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Vacuum impregnation of fish gelatin combined with grape seed extract inhibits protein oxidation and degradation of chilled tilapia fillets

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ABSTRACT

The present study studied the effects of fish gelatin (FG) incorporated with grape seed extract (GSE) through vacuum impregnation (VI) on refrigerated tilapia (*Oreochromis niloticus*) fillets over 12 days. The VI of FG-GSE significantly improved the quality of the fish by decreasing drip loss, texture changes, and microbial survival. It also delayed protein oxidation by inhibiting the formation of disulphide bonds and carbonyl groups, and maintaining a higher sulfhydryl content and Ca^{2+}-ATPase activity. Regarding myofibril degradation, FG-GSE maintained their secondary structure by increasing the ratio of α-helices and β-sheets (70.88–75.51%). Atomic force microscopy further revealed that the FG-GSE coating preserved the myofibril nanostructure by maintaining their length, width, and height. Overall, the synergistic effects of VI with 3% FG and 0.9% GSE suggested a promising approach for fillet preservation.

1. Introduction

With a yield of 3,700,000 tons in 2014, tilapia (*Oreochromis niloticus*) is one of the most abundant freshwater fish species in the world. Tilapia is widely consumed or used to produce ready-to-eat and high quality salt fish products because of its attractive taste and high nutritional value (rich in protein) (Shi et al., 2018). However, they are susceptible to deterioration during storage because of enzymatic and/or microbial activities, such as drip loss, oxidation, protein degradation, microorganism proliferation, and changes in texture (Farvin, Grejsen, & Jacobsen, 2012). Recent changes in consumer lifestyles have shifted demand toward safe and high-grade foods. Thus, freshness and safety are the principle quality features of fish products for consumers. To prevent the decrease in fish quality during storage, edible coatings based on natural preservative agents have been studied widely and applied recently. As a novel preservation technique, an edible coating is very cost-effective because no advanced equipment is required. Edible coatings can act as barriers to moisture, gases, and flavours, and act as carriers for bioactive agents (e.g. antioxidants, antimicrobials, or nutrients) to protect foodstuffs from deterioration (Murrieta-Martínez et al., 2018).

Fish gelatin (FG), as an alternative to mammalian gelatin, has gained popularity in the food industry for its good film-formation and biodegradable ability (Jiang, Yu, & Liu, 2014). Applying FG can improve the stability, quality, and safety of seafood. Nevertheless, some problems restrict the application of FG, such as its low antioxidant and antimicrobial ability, as well as its relatively high moisture sensitivity (Kchaou et al., 2018). Modifications of gelatin using chemical methods or combining it with other effective coating materials have been developed to overcome such limitations and to broaden its application (Zhao, Wu, Chen, & Yang, 2019). Recent studies have demonstrated the efficacy of multilayer or bilayer coatings combining plant extracts with gelatin to improve fish quality and safety (Zhao, Wu, Chen, & Yang). Grape seed extract (GSE) is a natural additive that is obtained as a by-product of the grape wine and juice industry, and contains abundant polyphenol compounds, such as catechin, epicatechin, proanthocyanidins, monomeric flavanols, and gallic acid. GSE shows significant antibacterial, antioxidant, and antiviral activities (Alves et al., 2018). Therefore, the addition of GSE into FG-based coatings could enhance their quality, inhibit oxidation, and prolong the shelf life of fish products.

Previous coating studies mainly dipped or immersed the fish in coating solutions; therefore, only the surface part was coated and the coating efficiency was quite low (Feng, Ng, Mikš-Krajnik, & Yang, 2017). Vacuum impregnation (VI) is a promising technique to improve food quality by introducing various materials into porous tissues in a rapid and direct way. It involves removing the gas and liquid inside pores under vacuum conditions and then replacing them with VI

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solutions under restored atmospheric pressure. Various compounds can be impregnated via VI, such as cryoprotectants, antioxidants, bio-preservatives, enzymes, and probiotics. In the fishery industry, this technology has been used to develop fish and seafood with high structural and sensorial properties, as well as prolonged shelf life (Zhao et al., 2019).

The aim of the present study was to test the effect of FG-based coatings containing GSE introduced by VI on chilled tilapia fillets. The changes in freshness (drip loss, texture, and microbial survival), protein oxidation (sulfhydryl groups, disulphide bonds, carbonyl groups, and Ca^{2+}-ATPase activity) and protein degradation (secondary structure and nanostructure) of refrigerated tilapia fillets during storage for 12 days were tested to determine the preservative mechanism of each treatment.

2. Materials and methods

2.1. Impregnation solution preparation

All the reagents for the coating solutions were of food grade. Commercial tilapia (*Oreochromis niloticus*) FG (200 bloom) was purchased from Jiangxi Kesheng Organism Co., Ltd (Yingtan, Jiangxi, China), and contained more than 80% protein. FG was soaked in deionised water produced from a Sartorius Arium 611 UV system at 4 °C overnight. The FG solution was placed in water bath (60 °C) for 30 min until it was completely dissolved and homogenised. GSE (containing 80% procyanidin and other minor components, such as catechin, gallic acid, and epicatechin) was purchased from Tianjin Jianfeng Natural Product Co., Ltd (Tianjin, China) and dissolved in deionised water using an Elmasonic S 60H sonicator (Elma Schmidbauer GmbH, Singen, Germany). The GSE solution and FG solution were mixed and homogenised by magnetic stirring. The concentration of FG was 3%, and the concentration of GSE was 0.3%, 0.6%, and 0.9%, respectively. Five coating solutions were prepared: (1) The control solution containing deionised water only, (2) 3% (w/w) FG solution, (3) 3% FG solution plus 0.3% (w/w) GSE, (4) 3% FG solution plus 0.6% (w/w) GSE, and (5) 3% FG solution plus 0.9% (w/w) GSE.

2.2. Fillet preparation by VI

Commercial tilapia were purchased from a local supermarket in Singapore. The average mass of an individual tilapia was 500 ± 5 g. The head, bone, and skin of tilapia were removed and the fillets were then transported to the laboratory within one hour under cold storage.

VI was carried out in a jacketed chamber connected to a vacuum pump (Model DZF, Beijing Yong Guang-Ming Medical equipment Co., Ltd, Beijing, China) that was controlled by a vacuum controller (IKA VC10, Guangzhou, Guangdong, China). Fillets (10 g) were immersed in the vessel with different coating solutions. The mass ratio of the fillet sample to the coating solution was kept at 1:3. A vacuum pressure of 5 kPa was applied for 15 min. Thereafter, the atmospheric pressure was restored within 10 s and the samples were maintained for 10 min. The solution adhering to the surface of the fillets was then removed using filter paper and the fillets were drained for 5 min (Mao et al., 2017). After this pre-preparation, all the samples were kept in ziploc bags (S.C. Johnson & Son Pte. Ltd, Singapore) and stored at 4 °C for 12 days. Further analyses were carried out every 3 days. Fresh fillets before coating treatment were also analysed (day 0).

