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CHARACTERIZATION OF HEAT CURED AND TRANSGLUTAMINASE CROSS-LINKED WHEY PROTEIN-BASED EDIBLE FILMS

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CHARACTERIZATION OF HEAT CURED AND TRANSGLUTAMINASE CROSS-LINKED WHEY PROTEIN-BASED EDIBLE FILMS

By

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ABSTRACT

CHARACTERIZATION OF HEAT CURED AND TRANSGLUTAMINASE CROSS-LINKED WHEY PROTEIN-BASED EDIBLE FILMS

By

Samir Amin

Edible whey protein films were produced using whey protein isolate (WPI), glycerol and candelilla wax. Films were also produced with the addition of microbial transglutaminase (TG) to the film forming solution after heating. Both sets of films were vacuum heat cured for 0, 12 and 24h at 90°C. The effect of heat curing and transglutaminase treatments on mechanical properties [tensile strength (TS), elongation at break (%E) and toughness], the free sulfhydryl, disulfide, and lysine contents and degree of cross-linking of WPI films were assessed. Heat curing increased the TS of WPI and WPI/TG films compared to uncured films. Heat curing films for 24h had increased %E compared to other films. Heat curing increased the toughness of WPI films. Heat curing decreased the free sulfhydryl content of WPI and WPI/TG films. The disulfide bond content of WPI films heat cured for 12h was higher than for films heat cured at 0 and 24h. The disulfide content of WPI/TG films heat cured for 12 and 24h was higher than for films at 0 and 48h. Heat cured WPI films had lower lysine content compared to uncured films. WPI/TG films heat cured for 24h had lower lysine content compared to films heat cured at 0 and 12h. SDS-PAGE was used to confirm heat curing and TG

treatment resulted in the formation of covalent cross-links, between whey protein fractions.

The water vapor permeability (WVP) of WPI and WPI/TG films heat cured for 0 and 24h was determined at 37.8°C and 85% RH as well as the oxygen permeability at 23°C and 0% RH. Moisture sorption isotherms (MSI) of films were determined at 23°C and 5°C using the Guggenheim-Anderson-de Boer equation (GAB). Heat curing WPI/TG films reduced the WVP compared to uncured WPI, WPI/TG and cured WPI films. The addition of TG reduced the oxygen permeability (O_2P) compared to films without TG. Heat curing reduced the O_2P of films compared to uncured films. Heat curing of WPI/TG films produced the greatest reduction in O_2P of films tested. The moisture sorption isotherm of WPI and WPI/TG films heat cured for 0 and 24h at 90°C were found to closely follow the GAB model at 5 and 23°C. The films MSI, at 5°C were higher than the MSI of films at 23°C.

The ultraviolet and visible light transmission and the color characteristics of WPI and WPI/TG films heat cured for 0 and 24h were assessed. Heat curing increased the yellowness of films. All films had low transmission of ultraviolet light.

The effect of heat curing and transglutaminase treatments on the thermal properties (melting onset temperature, peak melting temperature and enthalpy of fusion) of whey protein isolate films with and without transglutaminase, and heat cured at 90°C for 0, 12 and 24h was assessed. The peak melting temperatures for films increased with heat curing and time.

This dissertation is dedicated to Theresa, Chandrakant and Marilyn

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"If I have seen further it is by standing on the shoulders of giants."

Isaac Newton, *Letter to Robert Hooke, February 5, 1675*
English mathematician & physicist (1642 - 1727)

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For those I have not acknowledged by name, thank you for your support both professional and personal.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED IN THIS THESIS

ASTM	American Society for Testing and Materials
AOAC	Association of Analytical Communities
CW	Candelilla Wax
DTNB	5, 5'- dithiobis (2-nitrobenzoic acid)
DSC	Differential scanning calorimetry
%E	Percent elongation
EMC	Equilibrium moisture content
GAB	Guggenheim-Anderson-de Boer
IMC	Initial moisture content
MSI	Moisture sorption isotherm
$NTBS^{-2}$	disodium 2-nitro-5 thiosulfobenzoate
NTB^{-2}	2-nitro-5-thiobenzoate anion
O_2P	Oxygen permeability
PAGE	Polyacrylamide gel electrophoresis
RH	Relative humidity
SDS	Sodium dodecyl sulfate
TG	Microbial transglutaminase
T_g	Glass transition temperature
T_m	Melting temperature
T_o	Onset transition temperature

TS	Tensile strength
WPI	Whey protein isolate
WVP	Water vapor permeability

INTRODUCTION

During the past two decades there has been a renewed interest in the use of edible films as a packaging material, with a number of materials emerging as potential candidates. Edible films based on proteins possess good mechanical and oxygen barrier properties, but are poor moisture barriers. Edible films could serve as a secondary packaging material to reduce the amount of packaging waste produced. In 2000 the total plastic packaging waste generated in the US was 11.2 million tons (EPA 2001). Recycling recovered only 8.9% of the plastic packaging waste which is much lower than the 45.0% recovery of aluminum containers. One of the main challenges to increase the amount of plastic packaging waste recovered through recycling is to find secondary markets for it. Some of the barriers that must be overcome include the lack of economic incentives for people and companies to recycle, and the hidden taxes associated with the municipal collection and disposal of the material (EPA 2000). When used in conjunction with a primary package material, edible packaging can reduce the amount of waste generated. This could also allow packers to change and/or reduce the type of primary packaging used.

Many cities have or are reducing their recycling programs as a way to help reduce budget deficits (O'Connell 2002). The costs associated with recycling are significant. In 2002, New York City paid up to $240 per ton for its glass, metal, and plastics recycling program. On the other hand, it would only cost the city $65-$85 per ton to dispose of the waste in landfills (O'Connell 2002). The disposal of all

waste in landfills would save the city ~$57 million. By reducing the amount of packaging waste generated, the amount of waste entering landfills would be reduced.

The main function of edible films is to act as a barrier to mass transfer into and out of the product. By limiting the migration of moisture, oxygen, aroma, and oil, both the shelf life and quality of the food product will be improved (Krochta 2002). In this capacity edible films are intended to work in conjunction with traditional packaging materials to decrease the amount of traditional packaging used or and/or allow a packaging material that is more environmentally friendly to be used. In 2002, 1.05 billion pounds of dry whey was produced for human consumption, an increase of 7.5% over the previous year. Of this, only 608.8 million pounds were sold in 2002, leaving a surplus of 441.2 million pounds of dry whey in the U.S (USDA 2002). This surplus and the functional properties of whey proteins make them an attractive choice as a possible raw material for the production of edible films.

Kim (2000) produced films using either WPI or whey protein concentrate. She found that WPI films possessed superior mechanical and barrier properties compared to whey protein concentrate films. Kim (2000) investigated the addition of lipids on the mechanical, barrier and thermal properties. She observed that the addition of candelilla wax enhanced the barrier properties of films. Mert and Ustunol (2004) investigated the effect of chemical cross-linkers on WPI films properties. They observed increased tensile strength and a decrease in oxygen

permeability for cross-linked films. The main concern in using chemical cross-linkers is that they are not suitable for human consumption.

Both heat curing and transglutaminase have been used to cross-link protein – based films (Mahmoud and Savello 1990; Gennadios et al. 1996; Miller et al. 1997; Yildirim and Hettiarachchy 1998; Kim et al. 2002; Kim et al. 2002). The limited availability and the high cost of transglutaminase has restricted its use in the past. However with the availability of transglutaminase from a microbial source this has become a viable option for cross-linking films. Preliminary studies on heat curing WPI films have been shown to enhance their mechanical properties (Appendix A). Treating WPI films with transglutaminase and heat curing also enhanced the mechanical properties of the films (Appendix B).

I hypothesize that heat curing and enzymatic cross-linking with transglutaminase can be used to modify whey proteins by enhancing disulfide bond formation and inducing the formation of Lys-Gln cross-links. By enhancing the formation of these bonds, whey protein-based edible films can be produced with improved mechanical, barrier, thermal, and optical properties.

The objectives of my research are:

1. To compare the mechanical properties (tensile strength, elongation at break and toughness) of whey protein isolate films with and without transglutaminase, and heat cured at 90°C for 0, 12 and 24h.

2. To determine the sulfhydryl, lysine and disulfide content of WPI films with and without TG heat cured for 0, 12 and 24h.
3. To determine the water vapor permeability and oxygen permeability of WPI films with and without TG, and heat cured at 90°C for 0 and 24h.
4. To characterize the moisture sorption isotherms of the films at 5 and 23°C.
5. To determine the color characteristics and light transmission of WPI films with and without TG, and heat cured at 90°C for 0, 12 and 24h.
6. To compare the color values for the films to commercially available plastic packaging materials.
7. To determine thermal properties (melting onset temperature, peak melting temperature and enthalpy of fusion) of whey protein isolate films with and without transglutaminase, and heat cured at 90°C for 0, 12 and 24h.

Chapter 1

LITERATURE REVIEW

1.1 Edible Films

1.1.1 Definition and Historical Background

Edible films are defined as a thin layer of edible material formed separately from a food product which can act as a barrier to mass transfer and/or as a carrier for food ingredients or additives. The first free standing edible films, produced from boiled soymilk, were used in 15th century Japan for preservation and to improve the appearance of food products (Guilbert and Biquet 1996). Even though the Japanese have used edible films since the 15th century, there have not been many commercially successful edible films.

Conventional packaging is used to prevent the mass transfer of moisture, oxygen, aroma, or oil between a food product and the environment and to protect the product from mechanical forces encountered during storage and distribution. Edible films are used to prevent mass transfer between a food product and the environment. This leads to an increase in the quality and shelf life of the product. Thus, edible films are not intended to replace conventional packaging (Krochta 2002). Edible films are to be used in conjunction with conventional packaging to allow a less complex package to be used. Edible films can provide additional protection to a product after the package is opened.

Haugaard and others (2001) found that there were few examples of bio-based (non-synthetic) materials used commercially as packaging material. Potential applications in dairy, home meal replacement, and dry food categories were identified. These include antimicrobial edible casings made from whey protein for hot dogs to inhibit *Listeria monocytogenes* (Cagri et al. 2001). The casings were effective in inhibiting the growth of *L. monocytogenes*. Antimicrobial edible whey protein films as a wrap for sliced summer sausage and bologna were also investigated by Cagri and others (2002). The films were effective in reducing the growth of *L. monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella enterica* subsp. *Enterica* serovar Typhimurium DT104 by 3.1 – 4.1 logs. Kim (2000) was able to produce heat sealed pouches from whey protein-based edible films filled with various dry food products such as oatmeal, mashed potato flakes, and hot cocoa mix. She also investigated the possible use of edible whey protein-based films as a wrap for processed cheese slices. The films, however, were found to be ineffective as a cheese wrap. The cheese slices wrapped in whey protein-based films had a significant decrease in moisture content compared to commercially wrapped cheese.

Edible films could serve as a secondary packaging material to reduce the amount of packaging waste produced. In 2000 the total plastic packaging waste generated in the US was 11.2 million tons (EPA 2001). Recycling recovered only 8.9% of the plastic packaging waste which is much lower than the 45.0% recovery of aluminum containers. One of the main challenges to increase the amount of

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consumption, an increase of 7.5% over the previous year. Of this, only 608.8 million pounds were sold in 2002, leaving a surplus of 441.2 million pounds of dry whey in the U.S (USDA 2002). This surplus and the functional properties of whey proteins make them an attractive choice as a possible raw material for the production of edible films.

1.1.2 Formation of Protein-Based Films

The formation of protein-based films usually follows three basic steps. The first step is the dispersion of high molecular weight polymer and other film forming constituents in a solvent. The second step is to cast the solution on a smooth level surface or to coat the solution on to a food item. The third step is the drying process where the solvent is evaporated which allows the protein to develop a matrix thereby forming a free standing film or coating (Cuq et al. 1995).

1.1.2.1 Manufacture of Edible Films

Solvent casting is commonly used to produce edible films. In this process, the film-forming material is dispersed in a solution (generally this solution is water or ethanol). The film forming solution is cast by spreading or pouring in a thin layer on a surface coated with polyterafluorethylene (Teflon®), and then allowing the solvent to be removed through evaporation. The dried film can be detached from the surface to produce a free-standing film.

Solvent casting is not widely used in the commercial production of self-supporting films. The most common process for production of films is extrusion. In this process the solid polymer resin is converted into a melt. The melt is then

shaped using heat, shear and pressure. During the extrusion process the melt is conveyed through the extruder barrel using a screw. The screw is usually divided into three sections: conveying, compression or melting, and a metering section. At the end of the extruder barrel the material is forced through a die and formed into a film (cast or blown) (Hernandez et al. 2000). Extrusion has been used in the production of sausage casing from collagen (Osburn 2002) and corn zein (Ha and Padua 2001). Currently, these are the only protein-based films to be produced using extrusion.

1.1.2.2 Components of Protein-Based Films

The basic requirement for the formation of an edible film is the presence of a high molecular weight polymer. The long chain polymeric structure provides the cohesive strength required for the formation of a structural matrix (Banker 1966). The two types of high molecular weight polymers commonly used in the formation of edible films are hydrocolloids (polysaccharides and proteins). Both polysaccharides and proteins have better mechanical strength than lipid–based films, while lipid films have better moisture barrier properties.

Proteins used in edible films include soy, wheat, corn, whey and collagen (Gennadios 2002). Of these, only collagen has become commercially successful. It is used in the processed meat industry primarily as an edible alternative to natural casings (Osburn 2002) and a wrap for meat products.

By themselves, proteins form brittle films. The brittleness of the films can be reduced by the addition of a plasticizer. Plasticizers reduce the intermolecular

interactions between the polymer chains. Plasticizers commonly used in edible films include glycerol, sorbitol, mannitol, xylitol and polyethylene glycol (McHugh et al. 1994; McHugh and Krochta 1994; Tanaka et al. 2001; Shaw et al. 2002; Shaw et al. 2002).

Due to the hydrophilic nature of proteins, they are poor moisture barriers. The addition of lipids to protein films can improve their moisture barrier properties. Waxes such as candelilla, carnauba and beeswax are commonly incorporated in film-forming solutions (McHugh and Krochta 1994; Shellhammer and Krochta 1997; Debeaufort et al. 2000; Gallo et al. 2000; Kim 2000; Perez-Gago and Krochta 2001; Chick and Hernandez 2002; Gallstedt and Hedenqvist 2002).

1.1.2.3 Forces Involved in the Formation and Stability of Protein-Based Films

Protein structures are stabilized through two groups of interactions: intramolecular forces inherent to the protein molecule and intermolecular interactions arising from the surrounding solvent or other molecules. Peptide bonds, steric strain, and van der Waals interactions are intramolecular forces inherent to protein and hydrogen bonding; electrostatic and hydrophobic interactions can be both intramolecular and intermolecular forces (Damodaran 1996). Interaction among protein chains influence the characteristics of the film produced. The ability of proteins to form films is influenced by the primary structure of the protein and the formation of ionic cross-links, hydrogen bonds, and inter- and intramolecular disulfide bonds (Gennadios 2002). The application of

heat to protein-film-forming solutions enhances the film-forming properties (Gennadios et al. 1994; Roy et al. 1999; Perez-Gago and Krochta 2001). Heating the protein solution increases the denaturation of the protein, which exposes hydrophobic and sulfhydryl groups that were previously buried. During drying, these groups interact to form high – molecular – weight aggregates. These aggregates enhance the mechanical properties of the films.

Hydrogen bonds are formed between a hydrogen atom covalently bonded to an electronegative atom (usually N or O) and another electronegative atom. Hydrogen bonds are one of the most important forces affecting the stability of proteins. Hydrogen bonds are classified as a strong permanent dipole-dipole interaction (Damodaran 1996). The bond energies of commonly found hydrogen bonds in proteins are shown in Table 1.1.

Fairley and others (1996) investigated the role of sulfhydryl/disulfide interchanges in whey protein films. By inhibiting the interchange of sulfhydryl and disulfide bonds they observed an increase in film solubility. The increase in solubility was unexpected and attributed to the protein adopting a conformation that increased hydrogen bonding. Subirade and others (1998) investigated the change in conformation in glycinin in both an aqueous solution and films. They observed a decrease in the percentage of β-sheet and unordered structure found in the film-forming solution and an increase α-helix conformation. Another conformational change occurred while drying the solutions and that resulted in the forming of intermolecular hydrogen bonded β-sheets.

Table1.1 Bond energies of typical hydrogen bonds found in proteins (Rodriguez 1996).

Hydrogen Bonds	Typical Bond Energy (Kcal/mol)
O-H—O	3 to 6
O-H—N	3 to 6
N-H—O	4
N-H—N	3 to 5

Table 1. 2: Common bonds found in proteins and their average bond energies (Rodriguez 1996).

Bond	Ave. Bond Energy (Kcal/mol)
C-C	83
C-O	84
C-H	99
C=O	171
C-N	70
N-H	93
N=C	147
S-S	51
C-S	62
S-H	81
O-O	33
O-H	111

The conformational changes induced in wheat gliadins were studied by Mangavel and others (2001). They found that there was an increase in β-sheet formation in the film-forming solution. Drying the solution induced major conformational changes in the protein. The conformational changes were attributed to antiparallel hydrogen-bonded β-sheets and protein aggregates. The increase in hydrogen-bonded β-sheets led to an increase in tensile strength of the dried films. Even though hydrogen bonds are relatively weak, their abundance significantly contributes to the stability of proteins. Increasing the amount of hydrogen bonding can increase the mechanical strength of a protein film.

Disulfide bonds are formed when two cysteine residues are brought in close proximity. The sulfhydryl groups undergo oxidation in the presence of molecular oxygen which results in the formation of a disulfide bond. Disulfide bonds can be formed both inter-molecularly and intra-molecularly in proteins. Disulfide bonds help to stabilize the folded protein and are important in the stabilization of protein films. Even though disulfide bonds are important in stabilizing the structure of a protein; they are relatively weak compared to other covalent bonds found in proteins (Table 1.2). It is thought that the proteins in film-forming solutions denature through the reduction of S-S bonds and reveal buried SH groups. During the drying process, covalent S-S bonds are formed by air oxidation from the exposed SH and sulfhydryl-disulfide interchange reactions (McHugh, Aujard et al. 1994; Roy, Weller et al. 1999).

Perez-Gago and others (1999) investigated the solubility of native and denatured whey protein isolate (WPI) films and found that denatured WPI films had lower solubility in water then native WPI films. The decrease in solubility was attributed to the denaturation of the protein, the exposure of SH groups, and the formation of intermolecular disulfide bonds. Kim (2000) observed that the solubility of WPI films differed when incubated in different bond dissociating agents. Films incubated in β-mercaptoethanol (BME) had higher solubilities than those in SDS. These results suggest that disulfide bonds play a role in stabilizing the film network. The solubility of films incubated in urea was higher than in SDS, indicating that hydrogen bonds are also important in stabilizing WPI film networks (Kim 2000).

Kayserilioglu and others (2001) determined that wheat gluten film solutions at pH 11 produced films that were cross-linked through the formation of lysinoalanine. The films showed no change in protein solubility with the addition of sodium dodecyl sulfate (SDS), indicated that hydrophobic interactions were not as important to the stability of the films.

1.2 Composition and Properties of Whey Protein Isolate

Bovine milk contains ~3.5% protein, and of this, whey proteins account for 18% (w/v) of the total protein content. Whey protein can be defined as the proteins that remain soluble after the pH of milk has been adjusted to 4.6 (Fox and McSweeney 1998). Liquid whey from cheese production contains lactose, lipids and minerals along with the soluble proteins. Cheese whey is further processed to remove

lactose, lipids and minerals to increase the protein concentration. Whey proteins are used commercially as ingredients in baked goods, fortified beverages, dairy and frozen desserts, dressings, sauces, dietetic products and processed meats (Kinsella and Whitehead 1989).

1.2.1 Manufacture of Whey Protein Isolate

Due to the high moisture and low solids content of cheese whey it tends to spoil rapidly. Moisture is removed through high velocity and low temperature spray drying. High temperatures (>75°C) need to be avoided to minimize the denaturation of whey proteins which alters their functional properties (Morr and Ha 1993).

The production of whey protein isolate begins with the pasteurization and clarification of liquid whey. The whey is clarified using a large scale centrifuge (clarifier) to remove casein and cheese particles. The removal of lactose and minerals are accomplished using ultrafiltration, which concentrates the protein and fat. Ultrafiltration is used to produce whey protein concentrate (WPC) having a protein content of 30-50%. Diafiltration is used to further concentrate the WPC to 80% protein (Morr and Ha 1993).

Two additional steps are required to produce whey protein isolate. After clarification, fat and lactose are removed using microfiltration. These processes are then followed by ultrafiltration and diafiltration. Once these processes are completed, spray drying is used to produce a fine white powder with low-fat and

lactose content. The composition of WPI is <90% protein, >1% fat, and 1-4% lactose and ash (Huffman 1996).

1.2.2 Protein Fractions in Whey

Whey proteins account for 15-25% of the total protein content of milk. Whey proteins are globular and composed of β-lactoglobulin, α-lactalbumin, bovine serum albumin, proteose-peptones, lysozyme and lactoferrin (Brunner 1981; Kinsella and Whitehead 1989).

