JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 5393−5399 **5393**

Blends of Cysteine-Containing Proteins

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Proteins such as keratin, lactalbumin, and gluten can be obtained from agricultural sources. These proteins contain the amino acid cysteine. Cysteine allows for the formation of inter- and intramolecular sulfur−sulfur bonds. It was found that cysteine-containing proteins have varied properties and can be blended together to form materials with the attributes of each polymer. The addition of wheat gluten to other proteins increases the strain to break or "toughness". The addition of lactalbumin increases the modulus and strength of blends. Birefringence shows that lactalbumin contains an added "structure" not found in the other proteins. Permeability studies reveal that one protein may dominate the transport of small molecules through the blend. Scanning electron microscopy shows that blends contain features of each protein and correlate with observed tensile properties.

KEYWORDS: Proteins; mechanical properties; permeability; birefringence

INTRODUCTION

There has been much interest over the years in utilizing agriculturally derived polymers in commodity plastics applications such as packaging. Agriculturally derived polymers would be sustainable and typically biodegradable and therefore advantageous over petroleum-derived polymers. There is much literature on proteins and carbohydrates derived from plants and animals and polyesters made from the fermentation of plant-derived feedstocks. The focus here is on proteins, which can be derived inexpensively from agricultural sources and have much versatility because of the various amino acid sequences found in nature. Naturally derived proteins from gelatin (*1*), soybean (*2*−*6*), wheat (*7*−*14*), sunflower (*15*−*17*), corn (*18*−*23*), fish (*24*), milk (*15*−*27*), peanut (*28*), wool (*29*−*31*), and poultry feathers (*32*−*34*) have been processed into polymeric materials using a variety of techniques. These techniques included solvent-cast and thermally processed materials. Solvent casting is tedious, and the use of volatile organic compounds would defeat the purpose of environmental friendliness. Thermal processing is simpler, and the method is currently embraced by the polymer industry. If proteins from sustainable resources are to be used commercially, the proteins have to be processed through preferred processing methods. Proteins contain a fair amount of hydrogen-bound water. The bound water stabilizes the protein structure (*35*). Loss of that water at high temperatures will destabilize the protein structure and cause a rearrangement or "denature" the protein. Therefore, proteins are temperature sensitive and require thermal processing at reduced temperatures. The use of plasticizers, such as glycerol, poly(ethylene glycol), propylene glycol, or sorbitol, can lower thermal transition temperatures to acceptable levels. There is also evidence that plasticizers play a similar role as water in stabilizing the protein (*34*).

Gluten is an amorphous protein obtained from wheat with properties described in **Table 1**. Wheat gluten (WG) can contain gliadin (M_w = 40000 g/mol) and glutenein (M_w = 40000−100000 g/mol) (*36*). Lactalbumin (LA) is a semicrystalline protein derived from whey following milk processing (*37*). Keratin is a semicrystalline protein found in hair, nails, hooves, horns, and feathers (*38*−*41*). Poultry feather keratin (FK) is used here. There are potentially billions of pounds of these proteins available for commodity polymer applications. However, total worldwide sales of commodity polymers are still several orders of magnitude larger than the amount of these proteins that are available. Therefore, the proteins listed could only serve a niche market at best. In addition, each protein has advantages and disadvantages that can be optimized through blending, a method routinely used by the synthetic polymer industry to optimize properties without costly chemical routes.

All of the proteins studied contain the amino acid cysteine. Cysteine (C) is a sulfur-containing amino acid and can form sulfur−sulfur (S−S) cystine bonds with other intra- or intermolecular cysteine molecules. Intermolecular cystine bonds are referred to as "cross-links". The cross-links plus other protein structural features, like crystallinity and hydrogen bonding, usually give the protein high strength and stiffness (*35*). The amount of cysteine varies in each protein as shown in **Table 1**. Little is known about how much intermolecular vs intramolecular cystine bonding exists in each protein. However, some indirect observations have been made. For example, FK is difficult to process without the use of reducing agents to reduce cystine bonds. Therefore, FK probably contains a lot of intermolecular cystine bonding forming a network structure. A