2.3. Determination of yields and drip losses

The weight of the fillet (n = 3) was monitored at the beginning and end of the VI process. The yield (weight gain, %) of coating by VI and the drip loss (%) during storage were calculated using Eqs. (1) and (2), respectively:

$$\text{Yield}\ (\%) = m_1/m_0 \times 100\% \quad (1)$$

$$\text{Drip loss}\ (\%) = (m_1 - M_i)/m_1 \times 100\% \quad (2)$$

where m_0 is the weight of the fish fillet before VI, m_1 is the mass of the fish after VI. M_i is the weight of the coated fillet at a particular day of storage before analysis (i = 3, 6, 9 or 12).

2.4. Texture measurement

The texture of the tilapia fillets was measured using a TA. XT. Plus Texture Analyzer (Stable Micro155 Systems, Goldaming, Surrey, UK) using a P30 probe. All samples were cut into small cubes (1.5 cm × 1.5 cm × 1.5 cm) and the samples were prepared and compressed to 30% deformation. The testing parameters are as follows: pretest speed: 2.0 mm/s; test speed: 1 mm/s; post-test speed: 1 mm/s; a penetrating distance of 4.0 mm; and a trigger force of 0.05 N. Texture parameters, including hardness, springiness, cohesiveness, and chewiness of each fillet were obtained from the texture profile analysis (TPA) curve (Feng et al., 2017).

2.5. Analysis of myofibrillar proteins

2.5.1. Extraction of myofibrillar proteins

Myofibrils were extracted from fillet samples according to the method described by Feng et al. (2017) with some modifications. Solution A was freshly prepared before the start of each extraction, which comprised 0.10 M KCl, 1 mM phenylmethylsulfonylfluoride, 0.02% NaN_3, 20 mM Tris–HCl buffer pH 7.5. Solution B consisted of 0.2 M Mg $(CH_3COO)_2$, 0.45 M KCl, 1 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N′, N′-tetraacetic acid, 5 mM β-mercaptoethanol, and 20 mM Tris–maleate buffer pH 6.8. Fillets samples were homogenised and then added to solution A at a weight ratio of sample to solution of 1:10 (w/v). The mixture was gently blended using a magnetic stirrer, kept at 0 °C for 60 min and centrifuged at 10,000 × g at 4 °C for 10 min. The supernatant was discarded. The pellet was diluted in solution B (1:5, w/v), and added with 10 mM ATP. After stirring for 60 min at 0 °C, the mixture is centrifuged at 10,000 × g for 10 min. The supernatant was collected as the myofibrillar proteins. The extract was stored at −20 °C before further analysis.

2.5.2. Fourier transform infrared spectroscopy analysis of secondary structure

Myofibril samples were freeze-dried and ground with potassium bromide (1:100, w/w) (n = 5) to form a homogeneous powder, pressed into a sheet, and scanned from 4500 to 500 cm^{-1} using a Spectrum One Fourier Transform Infrared (FTIR) spectrometer (PerkinElmer, Waltham, MA, USA). The resolution was 4 cm^{-1} and the number of scans was 32 (Sow, Toh, Wong, & Yang, 2019). At least five repetitions were carried out for each sample. The spectra in the amide I region (1600–1700 cm^{-1}) was deconvoluted using the Origin Pro 9 software (OriginLab, Northampton, MA, USA) and the Gaussian curve fitting function. The final concentration of each protein structure component was calculated according to the area of the component peak and the overall area of the amide I region after deconvolution.

2.5.3. Analysis of nanostructure profiles of myofibril using atomic force microscopy (AFM)

The myofibrils extracted from each fish fillet was diluted with deionised water 10 times and vortexed. Samples (20 μL) were pipetted onto a freshly peeled mica sheet that was attached to a magnetic specimen disc. The sample was air-dried at 25 °C in a laminar flow cabinet for 4 h before scanning. Scanning was performed using a TT-AFM (AFM workshop, Signal Hill, CA, USA) equipped with a Sensaprobe TM190-A-15 tip (Applied Nanostructures, Mountain View, CA, USA) to analyse the nanostructure profiles of the myofibrils of each treatment. The

testing parameters were as follows: a resonance frequency of 145–230 kHz; a force constant of 25–95 N/m; and the Z scanner at 0.2–0.4 Hz. The images were scanned using the tapping mode. The scan rate and scan line were set at 0.4 Hz and 512, respectively (Chen et al., 2018). The vibration mode was selected. Gwyddion software (http://gwyddion.net/) was used to analyse the dimensions in the image, including the length, height, and diameter of the myofibrils under different treatments. To obtain typical and ideal results, at least 10 images and 20 measurements for each sample were carried out for further analysis.

2.6. Protein oxidation measurement

The level of protein oxidation of the fish fillets was analysed by investigating the sulfhydryl groups, disulphide bonds, carbonyl groups, and the Ca^{2+}-ATPase activity. Before analysis, the myofibril sample was adjusted to 4 mg/mL with 0.6 M KCl.

The sulfhydryl group and disulphide bond contents were determined using 5, 5′-dithio-bis (2-nitrobenzoic acid) according to a previously described method (Zhou & Yang, 2019). Carbonyl groups were determined by reactivity with 2,4-Dinitrophenylhydrazine to form protein hydrazones. The results were expressed in nmol of carbonyl per 1 mg of total protein (Liu, Chen, Kong, Han, & He, 2014). The Ca^{2+}-ATPase activity was detected using the method of Lu, Wang, and Luo (2017). The activity of Ca^{2+}-ATPase was expressed as mmol inorganic phosphate released by 1 mg protein in 1 min.

2.7. Microbial analysis

The spread plate method was used for the microbial analysis of fish samples, with some modifications. Each sample (10 g) was put in a sterile stomacher bag, homogenised with sterile peptone water (0.1%, 90 mL), and serially diluted. Samples at each dilution (100 μL) were used for plate spreading. The aerobic mesophilic count and aerobic psychrotrophic count were calculated from standard plate count agar, which were incubated at 36 °C for 2 days and at 4 °C for 7 days, respectively. Yeasts and moulds were calculated based on their growth on potato dextrose agar with incubation at 25 °C for 3 days. The results were expressed as log colony forming units (CFU)/g sample (Feng et al., 2017).

2.8. Statistical analysis

Data were analysed statistically using analysis of variance (ANOVA) of at least three replicates. Means were compared using the least significant difference (LSD) method with the software IBM SPSS Statistics Version 23 (International Business Machines Co. Armonk, NY, USA) to assess the effects of different treatments on the fillets. Differences with $P < 0.05$ were regarded as significant.

3. Results and discussion

3.1. Quality and freshness of fillets

3.1.1. Yield and drip loss

Fish is a commercial product sold on the basis of mass and the water concentration of fish plays a major role in evaluating its quality. Table S1 shows the yield and drip loss of tilapia fillets during storage after vacuum impregnation with different coating solutions. All coatings introduced through the VI process increased the yield of tilapia fillets, ranging from 2.02% to 3.01%. Coating with FG only or with FG combined with GSE resulted in a significantly higher yield compared with that in the control group, which was impregnated with deionised water only ($P < 0.05$). However, no significant effect of GSE addition on yield was found. The FG-based coating increased the fillet's weight by infiltrating into the extracellular space of the fish tissue or attaching to the surface of the fillets (Rodriguez-Turienzo et al., 2011).