1.2.2.1 β-Lactoglobulin

β-lactoglobulin (β-Lg) is the major protein fraction found in whey. It accounts for 50% of the whey protein and has a molecular mass of 18,600 Daltons. In its native state and in solution β-Lg exists as a dimer due to electrostatic interactions between Asp^{130} and Glu^{134} with the corresponding lysyl residues of another monomer (Cramer et al. 1983). Native β-Lg possesses 2 pairs of S-S between Cys^{66}-Cys^{160} and Cys^{106}-Cys^{119} and a free SH group on Cys^{121}. The free SH is buried and inaccessible to solvents at neutral pH (Papiz et al. 1986). Bovine β-Lg exists in seven different variants; of these seven variants A and B are the most common. Variant A and B differ in their AA composition at residues 64 and 118. Variant A has Asp and Val at these residues and variant B has Gly and Ala (Cayot and Lorient 1997). Bovine β-Lg shows a high degree of secondary and tertiary structural organization. It contains approximately 51% β-sheet, 15% α-helix, 17% reverse turns and 17% random coils (Cramer, Parry et al. 1983).

Bovine β-Lg exists as a dimer at pH 5-8. Between pH 3-5 β-Lg undergoes a pH dependant association to form octamers. Below pH 2 and above pH 8 β-Lg dissociates into monomers (Pessan et al. 1985). Heating of β-Lg above 55°C under alkaline conditions induces denaturation and aggregation. Denaturation of β-Lg exposes the SH at Cys^{121} which undergoes S-S/SH exchange which leads to irreversible aggregation of the protein (Boye et al. 1998).

1.2.2.2 α-Lactalbumin

α-lactalbumin (α-La) is the second most abundant protein in whey, and accounts for ~19% of the whey protein (Cayot and Lorient 1997). The molecular mass of α-La is ~14,000 Da, and the protein contains 4 disulfide groups (cystine) and no free sulfhydryl (cysteine). α-La contains 123 amino acid residues and shows a strong homology to lysozymes (Fox and McSweeney 1998).

α-La is a globular protein and exhibits little organized secondary structure. The secondary structure of α-La consists of 26% α-helix, 14% β-sheet and 60% unordered structure (Kinsella and Whitehead 1989). α-La has an ellipsoidal tertiary structure with a deep cleft dividing the molecule into two lobes. One lobe is composed of 4 helices and the other lobe has 2 β- sheets and a loop chain structure (Wong et al. 1996).

α-La is the most heat stable whey protein. α-La will undergo renaturation after heat denaturation. However, in the presence of β-Lg, heated α-La will undergo irreversible denaturation and form aggregates. Aggregation occurs

through the formation of disulfide bonded copolymers of α-La and β-Lg (Gezimati et al. 1997).

1.2.2.3 Bovine Serum Albumin

Bovine serum albumin (BSA) accounts for ~5% of the whey proteins (Cayot and Lorient 1997). BSA is a single peptide with a molecular mass of ~66,000 Da and is comprised of 582 amino acid residues (Kinsella and Whitehead 1989). BSA contains 17 disulfides and 1 sulfhydryl group. The BSA molecule is elliptical and is divided into three domains each with a different net charge (-10, -8, 0) (Fox and McSweeney 1998). The denaturation of BSA occurs similarly to β-Lg and the protein can be denatured by heat, acid or base treatment (Cayot and Lorient 1997). BSA forms disulfide-bonded polymers and aggregates through hydrophobic interactions upon heating (Gezimati, Creamer et al. 1997).

1.2.2.4 Immunoglobulins and proteose-peptone

Immunoglobulins are made up of high molecular weight proteins, such as IgG_1, IgG_2, IgA, IgM, and IgE, all of which have antibiotic properties. The molecular mass of immunoglobulins range from 15,000-1,000,000 daltons (Kinsella and Whitehead 1989). Immunoglobulins account for ~13% of the whey protein (Cayot and Lorient 1997). The function of immunoglobulins is to impart various types of immunity to the body. In bovine milk ~80% of the immunoglobulins are IgG (Fox and McSweeney 1998). Immunoglobulins are comprised of two light polypeptide chains and two heavy polypeptide chains. Both light and heavy polypeptide chains are cross-linked by disulfide bonds (Brunner 1981).

Proteose-peptones account for ~10% of the whey proteins. The molecular mass of proteose-peptones range from 4,000 – 40,000 daltons (Kinsella and Whitehead 1989). Proteose-peptones remain soluble after the precipitation of casein at pH 4.6 and heat coagulation of β-Lg and α-La at 95°C. Proteose-peptones are defined as a mixture of acid-soluble and heat stable phosphoglycoproteins (Girardet and Linden 1996).

1.3 Properties of Whey Protein-Based Films

1.3.1 Mechanical Properties

Mechanical property requirements have practical importance in selecting applications for which edible films are best suited. The two most studied mechanical properties of whey protein films are tensile strength (TS) and elongation-at-break (%E). Tensile strength is a measure of the maximum stress (force per unit area) that a material can sustain before breaking. Elongation-at-break is a measure of the maximum amount of strain (dimensionless fractional length increase) a material can sustain before fracture (Hernandez, Selke et al. 2000).

The mechanical properties of whey protein-based (WPI) films have been extensively studied (Table 1.3). Mechanical properties of WPI films heat cured 80, 90 and 100°C for 0, 12, 24, 48 and 72h treated with and without transglutaminase is shown in Appendix A and Appendix B. Various compounds and treatments such as plasticizers, denaturation, hydrolysis, chemical modification, drying conditions, and

Table 1.3: Mechanical properties of whey protein and β-lactoglobulin based edible films as influenced by various treatments.

Film[2]	Film composition[3]	Treatment	Tensile Strength[4]	Elongation[5]	Elastic modulus[6]	Reference
WPI/Gly	10:1.5	Glycerol	29.10	4.10	-----	McHugh and Krochta 1994[1]
WPI/Gly	10:3.0	Glycerol	13.90	30.80	-----	McHugh and Krochta 1994[1]
WPI/Sor	10:3.0	Sorbitol	14.00	1.60	-----	McHugh and Krochta 1994[1]
βLg/Gly	10:5.8	Glycerol	16.01	76.46	705.60	Sothornvit and Krochta 2001[1]
βLg/Sor	10:11.65	Sorbitol	10.06	65.85	383.80	Sothornvit and Krochta 2001[1]
βLg/PEG 200	10:12.8	Polyethylene Glycol	6.46	77.09	255.20	Sothornvit and Krochta 2001[1]
βLg/Suc	10:21.9	Sucrose	9.71	89.41	340.80	Sothornvit and Krochta 2001[1]
βLg/PEG 400	10:25.6	Polyethylene Glycol	2.88	32.31	117.20	Sothornvit and Krochta 2001[1]
WPI/Gly	10:5.0	Glycerol	3.25	35.00	100.00	Shaw and others 2002b[1]
WPI/Gly	10:8.0	Glycerol	2.00	32.00	25.00	Shaw and others 2002b[1]
WPI/Gly	10:10	Glycerol	1.50	47.00	12.00	Shaw and others 2002b[1]
WPI/Sor	10:5.0	Sorbitol	9.00	10.00	325.00	Shaw and others 2002b[1]
WPI/Sor	10:8.0	Sorbitol	3.50	15.00	150.00	Shaw and others 2002b[1]
WPI/Sor	10:10	Sorbitol	3.30	25.00	100.00	Shaw and others 2002b[1]
WPI/Xyl	10:5.0	Xylitol	8.00	15.00	225.00	Shaw and others 2002b[1]
WPI/Xyl	10:8.0	Xylitol	4.50	7.00	175.00	Shaw and others 2002b[1]
WPI/Xyl	10:10	Xylitol	1.00	2.00	100.00	Shaw and others 2002b[1]
WPI/Gly	5.0:3.5	Heat denatured	6.90	41.00	199.00	Perez-Gago and others 1999[1]
WPI/Gly	5.0:3.5	Native WPI	3.10	7.00	88.00	Perez-Gago and others 1999[1]

Table 1.3 cont.: Mechanical properties of whey protein and β-lactoglobulin based edible films as influenced by various treatments.

Film[2]	Film composition[3]	Treatment	Tensile Strength[4]	Elongation[5]	Elastic modulus[6]	Reference
WPI/Gly	10:2.5	5.5% degree of hydrolysis	1.00	35.00	100.00	Sothornvit and Krochta 2000[1]
WPI/Gly	10:2.5	10% degree of hydrolysis	2.00	2.50	25.00	Sothornvit and Krochta 2000[1]
WPI/Gly	10:5.5	5.5% degree of hydrolysis	----	-----	------	Sothornvit and Krochta 2000[1]
WPI/Gly	10:5.5	10% degree of hydrolysis	1.75	60.00	25.00	Sothornvit and Krochta 2000[1]
WPI/Gly	10:3.3	0.0 mmol NEM	6.50	20.00	325.00	Fairley and others 1996[1]
WPI/Gly	10:3.3	0.01 mmol NEM	7.00	23.00	375.00	Fairley and others 1996[1]
WPI/Gly	10:3.3	0.02 mmol NEM	7.50	20.00	325.00	Fairley and others 1996[1]
WPI/Gly	10:3.3	0.03 mmol NEM	7.00	15.00	370.00	Fairley and others 1996[1]
WPI/G	10.0:10.0	Microwave drying	2.23	36.10	27.08	Kaya and Kaya 2000[1]
WPI/G	10.0:10.0	Room conditions	1.94	26.10	20.87	Kaya and Kaya 2000[1]
WPI/Gly	11.5:11.5	dried at 21C & 50% RH	0.80	42.00	23.90	Alcantara and others 1998[1]
WPI/Gly	11.5:11.5	dried at 95C & 30% RH	1.40	32.10	42.80	Alcantara and others 1998[1]
WPI/Gly/BW	10.0:3.3:40	dried at 25C & 40%RH	3.75	2.50	225.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/BW	10.0:3.3:40	dried at 40C & 40%RH	3.50	1.75	275.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/BW	10.0:3.3:40	dried at 80C & 40%RH	5.00	10.50	270.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/AMFF	10.0:3.3:40	dried at 25C & 40%RH	6.00	3.50	300.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/AMFF	10.0:3.3:40	dried at 40C & 40%RH	5.00	9.50	225.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/AMFF	10.0:3.3:40	dried at 80C & 40%RH	4.50	9.00	250.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/CW	10.0:3.3:40	dried at 25C & 40%RH	----	----	----	Perez-Gago and Krochta 2000[1]

Table 1.3 cont.: Mechanical properties of whey protein and β-lactoglobulin based edible films as influenced by various treatments.

Film[2]	Film composition[3]	Treatment	Tensile Strength[4]	Elongation[5]	Elastic modulus[6]	Reference
WPI/Gly	10:2.5	5.5% degree of hydrolysis	1.00	35.00	100.00	Sothornvit and Krochta 2000[1]
WPI/Gly	10:2.5	10% degree of hydrolysis	2.00	2.50	25.00	Sothornvit and Krochta 2000[1]
WPI/Gly	10:5.5	5.5% degree of hydrolysis	---	---	---	Sothornvit and Krochta 2000[1]
WPI/Gly	10:5.5	10% degree of hydrolysis	1.75	60.00	25.00	Sothornvit and Krochta 2000[1]
WPI/Gly	10:3.3	0.0 mmol NEM	6.50	20.00	325.00	Fairley and others 1996[1]
WPI/Gly	10:3.3	0.01 mmol NEM	7.00	23.00	375.00	Fairley and others 1996[1]
WPI/Gly	10:3.3	0.02 mmol NEM	7.50	20.00	325.00	Fairley and others 1996[1]
WPI/Gly	10:3.3	0.03 mmol NEM	7.00	15.00	370.00	Fairley and others 1996[1]
WPI/G	10.0:10.0	Microwave drying	2.23	36.10	27.08	Kaya and Kaya 2000[1]
WPI/G	10.0:10.0	Room conditions	1.94	26.10	20.87	Kaya and Kaya 2000[1]
WPI/Gly	11.5:11.5	dried at 21C & 50% RH	0.80	42.00	23.90	Alcantara and others 1998[1]
WPI/Gly	11.5:11.5	dried at 95C & 30% RH	1.40	32.10	42.80	Alcantara and others 1998[1]
WPI/Gly/BW	10.0:3.3:40	dried at 25C & 40%RH	3.75	2.50	225.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/BW	10.0:3.3:40	dried at 40C & 40%RH	3.50	1.75	275.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/BW	10.0:3.3:40	dried at 80C & 40%RH	5.00	10.50	270.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/AMFF	10.0:3.3:40	dried at 25C & 40%RH	6.00	3.50	300.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/AMFF	10.0:3.3:40	dried at 40C & 40%RH	5.00	9.50	225.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/AMFF	10.0:3.3:40	dried at 80C & 40%RH	4.50	9.00	250.00	Perez-Gago and Krochta 2000[1]
WPI/Gly/CW	10.0:3.3:40	dried at 25C & 40%RH	---	---	---	Perez-Gago and Krochta 2000[1]

Table 1.3 cont.: Mechanical properties of whey protein and β-lactoglobulin based edible films as influenced by various treatments.

Film[2]	Film composition[3]	Treatment	Tensile Strength[4]	Elongation[5]	Elastic modulus[6]	Reference
WPI/Gly/CW	10.0:3.3:40	dried at 40C & 40%RH	-----	-----	-----	Perez-Gago and Krochta 2000[1]
WPI/Gly/CW	10.0:3.3:40	dried at 80C & 40%RH	-----	-----	-----	Perez-Gago and Krochta 2000[1]
WPI/Gly/soya oil	10:5.0:4.0	Soya oil	4.06	44.05	105.29	Shaw and others 2002[1]
WPI/Gly/soya oil	10:5.0:3.0	Soya oil	4.28	85.42	118.54	Shaw and others 2002[1]
WPI/Gly/soya oil	10:5.0:2.0	Soya oil	3.98	60.75	130.19	Shaw and others 2002[1]
WPI/Gly/soya oil	10:5.0:0.0	Soya oil	4.26	33.63	159.49	Shaw and others 2002[1]

[1]Films tested according to ASTM 882
[2]WPI=whey protein isolate; Gly=glycerol; Sor=sorbitol; Xyl=xylitol; G=glycerine; BW=beeswax; AMFF=anhydrous milkfat fraction; CW=candelilla wax; βLg=β-Lactoglobulin
[3] %protein: %plasticizer: %lipid (w/w/w); [4] MPa; [5] %; 6 Mpa

the addition of lipids affect the mechanical properties of WPI films. Plasticizers enhance the mechanical properties of protein-based edible films, which otherwise tend to be brittle by themselves. The addition of plasticizers decreases the intermolecular interactions, which leads to an increased pliability in the films. Common plasticizers used for edible protein-based films include glycerol, sorbitol and polyethylene glycol (Gennadios 2002). Glycerol is the most effective plasticizer, due to its smaller size and ability to interact with the polymers. Increasing the level of plasticizers in the film solution has been shown to decrease the TS and increase the %E of WPI films (McHugh and Krochta 1994; Sothornvit and Krochta 2001; Shaw, Monahan et al. 2002) by reducing the intermolecular attraction between the polymer chains. Increasing the molecular weight of the plasticizer will affect the mechanical properties of WPI by decreasing the elongation at break and by increasing the tensile strength (Sothornvit and Krochta 2001; Shaw, Monahan et al. 2002). The higher molecular weight plasticizers are less effective in disrupting the intermolecular attraction between the polymer chains.

Heating WPI solutions causes the proteins to denature and exposes sulfhydryl groups (SH) in the protein interior. Exposing the SH increases the number of intermolecular disulfide bonds that may be formed during the drying process and leads to increased intermolecular cross-linking. The increased TS of denatured WPI films have been attributed to the formation of covalent cross-links during the heating process. Enzymatic hydrolysis of whey protein resulted in films

that had lower TS compared to unhydrolyzed WPI (Sothornvit and Krochta 2000). The decrease in TS in hydrolyzed WPI was attributed to the weaker protein network formed by the shorter chains. The blocking of disulfide bond formation by free sulfhydryl using N-ethylmaleimide (NEM) had no effect on the mechanical properties of WPI films (Fairley, Monahan et al. 1996). Conformational changes induced through the heating of protein film solutions during the drying process enhanced the mechanical properties of the film. Increasing the drying temperature and decreasing the humidity increases the TS and decreases the %E of WPI films (Alcantara et al. 1998). Drying of WPI films by microwave radiation was shown to increase the TS of films compared to films dried at room temperature (Kaya and Kaya 2000). The addition of lipids to the film forming solutions decreased the TS of WPI films (Perez-Gago and Krochta 2000; Shaw, Monahan et al. 2002). Drying WPI films at different temperatures and a constant humidity had no effect on the mechanical properties of WPI films containing lipids (Perez-Gago and Krochta 2000).

1.3.2 Barrier Properties

Barrier properties measure the resistance of a material to diffusion or sorption of a substance (Hernandez, Selke et al. 2000). Permeability of a material combines the effects of diffusion and sorption into a single value. Two important barrier properties of WPI films are water vapor permeability (WVP) and oxygen permeability (O_2P) (Table 1.4). Another barrier property that is important, but less studied, is sorption of various volatile compounds. Protein based films in general

Table 1.4: Barrier properties of whey protein and β-lactoglobulin based edible films as influenced by various treatments.

Film[4]	Film composition[5]	Treatment	O_2P[6]	WVP[7]	Reference
WPI/Gly	10:1.5	Glycerol	18.50	----	McHugh and Krochta 1994[1]
WPI/Gly	10:3.0	Glycerol	76.10	----	McHugh and Krochta 1994[1]
WPI/Sor	10:3.0	Sorbitol	4.30	----	McHugh and Krochta 1994[1]
WPI/Gly	10:5.0	Glycerol	----	118.00	Shaw and others 2002b[3]
WPI/Gly	10:8.0	Glycerol	----	118.00	Shaw and others 2002b[3]
WPI/Gly	10:10	Glycerol	----	150.00	Shaw and others 2002b[3]
WPI/Sor	10:5.0	Sorbitol	----	85.00	Shaw and others 2002b[3]
WPI/Sor	10:8.0	Sorbitol	----	85.00	Shaw and others 2002b[3]
WPI/Sor	10:10	Sorbitol	----	110.00	Shaw and others 2002b[3]
WPI/Xyl	10:5.0	Xylitol	----	90.00	Shaw and others 2002b[3]
WPI/Xyl	10:8.0	Xylitol	----	90.00	Shaw and others 2002b[3]
WPI/Xyl	10:10	Xylitol	----	90.00	Shaw and others 2002b[3]
WPI/Gly	5.0:3.5	Heat denatured	----	119.04	Perez-Gago and others 1999[3]
WPI/Gly	5.0:3.5	Native WPI	----	121.44	Perez-Gago and others 1999[3]
WPI/Gly	10:2.5	5.5% degree of hydrolysis	25.00	----	Sothornvit and Krochta 2000[1]
WPI/Gly	10:2.5	10% degree of hydrolysis	25.00	----	Sothornvit and Krochta 2000[1]
WPI/Gly	10:5.5	5.5% degree of hydrolysis	300.00	----	Sothornvit and Krochta 2000[1]
WPI/Gly	10:5.5	10% degree of hydrolysis	300.00	----	Sothornvit and Krochta 2000[1]
WPI/Gly	10:3.3	0.0 mmol NEM	----	90.00	Fairley and others 1996[3]

Table 1.4 cont: Barrier properties of whey protein and β-lactoglobulin based edible films as influenced by various treatments.

Film[4]	Film composition[5]	Treatment	O_2P[6]	WVP[7]	Reference
WPI/Gly	10:3.3	0.01 mmol NEM	---	84.00	Fairley and others 1996[3]
WPI/Gly	10:3.3	0.02 mmol NEM	---	84.00	Fairley and others 1996[3]
WPI/Gly	10:3.3	0.03 mmol NEM	---	84.00	Fairley and others 1996[3]
WPI/G	10.0:10.0	Microwave drying	---	45.60	Kaya and Kaya 2000[3]
WPI/G	10.0:10.0	Room conditions	---	50.16	Kaya and Kaya 2000[3]
WPI/Gly/BW	10.0:3.3:40	dried at 25C & 40%RH	---	54.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/BW	10.0:3.3:40	dried at 40C & 40%RH	---	54.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/BW	10.0:3.3:40	dried at 80C & 40%RH	---	36.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/AMFF	10.0:3.3:40	dried at 25C & 40%RH	---	60.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/AMFF	10.0:3.3:40	dried at 40C & 40%RH	---	48.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/AMFF	10.0:3.3:40	dried at 80C & 40%RH	---	36.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/CW	10.0:3.3:40	dried at 25C & 40%RH	---	78.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/CW	10.0:3.3:40	dried at 40C & 40%RH	---	72.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/CW	10.0:3.3:40	dried at 80C & 40%RH	---	60.00	Perez-Gago and Krochta 2000[3]
WPI/Gly/soya oil	10:5.0:4.0	Soya oil	---	126.41	Shaw and others 2002[3]
WPI/Gly/soya oil	10:5.0:3.0	Soya oil	---	111.62	Shaw and others 2002[3]
WPI/Gly/soya oil	10:5.0:2.0	Soya oil	---	118.32	Shaw and others 2002[3]
WPI/Gly/soya oil	10:5.0:0.0	Soya oil	---	115.72	Shaw and others 2002[3]
βLg/Gly	10:1.5	Glycerol	10.00	55.92	Mate and Krochta 1996[2,3]

Table 1.4 cont: Barrier properties of whey protein and β-lactoglobulin based edible films as influenced by various treatments.

