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Formulation And Functional Properties Of Whey Protein Based Tissue Adhesive Using Totarol As An Antimicrobial Agent

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FORMULATION AND FUNCTIONAL PROPERTIES OF WHEY PROTEIN BASED TISSUE ADHESIVE USING TOTAROL AS AN ANTIMICROBIAL AGENT

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ABSTRACT

Tissue adhesives have been widely used in surgical procedures. Compared to traditional surgical sutures, tissue adhesives provide fast bonding experiences and full closure of wounds. However, current tissue adhesives are mostly fossil-based synthetic products. Therefore, it is of great significance to explore the use of natural polymers in tissue adhesives. Whey is a low-end byproduct of cheese making. Whey protein consists of a group of small globular proteins. They can exhibit adhesive properties if their structures are modified by physical or chemical means. The objectives of this study were to investigate the formulation and functional properties of whey protein based tissue adhesive with an antimicrobial agent, totarol. Whey protein isolate (WPI) solutions (25-33% protein) were mixed with different levels (0.1%-0.3%, v/v) of totarol. The total plate counts and yeast and mold counts in the mixtures were negative except the control and the low dosage of totarol. The lap-shear bonding strength was tested after the WPI-totarol solutions were mixed with the crosslinking agent. The lap-shear bonding strength of the optimal tissue adhesive was about 20 kPa, which is comparable to that of a commercial BioGlue®. The microstructures of the mixtures were also examined by Scanning Electron Microscopy (SEM).

Key words: tissue adhesive; whey protein; totarol; functional properties

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

1.1 INTRODUCTION

Interest in sealing wounds and stopping hemorrhages can be dated back to thousands of years ago. Over time, sutures have become the standard of wound closure. However, there are several drawbacks to sutures, such as the high infection rate, physical pain, and long healing time (Quinn, 2005). Therefore, interest in tissue adhesives has grown over the last century. Like sutures, tissue adhesives can be used to close wounds and stop bleeding. In recent years, the use of proteins as a component in tissue adhesives has been widely studied and whey protein has been proven to have a demonstrated potential as a functional polymer component of tissue adhesives (Liu, 2015).

Whey is the by-product of cheese-making. For decades, preventing pollution caused by disposal of whey and recovering its nutritional value have been two major goals of interest in creating whey supplements. For a long time, the main applications of whey have been animal feed, fertilizers, and ingredient for human foods. Recently, though, whey protein has been used for formulation of wood and paper adhesives (Guo & Wang, 2016; Zhao et al., 2011), and the development of a tissue adhesive would be another value-added application of whey protein. Currently, adhesive products used in households, offices and schools are low-priced, fossil-based synthetic products; however, due to the decreasing availability of non-renewable fossil resources, there is growing concern about the long-term supply of synthetic adhesive raw materials.

In addition, fossil-based adhesives are not biodegradable, thus contributing to an unwanted accumulation of waste on the planet (Gao et al., 2011). Therefore, using renewable natural materials like whey protein to develop novel glue products is drawing more and more attention from both academia and industry.

1.2 WHEY PROTEINS

1.2.1. Whey Production and Application

Milk and milk products are consumed extensively in human history. Dairy foods provide not only nutrients but also a variety of functional properties for humans. Milk proteins have been in extensive demand for human health benefits. In bovine milk, approximately 80% of milk protein is casein (Grandison, 1990) and the other major protein is the whey protein (Guo & Wang, 2016).

Whey is the by-product of cheese making, it is obtained by separating casein from bovine milk. Whey is a nutrient dense product containing about 50% of milk solids; however, whey was considered a waste for a long time in history, especially when mass production of cheese started during the industrial revolution in the nineteenth century (Guo, 2019). The waste of whey has a biochemical oxygen demand (BOD) of 35-45kg/m^3 and a chemical oxygen demand (COD) of 60-70kg/m^3 (Mawson, 1994). Whey waste was the most polluted waste of the dairy industry (Guo, 2019).

To obtain whey protein, the whey concentrating and drying techniques were invented. The long-tube multiple-effect evaporator and the spray drier were two important inventions in the early twentieth century in the whey industry and the spray

drier is still used in modern dairy industry (Onwulata & Huth, 2008). Membrane filtration technologies including microfiltration, ultrafiltration, nanofiltration, and reverse osmosis were developed and applied to obtain dry product of whey protein (Guo, 2019).

Whey protein concentrate (WPC) and whey protein isolate (WPI) are the two major whey protein products. Ultrafiltration is used in their processing procedures to separate lactose and minerals from whey protein in liquid whey (Guo, 2019). WPC and WPI have high concentrations of whey protein and are widely used. WPC may contain 20% to 90% of protein and WPI contains at least 90% of protein. WPC's production reached 211,000 tons in 2016, meanwhile the production of WPI reached 50,000 tons in 2016 in the United States (U.S. Dairy Export Council, 2018). In food applications, WPC and WPI have the advantages of high protein and amino acid content, abundant availability, and lack of pathogens, toxic compounds and antinutitional factors (Onwulata & Huth, 2008).

1.2.2. Whey Protein Chemistry

Whey protein is a mixture of various secreted proteins. The major whey proteins are β-lactoglobulin (β-LG), α-lactalbumin (α-LA), and bovine serum albumin (BSA). Other components include lactoferrin (Lf), immunoglobulins (Ig) and minor proteins (Smithers et al., 1996)