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10.1021/jf053238l
Published on Web 06/23/2006

Table 1. Protein Properties

protein	mol. wt (g/mol)	% cysteine	melt temp (°C)
FK[a]	10500	7.3	238
LA[b]	14174	5.8	232
WG[c]	40000–100000	2.5	167 (T_g)

[a] Refs *38–41.* [b] Ref *36.* [c] Ref *37.*

network structure can be defined as one where "more than two functional groups per molecule are present" so that "cross-linking can take place with the formation of network structures where a branch of one molecule attaches to another molecule" (*42*). In this case, the amount of cysteine is assumed to be the functionality, f, and all proteins have $f > 2$. In contrast, WG contains enough cysteine to theoretically form a network structure but can easily be processed without reducing agents. In literature on WG and bread dough, cystine bonding seems to result in a high molecular weight fraction of the glutenin fraction of gluten giving dough its elasticity (*36*, *43*). Redox reagents, most notably sodium sulfite, have been used successfully to reduce cystine bonds to make proteins with high levels of cysteine easily processable (*15*, *44*).

In this study, WG, LA, and FK were blended in various ratios in the presence of glycerol and sodium sulfite to aid processing. In the polymer industry, blending is viewed as a simple and cost-effective way to elicit new material properties without expensive new polymerization routines. The tensile properties and permeability of the blends were characterized as well as structural features shown through birefringence and scanning electron microscopy (SEM) analysis.

MATERIALS AND METHODS

Proteins. FK was obtained from Featherfiber Corp. (Nixa, MO) and ground and fractionated according to a procedure described previously (*34*). The FK was a combination of chopped quill and feather fibers. It was shown previously using Raman spectroscopy that the feather fiber fraction was 41% α-helix and 38% β-sheet protein structures with the balance being disordered structures while the quill fraction was 21% α-helix and 50% β-sheet with the balance being disordered structures (*45*). WG and LA were obtained in powder form from Aldrich Chemical.

Preparation of Blends and Sheets. Blends of 60:30:8:2 wt % dry protein powder:glycerol:deionized water:sodium sulfite were mixed on a Brabender mixing head. Mixing proceeded at 40 °C and 50 rpm for 15 min. The total material occupied 70% of the volume of the mixer. For mixtures of proteins, the stated ratio of one protein to another referred to the amount in the protein fraction so that a "50:50 wt % blend of FK to WG" was 30:30:30:8:2 wt % FK:WG:glycerol:deionized water:sodium sulfite in the total mixture. Following mixing, 5 g of sample was sandwiched between Teflon-coated aluminum foil and pressed into sheets in a Carver Press Autofour/30 model 4394 at 140 °C and 88964 N for 2 min.

Mechanical Testing of Sheets. Test samples were prepared according to ASTM D882 for thin plastic sheets. The samples were 2.54 cm wide by 10.16 cm long, and a 5.08 cm gauge length was employed. All sheets were of similar thickness of 0.040 ± 0.005 cm. All sheets were measured with a Fisher Scientific Electronic Digital Caliper and were the result of five measurements of each dimension. Mechanical testing of the sheets was performed at a crosshead speed of 2.54 cm/min using a Com-Ten Industries 95 RC Test System. A minimum of 15 sheets were tested, and results were reported as average values with standard deviations given. Sheets were tested immediately after preparation at ambient conditions of 21 °C and 50% relative humidity (RH).