Film[4]	Film composition[5]	Treatment	O_2P[6]	WVP[7]	Reference
βLg/Gly	10:3.0	Glycerol	25.00	93.60	Mate and Krochta 1996[2,3]
βLg/Gly	10:4.0	Glycerol	50.00	138.72	Mate and Krochta 1996[2,3]
WPI/Gly/BW	5:3.3:0	Beeswax & pH 4	----	120.00	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:2.0	Beeswax & pH 4	----	96.00	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:4.0	Beeswax & pH 4	----	----	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:0	Beeswax & pH 6	----	126.00	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:2.0	Beeswax & pH 6	----	60.00	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:4.0	Beeswax & pH 6	----	60.00	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:0	Beeswax & pH 8	----	120.00	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:2.0	Beeswax & pH 8	----	48.00	Perez-Gago and Krochta 1999[3]
WPI/Gly/BW	5:3.3:4.0	Beeswax & pH 8	----	48.00	Perez-Gago and Krochta 1999[3]
WPI/Gly	10:2.5	5.5% degree of hydrolysis	----	78.00	Sothornvit and Krochta 2000b[3]
WPI/Gly	10:3.0	5.5% degree of hydrolysis	----	102.00	Sothornvit and Krochta 2000b[3]
WPI/Gly	10:3.5	5.5% degree of hydrolysis	----	108.00	Sothornvit and Krochta 2000b[3]
WPI/Gly	10:2.5	10.0% degree of hydrolysis	----	96.00	Sothornvit and Krochta 2000b[3]
WPI/Gly	10:3.0	10.0% degree of hydrolysis	----	99.60	Sothornvit and Krochta 2000b[3]
WPI/Gly	10:3.5	10.0% degree of hydrolysis	----	120.00	Sothornvit and Krochta 2000b[3]

[1]Films tested using ASTM 3985 at 23°C and 50%RH
[2]Films tested using ASTM 3985 at 23°C and 40%RH
[3]Films tested using ASTM E96 modified according to McHugh and others 1993
[4]WPI=whey protein isolate; Gly=glycerol; Sor=sorbitol; Xyl=xylitol; G=glycerine; BW=beeswax; AMFF=anhydrous milkfat fraction; CW=candelilla wax; βLg=β-lactoglobulin
[5] %protein: % plasticizer (w/w); [6] Oxygen permeability (cm^{3}* mm/m^{2}*d*KPa); [7] Water vapor permeability (g mm/d m kPa)

and WPI specifically are poor barriers to moisture but are good barriers to oxygen (Krochta and De Mulder-Johnson 1997). Their inadequacy as moisture barriers has been attributed to their hydrophilic nature. The O_2P of protein films is dependant on the relative humidity (RH) during testing; as RH increases the O_2P of the film increases (Miller and Krochta 1997). Decreasing the WVP and O_2P of WPI films will increase the likelihood of commercialization.

The plasticizer used has an effect on the permeability of WPI films by increasing the intermolecular distance between the proteins. This increases the diffusion of water or oxygen through the film (Hernandez, Selke et al. 2000). Films which included glycerol had higher WVP compared to sorbitol plasticized films (Shaw, Monahan et al. 2002). The hygroscopic nature of glycerol increases the adsorption of water, with the water acting as an additional plasticizer (Anker et al. 2000; Hernandez, Selke et al. 2000). Increasing the temperature and reducing the RH during drying of films leads to a decrease in WVP (Alcantara, Rumsey et al. 1998; Perez-Gago and Krochta 2000). The addition of lipids to WPI films has been investigated as a means to improve the WVP of the films. However, Shaw and others (2002) observed no effect on the WVP of WPI films to which soya oil had been added. The incorporation of lipids and a reduction in the level of plasticizer can be used to improve the WVP of protein-based films. Perez-Gago and Krochta (1999) investigated the effect of pH on the WVP of WPI beeswax emulsion films. As the pH decreased to the isoelectric point ($\approx$ 5), the WVP of the

films increased. The increase in WVP was attributed an increase in viscosity of the film solution from protein aggregation and decreased lipid mobility.

Mate and Krochta (1996) compared the O_2P of WPI films and β-lactoglobulin films. They observed that despite the differences in molecular structure, hydrogen bonds, hydrophobic interaction and disulfide bonds, there was no difference in O_2P between films. They did observe that the amount of glycerol was more important in lowering O_2P than the structure of the protein.

Edible protein-based films are sensitive to changes in humidity due to the hydrophilic nature of the proteins. However, there is little literature relating to the moisture sorption isotherms of edible films (Coupland et al. 2000; Kim and Ustunol 2001). The type and level of plasticizer influences the moisture sorption characteristics of protein based films. Films plasticized with glycerol had higher equilibrium moisture content compared to sorbitol films. This is due to the more hygroscopic nature of glycerol compared to sorbitol. An increase and/or decrease in the relative humidity affect the mechanical and barrier properties of protein-based edible films. Cho and Rhee (2002) investigated the relationship between the moisture sorption characteristics of soy protein films and their mechanical properties. They observed that the TS decreased as RH% increased, and attributed this to the higher moisture content of the films; this correlated to the higher equilibrium moisture content of the films at higher water activities.

1.3.3 Thermal Properties

Thermal properties such as melting temperature (T_m) and glass transition temperature (T_g) are important in the determination of processing conditions of polymer films. The T_m of a polymer is the temperature at which the polymer molecules are free to move past each other and the crystalline regions begin to break up (Rodriguez 1996). The T_m of films can also be used to determine an optimal temperature range for film extrusion. For crystalline plastics, processing temperatures are about 50°C above their T_m (Hernandez, Selke et al. 2000). The T_g of a polymer is the temperature at which the polymer begins to have segmental mobility. Above the T_g polymers are soft and flexible, while below T_g polymers are stiff and rigid (Hernandez, Selke et al. 2000). The T_g of a polymer is influenced by the intermolecular forces. As the intermolecular forces increase, the T_g and crystallinity of the polymer increases.

The T_g of plastic films are influenced by various parameters (Hernandez, Selke et al. 2000). These include the primary structure, intermolecular forces, and the film additives. The T_g of protein films is also, influenced by these same parameters. The most common additives used in edible films are plasticizers, usually polyols such as glycerol and sorbitol. The effect of different plasticizers on the T_g of WPI films was investigated by Shaw and others (2002). They observed that T_g's were lower for glycerol-plasticized films than for xylitol- and sorbitol-plasticized films. This was attributed to the higher solubility of glycerol in water compared to the other plasticizers. Shaw and others (2002) investigated the

effects of glycerol concentration and addition of soya oil on the T_g of composite WPI films. As the concentration of glycerol increased, the T_g of the films decreased. Increasing the soya oil concentration decreased the T_g of the films (Table 1.5).

Differential scanning calorimetry (DSC) can be used to determine the optimum heat seal temperature for WPI films. The optimum seal temperature for the films corresponds to the onset transition temperature T_o (Kim and Ustunol 2001). Heat sealing at temperatures above T_o resulted in distorted seals and decreased seal strength. Kim and Ustunol (2001) proposed that heat seal strength may be due to covalent bond formation between the ε-NH_2 group of lysine and the carboxyl side groups of asparagine or glutamine.

1.4 Methods to Improve the Properties of WPI Films

Protein based edible films have good mechanical and oxygen barrier properties, but are poor moisture barriers. The incorporation of lipids into the film-forming solution produces a protein/lipid emulsion film. Cross-linking the proteins can enhance their mechanical and barrier properties. Proteins can be cross-linked through enzymatic, chemical or physical treatments.

1.4.1 Chemical

Chemical agents that are commonly used as cross linking agents include gluteraldehyde, glyceraldehydes, formaldehyde, glyoxal and dialdehyde starch (Gennadios 2002). Wheat gluten films treated with formaldehyde vapors led to an increase in tensile strength but had no effect on water vapor permeability

Table 1.5: Glass transition temperature of whey protein based edible films as influenced by various treatments.

Film	Film composition	Treatment	Tg (K)	Reference
WPI/Gly	10:5.0	Glycerol	193.00	Shaw and others 2002b[1]
WPI/Gly	10:8.0	Glycerol	193.00	Shaw and others 2002b[1]
WPI/Gly	10:10	Glycerol	183.00	Shaw and others 2002b[1]
WPI/Sor	10:5.0	Sorbitol	233.00	Shaw and others 2002b[1]
WPI/Sor	10:8.0	Sorbitol	233.00	Shaw and others 2002b[1]
WPI/Sor	10:10	Sorbitol	233.00	Shaw and others 2002b[1]
WPI/Xyl	10:5.0	Xylitol	223.00	Shaw and others 2002b[1]
WPI/Xyl	10:8.0	Xylitol	223.00	Shaw and others 2002b[1]
WPI/Xyl	10:10	Xylitol	223.00	Shaw and others 2002b[1]
WPI/Gly/soya oil	10:5.0:4.0	Soya oil	193.89	Shaw and others 2002[1]
WPI/Gly/soya oil	10:5.0:3.0	Soya oil	192.65	Shaw and others 2002[1]
WPI/Gly/soya oil	10:5.0:2.0	Soya oil	191.05	Shaw and others 2002[1]
WPI/Gly/soya oil	10:5.0:0.0	Soya oil	191.01	Shaw and others 2002[1]

[1]Films tested using DSC and analyzed according to ASTM D3418

(Micard et al. 2000). Galietta and others (1998) cross-linked whey proteins with formaldehyde and reported an enhancement in mechanical properties and a decrease in water solubility. Under alkaline conditions formaldehyde and gluteraldehyde form short methylene cross-links between lysine residues. The abundance of lysine in β- lactoglobulin and α-lactoglobulin (12% and 15% respectively) makes the formation of methylene cross-links as a potential source to enhance the mechanical properties of WPI films. Both formaldehyde and gluteraldehyde are chemically reacted during cross-linking, though there are concerns of residual toxicity which limits their acceptability in the production of edible protein-based films (Gennadios, McHugh et al. 1994). Dialdehyde starch has been studied as an alternative to glyceraldehydes and formaldehyde. Dialdehyde starch is obtained by reacting native starch with periodic acid to produce a polymeric aldehyde. Soy protein films with dialdehyde starch were essentially insoluble in water, 0.01 N HCl, 0.01 N NaOH, 4 M urea, and 0.2 M 2-mercaptoethanol (Rhim et al. 2000). The addition of dialdehyde starch was shown to be an effective cross-linker for the production of egg white films (Gennadios et al. 1998). An increase in the band intensity of higher molecular weight aggregates was found using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with increasing amounts of dialdehyde starch; this was coupled with a reduction in total soluble matter of the films. Calcium chloride was used as a cross-linker for caseinate-based edible films (Avena-Bustillo and Krochta 1993).

Calcium chloride induced cross-linking in calcium caseinate films, which resulted in decreased water vapor permeability. Calcium salts were added as a way to induce cross-linking of soy proteins films to enhance their mechanical properties (Park et al. 2001). Films with calcium sulfate had higher TS than those with calcium chloride. Due to the pH of the film solution, the protein side chains had a predominantly negative charge. The increase in TS was attributed to the bonding of divalent calcium ions to carboxyl groups of the protein. In the same paper, Park and others (2001) investigated the effects of glucono-δ-lactone (GDL) on the mechanical properties of soy protein films. They observed that the addition of GDL increased the TS and puncture strength, and decreased the %E of soy protein films. The formation of soy protein films using GDL occurred because of hydrogen bonding, while electrostatic interactions and disulfide bonds were involved in the heat formation of soy protein films.

1.4.2 Enzymatic

Transglutaminase (TG) forms covalent cross-links between lysine and glutamine. Mahmoud and Savello (1993) investigated the effects of TG on the solubility and hydrolyzability of WPI films. Cross-linking using TG produced a polymerized network of whey proteins. The solubility of films increased in the presence of urea compared to β mercaptoethanol (BME). Yildirim and Hettairachchy (1998) examined the effects of TG on the solubility of WPI films with and without soy protein in a bond dissociating agent. Mechanical properties and water vapor permeability were also studied. Films were found to be soluble in

solutions of urea and sodium dodecyl sulfate (SDS) than in BME. They concluded that hydrogen bonding and hydrophobic interactions are more important than disulfide bonds in maintaining integrity of TG cross-linked films. This is to be expected since transglutaminase induces cross-links through an acyl transfer reaction with the γ-carboxyamide group of glutamine and ε-amino group of lysine (Folk and Cole 1966). WPI films cross-linked with TG had greater tensile strength then control films, but TG had no effect in lowering WVP. The two major proteins found in whey are β-lactoglobulin and α-lactalbumin; both of these proteins are composed of ~ 9.0% lysine residues and ~3.0% glutamine residues (Kinsella and Whitehead 1989). In the past TG was only obtained through animal sources which limited its availability and use. Currently, TG from a microbial source, *Streptoverticillium mobaraense*, is marketed under the name Activa™ TG through Ajinomoto Company Inc. (Japan) and has been approved as a GRAS substance. The production of TG from a microbial source has reduced its cost, which has increased the feasibility of using TG in commercial production of edible protein films.

1.4.3 Physical

Physical treatments such as γ-irradiation, ultraviolet (UV) radiation and heat curing have been shown to improve mechanical and barrier properties of protein films. Rhim and others (1999) showed that UV radiation increased tensile strength in corn zein, egg albumen and wheat gluten films. Exposure to UV radiation has been shown to decrease the solubility of sodium caseinate films without apparent

improvement in its tensile strength (Rhim, Gennadios et al. 1999). UV radiation causes free radical formation among the aromatic amino acids. These free radicals are then involved in the formation of intermolecular covalent bonds. Neither α-lactalbumin or β-lactoglobulin have high amounts of these amino acids (Rhim, Gennadios et al. 1999). Cross-linking of these proteins using UV radiation will likely not be as effective as other physical treatments.

Caseinate films had increased resistance to puncture and moisture when cross-linked by γ-irradiation (Brault et al. 1997). Cross-linking due to irradiation was also enhanced in the presence of $CaCl_2$. The use of γ - irradiation on cast wheat gluten films resulted in an increase in tensile strength (43%) and a decrease in elongation (79%). This was attributed to the formation of ditryosine, which resulted in cross-links within the polypeptide macromolecule (Micard, Belamri et al. 2000). Sabato and others (2001) examined the effect of γ - irradiation on a whey protein/soy isolate film. They found that γ-irradiation was effective in increasing mechanical strength. Vachon and others (2000) treated milk proteins, casein, and whey protein (isolate and concentrate) both individually and as a mixture with γ-irradiation. They observed that γ-irradiation was more effective on casein than whey proteins. The lack of effectiveness of γ-irradiation on the whey proteins was attributed to the low tyrosine content.

Heat curing is a common method used to induce cross-linking in synthetic thermoset polymers. Heat curing is the exposure of a film to an increased temperature that allows the molecules to relax and/or rearrange. This leads to a

decrease in internal stresses (Soroka 1995). Heat curing has been shown to increase tensile strength and reduce elongation-at-break. Heat curing decreases water vapor permeability of protein-based edible films (Gennadios et al. 1996; Miller, Chiang et al. 1997). By exposing whey protein based edible films to a heat curing process the tensile strength can be increased, but elongation-at-break is reduced. Yannas and Tobolsky (1967) reported that non-plasticized gelatin films had lower solubility when heat cured under vacuum. They attributed this to the formation of a three dimensional network developed from interchain cross-linking. Kim and others (2002) studied the effects of heat curing soy protein films at atmospheric and sub-atmospheric conditions. Heat curing films under vacuum enhanced the barrier and mechanical properties more rapidly than heat curing at atmospheric conditions.

By gaining a better understanding of the effects physical and enzymatic treatments on the mechanical, barrier, and thermal properties of edible films material can be produced that will have wider commercial application. The effect of physical treatment such as heat curing, or the combination of heat curing with enzymatic cross-linkers, on the molecular interaction of WPI films have not been extensively studied

Much work has been done the TS, %E, WVP, and O_2P of WPI films. There is little or no literature reporting on other properties of WPI films that have been heat cured or treated with TG. These other properties include: toughness, moisture sorption, melting temperature and heat of fusion.

Chapter 2

MECHANICAL AND MOLECULAR PROPERTIES OF HEAT - CURED WHEY PROTEIN ISOLATE - BASED EDIBLE FILMS AND MICROBIAL TRANSGLUTAMINASE HEAT - CURED WHEY PROTEIN - ISOLATE BASED FILMS.

2.1 Abstract

Edible whey protein films were produced using whey protein isolate (WPI) (5% w/v), glycerol (3.5% w/v) and candelilla wax (0.8% w/v). Films were also produced as described above with the addition of microbial transglutaminase (0.001g/g WPI) to the film-forming solution after heating. Both sets of films were vacuum-heat-cured for 0, 12 and 24h at 90°C. The effects of heat curing and transglutaminase treatment on mechanical properties (tensile strength (TS), elongation at break (%E) and toughness) of WPI films were assessed. The effects of heat curing and transglutaminase treatment on the free sulfhydryl, disulfide, and lysine content and degree of cross-linking were also determined. Heat curing increased the TS of WPI and WPI/TG films compared to uncured films. Heat curing films for 12h decreased %E while films at 24h had higher %E compared to films heat cured for 0 and12h. Heat curing increased the toughness of WPI films with 24h films having the highest toughness of all films tested. All WPI films had lower free sulfhydryl, disulfide and lysine content compared to whey protein isolate. Heat curing decreased the free sulfhydryl content of WPI and WPI/TG films. The

disulfide bond content of WPI films heat cured for 24h was higher than for films heat cured at 0 and12h. The disulfide content of WPI/TG films heat cured for 12 and 24h were higher than films at 0h. Heat cured WPI films had lower lysine content compared to uncured films. WPI/TG films heat cured for 24h had lower lysine content compared to films heat cured at 0 and12h. SDS-PAGE was used to confirm heat curing and TG treatment resulted in covalent cross-links.

2.2 Introduction

Edible films consist of a thin continuous layer of edible material which can act as a barrier to mass transfer and/or as a carrier for food ingredients or additives. During the last 20 years a renewed interest has developed in the potential use of edible films as a packaging material, with a number of materials emerging as potential resources. Edible films based on proteins possess good mechanical and oxygen barrier properties, but are poor moisture barriers. Proteins used in edible films come from soy, wheat, corn, whey and collagen (Gennadios 2002). Of these, only collagen has become commercially successful. It is used in the processed meat industry primarily as an edible alternative to natural casings (Osburn 2002) and as a wrap on meat products.

Mechanical properties have practical importance in determining which edible films are best suited for a particular application. The two most studied mechanical properties of whey protein films are tensile strength (TS) and elongation-at-break (%E). Tensile strength is a measure of the maximum stress that a material can sustain. Elongation-at-break is a measure of the maximum

amount of strain a material can sustain without breaking (Hernandez, Selke et al. 2000). Toughness, another mechanical property, is the energy absorbed by a material before it breaks. It is the area under a stress-strain curve and materials range from soft and weak to hard and tough (Hernandez, Selke et al. 2000). Toughness provides another measure of the mechanical properties which is different from either TS or %E. The toughness of protein-based edible films has not been previously published.

Cross-linking proteins through enzymatic or physical treatment can enhance mechanical properties. Transglutaminase has been investigated as a method to induce cross-linking in protein films (Mahmoud and Savello 1992; Yildirim and Hettiarachchy 1998; Lim et al. 1999; Larre et al. 2000). Treating film solutions with TG increased the tensile strength compared to control films. The increase in tensile strength was attributed to cross-linking of proteins. The enzyme transglutaminase induces cross-links through an acyl transfer reaction with the γ-carboxyamide group of glutamine and ε-amino group of lysine (Folk and Cole 1966). In the past TG, was only obtained from animal sources limiting its availability and use. Currently, TG is available from a microbial source, *Streptoverticillium mobaraense*, and is marketed under the name Activa™ TG by Ajinomoto Company Inc. (Japan). It has been approved as a GRAS substance. The production of TG from a microbial source has reduced its cost. The reduced cost increases the feasibility of using TG in commercial production of edible protein films.