β-LG is the predominant component of whey proteins, comprising 50% of the whey protein in bovine milk (Liang et al., 2016). β-LG is a small water soluble protein with the molecular weight ranging from 18.20 to 18.40 kDa and total amino acid

residues of 162 (Yadav et al., 2015). The radius of the β-LG molecule is 2 nm and isoelectric point of pH 5.2 (Bolder et al., 2007). There are seven different genetic variants of β-LG, in which β-LG A and β-LG B are the two major variants, they are associated with milk production and composition, which can vary between dairy breeds (Mezanieto et al., 2012). The two variants also differ in thermal stability, charge, the reactivity of certain groups, heat induced aggregation behaviors such as aggregation rate, the size of aggregates and the subsequent formed gel strength (Schokker et al., 2000). Bovine β-LG have functions of transporting nutrients, controlling cell regulation, pheromone transport, cryptic coloration, and the enzymatic synthesis of prostaglandins (Adams et al., 2006). β-LG can interact with several hydrophobic ligands such as fatty acids, hemin, ellipticine, aromatic hydrocarbons, and carcinogenic hydrocarbons (Divsalar et al., 2009). β-LG is an excellent nanoparticle building conponent and microencapsulation wall material because of the presence of at least two hydrophobic binding sites in the monomer of it (Paul et al., 2014). β-LG is a major allergen in whey fraction and it can induce allergic reactions at very low concentrations (Yong et al., 2010). Native β-LG is resistant to degradation by pepsin hydrolysis in the stomach due to the compact structure (Reddy et al., 1988). Most of the hydrophobic and aromatic amino acid side chains of β-LG are buried in the β-LG core and are not exposed to the enzymes' action (Hernandez-Ledesma et al., 2006). Heat treatment of β-LG solutions at 90-100 °C for 5 or 10 minutes caused changes in the structure or conformation of the protein that rendered it accessible to pepsin and enhanced the extent of proteolysis by trypsin (Guo et al., 1995). β-LG possesses antibacterial and antiviral effects. It was proposed that positive charge and

hydrophobic properties might be the reason for β-LG's bactericidal activity (Pellegrini, 2003). β-LG is also a key antioxidant in milk. The antioxidant activity of β-LG includes both free radical scavenging by amino acid residues chelation of pro-oxidative transition metals (Guo, 2019). Enzymatic hydrolysis of β-LG leads to a high antioxidant activity than the intact β-LG. Chymotryptic hydrolysates of β-LG are more effective than an equivalent concentration of β-LG in retarding lipid oxidation reactions when dispersed in the continuous phase of an oil-in-water emulsion (Elias et al., 2006).

α-LA is a small, compact globular protein with molecular weight of about 14 kDa and isoelectric point ranging from pH 4.2 to 4.5 (Guo, 2019). It consists 123 amino acids in a single peptide chain (Konrad & Kleinschmidt, 2008). Human milk and bovine milk have different percentages of amino acids for α-LA and the protein composition is one of the most important differences between human and bovine milks (Heine et al., 1991). α-LA is a strong calcium binding protein. It has a strong calcium binding site, which is formed by oxygen ligands from carboxylic groups of three Asp residues and two carbonyl groups of the peptide backbone (Permyakov & Berliner, 2000). α-LA has several physiological functions in human. It participates in lactose synthesis and facilitates milk production and secretion in the mammary gland (Stanciuc & Rapeanu, 2010). It has a high nutritional quality (protein efficiency ratio of 4.0, compared to 3.6 for whey protein and 2.9 for casein) and an excellent biological value (Lucena et al., 2006). It can be used in infant formulas because of its high tryptophan content (Arunkumar & Etzel, 2014).

BSA is a single-chain globular nonglycoprotein. It is one of the major

components in plasma protein (Liu et al., 2004). The primary structure of BSA is very similar to that of human serum albumin (HSA). BSA has 583 amino acid residues while HSA has 585. Both BSA and HSA have 35 Cysteine residues which form 17 disulfide bonds and one free sulfhydryl group is left (Guo, 2019). Serum albumins are the major soluble proteins in the circulatory system and they have many physiological functions. They contribute to colloid osmotic blood pressure and are in charge of the maintenance of blood pH (Klajnert & Bryszewska, 2002). Albumin is capable of binding with a variety of ligands reversibly (Carter & Ho, 1994). It is also a carrier of fatty acids in serum (Sklar et al., 1977).

1.2.3. Functional Properties of Whey Protein

In order to improve whey protein functionality, its structures can be modified by physical, chemical and other means. Denaturation of proteins changes their secondary and tertiary structures. High temperature, high pH, and the presence of certain chemicals can induce the denaturation of proteins. Denaturation is reversible until the aggregation of proteins occurs. Whey proteins can aggregate and gel under thermal treatment. The protein structures and properties may change when treated at temperatures of 60 °C or higher. Irreversible changes require a temperature of 70 °C or higher. The color, viscosity, pH, solubility of whey protein may be changed by denaturation. Protein-protein interactions occur at high temperature and these reactions can crosslink peptide chains and cause polymerization. Proteins may polymerize into larger molecules at higher temperatures (Liu, 2015). Denatured whey protein, or polymerized whey protein (PWP) shows improved gelation properties and film forming properties; therefore, it is widely

used in the food industry as thickening agents, stabilizers, microencapsulation wall materials and coating to improve texture and quality in various foods, such as sausages, dairy products, desserts, bakery products, cold sauces (Elofsson et al., 1997), beverages, bars, and fruits (Implvo et al., 2007).

Other than food applications, whey protein can also be used to manufacture many biobased products such as wood adhesives (Gao et al., 2011), paper adhesives (Wang & Guo, 2014), and tissue adhesives (Wang et al., 2018). The functional groups of whey protein, such as the amino, hydroxyl and carboxyl groups, can be chemically modified and crosslinked so that the whey protein molecule weight can increase to form three-dimensional networks, which may offer the product desired mechanical properties, bond strength, and water resistance. As adhesives, whey protein has two major advantages, thermal-induced gelation and desired water solubility. When whey protein solution is heated to 60°C or higher, the molecules can be crosslinked to form three-dimensional networks through intra-molecular or inter-molecular thiol/disulfide (SH/S-S) interchange or thiol/thiol (SH/SH) oxidation reactions (Monahan et al., 1995; Dunkerly & Zadow, 1984). The heat-induced gelation enables whey protein as adhesives with good bond strength and durability because of the formation of three-dimensional networks. The solubility of whey protein in water can reach up to 40% (w/w). This makes more technological approaches possible, such as chemical modification, crosslinking, and blending. Since the adhesives prepared with whey protein alone have low bond properties, these modifications are necessary (Guo, 2019).

