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Article

Casein Films: The Effects of Formulation, Environmental Conditions and the Addition of Citric Pectin on the Structure and Mechanical Properties

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Abstract: Thin casein films for food packaging applications reportedly possess good strength and low oxygen permeability, but low elasticity and high sensitivity to moisture. Modifying the films to target specific behaviors depending on environmental conditions can enable a variety of commercial applications for casein-based films. The mechanical properties of solvent-cast (15% solids) calcium-caseinate/glycerol films (CaCas:Gly ratio of 3:1) were characterized as a function of processing and environmental conditions, including film thickness, solution formulation and ambient humidity (from 22% to 70% relative humidity (RH) at ~20 °C). At constant RH, the elongation at break (EAB) had a strong positive dependence on the film thickness. When RH increased, the tensile strength (TS) and modulus (E) decreased approximately linearly, while EAB increased. From 0.05% to 1% (*w*/*w*) of citric pectin (CP) was then incorporated into CaCas/Gly films following seven different formulations (mixing sequences), to alter the protein network and to evaluate the effects of CP on the tensile properties of CaCas/Gly/CP films. At constant film thickness and ~60% RH, the addition of 0.1% or 1.0% CP to the films considerably increased or decreased EAB, TS and E in different directions and to different extents, depending on the formulation, while optical micrographs also showed vastly differing network configurations, suggesting complex formulation- and stoichiometry-dependent casein-pectin interactions within the dried films. Depending on the desired film properties and utilization conditions, pectin may be a useful addition to casein film formulations for food packaging applications.

Keywords: high-methoxyl pectin; milk protein; edible film; food packaging

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1. Introduction

In recent years, interest has grown for edible packaging films as a replacement for synthetic plastic films driven by a societal desire for environmentally-friendly, biodegradable and sustainable food packaging. Edible packaging films are polymeric films based on edible materials that can provide mechanical protection, as well as physical, chemical and biological barriers between a food product and its environment [1]. Edible films can also enhance the appearance and nutritional value of foods, and deliver bio-actives (antioxidant or antimicrobial) to extend shelf life by acting as a carrier for different compounds [2–6].

Casein proteins constitute ~80% of the total proteins in milk [7]. Available in many different by-products from the dairy industry, including calcium and sodium caseinates (CaCas and NaCas), casein has great potential for producing protein-based edible films [8,9]. Edible casein films can form a good barrier to oxygen and other nonpolar molecules, because casein provides lots of polar functional groups, such as hydroxyl and amino groups, to the film matrix [10]. This property allows casein films to be used in combination with other packaging materials to protect products prone to oxidation. It is well known that pure casein films tend to shrink during drying and become brittle; therefore, edible plasticizers, such as glycerol, are used to increase the free volume of the polymer network and to render casein films more flexible, by lowering their glass transition temperature. Edible casein/glycerol films possess good tensile strength and moderate elasticity under normal conditions [11–15], but the presence of glycerol affects the barrier properties of casein films [16,17].

Several problems need to be solved before casein films can be widely commercialized. First of all, casein films are highly moisture sensitive. They easily absorb and release water molecules, which act as a plasticizer and greatly affect the mechanical properties of the films, and are mostly soluble in water, which limits their range of utilization. Secondly, casein/glycerol films are unable to provide both high mechanical strength and high elasticity, like synthetic films. Thirdly, their relatively high cost, limited raw-material source and complex manufacturing procedures can also cause processing problems [1,8,16,18].

A range of studies have been undertaken to try and solve these problems. β-casein, the most hydrophobic protein of milk, was studied for its potential to provide films with a lower water vapor permeability [15]. In prior investigations, Tomasula *et al.* developed a method to produce more hydrophobic casein through high pressure processing with CO_2, producing casein films with considerably lower solubility [14,16,19,20]. Hydrocolloids, such as gelatin, were used to enhance the mechanical strength, as well as the water vapor barrier properties. In the specific case of gelatin-casein films, transglutaminase was applied to crosslink the two materials. After enzyme treatment, a decrease in water vapor permeability (WVP) and an increase of elasticity were observed [21]. Cross-linking induced via physical treatment with UV or γ-irradiation was also investigated with some positive results [22,23]. Casein modification by the Maillard reaction before film casting improved the elongation of the films, but increased their hydrophilicity [24]. On the other hand, chemical methods, such as alkali-treatment or the addition of various cross-linkers, were reported to improve the elongation and strength and decrease the solubility of casein films [22]. Additives, such as stearic acid or various enzymes, were helpful to improve the water-resistance and mechanical properties of casein films, while DL-glyceraldehyde and glutaraldehyde also enhanced the microstructure of glycerol plasticized casein films [13,25,26].

Pectin, which can form complex interactions with casein [27–32], is an interesting additive with the potential to enhance some of the desired properties of casein films. Pectins are negatively charged polysaccharides. Their basic structure is a linear chain of poly-α-(1-4)-D-galacturonic acid with varying degrees of methyl esterification (DE) [33–35]. Depending on DE, pectins are divided between high methoxyl (HM) and low methoxyl (LM) pectin. HM pectins form gels mainly via hydrophobic interactions and hydrogen bonds in the presence of more than 55% sugar, while LM pectins can gel with only a small amount of sugar in the presence of calcium ions, Ca^{2+} [36]. The various interactions between pectin and casein are complex and are greatly affected by the stoichiometric casein/pectin ratio, the pH and the presence of salts, such as calcium [27,31,32,37]. For example, at neutral pH in milk, added pectin does not adsorb onto casein micelles and forms a separate, segregated phase [31]. When pectin is added to acidified milk or casein suspensions below pH 6, it adsorbs onto the casein micelles and forms different pectin/casein complexes, depending on pH and on the pectin:casein ratio. In the absence of calcium (Ca^{2+}), both LM and HM pectins cause bridging of simulated calcium-free micelles (casein-covered polystyrene beads) [29]. Bridging of casein micelles can lead to flocculation [31]. At lower pH, pectin-casein complexes become negatively charged, repulse one-another electrostatically and prevent aggregation [32], also preventing whey separation and affecting the particle size distribution in acidified milk [38]. Pectin/casein complex particles appear spherical after spray drying [28]. If the pectin concentration is high, pectin chains can completely cover casein molecules via electrosorption and hydrophobic interactions [27,30]. The adsorption is multilayered, and the thickness and number of layers increase when pH decreases between pH 5.0 and 3.5 [30]. Upon further pH reductions, the complexes begin to aggregate and become insoluble and, then, disassociate into soluble polymers [27]. Adding Ca^{2+} to casein/pectin systems (e.g., by utilizing CaCas instead of NaCas) induces cross-links between negatively-charged casein/pectin complexes and can trigger the formation of aggregates [32].

