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Protein-based bioplastics and their antibacterial potential

Alexander Jones,[1] Abhyuday Mandal,[2] Suraj Sharma[1]
[1]Department of Textiles, Merchandising & Interiors, University of Georgia, Athens, Georgia
[2]Department of Statistics, University of Georgia, Athens, Georgia
Correspondence to: S. Sharma (E-mail: ssharma@uga.edu)

ABSTRACT: The use of conventional petroleum-based plastics in many applications poses the risk of contamination, potentially causing infection when used in medical applications, and contamination when used in food packaging. Nontraditional materials such as protein are being examined for their potential use in the production of bioplastics for applications that require uncontaminated materials. The proteins of albumin, soy, and whey provide possible sources of raw material for bioplastic production, as they have already been utilized in the area of edible films and low-stress applications. We conducted this study to investigate the thermal, viscoelastic, and antibacterial properties of the albumin, soy, and whey bioplastics with the use of three plasticizers—water, glycerol, and natural rubber latex (NRL). *Bacillus subtilis and Escherichia coli* were utilized as Gram (+) and Gram (−) species, respectively, for antimicrobial analysis. Albumin and whey bioplastics exhibited similar thermal and viscoelastic properties, whereas soy bioplastics had varied viscoelastic properties based on the plasticizer used. In terms of antibacterial activity, the albumin–glycerol and whey–glycerol were the best bioplastics, as no bacterial growth was observed on the plastics after 24 h of inoculation. In terms of the future impact of this research, the aim will be to scale up production of the bioplastics for use in food packaging as well as biomedical applications. © 2015 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2015**, *132*, 41931.

KEYWORDS: biomaterials; biopolymers and renewable polymers; plasticizer

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INTRODUCTION

The cost of contamination through conventional plastics in numerous applications has been examined for the material being wasted, as well as the physical harm done to individuals. For instance, in 2002, 4.5 out of every 100 hospital admissions resulted in a hospital-acquired infection in the United States, with over 99,000 deaths being the end result.[1] There is also a fiscal cost to hospital-acquired infections, as a sustained illness will require additional hospital visit. In a study by Gould, an outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA) would result in a doubling of the cost of a hospital visit, with an overall cost between 1.5 and 4.5 billion dollars in the United States on a yearly basis.[2] Based on the findings by Neely and Maley, both MRSA and vancomycin-resistant enterococci (VRE) were able to survive at least 1 day when inoculated onto the surface of materials commonly used in healthcare applications, with some microorganisms being able to survive for more than 90 days.[3] It is because of these issues that materials that could provide antimicrobial properties are being examined for biomedical applications, as that would help in containing or reducing the hospital-acquired infections.

Another area in which contamination is a notable risk is the food packaging, where the material is in contact with food that will be consumed. According to a review study by Lau and Wang, there are five different aspects in which traditional plastics will contaminate food: the gradual degradation of the plastic that contains the food, volatiles such as benzene that are incorporated in the molecular structure of the plastic; contamination caused by the environment; contamination due to the processing agents used to produce the plastics; and other contaminants that are specific to the type of monomer utilized.[4] Food contamination by traditional plastics is caused by the use of a polymer that was not incorporated in the food product itself, leading to the migration into the food. There are three interrelated stages that occur when food becomes contaminated by the plastic packaging: diffusion that occurs within the polymer, solvation of the migrant at the food–polymer interface, and the dispersion of the migrant into the bulk of the food product.[4]

To determine alternative materials such as proteins to be used in plastics, thermal and viscoelastic analysis must first be conducted to determine their suitability for the given application. In a study by Sharma *et al.*, the protein albumin from egg white denatures at a temperature of 136.5°C ± 3°C, ensuring protein's ability to orient and form a bioplastic.[5] This alteration of the protein orientation was due to the breaking of hydrophobic

interactions and hydrogen bonds of the protein itself, allowing the bioplastic to form. Moreover, bioplastics undergoing cyclic loading multiple times did not cause failure, a phenomenon typically associated with conventional plastics.[5] Another protein that has been used extensively in the production of bioplastics is soy protein isolate (~90–95% protein). In a study by Paetau *et al.*, the optimal temperature of soy plastic thermomechanical molding was between 120 and140°C, as higher temperature led to thermal degradation and affected properties during molding.[6] The tensile and viscoelastic properties of the resulting bioplastics were highly dependent on the moisture content of the soy protein and the molding temperature. For instance, soy protein with a lower moisture content possessed greater tensile properties when molded at 120°C, whereas soy protein with a higher moisture content exhibited higher tensile properties when molded at 140°C.[6] Whey protein, byproduct of cheese production, would also be a suitable choice for bioplastic production, as it has been used extensively in the area of edible film.[7] For whey proteins, the minimum temperature of molding into a film was 104°C, with degradation starting above 140°C.[8]

It is because of the contamination issue with traditional plastics in applications where contamination is possible that biopolymers made from proteins are being examined for their potential use in medical applications. In a review conducted by Qiu *et al.*, it was found that biopolymers could promote antimicrobial activity in three ways: the creation of an antiadhesive surface, the disruption of cell–cell communication through antibacterial agents, or lysing the cell membrane to kill the bacteria.[9] Albumin protein (not in bioplastic film) has been studied for its antimicrobial in clinical research and treatment. Albumin is able to exhibit antimicrobial properties through its enzyme, lysozyme that utilizes a lysis reaction to kill cells.[10] Another protein that could be utilized in applications that require antimicrobial properties is whey. Whey has been found to contain immunoglobulins and glycomacropeptides, constituents that bind toxins and help prevent bacterial infection.[11] It is also possible to promote the antimicrobial activity of protein-based bioplastics through the use of additives, which possess antimicrobial activities. For instance, when additives such as grape seed extract and nisin were added to the soy protein during plastic production, the plastic inhibited microbial growth.[12] In another study, wheat gluten and egg white bioplastics loaded with bioactive agents, formic acid, and oregano essential oil demonstrated antimicrobial activity.[13] Also of note are the areas of antifouling and antiadhesive properties of plastic surfaces to prevent microbial adhesion to the surface.[14] Our objectives in this study were to determine the thermal and viscoelastic properties of albumin, soy, and whey bioplastics through the use of water, glycerol, and natural rubber latex (NRL) plasticizers, and to evaluate the antibacterial properties of these bioplastics.

MATERIALS AND METHODS

Materials

Albumin (purity $\geq$ 99%) and ultra-high-molecular-weight polyethylene powder (particle sizes of 53–75 μm) were obtained from Sigma-Aldrich Corporation (St. Louis, MO); the soy protein edible (protein content $\geq$ 72%) was acquired from MP (Solon, OH); and the biPro whey protein (purity $\geq$ 99%) was obtained from Davisco Foods Int'l (Le Sueur, MN). Plasticizers were purchased through various sources: deionized water was supplied by a water filtering system in the lab; glycerol was obtained from Sigma-Aldrich with a purity $\geq$99%. A 70% solid, 30% water mixture of NRL (pH = 10.8) was acquired from the Chemionics Corporation (Tallmadge, OH). In a study by Tarachiwin *et al.* on natural rubber from *Hevea brasiliensis*, the small rubber particles showed mean diameter <250 nm whereas larger rubber particles showed mean diameter >250 nm.[15] For antibacterial analysis, various materials were purchased for testing: bacto tryptic soy agar and broth from Bectin, Dickinson and Company (Sparks, MD); Dey-Engley neutralizing broth from Remel (Thermo Scientific, Suwanee, GA); agar–agar solution that consisted of granulated agar–agar from EMD (Gibbstown, NJ); sodium chloride from Baker (Phillipsburg, NJ); and phosphate-buffered saline solution from HiMedia (Mumbai, India). The bacterial species of *Bacillus subtilis* [Gram (+)] and *Escherichia coli* [Gram (−)] were provided through Dr. Jennifer Walker and the Department of Microbiology at the University of Georgia.