Images of fillets impregnated with different coating treatments are shown in Fig. S1. No significant difference was observed among different groups after coating (day 0). However, after 12-days of storage, the surface of fillets in the control group looked juicier and moister than the fillets treated with FG and GSE, indicating drip loss and decrease in water-holding capacity. For further analysis, drip loss during refrigerated storage was determined. As shown in Table S1, drip loss occurred during the whole refrigeration period, ranging from 2.09% to 11.27%. At the early period of storage (day 3), drip loss ranged between 2.09% and 2.69%, and could not be regarded as severe; therefore, this would not be a major issue in all of the fillet samples. An increasing tendency for drip loss in the five groups was observed after 6 days of storage. Coating with FG and GSE significantly slowed down the drip loss of fish fillets compared with that of the control group ($P < 0.05$). At day 12, the difference in drip loss between the control group and the groups treated with FG-based coating solutions reached 2.96%. However, the addition of GSE at different concentrations had no significant effect on the drip loss during cold storage ($P > 0.05$). These results were in accordance with the images shown in Fig. S1.

The water content of fish is very high (typically in the range of 45–85%) and is considered as one of the key factors that can influence sensory, nutritional, or processing characteristics of fish. Drip loss can directly lower the quality of fish by reducing the saleable weight, deteriorating its appearance, and further accelerating microbial growth. Drip loss involves both water (referring to the free and unbound water fraction) and water-soluble components (such as sarcoplasmic proteins) leaking from cells that are ruptured during storage (Przybylski et al., 2016). During cold storage, proteins are oxidised, causing inter-molecular and intra-molecular cross-linking and protein aggregation. The altered structure of the myofibrils had a passive effect on the water holding capacity and therefore increased the drip loss of fillets during storage (Bao, Boeren, & Ertbjerg, 2018). FG contains a majority of hydrophilic amino acids, such as proline (Sinthusamran, Benjakul, & Kishimura, 2014). These amino acids attract water, thus tending to hold water tightly inside the fillets. Previous studies confirmed that a gelatin solution, regardless of its concentration, was beneficial to control the drip loss of meat (Herring, Jonnalongadda, Narayanan, & Coleman, 2010).

3.1.2. Texture properties

The changes in the texture properties in each group during the storage are presented in Fig. 1, including hardness, springiness, cohesiveness, and chewiness.

Hardness, considered as the most important texture feature of fish, showed a decreasing trend with storage time in the samples (Fig. 1A). FG combined 0.3% GSE and 0.6% GSE maintained the hardness of tilapia fish fillets in the first 3 days. Significant reductions in the hardness value in 0.6% GSE treated group and other three groups occurred at day 6 and day 3, respectively ($P < 0.05$). However, there were no significant differences among the five groups from day 9 ($P > 0.05$). The reduction of hardness of fillets ranged from 33.27% to 37.81% at the end of storage. Similar results were also reported previously (Lu et al., 2017).

Springiness, also known as elasticity, represents the degree of recovery of fillet height after applying an external force. Within the first 3 days of storage, a significant loss of springiness was observed only in the control group ($P < 0.05$). Gradual decreases in springiness were observed thereafter, especially for the control group (36.84%). It is also notable that the fillets treated with FG and 0.6% GSE had higher springiness than the other four groups and showed no significant change during 12 days of storage ($P > 0.05$).

The cohesiveness of the fillets changed significantly during cold storage, showing a gradual decline ($P < 0.05$). Interestingly, the addition of GSE inhibited the decrease in cohesiveness after 12 days of storage when compared with the other two groups ($P < 0.05$). In terms

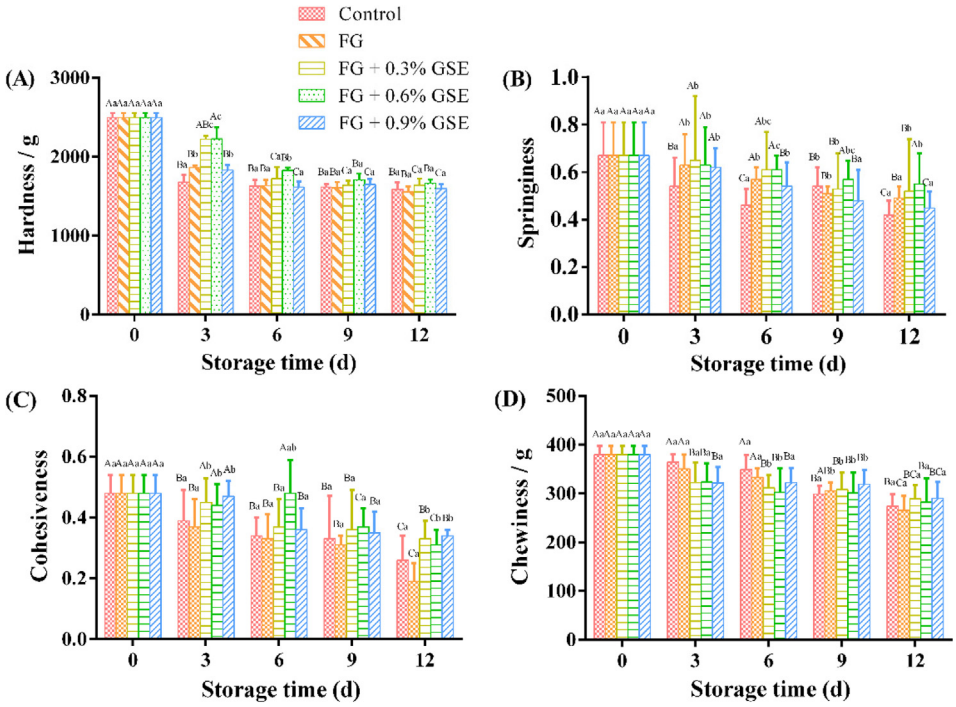


Fig. 1. Effects of vacuum impregnation of fish gelatin (FG)-based coatings incorporated with grape seed extract (GSE) on texture of tilapia fillets stored at 4 °C. (A) Hardness; (B) Springiness; (C) Cohesiveness; (D) Chewiness. Values with different lowercase letters at the same time point and uppercase letters of the same group indicate significant differences ($P < 0.05$), respectively.

of chewiness, there were no significant changes among the different groups after coating, which was in accordance with previous studies (Feng et al., 2017).

After death, fish undergo autolysis, which leads to softening of muscles and loss of elasticity. Furthermore, this autolysis process is facilitated by microbial activities (Lopez-Caballero, Gomez-Guillen, Pérez-Mateos, & Montero, 2005). In this study, FG coatings, especially the GSE added coatings, alleviated the decreasing tendency of texture properties, possibly by inhibiting the microbial activity in the fillets during storage. Researchers have identified a relationship between texture decline and microbial activity during refrigerated storage (Li et al., 2012). Moreover, the susceptibility of fish muscle to declining texture properties is closely related to the change in water holding capacity (Hultmann & Rustad, 2002).