While casein/pectin solutions have been broadly investigated, casein/pectin edible thin films have seldom been studied, in favor of abundant research on whey-protein/pectin films. In one prior study, Letendre *et al.* (2002) produced biodegradable CaCas films with added autoclaved pectin and agar, and cross-linked with a combination of thermal and radiative treatments. The pectin + agar additives improved the puncture strength and reduced the WVP of the films [39].

In this work, the tensile properties of hydrophilic CaCas/glycerol (Gly) films with a constant composition (75% CaCas, 25% Gly) were characterized on a wide range of formulations, film thicknesses and ambient humidities, to evaluate the sensitivity of CaCas/Gly films to these three critical criteria. The films were then used as a benchmark for comparison with and understanding of the complex CaCas/Gly/HM pectin film structures at neutral pH, using several different film-solution formulation protocols with low pectin contents, in isohume and iso-thickness conditions. At pH 7, the film formulation protocol strongly affected the macro-structure and tensile properties of the films, even at very low pectin contents, a phenomenon that can be exploited to tailor some of the properties of casein films in various ways to broaden their range of potential commercial applications, such as edible wrappers or soluble pouches for single-serve foods, packaging films for cheese or oxygen-sensitive foods, coatings for nuts and cereals or multi-layered packaging films.

2. Experimental Section

2.1. Materials

Calcium caseinate powder (CaCas) was purchased from American Casein Co. (Burlington, NJ, USA). It contained (as measured over 3 to 6 replicates) 4.0% moisture and 3.0% minerals, of which were 47.7% calcium, 29.6% phosphorus, 18.2% sodium, 3.8% potassium, 0.5% magnesium, 0.1% zinc and iron and trace amounts of copper and manganese. Mineral analysis was performed with an ICP-OES (inductively coupled plasma-optical emission spectrometer), model iCAP 6300 Duo (Thermo Fisher Scientific, Madison, WI, USA). Solutions of CaCas and glycerol (Gly) (ACS grade, Sigma-Aldrich, St Louis, MO, USA) were prepared with de-ionized (DI) water produced by a Milli-Q Synthesis water purification system (Millipore, Billerica, MA, USA). Pectin 1400, a citrus pectin (CP) with a high degree of methoxylation of 60% ± 1% and a molecular weight of 236,000 g/mol (236 KDa) was purchased from Danisco USA Inc. (Madison, WI, USA) as an additive for the casein/glycerol film-making solutions.

2.2. Preparation of Film-Making Solutions

Control casein/glycerol (CaCas/Gly) and casein/glycerol/pectin (CaCas/Gly/CP) film-making solutions were prepared with DI water to a total solids concentration of 15% (*w*/*w*). Solution compositions were calculated to keep a constant 3:1 CaCas:Gly ratio, while CP was added in various quantities as needed. Because CP can be hard to dissolve, it was first rehydrated into a thick, concentrated 3% (*w*/*w*) solution (CPsol) and stirred at 1200 rpm for 2 h. CPsol was then mixed with the other ingredients to produce the desired dry-films compositions.

The 4 different ingredients (CaCas, Gly, CP and DI water) were mixed together in varied order according to 7 different solution-formulation protocols (Table 1). Solutions were mechanically stirred at 500–1000 rpm for 1 h after the addition of each component (3 h total). “Control A” and “Control F” film solutions were prepared using Formulations A and F without any pectin.

The CP concentration in the dried films was calculated from 0.05% to 1.0% (*w*/*w*). At the end, two drops of liquid food coloring (assorted colors; McCormick & Co. Inc., Hunt Valley, MD, USA) were mixed into each film-making solution, to facilitate the observation of the appearance and structure of the dried films, and the pH was measured with a pH/ion meter (Mettler Toledo, Schwerzenbach, Switzerland). The pH of the solutions after mixing decreasing progressively with increased CP content, from pH 7.12 for control films to pH ~7.02 with 1% CP. Film-making solutions were then transferred to vacuum filtration flasks and connected to the laboratory vacuum system until degassed, to prevent air bubbles in the dried films.

Table 1. Different formulations used for calcium-caseinate (CaCas)/glycerol (Gly)/citric pectin (CP) film solutions. CPsol, CP solution.

Formulation	Components 1 + 2	Component 3	Component 4
Control A	Water + CaCas	Gly	-
A	Water + CaCas	Gly	CPsol
B	Water + CaCas	CPsol	Gly
E	Water + Gly	CaCas	CPsol
Control F	Water + Gly	CaCas	-
F	Water + Gly	CPsol	CaCas
G	Water + CPsol	CaCas	Gly
H	Water + CPsol	Gly	CaCas
K	CPsol + Gly	Water	CaCas

Solid fractions (Table 2) of the different ingredients in the solutions and dried films were calculated from the mass of each component added to the initial solution (adjusted with purities), divided by the total mass of ingredients in the solution, except water. The approximate number of moles of each component in solution was calculated from the average molecular weight and mass of each component; mole fractions were calculated as the number of moles of each component divided by the total number of moles of all ingredients, except water. Stoichiometric (molecular) ratios were calculated as the ratio of the mole fractions of two components.

Table 2. Steric and stoichiometric considerations for CaCas/Gly/CP mixtures.