Thermal Analysis of Raw Material

Thermal gravimetric analysis (TGA) was performed using a Mettler Toledo TGA/SDTA851e, with material examined from 25 to 500°C under a N_2 atmosphere with a heating rate of 10°C/min. Differential scanning calorimetry (DSC) was performed using a Mettler Toledo DSC821e, with materials examined from −50 to 250°C under a N_2 atmosphere with a heating rate of 10°C/min. For all sample testing, the weight of each sample was set between 2.0 and 4.0 mg to ensure consistent results and determine optimum plastic molding conditions.

Preparation of Compression Molded Samples

The molding of bioplastic blends was performed on a 24-ton bench-top press (Carver Model 3850, Wabash, IN) with electrically heated and water-cooled platens. Stainless steel molds were used to form dog bone-shaped bioplastics for antibacterial plastic analysis. To form the plastics, protein and plasticizers were mixed manually in predetermined w/w ratios to be placed into the molds (as indicated throughout the article). The mixture of protein and plasticizers was prepared in small batches of varying masses based on density of materials for dog bone plastics ($\leq$6 g for albumin and soy, $\leq$5 g for whey, and $\leq$4 g for polyethylene), while the DMA flexbars were made of 2 g of plasticized proteins. Subsequently, the mixture was filled into the flexbar and dog bone cavity of the stainless steel molds, with plungers placed on top of the molds to prevent the mixture from leaking. After covering with a plunger, the molds were then compressed for a 5-min molding time at 12°C, followed by a 10-min cooling period for the protein plastics. For the polyethylene plastics, a 20-min compression molding time at 150°C followed by a 10-min cooling period was used. Both the bioplastic and polyethylene samples were prepared under a pressure of at least 40 MPa, as a certain minimum amount of pressure must be applied in order to be able to mold a plastic.[16] After the samples were cooled for 10 min under pressure, the pressure was released and the samples were removed. The

plastic samples were conditioned at 21.1°C and 65% relative humidity for 24 h before characterization through dynamic mechanical analysis (DMA) and antibacterial testing.

Dynamic Mechanical Analysis

DMA flex bars of the protein plastics were analyzed for their viscoelastic properties through the use of DMA[17] by using a DMA 8000 Dynamic Mechanical Analyzer from Perkin Elmer. The analyzer examined the viscoelastic properties of the plastics by determining both the storage and loss modulus. The two types of moduli differ by which storage modulus (E') is an indication of the elastic region of the material where energy is stored, while loss modulus (E'') is the amount of energy that is dissipated through heat in the viscous region. The resulting moduli were then put in ratio form (E''/E') to calculate tan δ, which denotes the viscoelasticity of a given material.[18] DMA was conducted from 25 to 160°C, with a temperature ramp of 2°C/min. The settings of the analyzer were set to dimensions of $9 \times 2.5 \times 12.5$ mm^3 using a dual-cantilever setup at a frequency of 1 Hz with a displacement of 0.05 mm. Each sample type was analyzed in duplicate.

Mechanical Properties

The mechanical properties of the conditioned plastics were measured by using the Instron testing system (Model 3343) interfaced with the Blue Hill software. The test was performed according to the standard test method for tensile properties of plastics (ASTM D 638-10, Type I) with a 5 mm/min crosshead speed, a static load cell of 1000 N, and a gauge length of 4 cm. Samples were run in quintuplicate ($n = 5$) for each blend type in order to ensure precise measurement.

Antibacterial Testing of Plastics

The antibacterial properties of the conditioned plastics were measured using the ASTM E 2180-01 standard test method, in which the aqueous-based bacterial inoculum remains in close, uniform contact in a "pseudo-biofilm" state with the bioplastic. For each blend type, the Gram (+) specie *B. subtilis* and the Gram (–) specie *E. coli* were used as challenge bacterial cells to determine the efficacy of bacterial growth on the plastic surfaces. After equilibration of standardized culture banks of $1–5 \times 10^8$ cells/mL through the use of dynamic light scattering analysis, 1 mL of the culture was applied to 100 mL of agar slurry for inoculation. Once inoculated, the slurry was then applied to a 9-cm^2 area of the bioplastics that had been swabbed with phosphate-buffered saline to promote adhesion by reducing surface tension. After the appropriate time of application of agar (within 1 h for 0-h samples and at least 24 h for 24-h samples after incubation), the agar was removed through the use of neutralizing broth, followed by sonicating and vortexing each for 1 min. The neutralizing broth containing the agar was diluted five times in a 10^{-1} dilution set, and then the dilutions were applied to tryptic soy agar plates, which were incubated for 24 h at 37°C. After incubation, the culture plates were counted for microbial growth and averaged to determine colony-forming units (CFU)/mL. Samples were run in triplicate ($n = 3$) for each protein–plasticizer combination (as well as the polyethylene plastic control sample) in order to ensure accurate measurement.

Statistical Analysis

Statistical analyses were performed by fitting a regression model. For each plastic–plasticizer blend tested, bacterial growth for 0- and 24-h samples was analyzed by fitting two-way ANOVA using the statistical software of SAS and R. Box–Cox transformations were used to determine the appropriate transformations needed to satisfy the normality assumptions of the experimental errors. As the dataset has several very big and small values, Cook's distances were examined to ensure that no individual observation is an outlier that influences the conclusions.

RESULTS AND DISCUSSION

Material Analysis

Thermal Properties of Proteins and Bioplastics. An initial degradation peak (Figure 1) was observed for both soy and whey between 70 and 80°C, indicative of bound moisture loss, while for albumin it was between 220 and 230°C.[19] Much larger degradation peaks started at different temperatures for each of the proteins: 245–250°C for the albumin powder; 190–200°C for soy protein; and 200–210°C for the whey protein. At the end of the TGA run, 75% of the protein powders degraded, as the proteins were similar in the overall level of degradation due to the burning of the proteins (Figure 1). These results were similar to the results obtained in the work conducted by Sharma and Luzinov.[20] When compared to the optimum blends (Figure 2) of bioplastics, degradation peaks depended upon the plasticizer used, as plastics blended with water possessed similar thermal degradation peaks in comparison to plastics that did not contain any plasticizer. However, bimodal degradation peaks were witnessed in plastics prepared with glycerol and NRL, as the glycerol-based albumin and whey bioplastics possessed degradation peaks between 240 and 250°C (below protein degradation peaks between 300 and 315°C) while the NRL in albumin and soy bioplastics would degrade at temperatures higher than the proteins (~375°C). This occurs due to the glycerol[21] and natural latex[22] that are bound within the plastics to begin degrading at temperatures that differ to glycerol or NRL that is not bound within a plastic For the DSC data, endothermic dips occurred at varying temperatures: a small peak beginning at 75°C, with a broad peak at 120–125°C for albumin[19]; a narrow peak starting at 50°C and a broad peak at 85–90°C for soy; and a narrow peak beginning at 35°C, with a broad peak at 80–85°C for whey protein. These peaks indicated that the material had fully denatured at lower temperatures for soy and whey (80–90°C) due to higher bound moisture levels, whereas albumin denatured at a higher temperature between 120 and 125°C. An endothermic decomposition or pyrolysis peak occurred at 250°C for all the proteins, which exhibited the onset of degradation, as amino acids degrade at temperatures in this region. Therefore, the protein-based bioplastics were molded at 120°C to minimize thermal degradation while ensuring full denaturation leading to bioplastics. When these results are compared to bioplastics that have been blended with plasticizers (Figure 3), the curves are similar in shape and peak areas unless water was