1.3 TOTAROL CHEMISTRY

Totarol is a naturally occurring phenolic diterpenoid (Bendall & Cambie, 1995). *Podocarpus totara* is a conifer native to New Zealand and the wood of this tree is famous for its resistance to rotting (Shi et al., 2015). Hence, this tree was investigated for its antimicrobial properties. Totarol is extracted from the sap of *Podocarpus totara*. It exhibits a strong antibacterial activity against several gram-positive bacteria, including *Streptococcus mutans*, *Bacillus subtilis*, *Brevibacterium ammoniagenes* (Kubo et al., 1992), *Staphylococcus aureus* (both penicillin-susceptible and penicillin-resistant strains), and *Mycobacterium tuberculosis* (Muroi & Kubo, 1996; Constantine et al., 2001). For its antibacterial activity, totarol has been approved for application as an antimicrobial additive in several consumer products, including toothpaste and acne creams (Ma et al., 2017).

Podocarpus totara's history can be dated back up to 100 million years ago. This tree has a huge trunk and can live up to 1800 years. New Zealand natives Maoris regard *totara* as a symbol of strength and use the wood to make canoe because of its durability. The timber of *Podocarpus totara* was very valuable to early European settlers of New Zealand. They used it as wharf piles, fence posts and foundation blocks. The durability of *Podocarpus totara* comes from the antibacterial activity of totarol (Bendall & Cambie, 1995).

Totarol was first isolated from *Podocarpus totara*, but it has also been found in other plants including podocarp species, cypress, juniper, thuja, rosemary, and rimu. Totarol can be extracted from the dead wood, so there is no need to cut down the

living trees (Bendall & Cambie, 1995).

In search for the explanation of the mechanism of totarol's antimicrobial activity, Shi et al. (2018) proposed that the mode of action of totarol was the alteration in cytoplasmic membrane's integrity and permeability, which led to the leakage of cellular materials. Other studies have proposed that totarol functions by compromising the functional integrity of cell membranes (Micol et al., 2001). Haraguchi et al. (1996) investigated totarol's effects on *Pseudomonas* and found that it affects the oxidation of NADH in membrane preparation by inhibiting several NADH-related enzymes. Evans et al. (2000) suggested that totarol disrupts bacterial energy metabolism at high concentrations. Smith et al. (2007) argued that totarol was a potent efflux pump inhibitor in *S. aureus* and could reduce the biofilm formation.

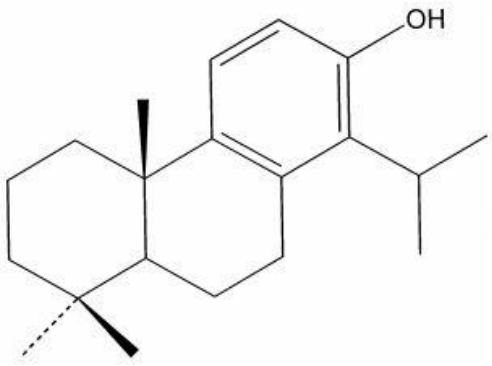

Figure. 1. Chemical Structure of Totarol (Micol et al. 2001)

Totarol has been identified as a non-hazardous ingredient. However, the manufacturers of totarol have brought up sveral potential health effects. If totarol is accidentally swallowed, it may produce localized irritation of the oral or gastrointestinal lining and induce vomiting and mild diarrhea. When used in liquid form, high totarol solution may cause eye irritation. The liquid may be miscible with fats or oils and may degrease the skin, causing non-allergic contact dermatitis. Repeated exposure to concentrated totarol may cause skin cracking, flaking or drying. Entry into the blood stream through cuts, abrasions or lesions may cause systemic injury with harmful effects. Low concentration of totarol is not considered to cause adverse health effects or irritation of the respiratory tract if accidentally inhaled (Liquid K7 totarol safety data sheet, 2014). Totarol has also been tested for safety in human applications. Low totarol solution (0.05%) was applied on 50 human subjects in a skin irritation test and the results showed no evidence of toxic effects (AMA Laboratories Inc, 2000).

1.4 TISSUE ADHESIVES

1.4.1. Overview of Tissue Adhesives

In modern medicine, suture is the most common and basic method for wound closure. However, the disadvantages of suture, such as physical pain, long healing time, and possibility of infection, led to the emergence of alternatives like surgical staples, tapes, and tissue adhesives. The World Wars stimulated the improvement of adhesives. With a large number of casualties, surgeons in World War I began to use sheets of fibrin during surgery to treat injuries (Sierra & Saltz, 1996). Since then, many

effective and convenient adhesive products have been used in different types of surgery. One of the advantages is that when tissue adhesives are used, there is no need for removal later. There is no risk of needle stick injury to the patients or surgeons. Tissue adhesives can also significantly reduce the pain of suture and provide the possibility of a surgery without the development of scars afterwards. Furthermore, most tissue adhesives are very convenient for clinical use and can be applied by a nurse without professional surgical training (Quinn, 2005).

1.4.2. Whey Protein Based Tissue Adhesives

Tissue adhesive is one of the value-added non-food applications of whey protein. Protein-based tissue adhesives have low toxicity and can be absorbed by human and animal tissue during the healing process (Hidas et al., 2006). The principle of protein adhesives is to use a protein polymer reacted with a crosslinking agent to adhere to tissue surfaces. Glutaraldehyde (GTA) is a commonly used cross-linker in tissue adhesives. The cross-linking of the protein based tissue adhesive to the tissue is the result of the reaction between the amino groups of tissue proteins and the terminal aldehyde groups, which are provided by the crosslinker. Proteins can form a soft and ductile barrier moving with the human body (Walt & Agayn, 1994). After bonding, the protein based tissue adhesive can be degraded by proteolysis (Ryou & Thompson, 2005). One of the FDA approved tissue adhesive in the United States is BioGlue®. Its major components are BSA (45%, w/v) as the protein polymer and Glutaraldehyde (GTA) (10% w/v) solution as the crosslinker (Quinn, 2005) and it provides a lap-shear bonding strength of 40.1 ± 12.2 kPa (Wang et al., 2018). Whey protein

contains a group of globular proteins including BSA. Most importantly, β-LG and α-LA are rich in ε-amino group just as BSA. So if BSA is a suitable protein polymer for tissue adhesives, whey protein should be a suitable tissue adhesive polymer as well.

Chapter 2: MATERIALS AND METHODS

2.1 MATERIALS

WPI powder with a protein content of 92% was purchased from Fonterra™ (Auckland, New Zealand). Totarol (1.21%) was purchased from Shanxi Undersun Biomedtech Co., Ltd (Xi'an, China). Glutaraldehyde (GTA) (50%) and pre-filled buffer (99 ml) were purchased from Fisher Scientific Inc. (Pittsburgh, PA). Aerobe count and yeast and mold count Petrifilms were purchased from 3M™. Scienceware™ Super Glue was purchased from Bel-Art™. Porcine skins were purchased from a local supermarket.

