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Physicochemical and structural characterization of sodium caseinate based film-forming solutions and edible films as affected by high methoxyl pectin

Mastaneh Jahromi^a, Mehrdad Niakousari^{a*}, Mohammad Taghi Golmakani^a, Mohammad Amin Mohammadifar^{b*}

^a Department of Food Science and Technology, School of Agriculture, Shiraz University, 71441-65186 Shiraz, Iran

^b Research Group for Food Production Engineering, National Food Institute, Technical University of Denmark, SøtoftsPlads, 2800, Kgs. Lyngby, Denmark

* Corresponding authors:

Mehrdad Niakousari

Tel.: +989173001030

E-mail address: niakosar@shirazu.ac.ir

Mohammad Amin Mohammadifar

Tel.: +4553331650

E-mail address: moamo@food.dtu.dk

Abstract

Present work focused on the role of high methoxyl pectin (HMP) in rheology and physical stability of sodium caseinate (CAS) film-forming solution as well as mechanical and structural characteristics of its edible film. CAS-HMP films were fabricated at different blend ratio. Incorporation of HMP into CAS solution decreased pH value of the solution, acting as a natural acidifier. Turbidity and particle size distribution measurements indicated that some level of protein-polysaccharide interactions developed in 50CAS:50HMP and 25CAS:75HMP. Analysis of steady-state shear viscosity of film solutions showed a transition from Newtonian to shear-thinning flow behaviour by addition of HMP. Dynamic viscoelasticity evaluations revealed that the elasticity of solutions decreased by increasing HMP ratio. Physical stability of solutions was assessed over time using a Turbiscanner. All

solutions showed distinct mechanisms of destabilization. 50CAS:50HMP and 25CAS:75HMP samples appeared to be the most stable solutions. Increasing CAS:HMP ratio from 100:0 to 25:75 increased the water solubility and elongation at break of edible films from 27.16 to 63.70% and 2.36 to 16.53%, respectively. SEM analysis exhibited a homogenous microstructure in 50CAS:50HMP and 25CAS:75HMP. DSC thermograms displayed lower glass transition temperatures in HMP-incorporated films. Presence of the interactions among biopolymers in biocomposites was confirmed by FTIR spectra.

Keywords: Sodium caseinate; Pectin; Composite film; Rheology; Mechanical properties; Physical stability

1. Introduction

Environmental concerns regarding the high production rate of non-biodegradable packages and waste disposal problems have led to a growing interest in the development of bio-based packaging materials and substitution of petrochemical-based films by these films [1-4]. Different natural polymers; proteins or polysaccharides; have been used to design environmentally-friendly films and coatings [5, 6]. However, the selection of biopolymers for packaging purposes is influenced by many factors, such as versatility, availability, cost, physical and mechanical characteristics, barrier effects, etc. [4].

Sodium caseinate is a water-soluble polymer with a random-coil structure. Its ability to form extensive molecular interactions (hydrogen, hydrophobic and electrostatic) makes it a good candidate to produce transparent edible films with good strength. However, the obtained films have mostly low flexibility and stretchability [7]. Most of the researches in food packaging segment have been targeted towards improving the multifunctionality of proteins by modifying them using physical, chemical, and biochemical methods as well as mixing with other biopolymers [8]. Pectin, the main constituent of all plants and biomass, is considered as a low-cost, abundant bio-resource which offers elegant technological properties in food-related applications [9]. Edible films and coatings developed by pectin have been recently proposed in food packaging by their excellent mechanical properties [4]. A number of studies have been already published with the purpose of characterizing composite films fabricated by the mixture of pectin and proteins [3, 6, 10-18]. Pectin-rich sources are suggested as a potential natural plasticizer to improve the stretchability of edible films [19]. Higher elongation at break in sodium caseinate films containing higher contents of low methoxyl pectin has been well-documented by Eghbal et al. [10, 11].

When proteins and polysaccharides solutions are blended together, several phenomena may occur in the system, including co-solubility, thermodynamic compatibility or incompatibility, and depletion flocculation [20, 21]. Coexistence of polysaccharide with protein may weaken or reinforce the protein network, consequently represents important impacts on engineering features of bio-based composites [22, 23]. Various factors such as pH, ionic strength, biopolymer charge density, protein/polysaccharide ratio, biopolymer concentration, and molecular conformation [6, 22] have a crucial effect in the compatibility of mixed biopolymer systems and development of polymer-polymer interactions or incompatibility and phase separation [18]. Achieving more insights into the stabilization/destabilization mechanism of film-forming solutions and involved parameters may promote the feasibility of developing novel tailor-made films and coatings with improved functionality for food packaging and drug delivery purposes. In this regard, Turbiscan optical analyser can be used as a promising and powerful tool for evaluation of the physical stability and phase separation kinetics of film-forming solutions. The greatest benefit of the method is its ability to detect the destabilization mechanisms (e.g. flocculation, clarification, sedimentation, coalescence) of various colloidal systems at an early stage, up to 50 times earlier than the naked eye, in a single experiment [24].

In this work, HM pectin was added to sodium caseinate film-forming solution with the aim to enhance the flexibility of caseinate edible film, while naturally providing a condition to promote protein-polysaccharide interactions by lowering the pH of film solutions. The present study was intended to widen experimental information about the impact of HM pectin on the film-forming properties of sodium caseinate. To our knowledge, relatively few studies have focused on the effect of pH-induced protein-polysaccharide interaction on the physical stability of film-forming dispersions, determination of underlying destabilization mechanisms of film-forming solutions, and the final quality of films. Therefore, a part of this work has been dedicated to evaluating physical stability of caseinate film solutions as affected by pectin using Turbiscanner. Rheological features of film-forming solutions, along with morphology, physicochemical, thermal and structural properties of caseinate films, were also characterized.

2. Material and methods

2.1. Materials

Sodium Caseinate (CAS) powder was supplied by Merck Co. (Darmstadt, Germany). High

methoxyl pectin powder (HMP) (GENU[®] Pectin, 50-75% esterification) was obtained from CPKelco (Lille Skensved, Denmark). Glycerol 87% (PlusOne Glycerol) was from GE Healthcare (Chicago, Illinois, USA). All solutions were prepared using distilled water.

