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Research Article

Enhancement of Hydrophobicity of Fish Skin Gelatin via Molecular Modification with Oxidized Linoleic Acid

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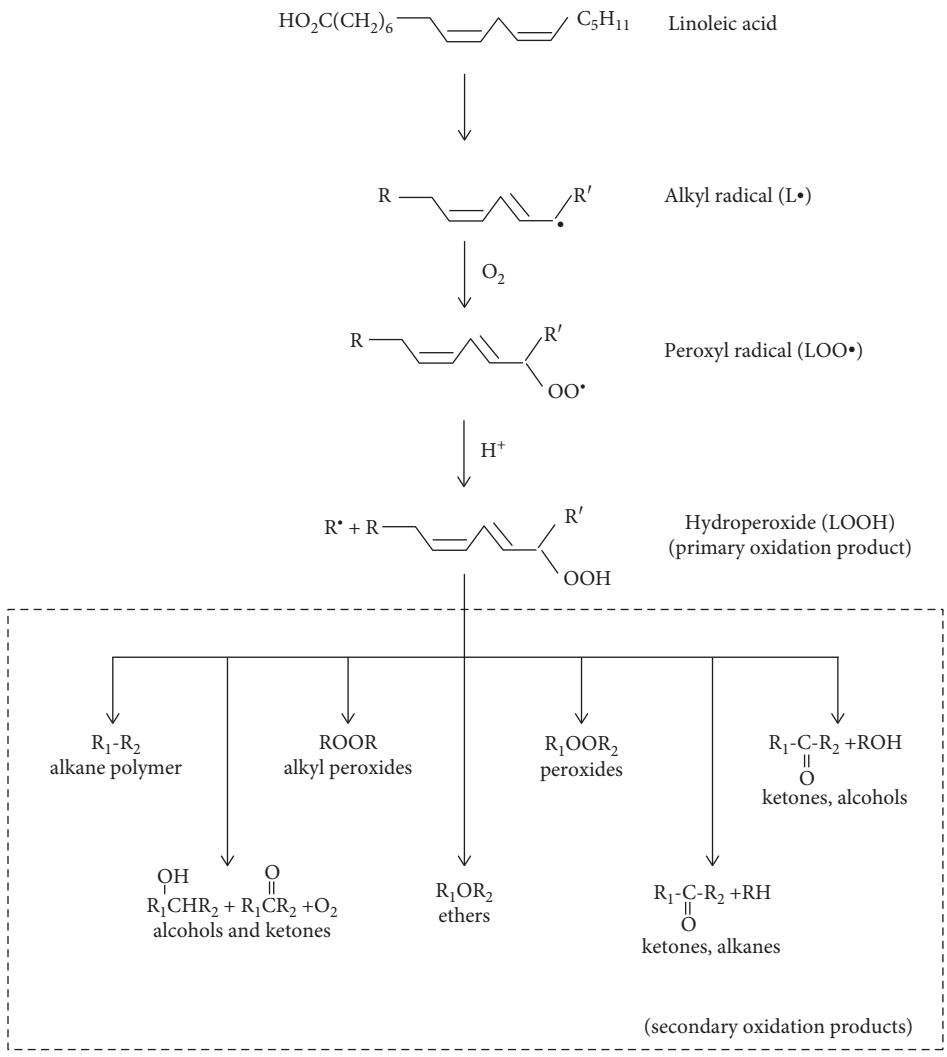
Fish gelatin possesses hydrophilicity in nature since it contains a few hydrophobic amino acids and a large portion of hydrophilic amino acids. Low hydrophobicity of fish gelatin results in poor water vapor barrier and water resistance of gelatin films. This can limit its usage as packaging material. To overcome this drawback, the molecular attachment of hydrophobic domains can effectively improve the hydrophobicity of fish gelatin. In this study, fish skin gelatin modified with oxidized linoleic acid (OLA) prepared under various conditions using different molar ratios of OLA/free amino group content was characterized. When OLA was prepared at 60 and 80°C for various oxidation times (0–24 h), the peroxide value (PV) increased continuously up to 9 and 12 h when reaction was performed at 80 and 60°C, respectively. Consequently, the PV decreased until the end of the reaction (24 h). Thiobarbituric acid reactive substances (TBARS) of OLA were increased sharply up to 12 h, regardless of reaction temperatures. Thus, primary and secondary lipid oxidation products mainly occurred within the first 12 h. As gelatin was modified with different OLA at various OLA/free amino group molar ratios, the one modified with OLA prepared at 60°C for 24 h at a molar ratio of 10 : 1 had the highest increases in surface hydrophobicity and carbonyl content with the coincidentally lowest free amino group content, compared with control gelatin (without OLA modification). Fourier transforms infrared (FTIR) spectra also reconfirmed the presence of fatty acid covalently attached to resulting gelatin. Therefore, OLA could be used to modify gelatin and increase its hydrophobicity.

1. Introduction

Gelatin is obtained by thermal denaturation or partial hydrolysis of collagen from the skin and bone. Nowadays, fish gelatin has gained increasing attention as the material for pharmaceutical, medical, and food industries [1]. Fish skin gelatin has several advantages, e.g., the lack of outbreaks of bovine spongiform encephalopathy (BSE) and religious constraint (Judaism and Islam) [2]. Nevertheless, gelatin contains a large portion of hydrophilic amino acids (lysine, serine, arginine, hydroxyproline, and aspartic and glutamic acids) with a low content of hydrophobic amino acids (proline and leucine) [3]. As a consequence, films or packaging based on gelatin exhibits poor water vapor barrier property, thereby limiting their applications [4].