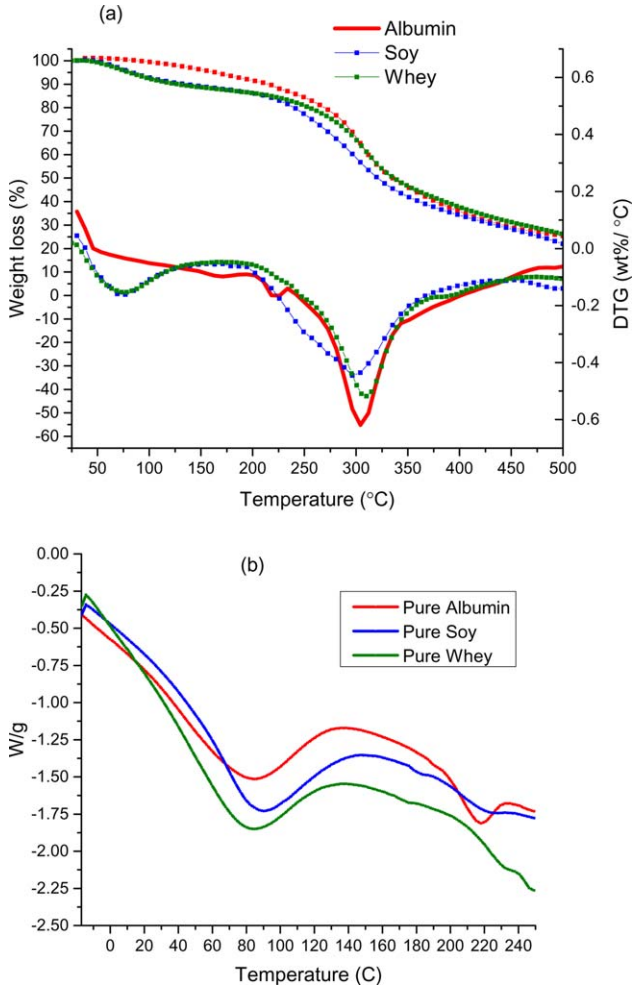

Figure 1. Thermographs of pure protein powders: (a) TGA and (b) DSC. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

utilized as a plasticizer. In this case, endothermic peaks in albumin and whey bioplastics occurred between 220 and 225°C, while in soy plastics the endothermic peaks occurred between 180 and 185°C. One potential reason for this lowering of glass transition and degradation temperatures is the addition of water in the plastic increased polymer–water interactions to the detriment of polymer–polymer interactions.[23] As it has been postulated that the effectiveness of plasticizers for bioplastics is highly dependent upon how they affect hydrogen bonding or hydrophobic interactions,[24] that may be why this property is witnessed only in water-plasticized bioplastics.

Dynamic Mechanical Analysis. In the albumin[19] and whey plastics, we found that the plastics made with the plasticizers of water and glycerol had similar properties, as each had tan δ peaks occurring at lower temperatures in comparison with plastics plasticized with NRL [Figure 4(a,c)]. While the albumin and whey bioplastics plasticized with water and glycerol possessed similar viscoelastic properties, the bioplastics plasticized with natural rubber possessed a lower initial tan δ, with the tan δ peak occurring at higher temperatures, as well as a higher initial modulus. These results point to higher levels of protein–glycerol or protein–water interactions and less protein–protein interactions in the thermoplastic hydrophilic polymers (albumin or whey), thereby shifting the tan δ peaks (glass transition) to lower

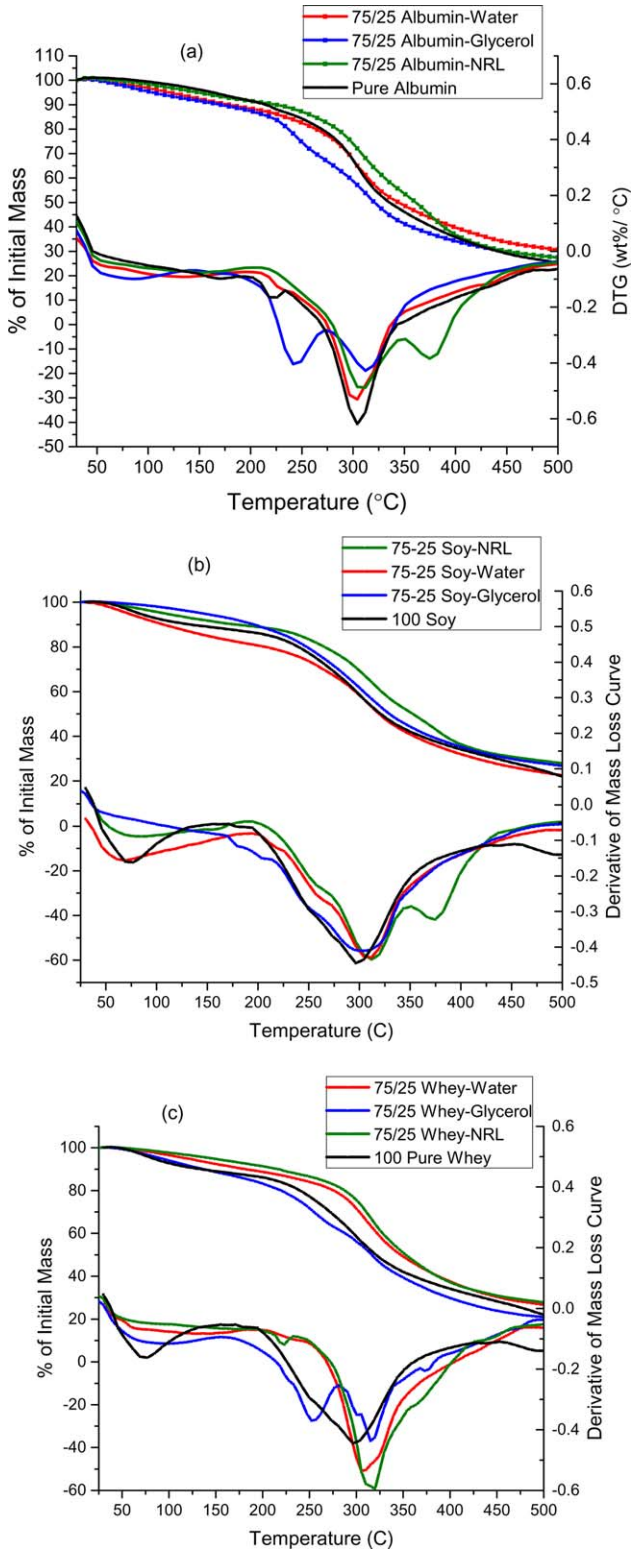

Figure 2. Thermogravimetric analysis of optimal protein plastic blends: (a) albumin, (b) soy, and (c) whey. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

