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Microbial Transglutaminase-Mediated Modification of Heat-Denatured Whey Proteins for the Preparation of Bio-Based Materials

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Abstract: This study sheds light on the potential of microbial transglutaminase (mTG)-mediated modification to enhance the properties of heat-denatured whey protein-based films. In this study, we investigated the biochemical modification of heat-denatured whey proteins (WPs) using mTG, an enzyme known for the ability of crosslinking reactions. By introducing ε-(γ-glutamyl)-lysine crosslinks via an acyl transfer reaction, mTG enhances the properties of bio-based materials. In this research, heated WPs were demonstrated to effectively serve as mTG substrates. The preparation of crosslinked bio-based material was achieved using a casting method under alkaline conditions (pH 12) in the presence of glycerol (40% *w*/*w*), which was added as a plasticizer to the film-forming solution (FFS). A comprehensive characterization of the FFSs and the resulting materials was carried out. The FFSs were quite stable as evidenced by Zeta potential values that were always around 30/40 mV regardless of the presence of the enzyme. The enzymatic modification increased the elongation at break of the materials from 10.4 ± 4.9 MPa to 27.6 ± 8.9 MPa, while decreasing both tensile strength and Young's modulus, thereby making the resulting material more extensible. On the other hand, the enzyme affected both the CO_2 and O_2 barrier properties, with permeability values for these gases being 0.90 cm^3 mm m^{-2} day^{-1} kPa^- and 0.26 cm^3 mm m^{-2} day^{-1} kPa, respectively, when the films were cast without the enzymatic treatment, but decreasing to 0.14 ± 0.02 cm^3 mm m^{-2} day^{-1} kPa (CO_2) and 0.02 ± 0.02 cm^3 mm m^{-2} day^{-1} kPa (O_2) in the presence of 24 U/g of mTG. These novel materials, prepared from renewable sources, could potentially be used in the food packaging field to replace/reduce the highly pollutant petroleum-based ones.

Keywords: microbial transglutaminase; whey proteins; crosslinked protein-based materials; film characterization

Academic Editors: Bruna Silva de Farias and Pinto Luiz Antonio de Almeida

Received: 29 November 2024
Revised: 6 January 2025
Accepted: 8 January 2025
Published: 9 January 2025

Citation: Abdalrazeq, M.; Aref, D.; Mariniello, L.; Giosafatto, C.V.L. Microbial Transglutaminase-Mediated Modification of Heat-Denatured Whey Proteins for the Preparation of Bio-Based Materials. *Coatings* **2025**, *15*, 66. https://doi.org/10.3390/coatings15010066

1. Introduction

The enzyme microbial transglutaminase (mTG, protein-glutamine γ-glutamyl transferase, E.C. 2.3.2.13) has been widely exploited to strengthen the structure of protein-based bioplastics over the last 15 years [1–8]. mTG is active in a wide range of pH (4–9) and introduces ε-(γ-glutamyl)-lysine crosslinks into proteins via an acyl transfer reaction. It has been proven to modify several food proteins, and among the milk proteins, both caseins and whey proteins (WPs) act as acyl donor and/or acceptor substrates for the enzyme [9–11]. mTG is resistant between 4 and 60 °C, it is commercially available, food grade, and useful

for modifying protein structure and biological properties [12–15]. In addition, the use of microorganisms for mTG production offers increasing economic benefits since the costs associated with microbial synthesis are significantly lower compared to the costs of producing TG from animal sources [16].

WP-based material is typically formed in aqueous solution in the presence of different plasticizers and their properties have been shown to be significantly influenced by the presence of mTG [17]. In particular, the use of mTG in WP-based films was demonstrated to induce an enhancement in film mechanical resistance and a reduction in its deformability. Even the barrier efficiency toward O_2 was found to be markedly improved in the crosslinked films, which also exhibited a lower permeability to water vapor [18–20].

