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Modification of Chemical and Functional Properties of Commercial Cold-Water Fish Gelatin by Oak Acorn (*Quercus. Castaneifolia*) Phenolic Extract

Seid Hamidreza Hashemi Kouchaksarayee, Hoda Shahiri Tabarestani*, Alireza Sadeghi Mahoonak, Mohammad Ghorbani, Mehran Alami

Faculty of Food Science, Gorgan University of Agricultural Sciences and Natural Resources, Golestan Province, Gorgan, Iran, Email: hoda.shahiri@gau.ac.ir

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ABSTRACT

Research Aim: Gelatin, a protein-based hydrocolloid widely used in food and drug industries, faces limitations when derived from cold-water fish species due to low gel strength and functional properties. In this regard, this study explores the potential of oak (Q.*castaneifolia*) phenolic extract as a natural modifier of commercial cold-water fish gelatin (CW-FG). The use of natural crosslinking additives especially oxidized forms of phenolic extract may be suggested as the modification strategy of gelatin hydrogel. Therefore, the objective of this study was to evaluate the potential of oak (Q.*castaneifolia*) extract as chemical and rheological modifier of commercial cold-water fish gelatin.

Materials and methods: The scope of the current work was to investigate the effects of different types of oak phenolic extract powder (oxidized (Ox-OPP) and unoxidized (OPP)), the concentration of oak phenolic extract powder (0.1 to 0.9 mg/g protein), and reaction pH values (7 and 9) on gel strength, crosslinking degree, color parameters, and swelling ratio of commercial cold-water fish gelatin. Further characterisation of the properties of different gelatin hydrogels was also conducted by their conformational changes using Fourier transform infrared (FTIR) analyses and scanning electron microscopy (SEM).

Findings: The Ox-OPP modified CW-FGs demonstrated higher gel strength, reduced swelling ratio, lower free amino group, and a more compact structure compared to the uncross-linked samples and those containing OPP at neutral pH. The addition of both types of oak acorn extracts at increasing pH levels led to a decrease in L*-value and an increase in a* and b* values. FTIR analysis revealed hydrogen bond formation as the primary molecular interaction, particularly in CW-FGs modified with Ox-OPP at pH 9. Additionally, free amino group could be controlled by adjusting the concentration of oak extracts. SEM analysis revealed that CW-FG gel containing Ox-OPP exhibited a non-uniform surface with varying pore sizes, demonstrating morphological differences from CW-FG gel containing OPP, particularly characterized by the creation of large empty spaces and fine filaments. These findings

collectively reveal the potential for improving CW-FG gel properties through the strategic use of oak acorn phenolic extracts at specific concentrations and pH levels, with Ox-OPP showing promising effects on the gel's chemical and functional characteristics.

Conclusion: Both oxidized and unoxidized oak phenolic extract showed high potential for modification of physico-chemical and functional properties of cold-water fish gelatin hydrogel. Also, crosslinking degree was controlled by concentration changes. Formation of higher molecular weight structures and hydrogen bonds between gelatin and phenolic compounds were determined as important factors for the observed results. These improved physicochemical properties of gelatin could lead to the development of products in the food industry that meet consumer demands.

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Introduction

Gelatin is widely used as a hydrocolloid in the food industry due to its properties related to gelling and surface behavior, flavor encapsulation, and edible film formation [1, 2]. This fibrous protein is a product of thermal, chemical and or physical denaturation of structural protein in connective tissues, most of which comes from the skin and bones of cows and pigs, commercially. However, for the reasons such as cultural considerations, halal/kosher food product consumer acceptance, and the concern of consumers for bovine spongiform encephalopathy (BSE), foot and mouth disease (FMD) and bird flu, marine source alternatives was introduced [3]. Furthermore, utilizing by-products of the aquatic processing industry for the production of gelatin as a natural food additive with high technological value is important for food waste management [3, 4]. However, it is important to note that fish gelatin gels (FG gel), especially those obtained from cold-water fish, have inferior gelling capacity, lower melting temperature, and reduced content of proline and hydroxyproline compared to mammalian gelatin gels [5]. Therefore, it is necessary to modify the structure of gelatin in order to improve its chemical and functional properties and increase its potential applications [6].

The simplest and most widely used methods to improve the properties of FG gel involve physical modifications such as adding electrolyte or non-electrolyte compounds, ultraviolet or gamma radiation, and high-pressure technology [7]. Researchers have also proposed protein polysaccharide complexation, crosslinking by aldehyde compounds, phosphorylation, enzymatic modification, and adding genipin, and natural plant compounds [3, 6]. Chemical cross-linking, which involves connecting polymer chains through covalent or non-covalent bonds to form a three-dimensional network, is one of the main methods for polymer modification. Cross-linking can occur intra- or inter-molecularly, and is primarily applied to proteins due to the multiplicity and variety of their functional groups [8]. Therefore, it is important to develop natural cross-linking agents that are reasonably economic, non-toxic, widely available, and easily accessible. In this regard, plant phenolic compounds are a good choice for natural cross-linking due to the abundance of their sources and easier extraction [9].

Phenolic compounds are a group of plant metabolites that possess one or more aromatic rings, as well as hydroxyl and carboxyl groups in their structure. These compounds have the ability to react with proteins both reversibly, through non-covalent forces, and irreversibly, through covalent bonds, under specific conditions [10, 11]. When polyphenols are oxidized, they can form covalent C-N bonds with the amino functional groups of proteins at alkaline pH, resulting in cross-linked networks [3]. The cross-linking between proteins and polyphenols can significantly affect the structure of protein, solubility, hydrophobicity, thermal stability, and isoelectric point. Additionally, the binding of phenolic compounds to proteins involves some amino acid residues and has important biological properties, including reducing digestibility and antioxidant capacity in certain cases [11-12].

