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Modification of gelatin from tuna skins by green tea polyphenols

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Abstract. This paper presents the results of studying the effect of modifying conditions on the properties and structure of tuna skin gelatin using green tea polyphenols. The results showed that gelatin modified by green tea polyphenols changed its gel strength, degree of cross-linking, molecular weight and gel structure. The appropriate parameters for gelatin modification using green tea polyphenols which indicated the best mechanical properties were determined as follows: temperature of 40 ^{o}C for 40 minutes, polyphenol content of 20 mg/g gelatin and gelatin solution of 20 %. As a result, modified gelatin showed a decrease in solubility, an increase in cross-linking degree (16.7 %), molecular weight (55 ÷ 72 kDa) as well as a larger protein structure in comparison with natural gelatin. The IR spectrograph of the modified gelatin did not show any evidence of the formation of new functional groups, only an apparent change in the absorption of infrared spectroscopy at the peak of the amide B region was found. The dried modified gelatin became insoluble in hot water.

Key words: fish gelatin, green tea polyphenol, modified gelatin, gel strength, molecular weight.

Classification number: 1.1.2, 1.2.5, 1.4.4, 1.5.4.

1. INTRODUCTION

The extraction process and the application of gelatin from seafood waste have attracted extensive research attention in the food, pharmaceutical, medical, cosmetic and photographic industries. Due to many sociocultural and religious reasons as well as diseases from porcine or bovine, the majority of commercial gelatin derived from mammals is gradually being replaced by alternative sources of gelatin from seafood waste. Many studies indicated that the production of gelatin from fish waste such as skin, scales, bones, bubbles, etc. provided a relatively high gelatin recovery and good quality, particularly the yield of gelatin recovery from the skin was the highest in comparison with other fish processing waste. However, gelatin obtained from the fishing industry exhibited lower values of viscosity, gel strength, molecular weight, melting temperature and film-forming ability than one obtained from mammalian sources. The application of marine gelatin as gelling agents or gelatin-based films and coatings in food

processing and packaging industries is hence limited [1]. In recent years, many scientists have focused on the improvement of those properties of gelatin, which intent to provide new insights into seafood gelatin in order to develop the technology of modified gelatin production from seafood residues, as well as to evaluate the applicability of marine gelatin in the food industry and other industries, thereby enhancing the economic value of products from seafood waste.

To improve the viscosity, gel strength, melting temperature and film-forming ability of gelatin, there have been different gelatin modification methods such as denaturing gelatin by biological agents (e.g. enzyme *Trangslutaminase*), chemical agents (e.g. caffeic acid, tannic acid, etc.) and physical agents (e.g. UV rays), which produce gelatin products with different properties [1, 2]. Compared to other methods, the most recent literature on the use of phenolic compounds from natural sources for modifying gelatin has demonstrated that these compounds can effectively improve the physical and mechanical properties of gelatin, making it cheaper and safer for consumers [2].

The objective of this study was to define the suitable paramters for gelatin modification by polyphenols extracted from green tea in order to enhance the mechanical properties of gelatin.

2. MATERIALS AND METHODS

2.1. Materials and chemicals

Gelatin processed from ocean tuna skin according to the procedure of Chau T H. [3] had a moisture of 11.53 %, a protein content of 95.34 % (dry basis), and a Bloom value of 102.8 g. Green tea polyphenols extracted from fresh tea leaves according to the procedure of Dang *et al*. [4] contained a polyphenol percentage of 64.88 % and a moisture content of 6.85 %. Other chemicals met analytical standards.

2.2. Methods

2.2.1. pH measurement: The pH of gelatin solution was determined using an Inlab Expert Pro-ISM benchtop pH meter (Mettler Toledo).

2.2.2. Viscosity measurement

The viscosity of gelatin solution was determined using a viscometer (DVEELVTJ0, Brookfield) at a temperature of 60 ^{o}C.

2.2.3. Evaluation of gelatine film solubility

Gelatine film was formed by drying gelatine solution in thin layer in a dryer at 45 ^{o}C for 24 hours. To evaluate the solubility, the film was submerged in hot distilled water at 60 ^{o}C contained in a 50-mL beaker conditioned in a water bath. The state of the sample was evaluated visually after 15 minutes of conditioning as soluble, partially soluble and insoluble depending on the remainder of the film.