Heat curing is a common method used to induce cross-linking in synthetic polymers. Heat curing is the exposure of a film to an increased temperature that allows the molecules to relax and/or rearrange which leads to a decrease in internal stresses (Soroka 1995). Heat curing has been shown to increase tensile strength and to reduce elongation-at-break. Water vapor permeability of protein-based edible films has also been shown to decrease due to heat curing (Gennadios, Ghorpade et al. 1996; Miller, Chiang et al. 1997). Heat curing may induce cross-linking in protein films. Yannas and Tobolsky (1967) reported that non-plasticized gelatin films had lower solubility when heat cured under a vacuum. They attributed this to the formation of a three dimensional interchain cross-link network. Kim and others (2002) studied the effects of heat curing soy protein films at atmospheric and sub-atmospheric conditions. Heat curing under vacuum enhanced the mechanical properties more rapidly than heat curing at atmospheric conditions.

The objective of this study is to compare the mechanical properties (TS, %E and toughness) of whey protein isolate films with and without transglutaminase, and heat cured at 90°C for 0, 12 and 24h.

2.3 Materials and Methods

2.3.1 Materials

Whey protein isolate (WPI; Provan 190) was obtained from Glanbia Ingredients Inc. (Monroe, WI). The whey proteins were extracted in a highly purified, undenatured form using membrane technology. Composition of the WPI

was determined using AOAC methods (AOAC 2000) and compared to values provided by Glanbia Ingredients (Table 2.1). Glycerol was purchased from J.T. Baker Co. (Phillipsburg, N.J.), and Candelilla wax was purchased from Stroh and Pitsch Inc. (West Babylon N.Y.). Microbial transglutaminase (Activa™ FP) was obtained from Ajinomoto Company Inc. (Japan).

2.3.2 Film preparation

Whey protein isolate (WPI) (5% w/v) and glycerol (3.5% w/v) were mixed in distilled water and the pH adjusted to 8.0 with 2N NaOH. These solutions were then heated at 90°C for 15 min while being stirred continuously. Candelilla wax (0.8% w/v) was added during the last 5 min of heating and allowed to melt into the WPI solution. The WPI solutions were homogenized for 2 min, using a Polytron PT 10/35 homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH). Each homogenized solution was then strained through a single layer of cheesecloth to ensure complete incorporation of the lipid and allowed to equilibrate at ambient temperature for 2 hours. Each solution was then degassed for 30 min and cast on to a Teflon-coated metal pan. Each solution was dried at 23±2°C and 35± 2%RH for 24h. Films were stored at 23±2°C and 50± 2%RH for 24h prior to heat curing.

2.3.3 Transglutaminase cross-linked films

Film forming solutions were prepared as above. Solutions were allowed to cool to ~ 50°C and TG was added (0.001g/ g of WPI) and allowed to incubate for 3h at 50°

Table 2.1: Protein, lactose, ash, and moisture content of whey protein isolate.

Composition	Experimental Values[1] (%)	Published values
Protein	92.1±0.65	92.0-95.0%[2,3]
Lactose	0.012±0.004	<1.0%[3]
Ash	2.6±0.01	<3.0%[3]
Moisture	4.1±0.33	<5.0%[3]

[1] n=3 for all components tested.
[2] Protein is on a dry basis and a nitrogen conversion factor of 6.38 was used.
[3] Values obtained from Glanbia

Each TG containing solution was then degassed under vacuum for 30 min and cast onto a Teflon-coated metal pan. The amount of TG used was based upon the technical specification received from Ajinomoto Company Inc. Each solution was dried at 23±2°C and 35± 5%RH for 24h. Films were stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

2.3.4 Heat curing

Films were wrapped in aluminum foil and heated in a vacuum oven at 90°C for 12 and 24h. After heat curing, films were allowed to equilibrate at 45± 5% RH for 24h prior to removal from foil. Films were conditioned at 23±2°C and 50± 5%RH for 24h prior to testing.

2.3.5 Mechanical Properties

The mechanical properties of uncured WPI, uncured WPI/TG, heat-cured WPI and heat-cured WPI/TG films were compared. Samples were cut into 2.54 cm-wide strips using a Precision Sample Cutter (Thawing Albert Instrument Company Philadelphia, PA). Film thickness was determined using a TMI model 549 M micrometer (Testing Machines, Inc. Amityville, NY). Measurements were taken at 5 locations along the sample and averaged. Tensile strength (TS) and percent elongation-at-break (%E) and tensile energy at break (toughness) were determined according to ASTM D 882(ASTM 1997). Samples were tested using an Instron Universal Testing Machine Model 5500 (Instron Corp., Canton MA) with

Merlin™ software to determine TS, %E and toughness. Initial grip separation was 50 mm and the crosshead speed was 500 mm/min.

2.3.6 Sulfhydryl and disulfide bond content

The free sulfhydryl group and disulfide bond content were determined according to Rangavajhyala and others (1997) based on the procedure developed by Thannhauser and others (1987). Ellman's reagent 5, 5'- dithiobis (2-nitrobenzoic acid) (DTNB) and disodium 2-nitro-5 thiosulfobenzoate ($NTBS^{-2}$) were used to determine the free sulfhydryl groups and disulfide bond content. Reaction of sulfhydryl groups and disulfide bonds with DTNB and $NTSB^{-2}$ (both colored reagents) will yield soluble 2-nitro-5-thiobenzoate anion (NTB^{-2}).

Since $NTSB^{-2}$ is not commercially available it was synthesized from DTNB according to Thannhauser and others (1987). DTNB was dissolved in 1M sodium sulfite at 38°C and pH was adjusted to 7.5. Oxygen was bubbled through the solution at 38°C until it changed from bright red to pale yellow.

To determine the free sulfhydryl content, 10mg of a sample was added to 1 mL of reaction buffer containing 8M urea, 10mM DTNB, 3mM EDTA, 1% SDS, and 0.2M Tris-HCl, pH 8.0. The mixture was allowed to react for 15 min at room temperature. The sample was centrifuged at 13,600 x g for 10 min to remove any particulate matter. The absorbance was measured at 412 nm, and the sulfhydryl content was calculated using the molar extinction coefficient of NTB (13,600 $M^{-1}cm^{-1}$).

To determine the disulfide bond content a 10 mg sample of WPI is added to 1 mL of reaction buffer containing 8M urea, 10mM $NTSB^{-2}$, 0.1M sodium sulfite, 3mM EDTA, 1% SDS, and 0.2M Tris-HCl, pH 9.5. The mixture is allowed to react for 25 min in a dark chamber at ambient temperature. The sample is centrifuged at 13,600 x g for 10 min. The absorbance is measured at 412nm which represents the total sulfhydryl content. The disulfide bond content is calculated as follows:

Equation 2:1 $$\text{Disulfide bonds} = \frac{\left(\left|\text{total sulfhydryl content - free sulfhydryl content}\right|\right)}{2}$$

2.3.7 Lysine content

Available lysine content was determined using o-phthalaldehyde (OPA) and N-acetyl-L-cysteine (NAC)(Medina Hernandez and Garcia Alvarez-Coque 1992). Reagent buffer (OPA-NAC) was prepared by combining 25mL of 0.05M OPA (EtOH), 25mL of 0.05M NAC (aqueous) and 200mL of 0.1M Boric acid-Borate buffer (pH 9.5). Sample product (~35mg) was heated in 1.5mL of 0.0625M Tris-HCl and 2% SDS (pH 6.8) at 90°C for 5 min. The material was then centrifuged at 13,600 x g for 10 min and 0.75mL of supernatant was transferred into a 10mL volumetric flask. To the flask 4.0mL of OPA-NAC solution was added to the flask and the volume was adjusted to 10mL with ddH_2O. The reaction was allowed to proceed for 10 min. The absorbance was determined at 335nm vs. a reagent blank. The concentration (μmoles of NH_2/ g of sample) was determined using a molar absorptivity of 6830 $M^{-1}cm^{-1}$.

2.3.8 Electrophoresis

The protein content was determined by using the bicinchoninic acid (BCA) protein assay (Smith et al. 1985) . Film samples were homogenized in 10 mL of 0.0625 *M* Tris, 3mM EDTA (pH 8.3) buffer for 20 sec. A 0.5 mL sample was then transferred in to a microcentrifuge tube with 0.5 mL of 10% SDS and 6.6M urea solution. The samples were heated at 50^{0}C for 20 min, vortexed and heated for an additional 5 min. After heating samples were centrifuged at 16,000 x g for 20 min. Samples were diluted with equal volume of water and mixed with BCA reagent. Samples were then heated at 37°C for 30 min. After heating samples were cooled to room temperature, absorbance was read at 562nm. Protein concentrations were calculated using a standard curve obtained using bovine serum albumen.

Molecular weight changes of the whey protein isolate, WPI and WPI/TG films (cured and uncured) were compared using electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), in a Mini-Protean II unit (Bio-Rad Laboratories, Hercules, CA) using a 4% stacking gel and a 12% resolving gel made from 37.5:1 acrylamide: bisacrylamide solution. Samples (20mg) were incubated in 5 mL of 10% SDS, 10% BME and 6.6M urea solution for 24h at room temperature. Samples were then centrifuged at 13,600 x g for 15min. Film samples (2 mg protein/mL) were mixed with denaturing sample buffer (0.0625 *M* Tris, pH 6.8, 2% SDS, 10% glycerol, 5% mercaptoethanol, and 0.2% bromophenol blue) and heated at 90°C for 3min. Each lane was loaded with 20μg of protein. The gel (7cm x 8 cm) was run at 200 volts

for 45min, stained with 0.1% w/v Coomassie blue (R250) dye in 50% v/v methanol and 10% v/v acetic acid, and de-stained with 50% v/v methanol and 10% acetic acid solution.

2.3.9 Statistical analysis

All experiments were replicated 3 times. Results were analyzed using the mixed model procedure in SAS (SAS Institute, Cary, NC). Treatment means were compared with significance defined as $p \leq 0.05$.

2.4 Results and discussion

2.4.1 Mechanical Properties

The mechanical properties of polymer films can be improved by increasing cross-linking between polymer strands which creates a three dimensional network. Both heat curing and TG are able to induce intermolecular cross-linking between protein strands. The effect of heat curing time, TG treatment and the interaction of time and TG had a significant impact on the TS and %E. Heat curing time significantly impacted film toughness (Table 2.2). Treating films with TG produced films with significantly different TS and %E compared to non-TG films. However, it had no effect on toughness. Films heat cured at 12 and 24h had a significant effect on the TS compared to 0h. Films heat cured for 24h had significantly different %E and toughness compared to the other films (Table 2.3).

2.4.2 Tensile Strength.

The mechanical properties of WPI films are shown in Table 2.4. Heat - curing WPI/TG films increased the TS compared to uncured WPI/TG films

($p<0.05$). Heat-curing WPI/TG films for 24h produced the highest TS ($p<0.05$) of all WPI/TG films. The TS of WPI/TG films decreased after 24h of heat curing. WPI films heat-cured for 24h were not significantly different from uncured films. WPI films heat-cured for 12h had the highest TS of all films tested ($p<0.05$). WPI/TG films heat cured for 12h were not significantly different compared to WPI films heat cured for 0 and 24h. WPI/TG films heat cured for 24h were not significantly different compared to WPI films heat cured for 0 and 24h. The treatment of protein film forming solutions with TG has been shown to increase their TS (Yildirim and Hettiarachchy 1998; Larre, Desserme et al. 2000). This is contrary to the results obtained. The previous studies used transglutaminase obtained from mammalian sources while transglutaminase from a microbial source was used in this study. Transglutaminase from microbial sources cross-linked only one lysine residue while mammalian transglutaminase cross-linked three lysine residues in α-lactalbumin (Lee et al. 2002). This difference in lysine cross-links may be responsible for the lower TS observed with these films. The initial heat treatment of the aqueous solution may have produced intermolecular disulfide bonds which decreased the accessibility of lysine. Miller and others (1997) reported heat curing increased the TS of whey protein films. They observed a linear relationship between TS and heat curing time as time increased. The results of this study show an initial increase in TS with increasing time then a decrease in TS. The difference in results from those reported by Miller and others can be attributed to a difference in heat curing conditions.

Table 2.2: Significance[1] of time and/or transglutaminase treatment on the tensile strength, elongation at break and toughness of heat-cured WPI films.

Source of Variation	DF[2]	TS[3]	%E[4]	T[5]
Time	3	<0.0001	0.0002	<0.0001
TG	1	<0.0001	0.0002	0.4560
Time x TG	3	<0.0001	0.0024	0.1082

[1] Probability values
[2] DF = Degrees of freedom
[3] TS = Tensile strength
[4] %E = Elongation at break
[5] T = Toughness

Table 2.3: Comparison of the significance[1] of heat curing time and transglutaminase treatment on the tensile strength, elongation at break and toughness of heat-cured WPI films.

Effects	Source of Variation			DF[2]	TS[3]	%E[4]	T[5]
TG	NoTG	Vs.	TG	40	<0.0001	0.4560	0.4560
	0h	Vs.	12h	40	<0.0001	0.6976	0.6976
Time	0h	vs.	24h	40	<0.0001	<0.0001	<0.0001
	12h	vs.	24h	40	0.2503	<0.0001	<0.0001

[1] Probability values
[2] DF = Degrees of freedom
[3] TS = Tensile strength
[4] %E = Elongation at break
[5] T = Toughness

Table 2.4: Mechanical properties of whey protein-isolate based films with and without microbial transglutaminase heat cured at 90°C for 0, 12, and 24h.

Treatment	Curing Time (h)	Thickness (mil)	Elongation at Break (%)	Toughness (kJ/m^3)	Tensile Strength (Mpa)
WPI/TG	0	7.78±0.81[a]	7.78±0.81[a]	22.5±8.72[a]	1.1±0.12[a]
	12	5.16±0.26[a]	5.16±0.26[a]	37.7±7.62[a]	3.8±0.14[b,g]
	24	7.27±0.76[a]	7.27±0.76[a]	75.6±17.07[b]	4.6±0.10[c,h]
WPI	0	7.88±0.51[a]	15.1±8.15[b,d]	40.6±12.92[a]	4.2±0.72[e,g,h]
	12	6.75±1.57[a]	15.0±3.08[b]	30.2±8.41[a]	5.5±1.60[f]
	24	6.88±1.14[a]	23.2±2.46[c,d]	67.4± 6.68[b]	4.1±0.14[e,g,h]

[a-h] means with different superscript are significantly different ($p<0.05$), Comparisons are made within each column, n=3 for all treatments

The previous study heat cured films under atmospheric conditions at temperatures below 90°C and at relative humidities of 40, 60 and 80%.

These lower temperatures and increased RH allowed the films to retain more moisture. Kim and others (2002) heat cured soy protein films under sub-atmospheric conditions and observed an initial increase in TS after 6 h of heat curing then remained relatively unchanged for the remainder on the time. The heat cured films had lower moisture content compared to uncured films. The difference in moisture content of heat-cured films influenced their mechanical properties. This was attributed to cross-linking which results in a structure that restricts moisture uptake.

2.4.3 Elongation at Break.

WPI films heat cured for 24h had the highest %E of all films tested ($p<0.05$). The %E of all WPI/TG films were significantly lower compared to WPI films. WPI/TG films heat cured for 24h and WPI films heat cured for 24h were not significantly different. The %E of films increased from 12 to 24h for both WPI/TG and WPI films. Kim and others (2002) and Miller and others (1997) observed a decrease in %E with an increase in heat curing time. In this research an initial decrease in %E was observed in WPI/TG followed by an increase in %E with increasing heat-curing time. This was not observed in WPI films. The difference between results and those reported previously may be attributed to the difference

in heat curing conditions. In this study the films were completely wrapped in aluminum foil, while the previous studies did not report wrapping the films during heat curing. Wrapping the films would allow more moisture to be retained which acts as a plasticizer and would increase %E.

2.4.4 Toughness.

Toughness is defined as the energy absorbed by the sample at failure (Rodriguez 1996). It is measured as the area under a stress-strain curve, and takes into account both the TS and %E. There was no significant difference between WPI/TG and WPI films heat cured at 0 and12h. Both films with and without TG and heat cured for 24h had the highest toughness and were not significantly different from each other. The toughness of edible film has not been previously reported. Films with moderate TS and high %E produced films with the highest toughness. Higher toughness films may be better able to withstand fill and seal conditions, and product distribution.

2.4.5 Electrophoresis

Cross-linking of heat cured WPI and WPI/TG films was verified using SDS-PAGE under reducing conditions (Figure 2.1 and 2.2). The three most abundant proteins found in whey are β-lactoglobulin, α-lactalbumin and bovine serum albumin (BSA) with molecular weights of 18,363 daltons, 14,174 daltons and 66,000 daltons respectively(Kinsella and Whitehead 1989).

The effect of heat curing on WPI films at 90°C for 0, 12 and 24h are shown in Figure 2.1. In the heat cured WPI films a decrease in the intensity of β-

lactoglobulin, α-lactalbumin and BSA was observed compared to WPI powder and uncured WPI films. This was accompanied by an increase in higher molecular weight polymers. In the case of WPI films heat cured for 12 and 24h high molecular weight aggregates were observed at the top of the stacking gel. These aggregates were too large to enter the stacking gel. Both uncured WPI films and WPI had similar electrophoretic patterns. The increase in higher molecular weight polymer (25-41kDa) and aggregates (200+ kDa) in WPI films heat cured for 12 and 24h suggests the formation of covalent protein cross-links other than disulfide bonds(Rangavajhyala, Ghorpade et al. 1997; Yildirim and Hettiarachchy 1998).

The effects of heat curing WPI/TG films at 90°C for 0, 12 and 24h are shown in Figure 2.2. In all of the WPI/TG films the formation of aggregates was observed at the top of the stacking gel. These aggregates were unable to migrate in to the stacking and resolving gels. The presence of protein aggregates in the uncured WPI/TG films indicates the formation of transglutaminase catalyzed cross-links. The formation of aggregates in the uncured WPI/TG was similar to those observed by Yildirim and Hettiarachchy (1998). The WPI/TG films heat cured at 90°C for 12 and 24h showed decreasing intensity in the β-lactoglobulin,α-lactalbumin and BSA bands with a corresponding increase in higher molecular weight polymers. The increase in the higher molecular weight polymers in the heat cured WPI/TG films indicates the formation of protein cross-links.

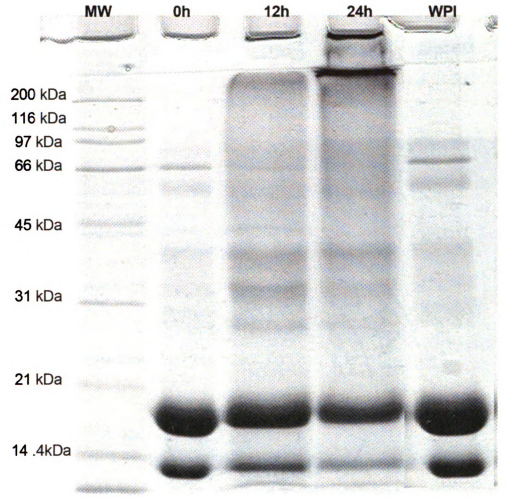

Figure 2.1: SDS-PAGE analysis of whey protein isolate (WPI) and whey protein isolate based films heat cured at 90°C for 0, 12, and 24h. Using a 4% stacking gel and a 12% resolving gel made from 37.5:1 acrylamide:bisacrylamide solution with 20μg of protein loaded in each lane.

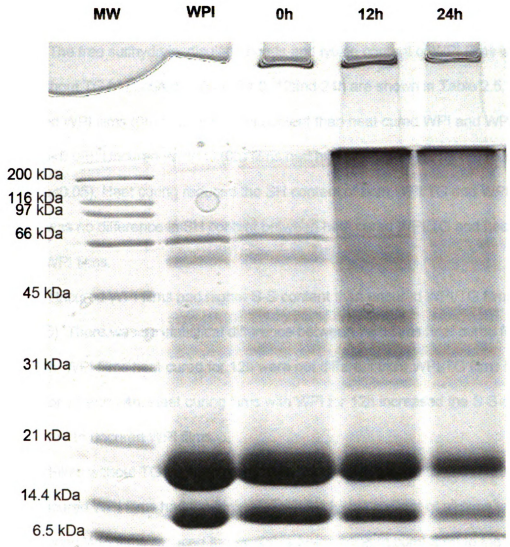

Figure 2. 2: SDS-PAGE analysis of whey protein isolate (WPI) and whey protein isolate based films with transglutaminase heat cured at 90°C for 0, 12, and 24h. Using a 4% stacking gel and a 12% resolving gel made from 37.5:1 acrylamide:bisacrylamide solution with 20μg of protein loaded in each lane.

2.4.6 Sulfhydryl group, disulfide bond and lysine content

The free sulfhydryls, disulfide bonds and lysine content of WPI films with and without TG heat cured at 90°C for 0, 12and 24h are shown in Table 2.5. Uncured WPI films (0h) had higher SH content than heat cured WPI and WPI/TG films ($p<0.05$). Uncured WPI/TG (0h) films had higher SH content than heat cured films ($p<0.05$). Heat curing reduced the SH content of both WPI/TG and WPI films. There was no difference in SH content between heat cured WPI/TG and heat cured WPI films.

