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Comparative Study on the Effect of Calcium Chloride on the Physicochemical Properties of Glycerol- and Sorbitol-Plasticized Pea Protein Isolate Films

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Abstract: Ca^{2+} can boost protein-protein interactions and, if present at an appropriate level, can potentially improve the physicochemical properties of protein gels and films. This study aimed to determine the effect of $CaCl_2$ (0, 0.01 0.025 0.05% w/w) on the optical, water affinity, and mechanical characteristics of glycerol (Gly)- and sorbitol (Sor)-plasticized pea protein isolate (PPI) films. The $CaCl_2$ caused darkening and a color shift of the films from yellow to yellow-green. Additionally, decreased light transmission, particularly in the UV range, and reduced moisture content were observed. The $CaCl_2$ decreased the water vapor permeability (WVP) of the Gly-plasticized film by an average of 20%, with no effect on the Sor-plasticized film. All films were completely soluble in water. The $CaCl_2$ negatively impacted the mechanical integrity of the films, reducing the tensile strength of the Gly- and Sor-plasticized films by ~16% and 14-37%, respectively. Further increases in $CaCl_2$ content (0.1% and 0.2% w/w) led to concentration-dependent cracks, suggesting protein over-crosslinking. In summary, the incorporation of $CaCl_2$ into PPI films did not provide significant benefits and actually worsened key properties such as transparency and mechanical strength. The type of plasticizer influenced how $CaCl_2$ affected some properties of the PPI film.

Keywords: edible films; pea protein; water vapor permeability; opacity; tensile strength

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1. Introduction

Recently, the European Union (EU) has implemented the single-use plastics directive [1], aimed at mitigating the environmental impact of single-use plastic products (including certain packaging) and promoting a shift towards a circular economy. This involves replacing single-use plastics with more sustainable alternatives, such as bioplastics. Among these, biodegradable materials derived from renewable resources present a particularly eco-friendly option. Using food-grade ingredients such as proteins, polysaccharides, or lipids, and employing suitable manufacturing methods, can result in packaging that is edible along with its contents. Edible packaging represents a niche alternative in eco-friendly packaging, ideal for scenarios where plastic packaging is restricted. Notable examples include collagen casings for sausages, gelatin, pullulan, starch, hydroxypropyl methylcellulose capsules, and certain food additives with coating-forming properties applied to fresh fruits [2].

Proteins offer a wider range of functions compared to polysaccharides, largely due to their structural complexity and dynamic nature. Their properties can differ significantly based on their origin and source. When selecting an ideal protein for packaging production, factors such as barrier performance, mechanical strength, optical properties, processability, availability, cost, as well as legal and environmental considerations must be taken into account. Additionally, structure-modifying methods and technologies could be considered to enhance or create novel attributes to protein-based materials.

Eco-design of packaging begins with the selection of raw materials. Approximately 80% of Earth's biomass consists of plants [3]. Growing plants requires less energy than raising animals. Moreover, plants absorb CO_2 during growth, helping to offset the carbon footprint [4]. Plants can be cultivated sustainably on a large scale, ensuring a continuous supply of raw materials. Legumes are considered an inexpensive source of high-quality proteins with potential for producing edible packaging [5]. Among them, pea protein is gaining popularity in the global food industry, mainly as an alternative to soy protein. The reason is that pea plants can be grown in more temperate climates than soy [6,7], and unlike many protein containing foods (e.g. cereals containing gluten, eggs, fish, peanuts, soybeans, milk, nuts, lupin (and products thereof, with some exceptions)), it is less likely to cause allergies, therefore is not listed as food that must be declared as allergens in the EU [8].

Previous studies have shown that factors such as plasticizer type and concentration, heating, pH, and the addition of lipids and polysaccharides significantly influence the barrier, optical, and mechanical properties of pea protein isolate (PPI)-based films [9, 10] [11, 12]. For instance, films plasticized with sorbitol (Sor) were less permeable to water vapor, stronger, and less stretchable compared to glycerol (Gly)-plasticized films. Thermal treatment of film-forming solutions (FFSs) improved the mechanical strength and transparency of PPI films. Increasing the pH from 7 to 11 enhanced the mechanical strength of Gly-plasticized films, but not those plasticized with Sor. At the microscopic level, films obtained at neutral pH showed PPI particles conglomerated into a continuous layer [9, 10]. As known, alkalization improves the solubility of pea proteins [13] and presumably cohesiveness of the films obtained from them [10]. Nevertheless, considering that films produced under alkaline conditions may not be safe for consumption (as they could irritate mucous membranes and the digestive tract), producing films under neutral pH conditions seems to be the most reasonable option.