3.1.3. Microbial survival

To evaluate the effect of coating solutions introduced by VI process, microbial populations (aerobic mesophilic bacteria, aerobic psychrotrophic bacteria, yeasts and moulds) in all fillets during 4 °C storage were investigated (Table 1). The results showed a significant increase in aerobic mesophilic counts for all five groups during refrigeration ($P < 0.05$). After 12 days of storage, the fillets treated with FG and 0.6% GSE showed the lowest mesophilic count (5.18 log CFU/g), followed by the 0.9% GSE group. The aerobic mesophilic bacteria count was significantly higher in the control group (5.99 log CFU/g), which still did not exceed 7 log CFU/g ($P < 0.05$). This was because the storage conditions (4 °C) were not quite suitable for the survival and proliferation of mesophilic bacteria.

Gram-negative, aerobic psychrotrophic bacteria are the dominant species causing fish spoilage at low temperature. The psychrotrophic bacteria count in each group increased during the 12-day storage period. The aerobic psychrotrophic count in the control group increased continuously from 4.14 log CFU/g to 7.52 log CFU/g. The FG and GSE treatments resulted in lower psychrotrophic bacterial populations. FG and 0.9% GSE treatment significantly ($P < 0.05$) decreased the cell counts by 1.19 log CFU/g at day 12 compared with that in the control group, followed by 0.6% GSE group (1.11 log CFU/g reduction). Based on the maximum limitation of total viable bacterial count (7 log CFU/g) set by the International Commission on Microbiological Specifications for Foods (Ojagh, Núñez-Flores, López-Caballero, Montero, & Gómez-Guillén, 2011), the fillets treated with 0.9% and 0.6% GSE were considered safe and acceptable at day 12.

Similar changes were observed for yeasts and moulds in the fillets. The initial counts of yeasts and moulds were 4.53 log CFU/g. After storage for 12 days, yeasts and moulds in the control group increased to 7.06 log CFU/g. FG and GSE treatment inhibited the increase in yeasts and moulds significantly ($P < 0.05$). FG combined with 0.6% GSE treatment was the most effective, decreasing the cell counts by 1.05 log CFU/g compared with the control group at the end of storage, followed by the 0.9% GSE group (0.86 log CFU/g reduction).

FG is not an antibacterial agent. However, it can restrain bacterial survival and proliferation by acting as a barrier to oxygen, thereby controlling the growth and proliferation of aerobic bacteria, as demonstrated by previous studies (Herring et al., 2010). GSE, as a natural plant extract, is significant not only because of its antioxidant ability, but also because of its antibacterial ability. Previous studies demonstrated the ability of GSE to reduce bacterial growth in foodstuffs, such as minced beef and salmon (Alves et al., 2018). Overall, medium to high concentrations of GSE (0.6% and 0.9%) combined with FG were more effective than the low GSE concentration (0.3%) in reducing microbial populations under vacuum conditions. Furthermore, the antibacterial abilities of the coatings introduced in the present study were more effective than those in previous studies, in which the fish were dipped directly into the coating solutions (Feng, Bansal, & Yang, 2016). This was probably because during the VI process, the vacuum conditions can effectively remove the oxygen inside the tissue and coating solutions, thereby suppressing the growth of aerobic microorganisms.

3.2. Effect of VI with FG and GSE on myofibril degradation

3.2.1. Secondary structure changes analysed by FTIR

Amide I (1700–1600 cm^{-1}) is the most predominant band in the myofibril spectrum, which is regarded as the most useful band to analyse the secondary structure of proteins because of its sensitivity to the hydrogen bonding pattern, dipole–dipole interaction, and the polypeptide backbone conformation. It is primarily influenced by the C═O stretching vibration, followed by the C−N and N−H stretching vibrations. To gain a deeper insight into changes in the secondary

Table 1
Effect of vacuum impregnation of FG-GSE coatings on microbial survival in tilapia fillets.

Storage time (d)	Control	FG	FG + 0.3% GSE	FG + 0.6% GSE	FG + 0.9% GSE
Aerobic mesophilic count (log CFU/g)					
0	4.09 ± 0.07^{aA}	4.09 ± 0.07^{aA}	4.09 ± 0.07^{aA}	4.09 ± 0.07^{aA}	4.09 ± 0.07^{aA}
3	4.50 ± 0.15^{aB}	4.34 ± 0.06^{abB}	4.34 ± 0.08^{abAB}	4.26 ± 0.09^{bAB}	4.31 ± 0.09^{abB}
6	4.75 ± 0.09^{aBC}	4.65 ± 0.24^{bC}	4.61 ± 0.26^{bBC}	4.41 ± 0.09^{cBC}	4.57 ± 0.16^{bC}
9	5.80 ± 0.22^{aBC}	5.82 ± 0.10^{aD}	4.94 ± 0.09^{bC}	4.52 ± 0.15^{cBC}	4.64 ± 0.24^{cC}
12	5.99 ± 0.12^{aD}	5.84 ± 0.03^{aE}	5.45 ± 0.11^{bC}	5.18 ± 0.15^{cC}	5.33 ± 0.16^{bC}
Aerobic psychrotrophic count (log CFU/g)					
0	4.14 ± 0.15^{aA}	4.14 ± 0.15^{aA}	4.14 ± 0.15^{aA}	4.14 ± 0.15^{aA}	4.14 ± 0.15^{aA}
3	5.07 ± 0.08^{abB}	5.26 ± 0.07^{aB}	4.99 ± 0.07^{bB}	4.67 ± 0.08^{cB}	4.76 ± 0.14^{bB}
6	5.52 ± 0.09^{aC}	5.37 ± 0.06^{aB}	5.11 ± 0.07^{bB}	5.05 ± 0.07^{bC}	4.96 ± 0.11^{cB}
9	6.72 ± 0.06^{aD}	6.59 ± 0.05^{aC}	6.54 ± 0.09^{aC}	6.30 ± 0.14^{bD}	6.24 ± 0.10^{bC}
12	7.52 ± 0.01^{aE}	7.07 ± 0.04^{bD}	7.03 ± 0.17^{bD}	6.41 ± 0.05^{cD}	6.33 ± 0.05^{cC}
Yeast and molds (log CFU/g)					
0	4.53 ± 0.07^{aA}	4.53 ± 0.07^{aA}	4.53 ± 0.07^{aA}	4.53 ± 0.07^{aA}	4.53 ± 0.07^{aA}
3	5.50 ± 0.10^{aB}	5.14 ± 0.06^{bB}	4.82 ± 0.13^{cB}	4.83 ± 0.11^{cB}	5.13 ± 0.65^{bB}
6	5.86 ± 0.23^{aC}	5.59 ± 0.06^{bC}	5.19 ± 0.08^{cC}	5.11 ± 0.03^{cC}	5.14 ± 0.09^{cB}
9	6.32 ± 0.02^{aD}	5.96 ± 0.01^{bD}	5.92 ± 0.02^{bD}	5.81 ± 0.03^{bD}	5.62 ± 0.03^{dC}
12	7.06 ± 0.04^{aE}	6.24 ± 0.07^{cE}	6.59 ± 0.14^{bE}	6.01 ± 0.07^{dD}	6.20 ± 0.20^{cD}

Values with different lowercase letters at the same time point and uppercase letters of the same group indicate significant differences ($P < 0.05$), respectively. Note: FG: fish gelatin; GSE: grape seed extract.

Table 2
Effect of vacuum impregnation of FG-GSE coatings on the protein secondary structures of tilapia fillets.

