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MECHANICAL, BARRIER AND STRUCTURAL PROPERTIES OF WHEY PROTEIN ISOLATE-BASED FILMS TREATED BY MICROBIAL TRANSGLUTAMINASE

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ABSTRACT

Nowadays, polymers obtained from edible resources such as polysaccharides and proteins have attained growing consideration to substitute petroleum-derived polymers. Edible films are a good alternative for the plastic packaging since these films are biodegradable, environmental-friendly and non-toxic. However, protein based edible films have poor water permeability and lower mechanical strength. In this study, edible films based on whey protein isolate (WPI) were produced with different microbial transglutaminase (MTG) concentrations (0, 5, 10 and 15 Unit/g of protein) and the effect of enzymatic treatment on the film properties was investigated. Results showed that as compared to control, the treatment with the lower concentration of MTG (5–10 U/g) significantly increased the tensile strength (TS) and decreased elongation at rapture (ER) values of WPI-films, while at the higher concentration of MTG (15 U/g), TS value slightly decreased and ER values significantly increased. By increasing the enzyme concentration from 5 to 10 U/g, water vapor transferability (WVT) and water soluble fractions (WSF) decreased significantly ($P \leq 0.05$). The MTG-treated films except the film treated with 15 U/g TG, had homogeneous and even surface without any crack or fracture. Based on results, treatment with MTG enzyme may be applied as an appropriate technique to modify the structural and barrier properties of WPI-based films.

Keywords: Biodegradable film, Enzyme modification, WPI, SEM

INTRODUCTION

Recently, concerns about the environmental pollution caused by plastics have led to attempts to produce biodegradable films using degradable components **(Pérez-Gago and Krochta, 2001)**. Edible films and coatings from biopolymers are biodegradable, non-toxic, environmental-friendly and in many occasions, they made with by-products obtained from food industry. Hence, they are good choices for the plastic packaging alternatives **(Rodriguez *et al.*, 2013)**. The expansion of recyclable films and coatings has received the most consideration since it results in the shelf life development of food **(Alvarez-Prez et al., 2015)**. The major components of edible films are proteins, polysaccharides and lipids or a combination of any of these macromolecules **(Atarés *et al.*, 2010)**. Food proteins have been broadly used to make edible films, because the films prepared by proteins have better mechanical and barrier characteristics as compared to polysaccharides- and lipids-based films **(Tang and Jiang, 2007)**. Amongst different proteins used in the edible film production, whey is one of the most favorable packaging materials **(Schmid *et al.*, 2012)**. Whey proteins are widely used in food industry in the various forms such as whey protein isolate (WPI), whey protein concentrate (WPC), whey protein hydrolysate (WPH) and fermented whey protein concentrate (FWPC) **(Jooyandeh and Minhas, 2009).** These ingredients have excellent functional and nutritive properties **(Jooyandeh *et al.* 2009)**. However, statistics indicates that solely in Europe 20 million tons of whey produced are disposed annually **(Bugnicourt *et al.*, 2010).** Therefore, numerous food researches focused on whey and whey derivatives as a crucial ingredient in food products and introduced its further applications. Many researches have been carried out to produce edible films or coatings constructed with whey proteins **(Pérez-Gago and Krochta, 2001; Osés *et al.*, 2009; Ramos et al., 2012; Soazo *et al.*, 2013; Kouravand *et al.*, 2016)**. Although edible films based on whey proteins have a good mechanical and oxygen barrier properties, their inadequate water vapor permeability and lower mechanical strength have limited their application in food packaging **(Miller and Krochta, 1997; Seydim and Sarikus, 2006)**. Among different methods to improve mechanical resistance and barrier properties of protein films, treatment with microbial transglutaminase (MTG) seems to be safe and effective technique **(Tang and Jiang, 2007)**.