2.2.4. Gel strength measurement (Bloom value)

Gelatin strength was determined on a 6.67 % hydrogel (w/v). The gelatin solutions were introduced into a cylinder with a diameter of 3.8 cm and a height of 2.7 cm, left at room temperature for 30 minutes and then cooled at 10 ^{o}C for 16 ÷ 18 hours to form a gelatin gel

structure. Gel strength, expressed in the maximum force (in grams) of a piston with a diameter of 12.7 mm penetrating the surface of a 4 mm sample with cross-head speed of 0.5 mm/s at 10 ^{o}C, was examined using a RheoTex SD-700II DP Rheometer **(**Sun Scientific, Japan) [1, 5].

2.2.5. Determination of molecular weight distribution by electrophoresis

Molecular weight distribution of gelatine before and after modification was determined by SDS PAGE according to Leamlli method [6] using 4 % stacking gel and 10 % running gel. Sample electrophoresis was run with a known molecular weight protein marker.

2.2.6. Determination of the microstructure by scanning electron microscopy (SEM)

Gelatin gels having a thickness of 2 - 3 mm were fixed with 2.5 % (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). The samples were then rinsed with distilled water for 1 hour and dehydrated in ethanol with a serial concentration of 50 %, 70 %, 80 %, 90 %, and 100 % (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold. The specimens were observed with an S-4800 scanning electron microscope (HITACHI) at an acceleration voltage of 10 kV [7].

2.2.7. Determination of infrared spectra by Fourier transformed infrared spectroscopy (FTIR)

The FTIR spectra of the films were scanned using a Nicolet™ iS50 FTIR Spectrometer - Thermo Fisher Scientific and evaluated as mentioned in the literature [7]. FTIR spectroscopy at 4000÷400 cm^{-1} was used to monitor changes in the structure of gelatin with and without modification. The FTIR spectra were recorded and analysed by Omnic software (OMNIC 8.2.).

2.2.8. Determination of the degree of cross-linking

The 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay was used to determine the complete cross-linking of gelatin with analysis of free amino acid content. At pH 8 ÷ 9, TNBS reacted with primary amines to form a highly chromogenic derivative, which was measured at 420 nm [8].

The degree of cross-linking (CLD) was determined by the following formula:

$$\mathrm{CLD}(\%) = \left(1 - \frac{\text{Absorption of gelatin after cross-linking}}{\text{Absorption of gelatin before cross-linking}}\right) \times 100.$$

2.2.9. Statistical analyses

MINITAB release 16 (Minitab Inc.) and Microsoft Office Excel 2010 (Microsoft Inc.) were used for statistical analysis of the data. The results were the means of replicates. Significant differences were determined at $p < 0.05$.

2.3. Modification of ocean tuna skin gelatin by green tea polyphenols

Ocean tuna skin gelatin was completely dissolved in distilled water with a ratio of 10 ÷ 25 %, adjusted to pH 9 with 1 N NaOH solution. Green tea polyphenols were added with 10 ÷ 25 mg/g gelatin at a modifying temperature

of 30 ÷ 45 ^{o}C. During the modifying process of protein, the sample was continuously aerated for 10 ÷ 60 minutes. After modification, the modified gelatin was analyzed for gel strength,

viscosity, cross-linking degree or dried down to a moisture content of 4 ÷ 5 % in thin film form for solubility evaluation [7, 9].

3. RESULTS AND DISCUSSION

3.1. Modification of ocean tuna skin gelatin by green tea polyphenols under different conditions

3.1.1. Effect of modifying temperature and time on gelatin characteristics

The effect of modifying temperature and time on viscosity increase, gel strength and cross-linking degree is shown in Figure 1.

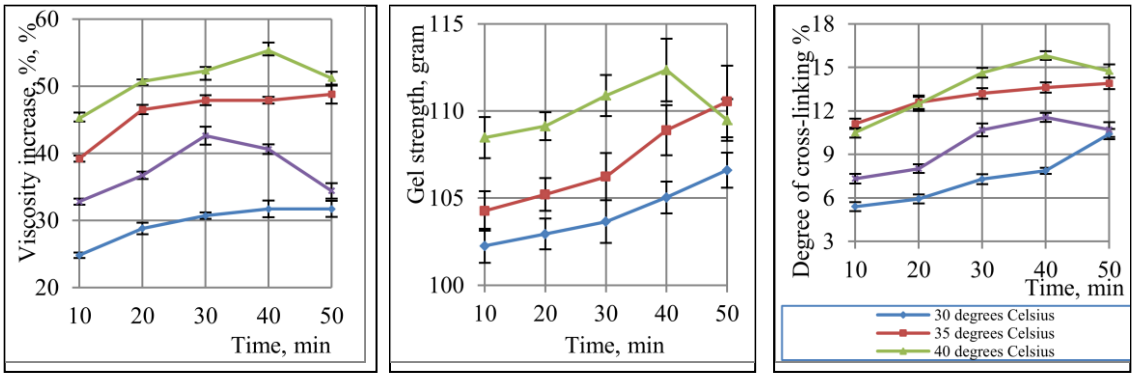

Figure 1. Viscosity increase, gel strength and cross-linking degree as a function of temperature and time. Error bars indicate standard deviation.