2.2 METHODS

2.2.1 Preparation of Adhesives

WPI powder was slowly dissolved in deionized water and mixed for 10 min. The final protein concentrations were 25%, 27%, 29%, 31%, and 33% (w/w), the samples (three trials each) were prepared in 100ml beakers. The solutions were then sealed and stored in the refrigerator overnight for defoaming. Totarol (1.21%) was added into the WPI solution and its concentrations were 0.1%, 0.2%, and 0.3% (v/v). The WPI-totarol solutions were mixed to reach homogeneity and stored for testing. When testing the adhesive property, the WPI-totarol solutions were mixed with GTA solutions, respectively.

2.2.2. Microbial Analysis

The WPI solutions and the WPI-totarol solutions were tested for microorganisms by 3M Aerobic Count Petrifilm and 3M Yeast and Mold Count Petrifilm. The samples were diluted to 10^{0}, 10^{-1}, 10^{-2}, and 10^{-3} with sterile buffer. Each dilution was applied on the Petrifilms according to the manufacturer's instructions. The top of the films was lifted and 1 ml of each sample was applied on the Petrifilms. The top was gradually rolled down and samples distributed evenly by a plastic spreader. Gas bubbles were also excluded by this process. After a few minutes, the Aerobic Count Petrifilms were put into the incubator at 37℃ for 48 hours and the Yeast and Mold Count Petrifilms were put at room temperature for at least 48 hours. On the first day of testing, the tests for aerobic bacteria were conducted every 2 hours to investigate the time needed for totarol to show antimicrobial effect.

Table 1. The Ratio of Protein to Totarol

Totarol (v/v) / Protein (w/w)	0 (control)	0.1%	0.2%	0.3%
33%	S1	S2	S3	S4

2.2.3 Shelf Life Tests

The microbial analysis was conducted every other day during an one-month storage of the adhesive. Gelation was also observed every other day during storage. If the WPI-totarol solutions solidified, then they are not suitable for use of tissue adhesives.

2.2.4 Lap-shear Bonding strength Tests

Bonding strength is mainly affected by the types of adhesives, surface of adherends and bonding process conditions (Li & Ren, 2011). In this study, five different factors, including protein levels, GTA levels, holding time, holding weight, and glue amount, were investigated for their influence on the adhesive property of the WPI/GTA adhesive. Single factor experiments, where one of the factors being the variable and others being fixed as constants, were conducted to determine the optimal condition which reaches the best bonding strength.

Table 2. Factors that Affect the Bonding Strength. A: Protein Levels; B: GTA Levels; C: Holding Time; D: Holding Weight; E: Glue Application Amount

A:

Protein levels (%, w/w)	25	27	29	31	33

B:

GTA levels (%, v/v)	6	8	10	12	14

C:

Holding time (s)	15	30	45	60	75

D:

Holding pressure (g)	400	600	800	1000	1200

E:

Glue Application Amount (μl)	100	140	180	220	260

ASTM standard (ASTM F2255-05) was applied to measure the lap-shear bonding strength. The Instron 5566 testing machine (Instron Corporation, Norwood, MA, USA) was used for the bonding strength testing. Porcine skin was purchased from a local market, sealed and stored at -20℃. Porcine skin has been shown to be the most similar to human skin. It is structurally similar to human epidermal thickness and dermal-epidermal thickness ratios. Pigs have similar hair follicle and blood vessel patterns to humans. Pigs also contain dermal collagen and elastic content that is more similar to humans than other laboratory animals. In addition, pigs have similar physical and molecular responses to various growth factors (Herron, 2009).

During the trials, frozen porcine skin was thawed at 23℃ for 2 hours and cut into 50mm×20mm×3mm pieces with a surgical scalpel (AD Surgical, Sunnyvale, CA, USA), and then wrapped by gauzes soaked with phosphate buffered saline to keep moist. The experiment was carried out in the environment chamber at 23℃ and 40% of humidity，Scienceware™ Super Glue was used to hold the porcine skin firmly on the aluminum blocks. WPI-totarol solutions and GTA solutions (ratio 4:1, v/v) were applied on the dermal side of the porcine skin carefully, two skin pieces were lapped together. Once again, the specimens were wrapped with gauzes soaked with phosphate buffered saline to keep moist.

The Instron 5566 was set at a mobile rate of 12.70 mm/min. The gripping force is supplied by two tightening knobs on the sides of the grips. These tightening knobs are hand-operated, and the jaw faces are self-aligning. The clamping force is adjustable by how hard the knobs are tightened. Specimens are fixed tightly in the loading cell and the maximum load (N) is recorded. The test ran until the two porcine skin pieces separated. Bonding strength is calculated by dividing the maximum load (N) by the bonding area (5cm × 2cm). Lap-shear bonding strength was tested under the optimal condition each week for one month.

2.2.5 Microstructure Analysis

On four separate occasions, scanning electron microscopy (SEM) was conducted to examine the microstructures of WPI solution, WPI-totarol mix, WPI-totarol crosslinked with GTA, and porcine skins bonded by the adhesive.

The samples of WPI solution and WPI-totarol solution were placed onto plastic coverslips and allowed to settle for 1 hour at room temperature. Then they are gently covered in Karnovsky's fixative and allowed to fix for 1 hour at room temperature. The samples were rinsed for 15 minutes for three times in 0.1M cacodylate buffer and stored in buffer at 4℃. Open ended beam capsules were placed in the center of wells of 6-well plate. The capsule was surrounded with warmed 2% SeaPrep agarose. The beam capsule would make a well in the agarose. Agarose is then chilled for 1.5 hours at 4℃. The beam capsule was removed to reveal the well, then placed 1-2 drops of agarose into well to make a base of agarose. 1-2 drops of samples were placed on top of the agarose base in the well and allowed to chill for 10 minutes at 4℃. A few drops of agarose were

placed on top of samples and allowed to chill overnight at 4℃. Agarose was submerged with samples in Karnovsky's fixative and stored at 4℃ overnight. The samples were rinsed for 15 minutes for three times in cacodylate buffer and stored in buffer at 4℃. For WPI-totarol crosslinked with GTA, the sample was submerged in Karnovsky's fixative and chilled at 4℃ for 1 hour, then rinsed for 15 minutes for three times in 0.1M cacodylate buffer and stored in buffer at 4℃. For the porcine skin with bonded adhesive, the whole piece was submerged in Karnovsky's fixative and stored at 4°C for 5 hours and then rinsed for 15 minutes for three times in 0.1M cacodylate buffer and stored in buffer at 4℃.