Some studies have shown that proteins can be customized using both chemical and enzymatic crosslinking agents to produce edible films with enhanced barrier and mechanical properties [14]. The use of calcium ions is a simple and cost-effective method to facilitate protein-protein interactions. Calcium-induced protein aggregation occurs through: (i) electrostatic shielding, (ii) crosslinking of anionic (carboxyl) groups via protein-Ca^{2+}-protein bridges, and (iii) ion-specific hydrophobic interactions [15]. Existing research on casein [16–18], soy protein isolate (SPI) [19], or whey protein isolate (WPI) films [20] indicate that the impact of calcium ions on the properties of protein-based films cannot be generalized, as it depends not only on the type of protein and calcium concentration but also on the type and amount of plasticizer used. It is worth mentioning that, in addition to enhancing the functionality of protein films, the inclusion of calcium (a nutrient crucial for the mineralization of bones and teeth) can also improve their nutritional value. This is particularly relevant as calcium's bioavailability increases when it is bound to proteins [21].

In the present study, we hypothesized that the interaction between Ca^{2+} and pea protein might improve some functional properties of the films through a protein crosslinking mechanism. The objective was to compare the effects of increasing concentrations of $CaCl_2$ on the optical, water affinity, and mechanical properties of Gly- and Sor-plasticized PPI films.

2. Materials and Methods

2.1. Materials

Propulse PPI (82 ± 2% protein content) was donated by Nutri-Pea (Portage la Prairie, Canada). Gly and D-Sor (min. 99.5%) were obtained from Sigma Chemical Co. Anhydrous $CaCl_2$ (analytical grade) was obtained from POCh (Gliwice, Poland).

2.2. Methods

2.2.1. Preparation and Conditioning of Films

The films were obtained from 10% (w/w) PPI solutions containing 5% (w/w) Gly or Sor and varying concentrations of $CaCl_2$ (0, 0.01, 0.025, 0.05, 0.1, and 0.2% w/w). The mixture (200 g) containing PPI, plasticizer (Gly or Sor), and distilled water was neutralized with concentrated NaOH solution to pH 7 and heated in a water bath at 90 °C for 20 min with constant stirring. Subsequently, $CaCl_2$ was added, and the FFSs were mixed using a H-500 homogenizer (Pol-Eko Aparatura, Poland) at 20,000 rpm for 5 min. The FFSs were then cooled to 25 °C with continuous stirring, rehomogenized at 14,000 rpm for 1 min, degassed, and cast onto leveled polystyrene Petri dishes (Nunc, Roskilde, Denmark). To maintain consistent film thickness, 1.65 g of total solids was cast over an area of 145 cm^2. The FFSs were dried at ~25°C and 50 ± 5% relative humidity (RH) for about 24 h. The resulting films were cut into samples, and their thickness was measured using a Mitutoyo No. 7327 micrometer

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(Mitutoyo, Tokyo, Japan). The samples were then conditioned at 25°C and 50% RH for 48 h in a Sanyo Versatile Environmental Test Chamber MLR-350H (Sanyo Electric Biomedical Co. Ltd., Oizumi-Machi, Japan).

2.2.2. Optical Properties

The color values (CIE L*a*b* and C*h°) of the film samples (1 × 4 cm) were measured with a colorimeter X-RiteColor 8200 (X-Rite Inc., USA) on the black background ($L^* = 25.63$, $a^* = -0.12$, $b^* = -0.47$). The total color difference (ΔE) was calculated by Eq. (1)

- $\Delta E = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{0.5}$ (1)

where Δ is difference between the color parameters of films without $CaCl_2$ (control) and those with $CaCl_2$.

The light barrier properties of the film samples (1 × 4 cm) were measured using a spectrophotometer (Lambda 40, Perkin–Elmer, Shelton, CT, USA) at selected wavelengths between 200 and 800 nm. The opacity (Op) of the films was calculated by Eq. (2):

- $Op = A_{600}/t$ (2)

where A_{600} is the absorbance at 600 nm and t is the film thickness (mm).