Several studies have successfully explored interaction between gelatin and pure phenolic compounds such as ferulic acid [13], caffeic acid [14, 15], tannic acid [16, 17], gallic acid, and rutin [18] to improve the functional properties of fish gelatin gels through both non-covalent and covalent bonds. For instance, Yan et al. [18] reported that the addition of rutin (8 mg/g) significantly improved the viscoelastic modulus and thermal stability of Alaska Pollock (*Theragra chalcogramma*) gelatin gel and reduced its swelling ratio compared to gallic acid (30 mg/g). Moreover, Kosaraju et al. [14] found that increasing the pH and concentration of caffeic acid significantly improved the gel strength, gelation temperature, and melting point of type B gelatin. Studies by Zhang et al. [19] showed that crosslinking gelatin with tannic acid (3%, w/w) at pH = 8 improved the mechanical properties and resulted in a stable cross-linked structure under boiling conditions. Several studies have explored

the potential of utilizing plant waste extracts rich in phenolic compounds to develop an economical and highly functional modified gelatin. Kaynarca et al. [20] investigated the use of grape pomace, Kaewdang & Benjakul [17] used ethanolic coconut husk extract, Temdee & Benjakul [21] utilized oxidized extracts of kiam wood/cashew bark, and Zhao et al. [2] take advantage of galla chinensis extract as essential natural additives to enhance the functional properties and structural characteristics of sea bream (*Sparus aurata*), yellowfin tuna, cuttlefish skin gelatin, and bovine bone gelatin, respectively. The researchers mentioned that the formation of a higher molecular weight structure, cross-linking, and hydrogen bond formation were important factors contributing to the improvement in the physico-chemical properties of fish gelatin.

Among the various phenolic extracts reported in literature, no report has been available on the use of phenolic extract of oak acorn fruit, *Quercus* as a green modifier of gelatin gel. *Quercus* is the dominant genus of plants in various regions of the world with the applications in animal feed, leather industries, and as a part of human diet in the forms of bread, cake, preparation of dry-roasted acorn drinks, and functional foods [22]. The fruits of oak Quercus is rich in phenolic compounds such as tannin, gallic acid and tannic acid all with high antioxidant effects [23]. The difference between oxidized and unoxidized oak phenolic extracts is an essential aspect of research in this area. Oxidized extracts may have different chemical compositions and properties compared to their unoxidized counterparts. Researchers were exploring the effects of oxidation on the bioactivity and sensory properties of these extracts. The previous research for oak acorn extract gave justifications for further investigations on its applicability as natural additive in food systems. In this regard, the main objective of this study was the development of CW-FG gel incorporated with oxidized (OX-OPP) and unoxidized (OPP) phenolic extracts of acorn (*Quercus. Castaneifolia*) under neutral and alkaline pH at different concentration (0.1, 0.5, and 0.9 mg/g gelatin) which is believed that may lead to changes in gel strength, free amino group content, color indices, swelling ratio, structural features, and morphological properties.

Material and methods

Materials and chemicals

Iranian oak acorn fruit (Q. *castaneifolia*) was collected from Nahar Khoran forest park (Gorgan, Iran). The sound and ripe fruits without cupule were selected and dried in a Memmert ULE 500 drying oven at 50°C for 12 h. Next, seed coat (testa) and the pericarp (fruit wall) were removed and the cotyledons were powdered by a high-speed electric mill (ROM2500G, China) and passed through a laboratory sieve (mesh size = 40). Teleostean gelatin (gelatin from cold-water fish skin) and 2,4,6-trinitrobenzenesulfonic (TNBS) acid were obtained from Sigma (St. Louis, MO, USA). Folin-Ciocalteu reagent was supplied by Qiangsheng Medicine Science Technology Co., Ltd. (Solarbio, China). All the remaining materials used in this study were bought from Merck Co. (Merck KGaA, Darmstadt, Germany). The solvents and the reagents were of analytical grade and used without further purification. The moisture, ash, and protein content of gelatin were measured according to the AOAC standard methods No. 950.46, 942.05, and 992.15 [24], and the mean values were determined to be 6.41, 2.93, and 91.3 (%), respectively.

Preparation of oak acorn phenolic extract powder (OPP)

Oak acorn phenolic extract powder was prepared by mixing oak cotyledon flour with 70% ethanol at a 1:15 ratio (w/v) and dispersing (IKA Ultra-Turrax T25 Homogenizer) for 2 minutes at 10000 rpm. The resulting homogenized mixture was then stirred for 12 hours at room temperature (25-27°C) using IKA™ RH basic 2 magnetic stirrer. Next, the mixture was centrifuged (Hettich EBA 200, Germany) at 5000 g for 10 minutes at room

temperature. The supernatant was filtered (whatman NO.1 filter paper), evaporated (IKA RV05 rotavapor) below 40°C and lyophilized (Laboratory freeze dryer VaCo 2 / Zirbus, Germany). The crude extract powder separated from oak acorn was referred to as OPP.

Preparation of oxidized oak acorn phenolic extract powder (Ox-OPP)

The oak acorn phenolic extract were oxidized using the method of Strauss and Gibson [25] with some modifications. A 0.05% (w/v) solution of OPP was prepared and the pH was adjusted to 7 and 9 using 6M NaOH or 6M HCl. The solutions were then placed in a water bath at 40°C and continuously oxygenated for 30 minutes by exposing them to a constant oxygen flow rate of 180 m^3/s to convert the phenolic compounds into quinone. This solution was dried by a freeze dryer, stored at 4°C, and labelled as Ox-OPP.