Transglutaminase-induced crosslinking treatment in different food proteins have enormously investigated **(Jooyandeh *et al.*, 2015; Kouravand *et al.*, 2018).** Polymerization of soy proteins **(Jiang *et al.*, 2007; Yildirim and Hettiarachchy, 1998)**, whey proteins **(Aboumahmoud and Savello, 1990; Eissa, 2004)**, casein **(Færgemand *et al.*, 1999)**, gelatin–calcium carbonate composite films **(Wang *et al.*, 2015)** and fish protein **(Rostamzad *et al.*, 2016)** using transglutaminase enzyme are some of these examples. The aim of present investigation was to evaluate the effect of MTG treatment at different enzyme concentrations on the structural and barrier characteristics of edible films based on whey protein isolate.

MATERIALS AND METHODS

Materials

Whey protein isolate (WPI) containing 97.5% protein was acquired from Arla Food Ingredient (Denmark). Microbial transglutaminase (MTG, with activity of 100 units per each gram of protein) was purchased from Ingredients BDF Natural Co. (Spain). Glycerol (as the plasticizer), Tween 80 and other chemicals were of analytical grade and were acquired from Merck (Darmstadt, Germany).

Film Preparation

Edible film samples were manufactured according to **Tang and Jiang (2007)** with a slight modification. For making film composition solutions, 5 g of WPI-powder was dissolved in 100 mL distilled water and 2 g of glycerol was added as a plasticizer. Following the addition of glycerol, a magnetic stirrer (IKA RH Basic 2) for 30 min stirred the mixture. Thereafter, the protein solution was placed in a water bath at 80 °C and was stirred for another 30 min. Heat treatment of the whey proteins is essential for the formation of intermolecular disulfide bonds. This treatment also is necessary to obtain a flexible film via covalent and non-covalent cross-linking that retains its integrity at high moisture environments **(Zinoviadou *et al.*, 2010)**. Solutions then quickly cooled to 45 °C by ice water bath to avoid further denaturation. After cooling to 45 °C, MTG at the level of 0 (as control film), 5, 10 and 15 units per gram of protein (U/g) added and solutions

incubated at 45°C for 1 h. Subsequently, to inactivate MTG enzyme, the solutions were heated at 85 °C for 10 min **(De Carvalho and Grosso, 2004)**. After cross-linking, ultrasonication by ultrasonic bath (Elmasonic P 60H, Germany) was performed for degassing of the film solutions as described by **Schmid *et al.*, (2014).** For making the film samples, the equal amount of solutions (~70 ml) were dispensed into poly methyl methacrylate plates (PMMP) and the film samples were prepared after drying at ambient temperature for 48 to 72 h. In this way, thickness differences between treatments were minimized. The casted dried films were separated from surface of PMMP and were placed in desiccator (25 ±2°C) with 50% relative humidity (RH) before analysis. Specimens of 2.54 × 7.5 cm rectangular strips used for tensile testing and 1 × 3 cm^2 for moisture content and water soluble fractions analysis.

Film Thickness Measurement

A digital micrometer (Mitutoyo No.293-766, Japan) was used to measure film thickness to the nearest 0.0001 mm **(Tang and Jiang, 2007)**. Three random positions on the film measured and the mean value used in tensile strength calculations.

Water content (WC) and Water Soluble Fractions (WSF)

WC and WSF of the films were measured according to **Ghasemlou *et al.*, (2011)**. Film samples were weighed, and the percentage of WC of the films were assessed by calculating the weightiness of the films before and after oven drying (Heraeus, Germany). The WSF of the film samples were determined by soaking the dehydrated weighed films for 6 h in distilled water at ambient temperature under continuous stirring. After separation of the remained pieces of the films by filtration, the WSF was assessed as follows (Eq. 1):

$$\text{TSM (\%)} = \frac{\text{initial dry weight} - \text{final dry weight}}{\text{initial dry weight}} \times 100 \quad (1)$$

Water Vapor Transferability (WVT)