Ratio (%)	Storage time (d)	Treatment				
		Control	FG	FG + 0.3% GSE	FG + 0.6% GSE	FG + 0.9% GSE
α-helix	0	56.19 ± 3.11^{aA}	56.19 ± 3.11^{aA}	56.19 ± 3.11^{aA}	56.19 ± 3.11^{aA}	56.19 ± 3.11^{aA}
	12	40.69 ± 8.42^{aB}	46.67 ± 2.91^{bB}	47.71 ± 4.09^{bB}	48.29 ± 0.88^{bB}	49.41 ± 3.88^{bcB}
β-sheet	0	19.10 ± 1.98^{aA}	19.10 ± 1.98^{aA}	19.10 ± 1.98^{Aa}	19.10 ± 1.98^{aA}	19.10 ± 1.98^{aA}
	12	21.93 ± 5.63^{aB}	22.68 ± 2.74^{abB}	23.17 ± 3.49^{bB}	24.18 ± 1.00^{bB}	26.10 ± 1.08^{cB}
β-turn	0	11.86 ± 1.49^{aA}	11.86 ± 1.49^{aA}	11.86 ± 1.49^{aA}	11.86 ± 1.49^{aA}	11.86 ± 1.49^{aA}
	12	18.19 ± 0.15^{abB}	17.95 ± 1.84^{aB}	17.94 ± 1.07^{aB}	17.72 ± 2.08^{aB}	19.28 ± 0.81^{cB}
Random coil	0	12.85 ± 2.21^{aA}	12.85 ± 2.21^{aA}	12.85 ± 2.21^{aA}	12.85 ± 2.21^{aA}	12.85 ± 2.21^{aA}
	12	15.02 ± 2.63^{cB}	10.00 ± 2.81^{aA}	12.42 ± 1.67^{abA}	11.43 ± 1.53^{aA}	12.13 ± 1.99^{aA}

Values with different lowercase letters at the same time point and uppercase letters of the same group indicate significant differences ($P < 0.05$), respectively. Note: FG: fish gelatin; GSE: grape seed extract.

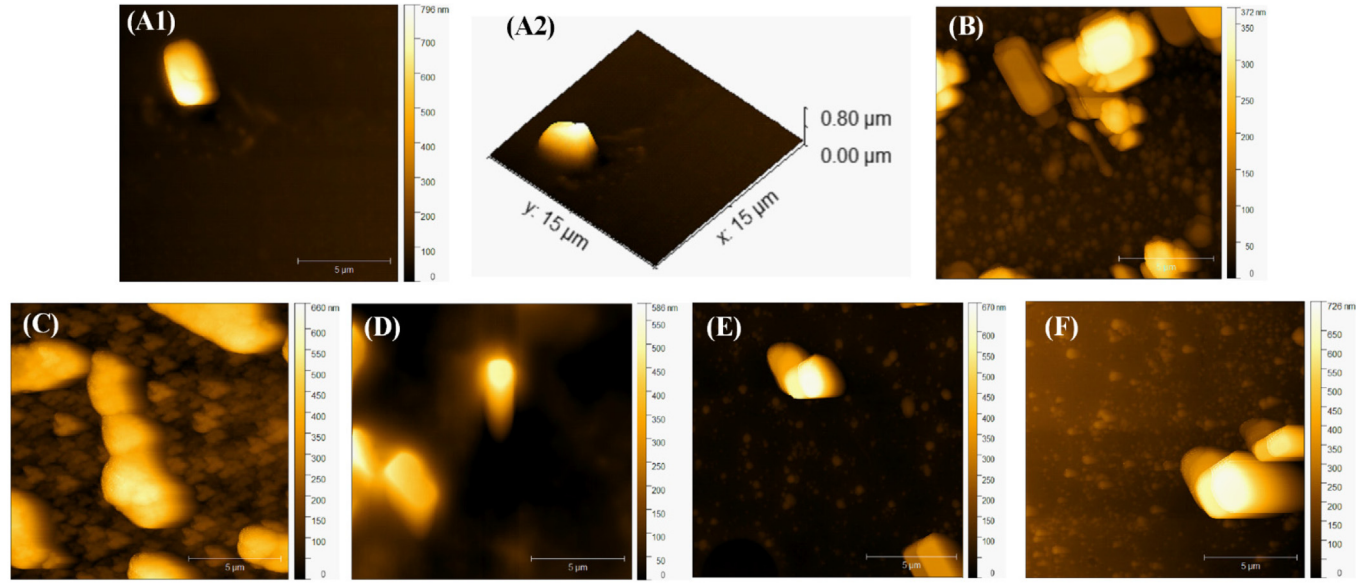
structure of the myofibrils during refrigerated storage, the amide I region was analysed using FTIR. Different peaks could be assigned to different secondary structures, including β-sheet (1611–1640 cm^{-1}), random coil (1642–1650 cm^{-1}), α-helix (1654–1662 cm^{-1}), and β-turn (1665–1693 cm^{-1} (Sow et al., 2019). Table 2 shows a quantitative analysis of the secondary structures of the myofibrils extracted from fillets under different treatments. The untreated fresh fillet contained 19.10% β-sheet, 12.85% random coil, 56.19% α-helix, and 11.86% β-turn. The ratio of α-helix decreased to 40.69–49.41%, while β-sheets increased to 21.93–26.10% after the storage period. Similar tendencies were found in previous studies, indicating the transformation of α-helices towards β-sheets under low temperature (Liu et al., 2010). The decrease in α-helices in the myofibrils might be related to the partial unfolding of helical structure under refrigeration during which the modification of non-covalent interactions occurs, including the hydrogen bonds that stabilise the secondary structures (Martínez et al., 2017). With the decline in the ratio of α-helix, more hydrophobic groups were exposed, resulting in an increase in surface hydrophobicity and consequently, a higher degree of drip loss during refrigeration.

The total ratio of more organised secondary structures (α-helices and β-sheets) in tilapia samples treated by FG-based coating solutions (69.35–75.51%) was significantly increased at the end of storage ($P < 0.05$) compared with those in the control group (62.62%). Moreover, a slightly looser conformation of the control group at day 12 was formed, which was also caused by the greater proportion of random coils (15.02%), which was significantly higher than that in the FG-treated groups (10.00–12.42%, $P < 0.05$). The results indicated that the FG coating resulted in stabilization of the myofibrillar protein structure during cold storage (Feng et al., 2017).

Furthermore, protective effects of GSE in inhibiting changes of myofibril secondary structures during cold storage were observed and were more pronounced for higher GSE concentrations. With the increase in the GSE concentration from 0 to 0.9%, a major increase of α-helices from 40.69% to 49.41%, and β-sheets from 21.93% to 26.10%, were observed. GSE is abundant in natural polyphenols, such as catechin, epicatechin, and proanthocyanidins. Previous studies indicated that the addition of such polyphenols led to increased stability of protein secondary structures upon protein-polyphenol interaction (Yang, Liu, Xu, Yuan, & Gao, 2014).