Determination of total phenolic content

The total phenolic content of the OPP and Ox-OPP were assessed using the Folin-Ciocalteu reagent, following the method described by Singleton et al.[26]. OPP and Ox-OPP were dissolved in double-distilled water (DDW) to create a concentration of 0.5 mg/ml. Aliquots of 100 ml from each sample were mixed with 2.0 ml of a 2% sodium carbonate solution. Subsequently, 100 µl of the Folin–Ciocalteau reagent (diluted 1:1 with water) were added to the mixture, which was then vortexed for 30 seconds. After allowing the mixture to stand for 30 minutes, its absorbance was measured at 760 nm using a T92+ UV-VIS Spectrophotometer (PG Instruments Limited, England). Tannic acid and Gallic acid were used as standards and the results are referred as mg gallic acid (GA)/tannic acid (TA) equivalent per gram dry matter. Total phenolic contents in OPP and Ox-OPP were found to be 408 mg of TAE/ gr and 138 mg GAE/ g, while those in OPP were determined to be 242 mg TAE/ g and 62 mg GAE/g, respectively.

Preparation of commercial cold-water fish gelatin gels without/with added OPP/Ox-OPP

To prepare commercial cold-water fish gelatin (CW-FG) gels with or without added OPP or Ox-OPP, the process involved first mixing CW-FG with distilled water (6.67% w/v) for 30 minutes until complete dissolution at 25°C using a magnetic stirrer, resulting in a hydrogel forming solution (HFS). Next, OPP and/or Ox-OPP freez-dried powder were added to the HFS and stirred for an additional 30 minutes at 45°C to obtain a homogenous hydrogel at concentrations of 0.1, 0.5, and 0.9 mg/g gelatin. The pH of the mixture was then adjusted to either neutral (pH 7) or alkaline (pH 9) conditions using 1 M HCl or 1 M NaOH, respectively. The resulting gelatin composites were named P7-C0.1-OPP, P9-C0.1-OPP, P7-C0.1-Ox, P9-C0.1-Ox, P7-C0.5-OPP, P9-C0.5-OPP, P7-C0.5-Ox, P9-C0.5-Ox, P7-C0.9-OPP, P9-C0.9-OPP, P7-C0.9-Ox, and P9-C0.9-Ox. The HFS without OPP was prepared in the same way and referred to as P7-C0 and P9-C0. The resulting mixture was then cooled to room temperature, conditioned at 4°C for 18 hours to form hydrogels, and subsequently freeze-dried. The resulting lyophilized samples were used in the subsequent tests.

Gel Strength Measurements

The gel strength was determined using a 6.67% (w/v) CW-FG gels, both with and without added OPP/Ox-OPP, prepared by dissolving dry gelatin powder in distilled water at 60°C. The solution was then cooled in a refrigerator to 7°C and allowed to mature at this refrigerated temperature for 16–18 hours according to the British Standard Institution method [27]. A Texture Analyzer (Koopa TA10Kg, Iran) equipped with a 12.7 mm diameter cylindrical probe was used for the measurements. The cross-head speed was set to 1 mm/s, and a load cell of 5 kN was employed. Gel strength was measured as the maximum force (in grams) required the cylindrical probe to penetrate 4 mm into the gelatin gel, which were set in bloom jar and was reported as the Bloom value.

Free amino groups

The amount of free amino groups of CW-FG gels was determined following the method outlined by Zhao et al. (2016). Briefly, 10 mg of each FG gel was immersed in a solution consisting of 1 mL of sodium bicarbonate buffer (4% w/v) and 1 mL of freshly prepared TNBS (2,4,6-trinitrobenzene sulfonic acid) solution (0.5% v/v). The mixture was then incubated at 40°C for 4 hours in the dark. Subsequently, 3 mL of 6 N HCl was added to each sample, and the mixture was heated for 30 minutes in a water bath at 60°C. Afterward, the final solution was diluted 2-fold with ultrapure water, and the absorbance value was measured using a UV/Vis spectrophotometer (T92+ PG Instruments Limited) at 346 nm, with the control sample as a reference (assuming it contains 100% of free amine groups). To estimate the amount of free amino groups, a calibration curve was generated using an L-Lysine solution, and the calculation was performed using Equation (1) as provided in the reference [2].

(1)

$$\frac{\text{moles } \varepsilon - \text{amino groups}}{\text{g gelatin}} = \frac{2 \times Abs \times 0.02L}{1.46 \times 10^4 \times b \times x}$$

where the molar absorption of Trinitrophenyl (TNP-Lys) is equal to 1.46×10^4 (liter/mol × cm), b is the length of cell path (cm), and x is the weight of the sample (g).

Swelling ratio

The swelling ratio was determined by the method of Strauss & Gibson [25]. For this purpose, 0.5 g of different gelatin samples of 6.67% (w/v) were transferred to a 50 ml container and dried in a vacuum oven at 40°C. Similarly, the aqueous suspensions of cross-linked samples were immersed in isopropanol and dried in a vacuum oven at 40°C. 20 ml of 0.05 M phosphate buffer at pH=7 was added to the dried samples and allowed to reach equilibrium for 4 hours. Then, the swollen samples were dried by filter paper and finally weighed. The weight of the swollen samples to the initial dry weight was used to calculate the swelling ratio.

Determination of color

The color coordinates of L* (lightness index), a* (red-green spectra), and b* (yellow-blue spectra) were determined in terms of the CIE scale using a handheld colorimeter (CR-400, Konica Minolta Inc., Osaka, Japan).

