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Preparation and Characterization of Pullulan-Soy Protein Concentrate Biocomposite Film

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ABSTRACT: The non-biodegradable nature of plastic packaging has brought about an attention in bio-based packaging materials. Edible composite films were prepared using soy protein concentrate (SPC) and pullulan (PUL) biopolymer at five different ratio (100:0; 70:30; 50:50; 30:70 and 0:100) with glycerol as plasticizer by casting-evaporation method. The thickness, mechanical properties, water vapor permeability (WVP), solubility and color of the film specimens were investigated. Increasing PUL content decreased the thickness, and tensile strength but the visual properties, elongation at break, WVP, and solubility in water of the films improved, simultaneously. In addition, the crystalline properties and microstructure of the bio-composites were characterized by X-ray diffraction and Scanning Electron Microscopy (SEM), respectively. Homogenous microstructure and crystalline properties of the films showed good affinity with mechanical and functional properties of the films. The optimum mechanical properties and WVP were obtained at the SPC to PUL ratio of 50:50. These findings support the potential of the equal proportion of SPC/PUL biocomposite as a food packaging material.

Keywords: *Functional Properties, Pullulan, Soy Protein Concentrate*.

Introduction

In recent years the wide use of petroleum based polymers have inappropriate effects on both the environment and consumer health. Waste disposal problems and high price of synthetic polymers can be solved to a certain extend by using of biopolymer-based packaging materials. The renewable and biodegradable agricultural material such as proteins and polysaccharides are well studied for food packaging (Hassannia-Kolaee *et al*., 2016; Shahabi-Ghahfarrokhi *et al*., 2015), which could be utilized to protect fresh and processed foods against contamination, aroma preservation, increasing shelf life, and quality maintenance (Siracusa *et al*., 2008).

According to FAO statistics, about 308 million tons of soybean seeds were cropped worldwide in 2014 (FAOSTAT, 2014). Soy protein contains two globulin type proteins, i.e. β-conglycinin and glycinin. The molecular mass of β- conglycinin and glycinin are 150 kDa and 350–400 kDa, respectively (Friesen *et al*., 2015). Soy protein concentrate (SPC) is a commercial form of soy protein that contains the high amount of protein (65-90%). Soy proteins

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have interested as a bio-based material for food packaging with good film forming properties (Kurose *et al*., 2007). Not only SPC is a good barrier against fats, oils and volatile compounds, but also possess a selective permeability for gases (González and Igarzabal, 2015; Su *et al*., 2010). However, there are some limitations for the application of this environment-friendly material for packaging due to its poor mechanical properties and high sensitivity to moisture as compared to their synthetic counterparts (Shahabi-Ghahfarrokhi *et al*., 2015). Therefore, different approaches such as chemical modification by changing the pH, the plasticizer, and making chemical cross-linking (Wihodo and Moraru, 2013), composition with other biopolymers (Su *et al*., 2012) composition with nano fillers were suggested to improve the drawbacks of biobased packaging materials (Hassannia-Kolaee *et al*., 2016). Looking converse side of the coin there are some concern about nanoparticles side effects on human body and toxic components residues (Hassannia-Kolaee *et al*., 2016). Due to these concerns modification methods that is the blending of polysaccharides and proteins to overcome the disadvantages of films properties has been recommended (Zolfi *et al*., 2014).

Pullulan (PUL) as a linear extracellular homopolysaccharide of glucose α (1-6), consisting mainly of maltotriose repeating units is produced by *Aureobasiduium pullulans* (Leathers, 2003). PUL based packaging films are suitable for food coatings because of their special properties i.e. odorless, tasteless, colorless, heat-sealable, transparent, highly flexible, and low permeability to oxygen and oils (Tong *et al*., 2008; Trinetta *et al.*, 2011). Despite many potential applications of PUL, usage in a pure form for films has been limited due to the high cost and some poor properties (Wu *et al*., 2013) therefore blending with whey protein (Gounga *et al*., 2007), caseinate (Kristo *et al*., 2007), alginate and carboxymethylcellulose (Tong *et al*., 2008) chitosan and carboxymethyl chitosan (Wu *et al*., 2013) have been proposed in previous researches.