WVT of the films was obtained using **ASTM E96 standard method (2000)**. This method determines WVT of the film as it withstand against of mass transfer in vaporous phase. The specimens of the film were cut to match the cup mouth with a surface area of 0.00066 m^2. The cups were contained anhydrous calcium chloride to reach 0% RH. The film was then fixed to the cup opening by parafilm. The cups were put into a desiccator with 75% RH (created by sodium chloride saturated solution at 25°C). This RH alteration amongst two edges of the films generates a vapor compression equivalent to 1753/55 Pa. After weighing up the cups regularly during 3 days, the slope of mass reduction against time was achieved by linear regression (R=0.99). Water vapor transmission rate (WVTR) was determined as follows (Eq. 2):

$$\text{WVTR} = \frac{slope}{A} \quad (2)$$

WVT was then measured via the subsequent calculation (Eq. 3):

$$\text{WVT} = \frac{WVTR \times X}{\Delta P} \quad (3)$$

A is the exposed surface area of the film, X is thickness and ΔP is pressure difference amongst two edges of the film.

Mechanical Properties

Texture analyzer (TA.XT.PLUS, Stable Micro System, UK) was used to calculate the most important mechanical properties of the edible film samples, i.e. tensile strength (TS) and elongation at rapture (ER). WPI-films samples were assessed for TS and ER analysis by using ASTM D882-00 method **(ASTM, 2000)** taking an average of three determinations for each sample. ER was measured by dividing the extension in length (elongation at the moment of film break) by initial gauge length expressed in percentage and TS value was expressed in mega Pascal (MPa) and was calculated as follows (Eq. 4):

$$\text{TS} = \frac{\text{Maximum force}}{\text{Film thickness} \times \text{Film width}} \quad (4)$$

Film Microstructure

Microstructure of the films were evaluated by using scanning electron microscopy **(**SEM, Philips XL30, Netherlands). WPI-based films were immersed in liquid nitrogen for fracturing and were mounted onto aluminum stubs and were coated with gold by using a sputter coater (SCD 050, Bal-Tec®, Switzerland). The SEM (Shojaee-Aliabadi et al., 2013) was used to observe the surface and cross-section microstructure of the films.

Fourier-Transform Infrared (FT-IR) Spectroscopy

FT-IR was used to determine molecular weight variations of WPI-based edible films treated without enzymatic treatment (control) and treated with 10 U/g MTG and to observe protein interactions. This level of MTG enzyme concentration was selected on the base on film characteristics obtained from experimental results. FT-IR was performed by using Fourier-Transform Infrared spectrometer (Bruker Instrument, France) according to **Jooyandeh *et al.*, (2018)**. Spectra were measured at 500 to 4,000 cm^{-1}. The FTIR spectrometer was attached with potassium bromide beam splitter, MICOR-ID sample changer compartment and deuterated triglycine sulfate detector. One mg of the film sample was milled with 300 mg of potassium bromide and pressed into a pellet (1 mm thickness and 13 mm diameter) for transmission infrared spectroscopy (Jooyandeh et al., 2018). Each spectrum was the averaged value of 20 scans in a transmittance mode. The spectra attained were deducted to attain the pure protein spectrum and the curve-fitted spectra were depicted for further analysis.

Statistical Analysis

Data were analyzed by one-way ANOVA analysis by using SPSS (version 20.0) software. Duncan's comparison test with a 95 % confidence level was used to determine statistically meaningful variations between the means.

RESULTS AND DISCUSSION

Film Thickness

Thickness of the films is listed in Table 1. As expected, thickness of WPI-films were affected by MTG treatment. Treated films with 10 and 15 U/g MTG had a significantly higher thickness as compared to the control and the film contained 5 U/g MTG (Table 1). By increasing enzyme concentration from 5 to 15 U/g, thickness of the films increased significantly from 0.101 to 0.120 mm ($p<0.05$). The same results have been reported by **Tang *et al.*, (2005)** who investigated the impact of transglutaminase on the properties of SPI films**. On the contrary, Tang and Jiang (2007) reported that modification by MTG (8 U/g) did not significantly affect the thickness value of edible films prepared from WPC (containing 84.7% protein).**