Birefringence. During tensile testing, sheets were observed between crossed polarizers oriented at 45 °C to the tensile axis. A white light source allowed for the observance of color change in the sample as a function of applied tensile strain. The strained polymer sample showed a change in its refractive index indicative of anisotropy in the polymer molecules. The "stress-optical law" phenomenologically described this through $\Delta n = C\sigma$, where Δn was the change in refractive index, σ was the stress, and C was a constant known as the "stress-optical coefficient" (*46*). The refractive index change manifested as a color change in the sample. Color was converted to retardance using a Michel-Levy chart (*47*). Retardance, R, was related to birefringence according to $\Delta n = R/h$, where h was sheet thickness. As the sheet was strained in tension, its thickness changed. Poisson's ratio, μ, was determined by $\mu = \Delta h/h\epsilon$, where ϵ was strain. So, the expression for Poisson's ratio related sheet thickness to strain and accurate stress-optical coefficients could be found by taking into account the change in sheet thickness. In all cases, Poisson's ratio was found to be $\mu \sim 0.3$.

Permeability Testing of Sheets. Oxygen transmission rates (OTRs) of sheets were measured following ASTM D3985-95. Sheet samples 6 cm by 6 cm were cut for oxygen permeability (OP) testing, and the average thickness was calculated from three measurements of each sample. OTRs were determined with an Oxtran 2/20 (Mocon Inc., Minneapolis, MN). Samples were placed between two stainless steel plates with an opening 5 cm in diameter. Testing conditions for both the nitrogen and the oxygen were 50% humidity at 25 °C. The test gas was set to 100% oxygen. OP was calculated by multiplying the measured OTR of each sample by its average thickness.

Following the ASTM E96-95, the water vapor transmission rate (WVTR) was measured for each sheet and used to calculate the water vapor permeability (WVP). Samples of sheets were cut into 6 cm by 6 cm pieces, and the average sheet thickness was calculated from three measurements using a digital caliper. Sheet samples were placed inside permeability cups with a testing area 5 cm in diameter. A test solution of distilled−deionized water was placed inside the cup to form a high RH environment (80−100% RH). To promote mass transfer through the sheets, the permeability cups were placed in a chamber with 0% RH (Drierite) with air circulation. Sheets made from agricultural proteins were only moderate moisture barriers, so a humidity gradient formed inside the permeability testing cups between the surface of the test solution and the underside of the sheets. A modification to the ASTM method described by McHugh et al. (*48*) accounted for this gradient in the calculation of WVP. The initial weight of each cup was taken, and then, the weight was measured at intervals that were at least 3 h long over a period of 24 h. Linear regression of time and weight data were used to calculate WVTR.

SEM. The fracture surfaces were excised from the failed tensile samples using a scalpel blade and transferred into a modified specimen carrier. The specimen carrier was known as an "indium vise" because the dissected pieces were clamped between sheets of indium metal and plunge-cooled in liquid nitrogen to −196 °C. The cooled holder was then transferred to an Oxford CT1500 HF cryopreparation system attached to a Hitachi S-4100 scanning electron microscope. The sample temperature was raised to −90 °C for 10 min to remove surface water from the sample surface. The sample was then cooled to below −120 °C and coated with approximately 5 nm of platinum metal using a magnetron sputter coater. Coated samples were transferred to the cold stage in the SEM at −170 °C and observed with an electron beam accelerating voltage of 2 kV.

RESULTS AND DISCUSSION

Mechanical Properties and Birefringence. Figure 1 shows data for FK, WG, and a 50:50 blend. A blend of FK and WG represented a blend of a lower molecular weight semicrystalline protein with a higher molecular weight amorphous protein. The addition of WG decreases the modulus, E, and the strength to break, σ_b, but increases the strain to break, ϵ_b. In this blend, the amorphous WG adds "toughness" to the blend but at the expense of stiffness and strength. The lines through the data represent a first-order polynomial fit to the data and show that the properties behave linearly with the addition of the second phase such that a "rule of mixtures" could be used. Mangavel et al. (*7*) saw slightly lower stress at break and slightly higher strain at break