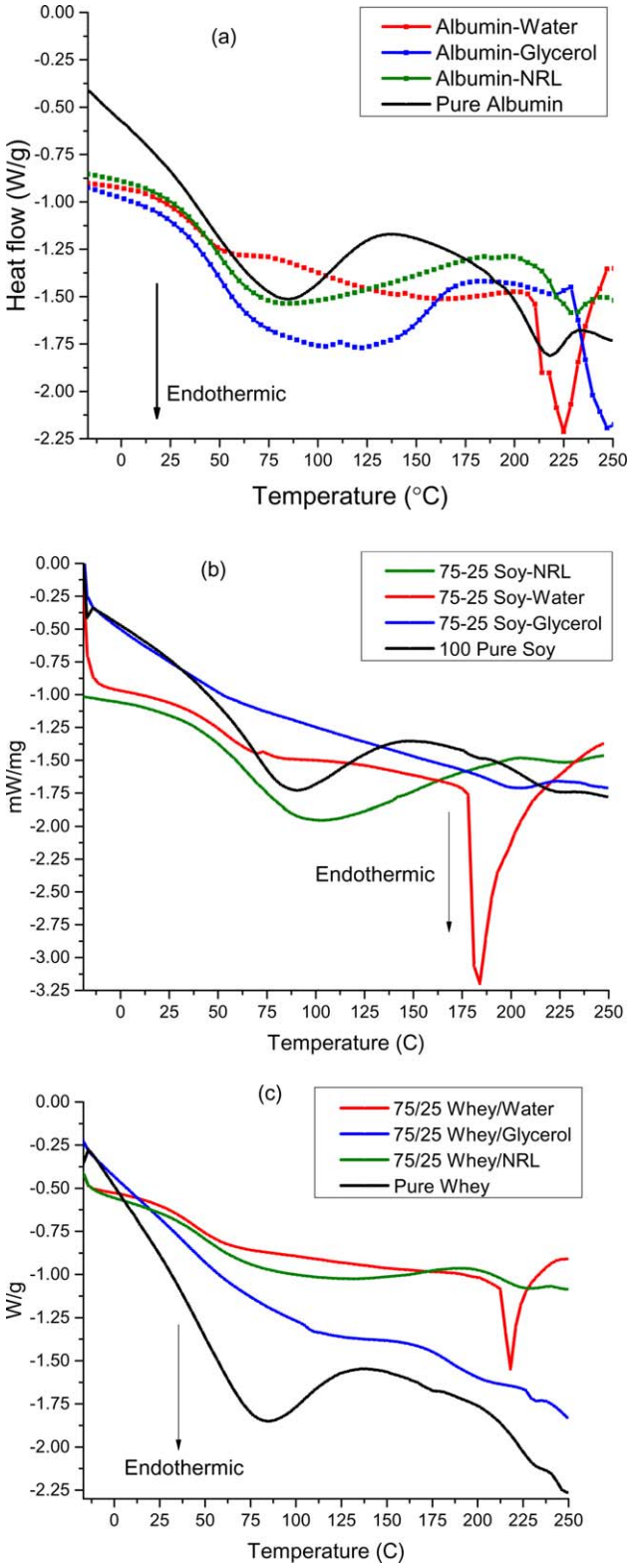

Figure 3. Differential scanning calorimetry of optimal protein plastic blends: (a) albumin, (b) soy, and (c) whey. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

temperature with higher initial tan δ values as well as dropping the elastic modulus (E') than plastics that do not possess any plasticizer.[25] Moreover, the bioplastics produced in the absence of plasticizers were stiff as evident from the higher elastic or storage modulus throughout the temperature of DMA testing. This phenomenon explains the breaking of protein–protein interactions

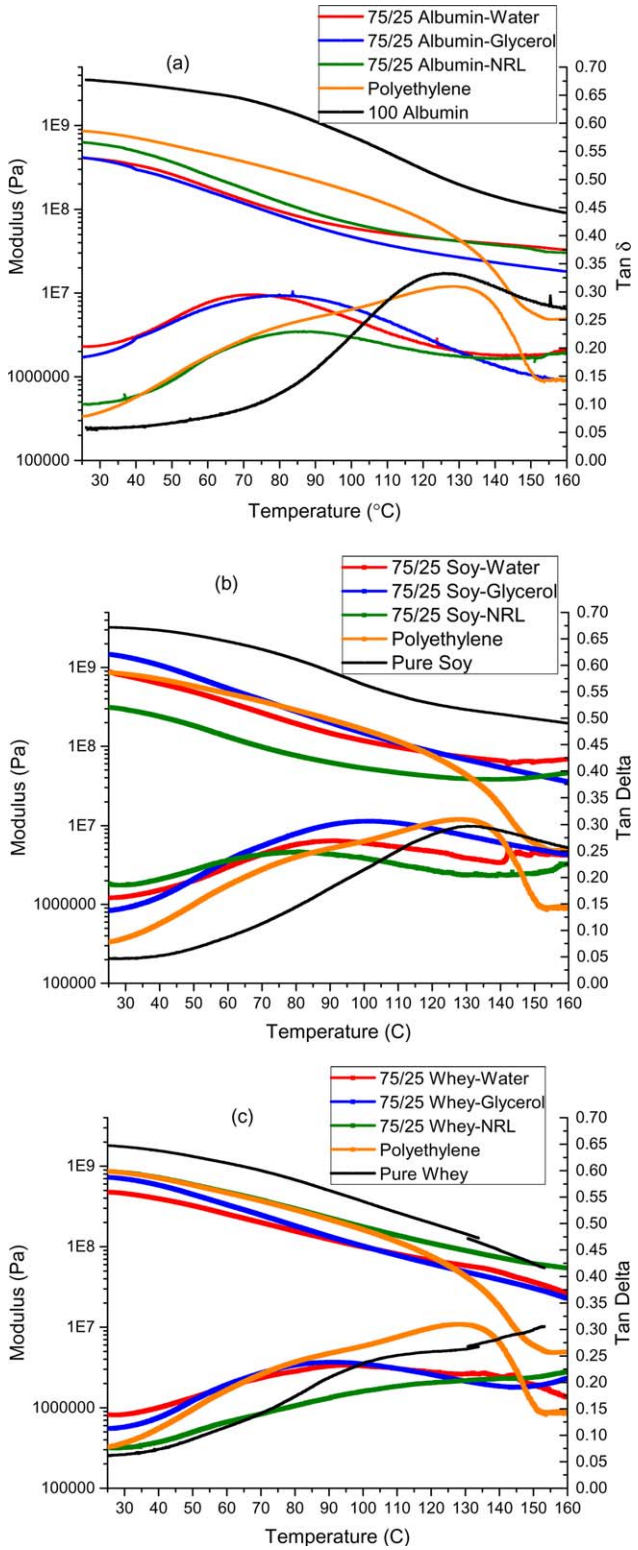

Figure 4. Dynamic mechanical analysis of optimal protein plastic blends: (a) albumin, (b) soy, and (c) whey. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