Hydrophobicity of gelatin can be enhanced by molecular incorporation of hydrophobic substances such as fatty acids into the gelatin molecules [5]. Attachment of fatty acid into protein including gelatin could improve the surface hydrophobicity, affecting solubility and hydrophilic/hydrophobic nature of the resulting modified gelatin. The covalent interaction between lipid and protein could be induced by using the oxidized lipids [6]. Oxidation of polyunsaturated fatty acid such as linoleic acid leads to the formation of radicals, lipid hydroperoxides, and various secondary lipid oxidation products, especially reactive aldehydes and ketones including malondialdehyde, 4-hydroxynonenal, and 4-hydroxyhexenal (Scheme 1) [7, 8]. These oxidation products could covalently modify side chains and the polypeptide backbone of protein molecules [6, 7]. The reaction between free amino groups of proteins



Scheme 1: Oxidation of linoleic acid (LA) into primary and secondary oxidation products [8].

and oxidized fatty acids has been successfully used as an effective means to improve surface hydrophobicity of protein [9]. Fatty acids oxidized under various conditions might modify the gelatin in different fashions. This might be associated with different types or amounts of reactive lipid oxidation products. However, a little information on the impact of oxidized linoleic acid (OLA) prepared with different conditions on fish gelatin exists. The aim of this study was to improve surface hydrophobicity of fish gelatin via molecular modification with oxidized linoleic acid (OLA) under various conditions.

2. Materials and Methods

2.1. Fish Gelatin and Chemicals. Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy). Linoleic acid, cumene hydroperoxide, 1-anilinonaphthalene-8-sulfonic acid (ANS), sodium dodecyl sulfate (SDS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), sodium sulfite, L-leucine, and malonaldehyde (MDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, trichloroacetic acid (TCA), butanol, hydrochloric acid, barium chloride ($BaCl_2$), and ferrous sulfate ($FeSO_4$) were procured from Merck (Darmstadt, Germany). Ammonium thiocyanate (NH_4SCN), ethanol, chloroform, and 2-propanol were obtained from Lab-Scan (Bangkok, Thailand). 2-dinitrophenylhydrazine (DNPH) was purchased from Wako Pure Chemical Industries Ltd. (Chuo-Ku, Osaka, Japan). All chemicals were of analytical grade.

2.2. Preparation of Oxidized Linoleic Acid (OLA) under Different Conditions. Oxidized linoleic acid (OLA) was prepared according to the method described by Aewsiri et al. [6] with a few modifications. Thirty mL of linoleic acid was added with 30 mL of water (HPLC grade). The mixture was then homogenized using a homogeniser (IKA Labortechnik homogenizer, Selangor, Malaysia) at a speed of 9,500 rpm for 2 min. The homogenate was obtained by centrifugation using a centrifuge (model z323k, Hermle Labor Technik, Wehingen, Germany) at 3,000 $\times g$ for 15 min to remove water-soluble oxidized lipids. To oxidize linoleic acid, the prepared fatty acid was flushed with air constantly and

incubated at different temperatures (60 and 80°C) in a controlled-temperature water bath for different times (0, 1, 3, 6, 9, 12, and 24 h). OLA samples were subjected to analyses as following.

2.2.1. Peroxide Value (PV). PV, an indicator of the initial stages of oxidation, was determined according to the method of Shantha and Decker [10] with a slight modification. OLA samples (0.3 mL) were mixed with 1.5 mL of chloroform/methanol (2 : 1, v/v) mixture. The mixture was vortexed for 10 sec, and 15 μL of 3.94 M ammonium thiocyanate and 15 μL of ferrous iron solution (prepared by mixing 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$ at a ratio of 1 : 1, v/v) were added. After 20 min of incubation at room temperature, the absorbance was measured at 510 nm. The concentration of hydroperoxides was calculated from a cumene hydroperoxide standard curve (0–500 mg/mL). The PV was expressed as mg hydroperoxide/kg sample.

2.2.2. Thiobarbituric Acid Reactive Substances (TBARS). TBARS, representing the secondary oxidation products, were determined according to the method of Buege and Aust [11]. The sample (1 mL) was added with TBA reagent (4 mL) (0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25 M HCl). The mixture was heated in boiling water for 10 min, followed by cooling in running tap water. The mixture was centrifuged at 3600 $\times g$ for 20 min at room temperature. The absorbance of the supernatant obtained was measured at 532 nm. The TBARS value was calculated from the standard curve of malonaldehyde (MDA) (2–10 ppm), and TBARS was expressed as mg MDA/kg sample.

2.2.3. Fourier Transform Infrared (FTIR) Spectroscopic Analysis. Linoleic acid was incubated at 60°C for different times (0, 6, 12, and 24 h). The OLA samples were transferred into an amber vial, flushed with nitrogen, and sealed tightly. The samples were stored at −40°C until analysis. FTIR spectra of OLA were obtained using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany). A small quantity of the OLA was accumulated with a Pasteur pipette between KBr disks, creating a thin film. The absorption of IR in the region of 650–4000 cm^{-1} was determined using 32 scans with a resolution of 4 cm^{-1}. These spectra were subtracted from the reference spectrum of air.

2.3. Preparation of Fish Skin Gelatin Modified with OLA Prepared under Different Conditions. Fish skin gelatin was modified with OLA according to the method of Aewsiri et al. [6] to obtain gelatin-OLA complexes via covalent interaction. First, OLA was prepared at 60°C for different times (6, 12, and 24 h). OLA was dissolved with ethanol at a ratio of 1/130 and 1/260 (v/v) to obtain the concentrations of 20 and 40 mM, respectively. Gelatin solution was separately prepared by dissolving gelatin powder in distilled water to obtain a final concentration of 1.1% protein (w/v) as determined by the biuret method [12]. To modify gelatin, the OLA solutions (1 mL) were added to gelatin solution (90 mL) at OLA-to-free amino group molar ratios of 5 : 1 and 10 : 1. The mixtures were then adjusted to 100 mL with distilled water to obtain the final concentration of 1% gelatin. The mixtures were stirred using a magnetic stirrer for 3 h at room temperature. Thereafter, the sample were dialyzed with a dialysis bag (MW cut-off = 14000 Da) with 20 volumes of 1% ethanol for 12 h at room temperature to remove free OLA (unbound to proteins). The dialysis was further performed using 20 volumes of distilled water for 12 h to remove ethanol. The dialysates were filtered through a filter paper no. 1 (Whatman, Germany), freeze-dried, and stored at −20°C prior to analyses. The control was prepared in the same manner, except that OLA was omitted. All gelatin samples were subjected to analyses.

