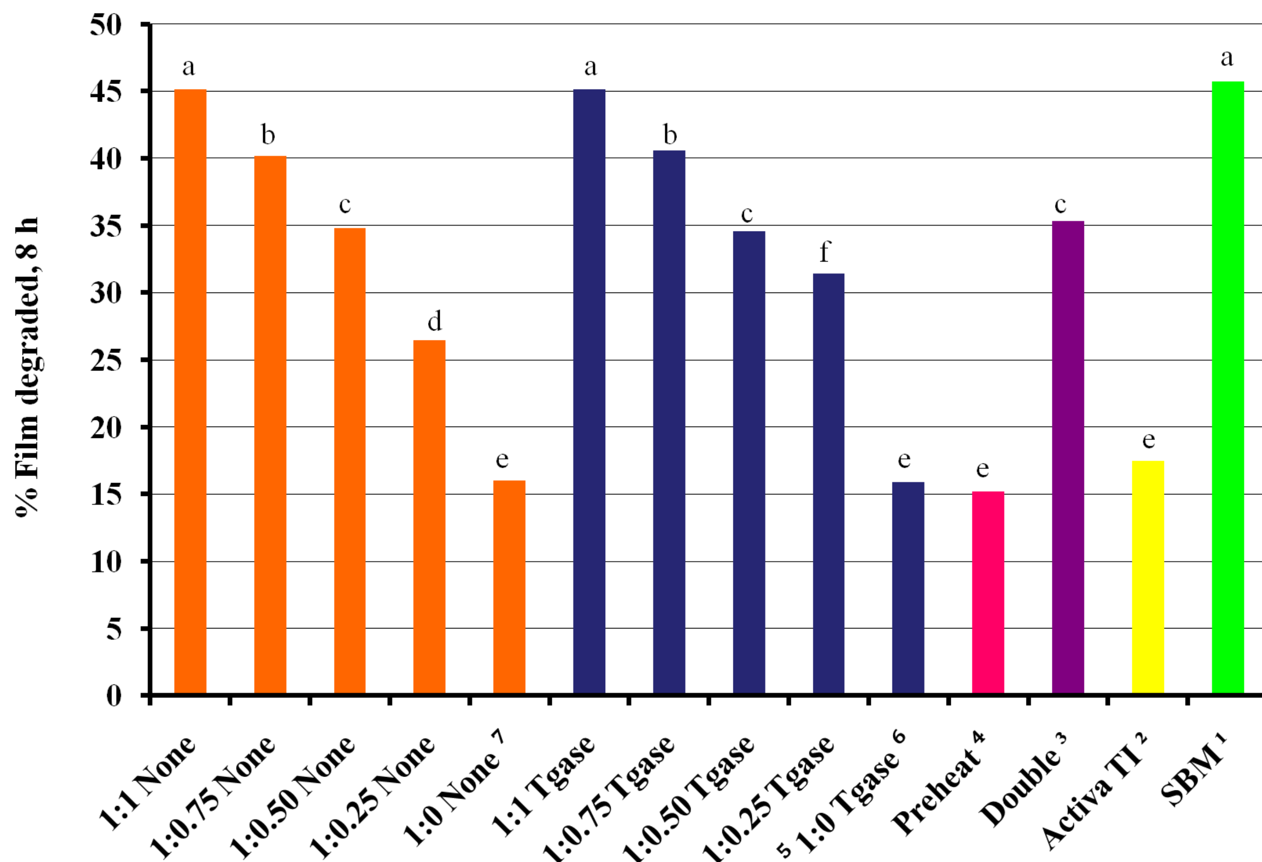


Figure 2.9 Effect of analytical grade transglutaminase with and without added lysine on degradability of films in the daisy incubator system

[SEM 0.65; linear effect of lysine, ($P < 0.05$); no Tgase effect ($P > 0.05$)]



[a,b,c,d,e,f] **Means with different superscripts differ ($P < 0.05$)**

[1] **= Soybean meal**

[2] **= Activa TI transglutaminase (Ajinomoto)**

[3] **= Double quantity of Tgase (Kinry Food Ingredients); with 1:0.5 Gln:Lys**

[4] **= Film solution heated to 80°C prior to reaction with Tgase (Kinry Food Ingredients)**