Component	Molecular Weight	Solid Fraction	Mole Fraction	Binding Potential
Caseins	19,000–25,000 g/mol	~73 wt%	~1.2%	Multiple functional groups, including –NH, and (+) and (−) charges. Assembly into micelles.
Glycerol	92 g/mol	~25 wt%	~89.7%	Small, polar, 3 –OH groups
Calcium ions	40 g/mol	~1.1 wt%	~9.1%	Ca^{2+}, 2 positive charges
Citric Pectin	236,000 g/mol	0 to 1 wt%	0 to 0.0014%	Long, linear, –OH and $–COO^{-}$ groups

2.3. Film-Casting Procedure

All films were cast on silicone baking mats (Weston Products LLC., Strongsville, OH, USA): silicone was selected after a study of various substrates for its good compatibility with CaCas solutions (good wetting) and the ease of peeling of the dried films. Silicone baking mats were cleaned with diluted Contrex detergent, wiped dry, then a 70/30 percent ethanol/DI Water solution was applied to remove any detergent residue.

To cast each film, a silicone mat was placed on a K-101 Control Coater apparatus (RK Print-Coat Instruments Ltd., Royston, UK) set to speed setting #3. A set of different spirally wound meter bars was used to spread the film solutions at controlled thicknesses, from 100 to 500 μm-thick (Bars #8, 150, 200, 300 and 500, RK Print-Coat Instruments Ltd., Royston, UK), to produce dried film thicknesses ranging from 0.010 to 0.18 mm. The volume of film-making solution cast on the silicone mat ranged from 3 to 40 g, depending on the meter bar chosen, to produce rectangular films of approximately 15 cm × 25 cm. After spreading, silicone mats were transferred to level tables, and films were left to dry for 1 or 2 days at 20 °C, depending on the current humidity. Throughout the year, relative humidity (RH) in the lab varied from 22% to 70%, and films cast at higher humidity needed more time to dry. After drying, films were peeled from the mats, placed between sheets of paper and stored in a chamber at 20 °C and 50% RH for at least 2 days before testing. Films were cast in at least 3 independent repeats.

2.4. Tensile Properties Measurement

After pre-conditioning at 50% RH, films were cut into 5 mm × 35 mm strips with a razor blade; then, the strips were allowed to equilibrate at ambient humidity (22% to 70% RH) overnight before mechanical testing. The thickness of an equilibrated film strip was measured at five points with ±0.001 mm precision using a Mitutoyo absolute thickness gage (Mitutoyo Corp., Kanagawa, Japan), and averaged. Film strips were mounted vertically onto a Tensile Grips clamp with a 25-mm gauge length, and stress/strain properties were recorded with a TA.XTPlus Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA) during the elongation of the strip with a cross-head speed of 0.2 mm/s (12 mm/min). For each film, 5–6 strips were tested. The data collected for each strip using the stress-strain curve, included: the tensile modulus, E (MPa), taken as the initial slope of the curve; the tensile strength, S (MPa), taken as the point of maximum stress on the curve; and the elongation at break, EAB (%), taken as the strain when the film begins to fail or snaps. The strength at break and elongation at maximum stress were also recorded.

All tensile data were compiled, analyzed and then averaged and graphed using Microsoft Excel 2010 (Microsoft Corp., Redmond, WA, USA). Polynomial approximations were performed using Excel's built-in "trendlines" tool.

2.5. Microscopy Imaging

Structural differences between the various films were visible to the naked eye. The macroscopic appearance of the films was recorded using a Nikon Eclipse E800 microscope equipped with a CoolSnap CF digital camera (Nikon Corporation Instruments Company, Tochigi, Japan). Film areas of 2 mm × 3 mm were magnified 4×, and digital images were captured using automatic exposure, an intensity target of 3000 and a camera speed of 20 KHz.

All figures were designed with Microsoft Excel 2010.

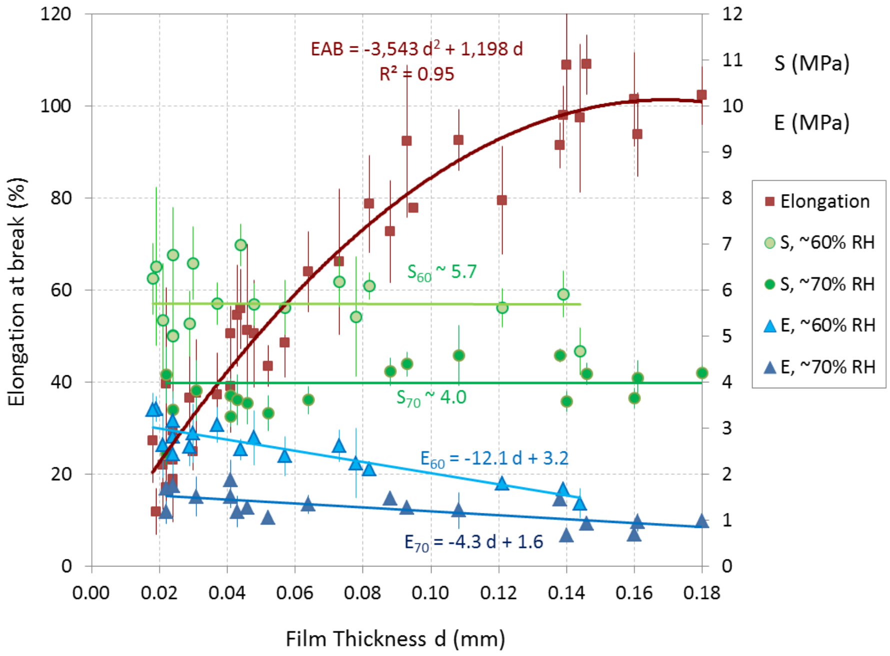
3. Results and Discussion

3.1. Control A and F Films: The Effects of Humidity and Thickness on Tensile Properties

The thickness, d, of control films (0% pectin) and the humidity in the laboratory varied greatly, both affecting the tensile properties. The relative humidity (RH) in the laboratory was not controlled and varied from 22% to 70% throughout the year and by as much as 20% from day to day, affecting the rate of drying of the films and the tensile test conditions. Factors affecting d included processing parameters, such as the spreading bar used, the rate of spreading and related solution-creep, the solution composition and formulation, the drying rate (which can change the structure of the film's network) and humidity-induced swelling. Numerous Control A and F films of different thicknesses (d = 0.01–0.18 mm) were repeatedly prepared throughout the year, and multiple samples were tested on many different days to take advantage of the entire range of RH available throughout the year, in order to quantify both the effects of RH and d on the mechanical properties of CaCas/Gly films and to characterize these films on the full range of environmental RH and thickness conditions that might be encountered during the commercial development and end-use of CaCas/Gly food-packaging films. All of the tensile results were then organized and analyzed according to the values (RH, d) recorded before each test.