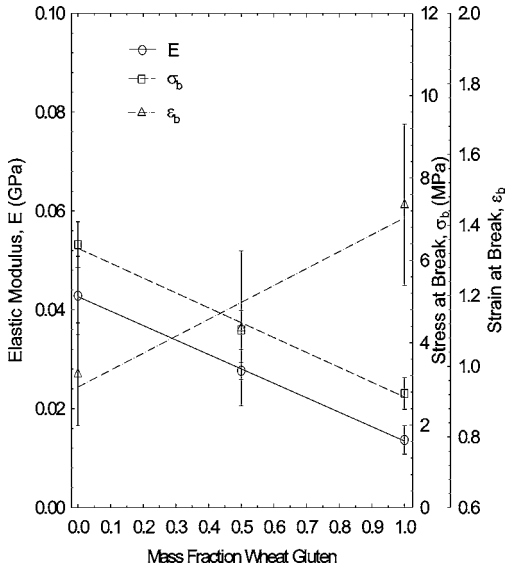

Figure 1. Tensile properties of FK, WG, and blends tested at 2.54 cm/min.

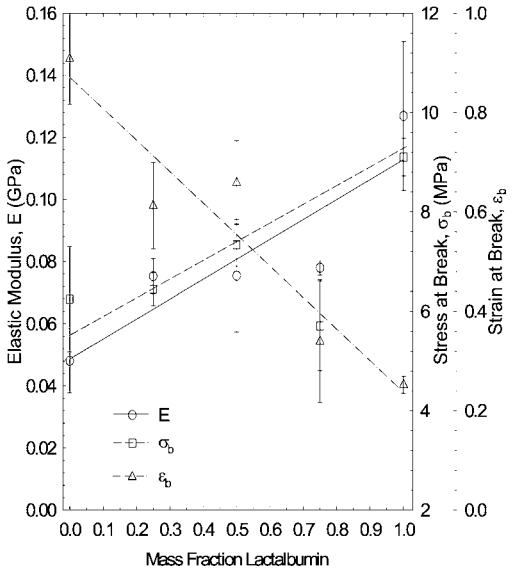

Figure 2. Tensile properties of FK, LA, and blends tested at 2.54 cm/min.

for WG films at similar glycerol loading as compared to results presented here. However, these polymers were cast from solution; therefore, the difference in properties most likely originates in the different processing routes. Irissin-Mangata et al. (*9*) made similar observations to Mangavel et al. on the tensile stress of WG but observed very low strains to break, even at high RH. Again, a solution process was used and was probably the cause of the difference. Clearly, polymer preparation methods for proteins are critical to final performance. Thermal processing of WG, while commercially preferred, may also give preferential cross-linking reactions resulting in the lower strain to break observed here over the results of Mangavel et al. (*10*).

Figure 2 shows the physical properties of FK, LA, and blends, which represented blends of lower molecular weight, semicrystalline proteins. Again, a simple rule of mixtures law applied for the blending of FK and LA.

Figure 3 shows representative stress−strain curves for WG, FK, and 50:50 wt % blends. Retardance data are also shown. All of the samples were of the same thickness so retardance can be viewed directly as birefringence. What was observed

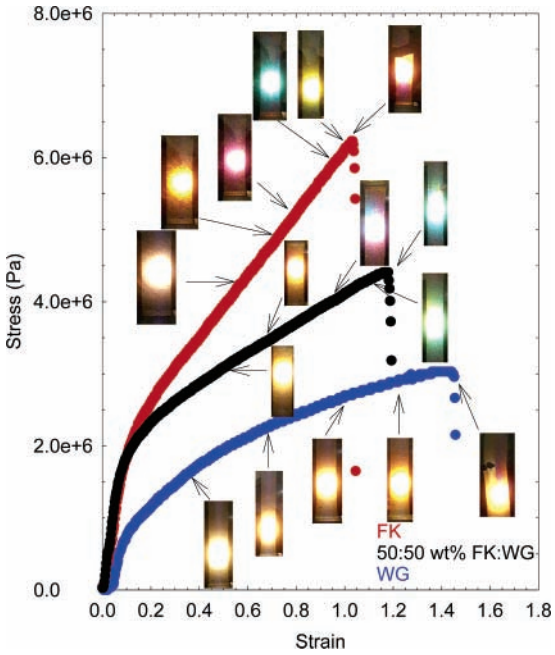

Figure 3. Representative stress–strain curves of FK, WG, and a 50:50 wt % blend with concurrent retardance images.