The main aim of the present study is to develop an inexpensive and an eco-friendly PUL/SPC composite film as food packaging material and characterize the functional properties of PUL/SPC composite film.

Materials and Methods

- *Materials*

Food grade PUL (PF-20-grade) with average molecular weight (Mn) = 200,000–250,000 Da, was purchased from Hayashibara Co. (Okayama, Japan); SPC with 85% protein content was supplied by Tianjing Co., China. Glycerol, sodium hydroxide, calcium chloride and magnesium nitrate were provided by Merck Chemical Co., Germany.

- *Preparation of SPC/PUL biocomposite films*

An aqueous solution of 5 wt% PUL was prepared and 70 wt% (dry base) glycerol was added to it as a plasticizer. Furthermore, an aqueous solution of 5 wt% SPC was prepared and pH was adjusted on 10 using NaOH (0.1 N). The SPC solution heated up to 90 °C for 30 minutes in order to denature soy proteins. 70 wt% (dry base) glycerol was added to SPC film solution as a plasticizer. For stopping soy protein denaturation the solution was cooled down to 40 °C and filtered through four layers of cheese cloth using a vacuum pump (Emiroğlu, Yemiş, Coşkun, and Candoğan, 2010).

The SPC and PUL solution were mixed at the following ratios: (100:0, 70:30, 50:50, 30:70, and 0:100) for 10 min to form a homogeneous film forming solution. After degassing, the film forming solutions were cast by pouring the mixture into plastic petri dishes. The casted solutions were dried at 30 °C and room relative humidity for 48 hours. All the peeled off dried film specimens were conditioned inside desiccator containing a

saturated magnesium nitrate solution to ensure a relative humidity of 55% at 25 ± 1°C for at least 48 h.

- *Scanning electron microscopy*

Microstructure analysis of the surface and cross-section films was conducted with a field emission scanning electron microscopy (FE-SEM) KYKY-EM3200 (KYKY, China) with the accelerating beam at 20kV. After mounting the films on aluminum stubs using double-sided tape, they sputtered with a thin layer of gold using a KYKY-SBC-12 sputter coater (KYKY, China). The cross-sections were prepared by breaking the films in liquid nitrogen.

- *Color*

Color of the film was determined using colorimeter (Hunter-lab model D25/DP-9000, USA). Film specimens were placed on a white standard plate and the lightness (*L*) and chromaticity parameters *a* (red-green) and *b* (yellow-blue) were measured. All colors can be described by *L* values range from 0 (black) to 100 (white); negative values of *a* (greenness) to positive values (redness); and negative values of *b* (blueness) to positive values (yellowness). All measurements were performed in five replicates. The total color difference (ΔE) and whiteness index (WI) were calculated using equations 1-2 (Shahabi-Ghahfarrokhi *et al*., 2015)

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

$$WI = 100 - \sqrt{(100 - L)^2 + a^2 + b^2} \quad (2)$$

Where L^*, a^*, and $b^*$are the color parameter values of a standard (L^*= 92.22, a^*= -1.28 and b^* = 1.17) and *L*, *a*, and *b* are the color parameter values of the specimen.

- *X-ray diffraction analysis*

The X-ray diffraction (XRD) analysis was recorded at ambient temperature over diffraction angle in the range of 2θ=5˚-40˚ with a step-scan of 0.02˚ by Cu Kα (λ = 0.15418 nm) at 40 kV and 30 mA using a Bruker Advance D8 (Karlsruhte, Germany).

- *Film thickness measurement*

Thickness was measured to the nearest 0.001mm at ten random locations on each specimen with a micrometer (Mitutoyo, Japan).