The most commonly encountered humidity range in our laboratory (Philadelphia, PA, USA) was a high RH range of ~58%–70% during approximately six months of the year. Figure 1 presents the static tensile modulus, E, tensile strength, S, and elongation at break, EAB (or "elongation"), of Control A films between 58% and 70% RH and d = 0.018–0.18 mm.

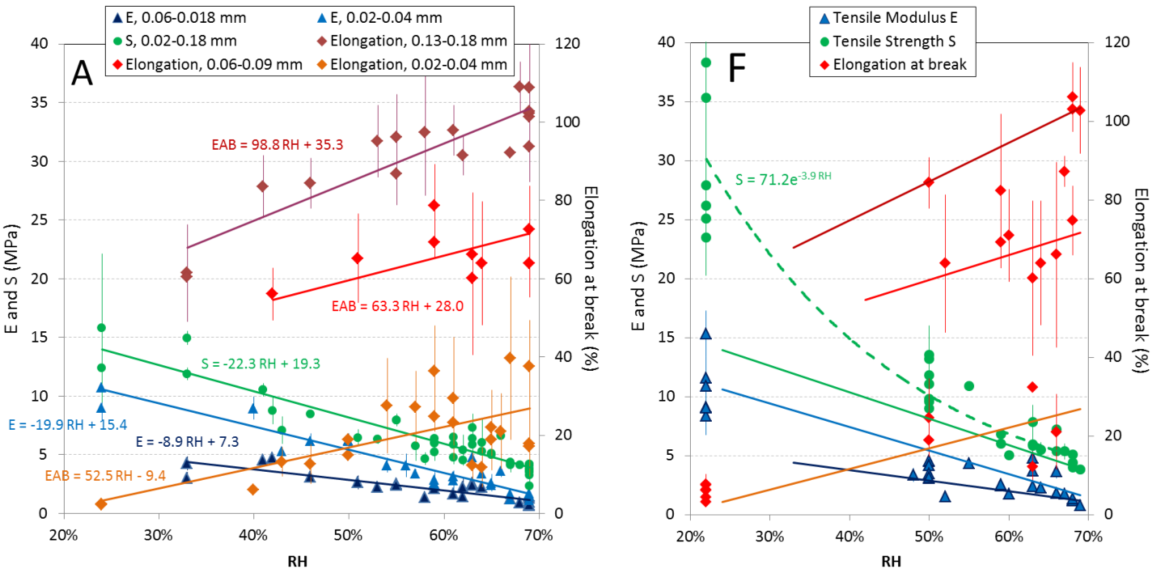
Figure 1. Control A films: tensile modulus (E), tensile strength (S) and elongation at break ("elongation", EAB) from 58% to 70% room humidity (RH), as a function of film thickness.



Elongation at break, EAB, was most affected by film thickness. EAB (%) was well fitted with a polynomial curve: EAB ~ $-3543d^2 + 1198d$, valid for d = 0.018–0.16 mm between 60% and 70% RH, with EAB~100% when d > 0.14 mm, down to EAB~22% when d ~ 0.02 mm. In contrast, commercial plastic wrap was measured as ~0.01 mm-thick with EAB ≥ 200%, but a tensile modulus similar to Control A films. Thickness had a small, approximately linear effect on the tensile modulus, E, of Control A films: thicker films showed lower E and higher EAB, possibly owing to greater molecular mobility in films with more bulk material and more space for polymeric chains to slide against each other and enable film stretching. Tensile strength, S, is mostly a function of the strength of the inter- and intra-molecular network bonds and did not appear to vary with film thickness. Both E and S decreased considerably from RH ~60% to ~70%, and testing data for E and S were split between two curves for samples tested near 60% RH or near 70% RH. The data scattering was due to imperfections in the films, differences in RH history during drying, differences in time of storage and, most of all, possible human error while cutting and loading thin film strips onto the texture analyzer. Scattering is greater with thinner films due to increased fragility and ease of damage during handling and the early breakage during testing.

The tensile properties of Control A films between 24%–69% RH and *d* = 0.02 to 0.18 mm all had an approximately linear, strong dependence on humidity, as presented on Figure 2A.

Figure 2. Effects of RH% at 0.02–0.18 mm of thickness for Control A and F films. (**A**) Control A films: tensile modulus, E, for two different thickness ranges; tensile strength, S, at all thicknesses; elongation at break for three different thickness ranges; and solid trendlines; (**F**) Control F films: data for E, S and elongation at break are plotted against the Control A trendlines of Figure 2A.



As mentioned above, S was apparently not affected by film thickness, and all data fit fairly well on a single curve, while results for E and EAB (*i.e.*, "elongation") were separated between two or three different curves, corresponding to different thickness ranges. Both S and E were very sensitive to humidity and decreased with increasing RH: S dropped from ~14 MPa at 24% RH to ~4 Mpa at 69% RH; and E decreased from ~10.5 MPa at 24% RH to ~1.5 MPa at 69% RH (for thicker films) or from ~4 MPa at 24% RH to ~1 MPa at 69% RH (for thinner films), demonstrating a high hydrophilicity of CaCas/Gly films, which soaked up atmospheric water and became strongly plasticized by it. Similarly, Somanathan (1996) showed that pure casein films absorbed moisture exponentially with increased RH, and water acted as a plasticizer that greatly modified the tensile properties of casein-based films [40]. When we extrapolate the linear fits of Figure 2A to 80% RH, both S and E became close to null at 80% RH at all thicknesses, corresponding to the total mechanical failure of the films, due to excessive water absorption and excessive dissolution of the various electrostatic and ionic bonds within the protein network. Cuq *et al.* (1997) also found that the functional properties of fish protein-based films rapidly changed at high RH, due to disruptive hydrogen bonding between water and proteins, which caused sharp decreases in S and E and increases in EAB [41]. Investigations on hydrophilic films from several other edible materials (corn zein, wheat gluten, methylcellulose and hydroxypropyl cellulose) also found that S decreased linearly with RH between 23% and 75% and increased quadratically with temperature between 5 and 45 °C [42].