2.2. Film-forming solutions preparation

Film-forming solutions were fabricated at five CAS:HMP ratios (100:0, 75:25, 50:50, 25:75 and 0:100). In order to prepare stock solutions of CAS and HMP at a concentration of 2% (w/w), the desired quantity of CAS and HMP were mixed with distilled water; followed by stirring for 4 h at room temperature. The solutions were then stored at 5 °C for 18 h to reach full hydration. Mixtures containing CAS and HMP were prepared in CAS:HMP ratios (75:25, 50:50, 25:75) from the stock solutions. The required amount of CAS solution was added to HMP solution in a dropwise manner while stirring at room temperature. The whole mixture was further stirred for 90 min to achieve a homogeneous blend. The pH value of prepared mixtures was measured using a pH meter (Metrohm 780 pH Meter, Metrohm AG, Herisau, Switzerland).

2.3. Characterization of film-forming solutions

2.3.1. Zeta potential and particle size distribution

The film-forming solutions were analyzed for zeta potential applying a Zetasizer Nano-ZS (Malvern Instruments, Worcestershire, UK). Distilled water at a pH similar to the pH of the solution was used to dilute the film solutions (0.004% (w/w)). The particle size distribution of the film solutions was determined in a Mastersizer 2000 (Malvern Instruments, Ltd., Worcestershire, U.K.) [16]. The solutions were added to the measuring unit filled with distilled water adjusted to pH values of the solutions. Results were reported as surface weighted (D[3,2]) and volume-weighted (D[4,3]) mean diameter; which were directly computed by the software provided with the apparatus using eq. (1) and eq. (2) :

$$D[3,2] = \sum n_i d_i^3 / \sum n_i d_i^2 \quad \text{eq. (1)}$$

$$D[4,3] = \sum n_i d_i^4 / \sum n_i d_i^3 \quad \text{eq. (2)}$$

where n_i is the number of particles of class “ i ” and d_i is the diameter of class “ i ”. All measurements were conducted three times.

2.3.2. Spectrophotometry

Turbidity of diluted solutions (0.14% (w/w)) was measured (in triplicate) as a function of

CAS-HMP ratio with a UV/Vis spectrophotometer (Hitachi u-1500, USA), at 600 nm and room temperature [20].

2.3.3. Rheological analysis

Rheological experiments were conducted in duplicate using a stress-controlled rheometer (DHR2, TA Instruments, New Castle, USA) equipped with a cone-plate geometry (diameter = 40 mm, cone angle = 2° and a gap = 51 µm) at 25 °C [25]. Steady shear flow measurement of the film solutions was performed at a shear rate ranged from 1–1000 s⁻¹. The Power-law model (eq. 3) was used to fit the rheological results:

$$\tau = m \dot{\gamma}^n \quad \text{eq. (3)}$$

where τ is the shear stress (Pa), m is the consistency coefficient (Pa.sⁿ), $\dot{\gamma}$ is the shear rate (s⁻¹), and n the flow behaviour index. Shear-thinning fluids indicate n values less than 1 ($n < 1$) and Newtonian fluids indicate n values equal to 1 ($n = 1$) [25].

In order to find the linear viscoelasticity range, an amplitude sweep measurement was carried out at the strain range of 0.01-100% and frequency of 1 Hz. Dynamic frequency sweep analysis was performed over a frequency range of 0.18-18 Hz at the linear viscoelastic region (the strain of 1%) [26]. Complex modulus (G^*) and $\tan(\delta)$ (G''/G') were reported as a function of measured frequency.

2.3.4. Physical stability

The stability or phase separation tendency of film-forming solutions was directly monitored by Turbiscan® Lab Expert (Formulation, Toulouse, France) using the dynamic light scattering method. The solution was poured into a glass tube, followed by placing in the Turbiscan tower. The analyzer was equipped with a source of NIR light ($\lambda = 880$ nm) and two synchronous optical sensors which scanned every 40 µm of sample length, obtaining transmission (T%) and backscattering (B%). The analysis was performed (in duplicate) as a function of time over 24 h at 25 °C. Results were reported as T%, variations of backscattering ($\Delta B\%$) and transmittance ($\Delta T\%$), as well as Turbiscan Stability Index (TSI) which is a specific parameter to estimate and compare the physical stability of various dispersions/solutions [27]. TSI was automatically calculated by the Turbiscan software using eq. (4):

$$TSI = \sqrt{\frac{\sum_{i=1}^n (x_i - x_{BS})^2}{n-1}} \quad \text{eq. (4)}$$

where x_i is the mean value of the scattered light intensity at each time of the instrument, x_{BS} is the average of x_i , and n is the number of scanning. Higher TSI indicates the less stability of the dispersion. TSI scale and the meaning of each scale is summarised in Table 1. The scale is based on an empirical analysis of a database of more than 500 measurements [28].

2.4. Film-casting procedure

CAS and HMP film solutions were prepared and mixed together in five CAS:HMP ratios (100:0, 75:25, 50:50, 25:75 and 0:100), as described in section 2.2. A volume of 40 ml of each film-forming solution containing glycerol (0.5% w/w) was cast into a plastic Petri-dish (90 mm diameter) and dried for 24 h at 40 °C [10]. Dried biopolymeric films were then peeled off and conditioned for 72 h at ambient temperature and relative humidity of 53% in a desiccator filled with saturated magnesium nitrate solution [29].

2.5. Film characterizations

2.5.1. Visual observation and colour parameters

The fabricated films were visually examined for uniformity, transparency, flexibility, and brittleness [20]. The colour properties of the films were evaluated (in triplicate) by a Minolta chromameter CR-200 on a white surface, and L^* , a^* , and b^* values were recorded [10].

2.5.2. Scanning electron microscopy (SEM)

The cross-section microstructure of the fabricated biopolymeric films was visualized by use of a Quanta FEG 3D scanning electron microscope (SEM) at an accelerating voltage of 10 kV. Cryo-fractured pieces of films were attached on SEM specimen by a double-sided carbon adhesive tape followed by sputtering them with a layer of gold (Leica Coater ACE 200) [1].

