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# Effect of radiation on polymerization, microstructure, and microbiological properties of whey protein in model system and whey protein based tissue adhesive development

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**EFFECT OF RADIATION ON POLYMERIZATION,  
MICROSTRUCTURE, AND MICROBIOLOGICAL PROPERTIES OF  
WHEY PROTEINS IN MODEL SYSTEM AND WHEY PROTEIN BASED  
TISSUE ADHESIVE DEVELOPMENT**

A Thesis Presented

by

Ning Liu

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements  
for the Degree of Master of Science  
Specializing in Nutrition and Food Sciences

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## ABSTRACT

Whey proteins are mainly a group of small globular proteins. Their structures can be modified by physical, chemical and other means to improve their functionality. The objectives of this study were to investigate the effect of radiation on protein-protein interaction, microstructure, and microbiological properties of whey protein-water solutions. Whey protein isolate (WPI) solutions (27-36% protein) were treated with different dosages (10-35 KGy) of gamma radiation. The protein solutions were analyzed for viscosity, turbidity, soluble nitrogen, total plate count, and yeast and mold counts. The interactions between whey proteins were also analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and scanning electron microscopy (SEM). The viscosity of protein solution (27%, w/w) was increased from 2.19 for the control to 4.78 mPa s for the sample treated at 25 KGy, respectively, and viscosity also increased during storage at 23 °C. The soluble nitrogen (10%, w/w) was decreased from 100% to 54.7% for control and the sample treated at 35 KGy. The effects of gamma radiation and storage time on viscosity of whey protein solutions were significant ( $p < 0.05$ ). Radiation treatment had significant impact on soluble nitrogen of whey protein solutions ( $p < 0.05$ ). SDS-PAGE results showed that intensity of the major protein band was reduced and it had smeared appearance for the treated samples and photographs of SEM also showed that protein-protein interactions induced by gamma radiation in the model system.

Key works: whey protein isolate, gamma radiation, sterilize, interaction.

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## **DEDICATION**

This is dedicated to my dear parents, Fengquan Liu and Yuzhi Sun, and my family members, for their wholehearted love. It is always my happiness to think you of you. I love you all my life. I dedicate this work to all of you.

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## **CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW**

### **1.1 INTRODUCTION**

Sealing wounds and stopping hemorrhages on the battleground have been going on for thousands of years. From then on, the suture was created and has become a common way to treat wounds in modern medical treatment. Currently, there are various types of absorbable and non-absorbable sutures of different sizes with different-size needles; and with more than forty different combinations are being used to close wounds and incisions (Quinn 2005). However, there are several manifest disadvantages to dealing with wounds by suture, such as high infection rate, physical pain, and long healing time. Therefore, alternative methods to suture have been evaluated including adhesive tapes, gums, and tissue adhesives.

Given the shortcomings of some wound closure devices, interest in tissue adhesives has grown in the last half-century and especially in the last decades (Quinn 2005). Tissue adhesives have been used to close wounds and stop bleeding. Substantial advantages make tissue adhesives widely used in clinics. During some surgeries, tissue adhesives can be used as a way to seal the vascular system and some organs like the stomach. The use of proteins as a component in tissue adhesives has been previously studied and whey protein

has potential to be used as a functional polymer component for adhesives.

Whey is the byproduct of cheese-making. Globally, dry whey and whey proteins (protein content 80% and below) exports totaled nearly 1.27 million metric tons in 2010, up 12.5% versus 2009 exports (Horizons 2012). However, the total amount of whey is not being fully used every year. The main utilizations of whey are for animal feed, fertilizers, or as an ingredient for human foods. The United States is one of the world largest whey producers, which also produced fluid whey not fully used by human and animals. Disposal of whey by dumping into rivers was frequently used in the United States before environmental regulations were enforced (Cryan 2001, Onwulata 2008). Preventing pollution caused by disposal of whey and recovering its nutrient value have been two major factors of interest of using whey for decades. At the University of Vermont's research lab, whey protein was used for formulation of wood and paper adhesives. It would be value-added applications of whey protein for developing other products

## **1.2 WHEY PROTEINS**

### **1.2.1 Whey Production**

Milk and milk products are used extensively because of their functional and nutritional properties. Milk provides diversified nutritional needs for human. The approximate composition of cow's milk is shown in Table 1

(Potter 1995). Historically, milk proteins were extensively demanded for human health benefits. Caseins, representing about 80% of the total nitrogen (Fox 1989), and whey proteins are the two major proteins in bovine milk.

**Table 1. Components of Cow's Milk**

<b>Components</b>	<b>%</b>
Water	87.1
Fat	3.9
Protein	3.3
Lactose	5.0
Ash	0.7
Total solids	12.9

Whey is obtained from separating casein from bovine milk during cheese manufacture. Whey can be divided into two categories: sweet whey (a minimum pH of 5.6) and acid whey (a maximum pH of 5.1). Whey contains many types of proteins and essential amino acids for human. Yet without being fully utilized, currently whey is an ample and burdensome byproduct for the U.S. dairy industry. Whey is as an essential ingredient in the modern food industry due to its high concentration of protein, amino acids, and minerals, which are all high quality of nutrients for human consumption. Whey proteins are used in the manufacture of diet supplements, infant formulae, bakery products, and sports drinks.

The utilization of whey and whey proteins has been in existence since antiquity. Portions of whey were used for humans and the rest was used for livestock forage and fertilizer. Even though the functional and nutritive values

of whey have been known for decades, whey as a byproduct is still not fully used. A substantial amount of whey is simply discarded every year. Whey has a biochemical oxygen demand (BOD) of 35,000-45,000 g/liter, and 100 liters of whey has a polluting strength equivalent to the sewage produced by 45 people (Fox 1982). However, proteins and nutrients in whey are too valuable to be unutilized.

Whey concentrating and drying are the two innovations to obtain the nutrients. The long-tube multiple-effect evaporator and the spray drier were the two important inventions in the early twentieth century in the whey industry and the spray drier is still used in modern dairy industry. Five types of membrane filtration (ultrafiltration, microfiltration, electrodialysis, nanofiltration, and reverse osmosis) were developed and applied to obtain a dry (<5% moisture content) product (Onwulata 2008). With the help of these processes, it is now possible and simple to obtain different concentrations of whey protein.

### **1.2.2 Whey Protein Concentrate (WPC) and Whey Protein Isolate (WPI)**

WPCs and WPI are important whey products because of their high concentrations of proteins and wide usage. WPC can be obtained from ultrafiltration (UF) of whey. WPC may contain from 20 to 90% protein; WPC with 35% protein (WPC-35) is a common product (Onwulata 2008). WPI has a high concentration of protein (at least 90%) from whey. The lactose in WPI

is totally separated. WPC 80 production was 86,000 tons in 2011 and WPI production was expected to reach 27,600 metric tons in 2011 in the United States (Horizons 2012).

The interest of using WPC and WPI is still growing for human health and benefits. Beneficial factors of WPC and WPI in food applications include its high protein and amino acid content; lack of pathogens, toxic compounds, and antinutritional factors; and abundant availability (Onwulata 2008). In the medicinal field and nutritional demands, products such as WPI supplements have also been developed from high concentrations of whey proteins using the processes of membrane filtrations.

### **1.2.3 Whey Protein Chemistry**

The nutritive components of whey have interested scientists for centuries. Lactalbumin is the solid got from heating whey. Lactoglobulin which was a precipitate showed in research to form by the addition of magnesium sulfate or ammonium sulfate at low pH (Creamer 2003, Onwulata 2008). Subsequently, bovine serum albumin, immunoglobulin, and other proteins were identified. Whey proteins are generally comprised of  $\beta$ -lactoglobulin ( $\beta$ -LG) (50%),  $\alpha$ -lactalbumin ( $\alpha$ -LA) (20%) (Fox 1989), bovine serum albumin (BSA) (6%), immunoglobulins (Ig) (10%), and proteose-peptones component glycomacropetides (12%) (Onwulata 2008). Whey also contains lactoferrin (LF) and lactoperoxidase (LP), but the concentrations are



at very low levels. There are other minimal components in whey proteins that are remained from the cheese making process. Table 2 shows the compounds and functions of whey proteins in bovine milk (Wit 1998).

**Table 2. Compounds and Functions of Whey Proteins in Cow's Milk**

Whey protein	Whey contribution (g/L of milk)	Biological function
Major		For the calf
		(Pro)vitamin A
β-LG	3.2	Transfer
α-LA	1.2	Lactose synthesis
BSA	0.4	Fatty acid transfer
IgG	0.8	Passive immunity
Bioactive		General
LF	0.2	Bacteriostatic agents
LP	0.03	Antibacterial agent
Enzymes (>50)	0.03	Health indicators
Proteose-peptones	≥1	

Even though WPC and WPI have higher protein concentrations, the amounts of various protein components such as β-LG and bovine serum albumin are not significantly changed.

WPC and WPI contain more than half of β-LG, so the properties of β-LG approximately reflect the properties of WPCs and WPIs. BSA and α-LA are the other two important proteins. β-LG and α-LA will be fractionated first and easily from whey proteins. Due to the binding ability and affinity for retinol of β-LG, it has the function of vitamin A transportation. The biological function of α-LA is to act as a specifier protein for lactose synthetase in the formation of lactose from galactose and glucose and α-LA is a calcium metallo-protein which Ca<sup>2+</sup> may have a second messenger function for the

binding of  $\alpha$ -LA to target proteins (Stuart 1986, Fox 1989). Bovine serum albumin (BSA), is a well-known transport protein for insoluble fatty acids in the blood circulatory system (Spector 1975, Fox 1989). Typical relative abundances, isoelectric points, molecular weights and denaturation temperatures of these proteins are listed in Table 3 (Wit 1981, Kinsella 1989). There are also many enzymes in whey and other minimal proteins.

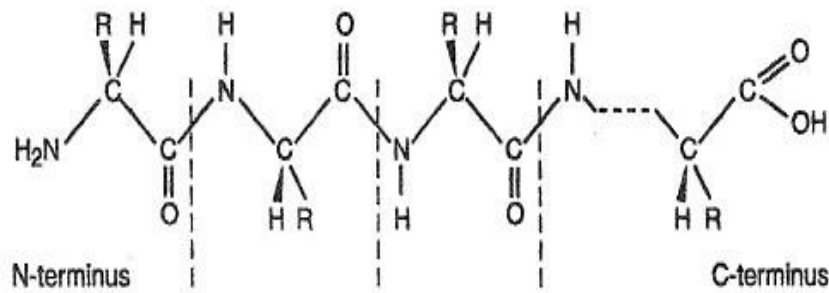
**Table 3. Physical Characteristics of the Major Whey Proteins**

Protein	pI	M.W.	%	T <sub>d</sub> <sup>a</sup>
$\beta$ -LG	5.2	18400	60	78
$\alpha$ -LA	4.8-	14200	22	62
BSA	5.1	66000	5.5	64
Immunoglobulins	4.8-	15-	9.1	72
	5.1	96 $\times$ 10 <sup>4</sup>		
	5.5-			
	6.8			

<sup>a</sup>T<sub>d</sub> represent the temperature of denaturation.