The tensile results for Control F films were plotted on Figure 2F using the Control A trendlines from Figure 2A, to compare the humidity and thickness-related mechanical properties of Control F and A films. Control F films had similar tensile modulus and elongation at break to Control A films, but apparently higher tensile strength at low RH, which would suggest a higher bond-density or the presence of stronger bonds within the casein network.

According to Figures 1 and 2, a comparison of the tensile properties (S, E and EAB) between different edible films should only be done at a similar film thickness and identical T and RH conditions. Additionally, the sample geometry and cross-head speed during tensile testing can also affect the tensile results greatly and should be chosen carefully. In two prior studies with identical sample geometry, but a lower crosshead speed of 5 mm/min, Tomasula *et al.* (1998, 2003) measured the tensile properties of 70% CaCas/30% Gly films, made with Formulation F and stored at 23 °C and 50% RH. Films with $d = 0.11–0.15$ mm had values of $S = 1.6–1.9$ MPa [43], which is much lower than $S \sim 10$ MPa in Figure 2F, possibly due to the higher glycerol content or lower crosshead speed; on the other hand, the results for EAB = 0.66%–0.76% fit well within the EAB data on Figure 2. CaCas films containing 20% glycerol had higher tensile strength, $S \sim 5.5$ MPa, but lower elongation at break, EAB ~ 40% [43]. In a second study, Tomasula *et al.* (2003) measured $S = 7.0$ MPa and EAB = 66 MPa for 70% CaCas/30% Gly films with $d = 0.1$ mm, which fits well within the data in Figure 2 [10].

The RH-dependent tensile results for Control A and F films suggest that they may be well-suited for dry commercial applications that require little elasticity, such as coatings for dry foods, like cereals or nuts, or for soluble applications, such as single-serve soup pouches. The thickness of the packaging/coating will considerably affect its properties and cost: thinner films will have higher stiffness and lower cost; thicker films will have better barrier properties (e.g., oxygen) and elasticity, but for a higher cost.

3.2. Incorporation of Citric Pectin (CP) to Calcium Caseinate Films

Citric pectin, a polysaccharide with a long, linear backbone-chain, methoxyl groups grafted to ~60% of its carboxyl groups and few side branches, was incorporated into the casein film formulations at various times and different amounts, to use as a linear-chain crosslinker and to potentially enhance the elasticity, strength and, later, water-resistance of casein films and to broaden the range of potential commercial applications. Table 2 lists the molecular weights and stoichiometric ratios (mass and molar) of the four main functional ingredients involved in the film-making solutions.

According to Table 2, several possible types of inter- and intra-molecular bonds of different strengths and densities may form within the CaCas/Gly/CP network, such as:

- Strong binding of $–COO^-$ from two pectin molecules with Ca^{2+}, forming a pectin gel,
- Ionic binding of Ca^{2+} with two negatively-charged groups on caseins,
- Electrostatic binding of Ca^{2+} with several –OH groups,

- Binding of –OH groups on glycerol with positively-charged groups on caseins,
- Binding of –OH and –COO$^-$ groups on pectin chains with positively-charged casein groups, such as –NH [44],
- Dipole-charge and/or dipole-dipole interactions [44],
- Other,
- Any combination of the above.

The wide variety of possible interactions to form pectin/casein complexes before and during film-casting will depend on the pH and concentration of the film-forming solution. Acidic casein/pectin complexes have been studied by others, but little information is available on the complexation of calcium caseinate (CaCas) with a diluted, high-methoxyl pectin, such as citric pectin (CP) at neutral pH, as is the case here.

The types of bonds favored and the resulting network configuration and mechanical properties were found to depend strongly on the order of mixing of the different components (see the seven different formulations, A through K, in Table 1). Because the molecules at play have widely different shapes, sizes, stoichiometric ratios and functional groups and charge distributions, different mixing sequences can lead to widely different molecular networks in the film-making solutions and the dried films. For example, CP may gel with itself in the presence of Ca^{2+}, or may wrap itself tightly around casein micelles, or individual casein molecules, or may interconnect casein particles loosely, *etc.* There are at least 850 casein molecules available per molecule of pectin, with a compact or spherical (micelles) shape, while CP looks more like a "string". In CaCas:Gly films with a 3:1 ratio, there are ~74 molecules of polar glycerol per molecule of casein or enough to fully coat casein, casein micelles and pectin, depending on the order of the addition of glycerol to the solution.

Experimentally, different formulations and CP amounts appeared to result in different network configurations with highly differing tensile properties. For example, Formulation E films prepared by adding 0.05 to 0.95% CP to Control F solutions as a third step (Table 1) and then dried at ~33% RH and tested at ~35% RH were compared to Control F films prepared and tested on the same days (thickness 0.02–0.03 mm for all). The tensile modulus and tensile strength of all of the E films (0.05%–0.95% CP) were higher than those of the Control F films (0% CP), with a bell shaped curve as a function of % CP and a maximum at ~0.4% CP, while the elongation at break of all E films was lower than that of Control F films and decreased with added CP content, as seen in Table 3. The addition of CP to thin casein films using Formulation E seemed to considerably increase the stiffness of the films at ~35% RH, rendering them more brittle.