2.5.3. Physical and mechanical characteristics

The fabricated films were analysed in terms of thickness [30], moisture content [31], and swelling index [20]. The water solubility of prepared films was measured based on the procedure described by Esteghlal et al. [20]. Dried film pieces ($2 \times 2 \text{ cm}^2$) at 105 °C for 24 h were weighted (M_i) and then immersed in 50 ml distilled water under regular shaking for 24 h at 25 °C. The rest of the film pieces were removed, followed by re-drying and weighting (M_f).

The solubility of the film in water was calculated according to the eq. (5):

$$\text{Solubility (\%)} = (M_i - M_f / M_i) \times 100 \quad \text{eq. (5)}$$

Mechanical parameters of prepared films, including tensile strength (TS) and elongation at break (EB) were evaluated employing a texture analyzer (TA.XT.plus, Stable Micro Systems, UK). Rectangular-shaped ($10 \times 90 \text{ mm}^2$) strips of pre-conditioned films were clamped between grips. The tensile test was applied at the initial grip distance of 50 mm and a cross-head speed of 50 mm/min [20]. All measurements were repeated three times.

2.5.4. Differential scanning calorimetry (DSC)

Glass transition temperature (T_g) of the prepared films was determined according to Sadeghi-Varkani et al. [32] with some modification using a differential scanning calorimeter (DSC 250, TA Instruments, Newcastle, Delaware, USA) equipped with Trios software and Refrigerated Cooling System 90. The cell was purged with 50 ml/min of dry nitrogen, and the instrument was calibrated with indium ($T_{\text{peak}} = 155.87^\circ\text{C}$, $\Delta H = 28.234 \text{ J/g}$) and sapphire for specific heat capacity. About 5 mg of each sample placed into Tzero aluminium pans and hermetically sealed. An empty pan of the equal weight and condition was used as a reference. The scanning temperature was ranged between -90 and 130°C at a ramp of 5°C/min with the modulated conventional procedure (amplitude $\pm 1^\circ\text{C}$, period 60 s). The T_g of the films was then obtained from the plotted thermic graphs using Trios software.

2.5.5. Fourier transform infrared (FTIR) spectroscopy

FTIR spectrum of the fabricated films was obtained by an FTIR spectrometer (Perkin-Elmer Spectrum 100, USA), based on a Universal Attenuated Total Reflectance sensor 125 (UATR-FTIR) in the transmission mode over a range of $4000\text{--}500 \text{ cm}^{-1}$ [33].

2.6. Statistical analysis

The obtained data were statistically analysed using one-way analysis of variance (ANOVA) by SPSS software (SPSS 23.0, SPSS Inc., Chicago, IL). The differences between means were evaluated by Duncan's Multiple Range test ($P < 0.05$).

3. Results and discussion

3.1. Physicochemical properties of film-forming solutions

Physicochemical characteristics of film-forming solutions (pH, zeta potential, turbidity, and

particle size distribution) are summarized in Table 2. 100CAS:0HMP and 0CAS:100HMP solutions showed the pH values of 7.00 and 3.23, respectively. pH value of commercial pectin has been reported in the range of 3-4 [34, 35]. Addition of HMP to CAS solution decreased the pH of the hybrid solutions to lower values ranged between 6.20 to 3.83, behaving as a natural acidifier.

Zeta potential measurements (Table 2) revealed that pure CAS and HMP biopolymeric solutions carried a net negative charge. It might be due to the deprotonation of carboxyl groups in CAS and HMP molecules [18] at pH values higher than isoelectric point (pI) of protein (≈ 4.6) [20] and pKa of HMP (≈ 2.8) [36], respectively. The density of negative charges in the film solutions decreased by increasing the HMP ratio ($P < 0.05$). Decreasing pH value in 75CAS:25HMP (pH 6.20), 50CAS:50HMP (pH 5.68), and 25CAS:75HMP (pH 3.83) samples, as influenced by HMP increment, could lead to the protonation of carboxyl and amino groups, and subsequently decline the number of negative charges [10, 20]. This phenomenon might result in carrying net positive charge by protein component in 25CAS:75HMP sample at pH value of 3.83 that was lower than pI of the protein. Thus, all positively charged groups of CAS were electrostatically interacted and saturated with negatively charged HMP; however, there were still excess amounts of HMP with negative charges in the solution which did not interact with CAS and kept the zeta potential of the system in the negative range. Silva et al. [3] found the zeta potential value close to zero for whey protein isolate solutions (pH 3) containing 0.5 and 1% pectin concentrations, however increasing pectin concentration to 2% resulted in a negatively charged solution more distant to zero.

According to the turbidimetric measurements (Table 2), the highest absorbance (0.686) was obtained for 50CAS:50HMP sample. The next highest absorbance (0.407) corresponds to 25CAS:75HMP sample. Film solutions of pure CAS and HMP were transparent with absorbance 0.092 and 0.071, respectively. Incorporation of 25% HMP in the CAS film solution (75CAS:25HMP) caused a slight increase in turbidity of the solution ($P > 0.05$). pH of 75CAS:25HMP dispersion was 6.20, thus no electrostatic interaction was expected to occur between negatively charged CAS and HMP molecules due to the predominant repulsive forces between them [37]. The higher turbidity in 75CAS:25HMP comparing to pure CAS is attributed to a weak hydrophobic interaction between CAS and HMP at this pH. It was previously reported that caseinate may have a weak association with xanthan gum [38, 39] and pectin [10] at neutral pH via hydrophobic interactions. Gorji et al. [37] found that the

weak hydrophobic association between the gum tragacanth and sodium caseinate at pH values close to 7 induced a rise in turbidity. The same observation was also reported by Eghbal et al.[10] for sodium caseinate-LM pectin film solutions at pH 7. Further increase in HMP ratio to 50 and 75% in film solution exhibited a different absorbance profile from that of CAS and HMP independently and induced an abrupt rise in turbidity of the solutions ($P < 0.05$), suggesting that some level of CAS-HMP complexation occurred. In 50CAS:50HMP (pH 5.08), with the pH value higher than CAS isoelectric point, most of the CAS side chains were negatively charged; however, locally there were some patches still carried positive charges on the surface [10, 21]. These positively charged moieties might attach CAS molecules to the external surface of a single negatively charged HMP chain and form primary complexes via electrostatic interactions, resulting in an increase in the size and number of particles and turbidity [10, 37]. Gorji et al. [37] obtained a continuous increasing trend in turbidity of mixed solutions of sodium caseinate and gum tragacanth by decreasing the pH from 6.50, reaching the highest value of turbidity at pH 4.15. The lower turbidity of 25CAS:75HMP in compared to 50CAS:50HMP might be due to the formation of smaller size complexes in the system.