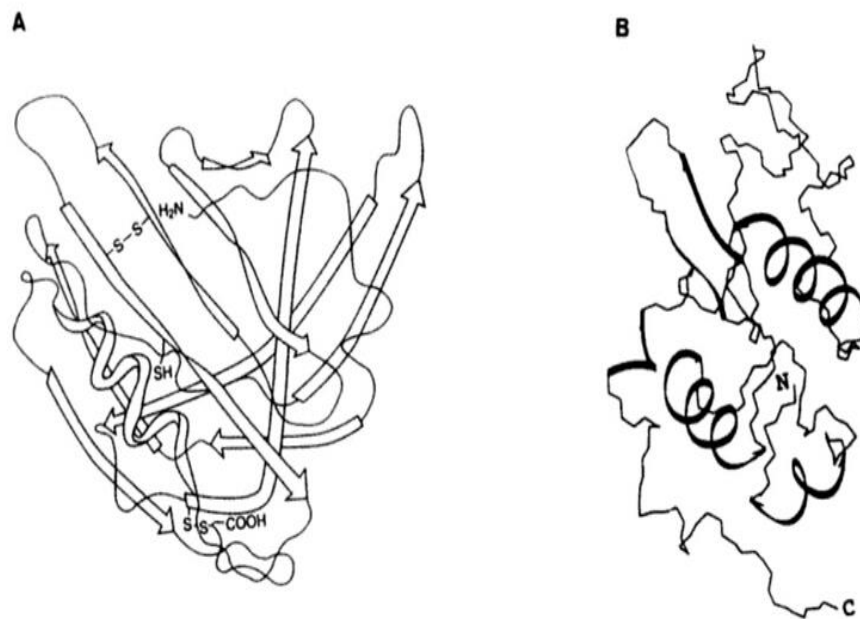
#### 1.2.4 Structures of Whey Proteins

Whey proteins are mainly groups of small globular proteins. The primary structure of protein is a peptide chain and amino acid residues (Figure 1) (Walstra 1999). Several amino acids can interact to form a linear peptide chain. Proteins are long chains that most are comprised of more than 100 amino acid groups. According to the primary structure, hydrogen bonds and hydrophobic interactions with a peptide chain can fold to obtain a specific structure.



**Figure 1. Peptide Chain**

The secondary and tertiary structures show a high degree of organization with a great proportion of beta sheets (43-50% of the residues) and only 10-15% of a helix and 15-20% of beta turn (Damodaran 1997). Domains are used to describe the tertiary structure of protein molecules. The tertiary structures of  $\beta$ -LG and  $\alpha$ -LA are shown in Figure 2 (Damodaran 1997). These molecules have their own distinguishing areas and functions. For example, domains have far-reaching impact on the denaturation. BSA has three specific domains for metal ion binding, lipid binding, nucleotide binding (Peters 1977, Fox 1989). The secondary and tertiary structures are important for protein functions so that the changes of these structures may cause the significant functional changes of protein properties.



**Figure 2. Tertiary Structures of  $\beta$ -LG (A) and  $\alpha$ -LA (B).**

### 1.2.5 Functional Properties of Whey Protein

Whey protein structures can be modified by physical, chemical and other means to improve their functionality. Factors including pH and ionic strength may have impact on the conformation of proteins.

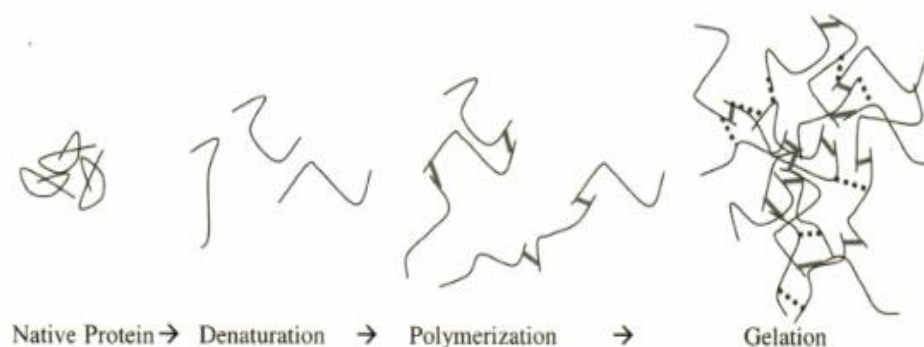
Denaturation of proteins means that the primary structure does not change but secondary and tertiary structures will be changed considerably. High pH, high temperature, some salts and other chemicals can be used for the denaturation of proteins. Occasionally, denaturation may be reversed to the original condition, but if aggregation occurs, it is an irreversible unfolding. Whey proteins can be aggregated and gelled when being thermal treated. The protein structures and properties may start to change at the temperature up to 60 °C and irreversible changes require higher temperatures

(above 70 °C). Color, viscosity, pH, solubility, and other physicochemical changes may occur caused by denaturation of whey proteins

Protein-protein interactions occur at high temperature and these reactions can crosslink peptide chains and cause polymerization. These changes also include changes in primary structure.  $\beta$ -LG dissociates into monomers and the non-covalent bonds are first to be cleaved and the SH groups are activated to form disulfide bridges (Sienkiewicz 1990). At higher temperature, proteins polymerize into larger molecular weight.

Gamma radiation can be used to sterilize WPI solutions without denaturing whey proteins by thermal treatment. However, there are some other side effects including gelation. Gelation occurs after gamma radiation and all the WPI solutions become more opaque. Except radiation, other factors like heat treatment, storage temperature, and total solids can also affect the degrees of gelation.

Gelation begins with protein unfolding interactions. Ionic and hydrophobic interactions and/or covalent disulfide bonding are critical intermolecular interactions (to a varying extent) for gel formation (Fox 1989). As the gelation continues, aggregation occurs to form a gel. A schematic representation of the gelation mechanism is shown in Figure 3 (Onwulata 2008). Gelation of whey proteins has its industrial values. For example, cheese curd is formed with cold gelation.



**Figure 3. A Schematic Representation of Gelation Mechanism**

Due to the functional and nutrient values of whey proteins, it is necessary and also possible to look into additional high value added applications of whey proteins. Moreover, WPI is cost inexpensive and readily available in the United States.

In the functional foods laboratory of the University of Vermont, we developed many whey protein products, like wood adhesives, paper adhesives, and stick glues. If we could develop a protein-based tissue adhesive, it would be a more valuable application of whey protein isolate.

### 1.3 TISSUE ADHESIVES

#### 1.3.1 Overview of Wound Closure

People in ancient time had the knowledge to deal with trauma or incision caused by injuries. Ancient Chinese people used medicinal herbs wrapped in sackcloth to treat bleeding. It was fatal for those with deep wound when they could not get a necessary treatment. Then the suture was created to

treat wounds and hemorrhage by sewing. Suture technique has become the common and basic method for closing wounds nowadays. Absorbable sutures with different-size needles are not a common technique in modern medicine.

Suture is the most popular method in clinic. Even when a few sutures are broken, other nearby sutures are not affected, resulting in an absent of total wound dehiscence. Suture are quick and easy to install and are generally non-allergenic. However, the disadvantages of sutures should be noticed. High infection rate may be an urgent problem. They also require the use of anesthetic and are a slow method of wound closure that is costly and difficult to master for some practitioners (Quinn 2005). It may take a long time to heal by sewing. Sutures can cause scars that may be of concern when the surgical site is on the face or neck. Leakage of gas or fluid can lead to a serious failure. The use of needles can cause many problems like anxiety and contaminations.

Therefore, alternatives to sutures are emerging for better wound care in clinics. Staples, clips, and surgical tapes are the common alternative techniques for wound closure including tissue adhesives. However, staples, clips and tapes all have their disadvantages. Therefore, using tissue adhesives to close and cure wounds has high possibility.

### **1.3.2 History and Background of Tissue Adhesives**

The use of tissue adhesives for closing wounds may have more than a thousand years. Due to the improvement of techniques and the shortcomings

of common closure devices, investigation in tissue adhesives has gained significantly since the middle of twentieth century. The sequential World Wars stimulated the improvement of adhesives. Faced with large numbers of casualties suffering severe blood loss, surgeons in World War I began to use sheets of fibrin during surgery for the treatment of injuries (Sierra 1996). Many effective, quick, and convenient adhesive products appeared in numerous different types of surgery. In modern medicine, tissue adhesive has already become an alternative method to seal wounds that are important in clinical applications. Comparing to sutures and staples, the use of tissue adhesives provides a fast bonding and fully closure.

### **1.3.3 Characteristics of Tissue Adhesives**

The use of tissue adhesives has shown numerous benefits in both clinical utilization and medical production. One of the advantages of tissue adhesives is the prevention of leaking of air or fluid. The surgical site of any vascular injury or a cavity organ surgery warrants an application for tissue adhesives. The use of tissue adhesives does not need to remove sutures later. There is also no risk of needle stick injury to the surgeon or assistant. Tissue adhesives also provide the possibility of surgery without development of scars after the healing process. The pinpricks of sutures make them a less attractive choice consideration in plastic surgery. Tissue adhesives also provide less time to heal comparing to sutures and significantly reduce the



pain of suture. Tissue adhesives act as topical wound sealants to provide an antimicrobial barrier to outside contamination, and to fixate structures where sutures and normal fixation devices such as plates and screws cannot be used (Quinn 2005). Most tissue adhesives are very convenient for clinical use and can be performed with the assistance of a nurse without professional surgical training. For example, some of them are applied in a matrix and can be activated by mixing in the surgical site just prior to use. Also the cost of tissue adhesives is not as expensive as sutures. With the enhancement of medical technology, there will be more effective adhesives for more extensive applications.

#### **1.3.4 Classifications of Adhesives**

Tissue adhesives in the United States can be categorized into five groups. There are several commercial products already available in the market for each category. Cyanoacrylate is the first one. They are not decomposed by our body. Cyanoacrylates can induce internal inflammation, tissue necrosis, and infection risks associated with foreign bodies (Reece 2001). Secondly, fibrin sealants are comprised of thrombin and fibrinogen. Fibrin sealants are biocompatible and biodegradable and they are not associated with inflammation, foreign body reactions, tissue necrosis, or extensive fibrosis (Ryou 2005, Spotnitz 2005). The composition of hydrogels is the third tissue adhesive that can be broken down, but they may require long time for reaction

under the conditions of light. Albumin-based compounds make the forth tissue adhesive that are glutaraldehyde glues which contain two compounds, albumin and crosslinker. BioGlue surgical adhesive is categorized in this group of tissue adhesive which is a two-component system. The last group is collagen-based adhesives. The composition is bovine collagen and bovine thrombin. This type of tissue adhesives is relatively expensive because it is comparatively new and more investigation may be needed for long-term effects.

### **1.3.5 Protein Based Tissue Adhesives**

The benefits of using tissue adhesives has stimulated new approaches and developments. The use of proteins in adhesives is not novel. Egg white was used as a binding agent in ancient time (Quinn 2005). The use of proteins in adhesives is when a protein polymer reacted with a crosslinking agent, usually glutaraldehyde (GTA), to adhere to tissue surfaces. The most significant difference of this class of adhesives and sealants from the fibrin-thrombin-based sealants is their independence of the coagulation cascade (Quinn 2005). Protein-based adhesives do not relate with coagulopathy or anticoagulation. The GR-Dial (Fehling, Germany), FocalSeal (Focal Inc., Massachusetts, USA), and BioGlue (CryoLife Inc., NW Kennesaw, GA, USA) are three examples for commercial tissue adhesives.

Protein-based tissue adhesives can be absorbed by human and animal tissue during healing procedures. Proteins also have low toxicity but they are

not without disadvantages. Premature dissolutions of the tissue adherences frequently occurred due to enzymatic influence (Schwarz 1983). Proteins can cause allergy for some infants and some adults. GTA as a crosslinker may need more toxicological tests to be approved by Food and Drug Administration (FDA). Avoiding these disadvantages and difficulties would be a significant step forward for tissue adhesives. Proteins react with GTA to neutralize the toxicological effects of GTA on human body to make GTA an appropriate crosslinker.