The optical analyses were conducted in five repetitions.

2.2.3. Water Affinities

The specimens (2 x 2 cm) were dried in an oven at 105 °C for 24 h. The moisture content (MC) was determined by calculating the percentage of water removed from the samples. Solubility (So) was expressed as the percentage of film solubilized in water containing 0.02% sodium azide. The specimens (2 x 2 cm) were shaken with 30 ml of distilled water in 50 ml Falcon test tubes in an ES-60 incubator (MIULAB, China) at 25 ± 1 °C and 170 rpm for 24 h. Both the MC and So analyses were conducted in quadruplicate

Water vapor permeability (WVP, $g\ m\ mm^{-2}\ day^{-1}\ kPa^{-1}$) was calculated as follows:

- $WVP = (WVTR \times t)/\Delta p$ (3)

where: WVTR is the water vapor transmission rate ($g\ m^{-2}\ day^{-1}$) measured gravimetrically based on the ISO 2528 method [22], t is the mean film thickness (mm), and Δp is the difference in the water vapor pressure (kPa) between two sides of the film.

Briefly, poly(methyl methacrylate) permeation cell cups with an internal diameter of 7.98 cm (exposed film area = 50 cm^2) and an internal depth of 2 cm were filled with 10 g of anhydrous calcium chloride (0% RH). The 10 cm diameter film samples were then placed over the circular openings and secured with O-ring rubber gaskets and screw tops. The cups were placed in a test chamber at 25 °C and 50% RH. The weight gain was monitored over 12 h, with weights recorded at 2-h intervals. The slopes of the steady-state (linear) portion of the weight gain versus time curves were used to calculate the WVTR.

The WVP analyses were performed in triplicate.

2.2.4. Mechanical Properties

Tensile strength (σ_{max}), elongation at break (ε_b), and elastic modulus (EM) were determined using a TA-XT2i texture analyser equipped with a 50 kg load cell (Stable Micro Systems, Godalming, UK) performed following the procedure outlined in PN-EN ISO 527-1, 2, 3:1998, with some modifications [10]. Dumbbell-shaped film samples were mounted on the analyser with an initial grip separation at 80 mm and stretched with a speed of 1 mm s^{-1}. The σ_{max}, ε_b, and EM were calculate d using equations (4)–(6), respectively:

- $\sigma_{max} = F_{max}/A$ (4)

where F_{max} is the maximum load for breaking the film (N) and A is the initial cross-sectional area (thickness × width, mm^2) of the specimen,

- $E = (\Delta L/L) \times 100$ (5)

where ΔL is the difference in the length at the moment of fracture and L is the initial gage length (mm),

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- $EM = (\sigma_2 - \sigma_1)/(\varepsilon_2 - \varepsilon_1)$ (6)

where ε_1 is a strain of 0.0025 (0.25%), ε_2 is a strain of 0.01 (1%), σ_1 (MPa) is the stress at ε_1, and σ_2 (MPa) is the stress at ε_2.

2.2.5. Statistical Analysis

Differences among the mean values of the data were tested for statistical significance at the $p < 0.05$ level using analysis of variance (STATISTICA 13.3, StatSoft Inc., Tulsa, OK, USA) and Fisher's test.

3. Results and Discussion

It was found that that coherent PPI films can be produced from FFSs containing up to 0.05% $CaCl_2$ (equivalent to a concentration of 4.5 mM). Increasing the $CaCl_2$ concentration to 0.1% and 0.2% w/w led to the formation of cracks in the films, with the severity depending on the concentration (data not shown). This issue may arise from over-crosslinking of the protein matrix (i.e., excessively strong bonding of polypeptide chains), leading to shrinkage of the FFS during evaporation. The detrimental impact of excessively high calcium ion levels on the integrity of protein-containing gels and films is a well-documented phenomenon [20, 23].

3.1. Optical Properties

Color and appearance are crucial factors that consumers consider when selecting a food product. The PPI films were transparent but had a yellow tint, which could limit their application possibilities. This color was due to the inherent creamy yellow hue of the PPI, indicating that some pea-origin color components, such as carotenoids and chlorophylls [24], were not removed during the isolation process. It's important to note that the final color properties of PPI also strongly depend on the drying method used [25].