Fourier-transform infrared spectroscopy (FTIR)

The infrared spectra of the CW-FG gel lyophilized powders were measured using a Cary 630 FTIR Spectrometer (Agilent, USA). The spectra of all samples were recorded in the wavenumber range 4000–600 cm^{-1}, with a resolution equal to 2 cm^{-1}. The peaks of amide I, amide II, amide A, and phenol compounds were identified by Agilent MicroLab Expert software and assigned according to values found in the literature.

Scanning electron microscopy (SEM) Images

The CW-FG gel solutions with/without OPP and Ox-OPP additions were poured into a thin plate, left to gelation and lyophilized. The small piece (2 mm × 2 mm) of the material was cut from the middle of the sample and mounted on an aluminium base (holder) by silver glue and covered with a thin layer of gold for better conductivity. The microstructures were observed using a SEM Tescan, Vegall, Germany at an acceleration voltage of 20 kV.

Statistical analysis

Experimental data were analyzed and presented as mean values and standard deviations of triplicate data. To assess the effects of oak acorn extract powder types (OPP/Ox-OPP), their concentrations, and pH values on the physical-chemical properties of CW-FG gels, a factorial experiment was performed. Results were subjected to analysis of variance (ANOVA), and post hoc comparison of the means was performed by Duncan's multi-

range at $p < 0.05$, using SPSS 22. Figures were generated using Excel 2013.

Result and Discussion

The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on gel strength

The gel strength, as measured by the bloom value, is a crucial quality criterion for gelatin that ultimately determines its commercial value [28] and is classified into low (<125 g), moderate (150-200 g), and high (>200 g) categories. The gelatines with higher bloom value have a wider range of applications [29-30]. Fig 1 provides some important data with respect to the effects of pH, oak acorn phenolic types, and concentration on gel strength. The most striking feature of the chart is that oak acorn phenolic extract (OPP: 0.9 mg/g protein) showed 39.4% (pH=7) and 54.3% (pH=9) increase in the bloom values of CW-FG gels ($p<0.05$). Moreover, higher bloom values of commercial gelatin with 102% (pH=7) and 134% (pH=9) increase was observed using Ox-OPP (0.9 mg/g protein), compared to the control sample ($p<0.05$). Similarly, Temdee et al. [31] found that gelatin gel with oxidized ethanolic extract of kiam wood (EKW) or cashew tree trunk (ECB) at pH=9 exhibited significantly higher gel strength than the control. Also, Erge and Eren [32] observed a 62.8% increase in the gel strength of chicken gelatin with the addition of 2.5% oxidized caffeine acid. On the other hand, the results showed that an increase in the amount of OPP and Ox-OPP (from 0.1 to 0.9 mg/g gelatin) increased the bloom value of the CW-FG gel ($p<0.05$). The specific impact of phenolic extracts is additional support to the gel matrix and formation of more extensive and denser gel, which can result in an increased bloom value. However, when the concentration of polyphenols exceeds 0.9 mg/g gelatin, gel strength reduced (data not shown). The reduction of gelatin mechanical properties may be due to precipitation of polyphenols through interaction with gelatin molecules, prevention of the formation of cross-links between chains due to the presence of numerous hydrogen bonds in a chain [9], or incomplete reactivity of phenolic compounds and gelatin [2]. Nevertheless, the impact of incorporating phenolic extracts on gelatin bloom value exhibited source- and concentration-dependent characteristics. For instance, studies have shown that the critical concentration threshold of coconut ethanolic extract for enhancing Yellowfin tuna gelatin gel strength is reported to be 0.5 mg/g [17]. Moreover, a concentration of 0.111 M caffeic acid per gram of hoki skin gelatin was used in the study by Mohtar et al. [15]. Additionally, Yasin et al. [33] employed 2 ml of aqueous extract from lemon grass stem and basil leaf per 100 g of chicken feet gelatin in their research.

According to the results, oak acorn phenolic extract oxidation has a more pronounced effect on increasing the gel strength of CW-FG at pH 9 and higher concentrations, compared to pH 7 (Fig 1). Similarly, Temdee et al. [31] reported that the highest gel strength of cuttlefish skin gelatin was achieved in the presence of oxidized catechin and gallic acid at pH 9. Also, Kosaraju et al. [14] observed that increasing the concentration of caffeic acid at high pH levels enhances the gel strength, gelation temperature, and melting point of type B gelatin. Cross-linking gelatin with tannic acid at pH 8 was also shown to be effective in enhancing the mechanical properties of gelatin [19].

As shown in Fig 1, P9C0.9OX had the highest significant increase in bloom value (205.97 g). The observed difference could be explained by the elevated levels of phenolic compounds, specifically tannic acid (408 mg/g) and gallic acid (138 mg/g), found in the OX-OPP, which were notably higher compared to the OPP (242 mg/g and 62 mg/g, respectively). The abundance of phenolic compounds is believed to have contributed to an augmented formation of quinones during oxidation under alkaline conditions. Quinones are known for their electrophilic nature, making them susceptible to reacting with amino nucleophilic groups present in proteins [34, 31, 21]. Furthermore, they have the capacity to create dimers through the formation of C-N or C-S bonds with

phenolic rings, linking sulfhydryl or amino side chains of peptides [21, 31].