2.3.1. Determination of Surface Hydrophobicity. Surface hydrophobicity was measured as per the method of Benjakul et al. [13] using 1-anilinonaphthalene-8-sulfonic acid (ANS) as a probe with a slight modification. Samples were diluted to 1, 2, 3, and 4 mg/mL with 10 mM sodium phosphate buffer (pH 6.0). To 2 mL of diluted solutions, 20 μL of 8 mM ANS in 0.1 M sodium phosphate buffer (pH 7.0) was added. The fluorescence intensity of the mixture was measured at an excitation wavelength of 374 nm and an emission wavelength of 485 nm. The initial slope of plot of fluorescence intensity vs. protein concentration was assigned to as S_0ANS.

2.3.2. Determination of Carbonyl Content. The carbonyl content was determined using 10 mM 2,4-dinitrophenylhydrazine (DNPH) following the method of Liu et al. [14]. To 0.5 mL of the sample solution (4 mg protein/mL), 2.0 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl was added. The mixture was allowed to stand for 1 h at room temperature. Thereafter, 2 mL of 20 mM potassium phosphate (pH 2.3) containing 0.6 M guanidine hydrochloride was added, and the mixture was mixed well until completely dissolved. The absorbance was read at 370 nm. The carbonyl content was calculated from a molar absorptivity of 22,400 $M^{-1}\cdot cm^{-1}$ [15]. Carbonyl content was expressed as nmol/mg protein.

2.3.3. Determination of Free Amino Group Content. The free amino group content was measured according to the method of Benjakul and Morrissey [16]. Reaction mixtures consisted of 125 μL of diluted samples and 2.0 mL of 0.2 M TNBS solution. The mixture was incubated at 50°C for 30 min in a temperature-controlled water bath (Memmert, Schwabach, Germany) and placed in the dark condition. To terminate the reaction, 2.0 mL of 0.1 M sodium sulfite was added. After cooling for 15 min at room temperature, the absorbance was read at 420 nm. The free amino group content was calculated from a L-Leucine standard curve (0.5–5.0 mM). The free amino group content was expressed as μmol/g protein.

2.3.4. Fourier Transform Infrared (FTIR) Spectroscopic Analysis. Fish skin gelatin without and with OLA modification was conditioned in a desiccator containing P_2O_5 for 2 weeks at room temperature to obtain the most dehydrated gelatin before analysis. Gelatin samples were scanned with an FTIR spectrometer, equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide, and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25°C as described by Nuthong et al. [17]. The spectra were corrected at 32 scans with the resolution of 4 cm^{-1}.

2.3.5. SDS-Polyacrylamide Gel Electrophoresis (SDS–PAGE). Protein patterns of gelatin with and without OLA modification were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [18] using 4% stacking gel and 10% running gel. Gelatin solutions were then mixed with sample buffer (20% (v/v) glycerol, 0.5 M Tris-HCl, pH 6.8 containing 4% (w/v) SDS, and 10% (v/v) β-ME) at the ratio of 1 : 1 (v/v). Samples (15 μg protein) as determined by the Biuret method [12] were loaded onto the gel, and electrophoresis was carried out at a constant current of 15 mA using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). Following electrophoresis, the gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid. After staining, the gel was destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight range protein markers were used to estimate the molecular weight of protein.

2.4. Statistical Analysis. All experiments were run in triplicates. Data were subjected to analysis of variance (ANOVA), and differences between means were tested by Duncan's multiple range test [19] using the SPSS program (SPSS 17.0 for windows; SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

3.1. Characteristics of OLA Prepared under Various Conditions

3.1.1. PV and TBARS. The changes in PV and TBARS value of linoleic acid oxidized at different temperatures for various times are illustrated in Figures 1(a) and 1(b), respectively. The PV demonstrates the primary lipid oxidation products, mainly peroxides, while TBARS value represents the secondary lipid oxidation products, including aldehydes, ketones, and epoxides as well as other carbonyl and hydroxyl compounds [20]. Increase in the PV of samples oxidized at 60°C was obtained when the oxidation time increased up to 9 h ($p < 0.05$). Subsequently, the PV decreased continuously for up to 24 h of reaction. At the higher reaction temperature (80°C), the increase in the PV was observed for up to 6 h ($p < 0.05$). Thereafter, a gradual decrease was found until the end of the reaction (24 h). At the same oxidation time, the higher PV was obtained in OLA prepared at lower temperature (60°C). The result suggested that the primary oxidation product, especially hydroperoxide, could be formed to a higher extent, but the rate of decomposition proceeded. As a result, the primary products were retained at a lower extent.

For TBARS values, the drastic increase in the TBARS value was observed for OLA prepared at both temperatures up to 12 h ($p < 0.05$). Subsequently, the sample prepared at 60°C had a slight increase in TBARS values. Nevertheless, the sample oxidized at 80°C had no changes in TBARS values ($p \geq 0.05$). After 24 h of reaction, the higher TBARS value was found in the sample incubated at 60°C. Therefore, both reaction temperatures and times played a major role in the formation and the loss of secondary oxidation products. In general, higher loss was attained when the reaction was carried out at a higher temperature (80°C). Both lipid oxidation products, primary and secondary products, developed under different reaction conditions in OLA could undergo interaction with gelatin to different degrees [6].

3.1.2. FTIR Spectra. Figure 2 shows the FTIR spectra of linoleic acid (LA) and oxidized linoleic acid (OLA) prepared at 60°C for various times (6, 12, and 24 h). The assignment of functional groups responsible for IR absorption peaks is presented in Table 1. The obtained IR spectrum of LA showed similar characteristic peaks as generally reported for nonoxidized fatty acid [21–23]. The spectrum of nonoxidized fatty acid consisted of the major bands with the wavenumbers of 3009 cm^{-1} (C-H stretching vibrations of the *cis*-double bond (=CH)); 2954 cm^{-1} (symmetric stretching vibration shoulder of CH_3); 2926 and 2854 cm^{-1} (asymmetric and symmetric stretching vibrations of CH_2, respectively); 1715 cm^{-1} (C=O stretching vibrations); 1465 and 1458 cm^{-1} (bending vibrations of the CH_2 and CH_3, respectively); 1398 cm^{-1} (bending in plane vibrations of the CH *cis*-olefinic group); 1377 cm^{-1} (bending vibrations of the CH_2 group); and 1238, 1163, 1120, and 1099 cm^{-1} (C-O stretching vibrations) [24].