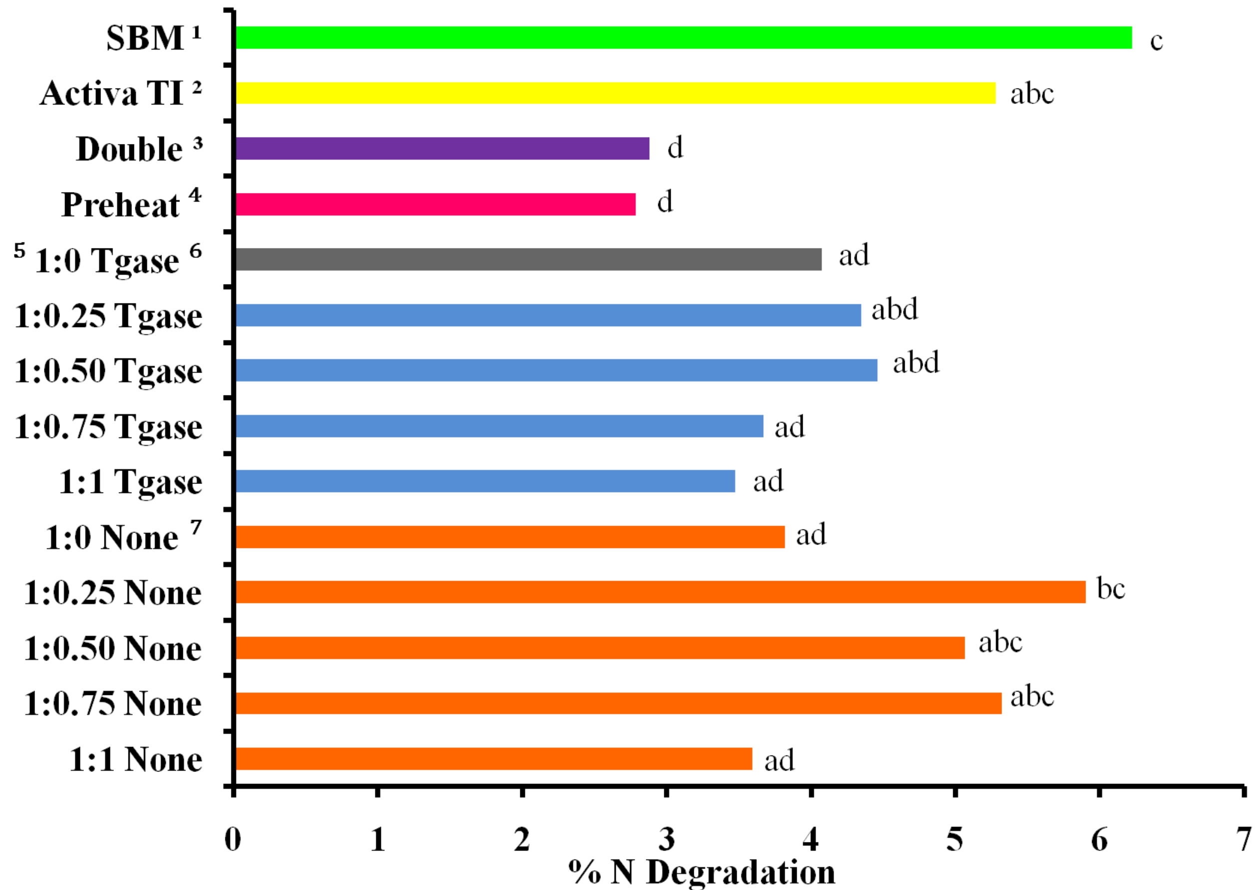
[5] **= Gln:Lys**

[6] **= With transglutaminase (Kinry Food Ingredients)**

[7] **= Without transglutaminase**

Figure 2.10 Effect of analytical grade transglutaminase with and without added lysine on degradability of films *in vitro* after 8 h incubation

[SEM 0.43; no effect of lysine, ($P > 0.05$); no Tgase effect ($P > 0.05$)]



[a,b,c,d] **Means with different superscripts differ ($P < 0.05$)**

[1] **= Soybean meal**

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[5] **= Gln:Lys**

[6] **= With transglutaminase (Kinry Food Ingredients)**

[7] **= Without transglutaminase**

CHAPTER 3 - Encapsulation Strategies with Wheat Gluten and Corn Zein

Abstract

Wheat gluten and corn zein were evaluated for their suitability as encapsulation materials for lysine with the aim to develop rumen protected lysine. In experiment 1, the three-step *in vitro* procedure (Calsamiglia and Stern, 1995) was carried out to determine abomasal digestion of wheat gluten films. To determine how wheat gluten would be digested in the abomasum to liberate lysine, films were either incubated in pepsin or only acidic medium. *In situ* degradability of wheat gluten films at 12 h was 28%. Digestion of wheat gluten films with pepsin was 59%. In experiment 2, gluten and zein film forming solutions were poured over soybean meal and left to dry on silicone baking trays as a method of encapsulation. Soybean meal was chosen as the core ingredient as lysine is soluble in gluten and zein solutions if left in this manner. In experiment 3, lysine was encapsulated with gluten or zein film forming solutions by spray drying. The degree of protection of both soybean meal and lysine was evaluated by *in vitro* ruminal degradation assays. Both gluten and zein encapsulated soybean meal demonstrated reduced degradability ($P < 0.01$) compared to unprotected soybean meal, although zein had a higher degree of protection compared to gluten ($P < 0.01$). Results from experiment 3 were inconsistent between time of incubation in ruminal fluid and the collector from which the spray dried encapsulates were collected (primary vs. cyclone). Neither gluten nor zein effectively protected lysine from ruminal degradation with the spray dry method of encapsulation. Scanning electron micrographs revealed gluten microcapsules from the cyclone collector with a particle diameter of approximately 100 µm. Zein microcapsules from the cyclone collector were approximately 50 µm in diameter. The type of encapsulation achieved by the spray dry method was a matrix encapsulation whereby many cores of lysine were embedded within the wall material. Further research is required to determine the optimal method for encapsulation of lysine with gluten or zein, though both look promising as encapsulating materials to prevent degradation in the rumen.

Introduction

There is considerable interest in protecting lysine and methionine from ruminal degradation as these are the amino acids that generally are considered limiting (Richardson and Hatfield, 1978; Schwab et al., 1992) to ruminant production. Other benefits of protected amino acids are claimed to be reduced nitrogen excretion and feed costs due to decreased crude protein in the diet. Solubility of lysine in water has been one of the most challenging factors to creating a rumen protected product. Lysine is an attractive target for protection based on the low success of companies in creating a viable rumen protected source of lysine. Another limitation to development of rumen protected amino acids has been cost, which is determined by the encapsulation material used and their high cost:benefit. To achieve effective rumen bypass, most encapsulated amino acids require a high percentage of encapsulating material relative to the core amino acid, and considering the sometimes low degree of ruminal protection, very little metabolizable amino acid is actually delivered to the small intestine. Other factors that have hindered optimum performance of encapsulated amino acids have been their susceptibility to acidic environments due to their need for core release in the abomasum. Acid sensitive coatings have been known to degrade in silage based rations for dairy cows (Kenna and Schwab, 1981). Wheat gluten and corn zein have been known for their ability to create coatings or films and for their insolubility in water due to their high content of nonpolar amino acids (Gennadios and Weller, 1990). Both are attractive proteins sources from a cost perspective, although gluten is less costly than zein (approx. $1 vs. $10/kg). Their complex protein structures are conducive to modification to yield properties that suit their application as protective barriers. The objective of this research was to develop an economical and effective means of encapsulating lysine for rumen bypass using wheat gluten or corn zein as the encapsulation materials.

Materials and Methods

Experiment 1: Estimating Abomasal Digestion of Wheat Gluten Films

A pepsin digestibility assay modified from Calsamiglia and Stern (1995) was carried out to determine abomasal digestibility of wheat gluten films. *In situ* digestion of wheat gluten films was measured after 12 h ruminal incubation. Kjeldahl analysis revealed the N content of the *in situ* residue. The *in situ* residue was weighed out to contain 15 mg of residual N into 50 mL centrifuge tubes. Blank tubes also were included without substrate. Ten mL pepsin solution (pH 1.9, 1g/L pepsin, 0.1 N HCl) or 10 mL 0.1 N HCl solution (pH 1.9, without pepsin) was added to each tube, vortexed and incubated for 1 h in a 38°C water bath. As the objective of this experiment was to estimate abomasal digestion, and not intestinal digestion, pancreatin was not utilized. Following incubation 1.5 mL of 100% (w/v) TCA solution were added to halt pepsin activity. Tubes were then centrifuged at 10,000 x g with a Beckman JA-14 rotor for 15 min. The supernatant was removed and 9 mL was subsequently analyzed for soluble N using the Kjeldahl method (AOAC, 1990).