- *Water vapor permeability*

The water vapor permeability (WVP) of films was measured gravimetrically according to ASTM E96 standard and corrected for the stagnant air gap inside the test cups (ASTM, 1995). Special glass vials with a diameter of 12.62 mm and a volume of 10 ml were used as test cups. The area of the vial mouth was 1.25×10^{-4} m^2, and the depth was 43 mm. The vials contained calcium-chloride desiccant (0% RH, assay cup) or nothing (control cup). Each vial was covered with a film specimen, which was sealed to the vial mouth using paraffin. Each vial was placed in a desiccator maintained at 75% RH with a saturated solution of sodium chloride. The difference in RH corresponded to a driving force of 1753.55 Pa, expressed as water vapor partial pressure. After the films were mounted, the weight gain of the whole assembly was periodically recorded (with an accuracy of 0.0001 g) every 1 h during the first 10 hours and finally after 25 h. The slope (S) of the time-weight plot ($R^2 \geq 0.986$) was divided by the effective film area (A) to obtain the water vapor transmission rate (WVTR), as shown in equation 3. This was multiplied by the thickness of the film and divided by the pressure difference between the inner and outer surfaces to obtain the WVP, as shown in equation.4:

$$WVTR = \frac{S}{A} \quad (3)$$

$$WVP = \frac{WVTR \times X}{\Delta P} \quad (4)$$

Where x is the average film thickness (m) and ΔP is the driving force (1753.55 Pa).

- *Water solubility*

Water-solubility (WS) was defined as percentage of film dry matter soluble after 6 h immersion in distilled water. Samples of films were cut (2 cm× 2 cm) and dried to constant weight in oven at 103 ± 2 ºC for approximately 3 h (m_1). Dried films were dropped into 50 ml distilled water with periodic agitation for 6 h at 25 ºC. The remaining pieces of films were taken out and dried at 103 ± 2 ºC to constant weight for approximately 3 h (m_2). WS was measured in triplicate order using the equation 5:

$$WS = \frac{m_2 - m_1}{m_1} \times 100 \quad (5)$$

- *Mechanical properties*

Tensile strength (TS) and elongation at break (EB) were evaluated by texture analyzer (Gotech, Taiwan) according to ASTM method D882 (ASTM, 2001). The films were cut into ribbons (10 mm × 80 mm). The desiccator containing saturated magnesium nitrate solution was used for conditioning film specimens in relative humidity of 55% at 25 ± 1°C at least 48 h. The initial grip distance and crosshead speed were set at 50 mm and 50 mm/min, respectively. The mechanical properties were carried out in triplicate order. TS and EB were calculated using equations. 6-7:

$$TS = \frac{F_{Max}}{A_{Min}} \quad (6)$$

$$EB = \frac{L_{Max}}{L_0} \times 100 \quad (7)$$

Where F_{max} is the maximum load, A_{min} is the minimum cross-section area, L_{max} is the extension at the moment of rupture, and L_0 is the initial length of specimen.

- *Statistical analysis*

Statistical analysis was carried out on a completely randomized design with the analysis of variance (ANOVA) procedure using SPSS software (Version 11.5; SPSS Inc., USA). Duncan's multiple range tests were used to compare the differences among mean values of film specimens' properties at $P<0.05$.

Results and Discussion

- *Microstructure*

The SEM micrographs of the surfaces of the PUL, SPC, and their biocomposite films are shown in Figure 1. As can be seen in Figure 1 (S-1) pure SPC film had smooth surface. Aalthough PUL film (S-5) had the smoothest surface morphology among all the tested films in this study. SEM observations of blend films with different PUL concentrations illustrated the structural differences. In composite films, the homogeneity of film surfaces was decreased with increasing the ratio of PUL. The surface of PUL and SPC pure films were smoother than their composites, especially in comparison PUL film that represented a continuous and homogeneous matrix. As can be observed here, if two polymers have appropriate miscibility, phase separation will not occur between them. Overall, the compact structure, free of air bubbles and pores and non- phase separation in the composite films is an indicator of the structural integrity and good miscibility of the two polymers. In other studies, the pure kefiran and starch films and their blend films micrographs were investigated. The observations have shown that the homogenous and smooth structure of kefiran films has changed by increasing starch content. Compact, rough, grainy and porous structure can be seen in 50:50 and 30:70 kefiran: starch blend film. Formation channels and granule structure of starch were the reasons of those observations (Motedayen *et al.*, 2013). Cellulose- SPI composite films made by hot surface casting technique indicated that the microstructure of pure SPI films was smoother and