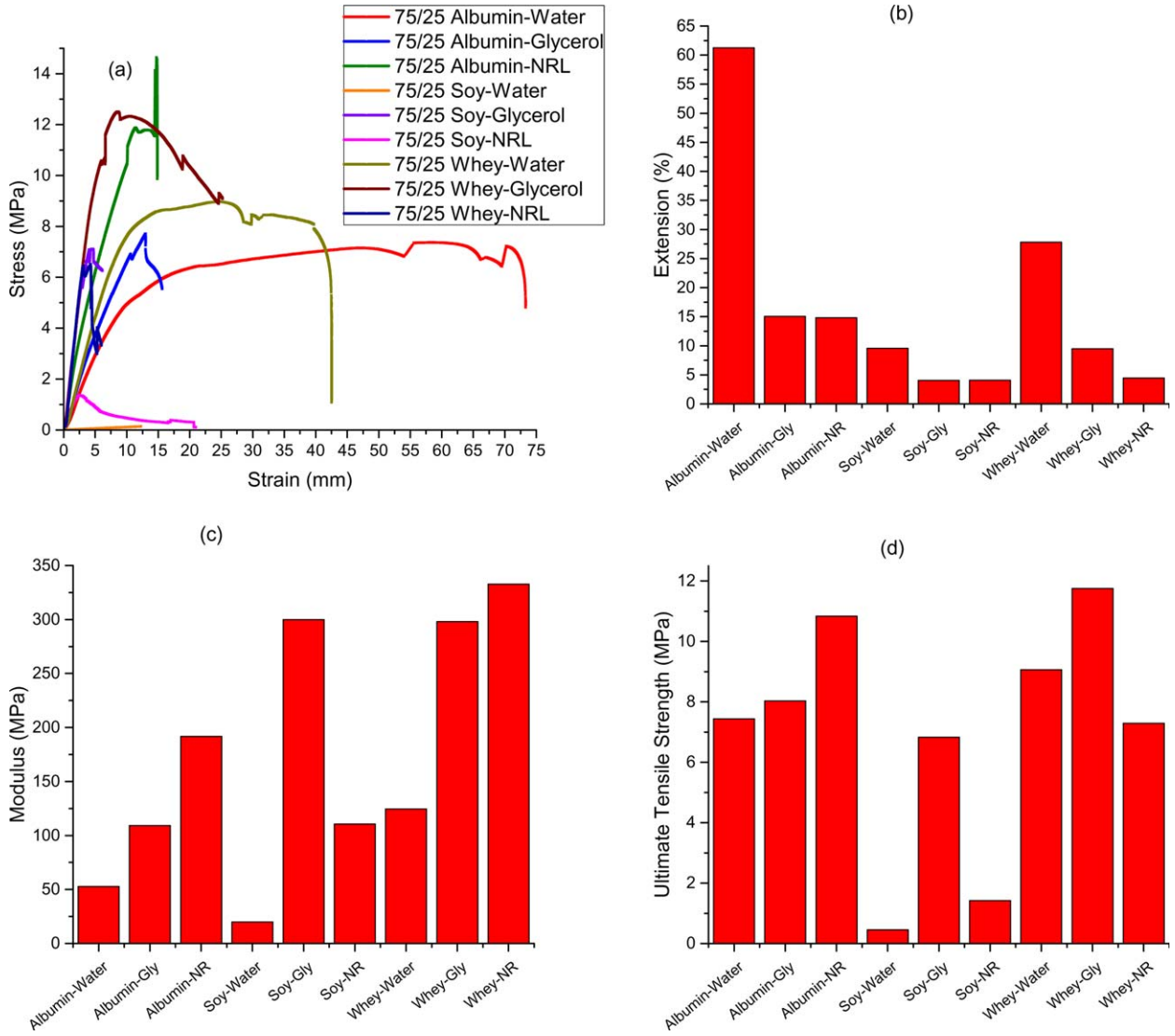

Figure 5. Tensile properties of optimal protein plastic blends: (a) stress–strain curves, (b) elongation, (c) modulus, and (d) ultimate tensile strength. Gly, glycerol; NRL, natural rubber latex. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and favoring the protein–plasticizer interaction, thereby producing flexibility in the resulting bioplastics. However, NRL seems less effective plasticizer for albumin or whey proteins as we see resulting bioplastics behaving more or less like stiff material with higher elastic modulus and lower tan δ values.

The soy–glycerol and soy–water plasticized plastics displayed the highest modulus and lowest initial tan δ, as well as the highest tan δ peak temperatures when compared to their counterpart proteins, albumin, and whey; these findings were consistent with the work by Zhang *et al.* Soy proteins possess strong intramolecular and intermolecular interactions, such as hydrogen bonding, dipole–dipole, charge–charge, and hydrophobic interactions, that promote stiffness or brittleness of soy plastics. Glycerol and water may be unable to break up intermolecular bonds to the same level as in whey- and albumin-based plastics.[26] However, the opposite was found for the soy–NRL plastics, as they possessed the highest initial tan δ values and lowest initial modulus, differing from the albumin–NRL and whey–NRL plastics [Figure 4(b)]. The possible explanation is that NRL-plasticized soy plastics had less dispersed rubber particles (or probably bigger phases of rubber particles), leading to a ductile material compared to NRL-plasticized whey and albumin plastics. These phenomena were also corroborated in the tensile performance as presented in the next section.

Tensile Testing. In terms of the amount of strain placed on the plastics, the albumin/water bioplastics were able to withstand the most strain by far, extending over 70% on average before a ductile break [Figure 5(a–d)]. When the plastics are compared based on protein content, the NRL-plasticized albumin bioplastics failed at the stress levels over 14 MPa, while the water- or glycerol-plasticized bioplastics failed near 8 MPa. These findings could have been due to increased hydrogen bonding that occurs during plasticization when plasticized with water or glycerol, while the NRL (because of more protein–plasticizer interaction) could serve as an additional load-bearing constituent in the plastic.[19]

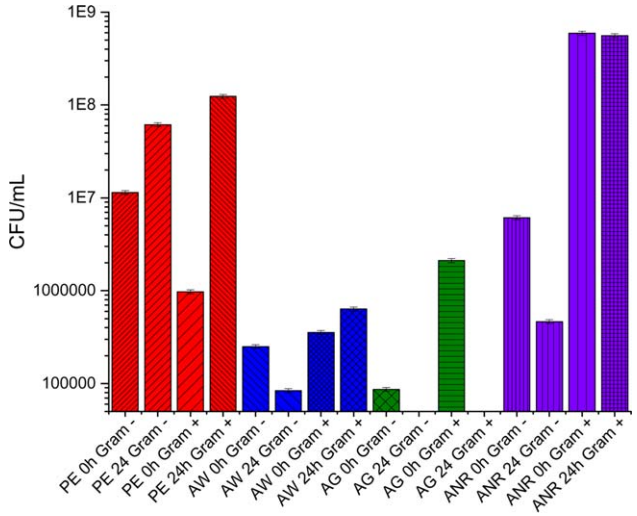

Figure 6. Antibacterial analysis of albumin protein plastic blends. PE, ultra-high-molecular-weight polyethylene; AW, 75/25 albumin–water; AG, 75/25 albumin–glycerol; ANR, 75/25 albumin–NRL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

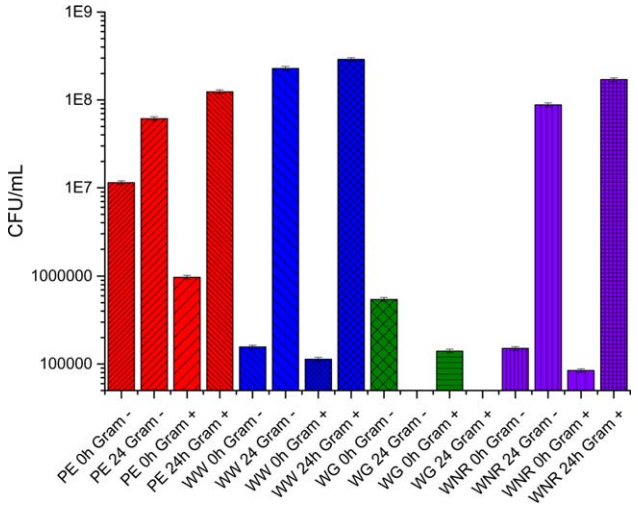

Figure 8. Antibacterial analysis of whey protein plastic blends. PE, ultra-high-molecular-weight polyethylene; WW, 75/25 whey–water; WG, 75/25 whey–glycerol; WNR, 75/25 whey–NRL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

For soy plastics, poor tensile properties were evident, as the plastic that was able to withstand the greatest amount of load (soy/glycerol) was only 7.5 MPa with brittle fracture, consistent with the findings made by Schilling *et al.*[27] This lack of ability for the soy plastics to undergo high stress or strain may be due to the soy protein lacking the ability to form a structure that possesses long-range orientation when plasticizers are utilized. As for the whey plastics, the whey plastics that have been plasticized with water performed similarly to the albumin/water plastics. The whey/water plastics were able to withstand only 27.5% of strain before breaking, but able to withstand over 8 MPa of stress. For whey protein it was found that when glycerol is used as a plasticizer, the plastic was able to withstand 12.5 MPa of stress and 9.8% of extension before failure, an extension that was similar to observe by McHugh and Krotcha.[28] When plasticized with NRL, the whey plastics possessed minimal tensile properties, as the protein may not be able to form a suitable structure during plasticization.

When the plastics are compared to each other based on elongation and modulus, we determined that the albumin plastics prepared with water possessed higher levels of elongation compared to any other plastic, but whey blended with NRL plastics possessed the highest modulus values [Figure 5(b)]. In comparison, the soy plastics possessed few tensile properties that would be comparable to the other proteins, as the modulus in the soy/glycerol plastics was the only tensile property that was similarly seen in other protein plastics.