Uncured WPI films had higher S-S content than uncured WPI/TG films ($p<0.05$). There was no statistical difference between WPI films heat cured for 0 and 24h. WPI films heat cured for 12h were not different from WPI/TG films heat cured for 12 and 24h. Heat curing films with WPI for 12h increased the S-S content compared to uncured WPI films.

Films without TG had more available lysine compared to films treated with TG. Uncured WPI films had the highest available lysine content of all films tested. Heat curing WPI films reduced the available lysine content compared to the uncured WPI films. Increasing the heat curing time did not significantly reduce the available lysine content. Treating the film solutions with TG prior to casting decreased the available lysine content compared to the untreated films. This was expected since TG catalyzes the formation of cross-links between lysine and glutamine. Heat cured films (24h) had the lowest available lysine content compared to the uncured films with and without TG.

Table 2.5: Free sulfhydryl, disulfide bonds and lysine content of whey protein-isolate based films with and without microbial transglutaminase heat cured at 90°C for 0, 12 and 24h.

	Heat Curing Time (h)	SH (μmol/g protein)	S-S (μmol/g protein)	Lys (μmol NH_2/g protein)
WPI	0	13.0 ± 1.67[a]	22.2 ± 1.12[a]	15.5 ± 0.60[a]
	12	3.0 ± 0.52[c]	25.9 ± 0.41[c]	2.1 ± 0.08[d]
	24	3.0 ± 0.30[c]	21.4 ± 0.29[a]	1.8 ± 0.11[b,d]
WPI/TG	0	8.9 ± 0.34[b]	19.7 ± 0.31[b]	1.4 ± 0.07[b]
	12	3.2 ± 0.68[c]	24.6 ± 0.27[c]	1.0 ± 0.03[b]
	24	4.5 ± 0.77[c]	24.4 ± 0.57[c]	0.3 ± 0.04[f]

[a-f] means with different superscript are significantly different ($p<0.05$), Comparisons are made within each column, n=3 for all treatments.

The alkaline condition of film formation in conjunction with heat curing may induce racemization of amino acid residues which can lead to the formation of covalent cross – links. An existing disulfide bond can form dehydroalanine through a base catalyzed elimination of persulfide. In cysteine the β – elimination of the SH will yield a dehydroprotein. The dehydroprotein is highly reactive and may form a lysinoalanine cross – link with the ε- amino group of lysine (Friedman 1999; Friedman 1999). For both WPI and WPI/TG films, heat curing may result in the formation of isopeptide cross-links. The formation of isopeptide cross-links can also occur between the ε- amino group of lysine and the amide group of asparagine or glutamine residues via a condensation reaction (Singh 1991). The formation of isopeptide cross-links in both WPI and WPI/TG films maybe the reason that the change in disulfide content was not proportional to the change in sulfhydryl content (Table 2.5). The formation of isopeptide cross-links during heat curing can be attributed to the decrease in lysine content observed in both WPI and WPI/TG films.

The electrophoresis patterns of the films heat cured for 24h showed higher molecular weight polymers compared to the other films (Figure2.1 and 2.2). The films with the lowest lysine content also coincide with the films that exhibit the highest toughness (Table 2.4). This suggests that covalent cross – links involving the ε – amino group of lysine are important in enhancing the mechanical properties of WPI and WPI/TG films.

2.5 Conclusions

The heat curing of WPI and WPI/TG films generally enhanced the mechanical properties of these films. Heat curing WPI and WPI/TG films for 24h produced films with the highest toughness of all films tested. Toughness has not been measured in previous studies. Toughness may relate to how a film withstands fill and seal conditions. SDS-PAGE indicated that heat curing the WPI films induced the formation of covalent cross-links, which were also observed in all WPI/TG film samples. Heat curing may promote the formation of isopeptide bonds involving the ε- amino groups of lysine.

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Chapter 3

BARRIER PROPERTIES OF HEAT - CURED AND MICROBIAL TRANSGLUTAMINASE HEAT - CURED WHEY PROTEIN - ISOLATE BASED EDIBLE FILMS.

3.1 Abstract

Edible whey protein films were produced using whey protein isolate WPI (5% w/v), glycerol (3.5% w/v) and candelilla wax (0.8% w/v). Additional films were produced containing the above ingredients with the addition of microbial transglutaminase (TG) (0.001g/g WPI) to the film forming solution after heating. Both sets of films were vacuum – heat – cured for 0 and 24h at 90°C. The water vapor permeability (WVP) of these films was determined at 37.8°C and 85% RH, while the oxygen permeability (O_2P) was determined at 23°C and 0% RH. Moisture sorption isotherms (MSI) of the films were plotted at 5°C and 23°C using the Guggenheim-Anderson-de Boer equation (GAB). Heat curing WPI/TG films reduced the WVP compared to uncured WPI, WPI/TG and cured WPI films. The addition of TG reduced the oxygen permeability compared to films without TG. Heat curing reduced the O_2P of films compared to uncured films. Heat curing WPI/TG films resulted in the greatest reduction in O_2P of films tested. The moisture sorption of films at 5°C decreased as follows WPI> WPI/TG> WPI, 24h> WPI/TG, 24h. At 23°C, the MS of films decreased as follows WPI> WPI, 24h> WPI/TG> WPI/TG, 24h. The films MS determined at 5°C were higher than the MS of films determined at 23°C.

3.2 Introduction

Conventional packaging is used to prevent the mass transfer of moisture, oxygen, aroma, and oil between a food product and the environment and protect the product from mechanical forces encountered during storage and distribution. Edible films can be used to prevent mass transfer between a food product and the environment, which can lead to an increase in the quality and shelf life of the product. Thus, edible films are not intended to replace conventional packaging (Krochta 2002) in all functions. Edible films can be used in conjunction with conventional packaging which can result in a more biodegradable and sustainable package. Edible films add a second layer of protection and thus can help maintain product quality when the package is opened.

Physical and enzymatic treatments such as heat curing and transglutaminase – catalyzed cross linking have been investigated as methods to enhance physical properties of protein based films (Mahmoud and Savello 1993; Miller, Chiang et al. 1997; Sabato, Ouattara et al. 2001; Kim, Weller et al. 2002; Kim, Weller et al. 2002). Protein – based films are good barriers to oxygen but are poor moisture barriers. Their poor performance as moisture barriers is due to the hydrophilicity of proteins and the use of hydrophilic plasticizers(Kester and Fennema 1986) in their composition. Heat curing has been shown to improve the moisture resistance of cast protein films from soy (Rangavajhyala, Ghorpade et al. 1997; Rhim, Gennadios et al. 2000; Kim, Weller et al. 2002; Kim, Weller et al. 2002) and whey protein (Miller, Chiang et al. 1997). Transglutaminase catalyzed

cross-linking in whey protein and soy was investigated by Yildirim and Hettiarachchy (1998). Other barrier properties such as oxygen and aroma permeabilities have not been as extensively studied. The effects of heat curing and transglutaminase treatment on the oxygen permeability and moisture sorption properties of protein-based films have not been extensively reported. Lim and others (1999) investigated the oxygen permeability of egg white protein films cross-linked with transglutaminase. They found that oxygen permeability increased with increasing relative humidity.

The objectives of this study were: 1) to determine the water vapor permeability (WVP) and oxygen permeability (O_2P) of whey protein isolate based edible films treated with and without transglutaminase, and heat cured at 90°C for 0 and 24h; 2) to characterize the moisture sorption isotherms of these films at 5 and 23°C.

3.3 Method

3.3.1 Materials

Whey protein isolate (WPI; Provan 190) was obtained from Glanbia Ingredients Inc. (Monroe WI). The whey proteins were extracted in a highly purified, undenatured form through membrane technology. Composition of WPI was determined using AOAC methods (AOAC 2000) and compared to values provided by Glanbia Ingredients. Glycerol was purchased from J.T. Baker Co. (Phillipsburg, N.J.). Candelilla wax was purchased from Stroh and Pitsch Inc.

(West Babylon N.Y.). Microbial transglutaminase was obtained from Ajinomoto Company Inc. (Japan).

3.3.2 Film preparation

Whey protein isolate (WPI) (5% w/v) and glycerol (3.5% w/v) were mixed in distilled water and the pH adjusted to 8 with 2N NaOH. Each solution was heated at 90°C for 15 min while being stirred continuously. Candelilla wax (0.8% w/v) was added during the last 5 min of heating and allowed to melt into the WPI solution. The WPI solutions were then homogenized for 2 min, using a Polytron PT 10/35 homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH). Each solution was strained through a single layer of cheesecloth to ensure complete mixing of the lipid, and than allowed to equilibrate at ambient temperature for 2 hours. The film solutions were degassed for 30 min and cast on a Teflon-coated surface, and allowed to dry at 23±2°C and 35±5%RH for 24h. Films were stored at 23±2°C and 50±5%RH for 24h prior to heat curing.

3.3.3 Transglutaminase cross-linked films

Film forming solutions were prepared as described. Each solution was cooled to ~ 50°C and TG was added (0.001g/ g of WPI) and allowed to incubate for 3h at 50°C. Each solution was then degassed under vacuum for 30 min and cast onto a Teflon-coated surface. The amount of TG used was determined from the technical specification received from Ajinomoto Company Inc. The films were allowed to dry at 23±2°C and 35±5%RH for 24h and then stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

3.3.4 Heat curing

Films were wrapped in aluminum foil and heated in a vacuum oven at 90°C films for 24h. After heat curing, films were allowed to equilibrate at 45±5% RH for 24h prior to removal from the foil. Films were then conditioned at 23±2°C and 50±5%RH for 48h prior to testing.

3.3.5 Water vapor permeability (WVP)

The water vapor transmission rate (WVTR) of the films was determined at 37.8°C and 85%RH using ASTM E 96-00 *"Standard test method for water vapor transmission of materials"* (ASTM 2000). Specimens were cut using a standard cutter and adhered with wax to standard size dishes containing dessicant. Samples were placed into an environmental chamber maintained at 37.8°C and 85% RH. The weight gain was recorded at 0, 0.5, 2.0, 3.0, 6.0 and 9.0h. The WVTR was calculated by linear regression of weight gain and time per area. To determine WVP from WVTR the following equation was used:

Equation 3:1

$$P = \frac{WVTR \times d}{\Delta p}$$

P = permeability
WVTR = water vapor transmission rate
d = thickness of film
Δp = difference of pressure between the internal and external sides of the film

3.3.6 Oxygen permeability

Oxygen permeability (O_2P) was determined according to ASTM D 3985 *"Standard test method for oxygen gas transmission rate through plastic film and sheeting using a coulometric sensor"* (ASTM 2000) using a Oxtran 2/21 (Mocon Minneapolis, MN). The testing surface area was reduced from 50 cm^2 to 5 cm^2 using aluminum foil tape. Film O_2P was determined at 23°C and 0%RH. All film samples were conditioned at test conditions for 10h prior to testing. The test gas was air (21% oxygen) and nitrogen was used as the carrier gas.

3.3.7 Moisture sorption isotherm

The hygroscopicity of a product relates to its ability to absorb moisture from its surrounding environment. To determine if heat curing affects the hygroscopicity of WPI films, moisture sorption isotherms (MSI) were experimentally derived for WPI and WPI/TG films at 5 and 23°C. Initial moisture content (IMC) was determined by weighing and then drying a 0.3g sample in a convection oven at 100°C for 24h. Samples were allowed to cool in a desiccator for 30 min and then weighed to determine loss of moisture. The IMC (dry basis) was determined using the following equation:

Equation 3:2

$$\text{IMC } (\%) = \frac{\Delta W}{W_f} \times 100$$

ΔW = change in weight
W_f = weight of sample before drying

To derive the MSI, samples were placed into containers with different humidity conditions using saturated salt solutions. Saturated salt solutions were prepared according to ASTM standard E-104 (ASTM 2000). Five humidity conditions were prepared, including $LiCl \cdot H_2O$ (12%), $MgCl_2$ (33%), $Mg(NO_3)_2 \cdot 6H_2O$ (50%), $NaNO_2$ (60%), and NaCl (75%). Samples were placed into each relative humidity condition (90 mil HDPE bucket) and allowed to equilibrate. Samples were weighed every 2 days until they reach a constant mass. The moisture sorption data was modeled using the Guggenheim-Anderson-de Boer equation as follows:

Equation 3:3

$$GAB = \frac{(W_m C K a_w)}{(1 - K a_w + C K a_w)}$$

GAB = equilibrium moisture content
W_m = BET monolayer moisture content
C = Guggenheim constant
K = factor correcting properties of the multilayer molecules corresponding to the bulk liquid
a_w = water activity

To determine the W_m, C, and K parameters the equation was transformed as follows:

Equation 3:4

$$\frac{a_w}{EMC} = \alpha(a_w)^2 + \beta(a_w) + \gamma$$

$\alpha = K/W_m (1/C-1)$
$\beta = 1/W_m (1-2/C)$
$\gamma = 1/W_m - (C \times K)$

The values for α, β and γ were determined by performing quadratic regression analysis on the experimental equilibrium moisture content values.

3.3.8 Statistical analysis

All experiments were replicated 3 times. Results were analyzed using the mixed model procedure in SAS (SAS Institute, Cary, NC). Pair – wise comparisons were made between treatment means using Student-Newmans-Kuhl with a significance of difference defined as $p \leq 0.05$.

3.4 Results and Discussion

3.4.1 Water Vapor Permeability

The WVP of WPI and WPI/TG films heat cured for 0 and 24h are shown in Table 3.1. Heat curing significantly ($p<0.05$) lowered the WVP of WPI/TG films. Heat curing lowered the WVP of WPI films but was not significantly different from the control. Only WPI/TG heat cured for 24h was found to have a significantly lower WVP than the other samples. The values obtained for the WVP of the WPI and WPI/TG films are higher than those reported by other researchers (Miller, Chiang et al. 1997; Yildirim and Hettiarachchy 1998; Kim 2000; Kim, Weller et al. 2002). The higher WVP values observed in this study may be due to the differences in testing conditions. The previous studies were conducted at lower temperatures (22-25°C) and relative humidity (50-80%RH) while in this study the testing conditions were 37.8°C and 85% RH. The higher temperature was chosen to study the WVP of the films under tropical conditions. An increase of ~10°C in temperature will increase the permeability by a factor of 2 (Hernandez, Selke et al. 2000). The increase in relative humidity allows for an increase in absorbed water.

Table 3.1: Water vapor permeability at 37.8°C and 85%RH and oxygen permeability at 23°C and 0%RH of whey protein-isolate based films with and without microbial transglutaminase and heat cured at 90°C for 0 and 24h

Treatment	Heat curing time (h)	WVP g*mm/m2*d*kPa	O_2P(x 10-2 cc*mm/m2*d*kPa
WPI/TG	0	33.7±2.9[a]	8.4±0.4[a]
	24	20.8±3.3[b]	5.2±0.4[b]
WPI	0	36.5±3.2[a]	11.1±0.1[c]
	24	30.7±4.6[a]	7.7±0.3[a]

[a-c] means with the same superscript are not significantly different ($p<0.05$). Comparisons are made within each column. n=3 for all treatments.

This absorbed water acts as a plasticizer and increases the free volume of the film (Hernandez, Selke et al. 2000).

3.4.2 Oxygen Permeability

The O_2P of WPI and WPI/TG films heat cured at 90°C for 0 and 24h are shown in Table 3.1. Heat curing both WPI/TG and WPI films significantly decreased the O_2P ($p<0.05$). WPI/TG films heat cured for 24h had the lowest O_2P ($p<0.05$) of all films tested. No significant difference between uncured WPI/TG films and WPI films heat cured for 24h were observed. Both TG and heat curing induce cross-linking in WPI films; thus the increase in cross-linking may reduce the O_2P of the films. The polar nature of cross-linked protein based films limits the motion of the polymer chains(Chick and Ustunol 1998). Increased cross-linking decreases the segmental mobility of the polymer, thus, decreasing the amorphous regions in the films. Permeability only occurs in the amorphous regions of a film (Hernandez, Selke et al. 2000).

3.4.3 Moisture sorption isotherms (MSI)

The GAB moisture sorption isotherm model has been shown to fit well the moisture sorption data of many food products and is the most accepted model for foods (Bizot 1983). The MSI for WPI and WPI/TG films heat cured at 90°C for 0 and 24h at 23°C (Figure 3.1) and 5°C (Figure 3.2) were determined. The equilibrium moisture content (EMC) of WPI and WPI/TG films heat cured at 90°C for 0 and 24h at 23°C increased slowly from 0.1 to 0.6 a_w followed by an exponential increase in the EMC of the films at higher a_w. The EMC of films at 5°C

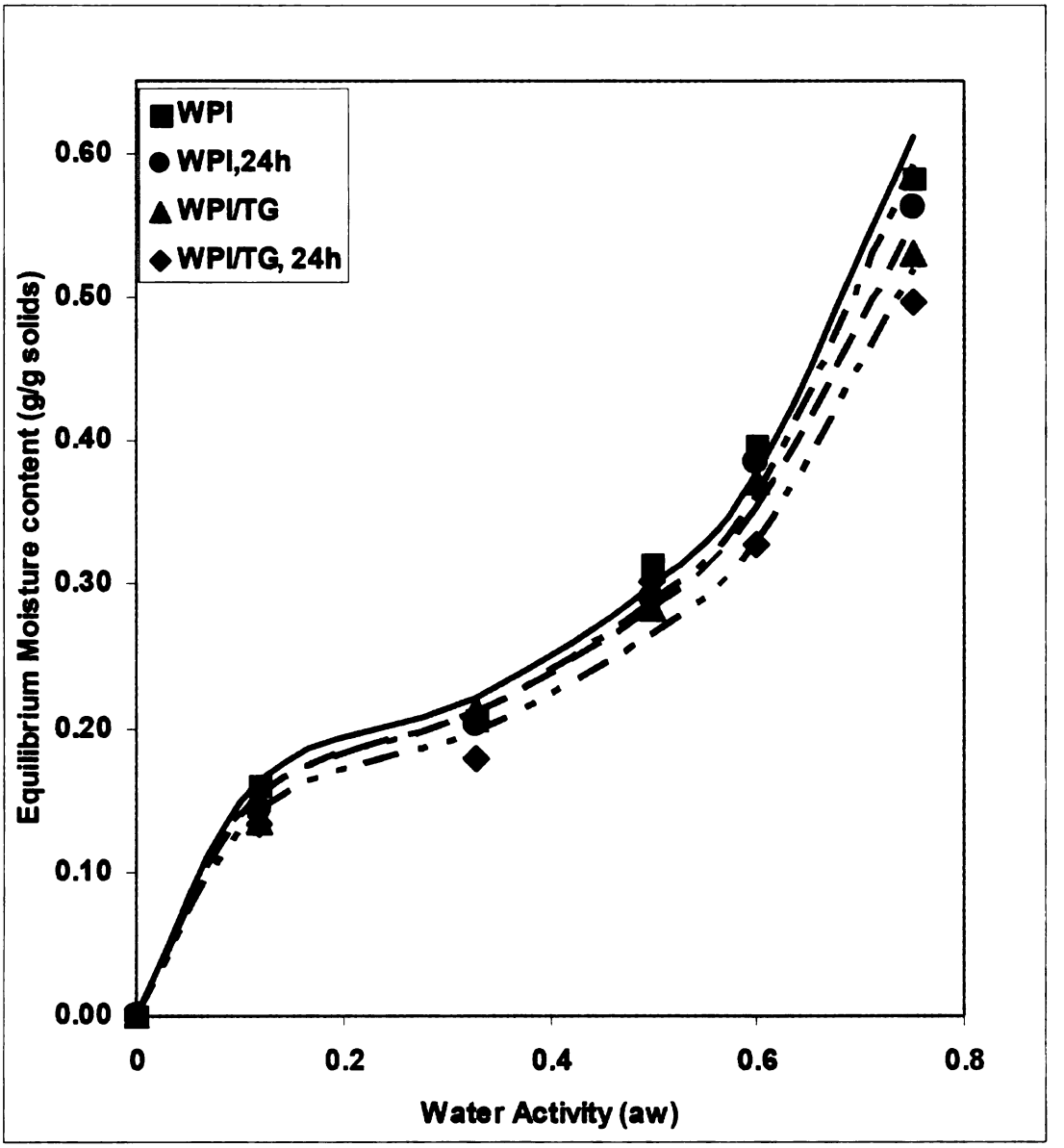

Figure 3.1: Moisture sorption isotherm at 23°C of whey protein-isolate based films with and without microbial transglutaminase and heat cured at 90°C for 0 and 24h.