The IR spectra of oxidized linoleic acid (OLA) obtained at various times exhibited noticeable differences as compared to those of nonoxidized LA, indicating the formation of functional groups as a result of oxidation. The absorption band in the range 3600–3200 cm^{-1} (representing stretching vibrations of OH and OOH [23, 25]) of OLA showed increased intensity as compared to that of nonoxidized LA. This was mostly associated with the formation of hydroperoxide and secondary products containing an OH moiety from the oxidation of LA. The amplitude of the vibrational peak at 3009 cm^{-1} of OLA at 12 and 24 h of oxidation was lower than that of nonoxidized LA. However, the OLA at 6 h, which was the early stage of oxidation, showed higher amplitude. The amplitude of this peak seemed to decrease with oxidation time, except for the OLA at 6 h. This indicated that the extent of oxidation reaction generally increased with time. As the oxidation proceeded, the loss of conjugated *cis*-double bonds occurred. This more likely led to isomerization of *cis*-double bonds to conjugated *trans*-double bonds or could generate secondary oxidation products, which were not formed during the initial stage of oxidation [25]. Moreover, the spectral region between 1800 and 1000 cm^{-1} (Figure 2(b)) underwent significant changes during the

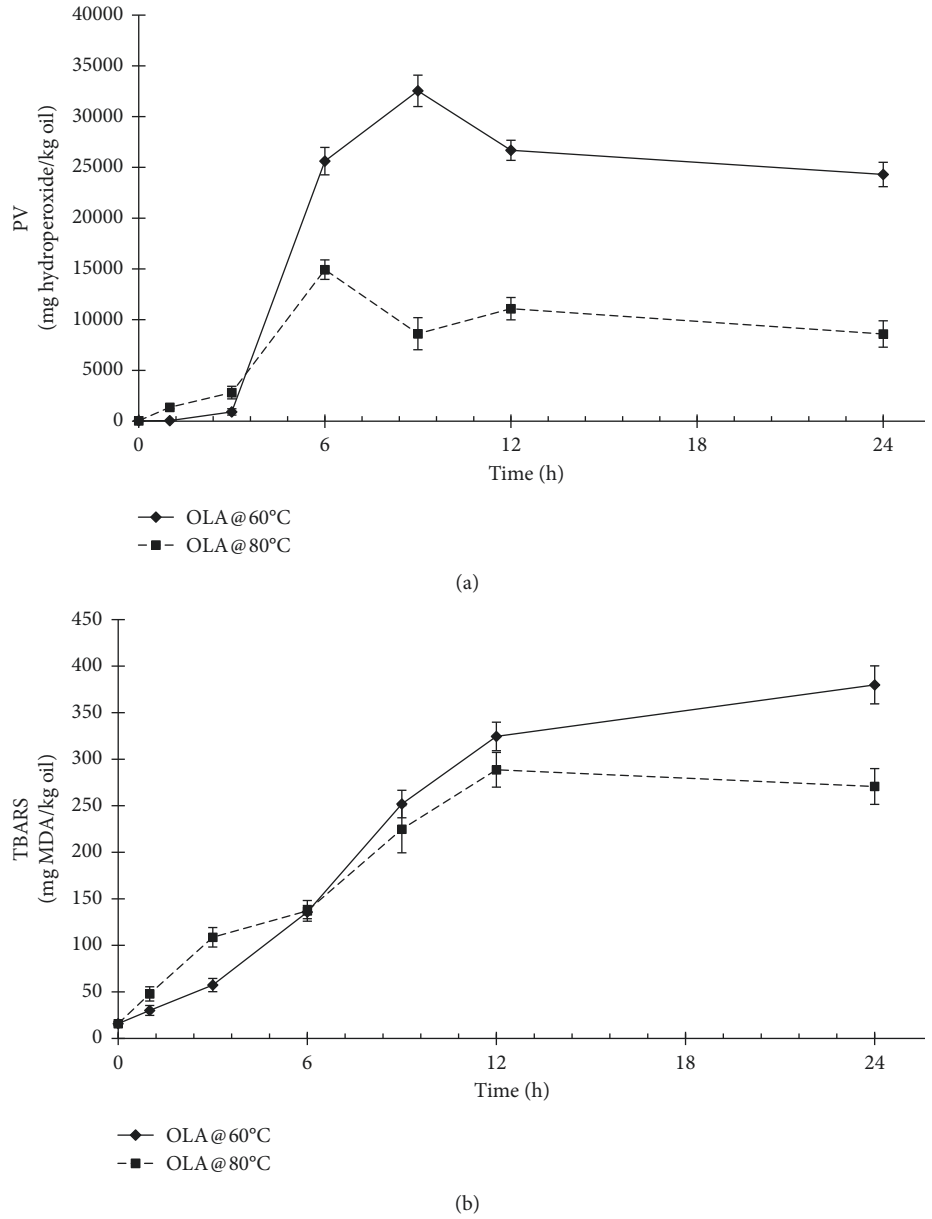


Figure 1: Changes in peroxide value (a) and TBARS value (b) of OLA prepared at different temperatures for various times. The bar represents the standard deviation ($n = 3$).