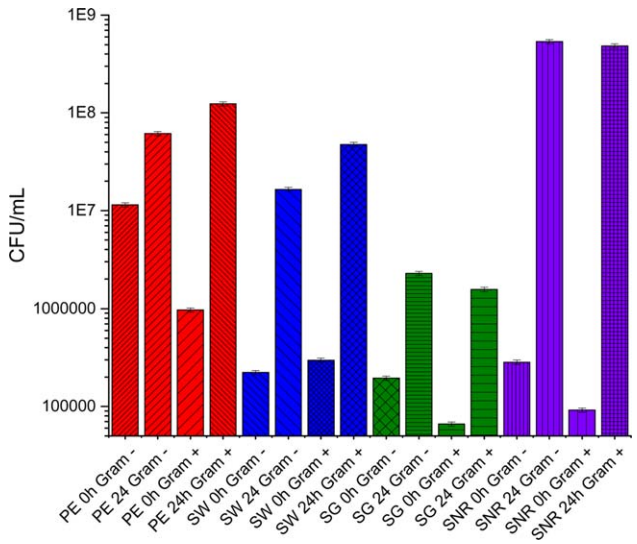

Figure 7. Antibacterial analysis of soy protein plastic blends. PE, ultra-high-molecular-weight polyethylene; SW, 75/25 soy–water; SG, 75/25 soy–glycerol; SNR, 75/25 soy–NRL. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

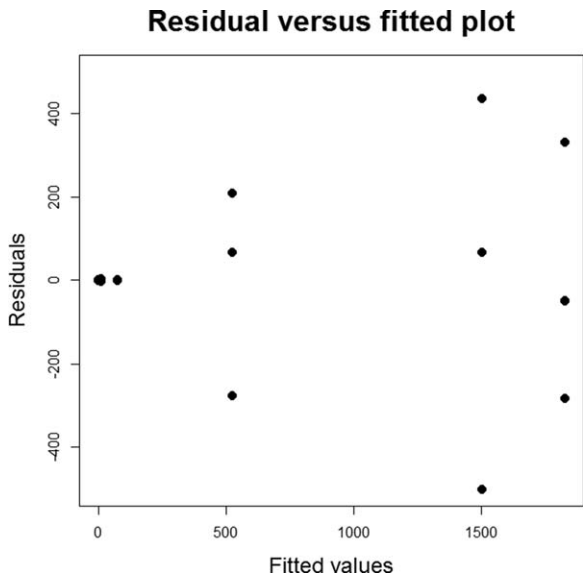

Figure 9. A residual versus fitted plot of the original Gram (−) data.

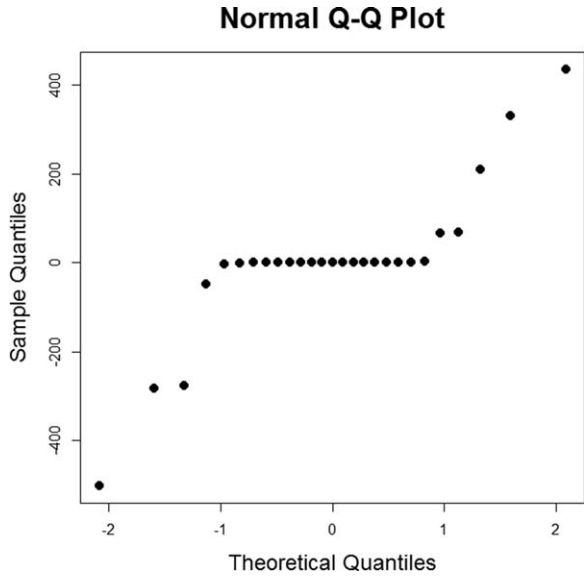

Figure 10. Normal Q-Q plot analysis of the original Gram (−) bacteria data.

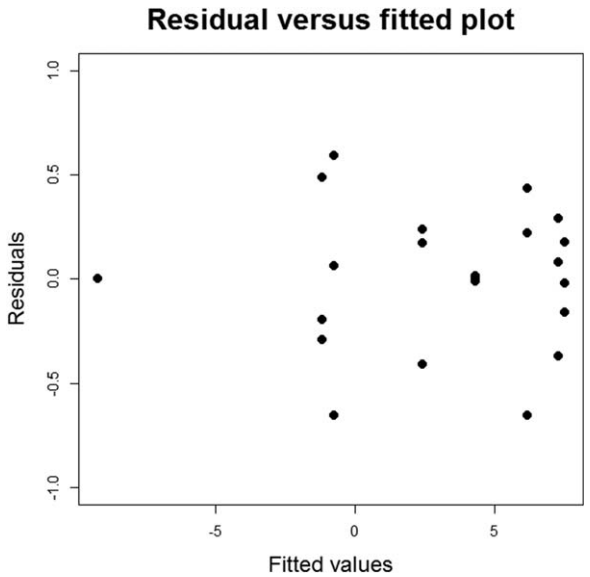

Figure 12. Residual versus fitted plot of Gram (−) when data is log-transformed.

Antibacterial Testing

Influence of Bioplastic Formulations. The above mentioned bioplastics produced using optimal level of various plasticizers were then evaluated for their antibacterial performance in comparison to a polyethylene (PE) control sample. For the polyethylene control samples a moderate level of growth (15.37%) by the Gram (−) and Gram (+) species was observed with a resulting CFU/mL value of 6.13×10^7 after 24 h (Figures 6–8). However, the result was statistically irrelevant at the 95% level, as neither the Gram (−) nor the Gram (+) contacted plastic samples possessed an α value <0.05. These findings were consistent with the analysis conducted by Seyfriedsberger *et al.*, as the promotion/inhibition of bacterial growth was marginal due to polyethylene not possessing any inherent properties to modify bacterial growth settings.[29]

In the albumin bioplastics, we found that the plastics made with plasticizers, water, and NRL showed similar properties, as each was able to reduce the amount of bacterial growth by both Gram (−) and Gram (+) bacteria (Figure 6). However, only the albumin plasticized by water was statistically significant in limiting Gram (−) bacterial growth at the 95% confidence level ($\alpha = 0.013$), as the albumin–water bioplastic decreased the CFU/mL level to 8.36×10^4 after 24 h of contact. The albumin–glycerol bioplastics in contrast possessed a strong inhibitive effect in antibacterial growth, as no growth occurred after 24 h [Gram (−) $\alpha = 0.002$, Gram (+) $\alpha = 0.004$]. This may be attributed to bioactive property of albumin due to lysozyme enzyme[30] plus the gradual leaching of glycerol from the plastic, as this creates an aqueous environment, preventing microbial adhesion and growth on the bioplastic. However, the glycerol leaching from

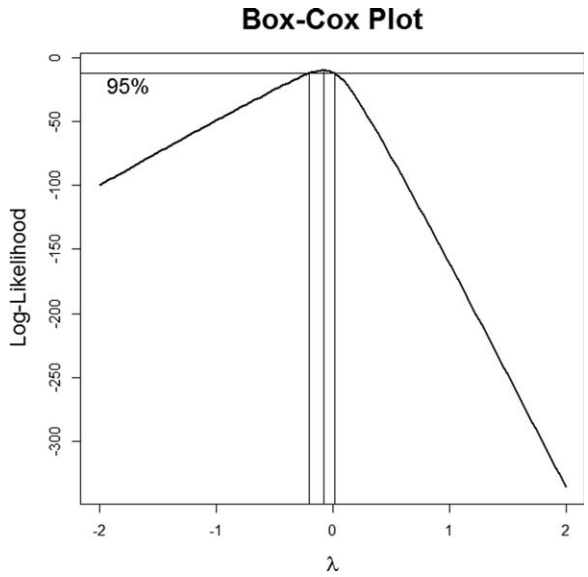

Figure 11. Box-Cox plot of the original data for the Gram (−) bacteria.

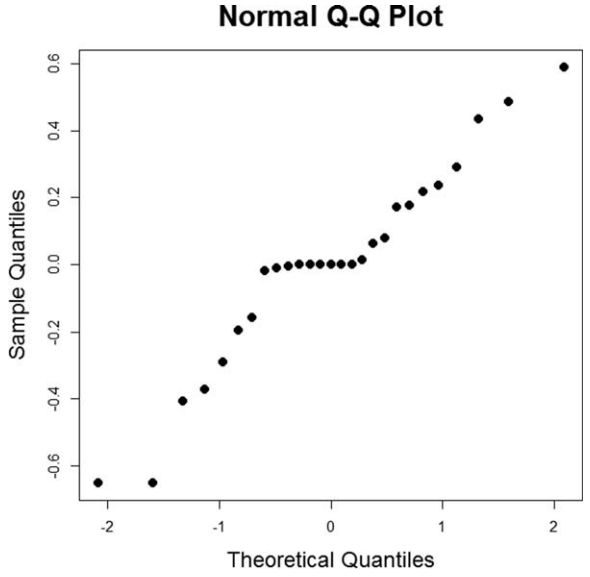

Figure 13. The normal Q-Q plot of Gram (−) data when log-transformed.