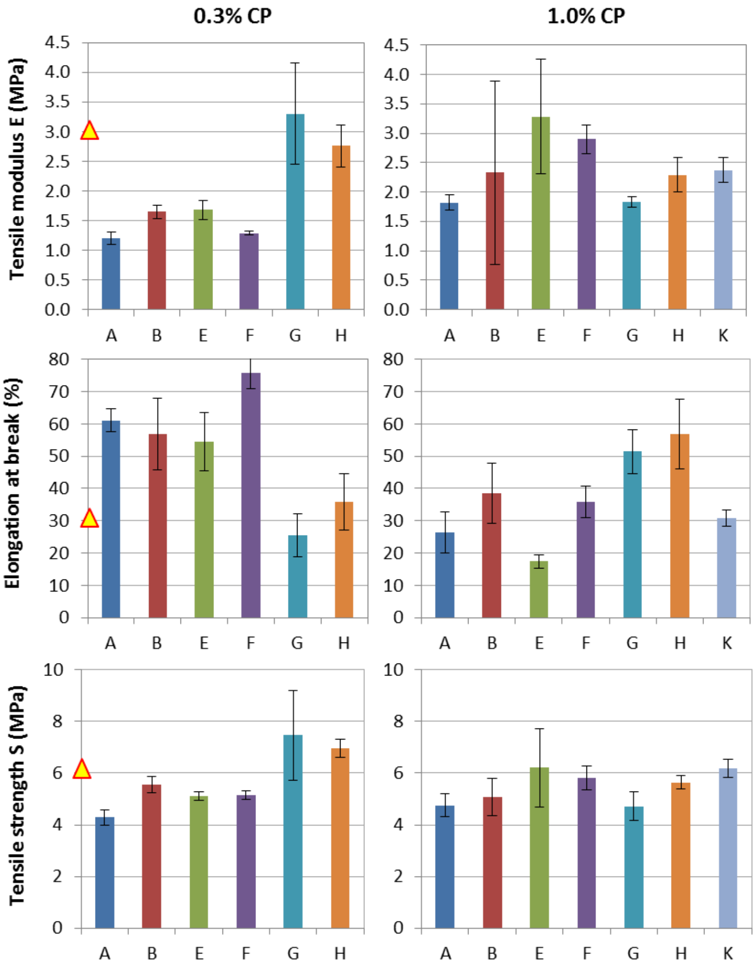
Table 3. The average tensile properties of E films at ~35% RH as a function of CP content.

CP Content	Modulus, *E*	Strength, *S*	Elongation, EAB
0% (Control F)	6 MPa	16 Mpa	20%
0.05%	10 Mpa	26 Mpa	12%
0.4%	13 Mpa	33 Mpa	7%
0.95%	8 Mpa	22 Mpa	3%

Figure 3 presents the average tensile properties for a series of films prepared using the different formulations in Table 1 by adding either 0.3% or 1.0% CP, dried at ~60% RH and tested at

54%–58% RH, with film thicknesses ranging from ~0.035 to ~0.05 mm, to compare the different formulations with each-other and to evaluate the effects of CP on the films' network.

Figure 3. Tensile properties for different film formulations containing 0.3% or 1.0% pectin, measured at 54%–58% RH, for film thicknesses *d* = 0.035–0.05 mm. Triangle symbol: average value for Control A films at ~56% RH and *d* ~ 0.4 mm, according to Figure 2.



The effects of CP content on the tensile properties of the films are strong and very different, depending on the formulation. With 0.3 wt% CP, A, B, E and F films all had comparatively low moduli, medium strength and high elongation at break; while G and H films had higher strength, much higher moduli and medium-to-low elongation at break. After increasing the CP content to 1.0 wt%, the strength and modulus of G films changed from being the highest, at 0.3% CP, to one of the lowest, at 1% CP, while their elongation at break approximately doubled; H films showed a behavior change similar to that of G, but to a lesser extent. On the opposite side, the tensile strengths of A, B, E and F did not vary much with the addition of more CP, while their tensile moduli increased considerably, and their elongations at break decreased greatly. These opposite behaviors suggest that there are indeed widely different molecular interactions at play within the CaCas-Gly-CP mixtures, depending on the mixing sequence of the ingredients, as mentioned above, as well as different reconfigurations of the polymeric network when more CP is added, which changes the stoichiometric ratios of the ingredients.

3.3. Micrographs of the Films at Different CP Contents

Micrographs of each film were captured to gain an understanding of the molecular interactions and network configurations in CaCas/Gly/CP films as a function of formulation and composition. Because dramatic differences in the films structures were visible to the naked eye, a low magnification of 4× was used to record the macroscopic structure of the films. Figures 4 and 5 show micrographs of Films A through K at 4× magnification with 0.3% CP and 1.0% CP, respectively, captured at ~60% RH. As is clearly visible in Figure 4, even though the composition of all of the films was identical, pectin intercalated itself within the CaCas/Gly network in different manners when a small amount (0.3% CP) was added to the film-making solution using the different formulations, to form structures of different sizes and shapes that were more or less densely distributed throughout the film.

Figure 4. Micrographs of film Formulations A through K containing 0.3 wt% CP, captured at ~60% RH with 4× magnification.