Generally, mTG has been used to improve the physiochemical properties of protein-based material and for reticulating the matrix of proteinaceous foods [16]. In fact, many scientific studies [17,21,22] proved that mTG notably enhances the protein-based material characteristics due to the crosslinking effect on the matrix. Cruz-Diaz et al. [21] used high-power ultrasound and mTG, individually and in combination, for the modification of WP concentrate-based edible films. The authors showed that ultrasound treatment increased the tensile and puncture strengths of films in comparison with untreated samples and that mTG addition only increased puncture deformation values and made films less green, more yellow, and darker than films without mTG. Further authors demonstrated that the tensile strength of WPs–soy protein films and WPs–chitosan added with mTG increased by 200% and 45%–63%, respectively [17], whereas the solubility of films decreased [22]. Therefore, this study, for the first time, investigated the effect of the crosslinking mediated by mTG on WPs-based hydrocolloid materials prepared via the casting method under pH 12, since by treating the solutions under these conditions, more transparent, flexible, and homogeneous films could be obtained [23]. Before film preparation, the effect of the reticulating enzyme on film-forming solutions (FFSs) viscosity, zeta potential, and particle size was investigated, as a previous study has shown that the enzyme may affect these properties [24]. In particular, our strategy to follow the high molecular weight crosslinks was based on size measuring methods by using both Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Dynamic Light Scattering (DLS), as the resulting isopeptide bonds contribute to the formation of a stable protein network. The mTG-crosslinked films were then studied for their mechanical, opacity, and gas barrier properties. In addition, such materials' gastric digestibility, carried out under physiological conditions [25], was also taken into account for their possible application in the food sector as edible films for protecting different types of foodstuffs.

2. Materials and Methods

2.1. Materials

Commercial WP isolate (~90% dry basis protein) was obtained from BioLine. Microbial transglutaminase (ACTIVA WM, Ajinomoto, Tokyo, Japan, specific activity 92 U/g) was purchased from Prodotti Gianni S.p.A. (Milan, Italy). Glycerol (Gly), used as a plasticizer for the preparation of the films, Trizma® base solution, Bis-Tris HCl, sodium hydroxide, and pepsin from porcine gastric mucosa for gastric digestion were acquired from Merck (Rome, Italy); acrylamide, coomassie brilliant blue, and all the products for gel electrophoresis were purchased from Bio-Rad (Segrate, Milan, Italy). All other chemicals and solvents used in this study were of analytical grade unless specified.

2.2. Microbial Transglutaminase (mTG)-Modification of WPs and Film Preparation

A 2.5% (*w*/*v*) stock solution of WPs was stirred for 2 h and then heated at 80 °C for 25 min. This treatment made the proteins more prone to mTG-mediated crosslinking, as previously demonstrated [25]. After cooling the solution, 24 U/g of mTG was added.

Following mTG modification, the pH was adjusted to pH 12 since, as reported in Abdalrazeq et al. [23], the alkaline pH facilitates the production of handleable, transparent, and more flexible films. GLY (30 and 40%, *w*/*w* of WP), used as a plasticizer, was finally added, and films were obtained by casting 500 mg WPs in a final volume of 50 mL and allowing them to dry in a climatic chamber at 25 °C and 45% RH for 24 h. The films were analyzed within 2 days.

2.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Preliminary enzymatic assays were carried out in order to investigate the effect of different concentrations of mTG on WP modification. In this respect, WPs were initially heated for 25 min at 80 °C and separately incubated for 1 and 2 h with increasing concentrations of the enzyme (0, 8, 16, 24, 32 U/g of WPs) at pH 7.5. Different experiments were also performed by treating the WPs at pH 12 before the enzymatic treatment at pH 7.5. The extent of enzymatic crosslinking was checked by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli [26]. Briefly, 5 µL of sample buffer (15 mM Tris-HCl, pH 6.8, containing 0.5% *w*/*v* SDS, 2.5% *v*/*v* GLY, 200 mM β-mercaptoethanol, and 0.003% *w*/*v* bromophenol blue) was added to 20 µL aliquots of each sample and analyzed by 4%–20% SDS-PAGE. The electrophoresis was performed at a constant voltage (80 V for 2–3 h), and the proteins were visualized by Coomassie Brilliant Blue R250 staining. Bio-Rad precision protein standards were used as molecular weight markers. SDS-PAGE gel images were acquired using a Bio-Rad ChemDoc Imager. The image analysis was carried out using Image Lab software (Bio-Rad, version 5.2.1), as described by Romano et al. [27].

For the preparation of crosslinked films, the pH of the reaction mixtures, following incubation with mTG, was adjusted to pH 12. GLY (30 and 40%, *w*/*w* of WP), used as a plasticizer, was finally incorporated into the FFSs, and films were obtained by casting 500 mg of WPs. After drying in a climatic chamber at 25 °C and 45% RH for 24 h, the obtained films were characterized within 2 days.