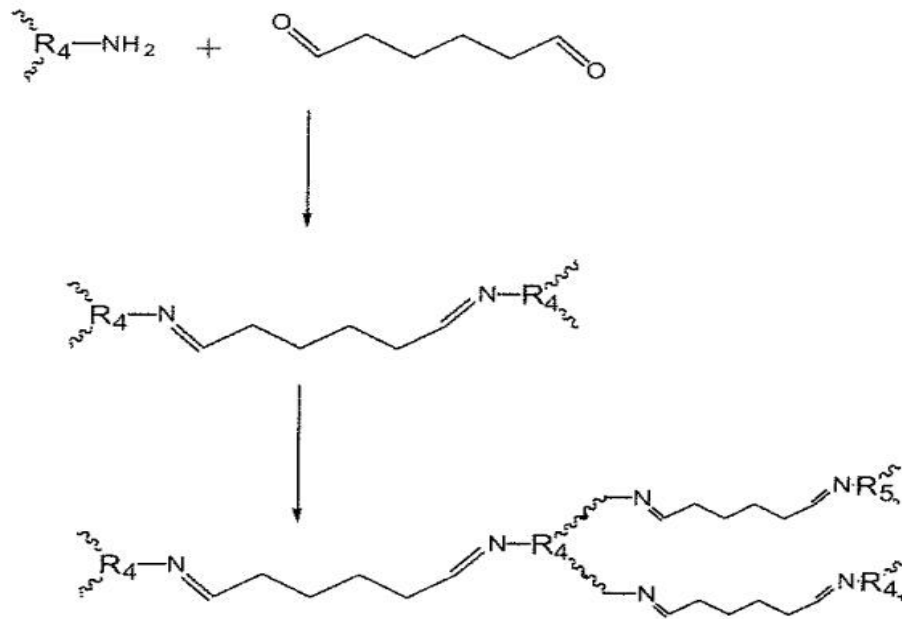
### **1.3.6 Principles of Protein Based Adhesives**

In this study, whey protein was used as protein polymer and GTA as a crosslinker. The linear structure of oily transparent GTA is a 5-carbon dialdehydes. GTA is a generally dependable material that invariably gives high yields of immobilized protein on the support (Walt 1994). GTA was used as a crosslinker in a tissue adhesive for hemostasis and urine leakage and also for decreasing blood loss during kidney surgery.

The cross-linking of a protein-based tissue adhesive to itself and to the tissue is the result of the reaction between the amine groups of tissue proteins and the terminal aldehyde groups which are provide by the bi-functional reagent GTA (Quinn 2005). The amines for this reaction are provided by the  $\epsilon$ -amino groups of the side chains of lysine residues with  $\epsilon$ -NH<sub>2</sub> in albumin and extracellular matrix proteins from the tissues (Hewitt

2001). Asparaginy, glutaminy, and arginy residues also provide amino groups crosslinked with GTA. The mechanism of reaction of GTA with BSA and cell surface proteins to form dimers and polymers which is shown in Figure 4 (Quinn 2005). Proteins are stable enough after reacting with GTA which also has the function of immobilization. Proteins can form a soft and ductile barrier moving with human body.

$\beta$ -LG,  $\alpha$ -LA, and BSA, respectively, contain 162, 123, and 583 total amino acid residues. The numbers of  $\epsilon$ -amino groups are 37, 27 and 113, respectively, in these three proteins (Brew 1967, Hirayama 1990, Fox 2003). There are enough functional  $\epsilon$ -amino groups for bonding tissue together. After bonding, the protein-based tissue adhesives can be degraded by proteolysis.



**Figure 4. Mechanism of Reaction of GTA with BSA and Cell**

### **Surface Proteins**

#### **1.3.7 Crosslinking Agents**

The choice of protein crosslinking agents usually affects the efficiency, toxicity, and bonding strength of tissue adhesives. There are usually three options used as protein crosslinking agents. GTA is the first one used as a cross-linking agent in tissue adhesives. BioGlue uses GTA as a crosslinker. The advantage of this approach is in the preservation of functioning renal tissue without compromising the oncologic outcome (Hidas 2006). GTA tissue adhesive can be used for hemostasis and decreasing the blood loss during nephron-sparing surgeries. The reaction of GTA with proteins is immediate, reaching full bonding strength within 5 minutes. The use of this tissue adhesive significantly reduced the volume and the rate of postsurgical bleeding, and

occasionally the inflammatory response. Although GTA is widely used in many tissue adhesives, the toxicity of GTA should be noticed. GTA can cause cytotoxic effects. Superior bonding and sealing capabilities to tissues as well as to synthetic graft materials allowing air-tight and blood-tight repair properties are claimed for BioGlue (Furst 2005).

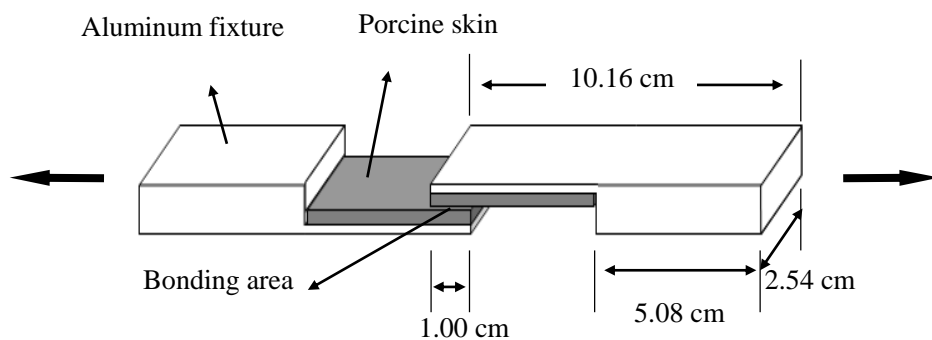
Genipin is another type of crosslinking agents. Genipin can react with chitosan and some peptides that contain primary amine groups. Genipin has the advantages of high bonding strength and low toxicity. It has recently provoked interest for its ability to crosslink chitosan and certain proteins containing residues with primary amine groups, particularly, gelatin and soya protein isolates (Butler 2003). Genipin can be widely used to crosslink gelatin in the study of tissue sutures, soy protein isolates in food applications, and so forth. The reason why genipin was used in these applications is that the cytotoxicity is much lower than other cross-linking agents such as glutaraldehyde. However, the bonding strength of genipin is not as sufficient as compared to glutaraldehyde. The reaction time for reaching full bonding strength between gelatin and genipin can be as long as 24 hours (Yao 2003).

Tartaric Acid Derivative (TAD) is relatively novel cross-linking agent that is widely used in many fields. TAD was prepared by reacting carboxyl groups of tartaric acid with N-hydroxysuccinimide in the presence of carbodiimide (Iwasashi 2009). It can reach full bonding strength within 5

minutes and the strength can be several times that of fibrin glue. TAD is an organic acid that can shorten bonding time and also has adequate bonding strength. TAD can be slowly dissolved and reacts with human serum albumin solution. Tartaric acid can be derived from fruit extracts and TAD tissue adhesives are less toxic. It has a limitation that rodent regeneration is better than in human beings, and the period for gel absorption and wound healing may differ in actual human use (Iwasashi 2009).

### 1.3.8 Testing of Tissue Adhesives

The physical bonding strength test is the primary test for evaluation criteria of any tissue adhesive development. Lap-shear bonding strength is tested for tissue adhesives according to the method of ASTM F2255-05. The fresh porcine skin graft is cut into the size shown in Figure 5. The bonding strength is tested by an Instron machine (Instron Corporation, Canton, MA, USA). The stress is needed to break the joint at the maximum load (N).



**Figure 5. Depiction of Lap-shear Bonding Strength Test Specimen**

### 1.3.9 Application of Tissue Adhesives

BioGlue<sup>®</sup>, a commercial tissue adhesive, is approved for clinical tissue bonding and vascular sealing by the US Food and Drug Administration (FDA) as a medical device. It has also been approved by the European Union and elsewhere for extensive clinical use. The major components of BioGlue are BSA (45%, w/v) as protein polymer and GTA (10% w/v) solution as crosslinker (Quinn 2005). BioGlue is provided in a double-tube syringe that is stored at temperature (23 °C). The ratio of BSA and glutaraldehyde is four to one. The syringe is inserted through an applicator which is shown in Figure 6. During a surgery, the cover on the top of the syringe is removed and a sterilized mixer is attached to the syringe ready for use. The compounds are mixed in the mixer when the applicator is applied by pushing forth and back. The pre-filled syringe is available in 2, 5, and 10 mL volumes for different requirements in surgery (Quinn 2005).



**Figure 6. Delivery System of Tissue adhesive**

A: Dispensing gun, B: Dual barrel cartridge system (4:1), C: Static mixer



BioGlue mainly contains albumin which can be degraded by proteolysis. The resorption of BioGlue is slow. BioGlue was approved for biocompatibility tests which contain toxicity, cytotoxicity, and mutagenicity studies. The cost of BioGlue is from \$50 to \$135/ml depending on different size (Quinn 2005).

If BSA is a suitable protein polymer for tissue adhesive, whey protein that contains a group of globular proteins including BSA and a large number of free amino groups, should be suitable for a tissue adhesive polymer as well. WPI as a protein polymer may reach full bonding strength comparable as BioGlue. Moreover, WPI is cheaper and more abundant than BSA. Also, the model system can be sterilized by gamma radiation easily and effectively and maintain aseptic.

## **1.4 GAMMA RADIATION**

### **1.4.1 Overview of Gamma Radiation**

The two types of radiation are electromagnetic and corpuscular. Electromagnetic radiation consists of self-propagating electric and magnetic disturbances (Urbain 1986). Corpuscular radiation contains massive particles with energy. Gamma rays are electromagnetic radiation of nuclear origin with short wavelengths in the region of  $3 \times 10^{-9}$  cm to  $3 \times 10^{-11}$  cm (Spinks 1976). Gamma rays are high frequency and energy photons. Gamma radiation is

ionizing so it is biologically hazardous. Gamma radiation is produced from gamma decay and the energy is emitted from high energy stage to low energy stage. When gamma radiation passes through matter, it is absorbed by processes which set electrons in motion and these electrons produce ionization of other atoms or molecules in the medium (Dendy 2012).

The absorbed energy from the radiation is the matter rather than wavelength. The energy can be described by wavelength shown in the equation where  $h$  is Planck's constant,  $c$  is the velocity of light, and  $\lambda$  is the wavelength (Spinks 1976). Cobalt-60 is a common source of radioactive isotope of gamma radiation.

$$E=hc/\lambda$$

Gamma radiation is described by one of the units called gray (Gy). Gamma radiation can cause damage at a cellular level such as skin burns. It also causes damage like DNA damage, cell death, and increasing incidence of cancer. Due to the protection of personnel safety away from hazard effects of gamma radiation, it must be surrounded by enough thick walls made from high density materials and is usually put underground. The properties of gamma radiation can be exploited in many applications. For example, gamma radiation can be used to kill microorganisms, to treat some types of cancer, and to form diagnostic images.

#### **1.4.2 Application of Gamma Radiation**

Food irradiation is another application of gamma radiation that employs an energy form to treat foods for sterilization. Some ionizing radiation is capable to pass through some foods. Through physical effects they interact with the atoms and molecules that make up the food and also those of food contaminants, causing chemical and biological consequences which can be utilized in beneficial ways (Urbain 1986). Food irradiation benefits include disinfection, inhibiting sprouting, and pathogen killing. Food irradiation also prolongs the shelf life of foods and delays ripening.