The particle size measurements (Table 2) indicated lower particle size in 75CAS:25HMP ($D[3,2] = 11.53 \mu\text{m}$, $D[4,3] = 32.55 \mu\text{m}$), compared to pure CAS ($D[3,2] = 14.22 \mu\text{m}$, $D[4,3] = 54.24 \mu\text{m}$) that might be explained by the partial replacement (25%) of CAS with small size HMP in the mixture solution. It is probable that addition of HMP to the system disintegrated some of the CAS large aggregates and split them into small aggregates. Weak hydrophobic interaction between some patches of HMP and CAS might partially inhibit the CAS self-aggregation. Consequently, the particle size reduced, and the number of small particles increased, which the latter raised the turbidity of the solution slightly. Increasing HMP ratio to 50% led to developing a solution (50CAS:50HMP, $D[3,2] = 21.30 \mu\text{m}$, $D[4,3] = 62.38 \mu\text{m}$) with particles substantially ($P < 0.05$) larger than pure CAS and HMP, proposing growth of CAS-HMP electrostatic complexation at pH 5.08. Increment of HMP level to 75% (25CAS:75HMP, pH 3.83) decreased the $D[3,2]$ ($12.76 \mu\text{m}$) and $D[4,3]$ ($24.44 \mu\text{m}$) values of the solution; however, the size of particles was still drastically larger than 0CAS:100HMP sample. The smaller particle size in this sample might correspond to the interaction of CAS with carboxylic groups of the HMP that led to the reduction of intermolecular repulsion and the formation of the complexes with less expanded, more compact structure and shrinkage of the molecule [37]. Gorji et al. [37] observed that the particle size of sodium caseinate and

gum tragacanth mixed solution decreased by lowering the pH to below 5, however, the particle size decrement was drastic at pH 4.

3.2. Rheological behaviour

Fig. 1a shows the bulk steady shear viscosity of CAS film-forming solutions blended with different ratio of HMP. 100 CAS solution represented the lowest viscosity value, whereas, the highest viscosity was obtained by 100 HMP sample. To better understand the rheological behaviour of solutions, the obtained data were fitted to the Power-law model. The values of m (consistency index) and n (flow index) is presented in Table 3. 100 CAS and 100 HMP solutions exhibited a Newtonian ($n = 1$) and shear-thinning behaviour ($n < 1$) over the assessed shear rate regime, respectively. Addition of HMP to CAS solution resulted in a transition from Newtonian to shear-thinning flow behaviour. The viscosity and consistency in HMP-incorporated CAS film-forming solutions were clearly higher (4.74-100 times) than in pure CAS solution. The possible cause for this flow behaviour in 75CAS:25HMP solution could be the coexistence of free HMP molecules, with extended structure, with CAS. In 50CAS:50HMP and 75CAS:25HMP, the observed rise in the viscosity might result from the electrostatic interaction of a part of HMP with CAS molecules, which developed a reversible network structure within the polymer solution. However, an extra HMP amount was still available in the system, which tremendously increased the viscosity of continues phase.

The complex modulus (G^*) and $\tan(\delta)$ (G''/G') of film solutions versus frequency are shown in Fig. 1b and c, respectively. 100 CAS solution showed the lowest G^* along the entire frequency range. However, the G^* of the solution increased as HMP content increased. 50CAS:50HMP (pH 5.08), and 25CAS:75HMP (pH 3.83) samples exhibited higher G^* values than pure CAS and pure HMP (at frequencies ≤ 5.6 Hz); which might be related to the formation of interpolymeric electrostatic interactions. Gorji et al. [21] found that by decreasing pH from 4.44 to 4.04 the shear viscosity, G' , G'' , and $\tan(\delta)$ of sodium caseinate-gum tragacanth solution increased owing to formation stronger electrostatic interactions between polymers. At frequencies ≤ 1 Hz, 100 CAS yielded higher values of $\tan(\delta)$ than other samples, however, the samples possessed no marked difference in $\tan(\delta)$. The elasticity of biocomposite solutions reduced by HMP increment, indicated by the increase in $\tan(\delta)$ at frequencies ≥ 2 Hz. Higher values of $\tan(\delta)$ point out the development of a more viscous network [21]. The $G'-G''$ intersection points and the corresponding frequency are shown in Table 3. It is indicated that 100CAS:0HMP sample reached a crossover point at 1.33 Hz.

However, there was no crossover point in 0CAS:100HMP sample. Replacement of CAS with 25 and 50% HMP in mixture film solutions resulted in reaching crossover at higher frequencies of 3.07 and 4.74 Hz, respectively, demonstrating predominantly viscous behaviour at low frequencies ($G' < G''$, $\tan(\delta) > 1$) and elastic behaviour at high frequencies ($G' > G''$, $\tan(\delta) < 1$). Similar to pure HMP solution, no crossover point was found in 25CAS:75HMP, representing a viscose-dominant behaviour in all measured frequencies ($G' < G''$ and $\tan(\delta) > 1$). It might be proposed that viscoelastic properties of film solutions in higher HMP ratios were dominated by the contribution of HMP.