Results were analyzed as a randomized complete block design using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with animal as the block. There were 2 animals with 2 replicates per treatment for each animal. Experimental unit was film. The fixed effect was treatment and random effect was animal. A P-value less than 0.05 was declared significant for this and subsequent experiments.

Experiment 2: Encapsulation of Soybean Meal with Wheat Gluten and Corn Zein Films

The objective of this experiment was to encapsulate soybean meal with either a wheat gluten or corn zein solution. Soybean meal was chosen as the core material as it possesses a relatively smooth surface, it's un-reactive with wheat gluten or zein film forming solutions, and a uniform particle size was easily obtainable using a Ro-Tap sieve shaker (W. S. Tyler, Mentor, OH). Soybean meal was thus chosen as a model core material to obtain proof of concept that

wheat gluten and zein could be utilized to effectively encapsulate a core particle. Approximately 0.25 g of soybean meal (mean particle diameter of 850 μm) was mixed with either 3 mL corn zein solution or 5 mL wheat gluten solution. A larger amount of the wheat gluten solution was required as it was a more viscous and less 'pourable' solution. This mixture was then poured onto a silicone sheet to dry at ambient air temperature.

The wheat gluten solution was made by mixing 54 g (13% w/v) of vital wheat gluten (Manildra Group, Shawnee Mission, KS) and 3 g of glycerol into 200 mL of 95% ethanol. Distilled water was then slowly added (100 mL) to the solution whilst stirring on a stirrer plate. The film forming solution was adjusted to pH 3 with 112.5 mL acetic acid and 0.6 mL HCl. The solution was then sheared for 8 min using a Polysciences CAT homogenizer (model X 120) on setting 4. Thereafter the sheared solution was stirred and heated to 75ºC on a hotplate under reflux. The temperature was monitored by a digital wire thermometer that was inserted into the top of the reflux unit and came into direct contact with the solution. The heated solution was held at the temperature for 10 min whilst stirring and then centrifuged at 959 x g for 5 min at room temperature (20°C) using a Beckman JA-14 rotor to remove any remaining insoluble gluten.

The corn zein solution was made by mixing 18 g zein (Sigma Aldrich, St. Louis, MO) and 2 mL glycerol in 108 mL 95% ethanol. Lastly, 14 mL of distilled water were added to the homogenous mixture. Unlike the wheat gluten film forming solution, the temperature and pH of the zein film forming solution was not altered.

An *in vitro* digestibility assay using a Daisy incubator (Ankom, Macedon, NY) was chosen as the method of analysis to evaluate effectiveness of wheat gluten and zein as protective coatings. The method of incubation was slightly modified according to the "In Vitro True Digestibility" guidelines described by the manufacturer. The films were weighed into Ankom filter bags that were previously rinsed in acetone to remove a surfactant that may inhibit microbial digestion. Each bag, including blanks without substrate, was sealed with a heat sealer and placed into the digestion jar. Ruminal fluid (1000 mL) that was strained and separated was combined with McDougall's buffer (1000 mL) and added to each digestion jar. The digestion

jars were purged with CO_2 for 30 seconds and placed into the Daisy incubator to incubate for 8 h. After 8 h the jars were drained of fluid and the bags rinsed with water until clear. The bags were then dried for 24 h in a 105°C oven. The dried bags were weighed to determine residual DM.

Results were analyzed as a randomized complete block design using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with day as the random effect. Block was by day of rumen contents collection. There were 2 replicates per treatment. The fixed effect was treatment.

Experiment 3: Encapsulation of Lysine with Wheat Gluten and Corn Zein by the Spray Dry Method

Wheat gluten film forming solution was previously prepared in the lab at Kansas State University and transported to the University of Nebraska-Lincoln (UNL) Food Processing Center. Zein-based film forming solution was prepared on day 2 at the Food Processing Center. Both wheat gluten and zein solutions were made in the exact manner stated in the previous experiment. Lysine was encapsulated by the respective solutions with the aid of the Henningsen Pilot Plant Tower, model T-20, spray drier (Henningsen Foods Inc., Omaha, NE) custom designed for the Food Processing Center. Inlet temperature of the spray dryer was 193 ± 5ºC and outlet temperature at 103 ± 5ºC. The spray nozzle was 2 mm in diameter with a pump flow rate of 47 g/min and an atomizing air pressure of 1.4 to 1.7 atm. Lysine was encapsulated with either wheat gluten or zein as the coating material at a ratio of 70% Lys and 30% coating by weight. Lysine was ground with a coffee grinder and mixed into the respective solution and subsequently fed into the spray dryer. The dried product recovered from the primary collector consisted of large particles, and that recovered from the cyclone collector consisted of small particles.

Due to the fine powder-like nature of the resulting dried product, an *in situ* or Daisy analysis of the product was not possible, as the product was likely to escape out the pores of the Ankom bags. For this reason an *in vitro* ammonia release assay was carried out on the spray dried product using a technique similar to that reported by Russell (2006). This study consisted

of a randomized complete block design with partial repeated measures as 24 and 48 h data were collected from the same tube. Data were analyzed as a 2×2×6 factorial plus one (the extra treatment was the control, unprotected lysine). Factor 1 was collector (primary or cyclone), factor 2 was protein (wheat gluten or corn zein), and factor 3 was treatment. There were 6 treatments, with 5 of them having varying ratios (100:0, 75:25, 50:50, 25:75, 0:100) of encapsulated Lys : unprotected Lys + spray dried film solution. The 6th treatment consisted of spray dried coating only (without lysine). In each case, 25 mg of total Lys were present per tube. For the primary collector, only the following treatment ratios were included in the assay: 100:0, 50:50 and 0:100.