At a low temperature of 30 - 35 ^{o}C, the longer the reaction lasted, the more the crosslinking was. The same tendency was also observed in gel strength and viscosity change. Perhaps the crosslinking between gelatine polypeptide fragments increased viscosity and gel forming ability when the size of gelatine fragments was bigger. But at higher temperatures (40 - 45 ^{o}C), hydrolysis of gelatine fragments took place significantly, two trends in change of gelatine fragment size happened. On the one hand, the fact that crosslinking made fragments joint together and became bigger increased viscosity and gel forming ability. On the other hand, hydrolysis made gelatine fragments smaller and thus decreased viscosity and gel forming ability. At the beginning, the effect of hydrolysis was not so significant and the increase in viscosity and gel strength was observed. Later on, the viscosity and gel strength decreased. In most cases of temperature, when increasing the modifying time, the viscosity, gel strength and degree of crosslinking tended to increase to a maximum and then decreased gradually. The gelatin lost its ability to form a gel if the modifying time and temperature exceeded 50 minutes or 40 ^{o}C, respectively. The highest values of viscosity increase, gel strength and crosslinking degree were 55.3 %, 112.4 grams and 15.8 %, respectively when the temperature was 40 ^{o}C and the reaction time was 40 minutes. The results were in accordance with those of gelatin modification with natural antioxidants studied by Li Jian-Hua *et al.* [9]. Hence, the modifying temperature of 40 ^{o}C and the modifying time of 40 minutes were chosen for further studies.

3.1.2. Effect of polyphenol content on gelatine characteristics after modification

The influence of polyphenol contents on viscosity, cross-linking degree, gel strength and

solubility of modified gelatin films is reported in Table 1.

Table1. Viscosity, cross-linking degree, gel strength and solubility of gelatin films as a function of polyphenol content(*).

Polyphenol content, *mg/g gelatin* / Characteristics	0	10	15	20	25
Viscosity, *cP*	55.0 ± 1.6^{d}	66.8 ± 2.0^{c}	71.3 ± 1.8^{b}	75.8 ± 1.1^{a}	73.6 ± 1.2^{ab}
Cross-linking degree, *%*	1.5 ± 0.3^{d}	11.7 ± 0.8^{c}	12.9 ± 0.9^{b}	15.9 ± 1.1^{a}	13.2 ± 0.6^{b}
Gel strength, *gram*	102.8 ± 1.1^{d}	108.6 ± 1.4^{c}	113.9 ± 1.0^{b}	117.2 ± 1.3^{a}	precipitated
Solubility of gelatin films	completely soluble	partially soluble	insoluble	insoluble	-

(*)*All data are mean values ± SD. The same letter in each row indicates no significant difference in value, P>0.05.*

From Table 1, it is obvious that the viscosity and the degree of cross-linking increased as polyphenol proportion increased, and thereafter reached a plateau of 75.8 cP and 15.9 %, respectively, at a polyphenol content of 20 mg/g gelatin.

In addition, the gel strength of modified gelatin solution slightly increased when the polyphenol concentration increased from 10 to 20 mg/g gelatin. At 25 mg polyphenol/g gelatin, gelatin solution precipitated and lost its gelling properties. It was proposed that polyphenols were oxidized to quinones by aeration during modification and then quinones reacted with the lysine residues of gelatin to form quinoneimine cross-linked by covalent bonds, leading to the larger molecular mass of gelatin, the increment of viscosity, gel strength and degree of gelatin cross-linking [10]. However, if the polyphenol content continued to increase, the phenolic compounds could adsorb and form a coating on the protein surface (as can be seen in Figure 3), resulting in precipitation of protein.

The dried modified gelatin lost its solubility in water at 60 °C. It might be because in the drying process, the use of high temperature (45 ^{o}C) and excessive exposure of polyphenol compounds to air contributed to a conversion of phenol groups to quinones and then a reaction of quinones with amines on gelatin to form a strong complex between protein - oxidized polyphenol (tannin), leading to loss of solubility of gelatin in water [10].

With the above results, further studies were undertaken with the polyphenol content of 20 mg/g.