Water content and Water Soluble Matter

Water content (WC) and water soluble fractions (WSF) of the films are shown in Table 1. Modified films had lower WC as compared to control film and this value for MTG treated films decreased by 20.66% as the enzyme concentration enhanced and reached to 15 U/g. **Jiang *et al.*, (2007)** have similarly reported that soy protein isolate (SPI) -based films treated with 10 U/g had significantly higher WC (21.6%) than control film (23.5%). As it is shown in table 1, the impact of MTG quantity on the WSF of the WPI films was multifarious. By increasing the MTG concentration from 5 to 10 U/g, WSF values of the films decreased meaningfully ($p < 0.05$) from 38.37% for control to 19.97% for sample containing 10 U/g MTG. However, the treatment with more MTG concentration, i.e. 15 U/g of enzyme contrarily increased the WSF value to some extent ($P>0.05$). In order to determine the effect of cross-linking of proteins by MTG, several investigations have been carried out and reported the reduction of WSF in the films because of MTG modification **(Yildirim and Hettiarachchy, 1998; De Carvalho and Grosso, 2004; Jiang *et al.*, 2007; Weng and Zheng, 2015; Rostamzad *et al.*, 2016)**. The decline in the WSF value of the treated MTG films is possibly due to an increase in the molecular weight of protein fractions caused by increasing the degree of cross-linking and formation of new intra- and intermolecular protein's bonds **(Carvalho and Grosso, 2004)**. However, as mentioned above, treated film with the highest enzyme concentration (15 U/g), had slightly ($p>0.05$) higher WSF value than sample containing 10 U/g MTG enzyme. These findings are in agreement with the data reported by **Jiang *et al.*, (2007)** who also showed that the treatment with over 10 U/g MTG on the contrary increased WSF values of SPI films and treated films with higher than 20 U/g MTG had even higher WSF than control.

Water Vapor Transferability (WVT)

Water vapor transferability (WVT) of the films is listed in Table 1. Many parameters can affect the water vapor transferability/permeability of films, such as the degree of plasticization **(Jooyandeh 2011),** film configuration **(Pérez-Gago and Krochta, 2000)** and the extent of cross-linking **(Færgemand *et al.*, 1999)**. In general, the films based on proteins are inadequate water vapor barriers owing to the intrinsic high hydrophilicity of proteins and the considerable degree of hydrophilic plasticizers added to protein-based films **(Yildirim and Hettiarachchy, 1998)**. The results of this study showed that treatment of WPI films with MTG significantly ($p<0.05$) improved WVT and sample treated with 10 U/g MTG had lower WVT than control (2.31 vs. 3.57). However, at the higher enzyme concentrations, i.e. 15 U/g, the WVT of the film increased by 8.4%, though this change was not significant ($p>0.05$). In agreement with our study, **De**

Carvalho and Grosso (2004) for gelatin-based films, **Wang *et al.*, (2015)** for gelatin-calcium carbonate composite films and **Rostamzad *et al.*, (2016)** for fish protein films have reported lower WVT as the result of cross-linking by MTG. The slight increase in WVT of treated films at the higher enzyme concentrations may be due to additional pores caused by the higher protein cross-linkages **(Yildirim and Hettiarachchy, 1998)**. In the other words, the increase in WVT of the MTG treated films caused by cross-linking reactions of proteins can result in the molecular orientation of proteins around themselves and formation of additional free space **(Yildirim and Hettiarachchy, 1998)**.

Table 1 Water content, water soluble fractions and water vapor transferability of WPI-based films cross-linked by MTG

Enzyme concentration (U/g)	Thickness (mm)	Water content (%)	Water soluble fractions (%)	Water vapor transferability ($\times 10^{-10}$ g m^{-1} s^{-1} pa^{-1})
0 (control)	0.097 ± 0.003^{c}	28.90 ± 0.62^{a}	38.37 ± 4.05^{a}	3.57 ± 0.16^{a}
5	0.101 ± 0.003^{c}	26.87 ± 1.46^{a}	28.03 ± 4.56^{b}	3.11 ± 0.33^{ab}
10	0.110 ± 0.005^{b}	24.20 ± 1.37^{b}	19.97 ± 1.68^{c}	2.31 ± 0.30^{c}
15	0.120 ± 0.004^{a}	22.93 ± 1.00^{b}	22.13 ± 1.60^{bc}	2.61 ± 0.28^{bc}