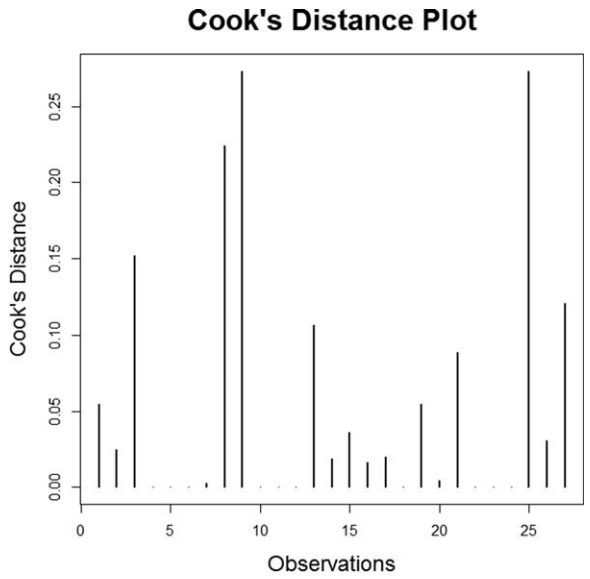

Figure 14. Cook's distance plot of Gram (−) data when log-transformed.

the plastic may only be bacteriostatic in nature, as concentrations of at least 28% of glycerol would be required for bacteriocidial properties.[31]

In the soy bioplastics, we found that none of the plastics were able to reduce the amount of bacterial growth by both Gram (−) and Gram (+) bacteria, as bacteria increased in growth after 24 h on the soy bioplastics (Figure 7). The soy plasticized by water was even statistically significant, as it promoted Gram (+) bacterial growth at the 99% confidence level ($\alpha = 0.008$), increasing the CFU/mL to 4.76×10^7. Of note is the soy bioplastics plasticized with glycerol, as overall lower rates of bacterial growth occurred in comparison to the soy plastics plasticized by water and NRL. We find these results were consistent with the findings of Padgett and coworkers, as they determined that soy bioplastics to be more suitable for an edible plastic application than antimicrobial application when bacterial inhibitors are not incorporated into the plastics.[30,32]

In the whey bioplastics, we found results were similar in relation to the soy bioplastics, as the plastics made with plasticizers, water, and natural rubber were unable to reduce the amount of bacterial growth by both Gram (−) and Gram (+) bacteria (Figure 8). Statistically the results were even more drastic, as the whey plastics promoted Gram (−) and Gram (+) bacterial growth at the 99% confidence level ($\alpha < 0.001$ for water-plasticized whey plastics, $\alpha < 0.002$ for natural rubber-plasticized whey plastics). However, the whey bioplastics were similar to the albumin bioplastics when plasticized with glycerol, as they possessed a strong inhibitive effect in antibacterial growth, as no growth occurred after 24 h [Gram (−) $\alpha = 0.002$, Gram (+) $\alpha = 0.019$]. This antibacterial activity may be attributed to certain peptides that are contained in the structure of whey protein, as the three peptides of secretory leukocyte protease inhibitor, trappin-2, and elafin have been found to possess antimicrobial activity.[33] Like in the albumin–glycerol bioplastic, this also may be due to the gradual leaching of glycerol from the plastic in the creation of an aqueous environment.

Table II. Estimated Values of Regression Coefficients for Some Parameters of Model (1) for Gram (−) Bacteria

Coefficients	Estimate	Std. error	*t* Value	*P* value
Soy (α_2)	5.50	0.29	18.7	3.04E−13
Whey (α_3)	8.45	0.29	28.8	<2E−16
Glycerol (β_2)	−8.03	0.29	−27.3	4.18E−16
NRL (β_3)	0.42	0.29	1.4	0.16737

Statistical Analysis of Antibacterial Property of Bioplastics. For the statistical analysis, response was the proportional change in count after 24 h:

$$y = \frac{\text{Count at 24 h} - \text{Count at 0 h}}{\text{Count at 0 h}} = \frac{\text{Count at 24 h}}{\text{Count at 0 h}} - 1$$

Mathematically, it was the same as considering $y = \frac{\text{Count at 24 h}}{\text{Count at 0 h}}$. We fit a linear regression model, separately for Gram (+) and Gram (−) bacteria, for the two-way layout given by

$$y_{ijk} = \eta + \alpha_i + \beta_j + \omega_{ij} + \varepsilon_{ijk} \quad (1)$$

for $i = 1$ (albumin), 2 (soy), 3 (whey); $j = 1$ (water), 2 (glycerol), 3 (NRL) and $k = 1, 2, 3$ were the three samples taken. Here, y_{ijk} was the response corresponding to the kth sample with the ith level of protein and the jth level of plasticizer. Note that in our model we have $1 + 3 + 3 + 9 = 16$ parameters (η, α_1, α_2, α_3, β_1, etc.). Here, α_i and β_j were the main effects of protein and plasticizer, respectively, and ω_{ij} was the protein–plasticizer two-factor interaction effect. The term "main effect of protein" means the effect of the individual protein (albumin, soy, or whey) irrespective of the effect of plasticizer. Similar interpretation is given for "main effect of plasticizer," which means the effect of the individual plasticizer (water, glycerol, or NRL) irrespective of the effect of the protein. Moreover, ω_{ij} term denotes the individual protein–plasticizer effects. For example, ω_{11}

Table I. Two-Way Analysis of Variance Corresponding to Model (1) for Gram (−) Bacteria

	df	Sum sq	Mean sq	*F* value	*P* value
Protein	2	327.2	163.58	1261.9	<2E−16
Plasticizer	2	513.2	256.61	1979.6	<2E−16
Protein × plasticizer	4	171.9	42.98	331.6	<2E−16
Residuals	18	2.3	0.13		

Table III. Two-Way Analysis of Variance Corresponding to Model (1) for Gram (+) Bacteria

	df	Sum sq	Mean sq	*F* value	*P* value
Protein	2	311.2	155.6	766.5	<2E−16
Plasticizer	2	635.8	317.9	1565.8	<2E−16
Protein × plasticizer	4	193.1	48.3	237.7	2.59E−15
Residuals	18	3.7	0.2		

Table IV. Estimated Values of Regression Coefficients for Some Parameters of Model (1) for Gram (+) Bacteria

Coefficients	Estimate	Std. error	*t* Value	*P* value
Soy (α_2)	4.81	0.37	13.1	1.23E−10
Whey (α_3)	7.59	0.37	20.6	5.59E−14
Glycerol (β_2)	−9.46	0.37	−25.7	1.21E−15
Rubber (β_3)	0.76	0.37	2	0.0543

represents the albumin–water interaction, and ω_{12} represents albumin–glycerol interaction. However, this model is overparamatized, so not all parameter values can be estimated uniquely. In order to overcome this problem, standard baseline constraints have been used.[34] In particular, we took $\alpha_1 = 0$ and $\beta_1 = 0$ so that albumin and water can be considered as baselines for comparison. The errors ϵ_{ijk} were assumed to be normal (Gaussian), identically and independently distributed with zero mean and some constant variance σ^2.

For both Gram (+) and Gram (−) datasets, after we fit the model, the residual versus fitted plot showed a clear "fanning out" pattern and the normal probability plot indicates departure from normality of errors. We considered the Box–Cox transformation and the corresponding plots (see Figure 11) indicated that the likelihood is maximized around $\lambda = 0$ suggesting the log transformation. Here we considered the response as $y + 10^{-4}$, that small positive term was added to make all the responses positive. After taking the log transformation, the improvements of the residual versus fitted plot and the normal probability plot were very apparent. Also the Cook's distances for the log-transformed data indicated that there were no influential points (see Figure 14), and the assumptions of linear regression could be considered to be satisfactorily met.