3.1.3. Effect of gelatin concentration on gelatine characteristics after modification

Table 2 summarizes the dependency of viscosity increase, gel strength cross-linking degree and solubility of gelatin films on gelatin concentration.

The viscosity increase, the degree of cross-linking and the gel strength gradually increased with increasing gelatin concentration and obtained the highest values of 177.7 %, 15.7 % and 116.4 g, respectively at a gelatin concentration of 20 % (Table 2). However, there were an opposite tendency to these values and an inability to gel when gelatin concentration exceeded 20 %. The enough amount of gelatin could make it easier to interact with polyphenols and to form cross-links between polypeptide chains [11]. However, at much higher concentrations of gelatin, the viscosity would increase, obstructing the gelatin interaction with other compounds and thus lessening the

cross-linking of gelatin molecules. These results are in agreement with those by Shantha Lakshmi Kosaraju *et al*. (2010) who used caffeic acid to modify gelatin solution of 10 ÷ 20 % [11]. Accordingly, the dried modified gelatin was water insoluble.

Table 2. Viscosity increase, cross-linking degree, gel strength and solubility of gelatin films as a function of gelatin concentration(*).

Gelatin concentration, % / Characteristics	10	15	20	25
Viscosity increase, *%*	89.6 ± 2.3^{d}	125.5 ± 2.4^{b}	177.7 ± 2.2^{a}	106.6 ± 2.1^{c}
Cross-linking degree, *%*	11.0 ± 0.8^{c}	13.6 ± 0.6^{b}	15.7 ± 0.7^{a}	15.2 ± 0.8^{a}
Gel strength, *g*	108.6 ± 1.5^{c}	112.9 ± 1.7^{b}	116.4 ± 1.9^{a}	precipitated
Solubility of gelatin films (**)	partially soluble	insoluble	insoluble	-

(*) All data are mean values ± SD. The same letter in each row indicates no significant difference in value, P>0.05.

To conclude, the appropriate parameters for modification of gelatin by polyphenols to improve viscosity, gel strength, and cross-linking degree were as follows: polyphenol content of 20 mg/g gelatin; gelatin concentration of 20 %; modifying time of 40 minutes and modifying temperature of 40 ^{o}C. Under the above conditions, the dried gelatin films were insoluble in water at room temperature or at 60 ^{o}C. It was predicted that the hydrophilicity of gelatin would be lost due to the losses of hydroxyl groups of polyphenols and amino groups of gelatin in the reactions of quinone-imine.

3.2. Characterization of ocean tuna skin gelatin modified by green tea polyphenols

3.2.1. Changes in gelatin molecular weight distribution

The SDS-PAGE electrophoretograms of unmodified and modified gelatin are shown in Figure 2.

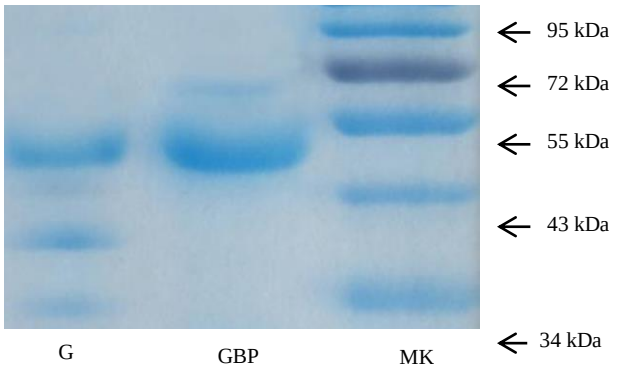

Figure 2. SDS PAGE electrophoretograms of gelatin. MK: molecular weight marker; GBP: modified gelatin; G: unmodified gelatin.

Unlike the natural gelatin sample (G) SDS-PAGE profiles of gelatin modified with polyphenols (GBP) appeared in several bands that indicated the molecular mass in the range of

55 ÷ 72 kDa; simultaneously, the low protein molecular weight of 34 ÷ 43 kDa was no longer present. It could be supposed that gelatin and green tea polyphenols might interact through weak bonds such as hydrogen bonds or hydrophobic interaction, resulting in an increase in gelatin molecular weight [12]. Low molecular weight of bands from natural gelatin samples also explained for its low Bloom value.

3.2.2. Changes in microstructure of gelatin film by scanning electron microscopy (SEM)

3D microstructures of gelatin film with or without modification with green tea polyphenols were examined by SEM (Figure 3).