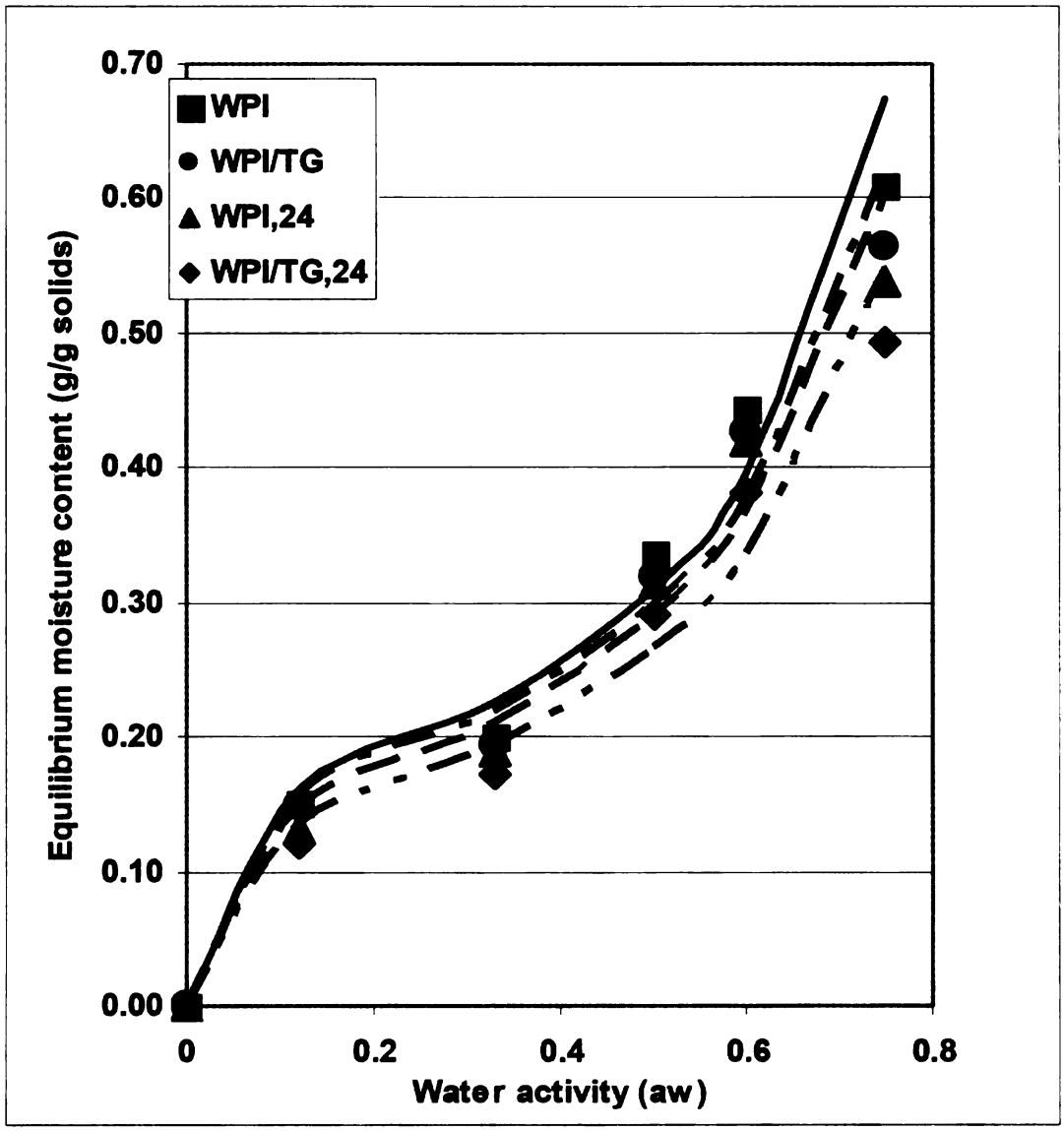

Figure 3.2: Moisture sorption isotherm at 5°C of whey protein-isolate based films with and without microbial transglutaminase and heat cured at 90°C for 0 and 24h.

showed a slow increase form 0 – 0.5 a_w followed by an exponential increase in EMC of the films. At both 5 and 23°C the MSI of WPI and WPI/TG films heat cured for 24h were less pronounced than their corresponding uncured films. The difference in EMC of WPI and WPI/TG films heat cured at 90°C for 0 and 24h were not noticeable for a_w from 0-0.5 at either 23 or 5°C. As a_w increased above 0.5 the EMC of samples at 5°C were higher than those at 23°C. The EMC of TG cross-linked egg white films was seen to increase with decreasing temperature (Lim, Mine et al. 1999). At 5°C and 23°C WPI and heat cured WPI films had sorption isotherms that showed higher moisture content at the same relative humidity (a_w) compared to WPI/TG and heat cured WPI/TG films at the same temperatures. The films with lower isotherms (WPI, 24h and WPI/TG, 24h) corresponded with films with lower lysine content (Table 2.5).By increasing the amount of cross-linking by heat curing produced films with lower sorption isotherms. The increased cross-linking in the heat – cured films will decrease the amorphous regions in the films. As with permeability, sorption occurs in the amorphous regions of the film.

The GAB constants were determined by fitting the GAB equation to the experimental isotherm data for the films at 23°C and 5°C (Table 3.2). The monolayer moisture content (W_m) of heat cured WPI and WPI/TG films were lower than uncured WPI and WPI/TG films at both 23 and 5°C. The W_m of WPI/TG films were lower than WPI films at both 23 and 5°C. At 23°C the Guggenheim constant (C), a correction factor for the enthalpy difference between the monolayer and

Table 3.2: Parameters used to fit the Guggenheim-Anderson-de Boer equation at 23°C and 5°C of whey protein-isolate based films with and without microbial transglutaminase and heat cured at 90°C for 0 and 24h.

Treatment	Heat curing time (h)	W_m[1]		C[2]		k[3]		RMS[4]	
		23°C	5°C	23°C	5°C	23°C	5°C	23°C	5°C
WPI/TG	0	0.146	0.148	96.90	91.44	0.980	1.016	5.13	2.72
	24	0.136	0.134	99.89	59.89	0.985	1.010	4.34	4.44
WPI	0	0.149	0.152	202.77	91.87	1.009	1.035	1.99	2.72
	24	0.143	0.146	138.87	60.63	1.012	1.013	3.34	4.38

[1]W_m = monolayer moisture content
[2]C = factor correcting enthalpy difference between monolayer and multilayer of water
[3]k = factor correcting enthalpy difference between free water and multilayer water
[4]RMS = root mean square values

multilayer of water, for WPI and heat cured WPI films was higher than for WPI/TG and heat cured WPI/TG films. At 5°C the C for uncured WPI and WPI/TG was higher than heat cured WPI and WPI/TG films. The k value, a correction factor for the enthalpy difference between free and multilayer water, was higher for uncured and heat cured WPI films than uncured and heat cured WPI/TG at both 23 and 5°C. The root mean square (RMS) was used to evaluate the quality of the fit between the experimental and calculated values for films at 23 and 5°C (Table 3.2).A lower RMS value indicates a good fit. The RMS values determined for the experimental and calculated values at both 23 and 5°C were in the low range. The MSI can be used to predict barrier and mechanical properties of films at various RH (Chick 1998). From the MSI of WPI and WPI/TG at 5°C films it could be predicted that the films would have increased water vapor permeability and elongation, and decrease in tensile strength compared to films at 23°C (Aydinli and Tutas 2000; Cho and Rhee 2002).

3.5 Conclusions

At higher humidity and temperature the effectiveness of WPI films as a moisture barrier is drastically reduced. Cross-linking WPI films with TG and heat curing can improve the water vapor and oxygen permeability of the films. The cross-linking produced using heat curing and TG inhibits the diffusion of both water vapor and oxygen through the films. Cross-linking decreases the segmental mobility of the polymer chains leading to a decrease in permeability. Heat cured WPI/TG films had the lowest WVP and O_2P of all films tested. Heat curing and TG

decreased the MSI of WPI films at both 23 and 5°C. The EMC of the films at 5°C were slightly higher than those at 23°C. The higher MS of films at 5°C could be a problem for products that require refrigeration. At refrigerated temperatures the films would have higher WVP permeability and lower mechanical properties.

Chapter 4

OPTICAL PROPERTIES OF HEAT - CURED WHEY PROTEIN - ISOLATE BASED EDIBLE FILMS AND MICROBIAL TRANSGLUTAMINASE HEAT - CURED WHEY PROTEIN - ISOLATE BASED FILMS.

4.1 Abstract

The transmission of both ultraviolet and visible light are important factors in considering possible applications for edible films. The color characteristics of the film can impact consumer acceptability of the packaging. Edible whey protein films were produced using whey protein isolate WPI (5% w/v), glycerol (3.5% w/v) and candelilla wax (0.8% w/v) with and without the addition of microbial transglutaminase (0.001g/g WPI) to the film forming solution after heating. Both sets of films were vacuum heat cured for 0, 12, and 24h at 90°C. The effect of heat curing and transglutaminase treatment on the optical properties (ultraviolet and visible light transmission and the color characteristics) of WPI films were assessed using spectrophotometer and a chromameter. Heat curing increased the yellowness of films. The yellowness of WPI films increased from 11.8±0.75 at 0h to 33.2±4.89 at 24h of heat curing. The yellowness of WPI/TG films increased from 9.2±0.15 at 0h to 33.8±3.11 at 24h of heat curing. All films showed low transmission of ultraviolet light, from 0-60% transmission between 190 - 400nm. WPI and WPI/TG films are good barriers to UV radiation.

4.2 Introduction

One of the main factors related to food quality is the absorbance of ultraviolet and visible light by the product. Light has been shown to affect the

flavor and nutritional content of milk (Bradley 1980). Color characteristics and light transmission are important properties in determining the acceptability of packaging materials. The color (light and transmission) characteristics can be critical in selecting the right film for a food application. By reducing the exposure of food products to ultraviolet and visible light the quality and shelf life of food products can be enhanced (Hernandez, Selke et al. 2000).

Even though ultraviolet radiation accounts for only 3% of the total radiation that reaches the earth it is able to cause chemical reactions in both polymers and food products. These chemical reactions can have an undesirable effect on the quality and shelf life of the food products. Ultraviolet radiation can be subdivided into three distinctive wavelength regions. These three regions are UV-A (400-315nm), UV-B (315-280nm), and UV-C (280-100nm). Visible light spans the wavelengths from 400-700nm and produce colors from violet, blue, green, yellow, and orange to red. There has been limited research on the color values of heat cured protein based films (Gennadios, Ghorpade et al. 1996; Rhim, Gennadios et al. 2000; Kim, Weller et al. 2002). The research was conducted soy protein based films not on whey protein based films.

The objectives of this study were to determine the color characteristics and light transmission of whey protein isolate based edible films with and without transglutaminase, and heat cured at 90°C for 0,12 and 24h, and secondly, to compare the color values for WPI films to commercially available plastic packaging materials.

4.3 Materials and Methods

4.3.1 Materials

Whey protein isolate (WPI; Provan 190) was obtained from Glanbia Ingredients Inc. (Monroe WI). The whey proteins were extracted in a highly purified, undenatured form using membrane technology. Glycerol was purchased from J.T. Baker Co. (Phillipsburg, N.J.), and Candelilla wax was purchased from Stroh and Pitsch Inc. (West Babylon N.Y.). Microbial transglutaminase was obtained from Ajinomoto Company Inc. (Japan).

4.3.2 Film preparation

Whey protein isolate (WPI) (5% w/v) and glycerol (3.5% w/v) were mixed in distilled water and the pH adjusted to 8.0 with 2N NaOH. These solutions were then heated at 90°C for 15 min while being stirred continuously. Candelilla wax (0.8% w/v) was added during the last 5 min of heating and allowed to melt into the WPI solution. The WPI solutions were homogenized for 2 min, using a Polytron PT 10/35 homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH). Each homogenized solution was then strained through a single layer of cheesecloth to ensure complete incorporation of the lipid and allowed to equilibrate at ambient temperature for 2 hours. Each solution was then degassed for 30 min and cast on to a Teflon-coated metal pan, where it was dried at 23±2°C and 35± 5%RH for 24h. Films were stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

4.3.3 Transglutaminase cross-linked films

Film forming solutions were prepared as described above. Solutions were allowed to cool to ~ 50°C and TG was added (0.001g/ g of WPI) and allowed to incubate for 3h at 50°C. Each solution containing TG was then degassed under vacuum for 30 min and cast onto a Teflon-coated metal pan. The amount of TG used was determined from the technical specification received from Ajinomoto Company Inc. Each solution was dried at 23±2°C and 35± 5%RH for 24h. Films were stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

4.3.4 Heat curing

Films were wrapped in aluminum foil and heated in a vacuum oven at 90°C films for 12 and 24h. After heat curing, films were allowed to equilibrate at 45± 5% RH for 24h prior to removal from foil. Films were conditioned at 23±2°C and 50± 5%RH for 48h prior to testing.

4.3.5 Optical Properties

The color values (L*, a*, and b*) for WPI and WPI/TG heat cured films were measured with a Minolta Chromameter CR-310 (Commission D'Edairerage (CIE) L*a*b*, Ramsey, NJ). The L* values measure the lightness and ranges from 0 (black) to 100 (white). The a* values measure the change from green (-) to red (+) and b* measures the change from blue (-) to yellow (+). Total color difference (ΔE) was calculated using equation 4:1:

Equation 4:1 $\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$

ΔE = **Total color difference**

$\Delta L^* = L^*_{sample} - L^*_{ref}$

$\Delta a^* = a^*_{sample} - a^*_{ref}$

$\Delta b^* = b^*_{sample} - b^*_{ref}$

The transmission of visible and UV light was determined for WPI and WPI/TG heat cured films. Transmission was measured using a Perkin Elmer Lambda 25 UV/VIS spectrometer equipped with a RSA-PE-20 Integrating Sphere for Transmittance and Reflectance. Samples were scanned at wavelengths between 190.0 to 900nm at 480 nm/min.

4.3.6 Statistical analysis

Color measurements were replicated 3 times. Results were analyzed using the mixed model procedure in SAS (SAS Institute, Cary, NC). Treatment means were compared with significance defined as $p \leq 0.05$.

4.4 Results and Discussion

4.4.1 Color

The color values for heat cured WPI and WPI/TG heat cured films are shown in Table 4.1. Both WPI and WPI/TG films heat cured for 0 and 12h did not have significantly different L* values. Heat curing films for 24h significantly decreased ($p<0.05$) the L* values compared to uncured films and films heat cured for 12h. There was no significant difference in L* values between WPI films treated with and without TG. No significant difference in a* values were observed

Table 4.1: The L*, a*, b* values of whey protein-isolate based films with and without microbial transglutaminase heat cured at 90°C for 0, 12 and 24h.

	Time	L*	a*	b*
WPI	0	85.8±0.29[a]	-5.3±0.06[a]	11.8±0.75[a]
	12	82.8±1.02[a,c]	-4.6±0.27[a,b]	24.7±3.33[b]
	24	77.9±2.40[c,e]	-1.8±1.29[c]	33.2±4.89[c]
WPI/TG	0	87.2±0.02[a]	-5.1±0.05[a]	9.2±0.15[a]
	12	83.5±0.16[a,b]	-4.8±0.07[a]	22.5±0.63[b]
	24	77.6±1.88[c,d]	-1.8±1.02[c]	33.8±3.11[c]

[a-e] means with different superscript are significantly different ($p<0.05$), Comparisons are made within each column, n=3 for all treatments values.

Table 4.2: Color difference between whey protein-isolate based films heat cured at 90°C 12 and 24h compared to films cured for 0h.

Time	ΔL*	Δa*	Δb*	ΔE
0	-	-	-	-
12	-2.92	0.71	12.89	13.24
24	-7.87	3.47	21.48	23.14

Table 4.3: Color difference between whey protein-isolate based films with microbial transglutaminase heat cured at 90°C 12 and 24h compared to films cured for 0h.

Time	ΔL*	Δa*	Δb*	ΔE
0	-	-	-	-
12	-3.72	0.25	13.28	13.79
24	-9.58	3.31	24.61	26.61

Table 4.4: The L*, a*, b* values of cellophane, polystyrene, low-density polyethylene and polyethylene terephthalate.

	L*	a*	b*
Cellophane	**88.96±0.10**	**-0.82±0.15**	**1.47±0.04**
PS[1]	**89.82±0.70**	**-0.76±0.05**	**0.51±0.05**
LDPE[1]	**89.75±0.10**	**-0.95±0.03**	**-0.70±0.04**
PET[1]	**88.15±0.20**	**-1.05±0.06**	**0.10±0.05**

[1] PS = polystyrene, LDPE = low density polyethylene, PET = polyethylene terephthalate

Table 4.5: Color difference between whey protein-isolate based films without microbial transglutaminase heat cured at 90°C for 0h compared to cellophane, polystyrene, low-density polyethylene and polyethylene terephthalate.

	ΔL*	Δa*	Δb*	ΔE
Cellophane	**-3.18**	**-4.43**	**10.31**	**11.67**
PS	**-4.04**	**-4.54**	**11.27**	**12.81**
LDPE[1]	**-3.97**	**-4.34**	**12.44**	**13.76**
PET[1]	**-2.34**	**-4.19**	**11.68**	**12.63**

[1] PS = polystyrene, LDPE = low density polyethylene, PET = polyethylene terephthalate

Table 4.6: Color difference between whey protein-isolate based films without microbial transglutaminase heat cured at 90°C for 12h compared to cellophane, polystyrene, low-density polyethylene and polyethylene terephthalate.

	ΔL*	Δa*	Δb*	ΔE
Cellophane	**-6.10**	**-3.73**	**23.21**	**24.28**
PS	**-6.96**	**-3.84**	**24.17**	**25.44**
LDPE[1]	**-6.89**	**-3.64**	**25.34**	**26.51**
PET[1]	**-5.26**	**-3.49**	**24.58**	**25.37**

[1] PS = polystyrene, LDPE = low density polyethylene, PET = polyethylene terephthalate

Table 4.7: Color difference between whey protein-isolate based films without microbial transglutaminase heat cured at 90°C for 24h compared to cellophane, polystyrene, low-density polyethylene and polyethylene terephthalate.

	ΔL*	Δa*	Δb*	ΔE
Cellophane	**-11.05**	**-0.96**	**31.80**	**33.68**
PS	**-11.91**	**-1.07**	**32.76**	**34.87**
LDPE[1]	**-11.84**	**-0.87**	**33.93**	**35.94**
PET[1]	**-10.21**	**-0.72**	**33.17**	**34.71**

[1] PS = polystyrene, LDPE = low density polyethylene, PET = polyethylene terephthalate

Table 4.8: Color difference between whey protein-isolate based films with microbial transglutaminase heat cured at 90°C for 0h compared to cellophane, polystyrene, low-density polyethylene and polyethylene terephthalate.

	ΔL*	Δa*	Δb*	ΔE
Cellophane	**-1.75**	**-4.23**	**7.77**	**9.02**
PS	**-2.61**	**-4.34**	**8.73**	**10.09**
LDPE[1]	**-2.54**	**-4.14**	**9.90**	**11.02**
PET[1]	**-0.91**	**-3.99**	**9.14**	**10.01**

[1] PS = polystyrene, LDPE = low density polyethylene, PET = polyethylene terephthalate

Table 4.9: Color difference between whey protein-isolate based films with microbial transglutaminase heat cured at 90°C for 12h compared to cellophane, polystyrene, low-density polyethylene and polyethylene terephthalate.

	ΔL*	Δa*	Δb*	ΔE
Cellophane	-5.47	-3.98	21.05	22.11
PS	-6.33	-4.09	22.01	23.26
LDPE[1]	-6.26	-3.89	23.18	24.32
PET[1]	-4.63	-3.74	22.42	23.19

[1] PS = polystyrene, LDPE = low density polyethylene, PET = polyethylene terephthalate

Table 4.10: Color difference between whey protein-isolate based films with microbial transglutaminase heat cured at 90°C 24h compared to cellophane, polystyrene, low-density polyethylene and polyethylene terephthalate.

	ΔL*	Δa*	Δb*	ΔE
Cellophane	-11.33	-0.92	32.38	34.31
PS	-12.19	-1.03	33.34	35.51
LDPE[1]	-12.12	-0.83	34.51	36.58
PET[1]	-10.49	-0.68	33.75	35.35

[1] PS = polystyrene, LDPE = low density polyethylene, PET = polyethylene terephthalate

between WPI and WPI/TG films heat cured for 0 and 12h. Films heat cured for 24h had significantly lower ($p<0.05$) a* values compared to films heat cured at 0 and 12h. Both(Kinsella and Whitehead 1989) uncured WPI and uncured WPI/TG films had significantly lower ($p<0.05$) b* values compared to heat cured WPI and WPI/TG films. Films heat cured for 12h had significantly lower ($p<0.05$) b*compared to films heat cured for 24h. The increase in yellowness of heat cured films was also observed in soy protein isolate films (Gennadios, Ghorpade et al. 1996; Rhim, Gennadios et al. 2000; Kim, Weller et al. 2002).

Tables 4.2 and 4.3 show the difference between heat cured WPI films treated with transglutaminase and uncured films treated with transglutaminase. In both cases the difference in the color values of the films increased as heat curing time increased. The increased ΔL^* observed in the heat cured indicated an increased lightness in the films compared to uncured films. The increased Δa^* in the heat cured films indicated an increased redness in the films compared to uncured films. The increased Δb^* observed in the heat cured films indicated and increased yellowness in the films compared to uncured films. The ΔL^* and Δb^* observed in the heat cured films resulted in the large ΔE obtained for the heat cured films. The increased yellowness observed in the heat-cured films may be from Maillard reactions occurring between whey proteins and residual lactose in the films.