Ruminal fluid was obtained from a cannulated steer fed a forage-based diet and strained through 4 layers of cheesecloth. The strained rumen fluid (SRF) was then filtered through eight layers of cheesecloth, and bubbled with carbon dioxide to eliminate oxygen. The SRF was transferred into a separatory funnel and allowed to stratify for 30 min. During this time, 40 mL McDougall's buffer (without urea) were added to each tube. Once the ruminal fluid had separated, 10 mL of the middle layer, devoid of fibrous matter and rich in microorganisms, was added to each tube before gassing with CO_2, vortexing, and topping with rubber stoppers. Yeast extract, trypticase, maltose and inhibitors were not included in this assay. Tubes were incubated for 0 and 48 h in a shaking water bath at 39°C. At the end of incubation, tubes were vortexed and 1 mL samples were transferred to micro-centrifuge tubes and acidified with 0.2 mL 1N HCl to cease fermentation. A 24 h sample also was taken from the 48 h tubes whilst gassing with CO_2. The samples were frozen until analysis for NH_3 on a BioTek PowerWave XS plate reader.

Results were analyzed using the Mixed procedure in SAS (version 9.2; SAS Inst., Cary, NC) with day as the random effect. Block consisted of day of rumen contents collection. There were 2 replicates per treatment and time. The fixed effects were protein type (wheat gluten or zein), treatment, time and all interactions. Given that there were fewer treatments for the primary than the cyclone collector, we decided to analyze the collectors separately using the same procedure.

Results

Experiment 1

Wheat gluten films were approximately 28% degraded *in situ*. When the film residue from *in situ* was incubated in pepsin solution (pH 1.9), 59% was digested (Figure 3.1). Degradation of the wheat gluten film in acid conditions (pH 1.9) for 1 h at pH 2, but without the addition of the pepsin enzyme, resulted in a low degradability of less than 2%. Digestibility was lower when incubated in acid rather than pepsin ($P < 0.001$) solution.

Experiment 2

Degradability of encapsulated soybean meal with wheat gluten and corn zein and the respective films alone, using the Daisy incubation system, are presented in Table 2. Zein was more resistant to microbial degradation compared to wheat gluten ($P < 0.01$), though wheat gluten itself as a coating material is only 11% more degraded than the zein film alone. Soybean meal was degraded more when it was not protected by either wheat gluten or zein ($P < 0.01$). By taking the weighted average of wheat gluten film (61%) and soybean meal (39%), we estimated that the amount of soybean meal degraded in the wheat gluten encapsulated soybean meal was approximately 35%. Considering that unprotected soybean meal was 44% digested in this experiment we can conclude that there is modest protection of soybean meal with wheat gluten. Zein encapsulated soybean meal was approximately 54% zein and 46% soybean meal by weight. Soybean meal was therefore estimated to be 28% degraded within a zein encapsulate, which, when compared to 44% degradability of unprotected soybean meal, demonstrates some protection of soybean meal.

Experiment 3

Ammonia release after 48 h for wheat gluten and zein encapsulates from the cyclone collector is displayed in Figure 3.3. There was a protein effect, whereby wheat gluten was more extensively degraded compared to zein ($P < 0.0001$). There were both treatment and time main effects ($P < 0.0001$). There was also a treatment × time interaction ($P < 0.0001$).

Discussion

Experiment 1

Watanabe et al. (2002) evaluated rumen protected lysine that was coated with a pH sensitive polymer and found that abomasal release of Lys in vivo was approximately 24%. Although in vivo studies were not done in our experiment, the *in vitro* studies look promising, as approximately 28% of the film is degraded in the rumen after 12 h, with almost 60% being degraded in a simulated abomasal environment with pepsin at pH 2 for 1 h (Figure 3.1). The extent of digestion should be sufficient for complete release of the core product in the small intestine. Although a 2% digestion of the wheat gluten films in acidic medium (without pepsin) suggests that wheat gluten films will not be susceptible to degradation when added to acidic diet ingredients such as silage, these results may underestimate the degradability. The addition of TCA in this procedure would have precipitated any partially degraded protein which could have resulted from the 0.1 N HCl treatment. Certain commercially available products, such as hydroxymethyl lysine (HML), have been suggested to be acid labile and will release the core product when mixed with silages (Kenna and Schwab, 1981). Considering that dairy diets often contain high percentages of silage, this is a major limitation of these products for dairy producers. Based upon visual assessment, as films were still intact after incubation with 1 N HCl compared to films incubated with pepsin, we conclude that 2% digestion with 1 N HCL is not unrealistic.

Experiment 2

The technique employed for crude encapsulation is similar to pan coating, which is one of the oldest encapsulation techniques, whereby particles (usually larger than 600 microns) are tumbled in a pan or drum with the coating material, thereby slowly covering the particles (Venkatesan et al., 2009). The coating procedure thus used in this experiment was a sure method for creating an encapsulate. The only downfall is that a lot of material was required (approximately 61% wheat gluten coating: 39% SBM and 54% zein coating: 46% SBM) to effectively coat the soybean meal, and therefore would not be desirable for industrial application where the least amount of coating is preferred due to cost considerations.

Experiment 3

Lysine encapsulated with wheat gluten from the secondary collector demonstrates protection to some extent, as there is a difference ($P < 0.05$) between the 0:100 and 100:0 treatment at 48 h, with the 0:100 treatment yielding a higher ammonia concentration. Irregularities between incubation time (24 vs. 48) and collector (primary vs. cyclone) suggest that encapsulation with wheat gluten provided only modest protection of lysine. Lysine encapsulated with zein at 48 h (cyclone collector) did not show the same trend as wheat gluten (when comparing 0:100 and 100:0 treatments), providing little evidence of a protective effect. Lopez-Alvarado et al. (1994) attempted to create a diet for marine larval fish by binding zein with a mixture of amino acids, but found that most of the amino acids leached after a 2 min immersion in water. They also concluded that microencapsulation of these amino acids using crab protein was a superior alternative, but lipid walled capsules yielded optimum retention of amino acids. In the present study, certain intermediary treatments, such as the WG 25:75 (containing 25% encapsulated lysine and 75% of the spray dried wheat gluten and lysine counterparts together), did not differ from the control WG 0:100. The reason to why this occurred is uncertain. Overall, zein was degraded to a lesser extent than wheat gluten. Gennadios et al. (1993) observed that when 20% of the wheat gluten was replaced with zein there was improved resistance (by about 23%) to water vapor permeability. Zein is generally viewed as a superior barrier to water when compared to wheat gluten (Aydt et al., 1991; Gennadios et al., 1993). This may be attributed to zein's high hydrophobicity, even higher than gluten (Taylor and Belton, 2002).

