Protein-phenolic interactions: Effects on the properties of biopolymer films

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Abstract. The targeted search for sustainable and safe packaging materials increases the interest in biopolymer films derived from natural ingredients. This study analyses the potential of protein-phenolic complexes as a material for the creation of multifunctional and environmentally friendly biopolymer films. The protein-phenolic complexes used in the study are of soy protein isolate and pomegranate peel polyphenol extract in three different ratios of the constituent components. The resulting films were analysed for physicochemical and mechanical properties and bioactive functionality. The results show that the protein-phenolic complexes can be used as a basis for obtaining biopolymer films, consistent with the goals of sustainable development of biodegradable and functional packaging and systems for the needs of the food and pharmaceutical industries.

1 Introduction

Modern man is increasingly aware that clean food and an unpolluted environment are indispensable elements in health care. Safe packaging materials made with care for the environment are increasingly appealing to him [1, 2]. For this reason, in recent decades, interest has grown in the development of sustainable, environmentally friendly biopolymer films for applications not only in the food industry (such as food packaging and edible coatings), but also in biomedical applications (such as controlled drug release systems). Protein-based films become noticeable among biopolymer films derived from natural renewable sources due to their excellent film-forming properties, biodegradability, and biocompatibility [3, 4]. Soy protein isolate (SPI) has a relatively low cost and is widely available plant protein with a complete set of essential amino acids that has the ability to form films with good barrier properties against gases and aromas [1, 5-7]. However, SPI films have inherent limitations for proteins, such as sensitivity to moisture, fragility, and insufficiently strong bioactive properties [1, 4]. This prompts researchers to look for ways to improve their characteristics, one of which is through the use of bioactive compounds.

Polyphenols are biologically active compounds that occur naturally in plants. They possess strong antioxidant, antimicrobial, and anti-inflammatory properties, making them ideal candidates for acquiring or improving the existing functionality of biopolymer films. Due to the existing interactions between phenolic compounds, they can not only add/enhance the bioactive properties of protein films, but can influence the formation of the protein film matrix by acting as plasticizers or as crosslinking agents [8]. It is known that protein-phenol interactions result in the formation of complexes or conjugates. This complexation can lead to the creation of multifunctional materials suitable for various applications. Furthermore, the use of renewable resources and natural crosslinking agents meets the goals of sustainable development of biodegradable and functional packaging and systems for the needs of the food and pharmaceutical industries.

Pomegranate is a fruit that is rich in phenolic compounds, known since ancient times for its health benefits [9]. In a literature, pomegranate extracts are considered additives that enhance the structural, mechanical, and biochemical properties of protein- and polysaccharide-based biopolymer films (e.g., zein, gelatin, chitosan, pectin, carrageenan, carboxymethyl cellulose) [10-12].

In our previous studies, we found that interactions between a model protein - soy protein isolate, and phenolic compounds in pomegranate peel extract lead to changes in the structure and functionality of the protein [13]. At the same time, interactions with the protein also change the biological activity of the phenolic compounds [14].

In this study, we used preformed complexes between soy protein isolate and phenolic compounds in pomegranate peel extract as a basis for the preparation of biopolymer films. The resulting films were investigated for physicochemical, mechanical, optical, and antioxidant properties.

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2 Material and methods

2.1 Materials

The soy protein isolate (SPI) (SUPRO® EX 37, protein content: 89.96 ± 0.02%, by Kjeldahl method, N x 6.25) used in the study was supplied by P.I.C.Co. (Sofia, Bulgaria). All used chemicals and reagents were from Sigma Aldrich and Alfa Aesar.

2.2 Preparation of water-ethanol extract of pomegranate peel

Ripe pomegranate fruits were purchased from local stores in Sofia, Bulgaria. After thorough cleaning and separation, the peels were dried at 30°C and ground to powder (sieve size of 0.5 mm; moisture content 8.37 ± 0.25 %). The extraction was carried out with a 50% water-ethanol solution and a sample-solvent ratio of 1:10, at a temperature of 40°C for 4 hours. The extract with the solvent was centrifuged (Beckman J2-21M, Beckman Coulter, USA) for 10 minutes at 3000 x g and filtered with Whatman paper (No. 1). Then the solvent was removed at 50°C using a vacuum evaporator (20 kPa, SPT-200 Vacuum Drier) and the resulting pomegranate peel extract (PGPe) was lyophilized (LYOBETA 6PL, Telstar, Barcelona, Spain).