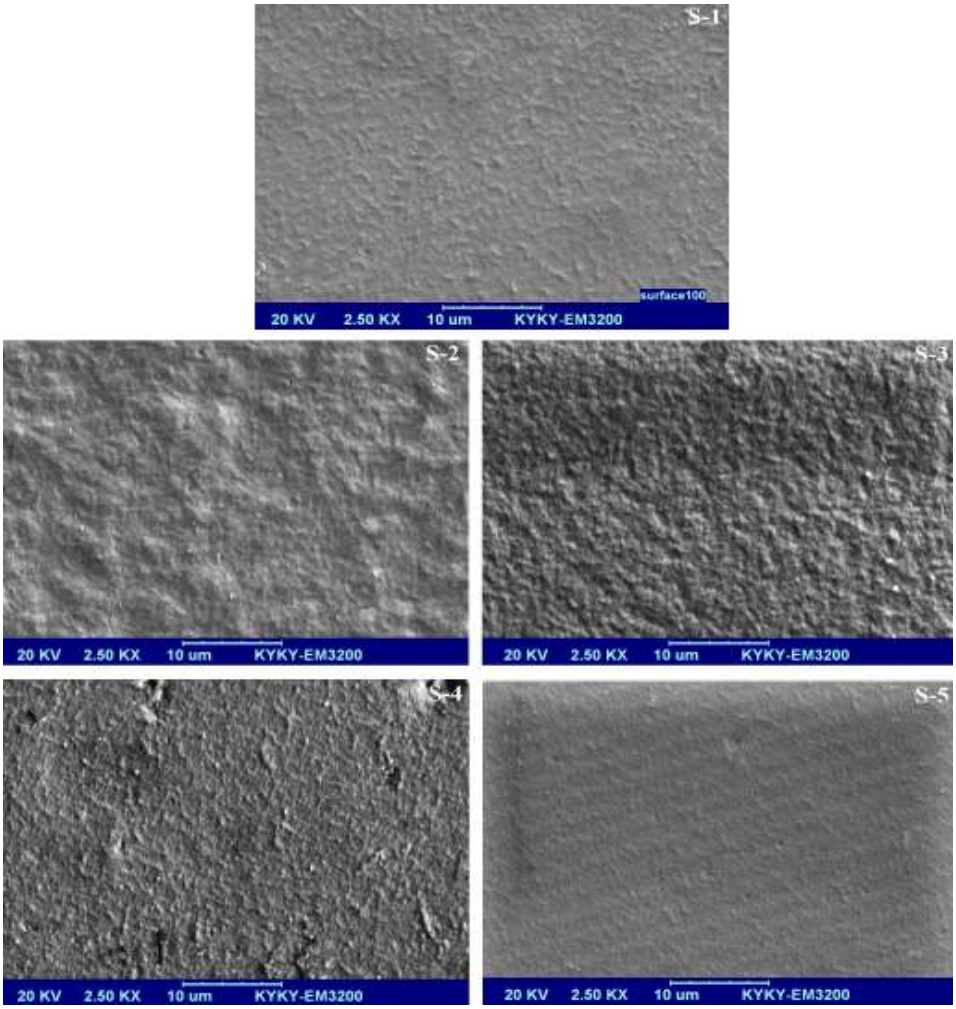

Fig. 1. Surface morphologies of SPC: PUL films (S-1) 100:0, (S-2) 70:30, (S-3) 50:50, (S-4) 30:70 and (S-5) 0:100

homogenous than the composite films (Jensen *et al*., 2015).

- *Color*

Color is one of the most important characteristic of a packaging. Consumers are highly influenced by the color and visual properties of the food packaging materials (Zhang *et al*., 2009). It is obvious that PUL film is more transparent as compared to the SPC film. Pullulan films are colorless, this property make this polysaccharide an ideal material for food packaging (Trinetta *et al*., 2011).