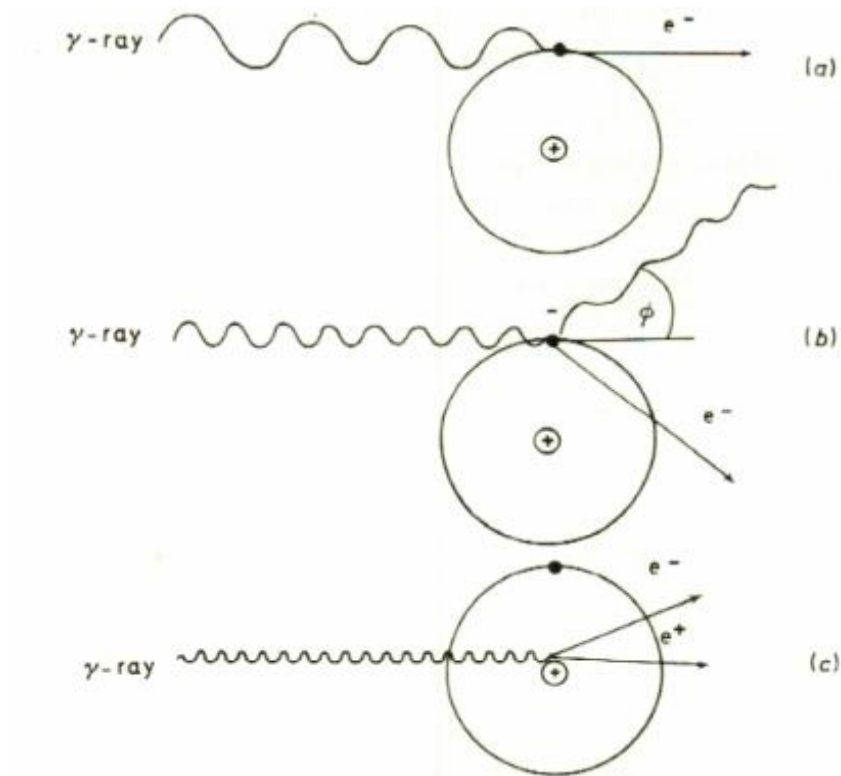
Furthermore, gamma-irradiation generates sterile biomaterials which could be used in pharmaceutical or biomedical applications (Ressouany 1998). In this study, gamma radiation was used to sterilize WPI solution as a part of tissue adhesive. The ideal tissue adhesive would have at a minimum the following capabilities which are safe and biodegradable, effective, easily usable, affordable, and approvable by government regulatory agencies for use in the United States (Sierra 1996). The sterilization of WPI solution is, therefore, very important for the safety of protein-based tissue adhesives. Thermal treatments, filtration, and irradiation are three commonly used methods for sterilizing food products, but heating can cause protein denaturation. Furthermore, WPI solutions in the desired range of concentrations (27%-36%) are too viscous to be filter sterilized. Gamma radiation may offer the only practical alternative method to sterilize WPI

solutions. Gamma radiation may offer an alternative and practical method to sterilize WPI solutions. However, gamma radiation can cause chemical and physical changes that could functional affect properties of WPI solutions.

Chemical changes in the proteins caused by gamma-irradiation include degradation, cross-linking, aggregation and oxidation by oxygen radicals that are generated in the radiolysis of water (Lee 2005).

### **1.4.3 Interaction of Matter with Gamma Radiation**

Gamma radiation interacts with orbital electrons of matter at the atomic scale. The energy absorbed is related to the density, the atomic cross-section, and thickness of the material. There are three situations including photoelectric effect, Compton scatter, and pair production, as shown in Figure 7 (Faires 1981).



**Figure 7. Interaction of Gamma Rays. (a) Photoelectric Effect. (b) Compton Scatter. (c) Pair Production**

When an individual gamma photon interacts with a massive object, such as shielding, the primary interaction takes place with one electron or the nuclear field of a single atom (Faires 1981). An electron is ejected and passes into the mass of the object where its energy is dissipated. In the photoelectric effect, a gamma photon strikes an electron and the energy is transferred to the electron and the photon is totally stopped. In Compton, scatter the photon interacts with an atomic electron and enough energy is transferred to the electron for the ejection from the atom (Faires 1981). The photon maintains some energy to become a new photon with a different direction and lower energy. The pair production situation happens at the absorption of a high

energy gamma photon. The photon collides with the electric field of a nucleus to generate a positron pair (Faires 1981). The ejected positron will bump into an electron and the annihilating process will generate two gamma photons.

### **1.5 OBJECTIVES**

The objectives of this study were to develop a method to sterilize whey protein solution using gamma radiation and to formulate a tissue adhesive using whey protein as the major polymer.

## **CHAPTER 2: MATERIALS AND METHODS**

### **2.1 MATERIALS**

WPI with a protein content of 90% was purchased from Fonterra™ (Auckland, New Zealand). Glutaraldehyde (GTA) (50%) was purchased from Fisher Scientific Inc. (Pittsburgh, PA). Aerobe count and yeast and molds count Petrifilms were purchased from the 3M™ Petrifilm™. Porcine skins were purchased from a local supermarket. Pre-filled buffer (99 ml) was purchased from Fisher Scientific Inc. (Pittsburgh, PA).

### **2.2 METHODS**

#### **2.2.1 Model System Preparation**

Protein tissue adhesive was comprised of WPI solution and GTA solution. WPI powder was dissolved in deionized water at concentrations of 27%, 30%, 33%, and 36% (w/w). The solutions were mixed with a blender for 15 minutes at 1000 RPM. WPI solutions were stored in a refrigerator at 4 °C overnight. Defoamed WPI solutions were poured into 10 ml centrifuge tubes and labeled for protein concentrations and irradiated dosages. Various concentrations of WPI solutions were treated by different dosages (10 – 25 KGy) of gamma radiation. Treatment of gamma radiation at different levels on

protein solutions were shown in Table 4. The treated WPI solutions were stored at room temperature. GTA (50%) was diluted with sterilized deionized water to 6.0%, 8.0%, 10.0% and 12.0% (v/v).

**Table 4. Treatment of Gamma Radiation on Different Levels of Protein**

		<b>Solution</b>			
Sample ID Protein Dosage		27%	30%	33%	36%
	10 KGy		S1-1	S1-2	S1-3
12.5 KGy		S2-1	S2-2	S2-3	S2-4
15 KGy		S3-1	S3-2	S3-3	S3-4
17.5 KGy		S4-1	S4-2	S4-3	S4-4
20 KGy		S5-1	S5-2	S5-3	S5-4
25 KGy		S6-1	S6-2	S6-3	S6-4

### **2.2.2 Microbial analysis**

Each sample (1 ml), respectively, was diluted to  $10^{-1}$  with 9 ml sterile buffer and another 1 ml of the sample was diluted to  $10^{-2}$  with 99 ml sterile buffer.  $10^{-3}$  dilution was prepared by adding 1 ml  $10^{-1}$  dilution to 99 ml of sterile buffer. Each different dilution was applied on aerobe count and yeast and molds count Petrifilms according to the manufacturer's instructions. Triplicates for each sample were tested for aerobe, yeast and molds. The top



of films was lifted to expose the surface and 1 ml of each dilution ( $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$ ) from each sample was added inside a circular foam barrier on Aerobe count Petrifilms and yeast and molds count Petrifilms. The petrifilm has a circular bottom with a barrier and a top to enclose the sample within the Petrifilm. Petrifilms were gradually rolled down and distributed evenly by a plastic spreader and gas bubbles were excluded by this process. WPI solutions prior to gamma radiation treatment were also tested by aerobe count and yeast and molds Petrifilms. After a few minutes of drying, aerobe count Petrifilms were put into incubator at 37 °C for 48 hours and yeast and molds count Petrifilms were put at room temperature for at least 48 hours. The readable plate count for Petrifilm is between 25 to 250 colonies forming units or CFUs. There will be splotches or spots surrounded by bubbles for molds if there are CFUs present.

### **2.2.3 Viscosity**

Apparent viscosity of various concentrations of WPI solutions before and after irradiation at various dosages was determined at room temperature (23 °C) using a Viscometer (Model DV-I Prime, Brookfield Engineering Labs Inc., Stoughton, MA, USA). Samples were tested with spindle # 3 at 100 RMP. The viscosity reading on the screen was recorded after the spindle has been activated for 20 seconds. Three replicates were performed for each sample.

#### **2.2.4 Gelation during Storage**

Gelation was observed every other week during the storage. If the WPI solutions showed solidifying properties then they are not suitable for use of tissue adhesives.

#### **2.2.5 Soluble Nitrogen**

The pH 4.6-soluble nitrogen of WPI solutions was tested by the method of International Dairy Federation (IDF 1964a). Four ml samples of each combination were weighed, respectively, and added into 10 ml centrifuge tubes. Four ml of distilled water was transferred into each tube. Acetic acid (10%, v/v, 0.4 ml) was added into every tube, mixed and kept for 10 minutes. Na acetate (1M, 0.4 ml) was added into every tube and mixed gently. All tubes were volumed up to 10 ml with deionized water. Total sample of mixture was filtrated through Fisher Q 8 filter paper. The precipitate was removed and a sample (2 ml) of filtrate was taken for nitrogen determination by the Kjeldahl method.

#### **2.2.6 Lap-shear Bonding strength**

Lap-shear bonding strength was measured with the method of ASTM standard (ASTM 2005). Fresh porcine skin was cut into 5.08 cm × 2.54 cm pieces with a No. 15 Uniblade™ surgical scalpel (AD Surgical, Sunnyvale, CA, USA). Two skin strips were adhered on two aluminum blocks with dermal side up with Gorilla® super glue (The Gorilla Glue Company, Cincinnati, OH,

USA). The test specimens were kept moist by wrapping with gauzes soaked with phosphate buffered saline (PBS) (Fisher Scientific, Fair Lawn, NJ, USA) and placed in an environmental chamber at 25 °C. 80 µl of WPI solution and 20 µl of GTA solution were applied on the skin dermal side and mixed with a small steel spatula and then the two porcine skin strips were lapped together by the fixture as showed in Figure 5. The bonding area was 2.54 cm × 1.0 cm. Glued specimens were kept for 30 min and tested by an instron 5566 machine (Instron Corporation, Norwood, MA, USA). Specimens were put tightly in the loading cell of the Instron and the test was started until the two porcine skin stripes separated. The maximum load (N) was recorded and the bonding strength (kPa) was calculated. The bonding strength (36% protein, 10% GTA) was also compared with a commercial tissue adhesive.

### **2.2.7 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)**

SDS-PAGE was conducted with separating and stacking gels according to the method of Guo et al. (Guo 1995). The glass plate sandwiches were assembled and sealed with Vaseline. Total volume of 80 ml sample buffer, separating gel, and stacking gel were prepared. 50µl sample were added into 10 ml sample buffer and mixed well. Equal amounts of samples were loaded on each lane for comparison. SDS-PAGE was performed at a constant current of 150 V for 10 minutes at 60 mA and then at 200 V mA at 25 mA for an additional 30-40 minutes at room temperature.

Gels were fixed with 25% (v/v) propan-2-ol and 10% glacial acetic acid overnight. Gels were stained with stained buffer and stored at room temperature for 5 hours. Then the gels were disdained with distained buffer for 2-3 hours until the blue color was washed off to make the gels transparent and the bands clearly visible.