oxidation of LA. The new absorption band observed as a shoulder at 1745 cm^{-1} in OLA spectra could be assigned to stretching vibrations of the C=O moiety in aldehyde, ketone, or ester, which are the secondary products from lipid oxidation [23]. The presence of this peak could result in widening or broadening of the carbonyl band at 1715 cm^{-1} of fatty acid. In addition, the peaks around 1160–1175 and 1238 cm^{-1}, associated with the stretching vibrations of the C-O in ester moiety [24] as well as 1055 cm^{-1} assigned to C-O bending were noticed. The absorption at around 1180 cm^{-1} due to C-O-O vibrations of hydroperoxides (ROOH) was also detected; this vibrational peak overlapped with C-O stretching vibrations [26]. It can be observed from the spectra that the amplitude of peaks at 1745 and 1238–1055 cm^{-1} increased with increasing oxidation time. This indicated the higher proportion of saturated aldehyde and saturated acyl groups of LA when oxidized at longer time [25]. Moreover, the absorption peaks appearing approximately at 975 and 680 cm^{-1} in IR spectra were related to =C-H out-of-plane bending of *trans* and *cis* isomers, respectively [22]. As the oxidation time increased, the intensity of the band at 680 cm^{-1} decreased, while that of the 975 cm^{-1} band increased. This result also indicated that isomerization of *cis*-double bonds to conjugated *trans*-double bonds more likely took place upon the progress of oxidation of LA. Therefore, the IR result reconfirmed that the oxidation carried out under conditions in this study was able to change the structure of linoleic acid, as indicated by the presence and increase in intensity of bands corresponding to saturated aldehyde groups and saturated acyl groups in OLA.

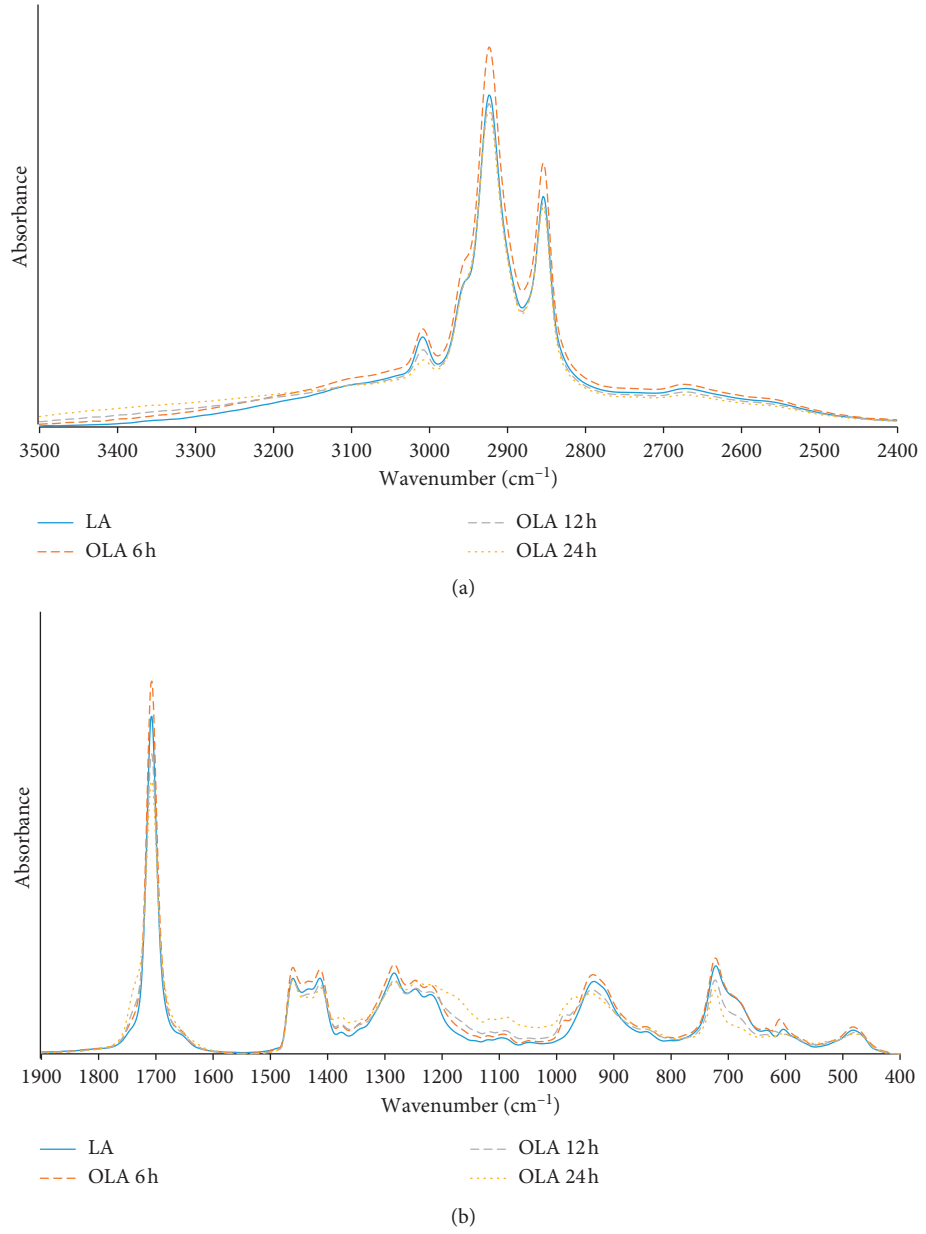


Figure 2: FTIR spectra in the wavenumber region of 3500–2400 cm^{-1} (a) and 1900–400 cm^{-1} (b) of linoleic acid (LA) and OLA prepared at 60°C for different oxidation times.

3.2. Characteristics of Gelatin Molecularly Modified with OLA

3.2.1. Surface Hydrophobicity. Surface hydrophobicity (S_0ANS) of native gelatin samples and those modified with OLA prepared from various oxidation times at different OLA/free amino group molar ratios is shown in Table 2. S_0ANS of all modified gelatin samples increased as the molar ratio and oxidation time of OLA used increased ($p < 0.05$). Increase in S_0ANS indicated that the hydrophobic domains of modified gelatin were increased, more likely due to OLA being attached to gelatin. The highest S_0ANS was observed for gelatin modified with OLA oxidized for 24 h using a ratio of OLA-to-free amino group of 10 : 1. It was noted that the gelatin modified with OLA showed the higher S_0ANS than the control gelatin. This might be due to the higher interaction between the free amino group of gelatin and reactive groups, especially carbonyl groups located in OLA. Similar results were obtained when soy protein was modified with acrolein (secondary lipid oxidation product), in which surface hydrophobicity increased [27]. The hydrophobicity of modified protein was increased with increasing number of carbonyl compounds on the protein surface [27]. When linoleic acid was oxidized, it became more electrophilic, which was then able to attach to the nucleophilic domain of gelatin. Those covalently attached domains mainly contributed to the increased surface hydrophobicity of modified gelatin.