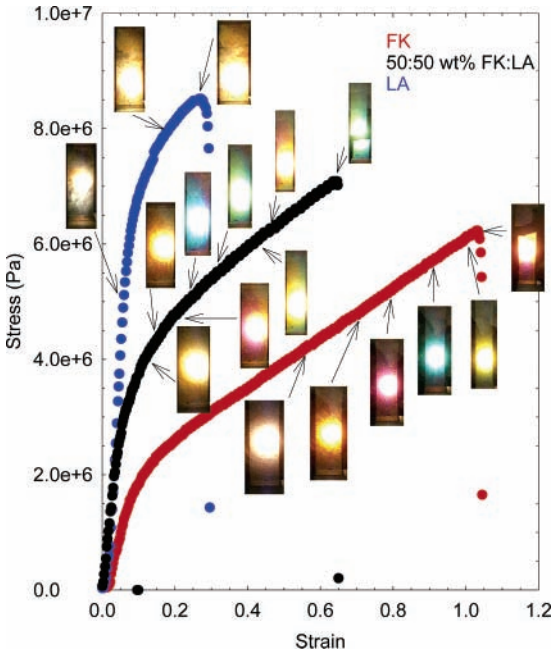

Figure 4. Representative stress–strain curves of FK, LA, and a 50:50 wt % blend with concurrent retardance images.

was that the FK sample went through two orders of retardation prior to breaking whereas the WG sample did not go through one order. So FK was more oriented than WG at break, but more importantly, FK was more oriented than WG at any value of strain. The blend showed an intermediate behavior with retardance going slightly over one order. There was then some load borne by the FK with WG contributing to the increased strain to break.

Figure 4 illustrates stress−strain curves for FK, LA, and a 50:50 wt % blend with concurrent birefringence. A different phenomenon was observed over the FK−WG behavior. LA showed a minimal increase in retardance with increased applied strain. However, a blend of LA and FK went through two orders of retardance.

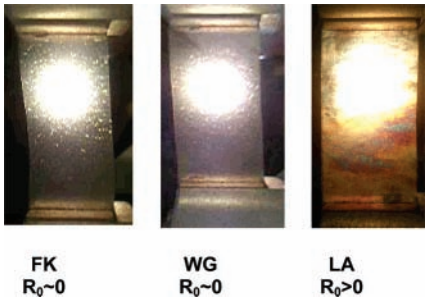

Figure 5. Images of initial retardance states, R_0, of FK, WG, and LA tensile test films.

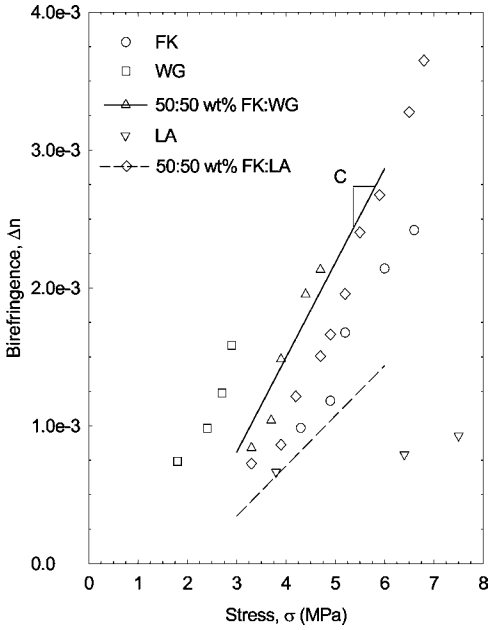

Figure 6. Calculated birefringence, Δn, as a function of generated tensile stress, σ. The slope of the line is the stress-optical coefficient, C.