2.3 Protein-phenolic complexes

Protein-phenol complexes were obtained according to our previous studies [13]. In summary, soy protein isolate was dissolved in distilled water, homogenized and kept at 90°C for 20 minutes. The pH of the samples was adjusted to 9.0 with 0.1 M NaOH. After mixing with the extract in a ratio of 1:1; 1:2 and 1:3 to the protein (10 mg/ml) (w/w), the pH of the reactive mixture was further adjusted to the appropriate values. The mixture was incubated for 18 hours at continuous stirring (70 rpm) at room temperature. After incubation, the samples were transferred to a dialysis membrane (3.5 kDa), dialyzed for 24 hours at 4°C against distilled water and lyophilized. The complexes obtained were c_1, c_2, and c_3, respectively, according to the above-mentioned ratio between protein and extract.

2.4 Preparation of biopolymer films

The films were obtained by the solvent casting method. A 2 % film-forming solution (FS) of SPI was used for pure films (FSPI) and of complexes c_1, c_2, and c_3 for films that are based on protein-phenolic complexes (respectively for films Fc_1, Fc_2 and Fc_3). Glycerol (0.5 %) was added as a plasticizer. FS were also treated with ultrasound (VEVOR TH-30A, China) for 20 minutes to remove air bubbles. Film solutions were cast into glass petri dishes and dried at 35°C (20 kPa, SPT-200 Vacuum Drier) for 14 hours. The resulting films were separated and stored at room temperature and 50 ± 5 % RH for subsequent analysis.

2.5 Thickness

The film thickness was measured with a digital micrometer Mitutoyo 293-832 (Japan) with an accuracy of 0.001 mm ± 5%, at five randomly selected points of each sample.

2.6 Water solubility

Pre-weighted samples of the films of equal size (d = 2 cm) were placed in flasks containing 50 mL of distilled water. The flasks were incubated in a shaker system ("Inkubations-Schüttelschrank BS-4 B.Braun", 100 rpm) at a temperature of 25°C for 24 hours. Undissolved substances were separated by filtration and dried at 105°C for 24 hours, then weighted. Water solubility (WS) was calculated by the eq. (1):

$$WS = \frac{wi-wf}{wi} \times 100 \quad (1),$$

where wi is the initial weight, wf - the weight after filtration and drying.

2.7 Light transmittance

A UV-visible spectrophotometer (UV-Vis Biochrom Libra S 22, USA) was used to analyze the light transmission of the films at wavelength at of 200, 300, 400, 500, and 600 nm at room temperature. The film was cut into 10 x 40 mm samples and attached to the side of a quartz cuvette and a blank cuvette was used for reference.

2.8 Degree of crosslinking

The determination of the degree of cross-linking was performed using a ninhydrin assay [15]. The ninhydrin solution was prepared from 2 g ninhydrin and 0.3 g hydrindantin dissolved in 75 mL dimethylsulfoxide (DMSO) and by adding 25 mL lithium acetate buffer (4 N, pH 5.2) while bubbling with nitrogen. For the assay 2 mL of distilled water and 2 mL of ninhydrin reagent were added to 2 g of the film. The mixture was heated to 80°C for 12 min, after which the resulting solution was cooled and diluted with 5 mL of 60 % ethanol. Absorbance was measured at 570 nm on a spectrophotometer against a blank without film sample. A standard curve was prepared using glycine (R^2 = 0.9994). All samples were tested in triplicate. The cross-linking degree was calculated using eq. (2):

$$Crosslinking\ degree\ (\%) = (1 - \frac{NHt}{NHo}) \times 100 \quad (2),$$

where NH_0 is the amount of free amino groups in the films before crosslinking and NHt is the amount of free amino groups after crosslinking.

2.9 Mechanical properties

Mechanical properties were determined according to BDS EN ISO 527 3:2003 with Universal Mechanical

and Tribological Tester, UMT:2M (Bruker-CETR, USA). The test was conducted at room temperature (20 ± 2ºC), speed - 0.017mm s-1, sensor - 1000 N. The results are presented as average values ± standard deviation SD of 5 measurements for each sample.