Particle size obtained from the primary collector was approximately 200 μm, while the powder collected from the secondary collector was approximately 100 μm in size. Scanning electron micrographs (SEM) revealed that particles for either collector were not uniform in size and were irregular in shape (Figure 3.2). Zein and wheat gluten microcapsules were clearly fractured in various places and large holes were visible in the coating. There could be multiple factors that resulted in a weak or compromised microcapsule. The ratio of encapsulating (wall) to core material may not have been high enough to achieve a barrier strong enough to withstand handling of the material. It is often stated that the typical ratio of wall to core material is 4:1 (Risch, 1995; Gibbs et al., 1999), although this is mainly for flavor encapsulation. Our ratio of wall to core material was much lower (1:2.3). Additionally, for optimum encapsulation, the wall

material must have no reactivity with the core product (Madene et al., 2006; Re, 1998), but in our case the lysine is soluble (although not readily) in both zein and wheat gluten solutions and since the wall and core materials were mixed prior to atomization, there may have been partial solubilization of core product. The wheat gluten solution was especially viscous and difficult to handle, and often plugged the tubing that led to the atomizing nozzle. For this reason, close monitoring of the process was required to ensure that the tubing did not burst. Viscosity of the solution has also been shown to impede the atomization process due to difficulty in droplet formation and the tendency to produce large droplets that affect drying of the product (Rosenberg et al., 1990). Other spray drying parameters that may have hindered effective encapsulation include atomization and drying conditions. Because atomization determines the distribution of droplet sizes this could have affected efficiency of encapsulation. Zein solutions were the most problematic from the standpoint that they most readily formed a film on the inside of the spray drying equipment when the spray dryer was left for a short period of time. This blocked flow of solution to the atomizer and was difficult to remove. If applied commercially, the spray drier unit would need to be cleaned after each use, before a dried film crust can form, and could be costly and time consuming. Rosenberg and Young (1993) encapsulated anhydrous milkfat with whey protein using the spray dry method and discovered that the 'large' capsules (appox. 25 μm) had holes in the whey coating. They attributed this to the high drying temperature (160°C) which can cause excessive pressure in the larger capsules whilst drying and lead to rupturing. This has been documented in other research and is known as the "ballooning temperature" (Re, 1998). As we dried the microcapsules at similar temperature to Rosenberg and Young (1993), and considering our particles were much larger, this seems a plausible explanation for the large holes. There are also numerous craters on the surface of the capsules, which could have resulted from shrinkage of the capsules during the beginning stages of the drying process (Re, 1998). The void inside the microcapsules is due to water, that when turned to vapor, causes expansion inside the particles during drying leading to formation of air pockets. Since the solubility of gasses in water decreases with an increase in temperature, dissolved gasses (air) diffuse out of the liquid during the drying process, also contributing to the formation of air pockets. This should therefore be encapsulation of the matrix type consisting of multiple cores of lysine embedded within the wall/coating (Re, 1998) instead of the 'hens egg' type of encapsulation (Desai and Park, 2005) which we expected.

Encapsulating lysine with zein by spray drying with the aim of protection against microbial degradation in the rumen has previously been attempted (Yoshimaru et al., 2000). Yoshimaru (2000), however, integrated lysine into porous starch before coating the product with Eudragit, AS-HF and finally, zein. The final percent lysine in the microcapsules was 25%, and they reported that the microcapsules, which were 20-30 μm, had an "almost smooth" surface. Incubated in phosphate buffer, the ruminal stability was between 83 and 88% after 48 h, whereas about 70% of lysine was released in a pH 3 citrate buffer and 90% released in a pepsin solution after 30 min. This observation proves that zein is acid sensitive, unlike wheat gluten; however, the exact procedure for producing the zein coating was not explained, therefore cannot be compared to our microcapsules. As their lysine microcapsules were triple coated and had a 45% higher load of coating material compared to our microcapsules, the fact that 90% of the lysine was released demonstrates zein's suitability for release of core materials in the abomasum.

Conclusions

In situ and pepsin digestion experiments confirm that wheat gluten films are approximately 72% resilient to microbial degradation in the rumen and sufficiently digested with pepsin to be able to release most, if not all, of the core materials under acidic conditions close to that found in the abomasum. Both wheat gluten and zein were effective at protection of soybean meal; however, further research is required in order to effectively encapsulate lysine. Zein was more resistant to ruminal degradation and provided a higher protection for soybean meal compared to wheat gluten. The drawback to zein is that it is more expensive than wheat gluten. Its digestion in pepsin has not yet been evaluated, although due to its similar properties and structure to wheat gluten, this should not present problems. Both wheat gluten and zein show potential for development of encapsulated products for ruminal protection although the preferred encapsulation process has yet to be identified and may differ between the proteins.

Studies anticipated in the future will be aimed towards optimization of the encapsulation process. Technologies such as fluidized bed will likely be investigated to determine if this is better suited to our goals. The coating thickness can be controlled to a greater extent and the coating can be sprayed directly onto the particles, unlike spray drying whereby a suspension of

the core and wall materials is required prior to spraying, thereby reducing the chances of solubilization of the core particle. As commercial encapsulated products typically are not utilized immediately after manufacture, the integrity and stability of the coating during storage is critical to ensuring the functionality of the product. Future studies will therefore include evaluation of the final encapsulated product during storage. Additionally, upcoming studies will include assessment of the bioavailability of the core nutrient by animal feeding trials. This would either include measurement of blood plasma levels of the core nutrient or animal performance indices, such as milk yield and milk protein.