The color differences (ΔE) between WPI films and commercial films are shown in Table 4.5 through 4.10. All film samples showed large degrees of color

difference compared to commercial films. As heat-curing time increased the ΔE values also increased. The greatest increase was observed between uncured films and those heat cured for 12h. As heat-curing time increased the increase in ΔE was smaller. The large ΔE values observed can be attributed to the high b* values of the WPI films. This was observed as an increase in yellowness of the heat cured films. The large difference between commercial plastics and WPI films may have a beneficial or a detrimental affect on consumer acceptance of products packaged in WPI films. More research needs to be conducted in this area to determine the acceptability of heat cured WPI and WPI/TG films.

4.4.2 Transmission of UV and Visible light

The transmission of UV and visible light are critical parameters in choosing the proper packaging applications for a film. The transmission of UV and visible light for WPI films with and without TG heat cured at 90°C for 0, 12, 24h are shown in Figure 4.1. All films showed low transmission in the UV-C and UV-B range. At ~300nm the transmission of UV light increased in uncured WPI and WPI/TG films. The increase in transmission continued through the visible spectrum from 400nm to ~550nm (Figure 4.1). Both WPI and WPI/TG films heat cured at 90°C for 12h did not show any increase in UV transmission up to~350nm. Increased light transmission then continued to ~550nm. The increase in UV and visible light observed in the WPI and WPI/TG films heat cured at 90°C for 24h was similar to films heat cured for 12h. The low UV transmission would allow WPI and WPI/TG films to be used to protect light sensitive products without the addition of UV

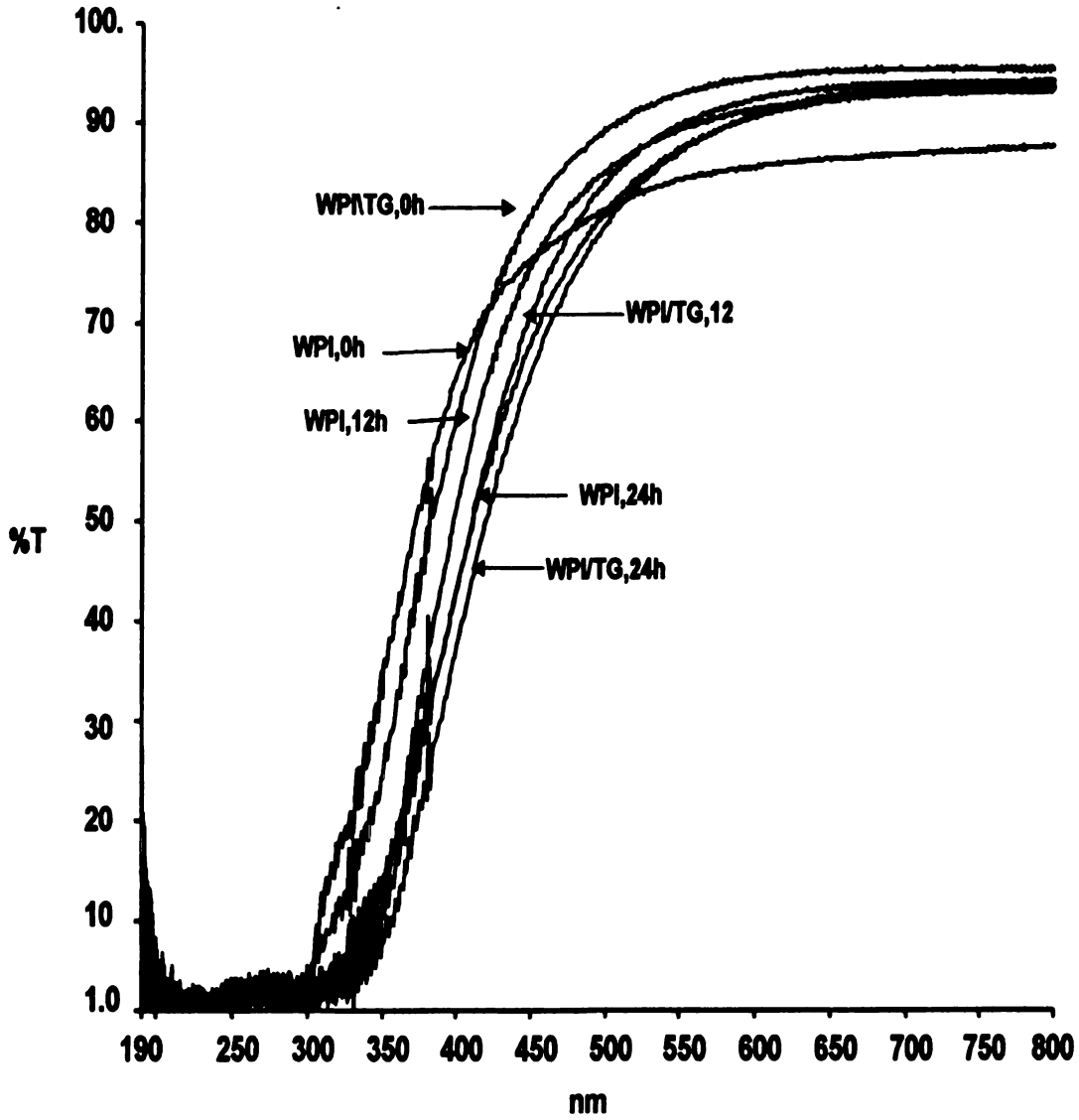

Figure 4.1: % transmission versus wavelength of whey protein-isolate based films with and without microbial transglutaminase heat cured at 90°C for 0, 12 and 24h.

absorbers. The low transmission of UV light observed by the films is to be expected since proteins absorb UV radiation in the 190 to 280 nm range.

4.5 Conclusions

Heat curing increased the yellowness of WPI and WPI/TG films. It also increased the redness of WPI and WPI/TG films. All WPI and WPI/TG films have low transmission of UV light which may impact oxidative rancidity of the food packaged therein. The increased yellowness of heat cured WPI and WPI/TG films will probably decrease their acceptability in certain packaging applications. The increased yellowness may lead consumers to view the package as old and that the product is past its prime. The increased yellowness may serve to enhance other food colors such as cured or smoked meat characteristic. The low transmission of UV light indicates that WPI and WPI/TG films could be used to protect light sensitive products. Based on the low transmission of UV radiation WPI and WPI/TG films may be used as a packaging material for products such as dairy foods and pharmaceutical products.

Chapter 5

THERMAL PROPERTIES OF HEAT - CURED WHEY PROTEIN ISOLATE - BASED EDIBLE FILMS COMPARED TO MICROBIAL TRANSGLUTAMINASE HEAT - CURED WHEY PROTEIN ISOLATE FILMS.

5.1 Abstract

Edible whey protein films were produced using whey protein isolate WPI (5% w/v), glycerol (3.5% w/v) and candelilla wax (0.8% w/v). Films were also produced as described above with the addition of microbial transglutaminase (0.001g/g WPI) to the film forming solution after heating. Both sets of films were vacuum heat cured for 0, 12 and 24h at 90°C. The effects of heat curing and transglutaminase treatments on the thermal properties (melting onset temperature, peak melting temperature and enthalpy of fusion) of the whey protein isolate films were assessed. The peak melting temperatures for films increased with heat curing and time. The peak melting temperatures of WPI films increased from 140.57°C at 0 h to 158.1°C at 24h of heat curing. The peak melting temperatures of WPI/TG films increased from 137.36°C at 0h to 157.2°C at 24h of heat curing. The increase in peak melting temperatures corresponded to an increase in cross-linking in the films. The higher peak melting temperatures observed for heat-cured films would indicate that higher processing temperatures would be needed to produce films and that the films have greater thermal stability.

5.2 Introduction

Thermal properties such as melting temperature (T_m) and glass transition temperature (T_g) are important in determining the processing conditions of polymer films. The T_m of a polymer is the temperature at which the polymer molecules are free to move past each other and the crystalline regions begin to break up (Rodriguez 1996). The T_m of films can also be used to determine an optimal temperature range for film extrusion. For crystalline plastics, film processing temperatures are about 50°C above their T_m (Hernandez, Selke et al. 2000).

Heat curing has been shown to enhance the mechanical and barrier properties of protein based films (Gennadios, Ghorpade et al. 1996; Miller, Chiang et al. 1997; Kim, Weller et al. 2002; Kim, Weller et al. 2002). Cross-linking proteins using transglutaminase has been shown to enhance the mechanical and barrier properties of protein based films (Mahmoud and Savello 1992; Mahmoud and Savello 1993; Yildirim and Hettiarachchy 1998; Lim, Mine et al. 1999; Larre, Desserme et al. 2000). There has been little research conducted on the thermal properties of heat-cured films and transglutaminase cross-linked films.

The objectives of this study were to determine thermal properties (melting onset temperature, peak melting temperature and enthalpy of fusion) of whey protein isolate films with and without transglutaminase, and heat cured at 90°C for 0, 12 and 24h.

5.3 Materials and Methods

5.3.1 Materials

Whey protein isolate (WPI; Provan 190) was obtained from Glanbia Ingredients Inc. (Monroe WI). The whey proteins were extracted in a highly purified, undenatured form using membrane technology. Glycerol was purchased from J.T. Baker Co. (Phillipsburg, N.J.), and Candelilla wax was purchased from Stroh and Pitsch Inc. (West Babylon N.Y.). Microbial transglutaminase was obtained from Ajinomoto Company Inc. (Japan).

5.3.2 Film preparation

Whey protein isolate (WPI) (5% w/v) and glycerol (3.5% w/v) were mixed in distilled water and the pH adjusted to 8.0 with 2N NaOH. These solutions were then heated at 90°C for 15 min while being stirred continuously. Candelilla wax (0.8% w/v) was added during the last 5 min of heating and allowed to melt into the WPI solution. The WPI solutions were homogenized for 2 min, using a Polytron PT 10/35 homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH). Each homogenized solution was then strained through a single layer of cheesecloth to ensure complete incorporation of the lipid and allowed to equilibrate at ambient temperature for 2 hours. Each solution was then degassed for 30 min and cast on to a Teflon-coated metal pan where it was allowed to dry at 23±2°C and 35± 5%RH for 24h. Films were stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

5.3.3 Transglutaminase cross-linked films

Film forming solutions were prepared as above. Solutions were allowed to cool to ~ 50°C and TG was added (0.001g/ g of WPI) and allowed to incubate for 3h at 50°C. Each solution containing TG was then degassed under vacuum for 30 min and cast onto a Teflon-coated metal pan. The amount of TG used was determined from the technical specification received from Ajinomoto Company Inc. Each solution was dried at 23±2°C and 35± 5%RH for 24h. Films were stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

5.3.4 Heat curing

Films were wrapped in aluminum foil and heated in a vacuum oven at 90°C for 12 and 24h. After heat curing, films were allowed to equilibrate at 45± 5% RH for 24h prior to removal from the foil wrap. Films were conditioned at 23±2°C and 50± 5%RH for 48h prior to testing.

5.3.5 Differential scanning calorimetry (DSC)

The melting temperatures T_m of uncured and heat-cured films were determined using a TA instruments Q 100 DSC, (TA instruments, Delaware) unit. Samples (~10 mg) were weighed and sealed in an aluminum pan with an encapsulating press. Samples were heated from room temperature (~22°C) to 250°C at a temperature increase of 20°C/min. An empty sample pan was used as a reference. The DSC cell was flushed with nitrogen at 20ml/min to maintain an inert atmosphere. The differential temperature and enthalpy change were calculated

using TA Universal Analysis 2000 software. Transition temperatures were determined according to ASTM standard D-3418.

5.4 Results and Discussion

The thermograms for WPI and WPI/TG films heat cured at 90°C for 0, 12 and 24h are shown in Figures 5.1 and 5.2. The onset temperature (T_o), peak melting temperature (T_m) and the enthalpy of fusion (ΔH) are shown in Table 5.1. In all samples two peaks were observed at around 60°C, these two peaks correspond to the melting temperatures of candelilla wax. The melting temperature of candelilla wax has been reported as 64.0°C (Donhowe and Fennema 1993).

The T_o of WPI and WPI films ranged from 98.6 to 153.1°C and the T_m ranged from 137.4 to 158.1°C. The WPI/TG films had lower T_o and T_m compared to WPI films at corresponding heat curing times (Table 5.1). As heat curing time increased a corresponding increase in T_o and T_m was observed in both WPI films with and without transglutaminase. The increase in transition temperatures can be attributed to an increase in cross-linking in the films (Galietta, Di Gioia et al. 1998). The cross-linking produced during heat curing may have increased the crystallinity of the films. The increase in crystalline regions in the heat-cured films required increased energy to break up. This was seen as an increase in T_m of heat-cured films as time increased.

Uncured WPI and WPI/TG films showed a broad endothermic peak over their melting range. As heat-curing time increased the endothermic peak narrowed in both the WPI and WPI/TG films (Figure 5.1 and 5.2). As heat curing time

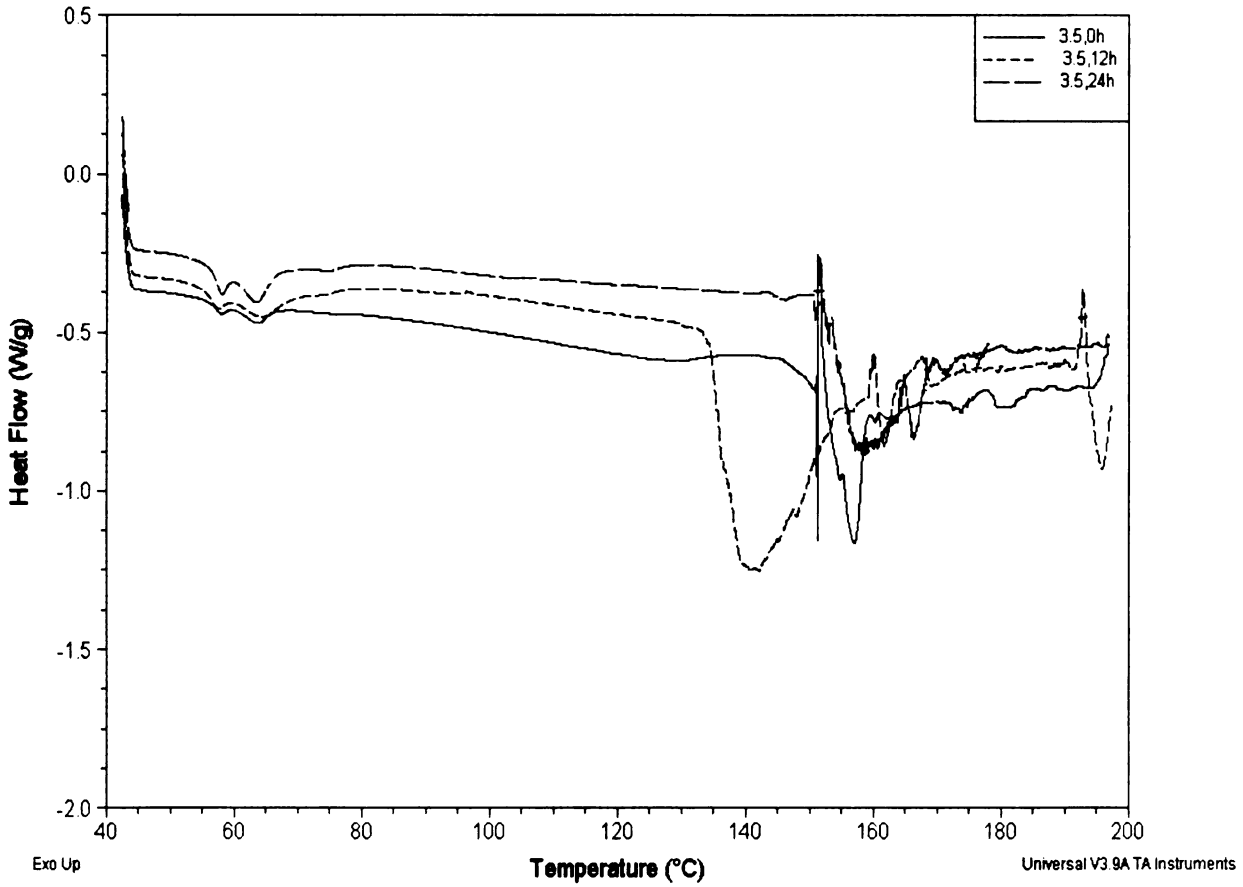

Figure 5.1: Differential Scanning Calorimetry thermogram of whey protein-isolate based films without microbial transglutaminase heat cured at 90°C for 0, 12, 24 and 48h.

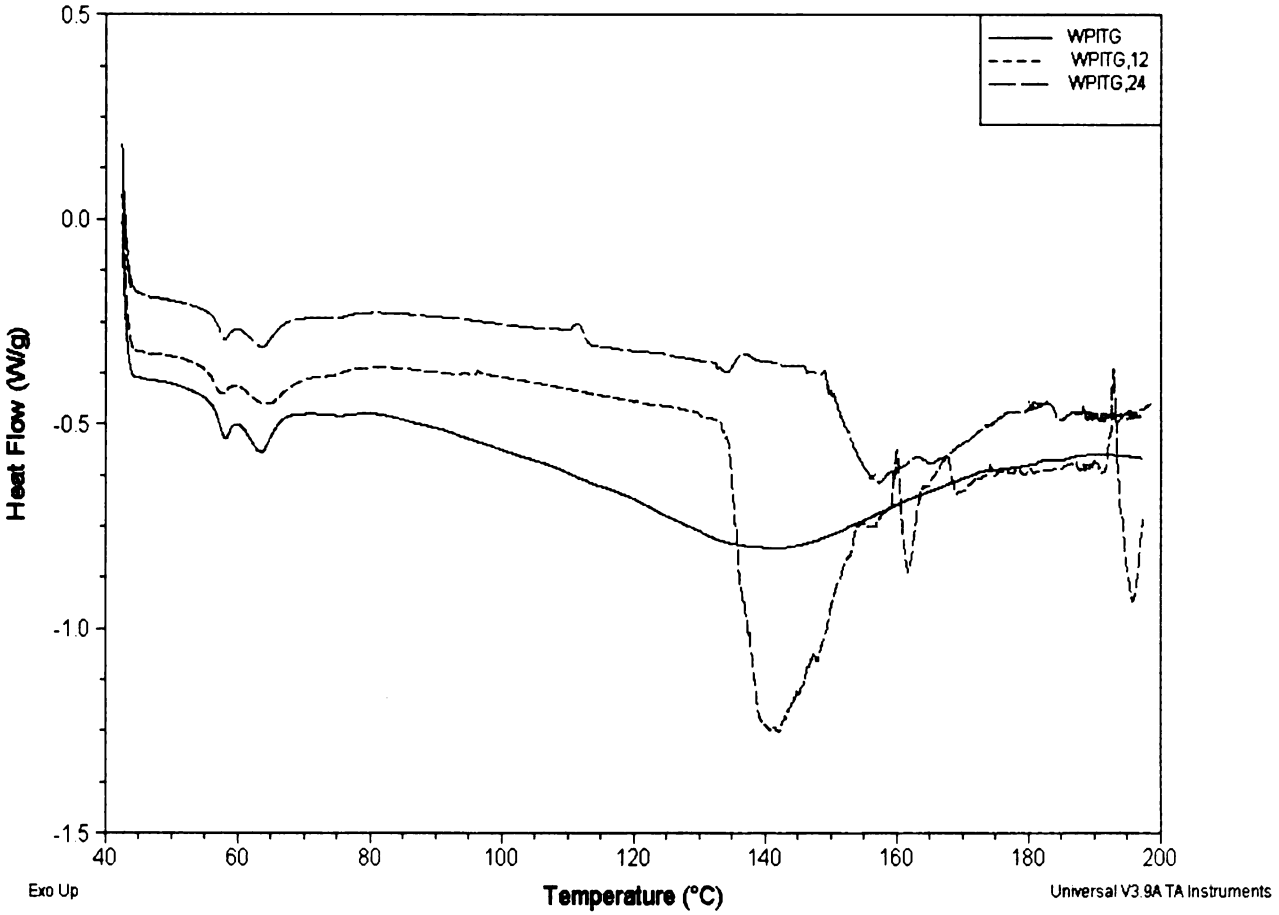

Figure 5.2: Differential Scanning Calorimetry thermogram of whey protein-isolate based films with microbial transglutaminase heat cured at 90°C for 0, 12, 24 and 48h.

Table 5.1: Selected thermal properties of whey protein-isolate based films with and without microbial transglutaminase heat cured at 90°C for 0, 12, 24 and 48h.

