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Design of Bioinspired Emulsified Composite European Eel Gelatin and Protein Isolate-Based Food Packaging Film: Thermal, Microstructural, Mechanical, and Biological Features

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Abstract: The study focused on the elaboration and the characterization of blend biofilms based on European eel skin gelatin (ESG) and protein isolate (EPI) and the assessment of European oil (EO) incorporation effect on their properties. Data displayed that the incorporation of EPI and EO to the gelatin formulation decreased the lightness and yellowness of composite and emulsified films, respectively, compared to ESG film. Moreover, ESG films exhibited improved mechanical properties than EPI films. FTIR analysis, all incorporated films with EO at the ratio 1:4 (oil/polymer) revealed similar characteristic bands as in free-oil films. Further, the SEM images of 100% ESG and 100% EPI films showed a smooth and homogenous structure, whereas the cross-section of blend film (at a ratio 50:50) displayed a rougher microstructure. In addition, emulsified film ESG100 revealed a smooth and homogeneous microstructure compared to that prepared using EPI/ESG 50/50 ratio. Furthermore, EPI or EO addition into the ESG matrix enhanced the blend films antioxidant activities.

Keywords: protein isolate; gelatin; blend films; antioxidant properties

1. Introduction

In order to reduce environmental pollution and to conserve fuel reserves, the biodegradable packaging based on safe biopolymers has been investigated in many researches [1]. Films based on eco-friendly biopolymer displayed advantageous properties compared to the traditional plastics, favoring their potential application, mainly their biocompatibility, non-toxicity, availability, and biodegradability [2]. Furthermore, biopolymer-based films behave as selective barriers to oxygen diffusion, lipid oxidation, moisture transfer, and loss of volatile aroma compounds which improve food shelf-life and quality [3].

Various biopolymers, such as proteins, polysaccharides, and lipids, could be used to produce biodegradable films [4–6]. Fish gelatin and protein isolate have been previously used for developing bio-films owing to their availability and interesting film-forming capacities [7,8]. Fish protein isolate extracted by alkaline solubilization could be a potential alternative for films properties enhancement [9].

The interactions between protein chains via disulfide (S–S) covalent bonding, electrostatic forces, hydrogen bonding, and hydrophobic bonding favored the appropriate mechanical characteristics properties of protein isolate-based films [10]. Nonetheless, protein isolate-based films are, to a variable extent, rigid, attributed to the presence of strong covalent bonding, particularly disulfide bonds [11]. Protein isolate may be combined with other compatible polymers as an alternative to improve the thermo-mechanical properties of resulting films. Mainly, combining biopolymers with the most desirable characteristics of each component are anticipated for developing materials with exceptional properties [12]. Therefore, blending gelatin with protein isolate could solve the problem of brittleness and improve mechanical properties of protein isolate-based films.

Lipids could advantageously be mixed with above-cited polymers to form dried emulsions or bilayer films [13] able to protect foods against lipid oxidation, a serious problem that leads to the loss of products' shelf-life [14]. Indeed, due to the hydrophobic nature, the incorporation of oils in the formulation of films improved films water barrier property [15]. Thus, emulsified films with a potential water barrier ability are requested for the development of innovative foods processed and the extension of the shelf life of food [13]. In our previous studies, protein isolate (EPI) was extracted by the shifting-pH technique from European eel fish muscle and was applied for the preparation fish protein gels [16] and microcapsules [17].

Therefore, the present research aimed to evaluate the influence of European eel gelatin and protein isolate combination on the functional and antioxidant properties of blend films and the assessment of the effect of European eel oil incorporation on the of the film properties.

2. Materials and Methods

2.1. Preparation of European Eel Protein Isolate

The protein isolate (EPI) was extracted from European eel muscle by the pH-shifting method according to the method previously described by Taktak et al. [16].

2.2. Preparation of European Eel Skin Gelatin

The skin was separated from muscle and viscera, rinsed with distilled water to remove salts and other contaminants. Gelatin was extracted from European eel skin as previously described by Jridi et al. [18].

2.3. Preparation of European Eel Oil

European eel oil (EO) was extracted from the upper layer, obtained in the first step of pH-shifting process, reported by Folch et al. [19] and the fatty acids composition was determined using gas chromatography (GC) analysis as previously described in our study [17].

2.4. Preparation of EPI/ESG Blend Films

To prepare film-forming solutions, ESG and EPI powders were dissolved separately in distilled water to achieve final concentration of 4% and 2% (*w/v*), respectively. The solubilization of ESG was carried out at 40 °C for 30 min. The optimal dispersion of EPI solution was obtained at pH 10.0 according to a previous study (Taktak et al., 2018). Then, ESG and EPI film-forming solutions were mixed gently at different EPI/ESG mass ratios (0/100, 25/75, 50/50, 75/25, and 100/0 (*w/w*)) at room temperature (25 °C) for 15 min. Glycerol was separately added as plasticizer to the ESG and EPI solutions at a level of 10% of total protein content (*w/w* polymer dry matter). The obtained films were named, respectively, ESG 100, EPI 25/ESG 75, EPI 50/ESG 50, EPI 75/ESG 25, and EPI 100.

Emulsified films (EF), prepared with the addition of EO, were noted EF-EPI 0/ESG 100, EF-EPI 25/ESG 75, and EF-EPI 50/ESG 50. Indeed, EO was added at a mass ratio of 1/4 (*w/w* polymer) after glycerol addition. Finally, a volume of 25 mL of each formulation was casted into plastic Petri dishes (13.5 cm diameter), and dried, peeled off from the surface and equilibrated at 25 °C and 50% RH.

2.5. Physical Characterizations of the Films

2.5.1. Moisture Content and Water Solubility

Water solubility (WS) of films was carried out referred to the method of Gennadios et al., [20]. Moisture content (MC) of the films was resolved by drying 100 mg of film samples at 105 °C in an oven until constant weight was reached. The moisture content of films is expressed as grams of moisture/100 g film.

2.5.2. Color and Ultraviolet-Visible Spectroscopy (UV-Vis)

A colorimeter (Chroma Meter CR-400; CIE, Konica Minolta, Osaka, Japan) was used to determine the color of blend films that was expressed as *L* (lightness/brightness), *a* (redness/greenness) and *b* (yellowness/blueness) values. To calibrate the chromameter, white standard color plate ($Y = 94.1$, $x = 0.3134$, $y = 0.3194$) was used. The total color difference (ΔE) was calculated referring to the EPI film as follows:

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

where ΔL , Δa and Δb are the differences between the color parameters of blend films and 100% EPI-based film.