3.3. Stability of film-forming solutions

Fig. 2a illustrates the transmission intensity of the film-forming solutions as a function of time. Pure HMP solution showed the highest intensity of transmission around 53%, keeping a constant value during the whole test. Pure CAS sample displayed the transmission intensity of 18.78% at the beginning of the test (Time = 0 s), and the value increased slowly to 32.17% over time. Addition of 25% HMP to CAS solution (75CAS:25HMP, pH 6.20) resulted in a solution with lower transmission intensity (7.09%) due to the HMP-CAS hydrophobic interactions. However, the transmission increased rapidly by the time, reaching to 29.68%. The 50CAS:50HMP (pH 5.08) and 25CAS:75HMP (pH 3.83) solutions exhibited time-independent, constant transmission intensity below 0.1% during the entire time, indicating the formation of a turbid solution caused by protein-polysaccharide electrostatic complexation.

Turbiscan Stability Index (TSI) values of film-forming solutions were plotted versus time (Fig. 2b). By comparing the TSI values of film solutions during the first 72000 s (≈ 20 h), a ranking order of 100CAS:0HMP > 75CAS:25HMP > 0CAS:100HMP > 50CAS:50HMP > 25CAS:75HMP on instability was found. Pure CAS exhibited a rapid initial rise in TSI during the first 22000s (≈ 6 h), followed by a slower further increase. In general, the addition of HMP to the film formulation reduced the TSI values, improving the stability of solutions. However, 75CAS:25HMP indicated a steadily increase in TSI until crossing the 100CAS curve at around 72300s, reaching to the higher TSI value than 100 CAS at the end of the examination. Both pure CAS and 75CAS:25HMP with TSI > 10 were ranked as solutions with high instability. 50CAS:50HMP and 25CAS:75HMP solutions appeared to be the most stable samples based on the lowest observed TSI values, with a slight increase in TSI by the time. Emerging, but not visible, destabilization in these solutions was confirmed by TSI values < 1.

To gain more insight into the destabilization mechanism of film-forming solutions, variations of backscattering (ΔB) and transmittance (ΔT) (Fig. 3) were used. In transparent solutions ($T > 0.2\%$: 100CAS:0HMP, 75CAS:25HMP and 0CAS:100HMP) transmittance profile was used to detect the destabilization mechanism. However, in turbid solutions ($T < 0.2\%$: 50CAS:50HMP and 25CAS:75HMP), the interpretation of data was conducted based on the backscattering signal. Pure HMP solution presented no substantial variation in transmittance profile by the time, showing a stable solution. In 100 CAS solution, a progressive increase in the transmittance profile was observed within the whole test tube. Particle size growth owing to aggregation/flocculation in the solution may occur, as the casein polymers tend to form aggregates in the aqueous medium via intermolecular hydrophobic interactions [40]. Similar to 100 CAS, $\Delta T\%$ increased throughout the solution in 75CAS:25HMP. On the basis of observations for the transmission intensity and TSI (Fig. 2), it is concluded that less extensive aggregation may occur in 75CAS:25HMP sample than sample containing 100% CAS for the first 20 h. However, the intensity of the transmittance signal was dramatically higher in the top to the middle of the tube. It was hypothesized that some level of depletion flocculation induced by pectin in the solution at pH $\approx$ 20. Although some of the HMP molecules hydrophobically interacted with CAS, there was still non-interacted HMP in the system which induced the depletion flocculation. The results found by Surh et al.[41] could support this assumption. They suggested that the presence of non-adsorbed pectin in the caseinate-stabilized emulsion at pH 6-7 was responsible for depletion flocculation and consequently aggregation in the system. This may explain the rapid increase in transmission intensity and TSI value of 75CAS:25HMP sample (Fig. 2). In 50CAS:50HMP and 25CAS:75HMP two phenomena were detected: a) clarification; which was identified by the negative peak of $\Delta B\%$ at the top of the tube ($< 10\%$ of tube length) and b) sedimentation; which was distinguished by increasing the BS signal at the bottom of the tube ($< 10\%$ of tube length). This might be attributable to the precipitation of dense insoluble complexes or coacervates. 25CAS:75HMP revealed smaller clarification and sedimentation zones in compared to 50CAS:50HMP; reflecting the slower migration velocity of particles towards the bottom. It has been mentioned that during clarification and/or sedimentation, the migration rate of particles toward the bottom is controlled by the viscosity of continuous phase as well as the size, shape, and density of particles [24]. The smaller size of particles in 25CAS:75HMP solution was a possible cause for slower sedimentation rate. Besides, the presence of an extra amount of HMP in the system increased the viscosity of the bulk phase and acted as a physical barrier to slow down the sedimentation rate of particles. No prominent change in $\Delta B\%$ was observed

in the middle of the tube in both 50CAS:50HMP and 25CAS:75HMP, implying the stability in the solutions. Existence of polysaccharide in the system could postpone the protein aggregation [37]. It could also be assumed that electrostatic repulsion forces between assemblies; which effectively provided by the negatively charged complexes [37], assisted solution stabilization. These outcomes highlight that combined effect of biopolymer ratio and pH have important impacts on the physical stability of biopolymeric solutions.

3.4. Visual, colour and microstructural properties of films

Dried film samples were peeled off from the casting plates. Pure CAS film was fragile and brittle, thus difficult to peeling off without cracking. However, peeling of the samples containing the higher proportion of HMP ($\geq 50\%$ HMP) was easier than pure CAS and 75CAS:25HMP films and they showed higher flexibility. All edible films had appropriate transparency. Some insoluble particles were observed in pure CAS film; however, the rest of the films exhibited a uniform appearance.

The influence of different CAS-HMP ratios on color attributes of the biopolymeric films are presented in Table 4. The results from L^* parameter measurement showed no dramatic difference ($P > 0.05$) between 100CAS:0HMP, 75CAS:25HMP, and 0CAS:100HMP samples; however the incorporation of the higher amount of HMP (50% and 75%) in CAS films decreased the brightness of biocomposite films significantly ($P < 0.05$), suggesting the formation complexes between CAS and HMP. At the same time, both a^* and b^* parameters increased noticeably ($P < 0.05$) with HMP increment, representing that prepared biopolymeric mixture films were less green and more yellow than the control.