Treatment	Heat Curing Time (h)	T_o [1] (°C)	T_m [2] (°C)	ΔH [3] (J/g)
WPI/TG	0	98.6	137.4	66.7
	12	135.0	140.6	48.1
	24	150.3	157.2	19.3
WPI	0	135.0	140.6	48.2
	12	151.1	156.8	12.5
	24	153.1	158.1	24.9

[1] T_o= onset transition temperature
[2] T_m= peak melting temperature
[3] Δh = Enthalpy of fusion

increased the T_o of the films became more pronounced. WPI films exhibited a sharper T_o compared to WPI/TG films. The sharper T_o observed in heat cured films can be attributed to an increase in crystallinity in the films. In amorphous polymers the transition from a solid to a liquid is not well defined; while crystalline polymers exhibit a well defined transition from a solid to a liquid (Hernandez, Selke et al. 2000).

Determination of proper seal temperature is important for the production of pouches. Kim and Ustunol (2001) determined that the optimum seal strength was obtained at a temperature that corresponded to the T_o of the films. WPI films would need a higher temperature to obtain an optimal seal compared to WPI/TG films. Heat curing of films would also, increase the temperature required to heat seal the films. The higher temperature need to seal the films can be attributed to the increase in crystallinity in the films as seen in the higher T_o.

5.5 Conclusions

Heat curing films produces films of higher transition temperatures. Increasing heat-curing time increased the transition temperatures of WPI films with and without transglutaminase. Increasing the cross-linking in WPI films increased the crystallinity of WPI films. The higher T_o of heat cured films would require higher temperatures to obtain proper heat seals.

CHAPTER 6

CONCLUSIONS

To investigate the effects of heat curing and transglutaminase treatments on the mechanical, molecular, barrier, optical and thermal properties of whey protein films a series of studies were conducted. The focus of these studies was to determine that heat curing induced covalent cross-linking in WPI films and to characterize the effect cross-linking on the properties of WPI films with and without transglutaminase.

1. The mechanical properties of whey protein films (WPI) treated with and without transglutaminase (TG) were influenced by the length of heat curing. WPI films had better tensile strength than WPI/TG, while WPI/TG films had higher elongation at break than WPI films. Heat curing both WPI and WPI/TG films for 24h produced films with the highest toughness. These films exhibited a balance between high tensile strength and elongation at break.

2. Heat curing WPI and WPI/TG films produced covalent cross-links as seen in an increase in higher molecular weight polymers in SDS-PAGE gels. The increase in higher molecular weight polymers corresponded to films with enhanced mechanical properties.

3. The sulfhydryl content of WPI films with and without TG were influenced by heat curing. Heat curing both WPI and WPI/TG films decreased the sulfhydryl content compared to uncured films. Treatment with transglutaminase did not affect the sulfhydryl content of heat-cured films.

4. The lysine content of WPI films with and without TG was influenced by heat curing. Heat curing both WPI and WPI/TG films decreased the lysine content compared to uncured films. Treatment with transglutaminase lowered the lysine content of heat-cured films compared to films only heat cured. Films with lower lysine content corresponded to films with the highest toughness. The formation of covalent cross-links by lysine plays a significant role in enhancing the mechanical properties of heat cured WPI and WPI/TG films.

5. Heat curing decreases the O_2P of WPI and WPI/TG films. Treatment with TG and heat curing produces films with improved O_2P compared to heat curing alone. Treatment with TG and heat curing improved the WVP of WPI films compared to heat curing or TG treatments alone. Increasing the amount of covalent cross-linking in WPI films can be used to improve both the O_2P and WVP.

6. The moisture sorption isotherm of WPI and WPI/TG films heat cured at 90°C for 0 and 24h determined at 23 and 5°C fit the Guggenheim-Anderson-de Boer model. Sorption isotherms for films at 5°C had higher equilibrium moisture contents at all relative humidity compared to films at 23°C.

7. The color characteristics of WPI films with and without TG, heat cured at 90°C for 0, 12 and 24h. Treatment of films with TG had no impact on the color values. Heat curing increased the yellowness of the films. Both WPI and WPI/TG films heat cured at 90°C for 0, 12 and 24h had a large ΔE (total color difference)

compared to polystyrene, low density polyethylene, polyethylene terephthalate.

8. WPI films with and without TG, heat cured at 90°C for 0, 12 and 24h had low transmission of ultraviolet radiation. Treating films with transglutaminase did not impact the VIS/UV transmission properties of the films. Heat curing films decreased the transmission of UV radiation.

9. Heat curing films produces films of higher transition temperatures. Increasing heat-curing time increased the transition temperatures of WPI films with and without transglutaminase. Increasing the cross-linking in WPI films increased the crystallinity of WPI films.

CHAPTER 7

Recommended Future Research

1. Currently WPI films are produced in small test batches by casting. Research needs to be conducted on methods to produce WPI films in a commercially viable manner.

2. Physical and molecular properties of commercially viable films need to be determined and compared to the research presented here.

3. Barrier properties of commercially viable WPI films need to be determined over a broader range of temperature and relative humidity.

4. The moisture sorption isotherms of commercially viable WPI films need to be determined and compared to the results obtained here.

5. The thermal properties of commercially viable WPI films need to be determined and used to determine correct conditions to produce sealed pouches.

6. Determine the sensory attributes along with the acceptability of commercially viable WPI films

APPENDIX A

Mechanical Properties of Microbial Transglutaminase Heat Cured Whey Protein Isolate Films

The objective of this study is to compare the tensile strength, elongation at break and the toughness of TG treated WPI films with varying levels of plasticizer and heat cured at 80, 90 and 100°C for 0, 12, 24, 48, and 72h.

Film preparation: WPI (5% w/v) and Gly (2.7, 3.3, or 3.5% w/v) was mixed in distilled water and the pH adjusted to 8 with 2N NaOH. Solutions were heated at 90°C for 15 min while being stirred continuously. CW (0.8% w/v) was added during the last 5 min of heating and allowed to melt into the WPI solution. The WPI/CW solutions were homogenized for 2 min, using a Polytron PT 10/35 homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH). Solutions were strained through a single layer of cheesecloth to ensure complete mixing of the lipid. Solutions were allowed to cool to ~ 50°C and TG was added (0.001g/ g of protein) and allowed to incubate for 3h at 50°C. Solutions were equilibrated to room temperature. Solutions were degassed for 30 min and cast on a Teflon-coated surface. Solutions were dried at 23±2°C and 35± 5%RH for 24h. Films were stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

Heat curing: Films were wrapped in aluminum foil and heated in a vacuum oven at 80, 90, or 100°C films were removed after 12, 24, 48 and 72h. After heat curing, films were allowed to equilibrate at 45± 5% RH for 24h prior to removal from foil. Films were conditioned at 23±2°C and 50± 5%RH for 48h prior to testing.

Mechanical Properties: Uncured WPI and heat-cured WPI films were compared. Samples were cut into 2.54cm-wide strips using a Precision Sample Cutter (Thawing Albert Instrument Company Philadelphia, PA). Film thickness was determined using a TMI model 549 M micrometer (Testing Machines, Inc. Amityville, NY). Measurements were taken at 5 locations along the sample. An average thickness value was used in the calculations. Tensile strength (TS) and percent elongation-at-break (%E) and toughness were determined according to ASTM D 882 (ASTM 1997). Samples were tested using an Instron Universal Testing Machine Model 5500 (Instron Corp., Canton MA) with Merlin™ software to determine TS, %E, and toughness. Initial grip separation was 50 mm and the crosshead speed was 12.5 mm/min.

Statistical analysis: All treatments were replicated 3 times. Results were analyzed using the mixed model procedure in SAS (SAS Institute, Cary, NC). Treatment means were compared with significance defined as $p \leq 0.05$.

Results

Table A.1: Tensile strength of whey protein-isolate based films cross-linked with microbial transglutaminase influenced by heat curing temperature, time and glycerol content.

Plasticizer	Time	80°C	90°C	100°C
3.5% Gly	0	1.15±0.05a	1.15±0.05^{a}	1.15±0.05^{a}
	12	4.90±0.75^{b}	3.92±0.16^{b}	3.77±0.15^{b}
	24	3.63±0.51^{b}	4.39±0.85^{b}	3.28±0.74^{b}
	48	3.83±0.62^{b}	6.35±0.92^{c}	3.88±0.84^{b}
	72	5.08±2.34^{b}	14.08±2.15^{d}	17.00±4.46^{c}
3.3% Gly	0	0.95±0.14^{a}	0.95±0.14^{a}	0.95±0.14^{a}
	12	2.93±0.63^{b}	3.28±0.35^{b}	3.35±0.40^{b}
	24	3.72±0.53^{b}	3.52±0.18^{c}	3.73±0.43^{b}
	48	4.33±0.29^{b}	5.85±1.83^{d}	5.15±0.41^{b}
	72	4.46±1.08^{b}	8.25±1.77^{e}	12.98±5.77^{c}
2.7% Gly	0	1.37±0.14^{a}	1.37±0.14^{a}	1.37±0.14^{a}
	12	1.91±0.15^{a}	5.53±0.96^{b}	3.58±0.68^{b}
	24	2.80±1.26^{a}	6.52±0.48^{b}	3.95±0.79^{b}
	48	2.60±0.51^{a}	9.43±3.38^{c}	10.47±4.51^{c}
	72	2.38±0.27^{a}	12.30±2.95^{d}	17.13±2.48^{d}

$^{a-e}$ means with the same superscript are not significantly different ($p<0.05$), n=3 for all treatments. Comparisons are within each glycerol level and temperature

Table A.2: Elongation at break (%) of whey protein-isolate based films cross-linked with microbial transglutaminase influenced by heat curing temperature, time and glycerol content.

Plasticizer	Time	80°C	90°C	100°C
3.5% Gly	0	11.50±1.17[a]	11.50±1.17[a]	11.50±1.17[a]
	12	11.67±1.08[a]	17.40±1.10[b]	10.92±1.08[a]
	24	12.12±5.05[a]	14.03±3.67[a]	6.23±0.82[b]
	48	10.63±2.55[a,b]	2.95±1.23[c]	4.88±0.85[b]
	72	6.80±2.65[b]	5.13±1.87[c]	1.15±0.18[c]
3.3% Gly	0	5.48±0.82[a]	5.48±0.82[a]	5.48±0.82[a]
	12	13.32±2.69[b]	12.08±2.88[b]	7.05±1.72[a]
	24	14.43±4.04[b]	15.87±1.79[c]	5.43±0.59[a]
	48	16.08±4.60[b]	11.50±3.77[b]	5.40±1.11[a]
	72	11.77±6.24[b]	6.20±1.84[a]	1.25±0.13[b]
2.7% Gly	0	20.25±3.81[a]	20.25±3.81[a]	20.25±3.81[a]
	12	10.43±3.42[b]	5.28±1.39[b]	7.58±2.45[b]
	24	9.26±3.39[b]	8.20±3.28[b]	6.77±2.12[b]
	48	11.18±2.26[b]	4.17±2.26[b]	1.38±0.13[c]
	72	9.78±3.21[b]	2.15±0.91[b]	1.40±0.37[c]

[a-c] means with the same superscript are not significantly different ($p<0.05$), n=3 for all treatments. Comparisons are within each glycerol level and temperature

Table A.3: Toughness (kJ/m^3) of whey protein-isolate based films cross-linked with microbial transglutaminase influenced by heat curing temperature, time and glycerol content.

Plasticizer	Time	80°C	90°C	100°C
3.5% Gly	0	8.71 ± 0.44	8.71 ± 0.44	8.71 ± 0.44
	12	37.97 ± 6.96	45.92 ± 1.65	26.82 ± 3.85
	24	28.00 ± 14.78	47.08 ± 9.82	12.54 ± 4.11
	48	28.75 ± 6.16	20.39 ± 11.24	10.41 ± 0.22
	72	16.73 ± 1.28	16.46 ± 9.21	7.43 ± 2.95
3.3% Gly	0	3.10 ± 0.48	3.10 ± 0.48	3.10 ± 0.48
	12	25.05 ± 8.09	18.21 ± 16.13	14.46 ± 5.31
	24	35.03 ± 8.91	35.61 ± 3.74	11.76 ± 1.15
	48	46.59 ± 16.16	41.93 ± 3.36	15.20 ± 1.12
	72	35.08 ± 12.47	21.47 ± 23.27	2.70 ± 2.34
2.7% Gly	0	17.73 ± 3.35	17.73 ± 3.35	17.73 ± 3.35
	12	12.20 ± 4.82	16.10 ± 2.28	16.24 ± 4.96
	24	11.36 ± 4.58	33.54 ± 16.08	16.98 ± 6.61
	48	18.33 ± 1.80	27.14 ± 16.44	4.60 ± 2.15
	72	15.08 ± 7.18	17.37 ± 13.28	6.60 ± 3.15

[a-b] means with the same superscript are not significantly different ($p<0.05$), n=3 for all treatments. Comparisons are within each glycerol level and temperature

APPENDIX B

Mechanical Properties of Heat Cured Whey Protein Isolate Based Edible Films compared to collagen and natural casings.

The objective of this study is to compare the tensile strength, elongation at break and the toughness of WPI films with varying levels of plasticizer and heat cured at 80, 90 and 100°C for 0, 12, 24, 48, and 72h to collagen and natural casings.

Film preparation: WPI (5% w/v) and Gly (2.7, 3.3, or 3.5% w/v) was mixed in distilled water and the pH adjusted to 8 with 2N NaOH. Solutions were heated at 90°C for 15 min while being stirred continuously. CW (0.8% w/v) was added during the last 5 min of heating and allowed to melt into the WPI solution. The WPI/CW solutions were homogenized for 2 min, using a Polytron PT 10/35 homogenizer with a PTA 20 TS homogenizing head (Tekmar Co., Cincinnati, OH). Solutions were strained through a single layer of cheesecloth to ensure complete mixing of the lipid. Solutions were degassed for 30 min and cast on a Teflon-coated surface. Solutions were dried at 23±2°C and 35± 5%RH for 24h. Films were stored at 23±2°C and 50± 5%RH for 24h prior to heat curing.

Heat curing: Films were wrapped in aluminum foil and heated in a vacuum oven at 80, 90, or 100°C films were removed after 12, 24, 48 and 72h. After heat curing, films were allowed to equilibrate at 45± 5% RH for 24h prior to removal from foil. Films were conditioned at 23±2°C and 50± 5%RH for 48h prior to testing.

Mechanical Properties: Uncured WPI and heat-cured WPI films were compared. Samples were cut into 2.54cm-wide strips using a Precision Sample

Cutter (Thawing Albert Instrument Company Philadelphia, PA). Film thickness was determined using a TMI model 549 M micrometer (Testing Machines, Inc. Amityville, NY). Measurements were taken at 5 locations along the sample. An average thickness value was used in the calculations. Tensile strength (TS) and percent elongation-at-break (%E) and toughness were determined according to ASTM D 882 (ASTM 1997). Samples were tested using an Instron Universal Testing Machine Model 5500 (Instron Corp., Canton MA) with Merlin™ software to determine TS, %E, and toughness. Initial grip separation was 50 mm and the crosshead speed was 12.5 mm/min.

Statistical analysis: All treatments were replicated 3 times. Results were analyzed using the mixed model procedure in SAS (SAS Institute, Cary, NC). Treatment means were compared with significance defined as $p \leq 0.05$.

Table B1: Tensile strength of whey protein-isolate based films as influenced by heat curing and glycerol content.

Heat curing Temperature (°C)	Glycerol Level (%)	Heat curing time (h)				
		0	12	24	48	72
80	2.7	6.50±2.17^{b}	7.27±0.42^{d}	7.70±1.15^{b,d}	9.57±1.66^{d}	9.12±4.46^{a,b,d}
	3.3	4.23±0.72^{b}	6.00±1.28^{a,b,d}	7.00±2.29^{a,d}	8.12±2.06^{d}	7.83±2.49^{a,b,d}
	3.5	4.03±0.75^{b}	5.37±1.31^{b,d}	4.95±0.59^{b,d}	6.45±1.78^{d}	7.08±1.65^{a,b,d}
90	2.7	6.50±2.17^{b}	8.95±0.97^{a,b,c}	10.00±1.95^{a,b,c,e}	14.02±4.44^{f}	15.40±2.21^{a,f}
	3.3	4.23±0.72^{b}	7.43±1.55^{a,b,c}	6.77±1.20^{a,b,c,d}	13.75±2.97^{a,e,f}	11.37±1.83^{a,c,f,g}
	3.5	4.03±0.75^{b}	5.50±1.60^{a,b,c,d}	6.05±0.65^{a,b,c,d}	16.82±1.80^{a,e,f,g}	14.23±3.96^{a,f,g}
100	2.7	6.50±2.17^{b}	16.40±4.45^{a,c}	11.43±0.32^{a,b,d}	22.26±2.60^{e}	26.23±5.98^{e}
	3.3	4.23±0.72^{b}	10.77±1.87^{a,b,d}	8.87±0.27^{a,b,d}	16.44±4.96^{c}	11.97±1.66^{d}
	3.5	4.03±0.75^{b}	9.10±1.38^{a,b,d}	9.52±1.22^{a,b,d}	11.35±1.20^{a,d}	22.85±5.29^{e}
Collagen	N/A	13.18±1.96^{a}				
Natural Casings	N/A	6.17±0.06^{b}				

$^{a-g}$ means with the same superscript are not significantly different ($p<0.05$), n=3 for all treatments. Comparisons are made within each temperature and against collagen and natural casings.

Table B2: Elongation at break (%) of whey protein-isolate based films as influenced by heat curing and glycerol content.

Heat curing Temperature (°C)	Glycerol Level (%)	Heat curing time (h)				
		0	12	24	48	72
80	2.7	6.85 ± 0.79^{b}	$17.67\pm0.65^{c,d}$	$21.05\pm4.44^{c,d}$	$19.87\pm5.64^{c,d}$	$15.53\pm6.45^{a,c,d}$
	3.3	15.22 ± 3.33^{e}	$18.43\pm1.62^{c,d,e}$	23.58 ± 9.13^{c}	$26.17\pm8.62^{c,d}$	$18.10\pm1.00^{a,c,d}$
	3.5	$15.05\pm8.15^{a,f}$	$17.57\pm6.01^{c,d,f}$	$18.30\pm6.81^{c,d,f}$	$19.08\pm8.21^{c,d,e}$	$22.33\pm7.93^{a,c,d,e}$
90	2.7	6.85 ± 0.79^{b}	17.40 ± 6.99^{c}	$20.87\pm5.01^{c,d,f,j}$	$14.25\pm7.80^{c,f,g,h}$	$8.38\pm5.97^{e,h,i,j}$
	3.3	15.22 ± 3.33^{k}	$24.75\pm5.00^{c,d,k}$	$21.17\pm4.63^{c,d,k}$	$13.88\pm3.07^{c,f,g,i,k}$	$14.62\pm3.77^{c,f,g,i}$
	3.5	$15.05\pm8.15^{a,j}$	$15.02\pm3.08^{c,e,k}$	$18.60\pm4.45^{c,b,k}$	$10.75\pm6.27^{c,i}$	$5.88\pm2.09^{i,b}$
100	2.7	6.85 ± 0.79^{b}	$8.95\pm1.53^{a,b,c}$	$10.82\pm1.30^{c,f,i}$	$6.62\pm4.56^{c,j,i}$	$4.06\pm0.66^{c,b}$
	3.3	15.22 ± 3.33^{j}	$10.35\pm4.68^{c,d}$	$15.62\pm0.53^{c,e,g}$	$9.53\pm3.15^{c,g,h}$	4.18 ± 2.55^{c}
	3.5	$15.05\pm8.15^{a,i}$	$19.68\pm7.34^{e,i}$	$17.24\pm3.02^{d,e,f,h,i}$	$9.86\pm1.33^{c,g,h}$	$4.72\pm0.41^{c,b}$
Collagen	N/A	10.75 ± 8.79^{a}				
Natural Casings	N/A	$5.93\pm0.49^{a,b}$				

[a-k] means with the same superscript are not significantly different ($p<0.05$), n=3 for all treatments. Comparisons are made within each temperature and against collagen and natural casings.

Table B3: Tensile energy to break (kJ/cm^3) of whey protein-isolate based films as influenced by heat curing and glycerol content.

Heat curing Temperature (°C)	Glycerol Level (%)	Heat curing time (h)				
		0	12	24	48	72
80	2.7	17.49±8.13	74.48±5.23	84.51±31.35	115.74±22.47	94.39±64.25
	3.3	35.13±16.27	70.75±8.27	70.16±23.11	132.26±16.11	86.56±16.24
	3.5	40.60±12.92	46.49±7.20	54.70±14.11	72.50±40.21	101.13±51.01
90	2.7	17.49±8.13	93.60±36.04	121.44±22.99	114.78±58.12	46.74±10.11
	3.3	35.13±16.27	116.63±25.36	80.85±24.35	74.74±18.98	65.33±9.30
	3.5	40.60±12.92	30.19±8.41	72.06±15.83	43.80±14.40	41.45±19.44
100	2.7	17.49±8.13	71.72±3.36	76.98±18.29	68.19±7.74	12.27±6.46
	3.3	35.13±16.27	43.65±30.10	91.49±5.10	34.60±4.47	6.69±2.23
	3.5	40.60±12.92	95.11±57.42	102.25±28.63	50.21±15.49	15.76±3.21
Collagen	N/A	24.21±10.43				
Natural Casings	N/A	13.90±3.85				

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