All samples were treated with 1% OsO_4 for 60 min at 4^oC and then rinsed for 15 minutes for three times in cacodylate buffer. The samples were then dehydrated in 35%, 50%, 70%, 85%, 95% ethanol for 15 minutes each and in 100% ethanol for 30 minutes. Then samples went through for critical point drying with liquid CO_2 and mounted onto aluminum stubs with carbon tape and colloidal graphite adhesive. Samples were sputter coated with gold and palladium and imaged at 10kV with JSM-6060 Scanning Electron Microscope (JEOL USA Inc). The SEM images were obtained from the University of Vermont Scanning Electron Microscopy Center.

2.2.6 Statistical Analysis

IBM SPSS® Statistics 25 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The data were processed by one-way ANOVA analysis. The p-value less than 0.05 was considered as significant differences.

Chapter 3: RESULTS AND DISSCUSSION

3.1 RESULTS AND DISSCUSSION

3.1.1 Effect of Totarol on Microbial Properties and Shelf Life

The results showed that 0.2% (v/v) of totarol had a long-lasting antimicrobial effect. It was indicated that totarol could eliminate the presence of viable microorganisms in 2 hours after addition. During the one-month period, there was no microorganism found in treated samples for both aerobic Petrifilms and yeast and mold Petrifilms.

In comparison, in samples without the treatment of totarol, microorganisms started to grow in the first day of testing. The results of colonies on aerobic count were $2.57 \times 10^4 \pm 5.03 \times 10^2$ CFU/ml and the yeast and mold count was 12.00 ± 2.65 CFU/ml.

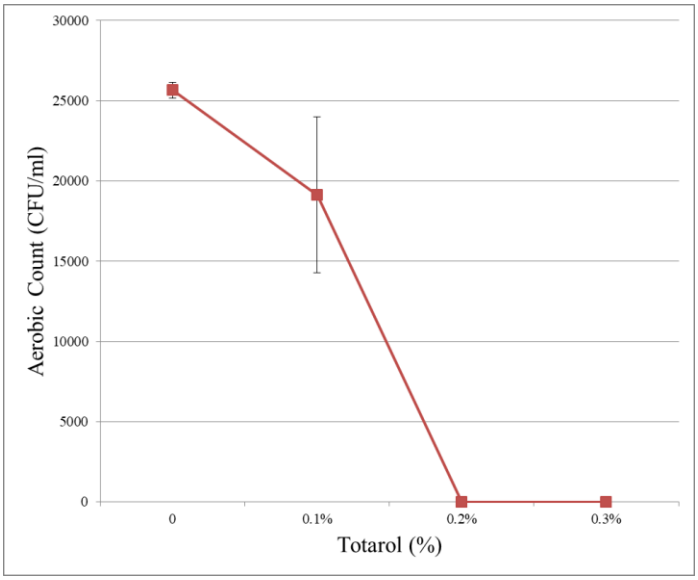

Figure 2. Effect of Totarol Level on Total Plate Counts

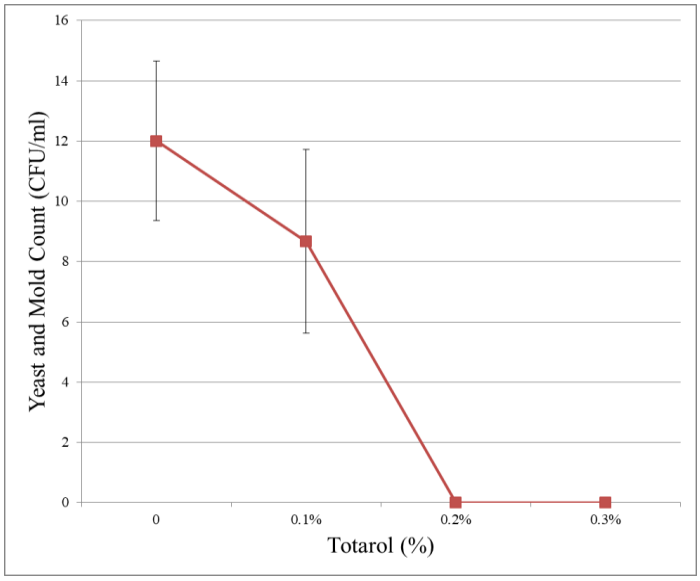

Figure 3. Effect of Totarol Level on Yeast and Mold Counts

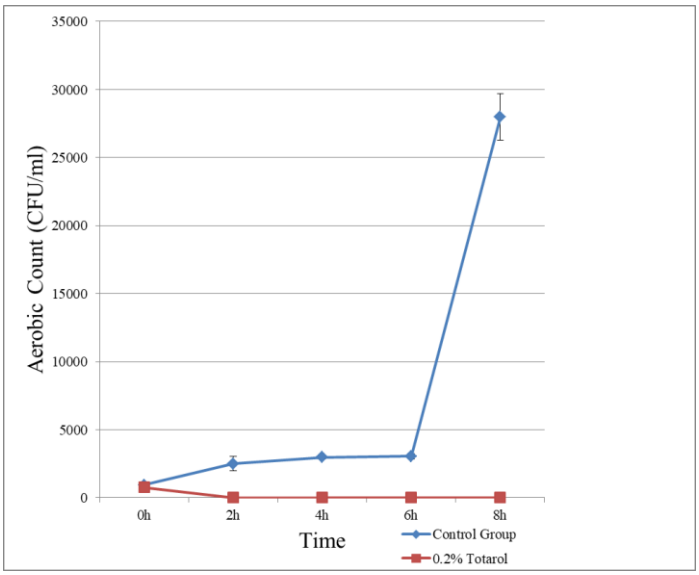

Figure 4. Time Needed for Antimicrobial Effect of Totarol

3.1.2 Lap-shear Bonding Strength

Results indicated that lap-shear bonding strength generally increased with higher concentration of whey protein and also with increasing concentration of GTA. When GTA concentration was 14%, the bonding strength of samples containing 25% (0.81 ± 0.41 kPa) and 27% (1.32 ± 0.12 kPa) of protein was lower than samples containing 29% (20.20 ± 0.17 kPa), 31% (20.09 ± 0.17 kPa), and 33% (20.81 ± 0.83 kPa) of protein. When protein concentration was 33%, the bonding strength of the sample containing 6% (0.80 ± 0.80 kPa) of GTA was lower than samples containing 8% (20.70 ± 0.92 kPa), 10% (20.08 ± 0.13 kPa), 12% (20.16 ± 0.25 kPa), and 14% (20.97 ± 0.84 kPa) of GTA.