2.4. Zeta-Potential and Particle Size Measurements

A Zetasizer Nano-ZSP (Malvern®, Worcestershire, UK) was used to measure the zeta potential and particle size of 1.0 mL of each WP-based FFS. A 4 mW helium-neon laser with a fixed wavelength of 633 nm (the wavelength of red laser emission) was installed in the apparatus. By delivering a 200 mV voltage and applying the Henry equation, the instrument software programmer determined the zeta potential through the electrophoretic mobility.

2.5. Film Forming Solution Viscosity

A typical Ostwald capillary viscometer was used to study the viscosity of FFSs, and the following formula was used to determine the specific viscosity values:

$$specific\ viscocity = \frac{(FFS\ flow\ time - water\ flow\ time)}{water\ flow\ time} \quad (1)$$

2.6. Film Thickness

A micrometer model HO62 Metrocontrol Srl (Casoria, Naples, Italy) was used to measure the thickness of the film at five different random locations throughout the film area. The values represent the mean ± standard deviation (SD) of five replicates.

2.7. Film Mechanical Properties

Film tensile strength (TS), elongation at break (EB), and Young's module (YM) were measured by using an Instron Universal Testing Instrument model no. 5543A (Instron Engineering Corp., Norwood, MA, USA). Film samples were cut using sharp scissors into 10 mm wide and 40 mm length strips, equilibrated for 48–72 h at 50% ± 5% RH and 23 ± 2 °C in an environmental chamber. Five specimens of each film type were tested (1 KN load and 1 mm/5 min speed), as previously reported [28] (ASTM D882-97, 1997).

2.8. Film Transmittance and Transparency

Each WP film was cut into 1 cm × 4 cm strips and placed in a quartz cuvette, and its whole light transmittance and absorbance spectra were obtained using a Shimadzu (Milano, Italy) UV-vis spectrophotometer in the range of 200–800 nm with a scan rate of 250 nm/min. The transparency analyses were performed as described by Galus et al. [29] by calculating film opacity as follows:

$$Opacity = A600\text{nm}/X \quad (2)$$

where $A600$nm is the absorbance at 600 nm, and X is the film thickness (mm).

2.9. Film Gas and Water Vapor Barrier Properties

Film permeability to O_2 (ASTM D3985-05, 2010) [30], CO_2 (ASTM F2476-13, 2013) [31], and water vapor (VWP) (ASTM F1249-13, 2013) [32] was determined in triplicate for each film using a TotalPerm apparatus (Extrasolution s.r.l., Pisa, Italy). Aluminium masks were used to reduce the film test area to 5 cm^2, whereas the testing was performed at 25 °C under 50% RH.

2.10. Film Moisture Content and Moisture Uptake

Weighed film samples (2 cm × 2 cm) were dried in an oven for 24 h at 105 °C. Each film underwent triple analyses, and the film moisture content was determined as follows:

$$Film\ moisture\ content\ (\%) = \left(\frac{(W_1 - W_2)}{W_1}\right) \times 100 \quad (3)$$

where W_1 is the film's original weight, and W_2 is its weight following 105 °C drying.

With minor adjustments, moisture uptake was determined gravimetrically in triplicate using the Sartori and Menegalli [33] approach. The samples were divided into squares with sides of 20 mm, dried for 24 h at 105 °C, and then conditioned with a saturated $Mg(NO_3)_2$ solution at 23 ± 2 °C in a desiccator with 50% relative humidity before being weighed. Ultimately, the moisture uptake was obtained as follows:

$$Film\ moisture\ uptake(\%) = \left(\frac{(W_s - W_d)}{W_s}\right) \times 100 \quad (4)$$

where W_s and W_d are the weights of swollen and dried films, respectively.

2.11. In Vitro Film Digestion

The digestibility of films prepared in the absence and presence of 24 U of mTG was assessed using the Infogest protocol and following the procedure described in Giosafatto et al. [25].

2.12. Statistical Analysis

JMP software 5.0 was used to perform statistical analysis on all of the data (SAS Institute, Cary, NC, USA). The Tukey–Kramer HSD test was used to compare the means after the data underwent analysis of variance. At $p < 0.05$, differences were deemed significant.

3. Results and Discussion

3.1. Modification of WPs by Means of mTG

Our experiments focused on using the enzyme mTG to improve the technological properties of WP-based materials. mTG is an enzyme that catalyzes the formation of intra- and inter-molecular isopeptide bonds between the endoprotein glutamine (Gln) and lysine (Lys) residues [25]. SDS-PAGE analysis was used to determine the degree of mTG-mediated crosslinking based on the reduction of the protein bands for α-lactalbumin (Mr~14.2 kDa) and β-lactoglobulin (Mr~17.4 kDa), as well as the concurrent rise of high molecular weight polymers and dark smearing. It is important to note that WPs could not function as an efficient mTG substrate when treated only with alkaline conditions (pH 12), which led to protein denaturation.