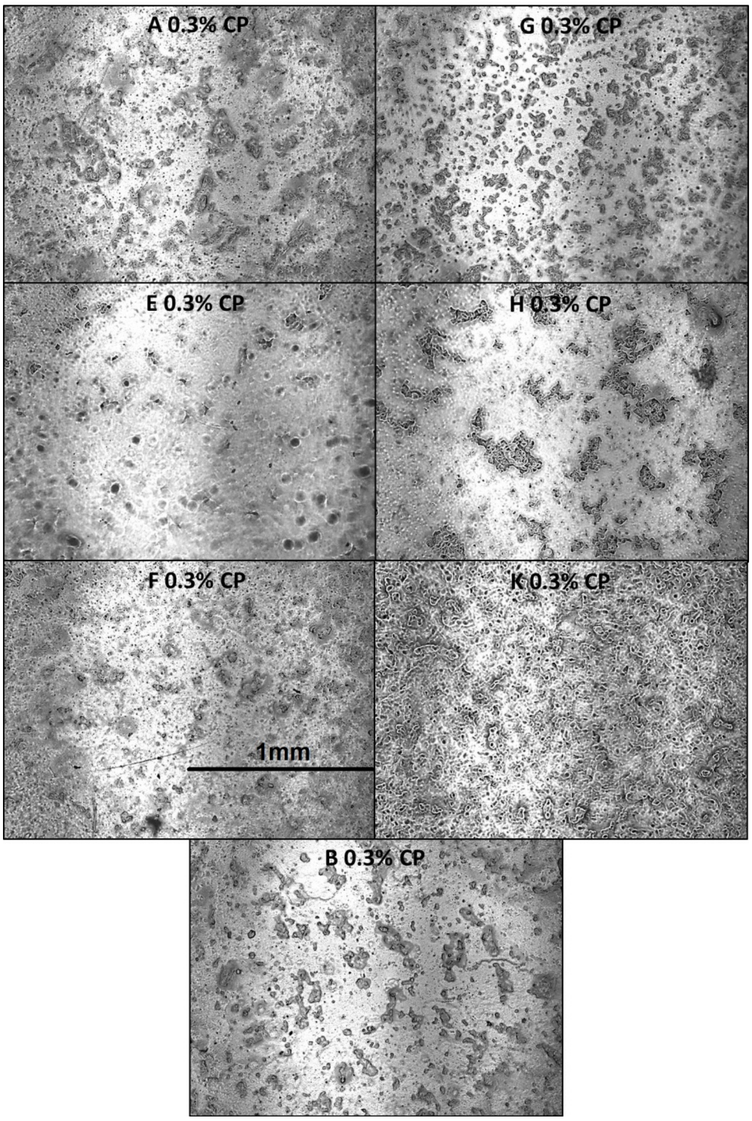
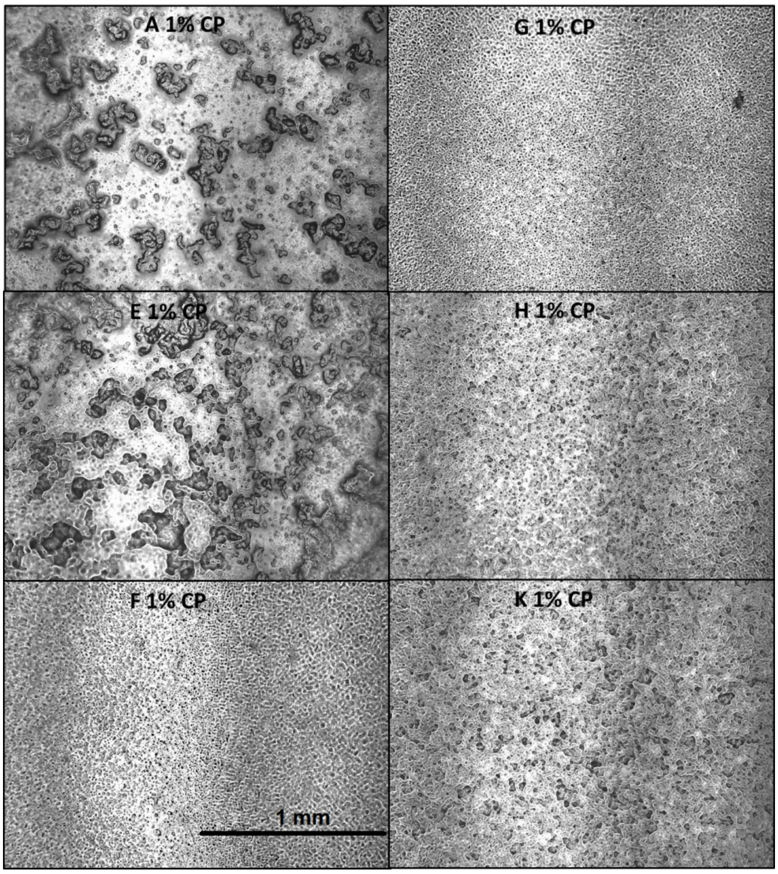


Figure 5. Micrographs of film Formulations A through K containing 1.0 wt% CP equilibrated at ~60% RH, with 4× magnification.

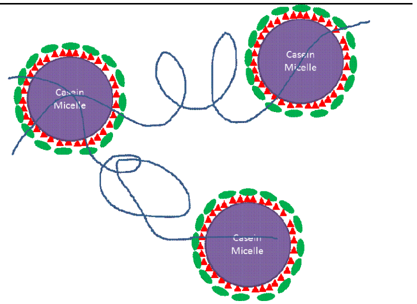
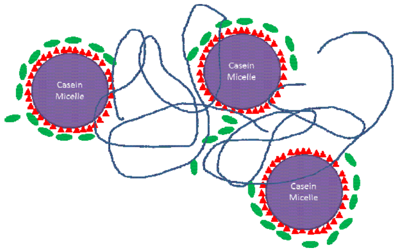
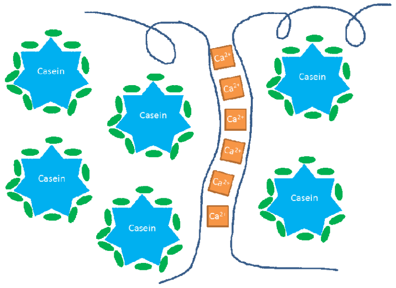


After adding 1.0% CP to all the film formulations, one of the most obvious differences between Figures 4 and 5 is that several formulations that formerly looked aggregated or segregated with 0.3% CP now appear as a tightly packed network of small homogenous particles. With film Formulations F, G, H and K, a larger pectin content seemed to favor a new equilibrium configuration composed of tightly knit, small particles. G seemed the tightest and most homogeneous network, followed by F, while H and K also appeared homogeneous, but with a more "porous" aspect. On the contrary, Films A and E contained lots of large pectin gel particles. Gel pockets appear unconnected to each other in Film A, causing mainly steric hindrance to the casein network; while the pectin gel pockets seem to form a somewhat continuous 3D network in Film E, which would impart Film E with some of the mechanical properties of a three-dimensional pectin gel instead of those of the casein polymer network and would explain the dramatic tensile properties changes between 0.3% and 1% CP in Figure 3, where E films with 1% CP were the strongest and stiffest of all.

3.4. Mechanistic Interpretation

According to Figures 3, 4 and 5, the following interpretation of the possible mechanisms of the network formation and the hypothetical structures of the films as a function of the formulation and pectin content was made and is presented in Table 4.

Table 4. The proposed mechanism of the formation and structure of CaCas/Gly/CP film networks, for Formulations A through G with 0.3% or 1.0% CP.