Figure 5 shows the beginning states of retardance prior to the application of tensile strain. FK and WG contained no intrinsic retardance prior to testing while LA contained an intrinsic level of retardance indicative of molecular anisotropy. The source of the intrinsic molecular anisotropy in the LA could be polymer chain orientation induced during processing. However, all of the samples were prepared similarly so it would be expected that all would show some intrinsic level of retardance if this were the case. LA appeared to have an intrinsic network structure formed after processing that confined the chains to a certain anisotropic conformation that further restricted chain stretching to a finite point that was much lower than the other two proteins. Large crystalline domains macroscopically visible in the initial birefringence may exist in the LA, which would correlate with the higher modulus observed over FK, a higher cysteine content protein, and be equally capable of forming cross-links. Crystals would trap portions of the polymer chains in extended conformations and limit extensibility.

Figure 6 depicts the relationship between birefringence, calculated from retardance values, and tensile stress generated in the FK−WG systems and the FK−LA systems. Some of the correlations do not go through the zero point, which may lie in the method to calculate birefringence. Instead of using actual intensity values, color was interpreted into retardance and then birefringence. Errors may arise in arriving at actual birefringence based on how color is defined between the observed retardance and the Michel-Levy chart. However, differences were observed between the samples that make comparisons interesting. The birefringence values for FK and WG were fit to first-order polynomials, and then, a "rule of mixtures" was applied to the 50:50 wt % FK:WG blend such that $\Delta n_{blend} = 0.5(\text{FK fit}) + 0.5(\text{WG fit})$ to yield the solid line in **Figure 6**, which shows a reasonable fit to the experimental data ($r^2 = 0.9967$). This analysis also shows that perhaps there is an intimate interaction between the WG and the FK with each contributing partially to the mechanical behavior and birefringence. Applying the same analysis to the 50:50 wt % FK:LA system does not yield the same result as depicted by the dashed line in **Figure 6**. The birefringence of the FK:LA blend was dominated by the FK sample. This may mean that the presence of FK disrupts the crystalline structure formed in pure LA sheets as no initial retardance was observed for the blend and allowed for more extensibility. The higher extensibility after loss of crystallinity would give higher birefringence as a function of applied stress, which was observed.

A simple comparison of the chain statistics of the different proteins, by no means rigorous, may provide a clue to the observed birefringence behavior. The Kuhn statistical length, $b_k = R_e/L$, is used to define the stiffness of a freely jointed polymer chain, where R_e is the mean square end to end distance and L is the maximum length of the end to end vector or the length of a fully extended chain (*46*, *49*). Birefringence measures molecular anisotropy or deviation of R_e from its most entropic state. So, $\Delta n \propto b_k$ or birefringence is developed as R_e approaches L. The freely jointed chain is made up of n segments of length l. The Kuhn statistical length is related to n, which is directly related to molecular weight, $b_k = n^{-1/2}$. Using molecular weight values for n, the mean square end to end distance of WG chains is 0.38% of L while the mean square end to end distance of FK chains is 0.98% of L. For WG, an intermediate molecular weight of 70000 g/mol was used. When each protein was equally strained, R_e was moving closer to L but FK was more oriented relative to its maximum end to end distance L. This resulted in higher levels of molecular orientation and therefore higher retardance or birefringence values. The higher orientation of FK molecules was probably also a contributing factor in the higher observed modulus values because as the FK molecule was more strained, the higher observed modulus and strength were more reflective of covalent bond strain.

For LA, R_e would be 0.83% of L. If motion in the chain were restricted by crystals, then L would be decreased to some fraction of its value based solely on molecular weight. If the reduced value of the maximum end to end vector is L', then the ratio of R_e/L' would be closer to 1 than R_e/L for LA resulting in the observed initial birefringence. In addition, because it was closer to 1, there was not much room for development of birefringence in the sample, which was observed. **Figure 6** may reveal information about the crystalline state of the proteins and possibly the molecular weights of the proteins. At a given level of stress, WG would achieve the highest birefringence, correlating with the much higher molecular weight and, therefore, room for extensibility. The lower values of birefringence for LA and FK may be related to their lower molecular weight and the fact that these proteins are semicrystalline, which would restrict chain motion.

