ABSTRACT
Whereas native whey protein films were totally water soluble, heat denatured films were insoluble. Heat-denatured whey protein films had higher tensile properties than native whey protein films. However, native and heat-denatured films had similar water vapor permeability (WVP). The pH of the film-forming solution did not have any notable effect on film solubility, mechanical properties, or WVP. Results suggest that covalent cross-linking due to heat denaturation of the whey protein is accountable for film water insolubility and higher tensile properties but does not affect WVP of the films.

Key words: whey protein, heat denaturation, water vapor permeability, film solubility, tensile properties

INTRODUCTION
EDIBLE POLYMER FILMS HAVE BEEN STUDIED FOR FOOD APPLICATIONS because of their ability to provide a barrier to mass transfer, carry food ingredients, and improve mechanical integrity of foods. Edible films can also enable reduction and simplification of the packaging material required for a food product.

Edible coatings and films can be based on lipids, polysaccharides, proteins, or their combinations. Techniques of film formation, properties, and applications have been reviewed (Guilbert, 1986; Kester and Fennema, 1986; Krochta et al., 1994; Krochta and De Mulder-Johnston, 1997; Debeaufort et al., 1998). Whey protein films produce transparent, bland, flexible, water-based edible films with excellent oxygen and aroma barrier properties at low relative humidity (McHugh and Krochta, 1994a; Miller and Krochta, 1997). However, whey protein films generally provide poor moisture barriers. Incorporation of lipids reduces their water vapor permeability (WVP) (McHugh and Krochta, 1994b; Shellhammer and Krochta, 1997).

McHugh and Krochta (1994a) studied optimization of film-forming conditions and found that, for the film formulations studied, heat treatment was necessary (e.g., 90 °C for 30 min) for formation of intact whey-protein-based edible films. The films were characterized by water insolubility, which could be beneficial in maintaining film and food integrity. Heat denaturation of the whey protein film-forming solution causes protein denaturation and exposure of internal sulfhydryl groups, promoting intermolecular disulfide bond formation (Shimada and Cheftel, 1998). These disulfide bonds have been hypothesized to be as partly responsible for film structure. Studies have shown that inhibition of intermolecular disulfide bonds with sodium dodecyl sulfate (SDS), inhibition of sulfhydryl-disulfide interchange with N-ethyl maleimide, or reduction of disulfide bonds with cysteine had no effect on formation or WVP of whey protein films (Fairley et al., 1996a, b).

For the current study, films were formed from native whey protein (NWP), as well as from heat denatured whey protein (DWP).

The cohesion of NWP films relies on non-covalent interactions, such as hydrogen bonding. However, intermolecular disulfide bonding is involved in DWP film formation and properties. In addition, whey protein exposed to different pH undergoes structural changes (De Wit, 1981; Leman and Kinsella, 1989) that may affect protein-protein interactions, film formation and properties. Our objective was to compare the WVP, solubility in water, and mechanical properties of NWP vs DWP films, including the effects of film-forming solution pH.

MATERIALS & METHODS
Materials
Whey protein isolate (WPI) (> 95% protein d.b.) was supplied by Davisco Foods International (Le Sueur, Minn., U.S.A.). Glycerol (Gly) (Fisher Scientific Inc., Fair Lawn, N.J., U.S.A.) was added as a plasticizer to all film-forming solutions. Sodium hydroxide and hydrochloric acid were purchased from Fisher Scientific Inc. and used for pH adjustment. Bicinchoninic Acid Protein Assay Kit, bovine serum albumin and potassium sorbate were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.).

Film formation
Heat-denatured WPI films were prepared according to the McHugh and Krochta method (1994). Aqueous solutions of 5% (w/w) WPI were prepared and degassed under vacuum. Based on preliminary experiments, this protein concentration was selected to avoid gel formation at the isoelectric point. To prepare heat-denatured films, WPI solutions were heated at 90 °C for 30 min in a water bath. Heated solutions were cooled to room temperature. Gly was added to all film-forming solutions as a plasticizer in a 70/30 ratio of WPI/Gly, and this ratio was kept constant throughout the study. Lower values of plasticizer gave films which were extremely brittle at pH 4 and 5. The pH of the WPI-Gly solution was adjusted, after heat treatment, with sodium hydroxide or hydrochloric acid to the desired pH in the range pH 3 to pH 8. The pH adjustment was monitored with a Corning pH-meter (Corning Inc., N.Y., U.S.A.). Before being cast, final solutions were degassed under vacuum to remove any dissolved air. Native films underwent the same film-forming process, with the exception of heat treatment. In order to compare WVP values with previous studies, a 10% WPI heat-denatured solution was prepared containing the same WPI/Gly ratio at the natural WPI pH (approximately 7).