SEM micrographs of the cross-section of the films (Fig. 4) displayed a non-uniform aggregated network in pure CAS and 75CAS:25HMP films. The 100 HMP film revealed a homogenous, dense, and smooth structure without any pores and cracks. By the increase in HMP content to 50 and 75%, the uniformity, smoothness and homogeneity of composites increased, reflecting high compatibility of CAS and HMP at mixture systems due to associative (electrostatic) interactions [29]. These morphological observations were consistent with the acquired results from the physical stability assessment of film solutions (Fig. 2 and 3). Eghbal et al. [10, 11] visualized a smooth and uniform structure in LM pectin film, but a rough and heterogeneous network in caseinate- incorporated LM pectin films.

3.5. Physical and mechanical properties of films

Physical characteristics (moisture content, water solubility, and swelling index) of the edible films are summarized in Table 5. The thickness of all fabricated films was around 0.11mm ($P > 0.05$). Same value for the thickness of sodium caseinate films was reported by Pereda et al. [30].

The moisture content of the obtained films ranged from 14.27 to 16.94% (Table 5). Moisture content increased by increasing the HMP ratio; however, the changes were not statistically significant ($P > 0.05$). The reduction of moisture content in caseinate/LM pectin films from 19.82% to 7.50% at pH 7 and 13.54% to 6.99% at pH 3 with increasing caseinate content was observed by Eghbal et al. [10, 11].

As presented in Table 5, 100CAS:0HMP film showed the lowest solubility in water (27.16%); whereas, 0CAS:100HMP film completely dissolved in water (100%). The partial hydrophobic nature of CAS [10] might be responsible for its low water solubility. Increasing HMP portion in the films to 25%, 50% and 75% increased ($P < 0.05$) the water solubility by 34.75%, 66.60% and 134.53%, respectively. The obtained results from swelling measurements (Table 5) also showed the same trend with water solubility. Increasing HMP content led to a substantial increase ($P < 0.05$) in swellability of the fabricated biofilms; which was correlated with the more hydrophilicity and water affinity of HMP. Silva et al. [3] observed higher water solubility in whey protein isolate-pectin films comprising higher concentrations of pectin.

Table 6 exhibits the mechanical properties of prepared films. Tensile strength (TS) and elongation at break (EB) of bio-films represent the film's mechanical resistance to break and stretching capacity or flexibility, respectively [35]. The film made of Pure CAS displayed the highest TS (33.94 MPa) and lowest EB (2.36%). Chevalier et al. [42] reported that the mechanical strength of casein extruded sheets ranged between 6 and 38 MPa, based on the casein polymer source and glycerol content. The 100% HMP film demonstrated the highest EB (22.21%). This value was close to what (EB 20%) obtained by Chakravartula et al. [35] for pectin film at pH 3.43. A considerable increase ($P < 0.05$) by 79.23- 600.42% was observed in the EB of films with increasing HMP portion. On the contrary, the TS of films decreased ($P < 0.05$) by 19.88-42.48% with increasing HMP amount. Therefore, a descending order of 100CAS:0HMP > 75CAS:25HMP > 0CAS:100HMP > 50CAS:50HMP > 25CAS:75HMP on TS was obtained. The TS of 50CAS:50HMP film was 21.30 MPa. Volpe et al. [43] obtained the tensile strength of 19 MPa for the caseinate-chitosan blend film

at the ratio of 50:50. Perone et al. [44] also found the tensile strength of 20 MPa for hydroxypropyl methyl cellulose-sodium caseinate film at the same ratio. Interactions between film components and strength of the inter- and intra-molecular network bonds can influence the mechanical properties of films [3, 17]. The lower values of TS in 50CAS:50HMP and 25CAS:75HMP than pure CAS and HMP films could be associated with the development of an inter-polymeric matrix via CAS-HMP electrostatic interactions with different strengths and densities. Presumably, these electrostatic interactions between CAS and HMP weakened the intermolecular forces between protein chains and decreased protein structural cohesiveness, resulting in lowering TS value and inducing film flexibility, as similarly addressed in literature [3]. Silva et al. [3] observed that by addition of 0.5 and 1% pectin to whey protein isolate at pH 3, the TS of films decreased by about 56.88% and 26.14%, respectively, due to electrostatic interactions between biopolymers. The enhanced extendibility of film in 75CAS:25HMP; which there was no electrostatic interaction between CAS and HMP, could be justified by the plasticizing effect of polysaccharides [45]. Incorporation of pectin, as a bulk and high molecular weight biopolymer, into the film matrices might lead to form an open structure with more space for biopolymer chains to move and slide against each other [17]. Mohal et al. [10] found higher Young's modulus and lower EB for caseinate-LM pectin films containing higher caseinate amount.

3.6. Thermal behaviour

Fig. 5a depicts the DSC profiles of fabricated biofilms. Thermograms showed the T_g of pure CAS film around 75.57 °C. Corak et al. [46] reported T_g of 78 °C for sodium caseinate film. Zhao et al. [47] also mentioned T_g of 73.4 °C for sodium caseinate powder. The glass transition temperature around -6.32 °C was detected for pure HMP film. The T_g values between -84.7 to -108.4 °C, at different RH% (33.3-75.2%), has been already reported by Pérez et al. [48]. Basu et al. [49] also documented the glass transition temperature of pectin in the range of 16.8 to -24.6 °C (water activity 0.12-0.91). Incorporation of 25, 50, and 75% HMP into CAS film led to shift T_g values to lower temperatures of 43.39, 40.24, and -2.75°C, respectively. Qiao et al. [50] found lower glass transition temperatures in gelatin films contained chitosan. Glass transition temperature is a structure-sensitive property which is greatly influenced by intra- and intermolecular interactions and steric effects. Increasing the polarity, rigidity, and size of the side chains in biopolymers increase the glass transition temperature [51]. Therefore, the high value of T_g in pure CAS film could be governed by its rigid structure. The T_g decrement in 75CAS:25HMP was probably due to the plasticization

function of HMP [52]. However, in 50CAS:50HMP and 25CAS:75HMP, complexation between HMP and CAS might also be the involved mechanism in lowering the glass transition temperature. Interpolymeric electrostatic interactions could partially suppress protein chain-to-chain interactions/attachments [3] and facilitate segmental mobility, leading to decrease T_g . These findings were in line with those obtained by mechanical testing of fabricated edible films (Table 6), which by increasing HMP ratio, the flexibility (EB) of the films increased. Jagannath et al. [53] observed more flexibility and ability to elongation in casein-based films with the lower glass transition temperature.