UV-Visible spectrophotometer (SAFAS, UV mc, Monaco, France) was used in order to determine the UV-visible barrier properties of films in the wavelength range from 200 to 800 nm. For this, films were cut into rectangle (1 cm × 3 cm) and placed in the test cell. An empty test cell was used as a reference.

2.5.3. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR profiles of samples were recording using a spectrometer (Agilent Technologies, Carry 630 series, Santa Clara, CA, USA) equipped with an attenuated reflection accessory (ATR), in frequency range from 650 to 4000 cm⁻¹. The OMNIC Spectra software (Thermo Fisher Scientific, Waltham, MA, USA) was used for the data analysis.

2.5.4. Mechanical Properties of Composite Films

The rheometer apparatus (Physica MCR, Anton Paar, GmbH, Les Ulis, France) equipped with a mechanical property measuring geometry were used for to elaborate the mechanical properties of films. Then, thickness of blend film (1.0 cm × 4.5 cm) was determined in the controlled temperature 25 °C and 50% RH for two weeks. The films retained in the extension grips, were exposed to a uniaxial tensile test, with a deformation rate of 5 mm/ min until breaking, based on the ISO standard (ISO 527-3:1995 [21]). Rheoplus software was used to estimate the mechanical properties (Tensile strength (TS) and elongation at break (EAB)). Average values from at least six measurements were reported.

2.5.5. Thermal Properties of Composite Films

The glass transition temperature (*T_g*) of the macromolecular materials was determined using differential Scanning Calorimeter (DSC) (Modulated DSC Q20, TA Instruments, Network Analyzers, NJ, USA) from 0 to 225 °C at a rate of 10 °C/min. After the precisely weight of films into aluminum pans and sealed, an empty capsule, which assists as an inert reference and the apparatus, was then calibrated using indium. Each pan was heated under a nitrogen flow rate of 50 mL/min. Finally, the TA Universal V4.5A software was used to analyze film thermograms.

2.5.6. Microstructure of Composite Films

The cross-section of ESG 100, EPI 100 and EPI 50/ESG 50 blend films and emulsified film were investigated using scanning electron microscopy (SEM, Hitachi S4800, Tokyo, Japan), at an angle of 90° to the surface and below an accelerating voltage of 2.0 kV, using different magnifications. The films

were cryofractured by engagement in liquid nitrogen. Before visualization, samples were cut and fixed through double side adhesive tape on the SEM support and observed at a magnification of 2000× and a pressure of 60 Pa.

2.6. In-Vitro Antioxidant Activity of the Films

The ability of films to reduce iron (III) was determined according to the method of Yildirim et al., [22]. The iron chelating effect of films was estimated by the method of Decker and Welch [23]. Total antioxidant capacity is based on the reduction of Mo (VI) to Mo (V) by the sample [24].

2.7. Statistical Analysis

Statistical analyses were performed with SPSS ver. 17.0, professional edition using ANOVA analysis at a (p -value < 0.05). Duncan's multiple-range test (p -value < 0.05) was used to detect differences among mean values of all the parameters analyzed for the different films. A standard deviation at the 95% confidence level was used to compare all parameters analyzed for the different film's ratios.

3. Results and Discussion

3.1. Protein Content and SDS-PAGE Profiles of EPI and ESG

3.1.1. Physicochemical Composition of EPI and ESG

Chemical compositions of fresh European eel muscle and freeze-dried EPI were described in our previous work [16]. Results show that alkali-aided process followed by centrifugation led to extract 94.33% of the total proteins. In addition, the highest solubility level of EPI (95%) was observed at alkaline pH 10.0. Furthermore, the protein molecular weight distribution of EPI showed typical fish protein bands, including molecular heavy chain proteins (>175 kDa) and actin bands (~45 kDa).

The proximate composition of skin and gelatin (ESG) from European eel skin was determined in Table 1. The skin was composed by 30.72% ± 0.94% protein, 2.08% ± 0.24% ash, 6.7% ± 0.86% fat, and 58% ± 2.25% moisture. ESG was characterized by high protein content (90.5% ± 0.70%) and a low amount of fat (1.12% ± 0.21%) suggesting the elimination of fat during gelatin extraction process [25]. The moisture content was in the vicinity of 6.1% which was within the limit prescribed for edible gelatin [26]. The ESG showed low water activity (0.33 ± 0.003), which is known to ensure microbiological stability.

Table 1. Physicochemical composition of skin (ES) and gelatin (ESG) from European eel.

Samples	Humidity (%)	Protein (%)	Lipid (%)	Ash (%)
ES	58 ± 2.25 ^a	30.72 ± 0.94 ^b	6.7 ± 0.86 ^a	± 0.24 ^a
ESG	6.10 ± 0.14 ^b	90.5 ± 0.70 ^a	1.12 ± 0.21 ^b	2.95 ± 0.07 ^a

Values are given as mean ± standard deviation. a and b: different letters in the same column indicate significant differences (p < 0.05); ES—European eel skin; ESG—European skin gelatin.

3.1.2. SDS Page of ESG

The subunit components of European eel skin gelatin were analyzed by polyacrylamide gel electrophoresis in the presence of SDS. The SDS-PAGE pattern of gelatin showed two major bands in the molecular weight range of 97–200 kDa (Figure 1). These bands represent α -chain and β -components. In addition, the SDS-PAGE pattern revealed number of low molecular weight bands which could be due to the degradation of α , β , and γ components during gelatin extraction [27]. The molecular weight of the extracted gelatin may be affected by the hydrolysis process that contributes to the dividing of the peptide bonds and also intermolecular cross links between peptide chains [28].

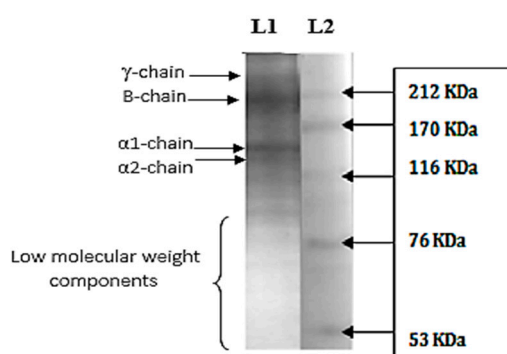


Figure 1. SDS-polyacrylamide gel electrophoresis of ESG, line 1(L1): ESG and line 2 (L2): high molecular weight marker.