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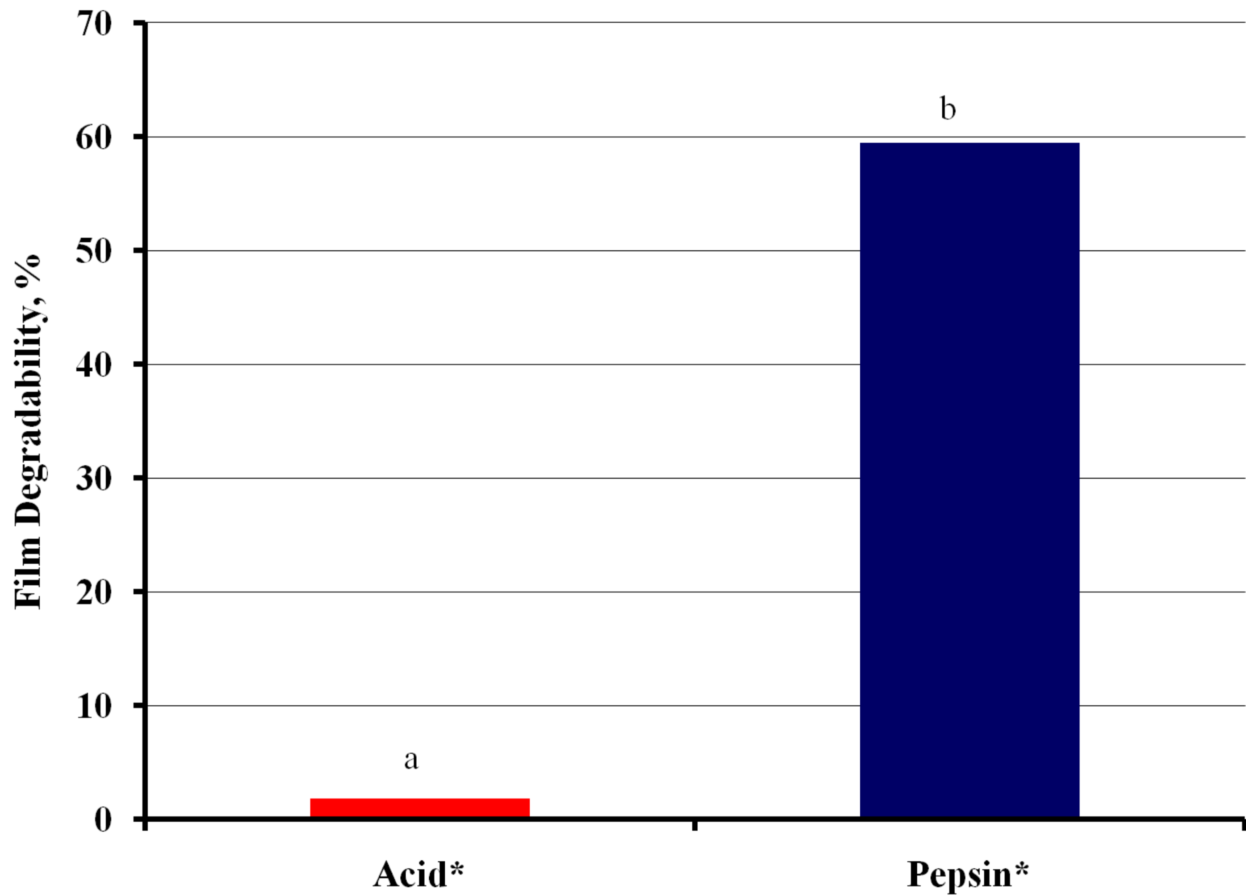
Figure 3.1 Acid and pepsin digestibility of wheat gluten film
[SEM 2.56; treatment effect, P < 0.05]



[a,b] **Means with different superscripts differ (P < 0.05)**

***Film degradability of acid and pepsin treatments (pH = 1.9) was measured from film portion that was rumen undegraded.**

Table 2. Degradability of wheat gluten and zein encapsulated soybean meal and wheat gluten and zein films after Daisy incubation in buffered ruminal fluid

Item	WG+SBM[1]	WG[2]	Zein+SBM[3]	Zein[4]	SBM[5]	SEM
% Degraded	24.57[a]	18.00[b]	16.48[b]	6.95[c]	44.21[d]	1.58

[a,b,c,d] Means in a row with different superscripts differ ($P < 0.05$)