Films were cast by pipetting whey protein solution onto rimmed, smooth plates resting on a leveled granite slab. For each property studied, different numbers of replicates were prepared. The films were allowed to dry at room temperature over 24 h.

Film thickness
Thicknesses of films were measured with a caliper micrometer (No. 7326, Mitutoyo Manufacturing Co. Ltd., Japan) at 6 random positions of the film, following WVP and preceding tensile tests. WVP and mechanical properties were calculated based on average thickness.
Water vapor permeability measurements

A modification of the ASTM E96-92 gravimetric method to determine the relative humidity (RH) at the film underside was used for measuring WVP (McHugh et al., 1993). Six films were cast from each treatment (5 replicates), onto 15.5 cm internal diameter Teflon® plates. Whey protein solutions were applied at 3 g total solids/plate to minimize thickness variations between treatments. After drying, one sample without defects was cut from each film. Distilled water (6 mL) was dispensed into flat-bottom Plexiglas® cups with wide rims. The rim of each cup was coated with silicon sealant (High Vacuum Grease, Dow Corning, Midland, Mich., U.S.A.). The film was sealed to the cup base with a ring using 4 screws symmetrically located around the cup circumference. The cups were placed in desiccator cabinets containing fans and held at 0% RH using anhydrous calcium sulphate (W.A. Hammond Drierite Co., Xenia, Ohio, U.S.A.). Weights were taken periodically after steady state was achieved and used to calculate the % RH at the film underside and the resulting WVP.

Film protein solubility

Films were prepared the same way as those used to measure WVP. Three films of each treatment were cast (3 replicates). Rectangular pieces (12) measuring 7.5 mm by 15.0 mm were cut from each film. Those pieces were dried in a vacuum oven at 70 °C and 34.5 kPa for 24 h to determine the initial dry weight of the film. Samples were weighed to the nearest 0.0001 g and placed into test tubes with 10 mL deionized water, containing 0.01% potassium sorbate to prevent microbial growth. The tubes were capped and placed on a rotating platform for 24 h at ambient temperature (approximately 23 °C). The soluble protein of films in water was determined by using the bicinchoninic acid (BCA) protein assay (Smith et al., 1985). Samples from the test tubes were added to the protein determination reagent, made by combining 1 part 4% copper (II) sulfate pentahydrate solution with 50 parts BCA solution, vortexed and heated to 37 °C for 30 min in a water bath. After cooling to room temperature, absorbances of mixtures were read at 562 nm using a UV-Vis Recording Spectrophotometer (UV-160A, Shimadzu Scientific Instruments Corp., Columbia, Md., U.S.A.). Protein concentrations were calculated from a standard curve obtained using bovine serum albumin.

The percentage of soluble protein (%SP) in the film was calculated by dividing the weight (wt) of soluble protein in the 10 mL of film soaking solution by the initial dry weight of protein in the film piece. The initial dry weight of protein in the film was calculated from the initial dry weight of the film, taking into account both the ratio of WPI:total solids in the film and the percent protein in the WPI powder (97.7%). The following equation was used:

%SP = \[\frac{\text{wt protein in 10 mL solution}}{\text{Initial wt film} \times 0.977 \times \text{Ratio of WPI:Total solids in film}}\] × 100

Film solubility (total soluble matter)

The remaining pieces of film after soaking were dried again in the vacuum oven at 70 °C and 34.5 kPa for 24 h to obtain the final dry weight of the film. The percentage of total soluble matter (%SM) of the films was calculated using the formula:

%SM = \[\frac{\text{Initial dry wt} - \text{Final dry wt}}{\text{Initial dry wt}}\] × 100