Table 1 shows hunter colorimeter values i.e. *L*, *a*, *b*, ΔE, WI of the SPC, PUL, and SPC: PUL biocomposite films, where *a* value of the films did not change and no significant differences between blend films were observed ($p>0.05$). By increasing SPC ratios, *L* and WI values of specimens were significantly decreased simultaneously and *b* and ΔE values were increased significantly ($p<0.05$). The yellow color and darkness of the films were increased by increasing SPC ratio in the film forming solution. The film with 30:70 (SPC: PUL) ratio had the lowest ΔE and *L* values. On the other hand, this ratio had the highest WI and *b* value among the blend films. Therefore, the blend film with 30% SPC had the best color and appearance. The same result was reported in previous study (Zhang *et al.*, 2009). Previous studies evinced the effects of pH

value, cross-linkage degree, plasticizer content, thermal treatment, protein ratio and fabrication process on color and visual properties of protein based films. Among all the factors, protein ratio was the most effective parameter in color and visual properties of protein based polymers (Su *et al*., 2012).

- *X-ray diffraction analysis*

XRD was used to determine crystal structure and distinguish the compatibility of SPI with PUL. XRD spectrum of SPC, PUL, and SPC/PUL composite films are presented in Figure 2. It is clear that SPC and PUL conformation were semi-crystalline to amorphous. SPC film has shown a broad peak at a 2θ angle of 20.54°, which corresponded to the crystalline structure (Su *et al*., 2010). On the other side a broad and weak peak was observed in PUL around 2θ=13.4°. The comparison of XRD pattern of SPC/PUL biocomposite films with pure films indicates that the intensity of 2θ=13.4° was decreased and disappeared by increasing SPC ratio. Simultaneously, the intensity of 2θ=20.54° was increased. It seems that strong interactions between functional groups of SPC and PUL has taken place and the crystalline structure of SPC has collapsed after increasing PUL content in biocomposite films. This is in agreement with previous study (Su *et al*., 2010).

Table 1. Hunter color values (*L*, *a*, and *b*), total color difference (ΔE), and whiteness index (WI) of SPC, PUL, and SPC/PUL biocomposite films[a,b]

SPC:PUL	*L*	*a*	*b*	ΔE	WI
100:0	72.76 ± 0.33^{e}	-2.56±0.13^{c}	21.53±0.19^{a}	28.19±0.35^{a}	65.18±0.36^{e}
70:30	78.23±0.06^{d}	-2.13±0.05^{b}	10.30±0.16^{b}	16.72±0.03^{b}	75.82±0.01^{d}
50:50	78.80±0.31^{c}	-2.10±0.03^{b}	9.46±0.16^{c}	15.79±0.34^{c}	76.69±0.34^{C}
30:70	83.53±0.07^{b}	-2.06±0.03^{b}	6.26±0.03^{d}	10.10±0.06^{d}	82.26±0.06^{b}
0:100	89.83±0.06^{a}	-1.00±0.02^{a}	2.00±0.03^{e}	2.54±0.06^{e}	89.58±0.06^{a}

[a] Data are presented as mean values ± standard deviation.
[b] Values with the same superscript letters within a column are not significantly different ($p < 0.05$).