3.2.2. Morphological changes detected by AFM

To better understand the underlying effect of VI-introduced FG and GSE, the nanostructural changes of myofibrillar proteins extracted from tilapia fillets under different treatments were analysed using AFM. The images showed a rod-like nanostructure of myofibrils from fresh tilapia fillets (Fig. 2A), which was in accordance with the myofibrils from golden pomfret fillets (Feng et al., 2017). After cold storage, the rod-like configuration of the myofibrils was maintained at day 12 in all groups (Fig. 2B–F). However, the myofibrils decreased in size, which was caused by the deterioration and degradation of myofibrils during refrigeration. Aggregation and cross-linking of myofibrils was markedly observed in the control (Fig. 2B) and FG (Fig. 2C) groups, which was



(G)

Dimension	Storage time (d)	Treatment				
		Control	FG	FG + 0.3% GSE	FG + 0.6% GSE	FG + 0.9% GSE
Length/μm	0	4.89 ± 0.18 ^{aA}	4.89 ± 0.18 ^{aA}	4.89 ± 0.18 ^{aA}	4.89 ± 0.18 ^{aA}	4.89 ± 0.18 ^{aA}
	6	1.76 ± 0.49 ^{aB}	3.77 ± 0.94 ^{bB}	4.08 ± 1.26 ^{bB}	4.46 ± 1.07 ^{cB}	4.61 ± 1.35 ^{cA}
	12	1.33 ± 0.12 ^{aC}	3.39 ± 1.04 ^{bB}	3.88 ± 0.33 ^{cC}	4.16 ± 0.70 ^{cC}	4.22 ± 0.84 ^{cB}
Width/μm	0	3.67 ± 1.17 ^{aA}	3.67 ± 1.17 ^{aA}	3.67 ± 1.17 ^{aA}	3.67 ± 1.17 ^{aA}	3.67 ± 1.17 ^{aA}
	6	2.44 ± 0.34 ^{aB}	2.35 ± 0.45 ^{aB}	2.52 ± 0.42 ^{aB}	3.00 ± 0.64 ^{bB}	3.53 ± 0.66 ^{cA}
	12	1.97 ± 0.13 ^{aC}	2.16 ± 0.36 ^{aB}	2.48 ± 0.34 ^{bB}	2.76 ± 0.48 ^{bB}	3.23 ± 0.82 ^{cAB}
Height/nm	0	708.00 ± 48.5 ^{aA}	708.00 ± 48.5 ^{aA}	708.00 ± 48.5 ^{aA}	708.00 ± 48.5 ^{aA}	708.00 ± 48.5 ^{aA}
	6	273.00 ± 39.49 ^{aB}	318.75 ± 11.05 ^{bB}	429.50 ± 22.97 ^{cB}	567.85 ± 47.02 ^{cB}	609.00 ± 22.03 ^{dB}
	12	170.10 ± 14.75 ^{aC}	300.50 ± 32.35 ^{bB}	417.00 ± 18.86 ^{cB}	552.70 ± 33.41 ^{cB}	567.70 ± 25.10 ^{cB}

Fig. 2. Atomic force microscopy (AFM) images of myofibrils under different treatments (A–F), and changes of length, width, and height (G). (A1) and (A2) represent the top view and 3-dimensional view of myofibrils from fresh tilapia before treatment, respectively; (B) control group at day 12; (C) fish gelatin (FG) group at day 12; (D) FG + 0.3% grape seed extract (GSE) group at day 12; (E) FG + 0.6% GSE group at day 12; (F) FG + 0.9% GSE group at day 12. Values in (G) with different lowercase letters at the same time point and different uppercase letters at the same group indicate significant differences ($P < 0.05$), respectively.

induced by oxidation and became more visible in the advanced oxidative conditions (Bao et al., 2018). Compared with the control group, the vacuum impregnated FG-GSE coatings enlarged the myofibrils and no small fragments were observed in these treatment groups.

To better understand the effect of the different treatments, quantitative analysis was conducted using the Gwyddion software, which revealed the detailed information of the myofibrils in each group, including the height, width, and length (Fig. 2G). The length, width, and height of the myofibrils from fresh fillets before any treatment were 4.89 μm, 3.67 μm, and 708 nm, respectively. After cold storage, the length for the control group decreased significantly to 1.76 μm at day 6 and 1.33 μm at day 12 ($P < 0.05$). Compared with the control group, the addition of FG-based coating solutions efficiently prevented the degradation of myofibrils during refrigeration. Specifically, the length of the FG treated myofibril was in the range of 3.77–4.61 μm at day 6 and 3.39–4.22 μm at day 12, which was much longer than that of the control group. No significant differences of myofibril length were observed among the three GSE combination groups at the end of the storage ($P > 0.05$).

For the control group, the width of the myofibril continually decreased, from 3.67 to 1.97 μm, during storage. The width of the myofibrils for the FG treated groups decreased in the first 6 days but not in the following 6 days ($P < 0.05$). Meanwhile, the FG + 0.9% GSE group had the largest width (3.23 μm) at day 12, which was comparable with that of the fresh fillet. A similar changing tendency was observed for the height of myofibrils. The height of the myofibrils in the control group decreased significantly from 708 nm at day 0 to 273 nm at day 6 and further declined to 170.1 nm at day 12 ($P < 0.05$). The height of myofibrils from the FG-treated groups was notably larger than that of myofibrils of control at the same day of storage, especially for the 0.6% GSE and 0.9% GSE groups.

The degradation of myofibrils during refrigeration may be related to the activities of endogenous enzymes and microorganisms. The proteolytic effect of endogenous enzymes (e.g. cathepsin B) may result in myofibril filament disassembly by decreasing their height, width, and length. However, slower and milder degradation of myofibrils were observed during the late storage period. This was probably caused by some endogenous proteases that took part in myofibrillar detachment only at the early stage of storage (Ge, Xu, Xia, Zhao, & Jiang, 2018).

The combination of GSE and FG resulted in reduced degradation of myofibrils, revealing a protective effect on their nanostructure. GSE and FG interacted with each other and formed a strong network that acted as a barrier to oxygen, water, and microorganisms, thus preserving the integrity of the myofibrils (Li, Miao, Wu, Chen, & Zhang, 2014). Furthermore, FG incorporated with GSE in the edible coatings protected the myofibrils by reducing the drip loss and stabilizing the hydrated water around them. Compared with the low GSE treatment, higher GSE concentrations (0.9%) showed a greater effect on protecting the integrity of myofibrils during chilled storage.