Tensile properties determination

Film solutions were cast onto square 30 cm × 30 cm, rimmed, smooth high density polyethylene (HDPE) plates, by applying 3 g total solids/plate to minimize thickness variations between treatments. After drying at room conditions, the films were conditioned at 58% RH in a chamber that contained saturated sodium bromide (Fisher Scientific Inc.) solution for more than 2 d. This preconditioning enabled ease of handling and cutting of the films. Test pieces of film were cut using a striking die (The Right Image, Sacramento, Calif., U.S.A.). The film samples presented a rectangular center section measuring 15 mm wide × 100 mm long, flaring to 25 mm × 35 mm square grips on each end, which provided a greater grip area. All film strips were equilibrated for 36 h to 53% RH in a cabinet using magnesium nitrate (Fisher Scientific Inc.) saturated solution at room temperature (23 ± 2 °C).

Tensile measurements were performed following the procedure outlined in ASTM method D882-91. The ends of the equilibrated film strips were mounted and clamped with rubber-lined aluminum grips on a Universal Testing Machine (Model 1122, Instron Corp., Canton, Mass., U.S.A.) with a 500 kg load cell. The initial gauge length was set to 115 mm, and films were stretched using a crosshead speed of 50 mm/min. Testing conditions were controlled throughout the measurements and held constant at 50 ± 5% RH and 23 ± 2 °C. Tensile properties were calculated from the curve of stress (tensile force/initial cross-sectional area) vs strain (extension as a fraction of the original length), using Series IX Automated Materials Testing System Software (Instron). Mechanical properties reported are maximum tensile stress (MPa), elongation at break (%), and elastic modulus (MPa).

Statistical analysis

Statistics on a completely randomized design were determined using StatView 4.0 (Abacus concepts, Berkeley, Calif., U.S.A.). Fisher PLSD multiple comparison tests were used to determine significance of differences between means. Significance of differences was defined at p ≤ 0.05.

RESULTS & DISCUSSIONS

Water vapor permeability

Films were prepared from both DWP and NWP solutions, adjusted in pH from 3 to 8. At pH 3, no intact films were formed for any of the samples, probably due to high electrostatic repulsions between proteins. When heat-denatured solutions were adjusted to pH 4 and 5 (pI range of whey proteins), solution viscosity increased rapidly resulting in a weak gel. However, this viscous solution could be transferred onto the casting plates to form a film. Resulting films were shrunken and curled, and they had to be conditioned in a high RH cabinet before being cut for WVP measurements. All films could be easily peeled off the casting plates.

The WVP of NWP-Gly vs DWP-Gly films and the effects of film-forming solution pH were compared (Fig. 1). Heat treatment of the film-forming solutions had no effect on WVP for pH 6, 7, or 8. At pH 4 and 5, DWP films had a significantly higher WVP than NWP films. The higher WVP for DWP films at these pH values was a consequence of the high viscosity of the film-forming solutions. As mentioned, at pH 4 and 5, the film-forming solution formed weak gels. This made difficult the removal of all air bubbles prior to film casting; hence the high WVP. Similar results had been reported in a previous work (Pérez-Gago and Krochta, 1999) where viscosity measurements showed that DWP solutions adjusted to the pI range had significantly higher viscosity compared to solutions adjusted to pH values greater than the pI. For NWP films, pH did not have any significant effect on WVP. Native whey proteins were soluble throughout the pH range, and no increase in solution viscosity was observed near the pI.

We also prepared 10% WPI films at the natural pH of aqueous WPI (pH approximately 7) and WVP was measured. WVPs of films from 10% WPI solutions were not different from the WVP of those formed from 5% solutions (Table 1). Films from both solutions have been prepared such that final solids level per plate was similar.