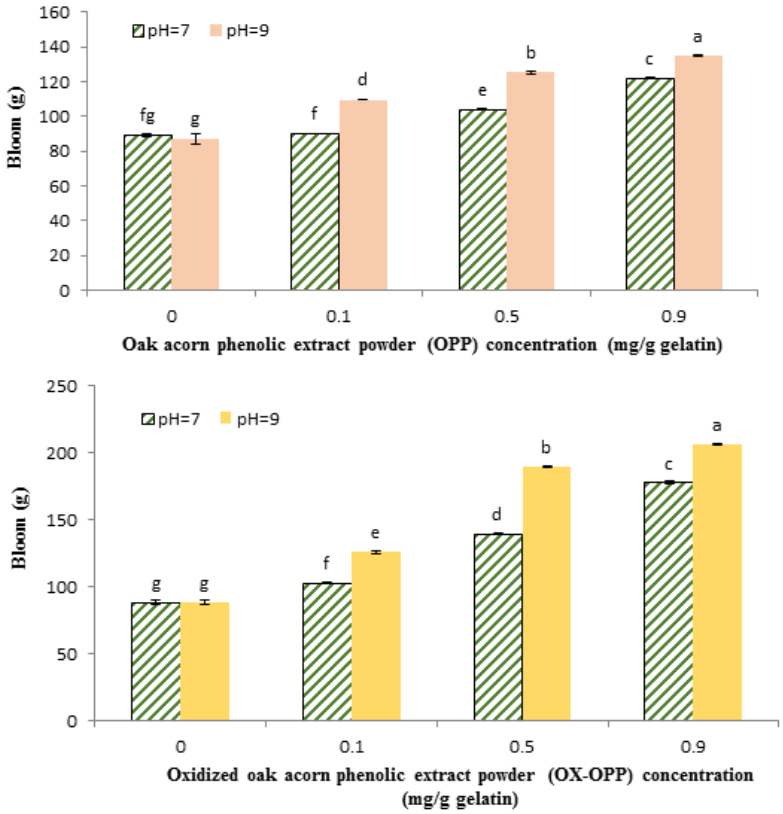

Fig 1. The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on CW-FG gel strength (bloom value).

The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on free amino groups

Fig 2 depicts the variation in the number of free amino groups (moles per gram of gelatin samples). It can be observed that as the pH and concentration of both OPP and Ox-OPP increase, the quantity of free amino groups decreases. The CW-FG sample (control) displayed the highest level of free amino groups, measuring 24 × 10^{-5} mol ε-free amino/ g gelatin ($p<0.05$). Subsequently, in Fig 2, the samples P7-C0.1-NOX, P9-C0.1-NOX, and P7-C0.1-OX exhibited varying significant ranges of free amino groups (ranging from 14.24 to 19.31 × 10^{-5} mol ε-free amino/ g gelatin). Notably, there was no significant difference in the amount of free amino groups between the samples P7-C0.5-NOX, P9-C0.5-NOX, and P9-C0.1-OX ($p>0.05$). According to the study conducted by Temdee et al. [31], the amount of free amino groups in cuttlefish gelatin gel (control) was reported to be nearly 2-fold higher than gelatin containing oxidized gallic acid and catechin. In another study, it was demonstrated that modified cuttlefish gelatin with both oxidized and non-oxide tannic acid had lower amounts of free amino groups compared to the control sample[16]. The reduction in free amino groups in the gelatin gel depends on the type of phenolic compounds used and their capacity to bind to more hydroxyl groups attached to the benzene ring [35].

The gelatin samples with the highest level of Ox-OPP and alkaline pH (P9C0.9OX) exhibited the lowest number of free amino groups, measuring 3.69 × 10^{-5} mol ε-free amino/ g gelatin (Fig 2). These findings align with the gel strength of the CW-FG gels (Fig 1), showing a simultaneous decrease in free amino groups and so an increase in crosslinking degree.

In general, the decrease in free amino groups signifies enhanced interactions between oxidized compounds and protein side chains, resulting in an overall increase in crosslinking. The presence of free amino groups makes them susceptible to reacting with quinones formed during oxidation, particularly under alkaline conditions. As these reactions occur, the gelatin's crosslinking degree is notably augmented, contributing to its overall structural and functional changes [21]. Moreover, under alkaline pH conditions, the oxidation of hydroxyl groups leads to the formation of free hydroxyl radicals, which can potentially react with the benzene ring and initiate polymerization [36]. The increase in the amount of oak acorn phenolic extract under various conditions corresponds to a higher degree of CW-FG gel crosslinking. It has been observed that the degree of crosslinking can be adjusted by modifying the concentration of these compounds. For instance, Zhao et al. [2] reported that the addition of 1 to 3% galla chinensis extract to gelatin gel resulted in a crosslinking increase ranging from 56.8% to 78.3%.

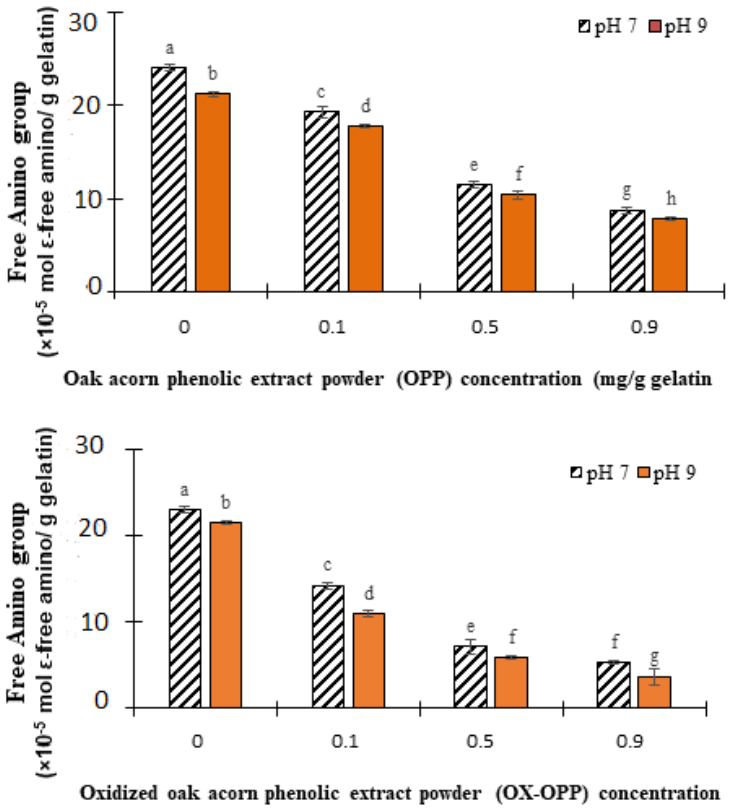

Fig 2. The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on free amino group content of CW-FG gel.