After the adhesive was applied on the porcine skin, the two pieces were lapped together and held for a certain period of time. When the skin pieces were held for less than 45 seconds, the results showed a less desirable bonding strength. When the holding time is 45 seconds or more, the bonding strength increased to a desirable level. Bonding strength increased when the weight applied on the skin pieces increased. For the size of skin used in this study (50mm×20mm×3mm), 140μl of adhesive was enough to be spread evenly on the skin surface. Too much adhesive would result in it flowing away from the bonding area before solidification.

During the one-month storage, the tests for bonding strength were conducted under optimal conditions. The concentration of whey protein was set at 33% (w/w) and the concentration of GTA was at 14% (v/v). The holding time and holding weight were 45 seconds and 1000 grams, respectively. For each test, 140μl adhesive was used. The results indicated that bonding strength did not significantly increase ($p=0.406$). The samples did not gell during storage, indicating that the whey protein based tissue adhesive with totarol as an antimicrobial agent can preserve functional properties for at least one month.

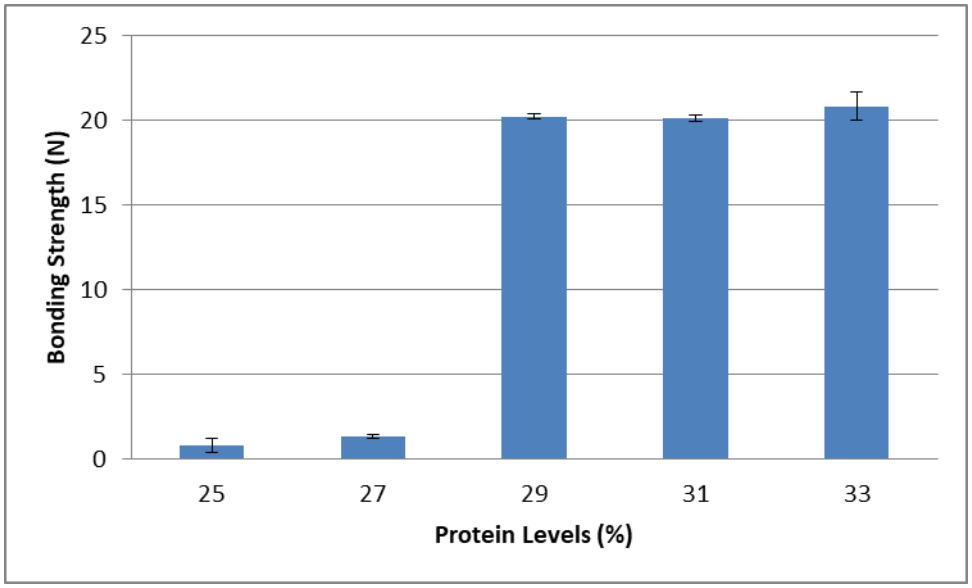

Figure 5. Effect of Protein Levels on Bonding Strength (GTA at 14%)

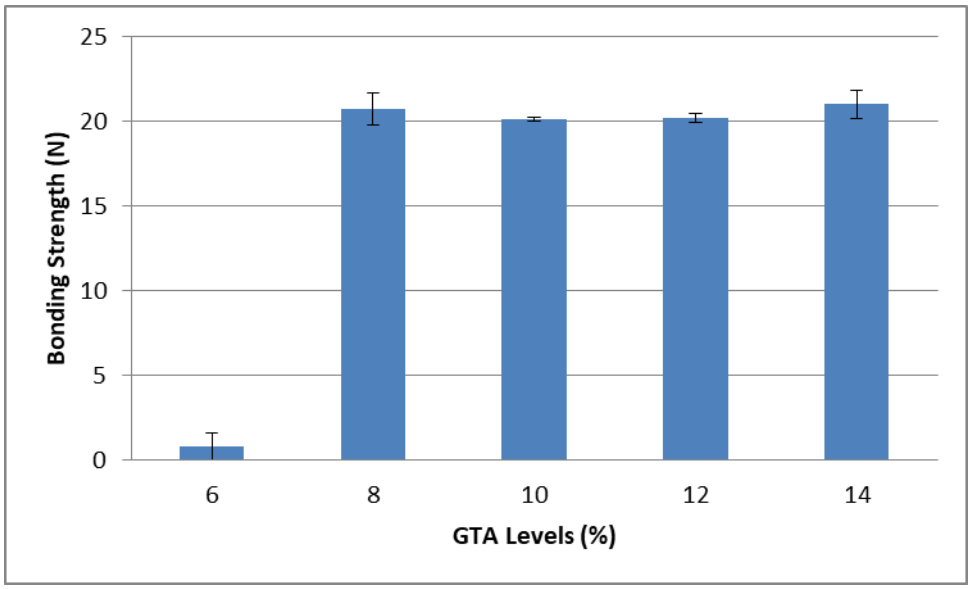

Figure 6. Effect of GTA Levels on Bonding Strength (Protein at 33%)