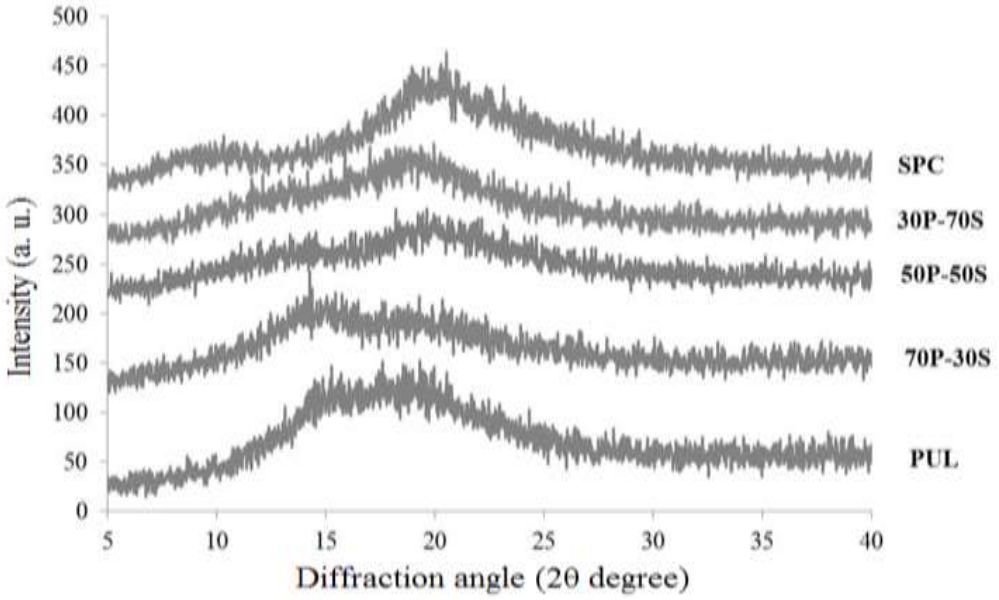

Fig. 2. XRD pattern of SPC, PUL, and SPC/PUL biocomposite films

- *Film thickness*

Film thickness affects WVP, mechanical properties, and color of a packaging system (Wu *et al*., 2013). Final thickness depends on preparation methods, film forming solution formulations and drying conditions (Galus *et al.*, 2012; Mahamadou Elhadji Gounga *et al*., 2007). The thickness of films specimens is presented in Table 2. The thickness of the films specimens was increased significantly by increasing SPC content ($p<0.05$). As shown in Figure 1, the microstructure of PUL was compacted and homogenised. By increasing PUL ratio of film forming solution, the compactness of films is increased, significantly ($p<0.05$) and consequently, the thickness of films specimens was decreased, drastically. Our results are in agreement with previous studies (Gounga *et al*., 2007).

- *Water vapor permeability*

WVP as the most important function of the biopolymers should be as low as possible. WVP shows the capability of polymer to avoid moisture transfer between food and the surrounding atmosphere (Zhang *et al*., 2009). Hydrophobicity of protein and polysaccharide based films is a critical effective factor on WVP. The sensibility of the biopolymers to water lead to structural changes in the network of the film and film-water interactions (Kristo *et al*., 2007).

WVP of SPC, PUL, and SPC/PUL biocomposite films are presented in Table 2. The WVP of the film specimens was decreased significantly by increasing SPC content in SPC/PUL composites ($p<0.05$). The lowest and the highest WVP were observed in SPC and PUL films, respectively. SPC contains 58% polar amino acids which enhances the hydrophilicity and moisture sensitivity in the film specimens (González and Igarzabal, 2013). On the other hand, PUL possesses many hydrophilic substituents and around 10% mono, di, and oligosaccharides which considerably enhances moisture sensitivity and polymer chain mobility (Stankovic, 2011). The penetration of water vapor in polymer matrix was explained by free volume theory. According to the theory, the effective factors on polymer mobility, i.e. plasticizers (water, glycerol, mono, and disaccharides), expand the free volumes and consequently increase WVP (Shahabi-Ghahfarrokhi *et al*., 2015). Although, previous researchers have shown that the mixing polysaccharides and proteins improved WVP of the films (Galus *et al*., 2012; Jensen *et al*., 2015; Su *et al*., 2010) but hyper-hydrophilicity of PUL brought about increased WVP in SPC/PUL biocomposite films.