Gram-Negative Bacteria. The ANOVA table (given in Table I) illustrated that all the main effects of protein, plasticizer, and protein–plasticizer two-factor interactions were strongly significant. The multiple R^2 for this model is 99.77%, indicating a good fit. In the regression fit, it is customary to consider baseline constraints which assumes the coefficients corresponding to Water and Albumin to be 0 (in other words, $\alpha_1 = 0$ and $\beta_1 = 0$). With respect to that, the coefficients of others (along with their *P*-values) are given in Table II. First, we note that the *P*-values of all the regression coefficients mentioned in Table II (except rubber) were very small and statistically significant. The estimate of the coefficients for soy (β_2) and whey (β_3) were 5.5 and 8.5, respectively, indicating albumin bioplastics showed fewer numbers of colonies as the coefficient of albumin (α_1) is set to 0, and that is smaller than both 5.5 and 8.5, consistent with the findings of Peters and Padgett.[30,35] Similarly, the estimate of coefficient of glycerol (β_2) is negative, which confirms that it prevents the growth of colonies significantly.

Gram-Positive Bacteria. The ANOVA table (given in Table III) illustrated that all the main effects of protein and plasticizer, as well as the protein–plasticizer two-factor interactions were strongly significant. The multiple R^2 for this model was 99.68%, indicating a good fit. The other results for Gram (+) bacteria were similar to those of Gram (−) bacteria (see also Table IV).

CONCLUSIONS

When comparing the thermal properties of the proteins, we found that the proteins had similar degradation rates, with soy and whey occurring at temperatures between 50 and 60°C lower than albumin. In terms of the viscoelastic properties, the albumin and whey exhibited similar properties based on the plasticizer used, while soy plastics exhibited a greater range of properties based on the plasticizer. As for antibacterial properties, we found that plasticizing either albumin or whey with glycerol produced the bioplastic with the strongest antibacterial properties. In terms of the statistical analysis, we found that the key determinant of antibacterial properties of a given bioplastic is the protein and plasticizer. With the knowledge gained in this study, there are different areas of interest that could be further studied. To determine if albumin or whey plastics could be utilized in medical settings, various testing would have to be conducted based on the intended end use in areas such as packaging medical products (ASTM F2097 – 10: Standard Guide for Design and Evaluation of Primary Flexible Packaging for Medical Products), as well as infection testing for medical applications [ASTM F813 – 07(2012): Standard Practice for Direct Contact Cell Culture Evaluation of Materials for Medical Devices]. Drug elution analysis would serve as another major area of interest, as the application of drugs over a period of time would be useful in treating patients in numerous settings. For food packaging applications, the testing of water and oxygen vapor permeability properties of the plastics would be crucial to determine, as these properties would determine whether they would be suitable for such applications. The further addition of different materials to the bioplastic blends, as well as the examination of other proteins would also be useful to examine, as it would serve to determine what blends and materials should be used to produce a bioplastic with the best combination of properties based on the application.

REFERENCES

1. Peleg, A. Y.; Hooper, D. C. *New Engl. J. Med.* **2010**, *362*, 1804.
2. Gould, I. M. *Int. J. Antimicrob. Agents* **2006**, *28*, 379.
3. Espert, A.; Vilaplana, F.; Karlsson, S. *Composites: Part A* **2004**, *35*, 1267.
4. Lau, O.-W.; Wong, S.-K. *J. Chromatogr. A* **2000**, *882*, 255.
5. Sharma, S.; Hodges, J.; Luzinov, I. *J. Appl. Polym. Sci.* **2008**, *110*, 459.
6. Paetau, I.; Chen, C.-Z.; Jane, J. *Ind. Eng. Chem. Res.* **1994**, *33*, 1821.
7. Gounga, M. E.; Xu, S.; Wang, Z. *J. Food Eng.* **2007**, *83*, 521.
8. Sothornvit, R.; Olsen, C. W.; McHugh, T. H.; Krochta, J. M. *J. Food Sci.* **2003**, *68*, 1985.
9. Qiu, Y.; Zhang, N.; An, Y. H.; Wen, X. *Int. J. Artif. Organs* **2007**, *30*, 828.
10. Baron, F.; Réhault, S. In Bioactive Egg Compounds; Anton, M., López-Fandiño, R., Huopalahti, R., Schade, R., Eds.; Springer-Verlag: Berlin/Heidelberg, **2007**; p 191.
11. Yalcin, A. S. *Curr. Pharm. Des.* **2006**, *12*, 1637.
12. Sivarooban, T.; Hettiarachchy, N. S.; Johnson, M. G. *Food Res. Int.* **2008**, *41*, 781.

13. Martínez, I.; Partal, P.; García-Morales, M.; Guerrero, A.; Gallegos, C. *J. Food Eng.* **2013**, *117*, 247.
14. Page, K.; Wilson, M.; Parkin, I. P. *J. Mater. Chem.* **2009**, *19*, 3819.
15. Tarachiwin, L.; Sakdapipanich, J. T.; Tanaka, Y. *Rubber Chem. Technol.* **2005**, *78*, 694.
16. Sue, H. J.; Wang, S.; Lane, J. L. *Polymer* **1997**, *38*, 5035.
17. Menard, K. P. Dynamic Mechanical Analysis: A Practical Introduction; CRC Press: Boca Raton, FL, **1999**.
18. Fried, J. Polymer Science & Technology, 2nd ed.; Prentice Hall: Upper Saddle River, NJ, **2003**.
19. Jones, A.; Zeller, M. A.; Sharma, S. *Prog. Biomater.* **2013**, *2*, 1.
20. Sharma, S.; Luzinov, I. *J. Polym. Environ.* **2012**, *20*, 681.
21. Rahmana, W. A.; Sina, L. T.; Rahmata, A. R.; Samadb, A. A. *Carbohydr. Polym.* **2010**, *81*, 805.
22. Rao, V.; Johns, J. *J. Thermal Anal. Calorim.* **2008**, *92*, 801.
23. Robertson, N.-L. M.; Nychka, J. A.; Alemaskin, K.; Wolodko, J. D. *J. Appl. Polym. Sci.* **2013**, *130*, 969.
24. Lunt, J.; Shafer, A. L. Polylactic Acid Polymers from Corn: Applications in the Textiles Industry, Cargill Dow Polymers, LLC, **2001**.
25. Galietta, G.; Gioia, L. D.; Guilbert, S.; Cuq, B. *J. Dairy Sci.* **1998**, *81*, 3123.
26. Zhang, J.; Mungara, P.; Jane, J. *Polymer* **2001**, *42*, 2569.
27. Schilling, C. H.; Babcock, T.; Wang, S.; Jane, J. *J. Mater. Res.* **1995**, *10*, 2197.
28. McHugh, T. H.; Krochta, J. M. *J. Agric. Food Chem.* **1994**, *42*, 841.
29. Seyfriedsberger, G.; Rametsteiner, K.; Kern, W. *Eur. Polym. J.* **2006**, *42*, 3383.
30. Han, I. Y.; Padgett, T.; Dawson, P. L. *J. Food Protect.* **1998**, *61*, 1330.
31. Werkman, C. H. In Glycerol; Miner, C. S., Dalton, N. N., Eds.; American Chemical Society: New York, **1953**; p 397.
32. Cha, D. S.; Chinnan, M. S. *Crit. Rev. Food Sci. Nutr.* **2004**, *44*, 223.
33. Wiesner, J.; Vilcinskas, A. *Virulence* **2010**, *1*, 440.
34. Wu, C. F. J.; Hamada, M. S. Experiments: Planning, Analysis, and Optimization, 2nd ed.; Wiley: Hoboken, NJ, **2009**.
35. Peters, T., Jr. All About Albumin: Biochemistry, Genetics, and Medical Applications; Academic Press: San Diego, CA, **1996**.