Regardless of $CaCl_2$ concentration, the Gly- and Sor-plasticized films did not show any significant differences in color parameters ($p > 0.50$) (Table 1). Even at the lowest concentration, $CaCl_2$ caused a noticeable decrease in brightness (L* parameter) and red color contribution (a* parameter). A ΔE value above 3 (Table 1) indicates that even an untrained observer could notice the difference [26] between films with and without $CaCl_2$. Increasing the concentration of $CaCl_2$ further reduced these parameters and additionally decreased the yellow color intensity (b* parameter). Consequently, the ΔE increased above 11, meaning that an observer would notice two distinctly different colors [26]. The films containing intermediate and the highest amount of salt did not differ in color (Table 1). Compared to the control, these films exhibited an increased h° (~96° vs. ~126°, Table 1), indicating a shift in color from yellow to yellow-green [27]. The darkening and reduced color saturation (C* parameter) of the $CaCl_2$-added films can likely be attributed to chemical reactions between the salt and the components of the material. It is known that the dissolution of $CaCl_2$ involves the hydration of each ion: Cl^- combine with protons (H^+) in water to form hydrochloric acid (HCl), a strong and corrosive acid, while Ca^{2+} combine with hydroxide anions in water to form calcium hydroxide ($Ca(OH)_2$), a weak but corrosive base. Notably, $Ca(OH)_2$ or calcium salts are used in the nixtamalization process (alkaline cooking), which causes maize kernels/flour (and resulting tortillas) to darken, depending on the processing conditions [28]. This discoloration is explained by formation of dark tones by phenolic compounds [29]. Additionally, calcium ions can interact with anionic components (e.g. proteins) [30], possibly also contributing to color changes.

Table 1. Effect of $CaCl_2$ concentration on the color parameters of glycerol- (Gly) and sorbitol (Sor)-plasticized pea protein isolate films.

Plasticizer	$CaCl_2$ (%)	L*	a*	b*	h (°)	C*	ΔE
Gly	0	41.39 ± 0.01^{cd}	-0.21 ± 0.03^{c}	2.13 ± 0.07^{cd}	95.77 ± 0.39^{a}	2.14 ± 0.07^{bc}	-
	0.01	37.60 ± 3.37^{b}	-0.69 ± 0.13^{b}	2.50 ± 0.16^{d}	106.41 ± 7.70^{a}	2.60 ± 0.18^{d}	3.97 ± 3.15^{a}
	0.025	30.87 ± 1.24^{a}	-1.00 ± 0.09^{a}	1.79 ± 0.51^{bc}	126.01 ± 17.71^{c}	2.06 ± 0.49^{bc}	10.56 ± 1.23^{b}
	0.05	29.80 ± 0.40^{a}	-1.04 ± 0.02^{a}	1.44 ± 0.20^{ab}	126.17 ± 4.42^{c}	1.78 ± 0.15^{ab}	11.64 ± 0.40^{b}
Sor	0	41.72 ± 0.04^{d}	-0.28 ± 0.02^{c}	2.30 ± 0.06^{d}	96.85 ± 0.32^{a}	2.32 ± 0.06^{cd}	-
	0.01	38.46 ± 3.23^{bc}	-0.77 ± 0.24^{b}	2.27 ± 0.48^{d}	108.40 ± 10.89^{ab}	2.42 ± 0.39^{cd}	3.36 ± 3.19^{a}

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	0.025	30.16 ± 0.62 ^{a}	-1.04 ± 0.08 ^{a}	1.80 ± 0.11 ^{bc}	126.69 ± 7.96 ^{c}	2.08 ± 0.05 ^{bc}	11.60 ± 0.61 ^{b}
	0.05	30.03 ± 0.44 ^{a}	-0.97 ± 0.08 ^{a}	1.25 ± 0.06 ^{a}	122.31 ± 7.80 ^{bc}	1.58 ± 0.06 ^{a}	11.76 ± 0.45 ^{b}

$^{a\text{-}d}$ Values with the different superscript letters within one column are significantly different ($p < 0.05$).