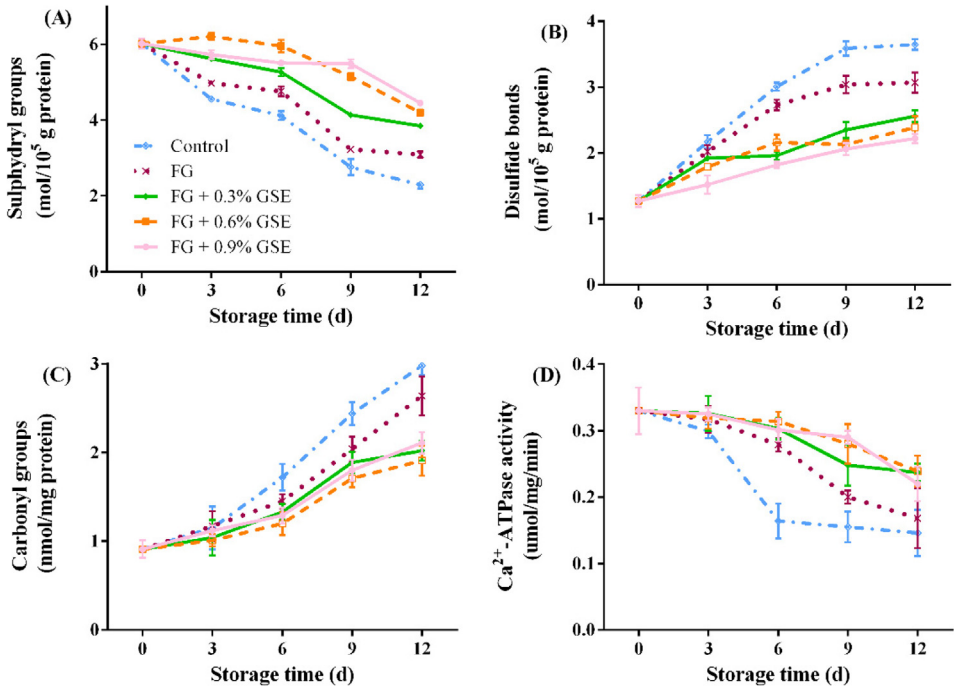


Fig. 3. Effects of vacuum impregnation of fish gelatin (FG)-based coatings incorporated with grape seed extract (GSE) on protein oxidation of tilapia fillets stored at 4 °C. (A) sulfhydryl groups; (B) disulphide bonds; (C) carbonyl groups; (D) Ca^{2+}-ATPase activity.

3.3. Effect of VI with FG and GSE on protein oxidation

3.3.1. Changes in sulfhydryl groups and disulphide bonds

The changes in the sulfhydryl groups and disulphide bond concentrations of different tilapia fillets stored under refrigeration are shown in Fig. 3A and Fig. 3B, respectively. The sulfhydryl group content decreased significantly from 6.02 mol/10^5 g protein in all treated groups to 2.29–4.45 mol/10^5 g protein after 12 days ($P < 0.05$). Research in various fish species has reported similar changing tendencies in the sulfhydryl group concentrations during refrigerated storage (Lu et al., 2017). It is notable that the addition of GSE resulted in a significantly higher sulfhydryl group content compared with that in the control group and FG-treated group at the same storage time ($P < 0.05$), especially for the 0.9% GSE group. Differences in sulfhydryl group levels were found for the different tilapia fillets, with the 0.9% GSE and 0.6% GSE treated fillets exhibiting up to 35.88% and 30.23% higher concentrations versus the other groups, respectively. However, no significant difference was observed between the 0.6% GSE and 0.9% GSE groups at the end of the storage. The protective effect on the sulfhydryl group content between the different treated groups could probably be attributed to the antioxidant capacity of the abundant phenolic compounds in GSE. Previous studies attributed the protective effect of different antioxidant agents or antioxidant foods (such as chitosan, green tea extract, and fermented soybeans) on sulfhydryl groups to oxidation reactions in myofibrils (Özen & Soyer, 2018).

Compared with sulfhydryl groups, disulphide bonds underwent an inverse change tendency, i.e., their number increased gradually but significantly during the storage period ($P < 0.05$). For the control group and the FG group, the content of disulphide bonds increased from 1.27 mol/10^5 g protein to 3.65 mol/10^5 g protein and 3.07 mol/10^5 g protein, respectively. The addition of GSE controlled this increasing trend by up to 113.60% (FG + 0.9% GSE group) at day 12. This phenomenon suggested that the decrease in total sulfhydryl groups and increase in total disulphide bonds might be caused by the transformation from sulfhydryl groups to disulphide bonds via oxidation or disulphide interchanges during cold storage (Thawornchinsombut & Park, 2005).

3.3.2. Changes in carbonyl groups

The carbonyl group is a major chemical product of protein oxidation. Protein oxidation results in the cleavage of the backbone, the formation of crosslinking, and the transformation of some amino acid residues towards carbonyl compounds (Wang, Li, Yuan, Lin, & Pavase, 2017). Therefore, to investigate the effects of different coating solutions introduced via the VI process on protein oxidation, the carbonyl level during refrigerated storage was analysed. The carbonyl group contents in all of tilapia fillets increased over the whole period of storage, as shown in Fig. 3C. Previous studies reported similar changes in carbonyl contents in fish under cool or supercool storage conditions (Liu et al., 2014). However, decreases or fluctuations in carbonyl content might occur during prolonged storage because carbonyl derivatives may degrade or interact with other cell components (Nikoo, Benjakul, & Xu, 2015). During first 3 days, there was no significant difference among the groups ($P > 0.05$). Thereafter, GSE began to take an effect. The rate of increase in the carbonyl content was markedly slower in the groups treated with GSE. The use of antioxidants is an effective strategy to control the formation of carbonyl groups during protein oxidation (Zhang, Li, Jia, Huang, & Luo, 2018). Similar results have been reported previously. For example, the application of potato peel extract in minced horse mackerel resulted in a slower increase in carbonyl groups during storage (Farvin et al., 2012). Interestingly, the effect of GSE on carbonyl groups was not concentration-dependent, thus a higher GSE content did not result in significant reduction in carbonyl when compared with a lower concentration. As a consequence of carbonylation caused by oxidative reactions, the water holding capacity of the myofibrils declined (Estévez, 2011). This was confirmed by Pearson Correlation analysis, which showed a significant correlation between the carbonyl content and drip loss during cold storage (r = 0.9825–0.9988, $P < 0.05$).

3.3.3. Changes in Ca^{2+}-ATPase activity

Ca^{2+}-ATPase activity is regarded as an indicator of the integrity of myofibrils and is responsible for the globular heads of myosin. As

shown in Fig. 3D, the Ca^{2+}-ATPase activity decreased during storage for all treated groups. In the present study, a decrease from 0.33 to 0.15 μmol/mg/min in Ca^{2+}-ATPase activity was observed in the control group ($P < 0.05$). GSE showed a protective effect on the Ca^{2+}-ATPase activity. The Ca^{2+}-ATPase activity of the GSE-treated fillets was significantly higher than that in the control group and FG group after 9 days ($P < 0.05$). At the end of storage, the Ca^{2+}-ATPase activity of GSE-treated fillets reached 0.24 μmol/mg/min (0.6% GSE), which was 1.6 times higher than that in the control group. The changing trend of Ca^{2+}-ATPase activity and protective effect of GSE on Ca^{2+}-ATPase activity were associated with changes in sulfhydryl groups and disulphide bonds. This might be because the reduction in Ca^{2+}-ATPase activity was associated with the oxidation of sulfhydryl groups on the myosin globular head and the aggregation of myosin (Kong et al., 2013). The formation and cross-linking of disulphide bonds between or within polypeptides may result in changes of protein conformation such as protein aggregation, and inhibite swelling of oxidised myofibrils, thus suppressing the Ca^{2+}-ATPase activity (Bao et al., 2018; Kong et al., 2013; Mathew & Abraham, 2006).