Form	Mixing Sequence	Hypothetic Mechanism	Possible Network Structure	Schematic
A	1. Water + CaCas 2. Gly 3. CPsol	1. Casein micelles + calcium ions. 2. Glycerol coats casein micelles. 3. Coiled pectin loosely binds to a few available micelles sites and strings micelles together, while excess pectin forms gels with calcium.	**0.3% CP:** coiled pectin acts as an elastic chain-extender for the micelles network ("pearl necklace" structure), increasing EAB and reducing S and E. **1% CP:** random, isolated pockets of CP/Ca^{2+} gel become larger. Steric disruption of micelles network increases S and E, greatly reduces EAB.	
B	1. Water + CaCas 2. CPsol 3. Gly	1. Casein micelles network + calcium ions. 2. Some coiled pectins interconnect casein micelles using the many available sites, while others form gels with calcium. 3. Glycerol coats the whole casein/pectin network.	**0.3% CP:** similar to A, but stiffer and less stretchy, because coiled pectins bind more strongly to uncoated micelles. **1% CP:** additional pectin chains both reinforce the micelles network and form more gel pockets, increasing E and S and moderately reducing mobility and EAB.	
E	1. Water + Gly 2. CaCas 3. CPsol	1. Casein molecules and/or micelles become fully coated with glycerol during mixing; calcium ions in solution. 2. Coiled pectin intercalates between caseins, with few available casein sites to bind with; at a higher CP concentration, pectin chains find each other and gel with calcium.	**0.3% CP:** coiled pectin disperses homogeneously and acts as chain-extender for casein network, increasing EAB; steric disruption of network reduces S and E. **1% CP:** narrow, interconnected pockets of hard CP/Ca^{2+} gel stiffen the network like bones and prevent mobility and elongation.	

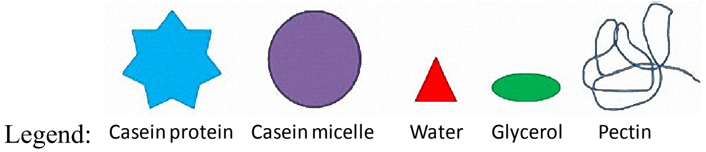
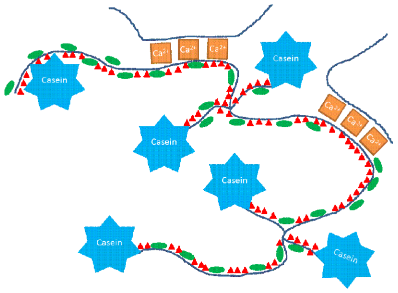
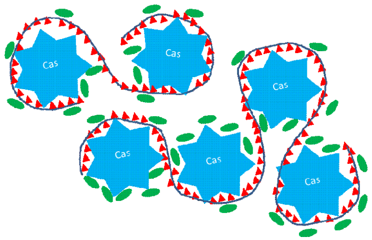
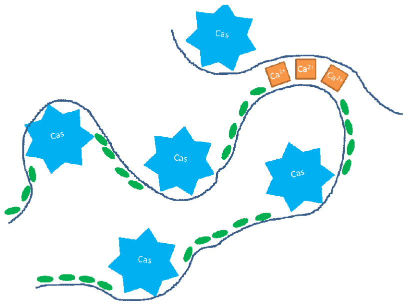


Table 4. *Cont.*

Form	Mixing Sequence	Hypothetic Mechanism	Possible Network Structure	Schematic
F	1. Water + Gly 2. CPsol 3. CaCas	1. Pectin chains become fully hydrated (uncoiled) and coated with glycerol. 2. Casein molecules attach to a few available sites on the pectin chains. Short pectin sections may gel together with calcium ions. 3. Excess caseins form micelle network.	**0.3% CP:** network of casein micelles, with segregated pockets of pectin grafted with caseins and crosslinked to other pectin chains by short gel sections; pectin chains provide mobility, increase elongation and reduce stiffness. **1% CP:** higher pectin:casein ratio produces a homogeneous network of casein-grafted pectins; denser gel-crosslinks increase stiffness and reduce mobility and elongation.	
G	1. Water + CPsol 2. CaCas 3. Gly	1. Fully hydrated, uncoiled pectin chains. 2. Pectin wraps around casein molecules tightly. Excess caseins form micelle network. 3. Glycerol coats pectin/casein complexes and micelles.	**0.3% CP:** network of casein micelles, with small segregated pockets of pectin/casein complexes that increase strength of the micelle network while slightly hindering mobility. **1% CP:** higher pectin:casein ratio produces a homogeneous network of pectin/casein complexes; less caseins per pectin chain form a looser network with increased mobility, lower stiffness.	
H	1. Water + CPsol 2. Gly 3. CaCas	1. Fully hydrated, uncoiled pectin chains, slightly coated with glycerol. 2. With fewer available sites, pectin wraps loosely around caseins. Excess caseins form micelle network. 3. Unbound pectin chain sections gel together with calcium ions.	**0.3% CP:** Similar to G; however, looser pectin/casein complexes with more gelled pectin chain sections form larger, looser segregated pockets in the micelle network and affect E, S and EAB less. **1% CP:** higher pectin:casein ratio produces a homogeneous network of loose pectin/casein complexes; less caseins per pectin chain form a looser network with increased mobility, lower stiffness.	

4. Conclusions

Film thickness and relative humidity during film storage and sample preparation have strong effects on the tensile properties of CaCas/glycerol films and must therefore be carefully controlled and recorded whenever measuring the mechanical properties of casein-based films in order to compare different compositions and formulations.

While much work must be done to fully understand and characterize the structure-property relationship of CaCas/Gly/CP films as a function of compositional and environmental conditions (T and RH) for each different formulation, it is already apparent that very small amounts of citric pectin (less than 1 wt%) can considerably modify the macrostructure of the casein/glycerol film network in various ways and, thereby, alter the mechanical properties of the films and, hypothetically, also the barrier properties, in opposite ways. Future studies should include the careful optimization of the formulation-composition relationship, adding higher pectin contents to the study, as well as pH and temperature changes to activate certain types of network bonds in the solution and during drying and to target films that possess both improved strength and elasticity or a reduced humidity and temperature sensitivity. Edible packaging films that are more mechanically versatile and environmentally stable would better resist real processing and end-use conditions and enable a variety of new commercial applications.

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Author Contributions

Peggy Tomasula and Laetitia Bonnaillie conceived and directed the research; Han Zhang and Serife Akkurt prepared casein/glycerol/pectin films, measured the physical and tensile properties, tabulated the results, and wrote part of the manuscript; Han Zhang captured the microscopic images; Kit Yam provided advisory and reviewed the manuscript; Laetitia Bonnaillie analyzed and interpreted the data, drew the figures and wrote the manuscript; Peggy Tomasula edited and reviewed the manuscript draft and approved the final version.

Conflicts of Interest

Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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