The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on the swelling ratio

The swelling ratio serves as a widely used quality control indicator to assess the water absorption properties and stability of biomaterials [33]. As depicted in Fig 3, the control samples exhibited a swelling ratio of 240%, which significantly decreased to 165.5% (with P9C0.9NOX) and 128.3% (with P9C0.9OX) in the final crosslinked gels ($p<0.05$). Furthermore, an increase in OPP/Ox-OPP concentration resulted in a significant reduction in the swelling ratio of gelatin (Fig 3). These findings highlight that both pH and concentration play crucial roles in altering the swelling ratio of gelatin ($p<0.05$). Similarly, Strauss and Gibson [25] demonstrated that the swelling ratio of cross-linked gelatin reduced with an

increase in the molar ratio of caffeic acid and grape extract. In other studies, Yasin et al. [33] reported a 5.04% and 4.6% reduction in the swelling ratio of chicken feet gelatin gel by adding aqueous extracts of basil leaves and seaweed stems (2 mL/100 g). Additionally, Zhao & Sun [9] observed a significant reduction in the swelling ratio for gelatin gel containing genipin (1690%) and polyphenol (1420%) compared to the control sample (1810%). Bastide et al. [37] found that the presence of a small amount of cross-linking agent in the polymer solution hindered solvent penetration into the polymer, and the swelling ratio was directly influenced by the level of cross-linking and the polymer-solvent compatibility.

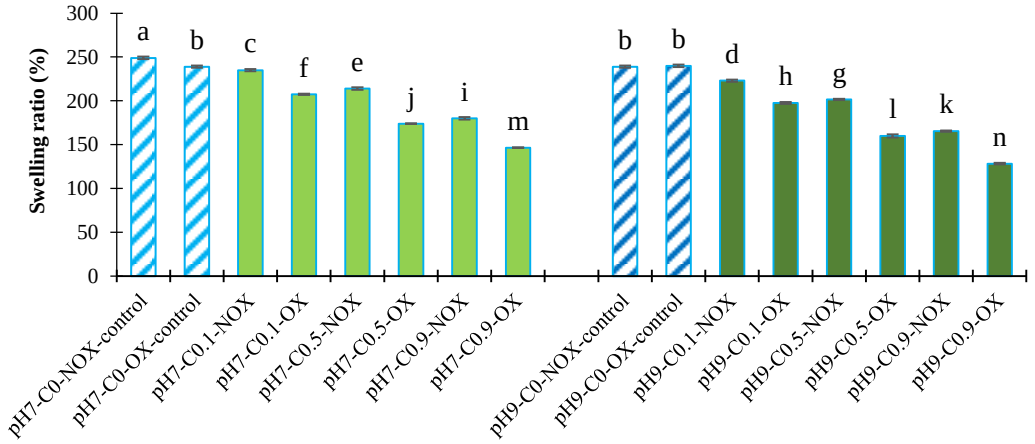

Fig 3. The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on the swelling ratio of CW-FG gel.

The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on color properties

Table 1 presents the color analysis of both the control and gelatin samples containing OPP/Ox-OPP. The impact of the cross-linking process on the color of CW-FG gel was evident, as all Ox-OPP modified CW-FG gels exhibited lower L* values within the range of 25.17-39.09 compared to the control (L*=51) ($p<0.05$). The L* value decreased with increasing pH and concentration of both types of oak acorn extract (Table 1). Notably, the lowest lightness value (25.17) was observed in gelatin samples containing P9C0.9OX (Table 1). This reduction in the L* value with increasing levels of Ox-OPP can be attributed to the oxidation process, leading to alterations in pigmentation and turbidity [21]. The use of oxidized extracts results in reduced light transmission and consequently a decrease in the L* value [31].

The cross-linking process, under alkaline conditions, leads to the oxidation of polyphenols and the formation of quinones in the presence of oxygen and amino groups of peptides, resulting in a brown color [38]. Similarly, Erge and Eren [32] demonstrated that increasing the concentration of caffeic acid and the oxidation period resulted in a decrease in the L* value of modified chicken gelatin. Hanani et al. [39] also observed a similar effect on gelatin films containing phenol-rich extracts from pomegranate skin. Balange and Benjakul [40] found that the lightness of mackerel surimi gels cross-linked with tannic acid, catechin, and caffeic acid decreased with an increase in the concentration of oxidized phenolic compounds. Furthermore, Casas-Forero et al. [41] reported an increase in the turbidity of gelatin with the addition of concentrated blueberry extract.