Permeability Studies. Hydrophilic sheets are known to have a dependence of WVP on thickness. **Figure 7** depicts this dependence for FK, LA, and a 50:50 wt % blend. **Table 2** summarizes the permeability data for ca. 0.02 cm thick films. WG had WVP that was about 40% higher than the other

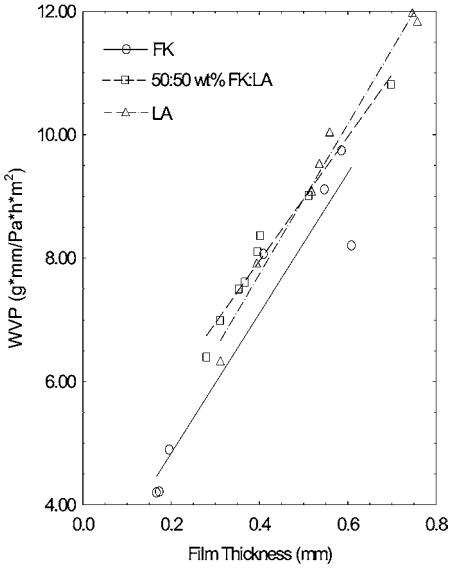

Figure 7. WVP as a function of film thickness for FK, LA, and a 50:50 wt % blend.

Table 2. WVP and OP Results

protein sample	WVP (g mm/Pa h m^2)	OP (cc μm/m^2 day kPa)
FK	5.2	64.4
LA	5.7	47.8
WG	8.3	>2000
50:50 wt % FK:LA	6.3	63.6
50:50 wt % FK:WG	5.8	>2000

proteins. McHugh et al. (*26*) and Sothornvit et al. (*50*) observed WVP of 15.8 for thermally processed whey protein films, 4.3 for solvent cast films, and 6.44 for solvent cast and thermally treated films. Because LA is a partial component of whey protein, the value observed here of 5.7 for thermally processed films for similar amounts of glycerol appears to agree with literature values with processing again contributing to differences. WVP for WG also appears to agree with literature values, with slightly lower WVP observed by Irissin-Mangata et al. (*9*) for solvent-prepared WG films with lower amounts of glycerol.

LA had the lowest OP of the group followed by FK. WG and its blends had a very high OP as compared to FK, LA, and their blends. Along with the WVP data, WG lacks structural features as compared to the other proteins that do not allow it to act as an impediment to water vapor or oxygen transport. WG was an amorphous polymer so of course the lack of crystallinity was a factor and this also manifested in lower modulus and strength. The OP data for FK and LA were significantly different, and the result for FK:LA blends was close to the FK result. This correlates with the birefringence result and may be further evidence of a disruption of the LA crystallinity upon blending. In FK:WG blends, the WVP results lie somewhere between FK and WG, similar to the observed birefringence, although a linear correlation was not observed.

The lack of a linear dependence between permeability and blend composition or the fact that the permeability appeared to be dominated by one protein means that some form of unique microstructure was formed. The glycerol content of the sheets was kept constant, and differences were noted that were protein-dependent. For FK−LA, FK seemed to disrupt the crystallinity in LA and increased permeability was observed over LA alone. The solubility of a permeant in the film also affects mass transfer. Blending proteins of different hydrophobicity would lead to changes in sorption and affinity between the penetrant and the film system (*51*). The WVP results for FK−WG may be from a combination of crystallinity and hydrophobicity in the FK fraction. However, the OP result for FK−WG was dominated by WG. This may mean that there was continuity of the WG through the sheets and OP was through that continuous WG. The WVP result for the blend then may be because of increased hydrophobicity from FK rather than crystallinity.