3.2. Characterization of EPI/ESG Blend Films

3.2.1. Color and Light Transmission of Films

Color of films is crucial parameter in food packaging, which influence the visual inspection and the acceptability of consumer. The color of EPI, EPG, and EPI/ESG blend films was presented in Table 2. EPI film showed the highest *L*-value (85.55) and the lowest *b* value (7.93). However, ESG film presented the lowest *L* value (80.91) and the highest *b* value (15.39) and ΔE value (8.78). Color results of blend films revealed that high amount of EPI leads to increasing *L* value (lightness) from 81.64 for EPI 25/ESG 75 to 84.24 for EPI 75/ESG 25 and considerably reducing *b* and ΔE values. Thus, the incorporation of ESG reduced *L* and increased *b* and ΔE values. The color changes of the obtained films were accredited to the important quantity of protein components (4%) existing in gelatin film compared to that of protein isolate film (2%). Consequently, the EPI integration in films based on gelatin influences significantly the color of resulting films.

Table 2. Color parameters of EPI, ESG, and EPI/ESG blend films. Color parameters of emulsified films (ratio EO:EPI/ESG = 1:4).

Films EPI/ESG (w/w)	Color Parameter			ΔE	
	<i>L</i>	<i>a</i>	<i>b</i>		
Not emulsified	100/0	85.55 ± 0.37 ^a	−0.67 ± 0.05 ^a	7.93 ± 0.95 ^c	–
	75/25	84.24 ± 0.73 ^{ab}	−0.61 ± 0.06 ^a	8.15 ± 0.43 ^c	1.33 ± 0.78 ^c
	50/50	83.09 ± 0.58 ^{bcA}	−0.58 ± 0.01 ^{aB}	13.32 ± 1.07 ^{bB}	5.93 ± 1.21 ^{bB}
	25/75	81.64 ± 0.71 ^{cdA}	−0.61 ± 0.08 ^{aB}	13.35 ± 0.01 ^{bB}	6.69 ± 0.40 ^{bB}
	0/100	80.91 ± 1.18 ^{dA}	−0.55 ± 0.02 ^{aB}	17.06 ± 1.62 ^{aB}	8.78 ± 0.90 ^{aB}
Emulsified	0/100	79.36 ± 0.25 ^{bA}	0.91 ± 0.16 ^{aA}	20.69 ± 0.09 ^{aA}	14.27 ± 1.01 ^{aA}
	25/75	80.55 ± 0.09 ^{aA}	0.41 ± 0.05 ^{bA}	18.83 ± 0.01 ^{bA}	12.04 ± 1.02 ^{abA}
	50/50	81.58 ± 0.50 ^{aA}	0.45 ± 0.09 ^{bA}	17.56 ± 0.38 ^{cA}	10.48 ± 1.02 ^{bA}

Values are given as mean ± standard deviation. a–d: different letters in the same column indicate significant differences between sample of the non-emulsified film and the sample emulsified film (separately), A–B: different letters indicate significant differences in the same ratio EPI/ESG between emulsified and non-emulsified films ($p < 0.05$); ESG: European skin gelatin; EPI: European eel protein isolate.

The color parameters of emulsified films were presented in Table 2. Indeed, with EO incorporation, lightness of emulsified films decreased and ΔE increased, compared to the control film. Emulsified ESG 100 based film showed the lowest *L* value (79.36) with unpredictably higher *b* (20.69) and ΔE (14.27) values. Furthermore, lightness of emulsified films increased with protein isolate addition, from 79.36 to 81.58, for ESG 100 and EPI 50/ESG 50, respectively, which is in accordance with the obtained color traits of non-emulsified films. These findings are in line with the previous study of Tongnuanchan et al. [29].

Comestible films should prevent UV light transmission to protect foods against oxidation and to enhance its stability during conservation. The light transmittance at a selected wavelength (200–800 nm)

of different films is presented in Table 3. ESG based film revealed the highest light absorbance reflecting its good UV barrier property, which allows it to protect the packaged foods against light oxidative deterioration [30]. The high UV barrier effect of gelatin film is mainly due to the high content of aromatic amino acids present in gelatin (phenylalanine and tyrosine) which absorb the UV light [31]. However, protein isolate addition increases the light transmittance of blend films. This data is correlated with the color of the added EPI component and approved the light barrier property of ESG film.

Table 3. Light transmittance (%) of not emulsified and emulsified films (ratio EO:EPI/ESG = 1:4).

Films EPI/ESG (w/w)		Light Transmittance (%)							
		200	280	350	400	500	600	700	800
Not Emulsified	100/0	0.1	0.06	15.82	29.24	46.22	52.88	57.76	60.60
	75/25	0.1	0.27	19.34	33.15	47.86	53.09	57.01	59.47
	50/50	0.08	0.14	11.43	20.71	31.57	35.68	38.65	40.31
	25/75	0.08	0.05	1.50	3.73	7.77	10.30	12.30	13.80
	0/100	0.03	0.04	0.56	1.34	3.01	4.32	5.57	6.62
Emulsified	0/100	0.05	0.04	0.58	1.81	4.40	6.33	8.10	9.50
	25/75	0.05	0.05	1.63	4.19	8.72	11.02	12.54	13.56
	50/50	0.06	0.08	0.51	1.38	3.12	4.04	4.68	5.11

These results could be related to the higher concentration of gelatin (4%) used for film production compared to that of protein isolate (2%). The light transmittance of the different emulsified films is presented in Table 3. Emulsified films displayed a lower light transmittance compared to the non-emulsified films ($p < 0.05$), suggesting that the incorporation of EO improved the UV barrier properties of films. This result is in line with previous study of Zhang et al. [32] who studied the effect of the addition of two types of waxes (beeswax and carnauba wax) on the functional properties of gelatin films and reported that the emulsified films have a higher opacity and UV-visible light barrier property than the wax-free film.

3.2.2. Moisture Content and Solubility of Films

The results of moisture content (MC) and water solubility of obtained films are presented in Table 4. Film containing only gelatin (ESG100) exhibited significantly higher water content (12.26%) than EPI100 film ($p < 0.05$). In addition, blend films presented intermediate MC values without significant differences among them ($p > 0.05$).