3.2.2. Free Amino Group Content. Free amino group contents of gelatins modified with OLA prepared at 60°C for

Table 1: Functional group assignment on major absorption bands occurring in the infrared spectra of nonoxidized linoleic acid (LA) and oxidized linoleic acid (OLA).

Wavenumber (cm^{-1})		Type of vibration and functional group
LA	OLA	
3350–3120		Stretching of OH in fatty acid
	3600–3200	Stretching of OH in aldehyde, peroxide coupled with OH of fatty acid
3009	3009	CH stretching of *cis*-double bond (=CH)
2954	2954	Asymmetric stretching vibration shoulder of the aliphatic CH_3 group
2926, 2854	2926, 2854	Asymmetric and symmetric stretching, respectively, of aliphatic CH_2 group
—	1745	Stretching vibration of C=O of aldehyde and ester
1715	1715	Stretching of C=O of fatty acid
1654	1654	C=C stretching vibration
1465, 1458	1465, 1458	Bending vibration of aliphatic CH_2, CH_3, respectively
1420	1420	Rocking vibration of CH bonds of *cis*-distributed olefins
	1238, 1175–1055	Stretching vibration of C-O ester groups
	1180	C-O-O vibration of hydroperoxide
	990–980	Bending of C-O of ester overlapping with CH bending of *trans*-double bond
975	975	Bending of C-O of acid
723	723	Overlapping of the CH_2 rocking vibration and the out-of-plane vibration of *cis*-distributed olefins
680	680	CH bending of *cis*-double bond

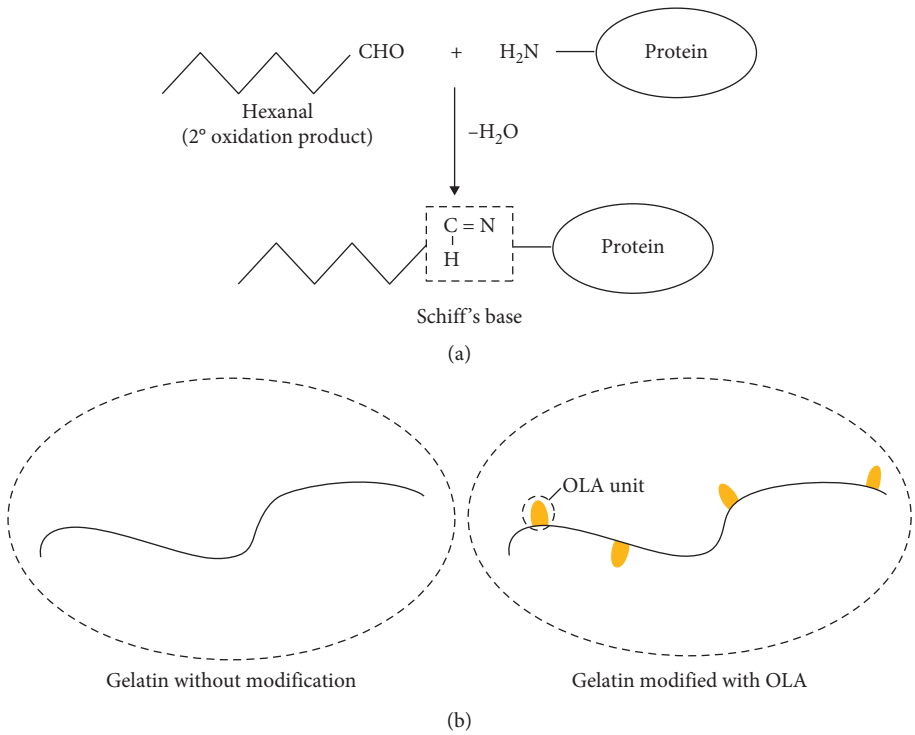
Table 2: Surface hydrophobicity, free amino group content, and carbonyl content of fish gelatin modified without and with OLA prepared at various oxidation times using different molar ratios of OLA/free amino group of gelatin.

Molar ratio of OLA/ free amino group	Oxidation time of OLA (h)	Surface hydrophobicity	Free amino group content (μmol/g protein)	Carbonyl content (nmol/mg protein)
Control	—	$2.06 \pm 0.01^{a*}$	4.39 ± 0.04^{a}	2.36 ± 0.01^{a}
At molar ratio 5 : 1	6	2.72 ± 0.08^{b}	4.27 ± 0.03^{ab}	2.59 ± 0.05^{b}
	12	2.94 ± 0.16^{c}	4.14 ± 0.07^{b}	2.76 ± 0.01^{c}
	24	3.16 ± 0.09^{d}	3.94 ± 0.06^{c}	2.96 ± 0.01^{d}
At molar ratio 10 : 1	6	2.97 ± 0.14^{c}	4.14 ± 0.07^{b}	3.04 ± 0.03^{e}
	12	3.22 ± 0.07^{d}	4.15 ± 0.13^{b}	2.95 ± 0.01^{d}
	24	3.36 ± 0.02^{e}	3.86 ± 0.12^{c}	3.55 ± 0.01^{f}

*Mean ± SD ($n = 3$). Different superscript letters in the same column indicate significant differences ($p < 0.05$). Control: gelatin without modification.