In protein-based films, protein-protein interactions determine the characteristics of the film. Film-forming ability may be influenced by amino acid composition, distribution, and polarity, conditions necessary for ionic crosslinks between amino and carboxyl groups, hydrogen bonding groups, and intramolecular and inter-
molecular disulfide bonds (Gennadios and Weller, 1991). Native and heat-denatured films differ in physical structures. Native whey proteins are globular proteins with most hydrophobic and sulfhydryl groups turned to the interior of the molecule. Heat denaturation of the whey proteins induces protein denaturation and exposure of internal sulfhydryl groups, promoting intermolecular disulfide bond formation (Shimada and Cheftel, 1998). Such differences influence the molecular structure of the final film. Thus, DWP films are made of cross-linked protein strands, whereas NWP films have a more random structure in which cohesion is mainly due to hydrogen bonding. These different structures could produce different permeability properties of the resulting films.

However, no significant difference in WVP was found between NWP and DWP films at pH values greater than the pl (Fig. 1). This suggests that covalent cross-linking due to heat denaturation of the whey protein did not affect WVP of WPI films. Fairley et al. (1996b) reported that reduction of covalent cross-linking in WPI films, by inhibiting sulfhydryl-disulfide interchange reactions, had no effect on film WVP. Those results were consistent with results of Maté and Krochta (1996), which showed no differences in WVP of WPI and heat-denatured films, by inhibiting sulfhydryl-disulfide interchange reactions at acidic pH. However, an increase in solution viscosity at pH 6 was also reported, which might have been the cause of the higher WVP value due to incomplete degassing.

The pH effect on WVP of other protein films has been shown to depend on protein type. Gontard et al. (1992) showed that the highest WVPs of gluten films were recorded at acidic pH. A pH effect on WVP of wheat gluten was reported by Herald et al. (1995), who compared films formed at pH 3.3 and pH 10.0. The pH 3.3 films showed higher WVP, presumably due to the unfolding of protein molecules causing exposure of hydrophobic groups. Brandenburg et al. (1993) reported that pH had an effect on mechanical and barrier properties only at pH 6 compared to pH 8, 10, and 12 for soy protein isolate films. At pH 6, films were not transparent, indicating incomplete solubilization of the protein. They suggested that the low barrier and mechanical properties at pH 6 might arise from the partial insolubility at that pH; whereas at alkaline pH, the protein was solubilized so that cross-linking could develop. Gnanasambandam et al. (1997) did not find any differences in WVP between rice bran films at pH 9.5 and pH 3.0. In the studies on wheat gluten and soy protein films, effect of increased pH was more pronounced probably due to the presence of alkali-soluble protein. However, the majority of rice bran proteins are albumins and globulins, which are water soluble and give similar barrier properties at both acidic and alkaline pH (Gnanasambandam et al., 1997). Native WPI consists of globular proteins that remain soluble throughout the whole pH range (Kinsella, 1984), giving barrier properties independent of pH. However, when WPI is denatured, protein solubility decreases as the solution approaches the isoelectric point, giving higher WVP in the pl range.

**Film protein solubility**

Solubility in water is an important property of edible films. Potential applications may require water insolubility to enhance product integrity and water resistance. However, in some cases film water solubility before consumption of the product might be beneficial.

Heat-denatured whey protein film pieces maintained their integrity throughout the film-soaking treatment, whereas NWP film pieces dissolved rapidly after coming in contact with water. The protein solubility and total soluble matter of DWP vs NWP films made from 5% WPI solutions at different pH were compared (Fig. 2). Protein solubility of DWP films was very low compared to NWP films. This indicates the high cohesion of the DWP matrix. The disruption of the native three-dimensional structure of the pro-

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**Table 1—Effect of heat treatment on WVP of WPI:Gly films (2.33:1) at pH 7**

<table>
<thead>
<tr>
<th>Film treatment</th>
<th>pH</th>
<th>Thickness (mm)</th>
<th>Test conditions (g mm/KPa h m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% WPI</td>
<td>7</td>
<td>0.115±a</td>
<td>25 °C, 0/77% RH 4.75b</td>
</tr>
<tr>
<td>5% WPI Heat Denatured</td>
<td>7</td>
<td>0.139±b</td>
<td>25 °C, 0/71% RH 4.96b</td>
</tr>
<tr>
<td>5% WPI Native</td>
<td>7</td>
<td>0.139±a</td>
<td>25 °C, 0/71% RH 5.06b</td>
</tr>
</tbody>
</table>

±Thickness means with different letters different (p < 0.05).