3.7. FTIR of fabricated films

FTIR spectra of the prepared films are presented in Fig. 5b. The spectrum of pure CAS film exhibited main bands at regions around 3278 cm^{-1} (amide A), 2931 cm^{-1} (amide B), 1631 cm^{-1} (amide-I), 1532 cm^{-1} (amide-II), and 1238 cm^{-1} (amide-III), assigned to NH-stretching coupled with hydrogen bonding, CH and NH_3^+ asymmetric stretching vibration, C=O stretching/hydrogen bonding coupled with COO^- (β -sheets), out-of-phase combination of CN stretch and in-plane NH deformation modes of the peptide group, and fluctuation in the plane of C-N and N-H groups of bound amide or fluctuation of CH_2 groups of glycine, respectively [29, 33]. The identified bands in approximately 1446, 1109 and 1043, and 923 cm^{-1} could be attributed to the carboxylate group ($\text{O}-\text{C}-\text{O}$), C-O stretching in C-OH bonds, and monocationic interactions with Na^+ , respectively [33].

FTIR spectrum of 100 HMP film showed the absorption at 3324 and 2935 cm^{-1} corresponded to $-\text{OH}$ and $-\text{CH}$ stretching regions, respectively [54]. The characteristic band at 1740 cm^{-1} was assigned to the esterified carboxylic groups and the shoulder at 1013 cm^{-1} referred to C-O-C stretching vibrations in polygalacturonic acid [54, 55]. The peak at 1612 cm^{-1} was characteristic of carboxylate ($-\text{COO}^-$) bonds [54]. The peaks at 922, 848, and 831 cm^{-1} could be attributed to the absorption of d-glucopyranosyl, β -glycosidic linkage, and the absorption of α -D-mannopyranose, as reported by Younis et al. [55].

The spectra of the composite films displayed the characteristic peaks of both CAS and HMP. The intensity of absorption peaks changed depending on the CAS-HMP ratio, which was more pronounced in 50CAS:50HMP and 25CAS:75HMP films. The amide A band in the 25CAS:75HMP film showed a redshift of 3 cm^{-1} (from 3278 to 3281 cm^{-1}), indicating stronger hydrogen bonds in the $-\text{OH}$ groups of mixed films compared to the pure CAS film. The intensity of the amide A also increased in 25CAS:75HMP film which might be

correlated to the existence the higher number of hydroxyl groups in the film containing the higher amount of HMP, as supported by Jantrawut et al. [54].

The band indicative of the amide-I group is the most used spectral region to determine the secondary structural changes of the protein backbone [56]. The obtained FTIR spectra showed a blueshift in the amide-I from 1631 to 1627 cm^{-1} in 75CAS:25HMP film, and 1625 cm^{-1} in both 50CAS:50HMP and 25CAS:75HMP films. The peak corresponding to amide-II shifted to higher wavenumbers (from 1532 to 1538 cm^{-1}) in 50CAS:50HMP and 25CAS:75HMP composites. The intensity of both amide-I and II bands also decreased in biocomposites by increasing HMP ratio. The peak associated with amide-III band shifted from 1238 cm^{-1} (100CAS:0HMP) to 1236, 1231, and 1228 cm^{-1} in 75CAS:25HMP, 50CAS:50HMP, and 25CAS:75HMP films, respectively. The changes in the intensity of the amide I and II indicate conformational changes occurring through interactions between protein and the polysaccharides [56]. Mohammadi et al. [29] detected a decrease in the intensity of the peak at 1640 to 1552 cm^{-1} in the gelatin film by raising the ratio of chitosan, suggesting the electrostatic interaction between amino and carbonyl groups resulted in decreasing the content of random-coil and single-helix structures in gelatin. The lower amount of protein in the mixture films could also lead to obtaining FTIR spectra with decreased intensity in the amide-I band [54].

4. Conclusion

This work aimed to study the effect of HM pectin on physicochemical and structural properties of caseinate-based film-forming solutions and edible films. Incorporation of 50 and 75% HM pectin into caseinate solution (50CAS:50HMP and 25CAS:75HMP) induced the formation of some level of protein-polysaccharide interactions by decreasing the pH value of the solution. Developing of electrostatic interactions and complexation between CAS and HMP improved the physical stability of film-forming solutions remarkably, and resulted in the formation of biocomposites with homogenous and uniform microstructure. HM pectin-incorporated caseinate films presented higher water solubility, swelling index, flexibility and lower glass transition temperature.

Naturally plasticizing and acidifying role of HMP makes it a good candidate for developing sustainable protein-polysaccharide biocomposites with improved flexibility while promoting interpolymeric electrostatic interactions. This could eventually reduce the usage of chemical acidic agents and synthetic plasticizers.

The evidence from this study points towards the idea to use of CAS-HMP biocomposites as tuneable biopolymeric matrices for various promising applications such as co-encapsulation of hydrophobic and hydrophilic bioactive compounds and/or designing tailor-made pH-responsive films and coatings. These composites could potentially be applied for the development of active/intelligent food packaging and controlled-release delivery systems. A wider level of research studies on the current topic is therefore recommended to elucidate the suitability of these natural films on food products and pharmaceutical industries.

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Table 1. Ranking the stability of dispersions according to turbiscan stability index (TSI) scale

TSI scale	Description
0-0.5	No significant variation, not visible
0.6-1	Emerging destabilization, not visible
1.1-3	Destabilization weak, potentially visible
3.1-10	Significant destabilization, visible
> 10	High destabilization, visible

Table 2. Physicochemical properties of film-forming solutions with different caseinate:HM pectin ratio

Property	CAS:HMP ratio				
	100:0	75:25	50:50	25:75	0:100
pH	7.00±0.00 ^a	6.20±0.02 ^b	5.67±0.02 ^c	3.83±0.03 ^d	3.23±0.01 ^e
Zeta potential (Mv)	-33.30±0.15 ^a	-29.70±0.23 ^b	-22.30±0.32 ^c	-18.70±0.25 ^d	-8.78±0.54 ^e
Absorbance (600nm)	0.092±0.00 ^c	0.097±0.00 ^c	0.686±0.00 ^a	0.407±0.06 ^b	0.071±0.00 ^d
D[3,2]* (µm)	14.22±0.35 ^b	11.53±0.26 ^c	21.30±1.58 ^a	12.76±0.65 ^c	0.15±0.00 ^d
D[4,3]** (µm)	54.24±3.02 ^b	37.55±2.15 ^c	62.38±9.13 ^a	24.44±0.78 ^d	2.63±0.31 ^e

*Surface weighted mean diameter

**Volume weighted mean diameter

Data are presented as mean ± standard deviation (n = 3).