The control Gly- and Sor- had similar the UV/VIS light barrier properties (Figure 1). Consistent with previous works [9, 11, 12], the films exhibited excellent UV-B (280–315 nm) and UV-C (100-280 nm) blocking properties. Namely, the transmittance of control films noted at the UV-B spectrum was below ~5% (Figure 1). Barrier properties of protein films against UV light are mainly due to the tyrosine and tryptophan absorption (~280 nm) and peptide group absorption (190–220 nm spectral range) [31]. It is worth noting that the films also provided a certain barrier against long-wave UV (UV-A), which is important, at this radiation is not absorbed by the ozone layer. The transmittance noted at this region was between ~5-60% (Figure 1). In this respect, the PPI films were better UV blockers than films made from porcine gelatin or whey protein isolate [32, 33]. The reason may be their yellow coloring (Table 1).

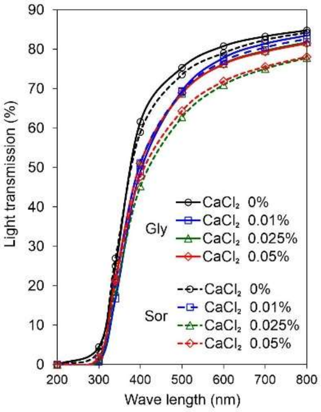

Figure 1. Effect of $CaCl_2$ concentration on the light transmission of glycerol- (Gly) and sorbitol (Sor)-plasticized pea protein isolate films.

The incorporation of $CaCl_2$ reduced light transmission, especially in the UV range (Figure 1). For example, at $\lambda = 400$ nm, the transmittance was reduced from approximately 60% to ~50% and ~46% for Gly- and Sor-plasticized films, respectively. Presumably, the light was multiply scattered by the Ca^{2+}-induced protein microaggregates and/or salt microcrystals that could be formed as a result of water evaporation during film formation. A decrease in light permeability was also observed by Fang et al. (2002) [20] after incorporating $CaCl_2$ (5 and 10 mM) into WPI films.

Regardless of $CaCl_2$ concentration, the Sor-plasticized PPI films were slightly less transparent compared to the Gly-plasticized counterparts ($p < 0.05$, Table 2). A possible explanation for this might be recrystallized Sor, which scattered light. Instantaneous recrystalisation of this polyol in some biopolymeric films, including the PPI-based, was observed previously [10, 33]. In various processes, such as crystallization or solidification, crystals can provide a template or surface for the formation of new crystals. Therefore, it is possible that Sor crystals could act as nucleation sites for $CaCl_2$ crystallization, or *vice versa*. Consistent with the present results, previous studies have shown that a gelatin-based film plasticized with sorbitol was more opaque compared to a film plasticized with glycerol [32, 34]. Comparatively, the transparency of the PPI films is similar to the Sor-plasticized SPI film [34], but lower than that of gelatin and WPI films plasticized with Gly [32].

Table 2. Effect of $CaCl_2$ concentration on the opacity (Op), moisture content (MC), water vapor permeability (WVP), and solubility (So) of glycerol- (Gly) and sorbitol (Sor)-plasticized pea protein isolate films.

Plasticizer	$CaCl_2$ (%)	Op (A_{600}/mm)	MC (%)	WVP (*)	So (%)
Gly	0	0.90 ± 0.02 ^{a}	29.46 ± 1.14 ^{d}	18.60 ± 0.88 ^{c}	100.00 ± 0.00 ^{a}
	0.01	1.09 ± 0.05 ^{bc}	26.88 ± 1.42 ^{c}	14.91 ± 0.77 ^{b}	100.00 ± 0.00 ^{a}
	0.025	1.19 ± 0.06 ^{d}	27.63 ± 1.32 ^{c}	14.43 ± 0.98 ^{b}	100.00 ± 0.00 ^{a}
	0.05	1.19 ± 0.05 ^{d}	27.34 ± 1.50 ^{c}	15.51 ± 1.93 ^{b}	100.00 ± 0.00 ^{a}

Sor	0	1.02 ± 0.08 ^{b}	11.50 ± 0.44 ^{b}	1.40 ± 0.04 ^{a}	100.00 ± 0.00 ^{a}
	0.01	1.13 ± 0.06 ^{cd}	6.71 ± 0.12 ^{a}	0.81 ± 0.20 ^{a}	100.00 ± 0.00 ^{a}
	0.025	1.40 ± 0.08 ^{e}	6.62 ± 0.42 ^{a}	1.19 ± 0.16 ^{a}	100.00 ± 0.00 ^{a}
	0.05	1.43 ± 0.01 ^{e}	6.69 ± 0.57 ^{a}	1.43 ± 0.07 ^{a}	100.00 ± 0.00 ^{a}