Table 4. Thickness, mechanical properties (Tensile strength (TS) and elongation at break (EAB)), moisture content, and solubility of films stored at the temperature 25 °C and the RH 50%.

Films EPI/ESG (w/w)		Thickness (μm)	TS (MPa)	EAB (%)	Moisture Content (g moisture/100 g film)	Film Solubility (%)
Not Emulsified	100/0	32.00 \pm 4.32 ^b	5.42 \pm 0.69 ^c	2.14 \pm 0.02 ^d	6.78 \pm 0.40 ^d	29.47 \pm 1.03 ^e
	75/25	50.78 \pm 3.03 ^a	10.69 \pm 1.20 ^b	8.55 \pm 0.61 ^c	7.58 \pm 0.24 ^c	41.91 \pm 1.86 ^d
	50/50	52.60 \pm 2.41 ^a	14.33 \pm 0.73 ^a	15.00 \pm 0.68 ^b	7.94 \pm 0.35 ^{cA}	64.99 \pm 2.07 ^{cB}
	25/75	56.25 \pm 4.79 ^a	16.34 \pm 0.30 ^a	16.05 \pm 0.62 ^b	8.87 \pm 0.03 ^{bB}	72.43 \pm 0.60 ^{bB}
	0/100	59.00 \pm 2.00 ^a	16.58 \pm 0.32 ^a	18.74 \pm 0.74 ^a	12.26 \pm 0.10 ^{aA}	93.01 \pm 0.54 ^{aB}
Emulsified	0/100	ND	ND	ND	10.12 \pm 0.60 ^{aB}	96.51 \pm 2.06 ^{aA}
	25/75	ND	ND	ND	10.36 \pm 0.99 ^{aA}	91.64 \pm 0.39 ^{bA}
	50/50	ND	ND	ND	8.36 \pm 1.35 ^{aA}	87.06 \pm 0.99 ^{cA}

Values are given as mean \pm standard deviation. a–e: different letters in the same column indicate significant differences between samples of the non-emulsified and emulsified films (separately) ($p < 0.05$), A-B: different letters indicate significant differences in the same ratio EPI/ESG between emulsified and non-emulsified films ($p < 0.05$); ESG: European skin gelatin; EPI: European eel protein isolate. ND: Not Determined.

Furthermore, amongst all films, EPI 100 film had the lowest solubility (29.47%), while ESG 100 film had the highest solubility level (93%). This finding is in line with previous works of Arfat et al. [33] and Kchaou et al. [7]. The obtained result indicates the poor water resistance of ESG film, owing

to the hydrophilic group of the gelatin. Thus, water solubility of films was reduced by increasing the amount of EPI. This finding could be related to the establishment of covalent bonds between EPI proteins such as disulfide covalent bonds [34]. Furthermore, film solubility is related to the surface hydrophobicity of the EPI, compared to the gelatin [35], subsequent in the lower hydrophilic sites accessible for absorbing water [36].

Regarding emulsified films, increasing EPI content in the emulsified film matrix reduced their solubility (Table 4). Indeed, EF-ESG 100, EF-EPI 25/ESG 75, and EF-EPI 50/ESG 50 had high-water solubility compared to the free oil-based films, reaching 96.51%, 91.64%, and 87.06%, respectively. Moreover, EF-ESG 100 had the highest solubility, followed by EF-EPI 25/ESG 75 and EF-EPI 50/ESG 50. The obtained results could be related to the higher stability of emulsion as presented in Figure 2, resulting in more uniform emulsion for EF-ESG 100.

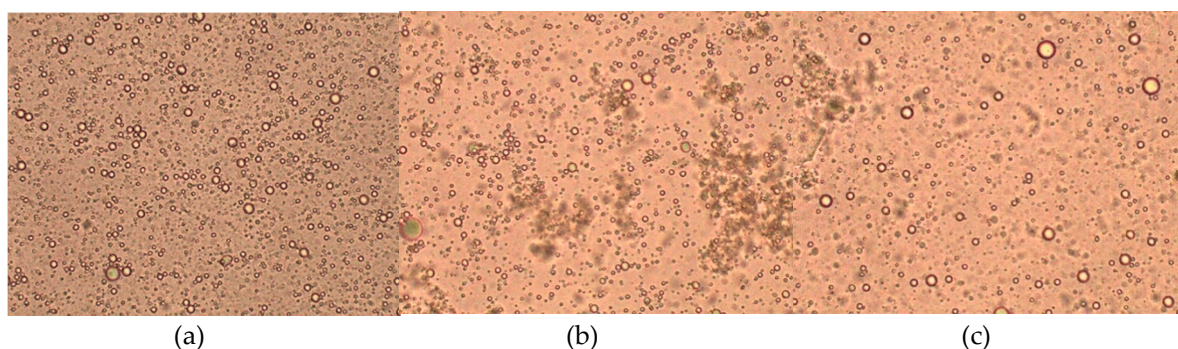


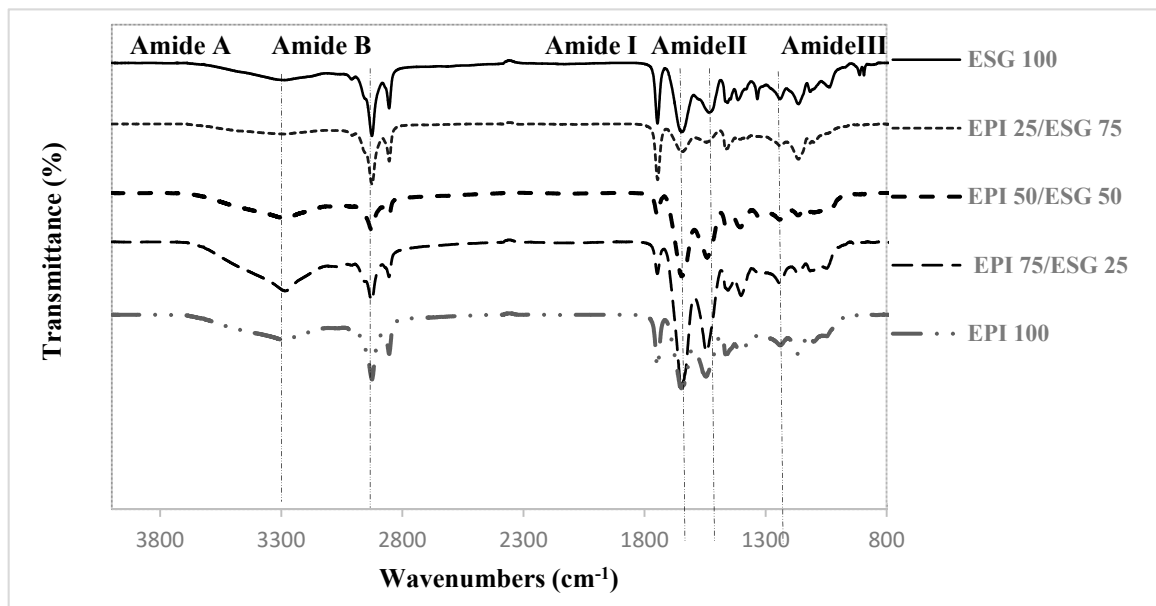
Figure 2. Microscopy optic observation of emulsions stabilized by protein isolate (EPI) film, gelatin (ESG) used for the preparation of emulsified films EPI/ESG: (a) 0/100, (b) 25/75, and (c) 50/50 using the optical microscope (Motic, ME 2000) (400 \times).