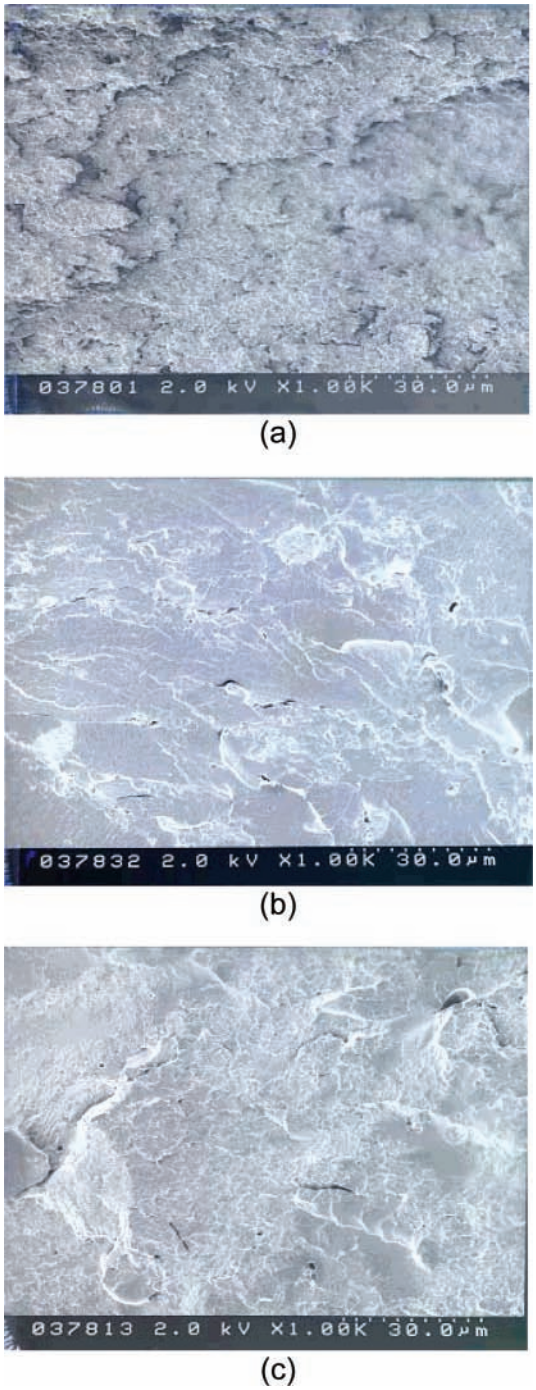

Figure 8. Scanning electron micrographs at 1000× of (**a**) LA, (**b**) FK, and (**c**) 50:50 wt % FK:LA blended with glycerol and sodium sulfite.

SEM. Scanning electron micrographs of the fracture surface of FK−LA blends show a distinct morphology in **Figure 8**. **Figure 8a** is a micrograph of a LA/glycerol/sodium sulfite blend. The fracture surface was flat, indicative of brittle fracture, with very small drawn-out areas. **Figure 8b** is a micrograph of a

FK/glycerol/sodium sulfite blend. The fracture surface has more topography and was indicative of ductile fracture, with large drawn-out areas. It was shown that FK blends failed at strains of over 100% while LA blends failed at strains of about 20%. **Figure 8c** is a micrograph of a 50:50 wt % FK:LA blend, which failed at a strain of about 65%. The fracture surface showed intermediate behavior to **Figure 8a**,**b** with small drawn-out areas on top of larger drawn-out areas (*52*).

Conclusions. The modulus followed the following pattern: LA > FK > WG. Higher molecular orientation in FK and LA seemed to contribute to higher modulus and strength values. LA contained an extra "structure" possibly in the form of crystals that resulted in the higher modulus and strength and in a low permeability of oxygen. Given the molecular weight values shown, entanglements may play less of a role in determining properties, as the highest molecular weight protein, WG, did not have the highest properties. Blending proteins is a method to elicit new properties easily, similar to methods used by the synthetic polymers industry. For instance, adding WG to FK increases the toughness of the FK at no cost to WVP.

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Received for review December 27, 2005. Revised manuscript received May 11, 2006. Accepted May 24, 2006. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

JF053238L