^{a-e} Different superscript letters in the same row show significant differences ($P < 0.05$).

Table 3. Flow and viscoelastic parameters of film-forming solutions with different caseinate:HM pectin ratio (T = 25 °C)

CAS:HMP ratio	Flow parameters			Viscoelastic parameters	
	Power-law model			Crossover point	
	m^* (mPa.s ⁿ)	n^{**}	R^2	$G' = G''$ (Pa)	Frequency (Hz)
100:0	1.60	1	0.99	0.03	1.33
75:25	7.59	0.97	0.99	0.26	3.07
50:50	70.00	0.83	0.99	2.44	4.74
25:75	160.00	0.78	0.99	-	-
0:100	230.00	0.80	0.99	-	-

*Consistency coefficient, **Flow index

Table 4. Colour properties of caseinate-HM pectin films

CAS:HMP ratio	L^*	a^*	b^*
100:00	73.16 ± 0.68^a	-5.13 ± 0.17^a	6.44 ± 0.24^c
75:25	72.76 ± 0.38^a	-5.13 ± 0.04^a	7.70 ± 0.59^b
50:50	71.84 ± 0.07^b	-4.88 ± 0.05^b	8.78 ± 0.16^a
25:75	72.48 ± 0.52^{ab}	-4.97 ± 0.08^{ab}	8.70 ± 0.47^a
00:100	73.03 ± 0.29^a	-5.02 ± 0.12^{ab}	8.52 ± 0.61^{ab}

Data are presented as mean $\pm$ standard deviation ($n = 3$).

^{a-e} Different superscript letters in the same column show significant differences ($P < 0.05$).

Table 5. Physical parameters of caseinate-HM pectin films

CAS:HMP ratio	Moisture content (%)	Water solubility (%)	Swelling index (%)
100:0	14.27 ± 1.11^a	27.16 ± 1.62^c	489.03 ± 12.25^c
75:25	15.42 ± 3.35^a	35.60 ± 3.36^d	734.03 ± 12.57^d
50:50	14.80 ± 2.45^a	45.25 ± 1.09^c	1135.99 ± 10.73^c
25:75	16.43 ± 1.08^a	53.70 ± 1.92^b	1214.45 ± 18.44^b
0:100	16.94 ± 2.49^a	100.00 ± 0.00^a	1264.33 ± 27.26^a

Data are presented as mean $\pm$ standard deviation ($n = 3$).

^{a-e} Different superscript letters in the same column show significant differences ($P < 0.05$).

Table 6. Mechanical properties of caseinate-HM pectin films

CAS:HMP ratio	Tensile strength (MPa)	Elongation at break (%)
100:0	33.94 ± 2.77^a	2.36 ± 0.37^e
75:25	27.19 ± 2.40^b	4.23 ± 1.00^d
50:50	21.30 ± 1.95^c	6.66 ± 0.35^c
25:75	19.52 ± 1.22^c	16.53 ± 0.39^b
0:100	26.17 ± 1.11^b	22.21 ± 0.20^a

Data are presented as mean $\pm$ standard deviation ($n = 3$).

^{a-e} Different superscript letters in the same column show significant differences ($P < 0.05$).

Figure captions

Fig. 1. Rheological profile of film-forming solutions prepared at different caseinate:HM pectin ratios (100:0, 75:25, 50:50, 25:75, 0:100) ($T = 25\text{ }^{\circ}\text{C}$): (a) steady shear flow viscosity, (b) complex modulus (G^*) and (c) $\tan(\delta)$ (strain = 1%).

Fig. 2. Effect of caseinate:HM pectin ratios (100:0, 75:25, 50:50, 25:75, 0:100) on (a) transmission intensity and (b) Turbiscan stability index (TSI) of film-forming solutions ($T = 25\text{ }^{\circ}\text{C}$).

Fig. 3. Backscattering (ΔB) and transmittance (ΔT) variation profile as a function of time ($T = 25\text{ }^{\circ}\text{C}$) for film-forming solutions prepared at different caseinate:HM pectin ratios (100:0, 75:25, 50:50, 25:75, 0:100).

Fig. 4. SEM micrographs (cross-section) of films prepared at different caseinate:HM pectin ratios, up: 100CAS:0HMP ($\times 1000$) and 0CAS:100HMP ($\times 1000$), down: 75CAS:25HMP ($\times 2500$), 50CAS:50HMP ($\times 2500$), and 25CAS:75HMP ($\times 2500$).

Fig. 5. Thermal and structural properties of films prepared at different caseinate:HM pectin ratios (100:0, 75:25, 50:50, 25:75, 0:100): (a) DSC thermogram and (b) FTIR spectra.

Author Statement

Mastaneh Jahromi :Formal analysis, Investigation, Methodology, Writing-original draft

Mehrdad Niakousari: Funding acquisition, Conceptualization, Investigation, Supervision, Writing-review & editing

Mohammad Taghi Golmakani: Investigation, Supervision

Mohammad Amin Mohammadifar: Resources, Validation

Highlights

- Effect of HM pectin on film-forming properties of caseinate was evaluated.
- Physical stability and destabilization mechanism of film-forming solutions were investigated using Turbiscan optical analyser.
- Interpolymeric electrostatic interactions improved the physical stability of film-forming solutions and developed biocomposites with homogeneous and smooth microstructure.
- Incorporation of HM pectin into caseinate increased the flexibility and water solubility of films.
- Obtained films were characterized for their thermal and structural properties.