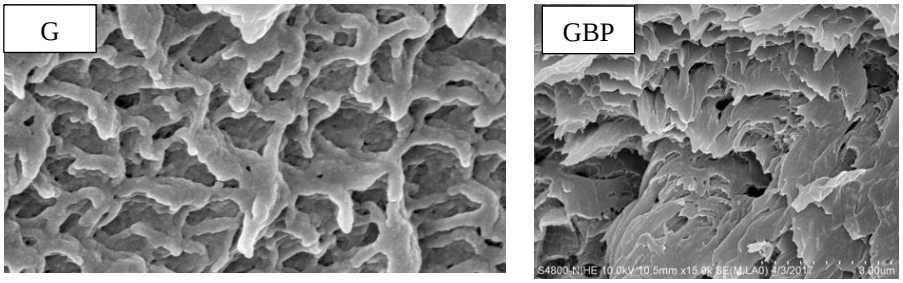

Figure3. Micrographs at 15000x magnification of unmodified gelatin (G) and modified gelatin (GBP).

Micrographs from Figure 3 show a clear difference between the structures of gelatin before and after modification. In the modified gelatin sample, there was no clear fibrous structure as compared to that in natural gelatin, but polyphenols covered the gelatin polypeptide strands to make them become smooth instead. The above results were consistent with the findings by Kaewdang Onouma *et al*. (2015) who reported that gelatin added with ethanolic extract from coconut husk exhibited a denser protein gel network and almost no void gelatin strands in comparison with natural gelatin [13].

3.2.3. Changes in structural characterization of gelatin by FTIR

FTIR spectra of gelatin with and without the modification with green tea polyphenols are illustrated in Figure 4.

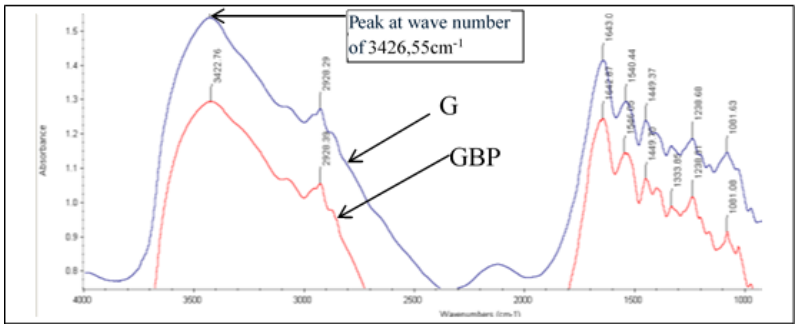

Figure 4. Fourier transform infrared (FTIR) spectra of unmodified gelatin (G) and modified gelatin (GBP).

Generally, slight changes in absorption band intensity were observed at each peak in FTIR

spectra of gelatin film incorporated with or without green tea extracted polyphenols. The addition of polyphenols did cause a marked decrease in the intensity of the amide B band, of which the absorption peaks were found at the wave numbers of 3422.76 cm^{-1} (GBP) and 3426.55 cm^{-1} (G). Compared to the natural gelatin film, green tea polyphenols incorporated gelatin films showed lower absorption band intensity and wave numbers in the amide B region, suggesting the interaction of the $-NH_3$ group between the peptide chains and the polyphenols.

4. CONCLUSION

Under certain conditions of modification of tuna skin gelatin by green tea polyphenols (temperature: 40 ^oC, period of time: 40 minutes, polyphenol content: 20 mg/g gelatin, gelatin concentration: 20 %), modified gelatin had significant changes in its mechanical properties and structure. In particular, there were increases in gel strength (from 102.8 g to 116.4 g), cross-linking degree (from 1.5 % to 15.7 %) and gelatin molecular weight (from 34 ÷ 55 kDa to 55 ÷ 72 kDa) as well as a denser gelatin film network with fewer voids. The modified gelatin film obtained after drying became insoluble in normal water and in hot water at 60 °C. The infrared spectrum of the gelatin after modification did not show the formation of new binding groups clearly but the only changes in infrared absorption intensity at the peak of the amide B region were found.

The remarkable effects of modifying gelatin with polyphenols extracted from green tea are the changes in solubility of gelatin films and possibly in other permeability of the gelatin films. The fact that modified gelatin films become more stable in a humid environment and have fewer void gelatin strands (as can be seen in SEM images) may lead to other potential applications for food packaging and preservation. Therefore, approaches to examine the water vapour permeability, oxygen permeability and antibacterial ability of modified gelatin films should be investigated together in future studies.

CRedit authorship contribution statement. Chau Thanh Hien: Experimentation, Writing – original draft; Dang Minh Nhat: Supervision, Writing – review editing; Mac Thi Ha Thanh: Formal analysis, Validation, Writing – review editing.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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