Data are the average ± standard deviations. Different small letters indicate significant differences between all samples ($p < 0.05$)

Mechanical Properties

The mechanical properties of the WPI-edible films are shown in Figure 1 and 2. Results showed that by increasing MTG concentration up to 10 U/g, the tensile strength (TS) of the films was increased significantly ($p<0.05$). However, at the higher amount of the enzyme, the TS value was slightly, but insignificantly decreased ($p>0.05$). These results are in agreement with the data reported by **Motoki et al., (1984)** for α_{s1}-casein, **Mahmoud and Savello (1993)** for whey protein and **Rostamzad *et al.*, (2016)** for fish protein who indicated the higher TS value in MTG treated films. Development of covalent iso-peptide linkages into the protein construction is the reason for the rising of TS value **(Yildirim and Hettiarachchy, 1998)**. On the contrary, Tang and Jiang (2007) reported insignificant changes in TS values (2.2 vs. 2.3 MPa) of the WPC-based film treated with MTG as compared to control. They illustrated that intrinsic whey proteins are globular proteins and are considerably less vulnerable to MTG as compared to other proteins, such as sodium caseinate and SPI, causing MTG treatment to be uselessness to improve the TS of resultant films.

Our results also showed that as the MTG concentration increased, the elongation at the rapture (ER) of treated films decreased significantly ($p<0.05$). This finding is in agreement with the data reported by **Wang *et al.*, (2015)** studied the effect of transglutaminase on the properties of gelatin-calcium carbonate composite films. They observed that as the MTG concentration increased from 4 to 8 U/g, elongation at rapture/break value decreased significantly. **De Carvalho and Grosso (2004)** demonstrated that the lower ER value of enzymatically treated films is due to decreasing the mobility of the film matrix because of whey protein cross-linking. These researchers also reported the higher TS and the lower ER values for treated films by MTG as compared to control (without enzymatic treatment). Like the other film properties, the ER of the films at the highest level of MTG, i.e. 15 U/g, changed adversely and to some extent increased (Figure 2). **Truong *et al.*, (2004)** reported similar results and declared that enzymatic treatment of whey protein films with a higher enzyme concentration undesirably led to the lower TS and higher ER values. The adverse mechanical behavior of the films treated at the highest MTG concentration (15 U/g) is probably due to the extensive intra- and inter-chain cross-linking formed by the enzyme. This extra polymerization causes an inappropriate network development, leading to the formation of weak gel **(Truong *et al.*, 2004)**. **Jiang *et al.*, (2007)** also indicated that enzymatic treatment of SPI-based film with 4-10 U/g of MTG resulted in higher TS value; however, at the higher enzyme concentration over 10 U/g, TS value was unfavorably diminished.