### **2.2.8 Scanning Electron Microscopy (SEM)**

On two separate occasions, scanning electron microscopy (SEM) was for analyzing the microstructure of WPI solutions. For the first trial, whey protein adhesives were prepared by loading in an applicator to mix and spread the components. The material was extruded out onto a piece of parafilm and cured at room temperature. Then the material was frozen in liquid nitrogen and fractured. The pieces were mounted on aluminum stubs with silver conductive paste and carbon coated and then sputter coated with Au/Pd (approximately 4 nm) (Hendricks 1983). Treated samples before and after irradiation were then scanned on a FEI Quanta 200 FEG ESEM Mark II scanning electron microscope (UMASS, Worcester, MA, USA) at an accelerating electron voltage of 10 kV. Images were recorded at 25,000 X magnifications. Second trial, SEM images were obtained at the University of Vermont. Samples were dehydrated and obtained at 10,000X magnifications. All images were recorded digitally using Scandium software (Olympus Corporate, Germany).

### **2.2.9 Statistical Analysis**

SPSS<sup>®</sup> Version 21 software for Linux (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data were analyzed by ANOVA which analyzes the effects of gamma radiation treatment and storage time. Significant differences between group means of the variations were observed in partitioned components. The p-value less than 0.05 is considered as significant differences.

## **CHAPTER 3: RESULTS AND DISCUSSION**

### **3.1. RESULTS AND DISSCUSSION**

#### **3.1.1 Effect of Radiation on Microbial Properties**

The WPI solutions were tested for aerobic microorganisms and yeast and molds by 3M™ Aerobic Count Petrifilm™ and Yeast and Molds Petrifilm™, respectively, for six months after gamma radiation treatment (10-25 KGy). No microorganism have been found during these six months for treated samples in both aerobic count Petrifilms and yeast and molds Petrifilms. The smallest dosage (10 KGy) of gamma radiation that was applied in this study sufficient to sterilize WPI solutions and to eliminate the presence of viable microorganisms. The tests for aerobic bacteria and yeast and molds before the treatment of gamma radiation were also conducted in Chinese Academy of Agricultural Sciences, Beijing, China. The colonies on aerobic count Petrifilms were 107, 43, and 93 for  $10^{-1}$  dilution so the mean result was 810 CFU/ml and the yeast and molds Petrifilms showed that the mean result was 200 CFU/ml.

WPI was dissolved with deionized water and stored at room temperature (23 °C). The conditions, which include water activity, temperature, oxygen, food, and acidity, are suitable for aerobic mesophilic bacteria to

generate and grow. Aerobic count Petrifilms were incubated for 48 hours at 35 °C and Yeast and Molds Petrifilms were incubated for 48-96 hours at room temperature that created optimum conditions. Similar as incubation conditions, microorganisms have not grown or generated during six-month storage. Spores, which are formed in unfavorable conditions and are able to develop into new organisms under favorable conditions, did not grow and generate in the systems.

### **3.1.2 Viscosity**

Viscosity is a measurement of pressure which comes from interactions or collisions of particles moving in different directions and velocities in fluid. Technically, with regard to homogeneous solution the higher concentration of the solution, the higher viscosity the fluid shows. Temperature significantly impacts viscosity. In this study, the temperature was kept constant at room temperature to eliminate interference.

The viscosity was used to measure the effects of gamma radiation on polymerization of WPI solutions. The viscosity of WPI solutions was influenced by the treatment of various dosages of gamma radiation. The viscosity before and after gamma radiation treatment was shown in Figure 8. The viscosity of protein solution (27%, w/w) was increased from 2.19 for the control to 4.78 mPa s for the sample treated at 25 KGy, respectively. For 30% protein solution, the viscosity was increased from 4.87 for the control to

12.72 mPa s for the sample treated at 25 KGy. The viscosity of protein solution (33%) was 11.2 for the control and then decreased to 10.14 and 11.15 mPa s at 10 KGy and 12.5 KGy but increased over 15 KGy. For protein solution (36%), the viscosity was from 10 KGy to 15 KGy and then increased to 71.6 mPa s at 25 KGy. Gamma radiation has the effects of fragmentation and aggregation. Proteins can be converted to higher molecular weight aggregates, due to inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, as well as the formation of disulfide bonds (Davies 1987, Lee 2005). However, at low dosage, gamma radiation exhibits the effect of fragmentation. Figures 9 to 12 also show the viscosity was increased during storage for six months after gamma radiation.

### **3.1.3 Gelation**

WPI solutions were treated by the highest dosage of gamma radiation. As long as WPI solutions begin to gel, they are not used for tissue adhesives any more. Protein solutions (27%) treated by 25 KGy gelled within six-month storage. Protein solutions (30 and 33%) treated by 15, 17.5, 20, 25 KGy all gelled. Protein solutions at 36% treated by 15, 17.5, 20, 25 KGy gelled in first 3 months. The one treated by 12.5 KGy got gelled in fifth month.

### **3.1.4 Soluble Nitrogen**

Gamma radiation affects functional and structural properties of



proteins including nitrogen solubility. Soluble nitrogen also measures the effects of gamma radiation on polymerization of WPI solutions. The nitrogen solubility was decreased by gamma radiation due to protein-based conformational modification, shown in Figure 13. The soluble nitrogen (10%, w/w) was decreased from 100% to 54.7% for the untreated sample and the sample treated at 35 KGy. The statistical analysis of soluble protein indicated that radiation treatment had significant impact on soluble nitrogen of whey protein solutions ( $p < 0.05$ ).

### **3.1.5 Lap-shear Bonding Strength**

Protein/ glutaraldehyde tissue adhesive was tested for bonding strength at the first week after gamma radiation treatment. On two different occasions, bonding strength was tested every other two month. Glutaraldehyde (8% and 10%) which was used as a crosslinking agent in this study reacted with various concentrations of WPI solutions before and after gamma radiation treatment. The results for bonding strength are shown in Figures 14, 15, and 16.

For the same whey protein solution, bonding strength was generally increased with increasing dosage of gamma radiation and also with increasing concentration of GTA. However, bonding strength is influenced by many other factors during the operation. For instance, after mixing WPI solution and glutaraldehyde, the tissue adhesive may flow away from the bonding area before solidification which may decrease the bonding strength.

Generally, providing the same percentage of glutaraldehyde, higher dosage of gamma radiation leads to higher bonding strength. Higher concentration of protein also increases the bonding strength. Increasing the percentage of protein also provides a method to enhance bonding strength when the concentration of glutaraldehyde and the dosage of gamma radiation remain constant.

During storage, bonding strength showed increasing. The 33% protein with 10% and 12% glutaraldehyde with 12.5 KGy gamma radiation bonding strength were 20.69 and 23.61 N, respectively. The results of bonding strength (36% protein) and commercial tissue adhesive are shown in Figure 17. The fifth month bonding strength was comparable to the commercial tissue adhesive. Overall, bonding strength increases during storage at room temperature. We conclude that, WPI solutions start to gel during storage. If gelation has already begun before gamma irradiation, proteins may be polymerized and denatured which can cause a lack functional and structural properties.

### **3.1.6 Protein Profile**

Polymerization caused by gamma radiation was also used SDS-PAGE to determine. On the base of electrophoretic mobility, proteins are conformed to several lines on the gel depending on their molecular weight. Sodium dodecyl sulfate (SDS) is used to form linear-structural proteins and cause a

negative charge so that proteins migrate from negative electrode to positive electrode.

Protein profiles of WPI solution were shown in Figure 18. The SDS-PAGE profiles (10% and 30%) both show that proteins were cross-linked in the samples treated by gamma radiation under the same amount of samples. The protein profiles show non-quantitatively that proteins were aggregated to higher molecular weights than samples untreated by gamma radiation. Any amino acid radical that is formed within a peptide chain could cross-link with an amino acid radical in another protein (Lee 2005).

### **3.1.7 Microstructure**

Micrographs of the treated WPI solution was shown in Figures 19 and 20. In Figure 19, treated sample (A) has disorder and non-regular pattern structure. It seems protein structure was fragmented by gamma radiation and produced some voids. In contrast, untreated sample (B) had clean bands. In Figure 20, treated protein bands were smeared.

Gamma radiation has two diametrically opposite effects on whey proteins aggregation and fragmentation. However, by high dosage of gamma radiation treatment, conformation of protein is generally aggregated to form high molecular weight.

## **3.2 FUTURE WORK**

Future work for this project would work on optimal dosage of gamma radiation to sterilize the model system and inhibit the growth of microorganisms for at least six months. This dosage should also have the minimal effects on protein-based functional and structural properties of whey protein.

Next attempts may be in the range from 30% protein to 45% protein. BioGlue has 45% BSA as a polymer.

Toxicological evaluations should also be included for the development of protein/glutaraldehyde tissue adhesives. Glutaraldehyde has been mentioned previously for its known toxicity. The percentage of glutaraldehyde did not only affect bonding strength, but also is a significant factor for toxicological testing. Animal experiments may be needed before the product enters human trial phases.

After sterilizing WPI solutions by gamma radiation, gelation may be a factor that affected shelf life. To extend shelf life of this development, preventing from gelation is essential. Other than the dosage of gamma radiation, gelation inhibitor or an appropriate procedure may be an alternative to slow down gelling for future work.