$^{a-d}$ Values with the different superscript letters within one column are significantly different ($p < 0.05$). * g m mm^{-2} day^{-1} kPa$^{-1.}$

3.2. Water Afinities

The control film plasticized with Sor exhibited about 2.5 times lower moisture content (MC) and 16 times lower WVP than the film with Gly ($p < 0.05$, Table 2). These differences can be attributed to the distinct water-binding capacities of the plasticizers. Although sorbitol has six hydroxyl groups, they are less accessible for binding with water compared to the three hydroxyl groups of the more compact glycerol. Specifically, at 25°C and 50% RH, the hygroscopicity and water-holding capacity of Sor are 1 and 21 H_2O mg/100 mg, respectively, while for Gly these values are 25 and 40 H_2O mg/100 mg, respectively [35]. Since the Sor-plasticized films absorb less moisture [9], they consequently allow less water vapor to pass through (Table 2). It is important to note, however, that hygroscopicity is a key factor but not the sole determinant of a material's WVP.

Regardless of the type of plasticizer used, the incorporation of $CaCl_2$ decreased the MC of the films in a concentration-independent manner ($p < 0.05$, Table 2). This reduction may be due to Ca^{2+}-induced protein-protein linkages, which consequently inhibited the possibility of protein-water interactions. It has been shown that Ca^{2+} can alter the molecular conformation of proteins, contributing to the formation of hydrogen bonds, disulfide bonds, and hydrophobic interactions during gelation. Consequently, it can affect the water-binding capacity of the gel system, either increasing or decreasing it, depending on Ca^{2+} concentration [36, 37]. To support this, Arabestani et al. (2013) [38] observed that the incorporation of $CaCl_2$ (0.1-1%, w/w) significantly increased the surface hydrophobicity of vetch protein film. However, only the highest $CaCl_2$ concentration (1%, w/w) caused a significant reduction in the MC. The authors speculated that since the pH of the FFS was above the isoelectric point of the protein, the predominant negative charges on the protein molecules were bound by the calcium ions to form compact network. These interactions might inhibit the charged sites from interacting with water, thereby enhancing the protein's hydrophobicity.

The salt addition did not affect the barrier properties of the Sor-plasticized film but reduced the WVP of the Gly-plasticized PPI film by an average of 20% (Table 2). The differential impact of $CaCl_2$ on the WVP of films containing glycerol and sorbitol suggests that the plasticizer type can influence the interactions between the salt ions and pea protein. Literature reports various effects of calcium ion addition on the WVP of protein films. Fang et al. (2002) [20] found that despite the cross-linking of the protein matrix, the addition of $CaCl_2$ (both 5 and 10 mM) to Gly-plasticized WPI films did not significantly influence WVP across the tested RH range (~30-75%). Moreover, doubling the Gly concentration, combined with the protein aggregation effect at 10 mM $CaCl_2$, increased WVP due to pore formation. Park et al. (2001) [19] found that the addition of $CaCl_2$ did not affect the water barrier properties of glycerol-plasticized SPI film, while a 30% reduction in WVP was observed with calcium sulfate incorporation. The authors attributed this to calcium bridges that maximized interactions between negatively charged soy protein molecules, enhancing network coherence. Also, the $CaCl_2$-induced tightening effect reduced the WVP by 7-18% and 42% in vetch protein and sodium caseinate-based emulsion film films, respectivetly [17, 38]. It should also be mentioned that the calcium caseinate-based film exhibited lower WVP than the sodium caseinate-based film, which is also indirect evidence that the presence of calcium ions contributes to enhancing the water barrier properties [17, 18].

All tested PPI films were completely soluble in water at 25°C (Table 2). A similar finding was also reported for the sorbitol-plasticized SPI film [34]. A note of caution is due here since two methods for film solubility determination are used by researchers. Namely, the heat-dried (residue after oven drying for moisture content determination) or conditioned (25°C, 50% RH) film samples are used. Solubility (also known as total soluble matter) values obtained using these two methods are different. This is because the dehydro-thermo-treating (at 105°C) of films develops tensional and compressional stresses in the material, which improves its integrity throughout the soaking procedure *[33].*

Therefore, only the second method, which was used in this study, shows an accurate behavior of films after coming into contact with water.