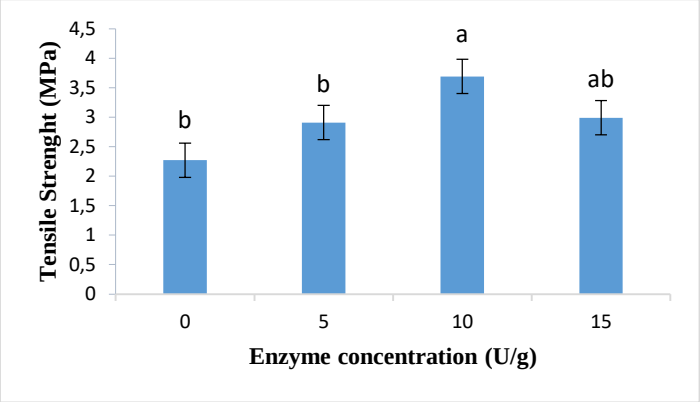

Figure 1 Effect of treatment with MTG on tensile strength of the films

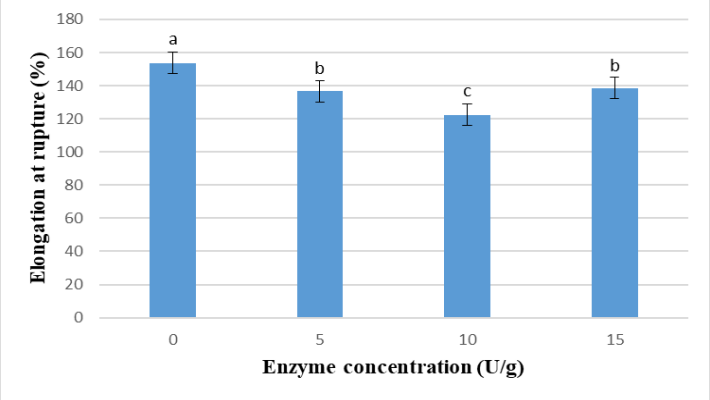

Figure 2 Effect of treatment with MTG on elongation at rapture (%) of the films

Film Microstructure

The scanning electron microscopy (SEM) micrographs of the surface and the cross section of WPI films treated by different concentrations of MTG are presented in Figure 3. As compared to the control film, the enzymatic-treated films with 5 and 10 U/g of enzyme had more compact cross-section and a smooth texture without any cracks.

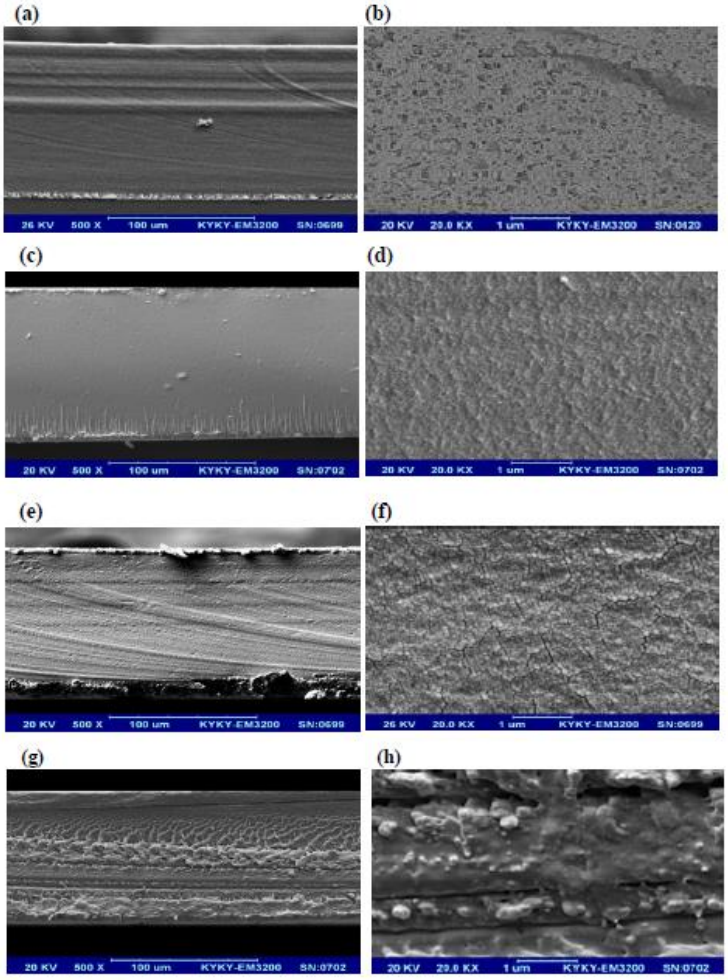

Figure 3 SEM micrographs of cross linked WPI-based edible films treated with different MTG enzyme concentrations; 0 U/g as control (a: cross-section; b: surface), 5 U/g (c: cross-section; d: surface), 10 U/g (e: cross-section; f: surface), and 15 U/g (g: cross-section; h: surface)