This finding could be related to the functional properties of ESG, such as its higher solubility and emulsifying activity. The strong correlation between solubility and the emulsifying property has been established [37]. In fact, high solubility level improves protein migration to the oil/water interface, allowing the appropriate rearrangement at the interfacial film [38]. In addition, the nature of incorporated lipids affects the permeability of some compounds in the emulsified films. Monedero et al. [39] stated that the apparent diffusion coefficient in whey protein films containing beeswax is lower than in the films containing oleic acid. Thus, the more the lipid is solid, such as waxes, the more the diffusion in the film will be limited.

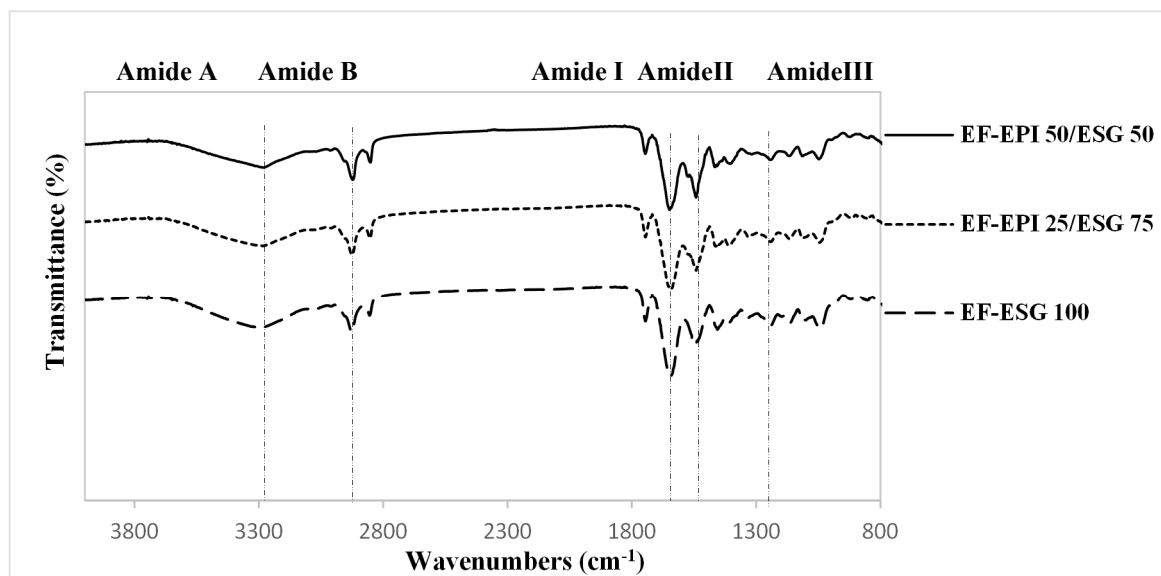
3.2.3. FTIR Spectra

As can be seen in Figure 3a, the different spectra of films showed characteristic pics of protein at approximately 1230–1340 cm^{-1} , 1540–1610 cm^{-1} , 1560–1680 cm^{-1} , 2920–2945 cm^{-1} , and 3300–3500 cm^{-1} correlated to amide-III (correlated to the vibrations in plane of C–N and N–H groups of bound amide or vibrations of CH_2 groups of glycine), amide-II (arising from bending vibration of N–H groups and stretching vibrations of C–N groups), amide-I (representing to C=O stretching/hydrogen bonding coupled with $-\text{COO}$), amide-B (asymmetric stretching vibration of $=\text{C}-\text{H}$ and $-\text{NH}^{3+}$) and amide-A (corresponding to NH-stretching coupled with hydrogen bonding), respectively [40]. In addition, all films presented an absorption band placed at the wavenumber of 1036–1039 cm^{-1} correlated to the interactions between film structure and OH group of glycerol used as plasticizer [36]. Furthermore, the absorbance peak of amide A, at 3286–3289 cm^{-1} regions, was ascribed to the interaction between polymers and water (Figure 3a). The decrease in N–H vibration band wavenumber and lengthening with ESG increasing content in the film may indicate the increase and reinforcement of interaction between gelatin and EPI via hydrogen bonding [36]. Kchaou et al., [7] stated that protein could also interact through hydrophobic bonds among hydrophobic aromatic amino acids of the cuttlefish

gelatin and protein isolate. According to Cao et al., [41], the interactions between the amino and carboxyl groups of soy protein isolate and gelatin could significantly affect the mechanical properties of composite films and hydrogen bonding. It is interesting to note a notable change in the absorbance of amide I, which attained 1607, 1694, and 1645 cm^{-1} for ESG 100, EPI 50/ESG 50, and EPI 100, and could be associated to gelatin secondary structure alterations [42]. Otherwise, compared to the gelatin film profile, the amplitude of amide III band was decreased for ESG 100, EPI 50/ESG 50, and EPI 100 by the EPI content increasing. This change could be related to electrostatic interactions establish between amino and carbonyl groups of polymers, as described by Gomez-Guillen et al. [43]. Thus, FTIR analysis proved the presence of interactions between ESG and EPI in the composite biofilms.



(a)



(b)

Figure 3. Fourier transform infrared spectra of (a) EPI film, ESG film, and EPI/ESG blend films and (b) emulsified films EPI/ESG.