This study has been unable to demonstrate that $CaCl_2$ can reduce the So of the PPI films (Table 2). Such a possibility, however, was observed for whey protein concentrate and vetch protein films, which, according to the authors, indicated some changes in the network cohesion due to the Ca^{2+}-induced crosslinking of protein chains [38, 39].

3.2. Mechanical Properties

The control film plasticized with Sor exhibited about 4 times higher σ_{max} and EM, but 2 times lower ε_b than the Gly-plasticized film ($p < 0.05$, Table 3). Presumably less hydrated Sor-plasticized film (11.50 vs. 29.46%, Table 2) had stronger and stiffer structure than the more hydrated Gly-plasticized film. Additionally, Gly as a smaller compound could more efficiently disrupt the intermolecular interaction among polypeptide chains than Sor, giving better plasticizing action and, thus weaker but more stretchable films (Table 3). The different physical states of the plasticizers were also likely significant; liquid Gly could be a better lubricant for the film matrix than solid Sor [10].

Table 3. Effect of $CaCl_2$ concentration on the of the tensile strength (σ_{max}), elastic modulus (EM), and elongation at break (ε_b) of glycerol- (Gly) and sorbitol (Sor)-plasticized pea protein isolate films.

Plasticizer	$CaCl_2$ (%)	σ_{max} (MPa)	EM (MPa)	ε_b (%)
Gly	0	1.70 ± 0.17 ^{b}	51.03 ± 3.35 ^{b}	73.79 ± 4.58 ^{d}
	0.01	1.43 ± 0.18 ^{a}	44.14 ± 8.00 ^{ab}	60.80 ± 4.89 ^{c}
	0.025	1.42 ± 0.16 ^{a}	42.52 ± 7.22 ^{ab}	57.52 ± 3.90 ^{c}
	0.05	1.41 ± 0.20 ^{a}	39.49 ± 4.00 ^{a}	59.49 ± 4.00 ^{c}
Sor	0	6.31 ± 0.24 ^{e}	219.15 ± 12.36 ^{e}	37.01 ± 3.70 ^{a}
	0.01	5.40 ± 0.27 ^{d}	193.52 ± 14.36 ^{d}	32.67 ± 2.72 ^{a}
	0.025	3.99 ± 0.26 ^{c}	129.23 ± 7.46 ^{c}	55.23 ± 3.06 ^{c}
	0.05	4.14 ± 0.25 ^{c}	133.39 ± 10.62 ^{c}	46.14 ± 8.88 ^{b}

$^{a\text{-}d}$ Values with the different superscript letters within one column are significantly different ($p < 0.05$).

$CaCl_2$ reduced the σ_{max} by ~16% and 14-37% (Table 3) in the Gly- and Sor-plasticized films, respectively, and also caused a reduction of about 13-23% and 12-41% in EM, respectively (Table 3). This finding was unexpected, as it is believed that Ca^{2+} favors electrostatic interactions between two adjacent carboxylic groups of different polypeptide chains and/or shields electrostatic protein-protein repulsions. This leads to a denser protein matrix structure, thus increasing the mechanical strength of gels and films [16, 36, 37, 40]. The negative result indicates that $CaCl_2$ destroyed the integrity of the PPI films. It is difficult to explain this result, but it might be related to the fact that the films consisted of incompletely dissolved PPI particles [9, 12], which were not very susceptible to calcium-mediated cross-linking. On the contrary, the salt presumably interfered molecular association of the PPI particles. Pea proteins are similar in several ways to soy proteins [41]. Park et al. [19] found that $CaCl_2$ did not affect the σ_{max} of SPI film, while improvement was observed with the use of calcium sulfate. The inefficiency of $CaCl_2$ was explained by differences in the solubility of these salts. Specifically, $CaCl_2$, being more soluble, probably coagulated proteins faster than calcium sulfate, resulting in a more irregular microstructure in the $CaCl_2$-added film. The heterogeneous structure did not offer the same strength as the uniform structure of the film with sulfate. This clearly indicates the importance of microstructural features in shaping the mechanical properties of the films. Fang et al. (2002) [20] demonstrated that the effectiveness of $CaCl_2$ depends not only on its concentration but also on the plasticizer content. Despite $CaCl_2$ at a concentration of 10 mM increased the mechanical strength of WPI emulsion film, the same concentration, but with twice the amount of Gly, resulted in films too fragile for tensile testing. Similarly, Mezgheni et al. [16] observed a weakening of film strength after the addition of $CaCl_2$ (at a concentration of 0.25%) for casein films, depending on the type and concentration of the plasticizer. Interestingly, lower concentration (0.125%) generally had no effect or improved the mechanical strength of casein films. Despite the assumed Ca^{2+}-induced crosslinking by formation of ionic bonds, the addition of $CaCl_2$ (0.05 g/100 g of dry matter) was insufficient to enhance the mechanical properties of the whey protein concentrate films, [39]. Similarly, $CaCl_2$ (at concentrations of 0.1–1%, w/w) was unable to affect the mechanical properties (both σ_{max} and ε_b) of the Gly-plasticized vetch protein film [38].