These findings are in agreement with the data reported by **Tang *et al.*, (2005)** who observed that the cross-section of SPI-films treated by MTG exhibited compact microstructure. This could explain the lower WSF, WVT and ER of MTG-treated films. The same results stated by **Mariniello *et al.*, (2003)** who found that the MTG treated films prepared from pectin-soy flour had a smooth appearance and homogeneous structure. However, as it is shown in Figure 3, the film treated by 15 U/g MTG had heterogeneous and uneven surface. The bumpy surface of this film as compared to other MTG-treated films are attributed to the extensive protein cross-linkages and formation of massive polymers that are unsuccessful to develop an appropriate protein network **(Truong *et al.*, 2004)**. This irregular film construction caused a loss of net integrity and subsequently resulted in the lower ER as compared to the lower MTG enzyme concentrations (Figure 2). **Wang *et al.*, (2015)** also reported that with increasing the MTG concentration, the gelatin–calcium carbonate composite films showed a rough and uneven surface.

FT-IR Spectroscopy

Proteins contain several configurations such as α-helix and β-sheet which are the most common secondary structural elements of protein. FT-IR spectroscopy is a major device to estimate protein secondary structures (**Kong and Yu, 2007**). Proteins are consisting of amino acids associated together by amide linkages. As it is shown in Figure 4, regarding to FT-IR analysis, there were substantial differences ($p<0.01$) in the amount of protein linkages between control and film samples treated with MTG enzyme. The spectra for films showed that the band was formed by different separable peaks; situated as amide-III (1200 to 1300 cm^{-1}), amide-II (1500 to 1600 cm^{-1}) and amide-I (1600 to 1700 cm^{-1}). Amid-I region of FT-IR spectra shows stretching vibrations of C=O and C–N groups and is straightly related with protein secondary buildings (**Kong and Yu, 2007**) while amide-II region represents N−H bending. These absorption bands, are confirmed the polypeptide and protein structure units. MTG treated films had the higher protein linkages than control film. MTG enzyme is an enzyme that catalyzes cross-link bio-molecular interactions thorough intra- and inter- linkages, resulting in a higher covalent connection and strengthen the protein network. Our findings are in agreement with the data reported by **Wang *et al.* (2019)** who observed similar FT-IR spectra for edible coatings prepared with using whey protein isolate.

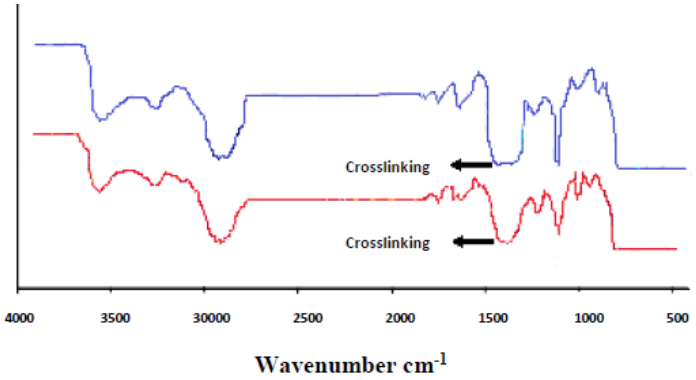

Figure 4 FT-IR spectra of WPI-based edibel films without enzymatic treatment (red curve) and treated with 10 U/g MTG (blue curve)

CONCLUSIONS

Results showed that the properties of MTG-treated films were influenced by enhancing the enzyme concentration. The treatment with a lower concentration of MTG (5–10 U/g) significantly increased the tensile strength (TS) and decreased elongation at the rapture (ER) values of WPI films ($P\leq 0.05$). Furthermore, enzymatic treatment by MTG resulted in the lower water soluble fractions (WSF) content and water vapor transferability (WVT) rate as compared to the control film. The MTG-treated films except the film treated by 15 U/g MTG had homogeneous and even surface. FT-IR analysis also revealed that treatment with 10 U/g of MTG enzyme enhanced the content of cross-linked **proteins** and thereby improved the edible film characteristics. These results suggest that the MTG cross-linking may uses as an efficient method to promote the mechanical and physical properties of WPI-based films. Overall, the best WPI film with the appropriate mechanical, structural and barrier aspects can be produced by using 10 unit MTG per g protein (U/g) and this biofilm meets all the requirements needed for food packaging.

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