Based on the findings presented in Table 1, the negative a* values (-3.25) for the control, P7C0.1NOX (-2.12), and

P9C0.1NOX (-0.37) indicate a tendency towards a green color, whereas positive a* values represent a reddish hue. Furthermore, oxidation at alkaline pH and higher OPP concentrations showed a significant increase in a* values (Table 1), with the highest value observed in P9C0.9OX. As for the b* values, the highest values were observed in samples containing P9C0.9OX and P7C0.9OX, respectively (Table 1). These parameters were similar in all Ox-OPP added CW-FG gel ($p>0.05$) and significantly higher than the control ($p<0.05$). Similarly, Erge and Eren [32] demonstrated that modifying chicken gelatin with increasing concentrations of caffeic acid and the oxidation period had significant effects on the redness and yellowness indices. Song et al. [42] reported similar results, showing an increase in the redness and yellowness indices in gelatin gels by adding 1000 mg/L of blueberry leaf extract. The color parameters are influenced by the type and concentration of phenolic compounds, protein source, and pH [43]. Importantly, it is worth mentioning that in the present study, the use of both types of oak acorn extract have significant effects on the natural color of commercial cold-water fish gelatin ($p<0.05$).

Table 1. The effect of oak acorn phenolic extracts types (OPP, Ox-OPP), concentration, and pH on the color indices of CW-FG gel.

pH	Concentration (mg/g gelatin)	Oak acorn extract type	L*	a*	b*
7	0.1	OPP	51.3±4 ^{a}	-2.12±0.7 ^{f}	18.7±2.1^{d}
		OX-OPP	46±4.6^{b}	1.27±0.96^{d}	15.1±2.6^{e}
	0.5	OPP	49.1±0.1^{a}	0.57±0.01^{e}	29.7±0.01^{c}
		OX-OPP	41.2±4.8^{c}	3.87±1.03^{ab}	39.8±4.3^{a}
	0.9	OPP	49.3±0.5^{a}	3.01±0.5^{c}	34.5±2.5^{b}
		OX-OPP	40.4±3.2^{c}	4.56±0.9^{b}	37.6±2^{a}
9	0.1	OPP	47.3±4.6^{b}	-0.37±0.01^{f}	30.9±3.4^{c}
		OX-OPP	39.1±3.6^{c}	2.84±0.02^{c}	38.7±3.7^{a}
	0.5	OPP	41.3±1.6^{c}	0.57±0.01^{e}	29.7±0.01^{c}
		OX-OPP	36.1±4.1^{d}	4.3±1.67^{b}	37.6±3.2^{a}
	0.9	OPP	35.3±2.6^{d}	2.89±0.2 ^{c}	35.4±1.2^{b}
		OX-OPP	25.2±4.6^{e}	5.43±1.7^{a}	38.6±3^{a}
Control			51±2.3	-3.25±0.5	18.25±3.1

FTIR Spectral Analysis

The major peaks observed in cold-water fish gelatin, along with the corresponding wavenumber positions based on infrared spectroscopy (Fig 4), are as follows: (1) Amide A at 3279 cm^{-1}, indicating the stretching vibration of NH or hydrogen bonding attached to NH, (2) Amide B at 2936 cm^{-1}, (3) Amide I at 1637 cm^{-1}, signifying the stretching vibration of C=O or hydrogen bonding attached to COO, (4) Amide II at 1508 cm^{-1}, representing the bending vibrations of NH groups and the stretching vibrations of C-N groups, and (5) Amide III at approximately 1234 cm^{-1}, suggesting the vibrations of the C-N plane and the N-H groups of the amide bonds or the vibrations of the C-H2 groups of glycine and the side chain of proline [44]. Furthermore, FTIR was employed to investigate the structural features of CW-FG gel containing OPP/Ox-OPP at neutral and alkaline pH. As shown in Fig 4, the band at 3279 cm^{-1} in the control CW-FG gel shifted to lower frequencies for all oak acorn extract modified CW-FG gels. Additionally, P9-C0.9-OX appeared to have a broader peak in the range of amide A compared to the CW-FG gel (Fig 4). The partial elimination of the peak and the decrease in the wavenumber of the amide A indicate the involvement of peptide NH

groups in hydrogen bonding [45]. This observation may indicate the presence of sufficient amounts of C=O and OH in the acorn extract to form both intramolecular and intermolecular hydrogen bonds, as evident from the stretching vibrations of the hydroxyl group at 3298 cm^{-1} (for OX-OPP) and 3301 cm^{-1} (for OPP), and the C=O stretching at 1636 cm^{-1}, which appeared only in Ox-OPP (Fig 4). The intensification of the amide I peak in oak acorn extract modified CW-FG gels might be attributed to the hydrogen interactions between the C=O group of gelatin and the OH of phenolic extracts. However, no change was observed in the Amide B absorption band, indicating the absence of hydrophobic interactions between the methylene groups of the oak acorn extract and the aliphatic chains of gelatin. Moreover, all other peaks and spectra followed an almost similar pattern. Therefore, it was found that hydrogen bonds play a dominant role in the stabilization of oak acorn extract modified CW-FG gel.

According to Manea-Saghin et al. [46], the ratio of $A_{1630}/A_{3300} = \frac{A_I}{A_{OH}}$ is used as an indicator for confirming cross-linking. In this regard, the highest value (3.3) was determined for CW-FG gel modified by P9-C0.9-OX, followed by P9-C0.9-NOX, P7-Co.9-OX, P7-C0.9NOX in the range of 2.5-3, and the lowset ratio of 1.58 for unmodified sample. This behavior is in accordance with the results of free amino group and gel strength, as previously reported in sections 3.1 and 3.2.