[1]WG+SBM = wheat gluten encapsulated soybean meal

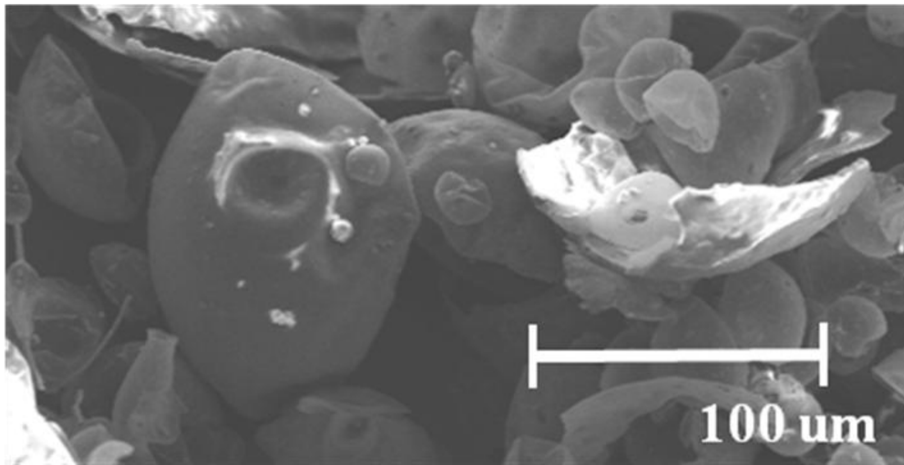
[2]WG = wheat gluten film

[3]Zein+SBM = zein encapsulated soybean meal

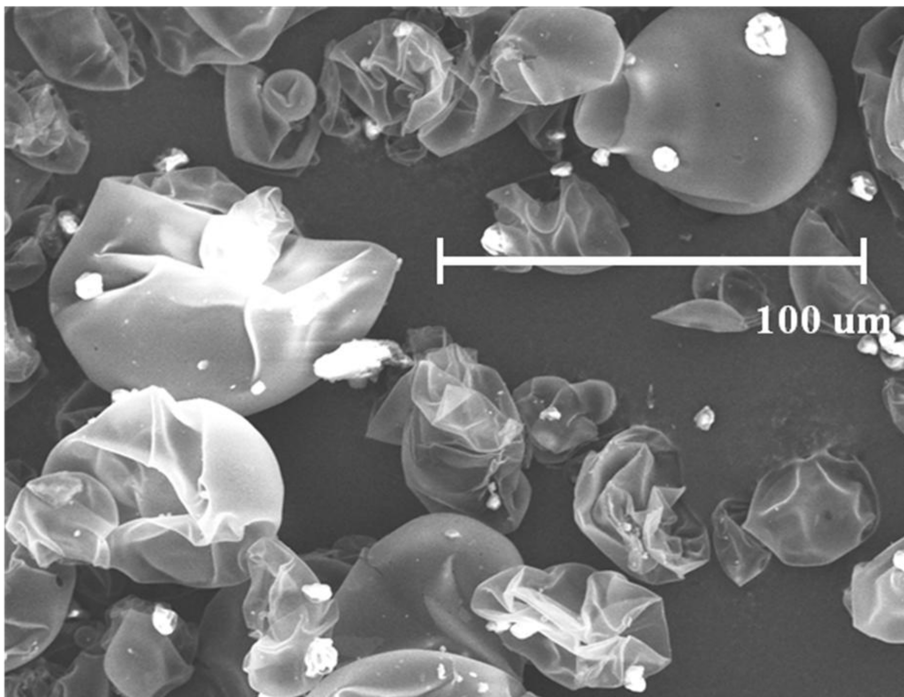
[4]Zein = zein film

[5]SBM = soybean meal

Figure 3.2 a. Spray dried wheat gluten without lysine core; b. Spray dried zein without lysine core; c. Wheat gluten encapsulated lysine (cyclone collector); d. zein encapsulated lysine (cyclone collector); e. Wheat gluten encapsulated lysine (primary collector); f. zein encapsulated lysine (primary collector).



a.



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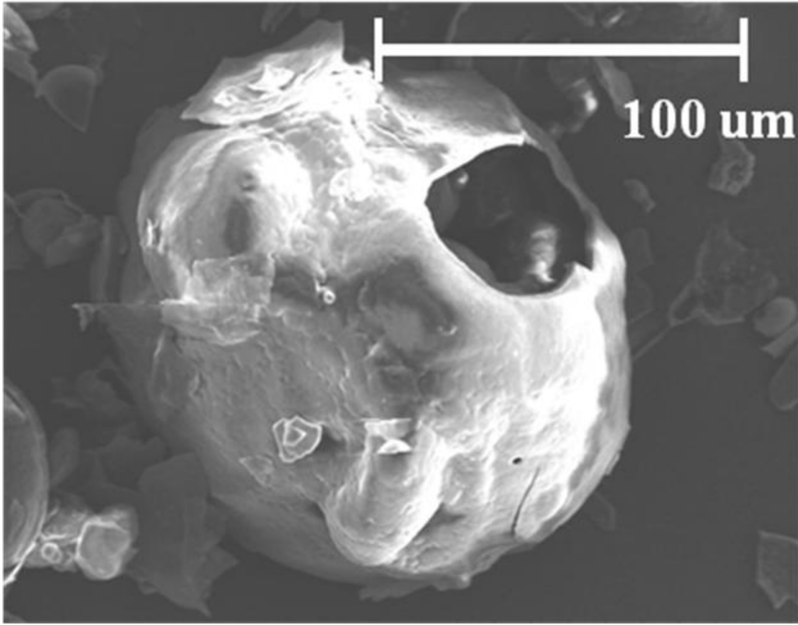
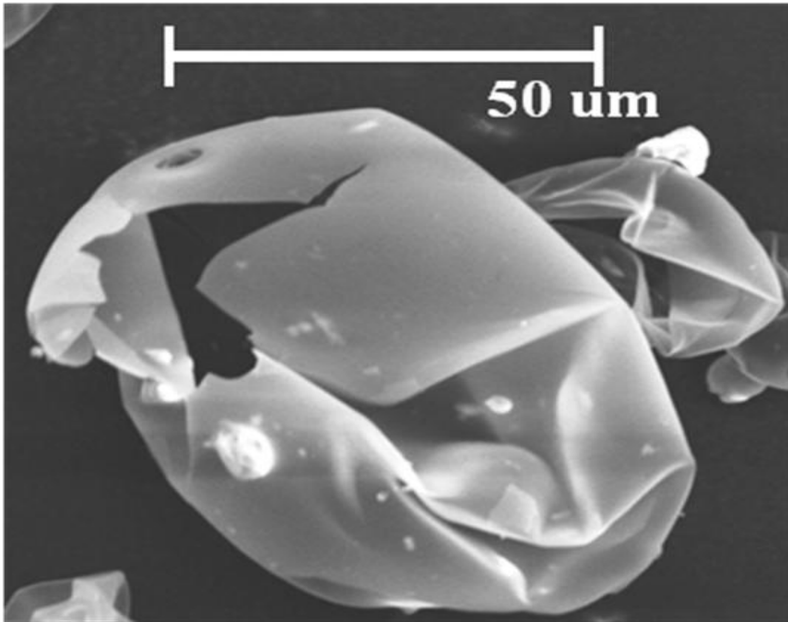
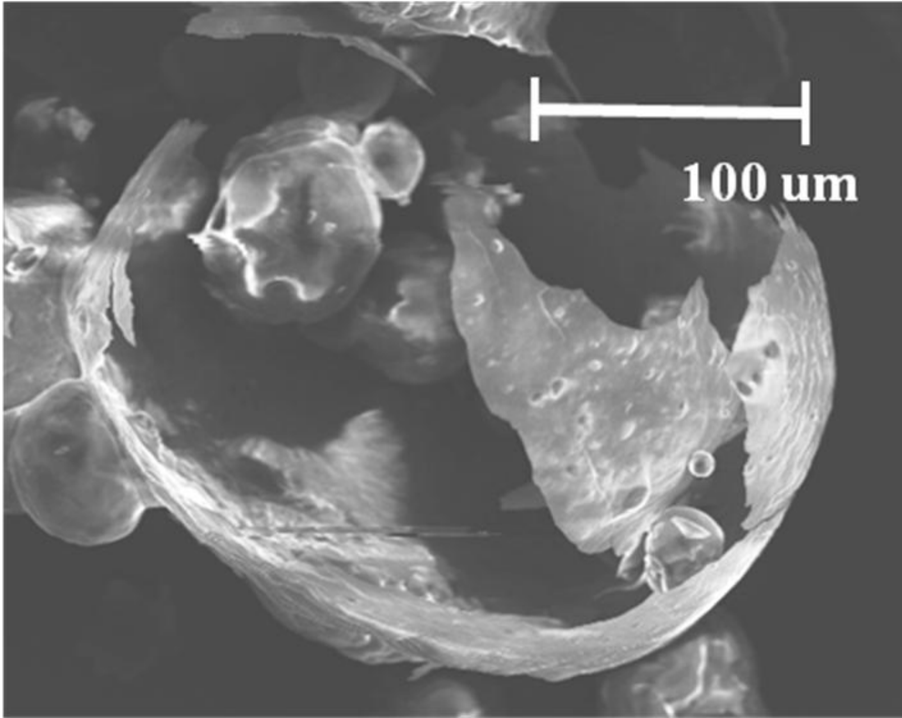