It is possible that observed reduction in σ_{max} and elasticity of PPI films (Table 3) could be attributed to $CaCl_2$ recrystallization. Notably, $CaCl_2$ caused a greater deterioration in σ_{max} and EM in Sor-plasticized film compared to Gly-plasticized films (Table 3). As suggested in Section 3.1, Sor crystals could act as nucleation centers, accelerating formation of $CaCl_2$ crystals, or *vice versa*.

Considering the above discussed results, it is somewhat surprising that $CaCl_2$ at concentrations of 0.025-0.05% enhanced the εb of the Sor-plasticized film ($p < 0.05$, Table 3) while the opposite effect was observed for the Gly-plasticized film. It is difficult to explain this result, but it might be due to the relaxation of the relatively strong and rigid structure of the Sor-plasticized film by the ions, which facilitated polypeptide chain slip movement. Most likely, the film plasticized with Gly did not exhibit this effect due to its already sufficiently high degree of plasticization (Table 3). In fact, $CaCl_2$ would apparently have an antiplasticization action in the Gly-containing PPI film. This outcome is contrary to that of Park et al. (2001) [19], who found that $CaCl_2$ significantly increased the ε_b of Gly-plasticized SPI film.

Comparing the mechanical properties of films produced by different researchers is challenging due to variations in several factors, including the concentrations of polymer in the FFSs, amounts and types of plasticizers used (i.e., polymer-to-plasticizer ratio), differences in the procedures for film preparation, and variations in testing methods. Nevertheless, considering the similar polymer:plasticizer ratio (i.e., ~1:0.5) and the method of preparation, the σ_{max} of the control Gly-plasticized PPI film (1.70 MPa, Table 3) is lower than that of the Gly-plasticized gelatin (11.36 MPa), WPI (6.10 MPa) [32], SPI (5.5 MPa, pH of the FFS = 9) [19], or vetch protein film (≈5.0 MPa, pH of the FFS = 11) [38]. In turn, the TS of the PPI film plasticized with Sor (6.31 MPa, Table 3) is comparable to that of the Sor-plasticized SPI film (5.4 MPa) [34]. The relatively low strength of the PPI film, particularly the Gly-plasticized one (Table 3), is partially likely due to the low cohesion of the matrix formed by incompletely dissolved proteins at pH 7 [9, 10].

5. Conclusions

Taking into account that $CaCl_2$ at concentrations of 0.1% and 0.2% w/w induces serious protein over-crosslinking, preventing the formation of a continuous PPI film, the amount of salt added should not exceed a critical concentration of 0.05% w/w in the FFS. $CaCl_2$ did not provide significant benefits to the PPI films and actually worsened their key properties such as transparency and mechanical strength. Light transmission and some other film properties were dependent on the concentration of $CaCl_2$, but not to an impressive extent, suggesting a low sensitivity of pea proteins to this salt. The results verified that the type of plasticizer differently affected how $CaCl_2$ influenced WVP and εb of the PPI film. Using pea proteins as an edible packaging ingredient requires methods beyond Ca^{2+}-induced crosslinking to address existing challenges and fully exploit their potential.

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