The results displayed in Figure 1 indicate that mTG hardly crosslinked WPs. Therefore, WP heat pretreatment, carried out for 25 min at 80 °C, was essential because it likely made Gln and/or Lys endoprotein reactive residues visible to the enzyme. The results obtained under these experimental conditions showed that both β-lactoglobulin and α-lactoalbumin were modified by heat-treated WPs when they were incubated at pH 7.0 for 2 h with mTG (Figure 2). In fact, under these conditions, a dark smear with a concomitant decrease in the intensity of the protein bands relative to α-lactalbumin (Mr~14.2 kDa) and β-lactoglobulin (Mr~17.4 kDa) protein bands was observed in the mTG-modified samples, which was completely absent in the control samples prepared without the enzyme. This result demonstrates that following heat treatment, mTG is able to catalyze ε-(γ-glutamyl)lysine bonds. The WP-based FFSs were then cast under alkaline conditions. Different authors have reported that the alkaline pH improved both the biological and technological properties of food proteins. For instance, Wang et al. [34] reported that alkaline pH treatment with heating disrupted protein subunits of hemp seed protein isolate, unfolded the tertiary structure, and increased hydrophobic groups, leading to enhanced solubility and emulsifying activity. Similarly, Wang et al. [35] observed a synergistic effect of alkaline treatment (pH 10) with heat treatment at 40 °C on peanut protein isolate functionalities, including solubility and gelling properties, compared to heat treatment alone.

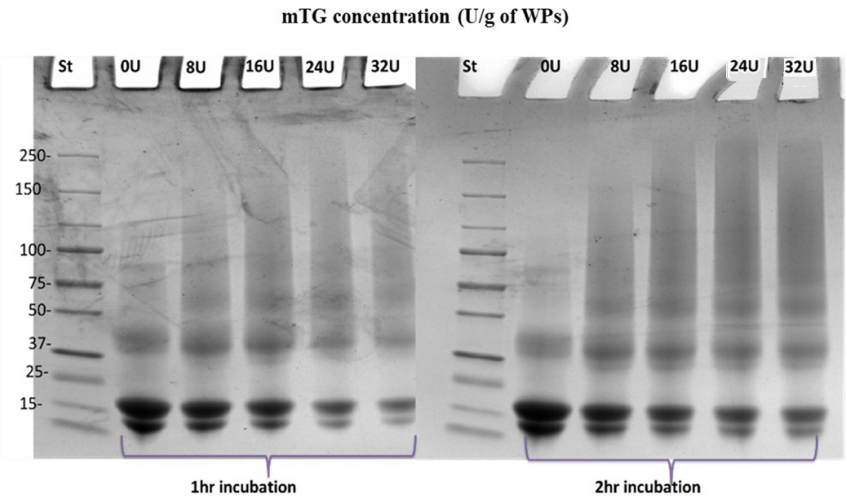

Figure 1. SDS-PAGE of WP (1%, *w*/*v*)-containing FFSs, previously treated under alkaline pH with different concentrations of mTG (U/g of WPs).

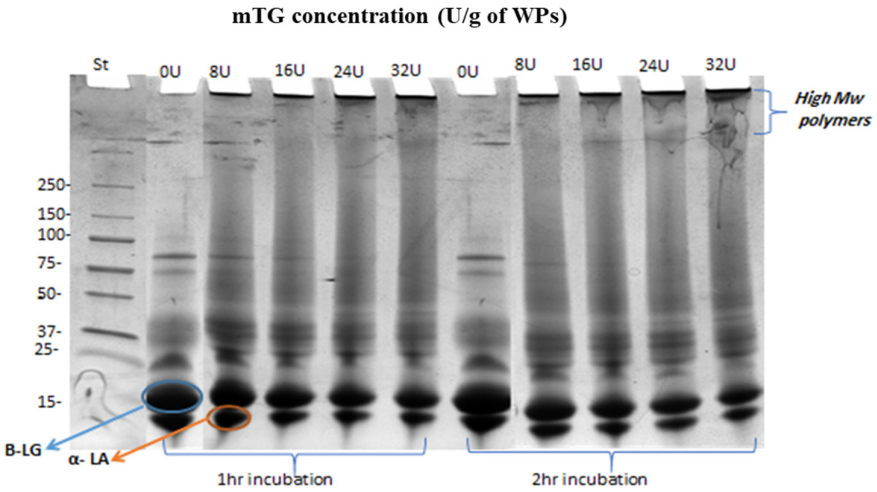

Figure 2. SDS-PAGE of heat-treated WPs (1%, *w*/*v*) incubated with different concentrations of mTG (U/g of WPs).