various oxidation times and using different OLA-to-free amino group molar ratios are shown in Table 2. The free amino group content of modified gelatin generally decreased with oxidation time of OLA and the molar ratio (OLA/free amino group of gelatin) used for modification ($p < 0.05$). Nevertheless, gelatin modified with OLA oxidized for 6 h with a OLA/NH_2 group molar ratio of 5 : 1 had no changes in the free amino group content ($p \geq 0.05$). The lowest free amino group content was observed for gelatin modified with OLA oxidized for 24 h ($p < 0.05$), regardless of the OLA/NH_2 molar ratio used. OLA might interact with gelatin molecules via ε-amino group of lysine in the gelatin [6], as shown in Scheme 2. More specifically, the reaction might involve the nucleophilic addition between the amino group of gelatin and the aldehyde carbonyl group of OLA [26]. The extent of gelatin-OLA reaction was higher by using OLA oxidized at longer time that contained a higher amount of aldehyde functional groups. As a consequence, gelatin modified using OLA with longer oxidation time had the lower free amino group content ($p < 0.05$) (Table 2). Liu and Xiong [28] also reported that free amino group might react with the carbonyl compounds. The result suggested that OLA having higher oxidation more likely interacted with gelatin to a higher extent. Therefore, the oxidation time of OLA and the molar ratio of OLA-to-free amino group of gelatin were important factors affecting the covalent attachment of OLA onto gelatin.

3.2.3. Carbonyl Content. Carbonyl contents of gelatins and those subjected to modification with different OLA at various ratios is presented in Table 2. The incorporation of OLA into gelatin resulted in the higher carbonyl content of resulting gelatin in comparison to the control gelatin. Increase in the carbonyl content was obtained when gelatin was modified with OLA prepared with the longer oxidation time. Higher carbonyl content was also found in gelatin as the OLA/gelatin molar ratio increased ($p < 0.05$). This result



Scheme 2: Reaction between secondary oxidation products of OLA and gelatin (a) and molecular model for gelatin chain without and with OLA modification (b).

suggested that gelatin could interact with OLA via free amino groups, particularly at ε-amino group of lysine [6]. Moreover, lipid oxidation products in OLA were postulated to accelerate the oxidation of gelatin, as indicated by the increase in carbonyl content. The increase in carbonyl content in gelatin modified with OLA might be mediated by Schiff's base process via reactive amino groups [29] (Scheme 2(a)). Wu et al. [27] also reported that the soy protein modified with aldehyde groups had decreases in lysine, cysteine, and histidine residues, as induced by the increased protein oxidation. Therefore, gelatin could be modified by OLA via carbonyl groups, mainly via Schiff's base but the degree of modification was dependent on the degree of oxidation of OLA used. Additionally, the OLA/free amino group of gelatin ratio was another factor determining the modification of gelatin.

3.2.4. FTIR Spectra. FTIR spectra of gelatin without and with modification by using various OLA at the ratios of OLA-to-free amino group of 5 : 1 and 10 : 1 are depicted in Figure 3. All gelatin samples exhibited the major characteristic bands of protein at ~3280 cm^{-1} (amide-A, illustrating NH-stretching coupled with hydrogen bonding), ~2934 cm^{-1} (amide-B, illustrating CH-stretching and -NH_3^+), ~1634 cm^{-1} (amide-I, representing the C=O stretching/hydrogen bonding coupled with COO^-), ~1523 cm^{-1} (amide-II, illustrating the NH bending coupled with CN stretching), and ~1234 cm^{-1} (amide-III, representing the CN and NH in plane bending of amide bonds or CH_2 of glycine) [6]. The absorption peaks of amide-I, amide-II, and amide-III of the gelatin appeared at 1634, 1523, and 1234 cm^{-1}, respectively. Amide-I and amide-II peaks increased and shifted to a higher wavenumber when the gelatins were modified with various OLA compared to the control gelatin. The shift was more pronounced when OLA prepared at longer oxidation time and a higher OLA/free amino group ratio was used. The incorporation of OLA into gelatin might lower interaction of these C=O bonds or NH along the chains of gelatin. It was noted that the amplitude of amide bands increased when OLA was used for modifications. OLA with higher oxidation caused increase in amplitude of those bands in the resulting gelatin. The shift to a higher wavenumber of amide-I peak could also be associated with the presence of an imine moiety (C=N) formed via Schiff's base reaction between gelatin and OLA [26] (Scheme 2(a)). The absorption peak of C=N was reported to occur around 1690 cm^{-1} (stretching), which presented at a higher wavenumber than C=O of amide. The vibration of the C=N group could merge or couple with that of C=O group of amide, resulting in broadening and shift of amide-I band to a higher wavenumber. This result could be indicative for the covalent attachment of OLA onto the gelatin chain.

For the amide-A peak, this peak represents the vibration of the NH group coupled with hydrogen bonding. The shift to lower wavenumbers indicated that the NH group of a peptide interacted with adjacent molecules via hydrogen bonds [30]. From the IR spectra, the amide-A peak of gelatin without modification appeared at a lower wavenumber than did that of OLA-modified gelatins. This was mostly due to the higher intermolecular interaction via hydrogen bonding between nonmodified gelatin molecules. However, the amide-A peak generally appeared at a higher wavenumber when gelatin was