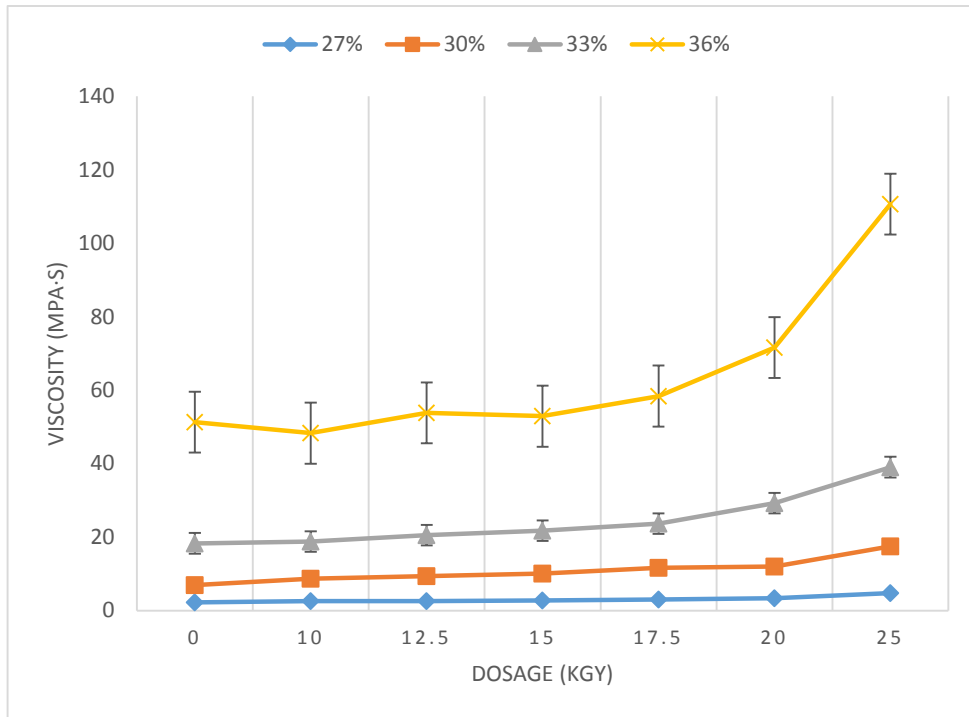
### **3.3 CONCLUSIONS**

Gamma radiation may cause protein-protein interactions resulting in

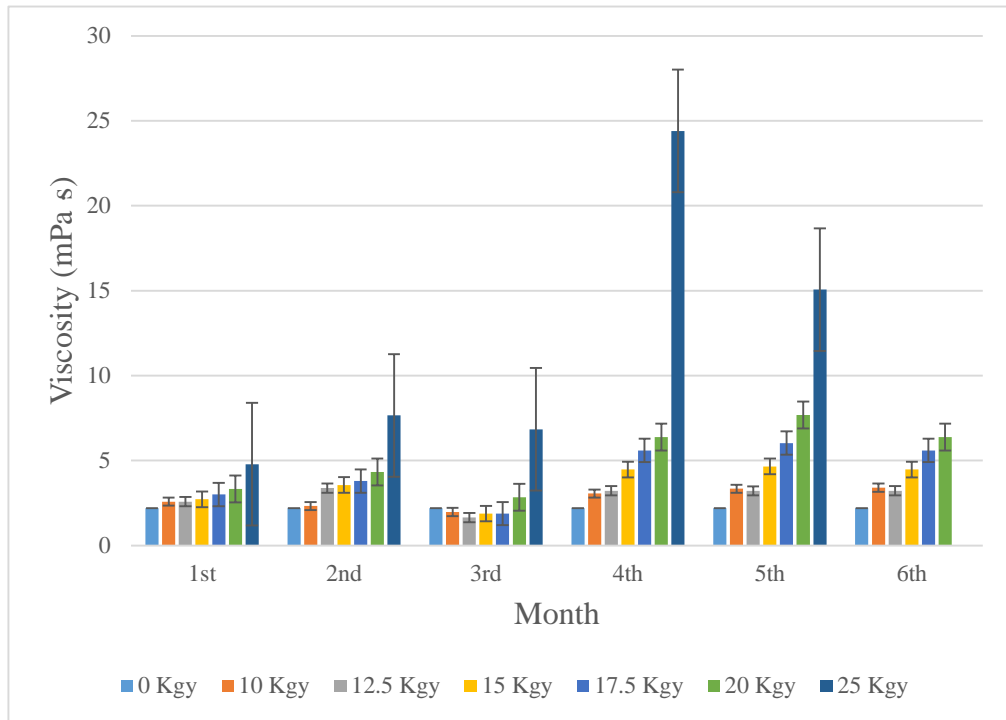
increase in viscosity, and bonding strength, decreasing pH 4.6 soluble N.

Results indicated that gamma radiation may be suitable for sterilizing the protein based adhesive system.