The different spectra of emulsified films presented in Figure 3b showed characteristic peaks of proteins and the presence of specific bands of EO at 1740, 1711, and 1640 cm^{-1} , corresponding to C=C, C=O, and C=C–C=C [44,45]. A specific absorption band of omega-3 in emulsified film, related to polyunsaturated fatty acid, was detected at 3012 cm^{-1} (Figure 3b), which corresponds to the C–H stretching of *cis*-alkene (–HC=CH–) [46].

3.2.4. Mechanical Properties

The mechanical properties of films were assessed through the determination of TS (MPa) and EAB (%) values, except for the emulsified films which were relatively sticky to be peeled off from the Petri dishes.

The results, illustrated in Table 4, showed that film prepared with 100% ESG exhibited the highest tensile strength (16.58 MPa) and elongation at break (18.74%) values. However, EPI-based film displayed the lowest EAB (2.14%) and TS (5.42 MPa) levels, which increase with the rise of ESG concentration. Thus, the increase of gelatin concentration ameliorates the mechanical properties of blend films, contrary to the EPI 100 based film which might not form a robust film network. This finding could be related to the lower reactivity of myofibrillar protein molecules in cross-linking or interaction between protein molecules [33]. Moreover, gelatin is cross linked with itself or with EPI in the film matrix and this interaction was made via hydrogen bonds [47]. This finding resulted to the improvement of mechanical properties of blend films. Consequently, the mechanical properties of ESG/EPI blend films were largely affected by the type of protein, the interaction and the ratio of used proteins as well as the number of interactions involved in the film matrix [36].

Thickness of EPI, ESG, and EPI/ESG blend films are presented in Table 4. EPI films had lower thickness (32 μm), compared to ESG film (59 μm). In fact, thickness increased with gelatin addition, which could be related to the higher concentration of gelatin (4%) used in the film's preparation based on the dry weight matter compared to that of protein isolate. However, Denavi et al., [35] found similar thickness in soy protein isolate and gelatin-based film, regardless of the proportion of SPI was used.

3.2.5. Thermal Properties

The glass transition temperature (T_g) of films, determined through the DSC curves, is reported in Table 5. As can be seen, ESG film showed higher T_g , by about 71.51 $^{\circ}\text{C}$, than the T_g of EPI film (50.98 $^{\circ}\text{C}$), which could be related to the triple helix structure of gelatin.

Table 5. Thermal properties of ESG, EPI, and ESG/EPI composite films; Thermal properties of ESG, and ESG/EPI blend emulsified films (ratio EO:EPI/ESG = 1:4) (Emulsified and not emulsified film were previously incubated at 25 $^{\circ}\text{C}$ and 0% RH).

	Film EPI/ESG (w/w)	T_g	Onset T ($^{\circ}\text{C}$)	Offset T ($^{\circ}\text{C}$)
Not Emulsified	100/0	50.98	50.58	51.18
	75/25	54.34	53.92	54.46
	50/50	57.43	46.15	59.43
	25/75	64.89	64.36	46.90
	0/100	71.51	70.36	72.37
Emulsified	0/100	86.24	86.12	86.72
	25/75	73.90	59.69	60.92
	50/50	60.38	71.63	76.86

The obtained T_g of ESG film was in line with those of triggerfish acidic gelatin (77 $^{\circ}\text{C}$) [48] and higher than that of gelatin extracted from skin cuttlefish (60 $^{\circ}\text{C}$) [7] and smooth hound (66.94 $^{\circ}\text{C}$) [49]. The difference of T_g among ESG and EPI films is mostly attributed to the different molecular structure, which resulted in different molecular arrangement and interactions [50].

For the blend films, the incorporation of EPI to the gelatin formulation reduced the Tg value of films. In fact, Tg decrease significantly with increasing EPI content from 71.51 °C for ESG 100 based film to 54.34 °C for EPI25/75ESG-based film. This finding was in line with the previous study of Akköse et al. [51] who find that addition of biopolymer levels significantly affected the transition temperature values. The DSC results, which were in accordance with the mechanical properties, confirmed the biocompatibility between the two polymers and the formation of hydrogen interactions between them. Thus, a positive correlation between Tg and TS values of the films could be noticed. This finding is in line with previous works of Arfat et al. [52] and Abdelhedi et al., [49] who found that TS for fish gelatin films increased with increasing Tg. TS increasing could be attributed to the formation of a denser matrix subsequently gelatin addition due to the interaction between the two biopolymers (EPI and ESG).

The thermal analysis of emulsified films, presented in Table 5, revealed that Tg of all emulsified films (71.51–86.24 °C) were higher than the Tg of not-emulsified films. Akköse et al. [51] reported that increasing the Tg by addition of biopolymers leads to extend the food shelf life and this approach would be feasible only for fabricated foods. Furthermore, the Tg of emulsified film increased with gelatin amount increasing, reaching 60.38, 73.90, and 86.24 °C, for EF-EPI 50/ESG 50, EPI 25/ESG 75, and EF-ESG 100, respectively. This finding approved that EO was appropriately incorporated in the emulsified film, which is in accordance with the enhancement of the UV barrier properties of emulsified films and the presence of a characteristic band at 3012 cm⁻¹, corresponding to the polyunsaturated fatty acid after the European oil incorporation. Thus, the increase of Tg value is related to the introduction of the cross-linking points which is responsible for reducing the mobility of chain segments in the matrix of the emulsified film compared to that prepared without oil [53]. Oil incorporation to the film matrix is a good alternative especially for the stabilization of frozen food as it protects against the water diffusion [54].

3.2.6. Microstructure of Films

Freeze-fractured cross-sectional images of EPI, ESG, and EPI/ESG blend films carried out via the scanning electron microscopy (SEM) are presented in Figure 4. Films elaborated with 100% ESG and 100% EPI had a smooth, compact, and homogeneous structure on the cross-section. These results were in accordance with findings obtained by Jiang et al., [55] and Pereda et al., [56], showing no porosity, smooth, and homogeneous film microstructure for gelatin-chitosan blends. The cross-sectional image of EPI 50/ESG 50 blend film showed continuous microstructure, indicating the compatibility and the enhancement of the molecular interaction of polymer in the film matrix.