Table 2. Effect of different ratio of pullulan (PUL) and soy protein concentrate (SPC) on thickness, WVP, solubility in water, and mechanical properties of pullulan-SPC films[a,b]

(SPC: PUL)	Thickness (mm)	WVP ($\times 10^{-11}$ $gm^{-1}s^{-1}Pa^{-1}$)	Solubility in water (%)	Tensile strength (MPa)	Elongation at break (%)
100:0	0.153±0.020[a]	2.98±0.02[d]	68.56±2.15[e]	6.55± 0.45[a]	98.38±2.85[d]
70:30	0.135±0.011[ab]	4.03±0.10[c]	76.61±0.78[d]	4.81± 0.43[b]	159.24± 2.33[c]
50:50	0.121±0.016[b]	5.06±0.23[bc]	81.13±3.43[c]	4.11±0.46[b]	223.31±3.19[b]
30:70	0.114±0.012[b]	5.91±0.17[ab]	86.74±2.06[b]	2.99± 0.44[c]	209.98±3.24[b]
0:100	0.091±0.013[c]	6.89± 0.08[a]	96.19±1.41[a]	2.43±0.36[c]	257.6±5.47[a]

[a] Data are presented as mean values ± standard deviation.
[b] Values with the same superscript letters within a column are not significantly different ($p < 0.05$).

- *Water-solubility*

Water resistance of packaging material is a critical property, which protects foods against spoilage. Table 2 showed the solubility of SPC, PUL, and SPC/PUL composite films in water. Solubility of SPC/PUL composite films was increased significantly by increasing PUL content ($p<0.05$).

Regular alternation of α (1→4) and α (1→6) bonds in PUL polymer chain and polar amino acids in SPC polymer chain bring about sensitivity of SPC/PUL films to water (Leathers, 2003; Su *et al*., 2010; Trinetta *et al*., 2011), but differences in the solubility of SPC and PUL in water was related to the materials structures. PUL has an amorphous structure (Figure 2) and is more hydrophilic than SPC therefore this led to the high solubility of PUL. Although, SPC and composite films have a semi-crystalline structure (Figure 2) therefore they are less soluble in water as compared to PUL. Similar results were reported previously by Zhang *et al*. (2009).

- *Mechanical properties*

Table 1 presented the results of TS and EB of SPC, PUL, and SPC/PUL composites. SPC and PUL film specimen showed the highest TS (6.55 Mpa) and EB (257.6%), respectively. TS of the SPC/PUL composite films were decreased significantly by increasing PUL content and EB was increased, simultaneously ($p<0.05$). The presence of α (1→6) bonds on the PUL structure and mono and disaccharide in PUL led the molecular motions to set around the three inter-residue bonds making a structure with extra flexibility (Leathers, 2003; Shahabi-Ghahfarrokhi *et al*., 2015). Furthermore, amino group in SPC enhances cohesiveness of SPC polymer matrix (Kristo *et al*., 2007), hence TS of films specimens were enhanced by increasing SPC ratio in SPC/PUL biocomposite films. As shown in Figure 2 PUL possesses an amorphous structure, therefore, increasing the PUL ratio in SPC/PUL biocomposite enhanced the flexibility and the EB of the film specimens. In pullulan- chitosan blend films TS improved by increasing the content of chitosan. This could be related to intermolecular hydrogen bonds and increase the number of amino groups by increasing the level of chitosan (Wu *et al*., 2013).

Conclusion

In this study three ratio of SPC and PUL were mixed together and compared with pure films of SPC and PUL. Composite films had a yellow appearance, flexible, and free of pores and cracks. The results indicated that incorporation of PUL significantly decreased thickness and tensile strength, increased elongation at break, enhanced water solubility and amplified WVP ($p<0.05$). The color of the composite films was changed depending on the ratio of incorporated PUL and SPC. By increasing PUL ratios b and ΔE were decreased significantly but WI and *L* were significantly increased leading to more discolored films ($p<0.05$). Based on the observation of microstructures by SEM, the SPC/PUL films micrographs were homogeneous without signs of phase separation and had good compatibility with each other. Through judicious mixing of SPC with PUL in the presence of glycerol as a plasticizer, composite films with optimal mechanical barrier and water-solubility can be synthesized for food packaging. Comparing to SPC, PUL is more expensive, and corporation of SPC into biopolymers is useful to reduce the cost of PUL-based edible films and can improve the properties of both materials. The optimum tensile and elongation are obtained at the SPC to PUL ratio of 50:50 suggesting that equal proportion of both materials could exhibit the best mechanical performance.

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