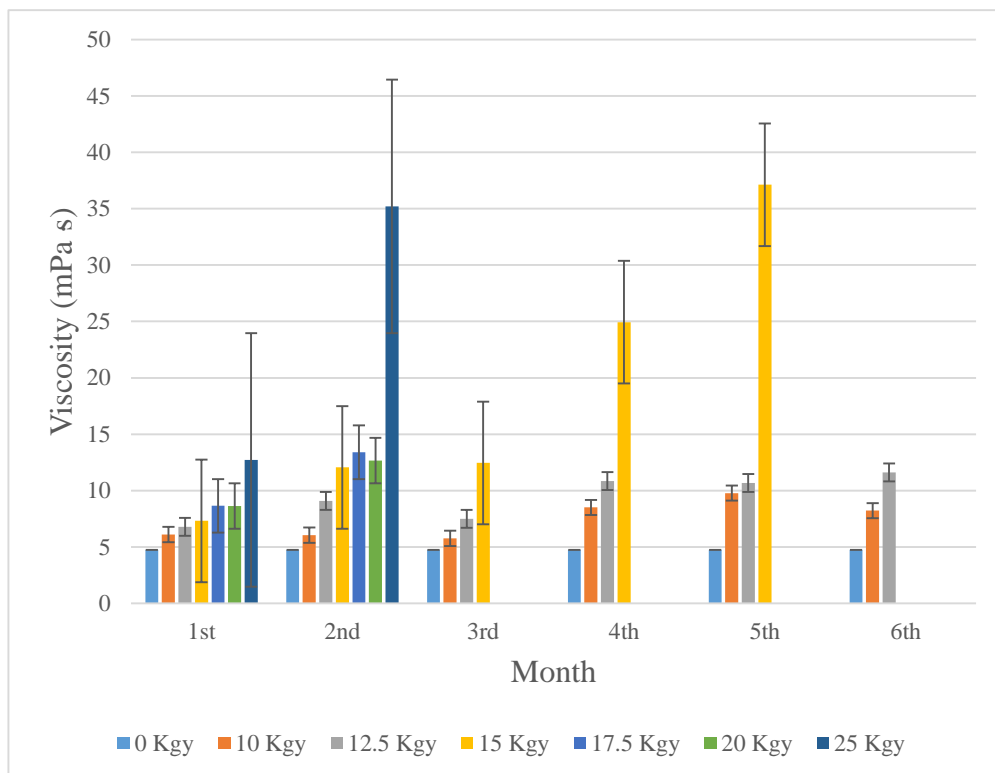
### 3.4 FIGURES



**Figure 8. Effect of Gamma Radiation on Viscosity of WPI Solutions**

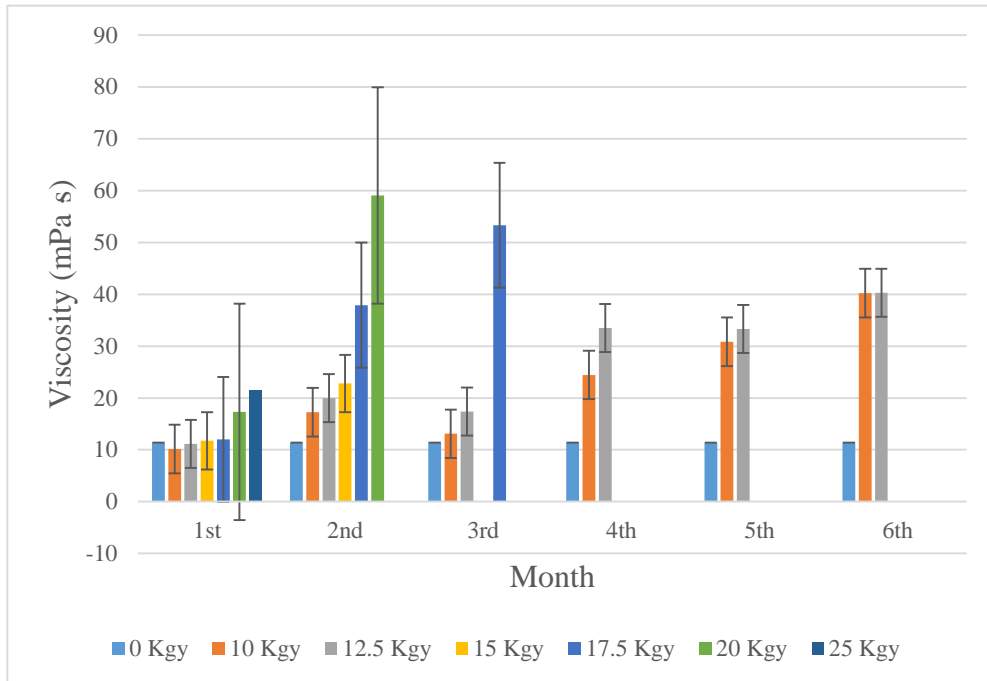


**Figure 9. Changes in Viscosity of WPI Solution (27%) during Storage**

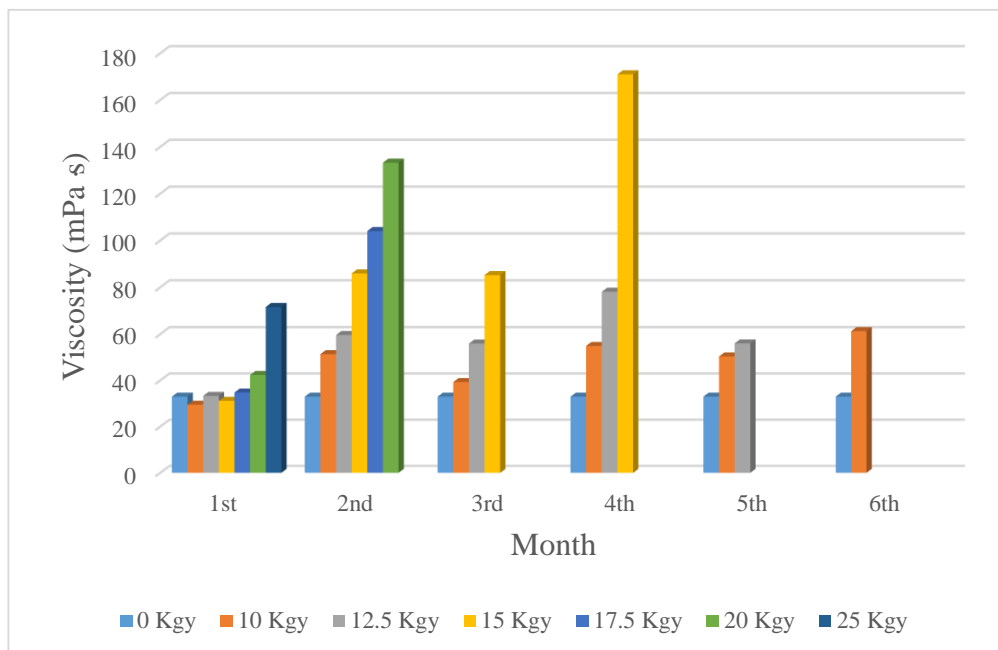


**Figure 10. Change in Viscosity of WPI Solution (30%) during Storage**

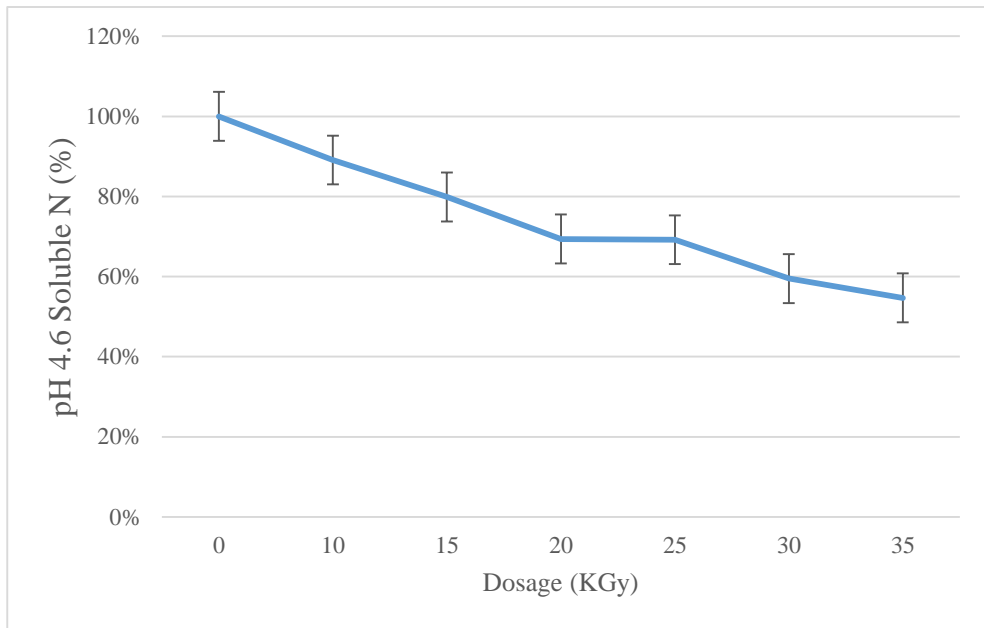




**Figure 11. Change in Viscosity of WPI Solution (33%) during Storage**

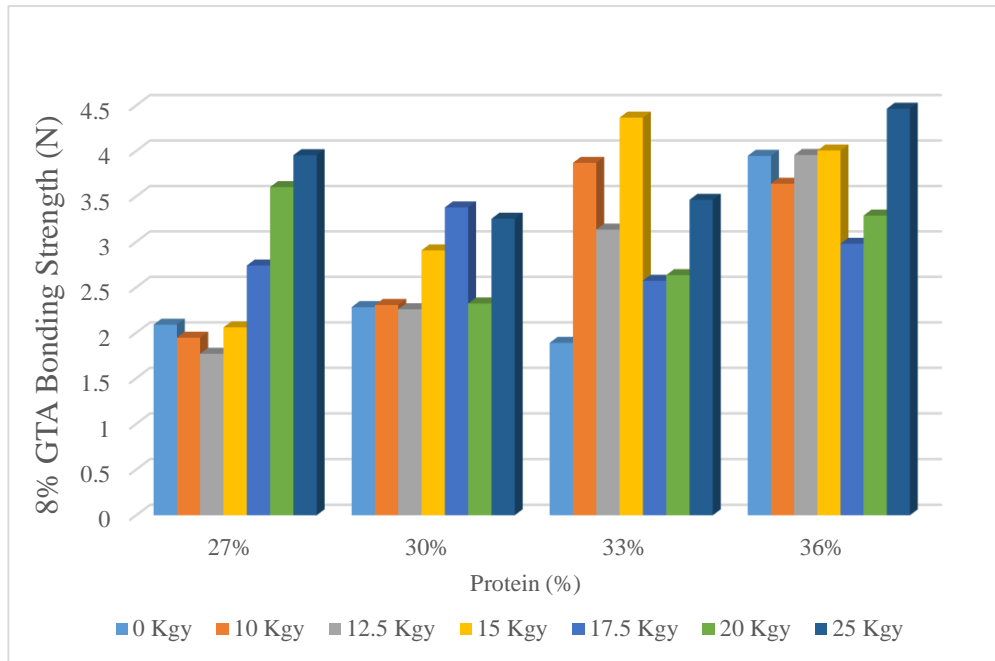


**Figure 12. Change in Viscosity of WPI Solution (36%) during Storage**

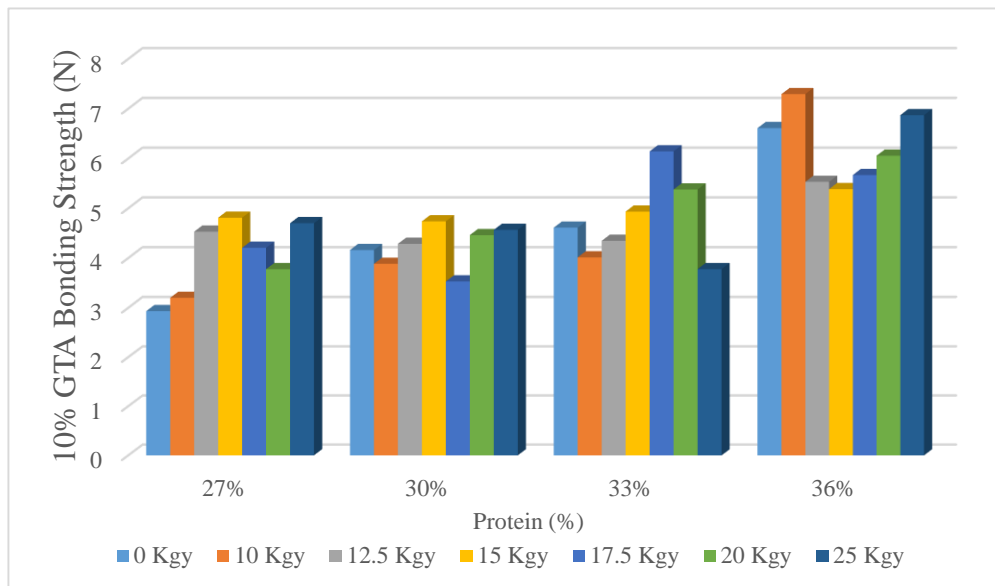


**Figure 13. Effect of Gamma Radiation on Soluble Nitrogen of WPI  
Solution (10%)**

A:

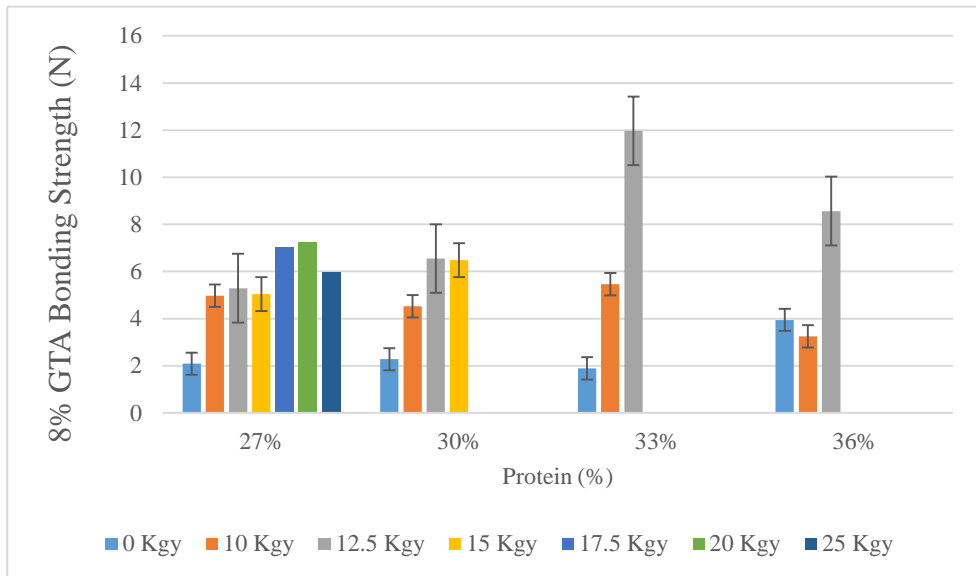


B:

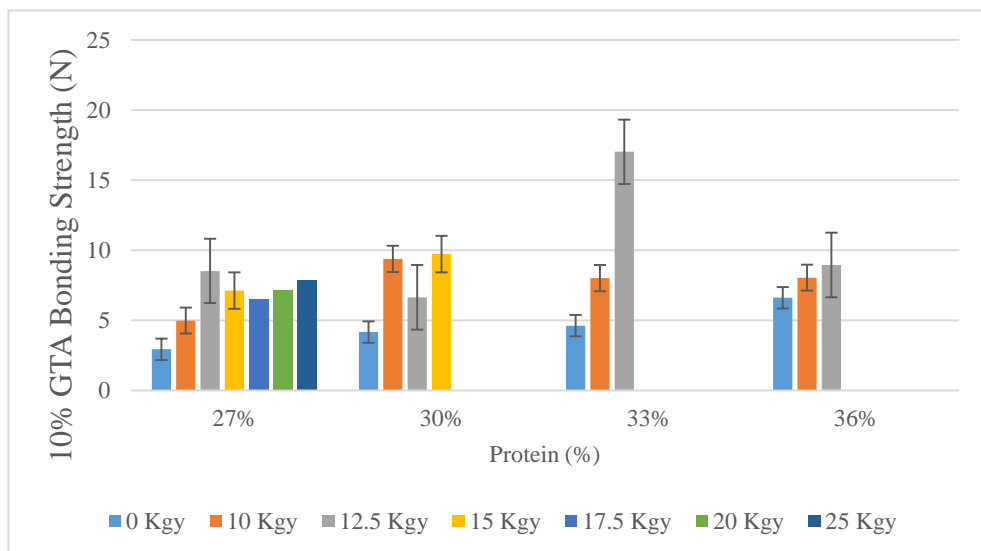


**Figure 14. Effects of Gamma Radiation on the First Month Bonding Strength of WPI Solutions with Different Level of GTA**

A:

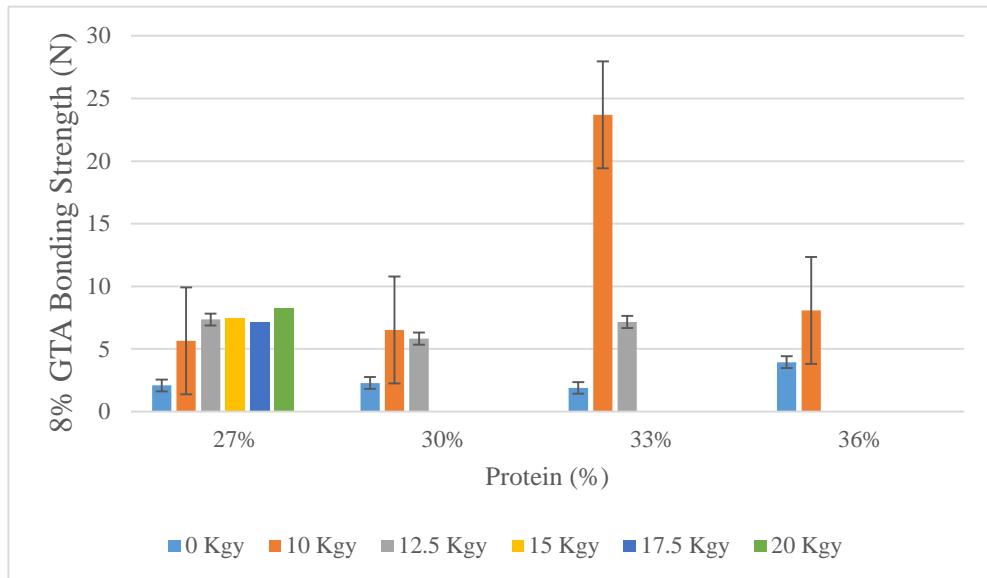


B:

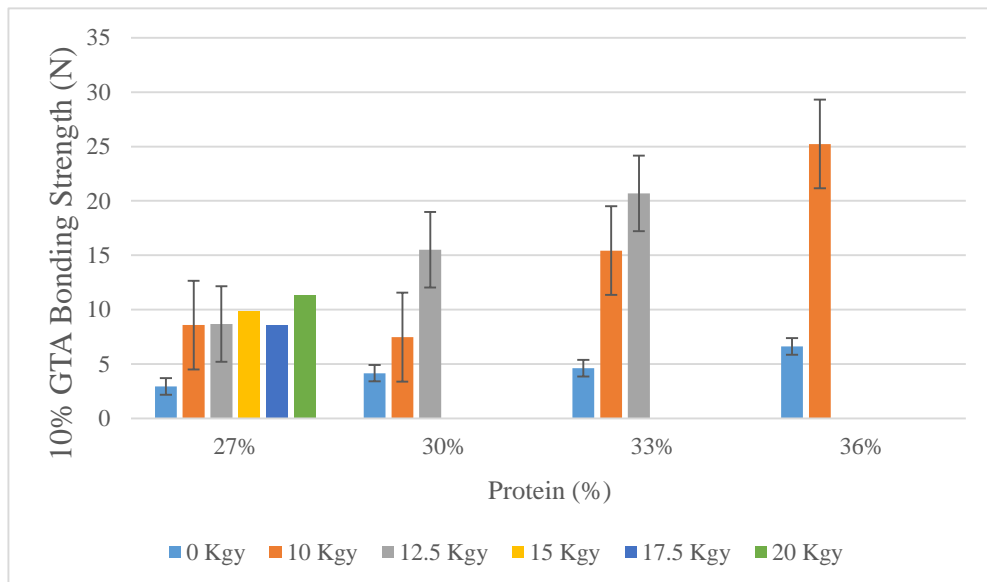


**Figure 15. Effects of Gamma Radiation on the Third Month Bonding Strength of WPI Solutions with Different Level of GTA**