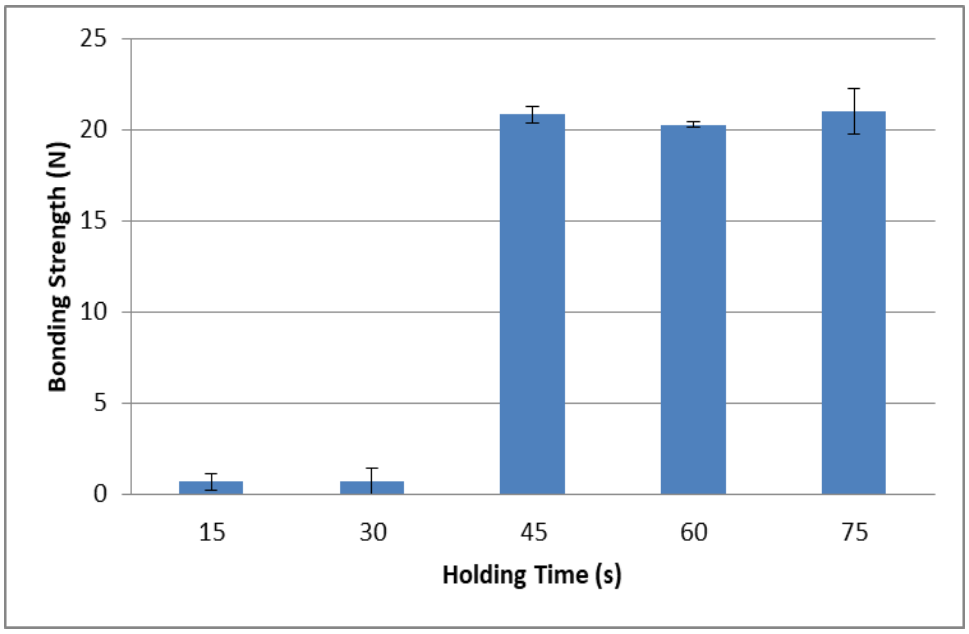

Figure 7. Effect of Holding Time on Bonding Strength

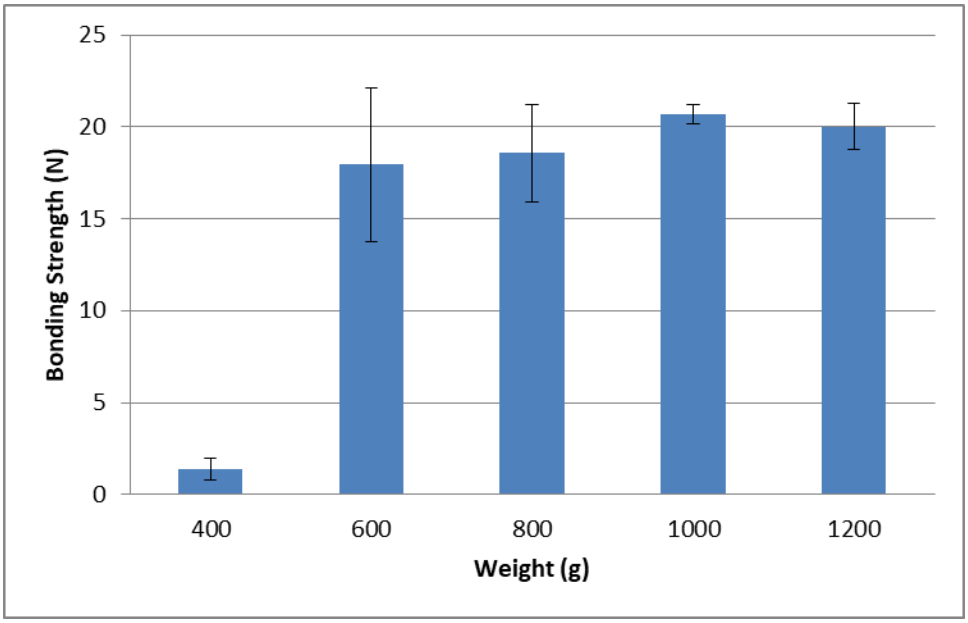

Figure 8. Effect of Holding Weight on Bonding Strength

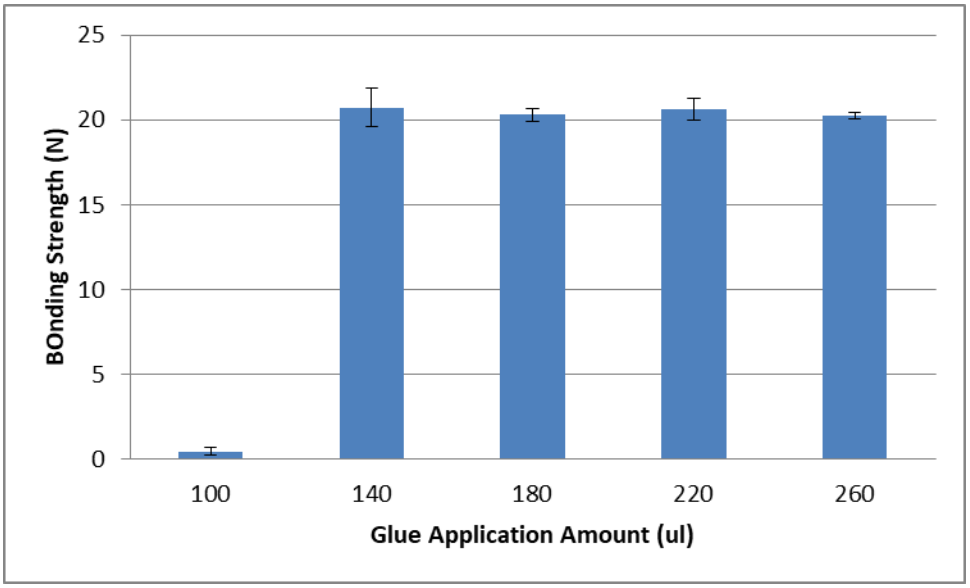

Figure 9. Effect of Glue Application Amount on Bonding Strength

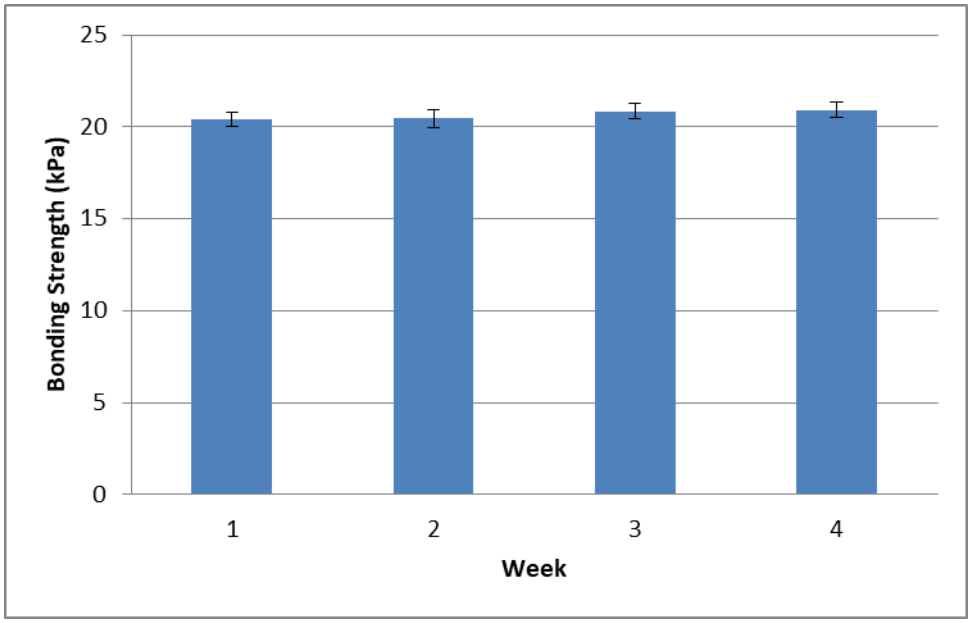

Figure 10. Changes in Bonding Strength of Whey Protein Adhesive during Storage at 23℃

3.1.3 Microstructure

The cross-linking of the whey proteins to themselves and to the tissue is the result of the reaction between the amine groups of proteins and the terminal aldehyde groups which are provided by the crosslinking agent GTA.

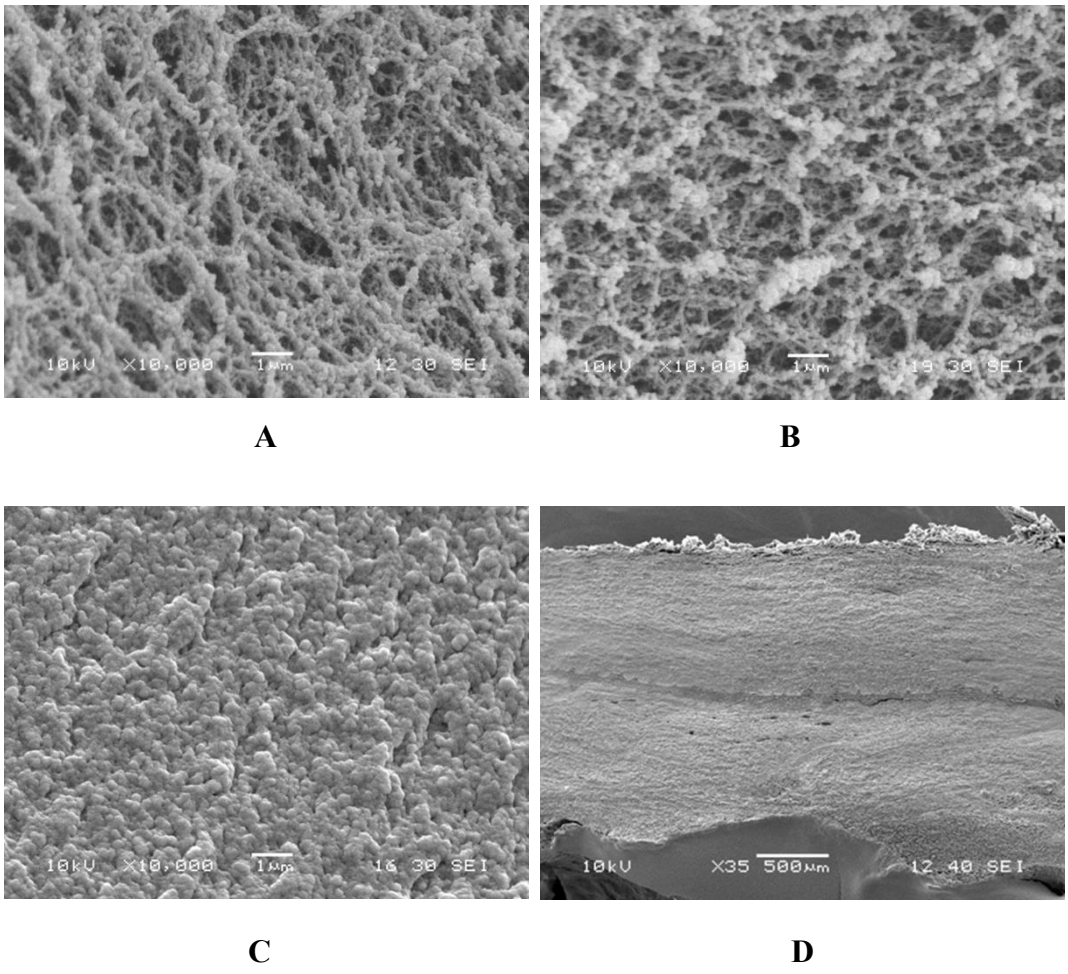

Figure 11. SEM Micrographs of WPI Solution (33% protein) (A), WPI Solution (33% protein) Containing 0.2% Totarol (B), WPI Solution (33% protein) Containing 0.2% Totarol Crosslinked by 14% Glutaraldehyde (C) at 10000 x Mag, and Porcine Skins Bonded by Tissue Adhesive at 35 x Mag (D)

Figure 11A and B showed the micrographs of WPI solution and WPI-totarol mixture. Totarol did not seem to affect the microstructure of whey protein. Figure 11C showed the micrograph of WPI-totarol mixture after crosslinking with GTA. GTA reacted with proteins, making them linked together, so the voids between the protein molecules disappeared and the structure became smoother. Figure 11D is the micrograph of the adhesive bonding with two pieces of porcine skin.

3.2 CONCLUSIONS

The results indicated that whey protein may be suitable for tissue adhesive formulation. The natural material totarol is a good antimicrobial agent for whey protein based tissue adhesive. Toxicological evaluations of GTA may be included in future studies. In order to further increase the protein content in the adhesive, totarol with higher purity is needed. In addition, a longer testing period is also needed so that the shelf life of the tissue adhesive could be extended.

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