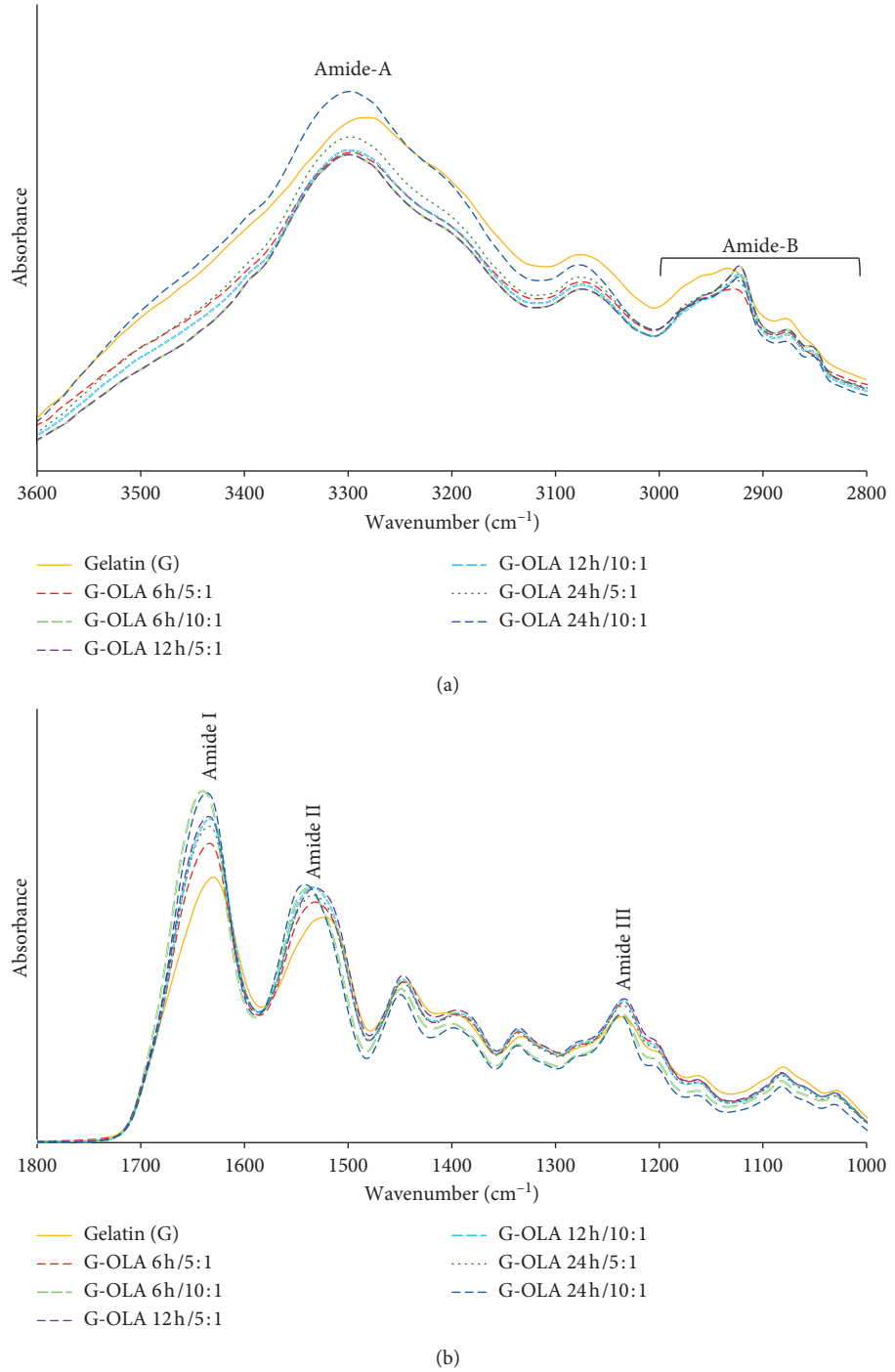


Figure 3: FTIR spectra in the wavenumber region of 3600–2800 cm^{-1} (a) and 1800–1000 cm^{-1} (b) of fish skin gelatin modified with OLA prepared using different oxidation times and using various OLA-to-free amino group molar ratios.

modified with OLA. This observation might be associated with that the long hydrocarbon chain of fatty acid of OLA attached to gelatin molecule could hinder the interaction via hydrogen bonding among adjacent gelatin molecules, as postulated in Scheme 2(b). Therefore, functional groups of fish gelatin were affected to some extent by molecular modification with OLA, and the OLA with different rates of oxidation as well as OLA-to-free amino group molar ratio affected the molecular property of the resulting gelatin.

3.2.5. Protein Patterns of Modified Gelatin and Control Gelatin. Protein patterns of fish skin gelatin modified with OLA having different oxidation rates and various OLA/NH_2 group molar ratios in comparison with gelatin are shown in Figure 4. All modified gelatin samples showed similar pattern to the control gelatin (without OLA modification). All samples had α_1- and α_2-chains as the main components. α_1- and α_2-chains have been reported as the dominant components in fish skin gelatin [31]. For all gelatins modified with

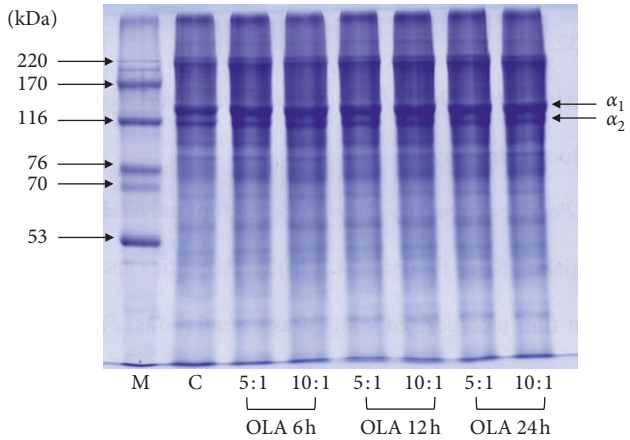


Figure 4: Protein patterns of fish gelatin modified with OLA prepared at different oxidation times and using various OLA-to-free amino group molar ratios. M: high MW protein marker and C: control gelatin (without modification).

OLA under different conditions, there were no differences in protein patterns. It was suggested that OLA mainly attached to gelatin without induction of intermolecular cross-linking between protein chains. Additionally, OLA with low molecular weight might not affect the molecular weight of modified gelatin. Consequently, OLA modification had no influence on the protein pattern of fish skin gelatin.

4. Conclusions

The covalent attachment of OLA onto fish skin gelatin increased surface hydrophobicity, as indicated by the increased carbonyl group content and decreased free amino group content. The degree of modification depended on the degree of oxidation of OLA used as well as molar ratios of OLA/free amino group of gelatin. The treatments used for modification did not affect the protein pattern of fish skin gelatin. This inferred that the modification resulted in the incorporation of linoleic acid into gelatin without induction of covalent bonded cross-linking of gelatin. Gelatin modified with OLA (oxidized at 60°C for 24 h) at a molar ratio of 10 : 1 for 3 h at room temperature was suggested to improve surface hydrophobicity of fish gelatin.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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