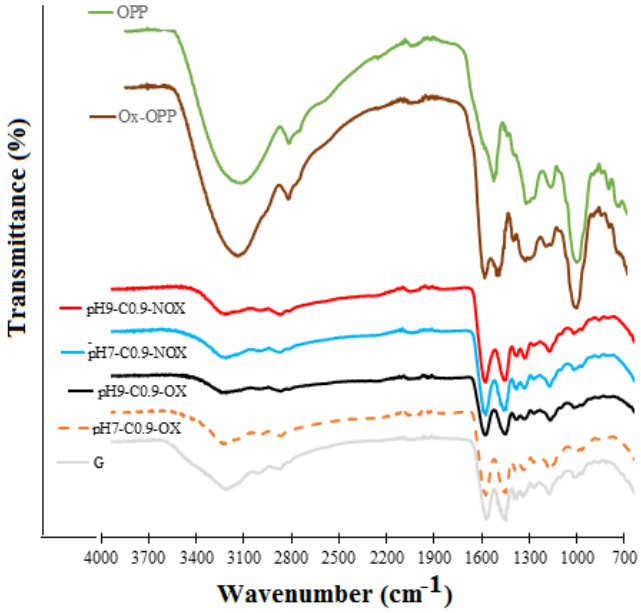

Fig 4. FTIR spectra comparison of CW-F gelatin (G), oak acorn extracts (OPP (NOX) and Ox-OPP), and CW-FG gels modified with OPP and Ox-OPP at 0.9 mg/g (pH 7 and 9).

SEM Analysis

The microstructure of CW-FG gel and OPP/Ox-OPP modified CW-FG gels is depicted through SEM images (Fig 5), with a focus on pore size and uniformity. As shown, the microstructure of CW-FG gel and P7-C0.9-OPP modified CW-FG gel displayed a non-uniform surface with pores of varying sizes, while P9-C0.9-OPP exhibited large empty spaces and fine filaments (Fig 5). Bertolo et al. [47] also observed a similar increase in pore size with the addition of phenolic compounds to gelatin-chitosan gel.

On the other hand, the addition of Ox-OPP affected the pore size differently. At neutral pH, the number and size of pores reduced (Fig 5), and in P9C0.9OX, a

compact, homogeneous, and regular structure, in accordance with its highest gel strength (section 3.1), was observed. Similarly, Erge and Eren [32] reported that chicken gelatin with the highest gel strength exhibited a tighter network with denser aggregates and irregular pores. In general, the gel strength of gelatin is directly related to the regular arrangement and connection of protein chains in the gel matrix [5]. Dense chains and finer structures are usually directly correlated with higher gel strength, higher melting points, and longer peptide chains [48].

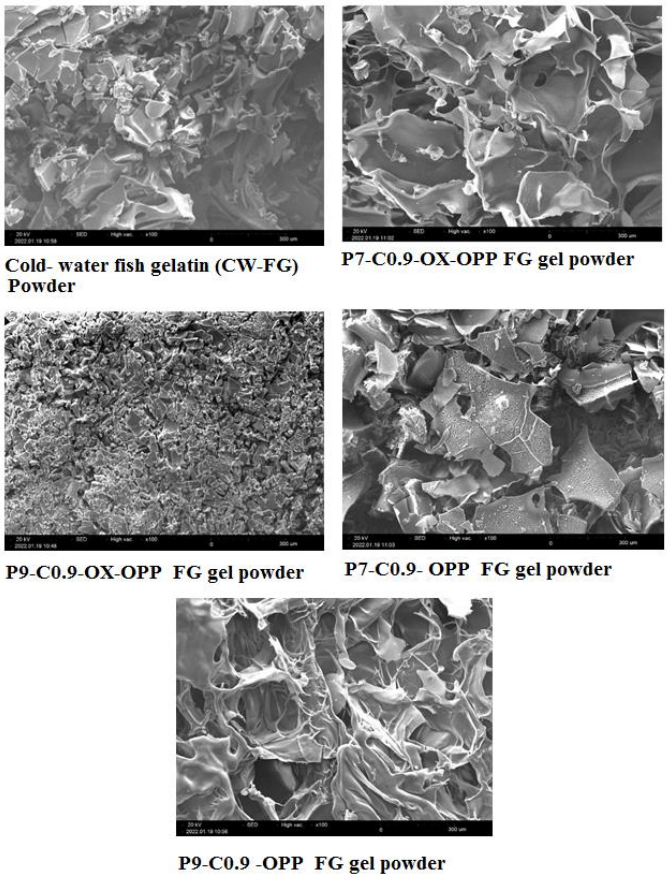

Fig 5. Scanning electron microscopy (SEM) of CW-FG and oak acorn extracts modified CW-FG gel.

Conclusion

This study investigated the effects of different pH levels and oxidized/non-oxidized oak acorn phenolic extracts at various concentrations on enhancing the chemical and functional properties of commercial CW-FG gel. The concurrent increase in Ox-OPP concentration and alkaline pH resulted in an elevation of both gelatin bloom and a* value, along with a reduction in swelling ratio, free amino groups, and L* value. Among the samples, P9-C0.9-Ox-OPP demonstrated the lowest free amino group and bloom value, followed by P9-C0.9-OPP, P7-C0.9-Ox-OPP, and P7-C0.9-OPP, in line with the FTIR results confirming the formation of hydrogen bonds in oak acorn extract-modified CW-FG gels, with the highest Amide 1630/Amide 3300 ratio observed for P9-C0.9-Ox-OPP, indicating crosslinking formation. SEM analysis revealed that CW-FG gel containing Ox-OPP exhibited a non-uniform surface with varying pore sizes, exhibiting morphological differences from CW-FG gel containing OPP, particularly characterized by the creation of large empty spaces and fine filaments. These findings collectively demonstrate the potential for improving CW-FG gel properties through the strategic use of oak acorn phenolic extracts at specific concentrations and pH

levels, with Ox-OPP showing promising effects on the gel's chemical and functional properties.

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