On the other hand, the cross-section combining gelatin with EPI in the ratio of 50:50 (*w/w*) displayed a rougher microstructure, which is in line with previous studies of Hoque et al., [36] and Abdelhedi et al., [49]. The roughness of blend films was probably due to the re-arrangements of protein molecules chains during films formation. Therefore, the microstructures could be related to the types of proteins and the interaction of proteins in film matrix in consort with the blend ratio of protein components designed for film preparation [36].

SEM micrographs of emulsified films cross-section revealed a smooth and homogeneous microstructure, with no phase separation, indicating high compatibility between EO and the gelatin [57]. The appropriate homogenous microstructure of the emulsified films with higher content of ESG (EPI/ESG 0/100 and EPI/ESG 25/75) is related to the interesting emulsifying property of ESG, allowing the encapsulation of the EO and the enhancement of the shrinkage of film network. However, the increasing of EPI content in the emulsified film (EF-EPI 50/ESG 50) resulted in the presence of cracks (Figure 4F) and droplets of oil floating towards the surface of film, suggesting that EPI 50/ESG 50 was not able to incorporate the EO in the film network [30].

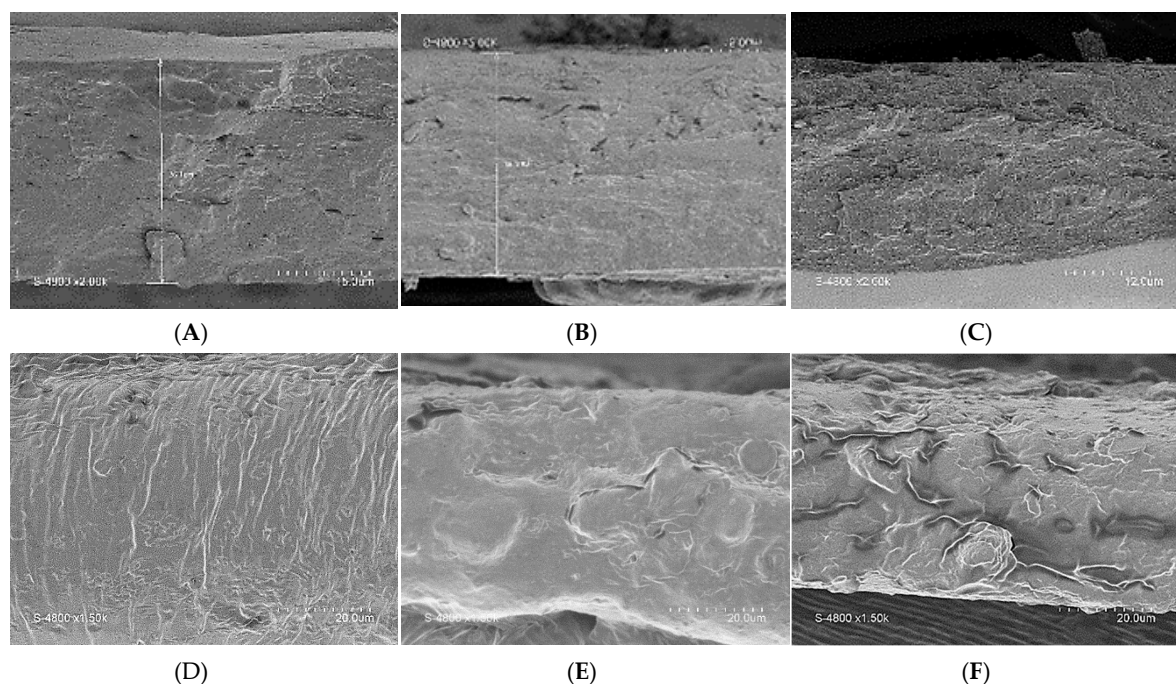


Figure 4. SEM micrographs (cross-section) of EPI/ESG: (A) 0/100, (B) 100/0, and (C) EPI 50/ESG 50 blend films and emulsified films EPI/ESG: (D) 0/100, (E) 25/75, and (F) 50/50.

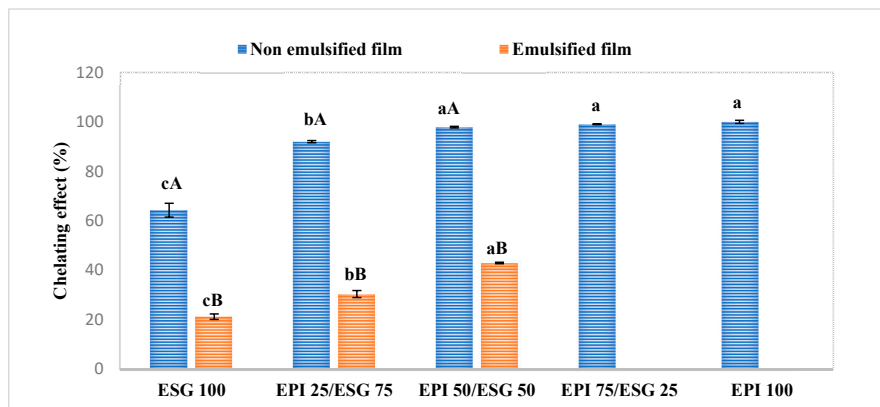
3.2.7. Antioxidant Activity of Films

Antioxidant packaging is considered as categories among the requested active packaging, owing to their promising effect on spreading foods shelf life, avoiding the oxidative stress caused by the free radicals, which is involved in the development of many human diseases [58].

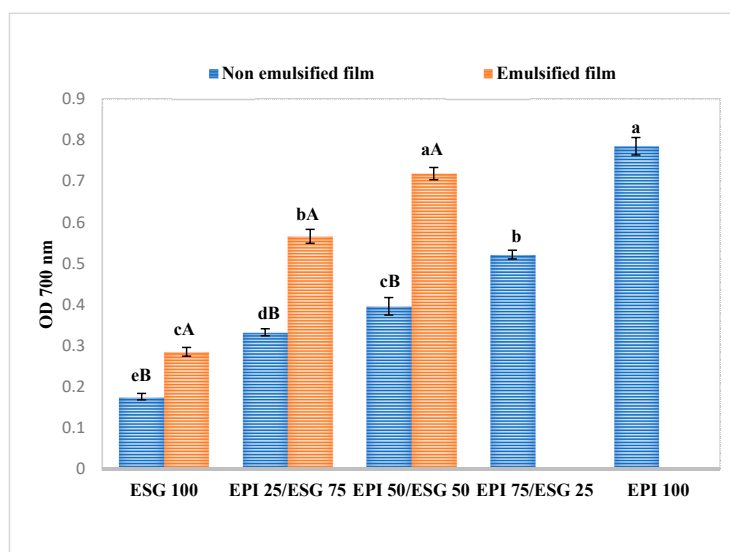
The chelating effect analysis, illustrated in Figure 5a, revealed an interesting activity for all films tested, ranged from 64% to 100%. In fact, EPI film displayed the highest chelating power (100%), while, ESG films exhibited the lowest antioxidant ability (64.30%). Thus, adding EPI to blend films enhanced their iron chelating effect, reaching 92.02%, 97.80%, and 99.04% for EPI 25/ESG 75, EPI 50/ESG 50, and EPI 75/ESG 25 films, respectively.