3.2. Zeta-Potential and Particle Size of Film Forming Solutions (FFSs)

Following mTG modification, the pH was adjusted to pH 12 since, as reported in Abdalrazeq et al. [23], the alkaline pH resulted in obtaining manageable, transparent, and more flexible films. GLY (40%, *w*/*w* of WP), used as a plasticizer, was finally added, and films were obtained by casting the obtained WP-based FFSs. Preliminary characterization of FFSs was carried out, including the evaluation of FFS stability via zeta potential (mV) analysis and of FFS particle size through the measurement of mean particle diameter (d., nm) (Table 1). Then, the derived films were prepared and characterized for their mechanical features (Table 2). While the mean particle size (d, nm) was affected by the enzyme addition, being higher for the samples enzymatically treated—most likely as a result of the formation of protein isopeptide bonds—zeta potential measurements (Table 1) showed that the FFSs, whether incubated with mTG or not, were fairly stable. Our results are in agreement with the results reported by Giosafatto et al. [25]. However, WP FFSs were more stable than the grass pea proteins studied by these authors.

Table 1. Zeta potential and mean particle size of WP (1%, *w*/*w*) FFSs containing different concentrations (U/g of WPs) of mTG in the presence of 30% GLY *.

WP FFSs	Mean Particle Size (d.nm)	Zeta Potential (mV)
−mTG	171 ± 7 ^{a}	−33 ± 2.5 ^{a}
+mTG (8 U/g)	268 ± 11 ^{b}	−38.8 ± 1.4 ^{b}
+mTG (16 U/g)	263 ± 17 ^{b,c}	−30.5 ± 1.8 ^{a}
+mTG (24 U/g)	182 ± 7 ^{a,c}	−41.8 ± 2.8 ^{b}
+mTG (32 U/g)	233 ± 12 ^{d}	−33.1 ± 2.2 ^{a}

* Values are expressed as mean ± SD; different letters indicate significant differences from the values reported in the same column (Tukey–Kramer test, $p < 0.05$).

Table 2. Mechanical properties of films obtained with WPs (500 mg) in the presence of 30% GLY and different concentrations (U/g of WPs) of mTG *.

WP Films	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
−mTG	2.4 ± 0.6 ^{a}	10.4 ± 4.9 ^{a}	164.7 ± 48.4 ^{a}	112 ± 28 ^{a,b}
+mTG (8 U/g)	1.1 ± 0.2 ^{b}	16.7 ± 5.2 ^{a,b}	33.9 ± 5.0 ^{b}	92 ± 6 ^{b}

Table 2. *Cont.*

WP Films	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
+mTG (16 U/g)	0.8 ± 0.2 ^{b}	19.1 ± 6.1 ^{a,b}	25.7 ± 4.5 ^{b}	150 ± 18 ^{a}
+mTG (24 U/g)	0.8 ± 0.2 ^{b}	27.6 ± 8.9 ^{c}	26.6 ± 4.8 ^{b}	122 ± 8 ^{a,b}
+mTG (32 U/g)	0.9 ± 0.1 ^{b}	17.9 ± 3.3 ^{b}	28.6 ± 6.3 ^{b}	156 ± 14 ^{c}

* Values are expressed as mean ± SD; different letters indicate significant differences from the values reported in the same column (Tukey–Kramer test, $p < 0.05$).

3.3. Film Characterization

As for film characterization, as shown in Table 2, the mTG-containing materials showed an increase in film thickness, likely due to an increase in the free volume inside the matrix caused by the isopeptide bond formation catalyzed by the enzyme. Mechanical properties analyses showed that mTG slightly enhanced the EB and reduced the YM, indicating that the enzyme was able to produce more flexible and less stiff films (Table 2).

From the results above, we can state that 24 U/g was the best concentration of the enzyme, as a reduction of EB was observed when a higher amount (32 U/g) was exploited (Table 2), most likely because the film structure became denser and more irregular as mTG increased [36]. In addition, from a macroscopical point of view, all the films, either untreated or treated with 24 U/g of the enzyme, were very similar, appearing in both cases transparent and handleable (Figure 3).