2.10 Antioxidant activity with DPPH

Film samples (0.20 g) were cut into small pieces and extracted with 10 ml of methanol under magnetic stirring for 5 hours. After subsequent centrifugation at 5000 rpm for 15 minutes at 4°C, the supernatant was collected and diluted with 80 % methanol. A mixture containing 0.6 ml of methanolic 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) solution (0.2 mM), 0.9 ml of methanol and 0.5 ml of the supernatant was incubated at room temperature in the dark for 60 minutes. Then the absorbance was measured at 517 nm. Methanol and DPPH solution were used as blank and control, respectively. The antioxidant activity (AA) was calculated as follows (3):

$$AA\ (\%) = \frac{Acontrol - Asampl}{Acontrol} \times 100 \qquad (3)$$

2.6 Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) and post hoc Tukey test by the Minitab 17.0. software. Values are expressed as mean ± standard deviation (SD); $p<0.05$ was considered as statistically significant.

3 Results and discussion

Biopolymer films obtained by solvent casting method and subsequent drying are homogeneous, flexible, without brittle areas or air bubbles. The results of the physicochemical analyses of the films are given in Table 1.

Moisture content (MC) is a parameter that affects the physical and mechanical properties of protein films. Its values can be influenced by the film formulation, preparation methods, and storage conditions [1-4]. The results in Table 1 show that the MC of the obtained films varies from 12 % to 13.65 %. Compared to pure SPI films - FSPI (where MC values are the highest), an initial significant decrease ($p < 0.05$) in MC is observed in films Fc_1 (12 ± 0.20) and Fc_2 (12.5 ± 0.60), after which MC increases again in Fc_3. According to data from other authors, when pomegranate peel extracts are added to films based on proteins [16] and polysaccharides [17], an increase in MC values is observed with an increase in the concentration of polyphenols. In our study, the interactions between SPI molecules and phenolic compounds in PGPe at c_1 and c_2 and c_3 change the hygroscopic nature of the protein matrix by reducing the presence of hydrogen bonds between hydroxyl groups and water.

Solubility is related to the structural properties of the matrix, the content of phenolic compounds, and the degree of their interaction. The results for WS in Table 1 show that the change in values has a trend similar to that for MC. The highest value is found in FSPI, with significant differences from the values of the films based on c_1 (49 ± 0.50), c_2 (49.72 ± 0.23), and c_3 (50 ± 0.65). The results cause us to consider the formation of a compact network structure in the films Fc_1, Fc_2, and Fc_3, due to which the penetration of water molecules is more difficult. Literature data show a decrease in the solubility of films after the inclusion of pomegranate peel extract [10, 18].

Table 1. Physical properties of soy protein isolate and protein-phenolic based films

Films code	Moisture content, %	Water solubility, %
FSPI	13.65 ± 0.05	51.00 ± 0.20
Fc_1	12.00 ± 0.20	49.00 ± 0.50
Fc_2	12.50 ± 0.60	49.72 ± 0.23
Fc_3	13.10 ± 0.07	50.00 ± 0.65

FSPI – film based on soy protein isolate; Fc_1 - film based on complex 1. of soy protein isolate with pomegranate peel extract; Fc_2 - film based on complex 2. of soy protein isolate with pomegranate peel extract; Fc_3 - film based on complex 3. of soy protein isolate with pomegranate peel extract. Values are mean ± SD (n = 7).

UV light has an adverse effect on the quality and safety of food products, especially by enhancing lipid oxidation. Therefore, the ability to block UV radiation is of great importance for food packaging materials [19]. As can be seen from the transmittance results in Table 2 in the wavelength range from 200 nm to 400 nm, all films block UV light, most clearly observed at 300 nm, where the transmittance is around zero. This ability is due to the amino acids such as tyrosine and tryptophan in SPI and phenolic compounds in PGPe, due to the content of aromatic rings with the presence of double bonds. In the visible region from 400 nm to 600 nm, the films of Fc_1, Fc_2, and Fc_3 retain their higher absorption ability compared to FSPI, with the differences being statistically significant. The results obtained at 600 nm show us the higher opacity of the films of Fc_1, Fc_2, and Fc_3 (compared to FSPI), which is in agreement with data in the literature for films with included plant extracts [20].