bWVP means with different letters different (p < 0.05).
Table 2—Mechanical properties of heat denatured vs native WPI films as affected by pH

<table>
<thead>
<tr>
<th>Film treatment</th>
<th>pH</th>
<th>Elastic modulus (MPa)</th>
<th>Tensile strength (MPa)</th>
<th>Elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% WPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heat Denatured</td>
<td>6</td>
<td>195a</td>
<td>6.5a</td>
<td>43a</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>199a</td>
<td>6.9a</td>
<td>41a</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>170a</td>
<td>6.0a</td>
<td>54a</td>
</tr>
<tr>
<td>5% WPI</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>4</td>
<td>102b</td>
<td>2.2b</td>
<td>3b</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>104b</td>
<td>2.9b</td>
<td>7b</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>88b</td>
<td>2.8b</td>
<td>9b</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>100b</td>
<td>3.1b</td>
<td>7b</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>105b</td>
<td>3.2b</td>
<td>10b</td>
</tr>
</tbody>
</table>

a—Elastic modulus, tensile strength, and percent elongation at break data are mean values. Elastic modulus, tensile strength, and percent elongation at break means with different letters are different (p < 0.05).

The proteins by heat denaturation, exposing SH groups formerly buried inside the molecules, enables formation of covalent intermolecular disulfide bonds, which are accountable for film water-insolubility. These results confirmed those of Fairley et al. (1996a), that inhibition of intermolecular disulfide bonds increases the protein solubility of WPI films.

The film protein-solubility of heat-denatured films at pH 4 and 5 was different (p < 0.05) from the solubility of other heat-denatured films (Fig. 2). This may have resulted from aggregation of the protein at this pH range, decreasing the amount of available protein sites reacting with the BCA reagent.

Film solubility (total soluble matter)

Once the native WPI films were exposed to the solution, they dissolved, and no film pieces were found after 24 h on the rotary platform. Hence, the total soluble matter was 100% (Fig. 2). No difference was found in total soluble matter of DWP films at different pHs. For the DWP films, glycerol would represent most of the soluble matter, since glycerol is very hydrophilic.

Thus, there was a consistent relationship in percentages of protein solubility to total soluble matter. Due to high energy intermolecular bondings, solubility of DWP films was lower than that of NWP films. The effect of cross-linking was also observed in the tensile properties.

Tensile properties

Edible films, to be useful, must maintain integrity during processing, shipping, and handling. Tensile strength, elastic modulus, and elongation help describe how the mechanical properties of such films materials relate to their chemical structures (Ninnemann, 1968).

Mechanical properties for native and heat-denatured films were compared as related to pH (Table 2). No data were obtained for heat-denatured films prepared at pH 4 and 5, since they shrank and curled after drying. Consistent with no effect on WVP, pH greater than pH had no effect on mechanical properties of either DWP or NWP films. However, NWP films showed lower elastic modulus, lower tensile strength, and lower elongation than DWP films. Thus, native films would be less stiff, weaker, and less extensible than DWP films. The low energy bondings and globular structure of whey proteins (Bryant and McClements, 1998) in the native films could be accountable for these results. The unfolded structure and covalent disulfide bonding of the DWP films produced stronger films and would enable the films to withstand greater deformations.

CONCLUSION

WHEREAS NWP PROTEIN FILMS WERE TOTALLY SOLUBLE, the DWP films were insoluble. The DWP films had higher elastic modulus, yield stress, and elongation than NWP films. However, heat treatment and pH of film-forming solutions had no effect on WVP at pH greater than the pI. In general, pH did not affect film solubility and mechanical properties for either the DWP or NWP films. Results suggest that covalent cross-linking due to heat denaturation of the whey protein is accountable for film water insolubility and increase in film tensile properties, but did not affect WVP of WPI films. Further research is needed to determine oxygen and aroma permeabilities of such films.

REFERENCES


Ms 0259 received 1/20/99; revised 5/17/99; accepted 6/25/99.

Maria B. Pérez-Gago was supported with a scholarship from the Spanish Agriculture Department. This research was also supported by Dairy Management Inc., through the California Dairy Research Foundation and California Dairy Foods Research Center.