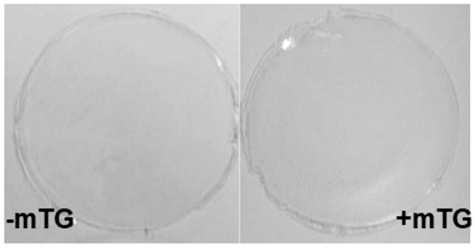

Figure 3. WP (500 mg)-based films prepared in the absence (**left**) and presence (**right**) of 24 U/g of mTG.

Furthermore, the results of the viscosity test, shown in Figure 4, indicate that a significantly increased value was obtained with the WP FFS containing 24 U/g of mTG. These data confirm the protein polymerization due to the formation of intermolecular isopeptide bonds between endo-protein Gln and Lys residues.

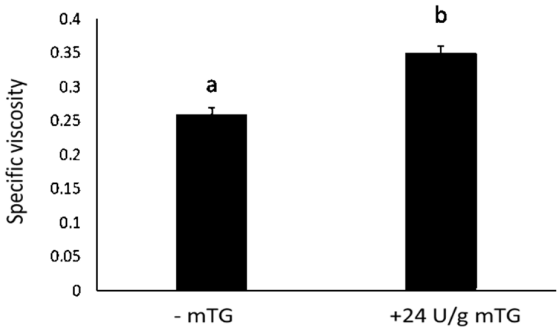

Figure 4. Viscosity of heat-denatured WP (1%, *w*/*w*) FFSs prepared either in the absence or presence of 24 U/g of mTG. Values are expressed as mean ± SD; different letters indicate significant differences (Tukey–Kramer test, $p < 0.05$).

Further experiments were carried out by using 24 U/g of mTG and by increasing GLY concentration up to 40%. The results reported in Table 3 show that the films prepared under these experimental conditions had an increased resistance and extensibility as GLY (a polyol) reduced the free space among the protein molecules and, in turn, facilitated their mobility in the film matrix [37].

Table 3. Mechanical properties of WP (500 mg)-based films obtained in the presence of 40% GLY after FFS treatment with 24 U/g of mTG *.

WP Films	Tensile Strength (MPa)	Elongation at Break (%)	Young's Modulus (MPa)	Thickness (μm)
−mTG	1.4 ± 0.2 [a]	34.3 ± 3.9 [a]	29.2 ± 4.9 [a]	79 ± 2 [a]
+mTG (24 U/g)	2.2 ± 0.1 [b]	49.8 ± 5.3 [b]	50.6 ± 2.6 [b]	77 ± 1 [a]

* Values are expressed as mean ± SD; different letters indicate significant differences from the values reported in the same column (Tukey–Kramer test, $p < 0.05$).

It was demonstrated that mTG pretreatment of WPs had a significant effect on the moisture content and uptake of the WP-based films (Table 4), likely due to the more homogenous and tighter structure created by mTG-catalyzed isopeptide bonds in the WP matrix.

Table 4. Functional properties of films obtained with heated WP (500 mg) cast at pH 12 in the presence of 40% GLY and containing 24 U/g of mTG *.

WP Films	Moisture Content (%)	Moisture Uptake (%)	Opacity (A_{600} * mm^{-1})	Permeability		
				CO_2	WV	O_2
				(cm^3 mm m^{-2} day^{-1} kPa^{-1})		
−mTG	22.1 ± 0.9 [a]	12.2 ± 0.9 [a]	1.9 ± 0.6 [a]	0.90 ± 0.06 [a]	0.04 ± 0.01 [a]	0.26 ± 0.02 [a]
+mTG (24 U/g)	18.8 ± 1.0 [b]	10.6 ± 0.5 [b]	1.2 ± 0.1 [b]	0.14 ± 0.02 [b]	0.04 ± 0.01 [b]	0.02 ± 0.01 [b]

* Values are expressed as mean ± SD; different letters indicate significant differences from the values reported in the same column (Tukey–Kramer test, $p < 0.05$).