Additionally, the results showed that the chelating activity of emulsified films decreased significantly with the incorporation of oil ($p > 0.05$). In fact, the EO addition to the EPI 50/ESG 50 film reduced its chelating activity from 97.80% to 42.93% (Figure 5a). This result is mainly due to the cross-linking points between polymer and EO, which reduce the mobility of chain segments and ions in matrix of emulsified film compared to that prepared without oil [53].

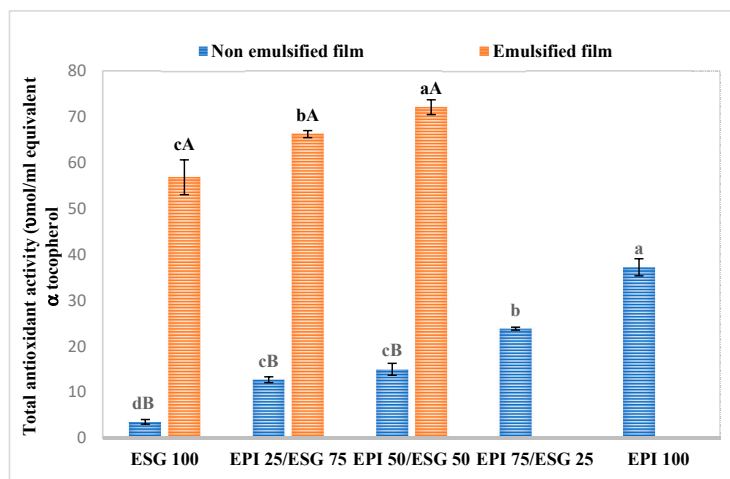
Reducing effect determines the reducing aptitude versus ferric ion (Fe^{3+}), indicating the ability to donate an electron by the active compounds to the ion [59]. The reducing power of the different films is expressed as the absorbance at 700 nm (Figure 5b). Among all samples, EPI100-based film displayed the highest ability (0.79) to reduce ferric ion (Fe^{3+}), while ESG100-based film presented the poor reducing power (0.18). Thus, EPI addition improved the reducing potential of combined films. On the other hand, the reducing ability of emulsified films was considerably higher, compared to the non-emulsified films. Furthermore, increasing EPI amount in the emulsified film improved the reducing power ability reaching $\text{OD}_{700} = 0.72$ for EF-EPI 50/ESG 50.



(a)



(b)



(c)

Figure 5. Antioxidant activity of protein isolate film (EPI) gelatin film (ESG) and EPI/ESG blend films and the emulsified film by the test of chelating power effect (a), ferric reducing activity (b), and total antioxidant activity (c). Values are assumed as mean \pm standard deviation. a–d: different letters indicate significant differences between sample of the non-emulsified film and the sample emulsified film (separately), A–B: different letters indicate significant differences in the same ratio EPI/ESG between emulsified and non-emulsified films ($p < 0.05$); ES/European eel skin; ESG: European skin gelatin; EPI: European eel protein isolate.

The antioxidant potential of enriched films comes from the bioactivity of the incorporated substances, which have proved their antioxidant effect as electron donors and chelating agents [49]. Likewise, total antioxidant capacity based on the reduction of Mo (VI) to Mo (V) expressed as a tocopherol equivalents (Figure 5c) showed the highest activity (37.24 $\mu\text{mol/mL}$) for EPI100-based film and the lowest one was obtained by ESG100-based film (3.69 $\mu\text{mol/mL}$). The antioxidant activity of composite and emulsified films increased proportionally to the EPI content incorporated in the gelatin film formulation (Figure 5c). Furthermore, the incorporated of EO in the films improved the total antioxidant capacity reaching a maximum of 72.15 $\mu\text{mol/mL}$ for EF-EPI50/ESG 50, compared to the non-emulsified EPI50/ESG 50 (15 $\mu\text{mol/mL}$). These findings are similar to the reducing ability and iron chelating result. Hence, all tests clearly indicated that the incorporation of EPI to ESG films enhanced the antioxidant activity of resulting blend films.

A previous study of Bao et al. [60] showed that specific amino acids (glycine and proline) are responsible for the antioxidant activity of gelatin which is improved considerably after protein isolate addition with is in line with the study of Kchaou et al. [7]. Moreover, antioxidant activity of the films is enhanced by the protein–protein interaction or hydrogen bonding forming the film network [61]. Furthermore, antioxidant activity of protein isolate is in correlation with the difference in the antioxidant efficiency of the added bioactive compounds present naturally in the protein. Additionally, antioxidant activity of peptide obtained after hydrolysis of protein isolate could be related to their sequence, composition, and hydrophobicity [62].

4. Conclusions

A characterization of blend biofilms based on European eel skin gelatin (ESG), protein isolate (EPI), and oil (EO) was released. The gelatin-based film displayed the highest tensile strength, elongation at break, and thermal properties, compared to EPI-based film and EPI/ESG blend films. Moreover, gelatin film presented the highest water solubility. Interestingly, the incorporation of EPI to the gelatin films matrix improved their antioxidant activity. The homogeneous microstructure was related with the better mechanical and physical characteristics of films. The cross-section combining gelatin with protein isolate (ratio 50:50) displayed a rougher microstructure correlated to the arrangements of protein molecules during films formation. ESG100 and EPI 25/ESG 75 films revealed a smooth and homogeneous microstructure. Additionally, the incorporation of EO improved the UV barrier properties of ESG/EPI films and their antioxidant activity, while their mechanical resistance was relatively affected. The different properties of the produced films give them the prospect of being effectively used in food industries as bioactive packaging to improve the shelf-life of bio-packaged foods.

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