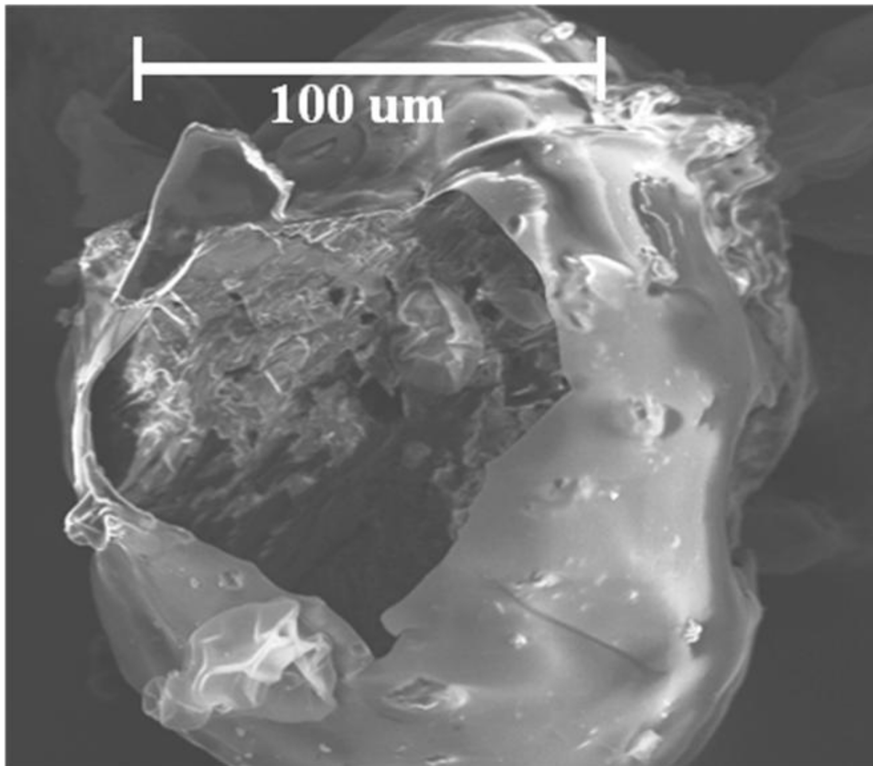
c.



d.

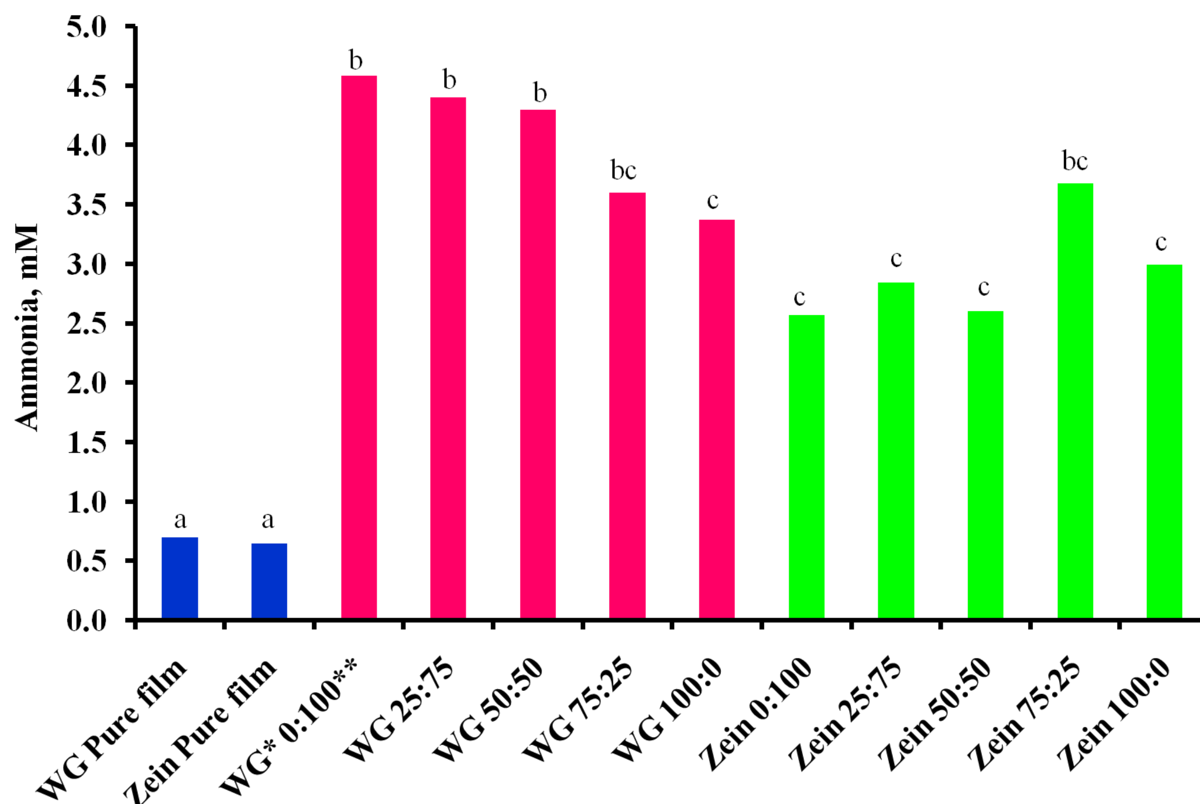


e.



f.

Figure 3.3 Ammonia release after 48 h for spray dried wheat gluten and zein encapsulates from the cyclone collector
[SEM 0.34; treatment effect, $P < 0.0001$; protein effect, $P < 0.0001$]



[a,b,c] Means in a row with different superscripts differ ($P < 0.05$)

***WG = wheat gluten**

****Ratio = encapsulated lysine : unprotected lysine + spray dried film**