Table 2. UV-Vis transmission of soy protein isolate and protein-phenolic based film**s**

Transmittance, %	Films code			
	FSPI	Fc_1	Fc_2	Fc_3
200 nm	48 ± 0.5	28 ± 0.14	27.8 ± 0.4	27.1 ± 0.15
300 nm	0.1 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.01 ± 0.00
400 nm	34 ± 0.17	4.3 ± 0.20	3.9 ± 0.23	3.1 ± 0.5
500 nm	65 ± 0.91	39 ± 0.50	38 ± 0.5	39.4 ± 0.63
600 nm	79 ± 0.76	61 ± 0.60	60 ± 1.10	64 ± 0.73

The degree of crosslinking of the protein films was estimated by calculating the difference in the content of free amino groups per gram of biopolymer between samples of the same protein matrix without and with PGPe. Table 3 shows the values for the degree of crosslinking, with the highest degree of crosslinking being found in Fc_2 and Fc_3, followed by Fc_1. The observed reduction in free amino groups in the proteins is a consequence of the interaction between the functional groups of the proteins and the phenolic compounds in the extract. Multiple interactions (covalent and non-covalent) determines the likely mechanisms of the process [21].

Table 3. Crosslinking degree and antioxidant activity of soy protein isolate and protein-phenolic based films

Films code	Degree of crosslinking, %	Antioxidant activity, %
FSPI	*	2.1 ± 0.5
Fc_1	55.25 ± 0.2 **	30.8 ± 1.2
Fc_2	58.32 ± 0.1 **	40.3 ± 0.74
Fc_3	58.34 ± 0.1 **	42.5 ± 0.21

*value is involved in calculation of **

The presence of biologically active substances with antioxidant activity in the formula of biopolymer films is of decisive importance for preserving the quality and freshness of food products. Natural antioxidants are preferred over their synthetic counterparts due to their safety and consumer acceptance [22]. The antioxidant activity results are given in Table 3. As can be seen, the antioxidant activity of films based on c_1, c_2, and c_3 is ~ 15 to 20 times greater than that of FSPI. The results obtained show that the presence of PGPe in the matrix increases the antioxidant activity of the resulting films and leads to an improvement in their functionality. Pure SPI film also showed some antioxidant activity, which can be explained by the high content of the amino acids cysteine, tyrosine, tryptophan, and histidine [23].

In Table 4 are shown the thickness of the obtained films. As can be seen from the results, the lowest values are for Fc_1, followed by Fc_2 and Fc_3. The difference in the values of the three films and the FSPI values is significant ($p < 0.05$). The complexation of the protein with polyphenolic compounds probably leads to a more ordered structure of the film. When including pomegranate peel extract in protein films, authors report an increase in thickness or unchanged values [10, 11].

Table 4. Thickness and mechanical properties of soy protein isolate and protein-phenolic based films

Films code	Thickness (mm)	Tensile strength (MPa)	Elongation at break (%)
FSPI	0.51 ± 0.004	0.61 ± 0.10	197.60 ± 2.19
Fc_1	0.24 ± 0.06	2.82 ± 0.31	104.7 ± 28.03
Fc_2	0.28 ± 0.08	3.23 ± 0.51	163.5 ± 13.48
Fc_3	0.32 ± 0.04	3.21 ± 0.46	171.4 ± 10.62

The mechanical strength and extensibility of protein films are of great importance for preserving the integrity and shelf life of packaged food products [3]. The mechanical properties of films are influenced by structural changes and modifications in the film components, the strength of intra- and intermolecular interactions between polymer chains, and the type and amount of plasticizers [3, 10, 11]. As can be seen from Table 3 value of tensil strength on the control film is 0.61 ± 0.10 MPa. This value is significantly increased ($p < 0.05$) by 530 % to 3.23 ± 0.51 for the Fc_2. Phenolic compounds are forming zones of connection within the matrix of the film through protein-polyphenol interaction, resulting in a stronger film matrix. These results are consistent with the improvement of the TS of biopolymer films after the addition of plant extracts to their composition [8]. The inclusion of PGPe initially reduced flexibility, as evidenced by reduction from the control film (197.60 %) to 104.7 % in Fc_1, after which elongation value increases again in Fc_2 and Fc_3 relative to the film based on c_1. This result indicates that the addition of PGPe in moderate and high concentrations (2–3 %) in complexes can promote not only protein crosslinking but also have a plasticizing effect on resulting films.

4 Conclusion

In this study, protein-phenolic complexes were used as a basis for the preparation of biopolymer films. The results show that in the films based on complexes, compared to the control group – a pure biopolymer, there is an improvement in the studied physical, mechanical, optical, and antioxidant properties. The study provides information about the effect of protein-phenolic interaction on the properties of the resulting films. This information can be used in the development of active films for packaging or drug delivery systems.

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