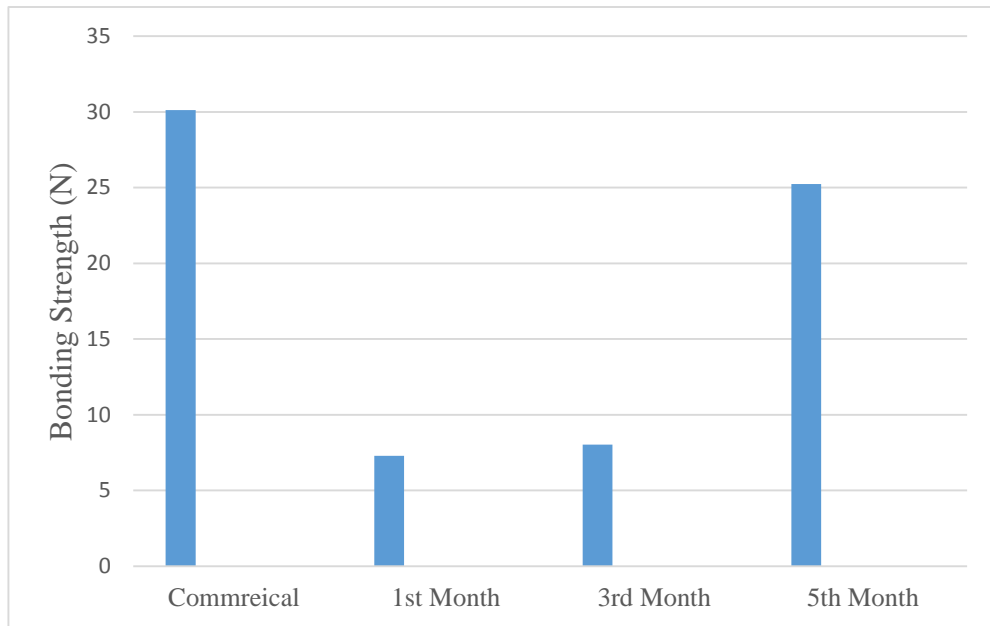
A:



B:

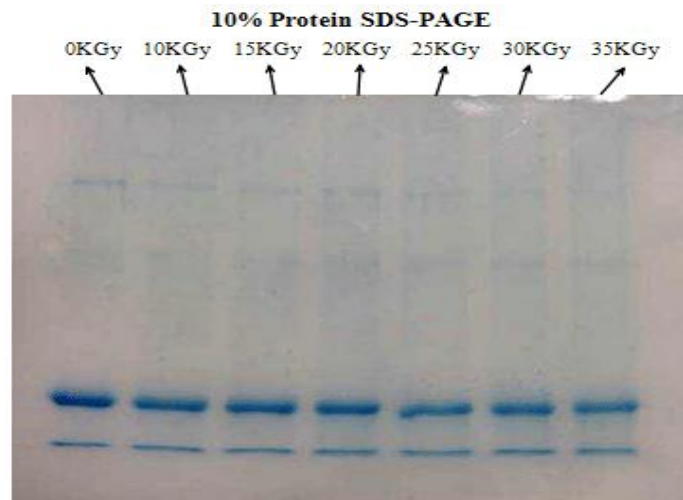


**Figure 16. Effects of Gamma Radiation on the Fifth Month Bonding Strength of WPI Solutions with Different Level of GTA**

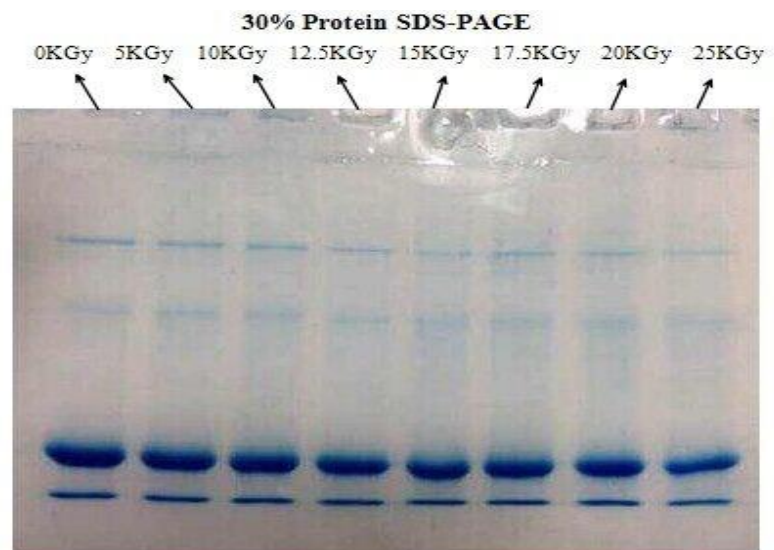


**Figure 17. Comparison of commercial tissue adhesive with 36% protein crosslinked 10% GTA**

**A:**



**B:**

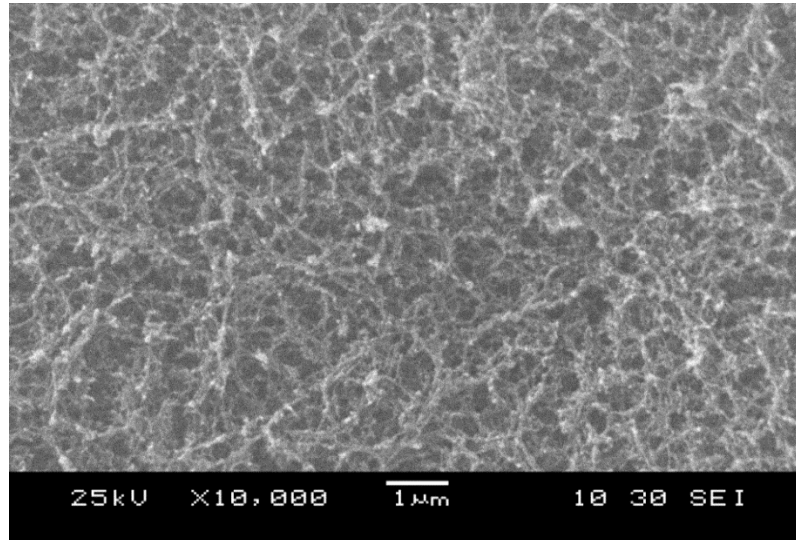


**Figure 18. SDS-PAGE Photographs of Whey Protein Profile before and after Radiation at Different Dosage (0-25 KGy) of 10% and 30% WPI**

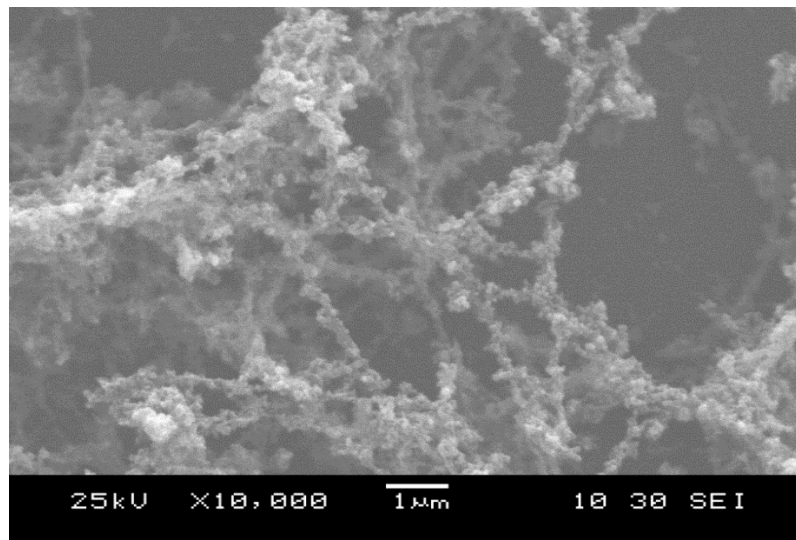
**Solution**



**A:**



**B:**



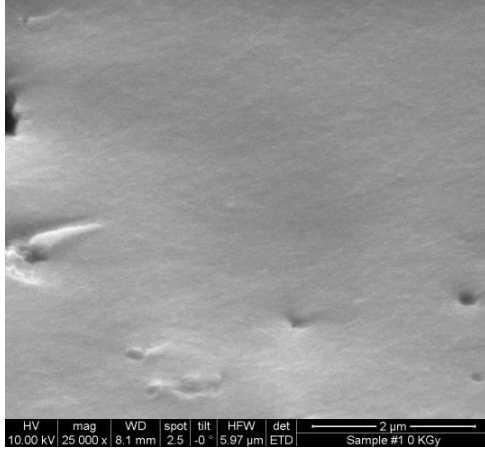
**Figure 19. Comparison of SEM Images of WPI Solutions (30% protein)**

**before and after Gamma Radiation Treatment. A: WPI Solution**

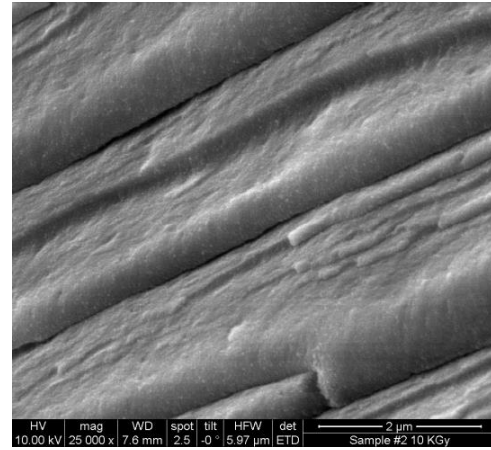
**Untreated at 10K Times; B: WPI Solution Treated by 15 KGy at 10,000**

**X Mag**

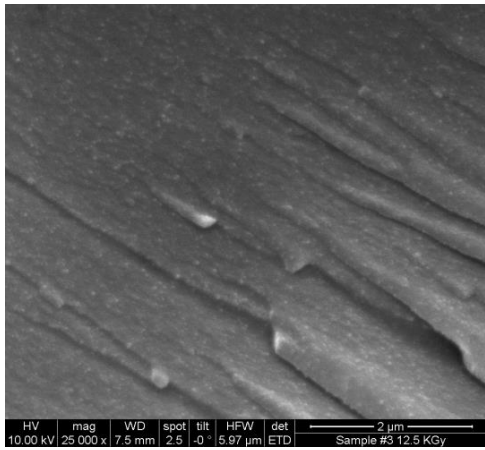
A:



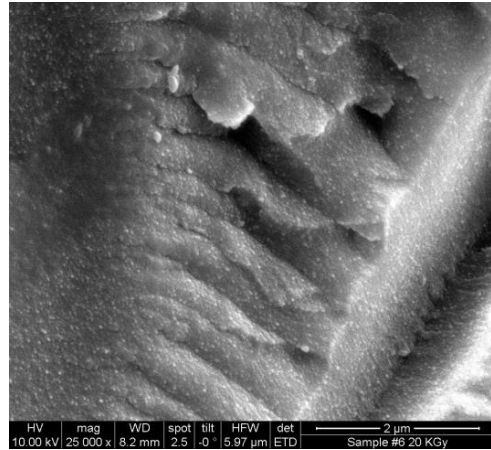
B:



C:



D:



**Figure 20. SEM Images of WPI Solutions (30% protein) Treated by 0 KGy (A), 10 KGy (B), 12.5 KGy (C), and 20 KGy (D) Gamma Radiation at 25,000 X Mag**

### 3.5 COMPREHENSIVE BIBLIOGRAPHY

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