3.4. Schematic illustration

A schematic illustration of the effect of FG-GSE coating introduced by VI on protein oxidation and degradation during chilled storage was proposed and is shown in Fig. 4. When tilapia fillets were treated with deionised water, free radicals present in fish tissues increased the degree of lipid and protein oxidation. Reactive oxygen species preferentially attacked side chains of amino acid residues, which led to the formation of carbonyl group and disulphide bond via sulfhydryl group oxidation. In turn, these reactions caused protein unfolding, aggregation, cross-linking, fragmentation of myofibrillar proteins, and, as a result, reduced protein solubility (He et al., 2018). After VI with FG-GSE coating, protein oxidation and degradation were inhibited. The antioxidant polyphenols contained in the GSE gradually released from FG into fillets and began to exert a preservative effect. It is reported that the antioxidant effect of plant phenolics against lipid oxidation is expected to reduce protein oxidation to some extent by minimising the formation of secondary lipid oxidation products, thereby blocking their pro-oxidant effects on proteins (Haak, Raes, & De Smet, 2009). Moreover, complicated interactions between FG and GSE existed. The polyphenol-protein interactions may take place via hydrogen bonding and hydrophobic interactions, resulting in a stronger network than control group and FG group. The concentration of GSE affected the interaction of it with FG. When the concentration of GSE was relatively low, FG and GSE might form complexes. With the increase of GSE content, FG-GSE aggregates began to form in the subsequent stage of association. However, when the concentration of GSE was high enough, aggregates coalesced and precipitation occurred (McRae, Falconer, & Kennedy, 2010). In this study, oxidative reactions in fillets treated with FG-0.9% GSE (no precipitation) were significantly retarded during 12-day storage as compared with other treatments. Under such inhibited oxidative stress, fewer sulfhydryl groups were exposed and oxidised. In

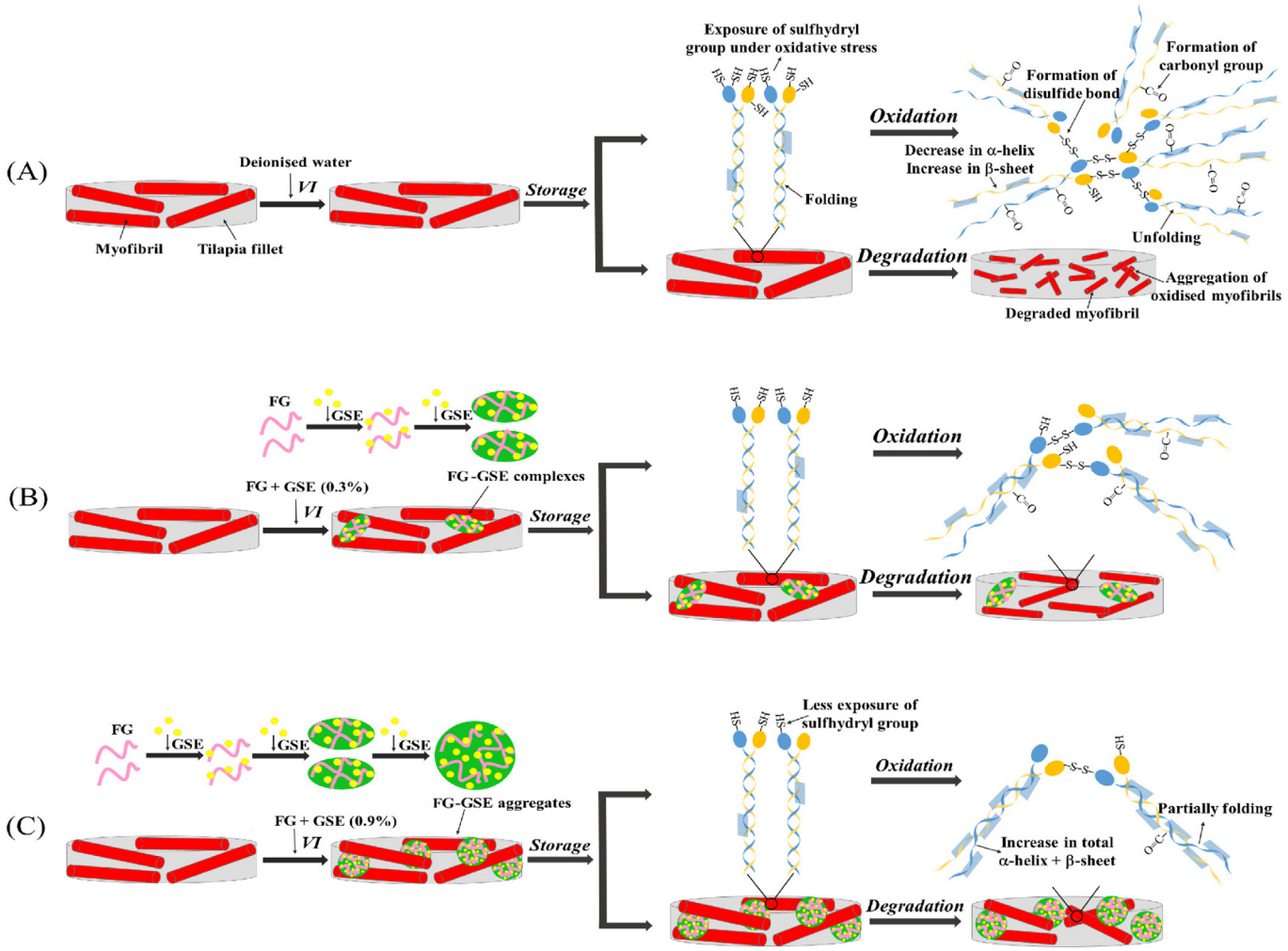


Fig. 4. Schematic mechanism of the effects of vacuum impregnation of fish gelatin (FG)-based coatings incorporated with grape seed extract (GSE) on myofibrils of tilapia fillets stored at 4 °C. (A) treatment with deionised water; (B) treatment with FG + 0.3% GSE coating; (C) treatment with FG + 0.9% GSE coating.

addition, the formation of carbonyl groups from basic amino acid residues was reduced. As a consequence of these inhibited chemical reactions, the balance between protein intramolecular interactions and protein-water interactions was better maintained, thus, inhibiting protein denaturation and unfolding (Estévez, 2011). Moreover, the interaction between FG-GSE aggregates and myofibrillar proteins may also be helpful to maintain a more organised secondary structure (α-helices and β-sheets).

4. Conclusion

The present study showed that the introduction of FG-based coatings incorporated with GSE using the VI process could maintain the quality and freshness of refrigerated fillets by reducing the drip loss, microbial growth, and adverse texture changes. Compared with the control group treated with deionised water only, FG-GSE treatment exerted significant protective effects against protein oxidation, including the prevention of carbonyl group and disulphide bond formation, a decrease in the total sulfhydryl groups, and a smaller reduction in Ca^{2+}-ATPase activity. Furthermore, decreased degradation of myofibrillar proteins during refrigerated storage was observed in the presence of GSE. Overall, 3% FG + 0.9% GSE coating introduced via VI showed the best preservative effect on the quality of fish fillet during cold storage and extending their shelf life. The results indicated that incorporation of edible GSE into FG improved the barrier ability of the pure FG coating and therefore, is a promising and effective way to extend the shelf life of food.

Declaration of Competing Interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with this manuscript. We have no financial and personal relationships with other people or organizations that can inappropriately influence our work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.05.054.

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