For evaluating customer acceptability, film transparency was also investigated. mTG treatment was also shown to significantly improve the transparency of the produced bioplastics, as demonstrated by the reduction of the film opacity (Table 4). These results indicate a significant improvement in transparency with mTG treatment. For comparison, common industry benchmarks for food packaging films include polypropylene (* 32.02 ± 3.35) and cellulose triacetate (* 0.54 ± 0.09), as described by Giosafatto et al. [25]. While the mTG-treated WP films are significantly more transparent than polypropylene, they exhibit slightly higher opacity compared to cellulose triacetate. The mTG-treated WP films fall within this range, demonstrating their potential for use in food packaging applications requiring transparency, such as visual appeal for product display. The UV-Vis spectral profiles of the WP films are presented in Figure 5. It is clear that there is no significant difference in the absorbance and transmittance profiles between WP films prepared with or without 24 U/g of mTGs. However, the film with mTGs showed approximately 7% higher transparency across the entire visible spectrum compared to the film without mTGs. Opacity was calculated from the transmittance at 660 nm, as described by Galus and Kandiska [29], and is summarized in Table 4, showing that the opacity of the film with mTGs is 36% lower than that of the film without mTGs. Additionally, the low transmittance of all the films at UV wavelengths is a noteworthy feature of the WP-based materials, offering potential protection against physicochemical alterations in coated or wrapped foods and drugs.

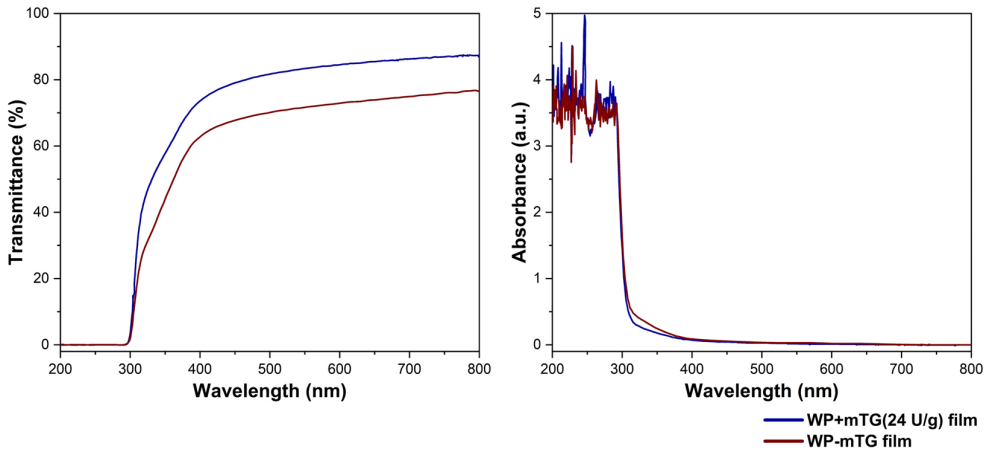

Figure 5. UV-vis spectra transmittance and absorbance of WP films prepared with and without 24 U/g of mTG.

Finally, since CO_2, O_2, and WV permeability properties are important parameters for assessing an industrial application of biodegradable materials, the barrier effects of the prepared films were investigated. The results summarized in Table 4 show that mTG strongly increased CO_2 and O_2 barrier properties of the mTG-crosslinked films, whereas WV permeability was not affected at all by the enzyme treatment. In fact, it was observed that the CO_2 and O_2 permeabilities for the uncrosslinked films were significantly higher than those for mTG-modified films, which could be attributed to the presence of a more compact structure due to isopeptide bonds on the surface of films prepared in the presence of the enzyme. In addition, it is worth noting that for the neat materials, O_2 permeability was lower than CO_2 permeability, indicating a selective effect of these films on gas permeability, which might be due to the higher solubility of CO_2 in the protein films (Table 4). As for the water vapor barrier property, protein films that contain hydrophilic polar groups interact with the water molecules, triggering deviancy in the permeability constant and, thus, leading to increased water vapor permeability (Table 4). Oxygen and carbon dioxide are important gases for the application of novel materials in food packaging. For example, the permeation of oxygen from the air into the package should be reduced since this gas can cause deterioration of products (encouraging the growth of microorganisms, resulting in mold and yeast growth, and activating oxidizing enzymes) during long-term storage. Since these materials do not act as an effective barrier to water vapor, they might be used for protecting oily foods, such as dried nuts. In addition, to reduce the amount of moisture uptake by the WP-based films, it might be convenient to store the films at a low relative humidity and temperature [38].

3.4. Film Digestibility

INFOGEST-based in vitro digestion was conducted to assess the film digestibility. The "INFOGEST" protocol is the most widely used static gastrointestinal (GI) digestion protocol. First published in 2014 by a network of scientists from over 45 countries [39], the INFOGEST protocol arose out of a European Cooperation in Science and Technology (COST) funding action to fill a critical gap in the harmonization of in vitro GI digestion methods across laboratories. In particular, the films were subjected to oral and gastric digestion experiments (adult model) [40]. At the end of digestion, the proteolysis was checked by means of SDS-PAGE (12%). As demonstrated by Arciello et al. [40], the alkaline treatment of the WPs seems to enhance the film's gastric digestion (Figure 6). In addition, it is noteworthy that the enzymatic modification did not lead to a decrease in digestibility in the stomach, unlike the observation by Leng et al. [41], who demonstrated that high-molecular-weight

aggregates of mTG-polymerized WPs are characterized by a more extended retention time in the gastric environment. Our result could be attributed to the fact that the enzymatic modification of WPs by means of intramolecular and intermolecular crosslinks may alter the protein structure, making some amino acid residues more exposed to proteolysis by pepsin. This is an interesting aspect for the application of these films as edible coatings, as digestibility might have serious implications for the nutritional quality of the proteins as well as the risk of food allergy.

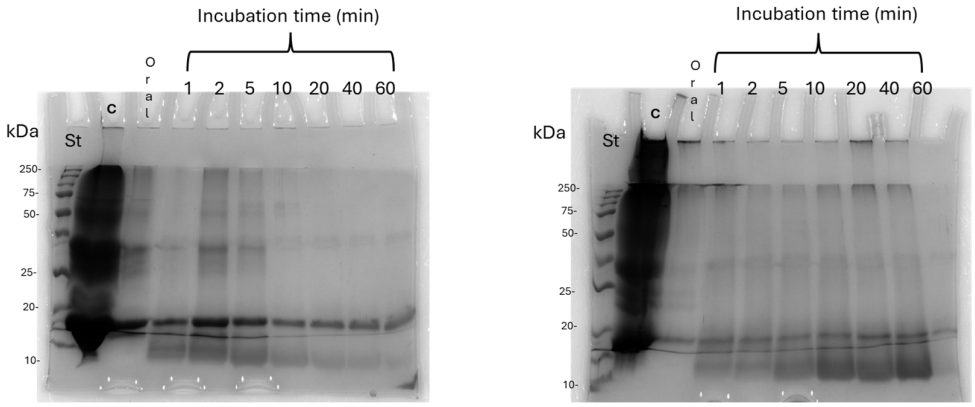

Figure 6. SDS-PAGE (12%) of oral and gastric digestion (1–60 min) of films prepared in the absence (**left** panel) and presence (**right** panel) of 24 U/g of mTG. C, electrophoretic pattern of the film; St, standard molecular marker.

4. Conclusions

The paper focuses on the use of mTG to modify WPs. mTG is a microbial enzyme able to catalyze the formation of isopeptide bonds between endoprotein glutamine and lysine residues. The enzyme was exploited in order to further improve the technological properties (such as mechanical and gas barrier properties, as well as hydrophilicity and opacity features) of WP-based materials. SDS-PAGE analysis has demonstrated that the enzyme-mediated modification of WPs was improved when the proteins were first pre-heated for 25 min at 80 °C, as this had an effect on protein denaturation, making Gln and Lys residues more prone to the enzymatic modification. The extent of mTG-mediated crosslinking has proved to influence the technological properties (mechanical and barrier properties) of the films by enhancing the EB and reducing the YM. At the same time, the mTG makes the materials more rigid, as evidenced by the decrease in TS. Moreover, the barrier properties against CO_2 and O_2 were improved following the mTG-catalysed crosslinking. In addition, it is worth underlining that the enzymatic modification did not influence the film gastric digestion, suggesting the possibility of using such materials in different industrial sectors, such as the food and pharmaceutical fields. In the near future, the biodegradability of these novel materials under soil-like conditions and/or in a marine environment will be investigated in order to assess their environmentally friendly properties.

Author Contributions: Methodology, M.A. and D.A.; investigation, M.A. and D.A.; writing—review and editing, M.A., D.A. and C.V.L.G.; supervision, C.V.L.G. and L.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Acknowledgments: The authors sincerely thank the University of Naples and the European Commission for supporting the inter-institutional agreement under Erasmus+ KA171 (South Mediterranean area). The authors also thank Maria Fenderico for her technical support in film preparation.

Conflicts of Interest: The authors declare no conflicts of interest.

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