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Characterization of a starfish gelatin film containing vanillin and its application in the packaging of crab stick

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Abstract To explore the use of starfish gelatin (SFG) films as a biodegradable material, SFG from starfish was extracted and used as a film material. In addition, to provide antimicrobial activity and enhanced flavor of SFG films, vanillin was incorporated. As the concentration of vanillin increased, the tensile strength of the films increased and water vapor permeability decreased. With regard to the structural characteristics of SFG films containing vanillin, the microstructure of the SFG films was not affected by the addition of vanillin. In addition, the SFG films containing vanillin exhibited antimicrobial activity against *Listeria monocytogenes*. As the application of the SFG films, crab sticks were packed with SFG films containing 0.05% vanillin. During storage, the populations of *L. monocytogenes* inoculated on crab sticks wrapped with SFG films containing vanillin were lower than those on the control sample, suggesting that SFG films containing vanillin can be useful in active food packaging.

Keywords: antimicrobial activity, physical property, protein film, starfish, vanillin

Introduction

Because of increasing environmental concerns related to plastic packaging, research attention has been directed toward biodegradable films with suitable physical properties that can act as barriers to moisture and gas (1). Biodegradable films can carry various active materials such as antioxidants and antimicrobial agents that can prolong the shelf life of foods (2). However, they also have several limitations, such as inadequate thermal resistance, inferior physical properties, and higher price than plastic films (3). Therefore, biodegradable films need to be developed using less expensive materials such as by-products of food processing or waste products.

Starfish (*Asterias amurensis* and *Asterina pectinifera*) is abundant in the West Sea and the East Sea in Korea, and the starfish population is growing. Starfish is usually collected and disposed of or used as a fertilizer because they can cause severe damage to fishery resources and marine ecosystems (4). In order to use them as a value-added source, their pharmacological compounds have been studied (5). In particular, several secondary metabolites such as alkaloids (6), steroid glycosides (7), peptides (8), and ceramides (9) have been identified in starfish. The body wall of starfish is known to be rich in collagen (10), an insoluble fibrous protein, that can be converted into gelatin by partial hydrolysis (11). Gelatin derived from starfish can be used as a reasonable film source for food packaging because it acts as a light and oxygen barrier and prevents lipid oxidation and moisture loss (12).

To enhance flavor of protein films, vanillin can be added into the film. Vanillin is obtained from the cured bean or pod of the tropical *Vanilla* orchid and is used as a food preservative and flavor enhancer as well as a cross-linking agent (13). Moreover, it has several functional properties, including antioxidant and antimicrobial activities (14,15). Because of the aldehyde group in the vanillin structure, the growth of pathogenic microorganisms can be suppressed effectively (16).

Based on the beneficial properties of vanillin, this study aimed to develop an antimicrobial starfish gelatin (SFG) film containing vanillin and apply it to the packaging of crab sticks. In particular, gelatin extracted from disposed and inexpensive starfish was used as a biodegradable film source for food packaging in this study, and this has never been reported previously.

Materials and Methods

Materials Starfish were obtained from Masan Happogu (Changwon, Korea). Sorbitol, tartaric acid, vanillin, and Tween 80 were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Crab stick was purchased from a local market (Daejeon, Korea).

Extraction of SFG SFG was extracted according to the method of Jongjareonrak *et al.* (17) with minor modifications. Dried starfish were cut into small pieces and blended into a powder. To remove non-collagenous proteins, ground starfish was soaked in 6 volumes

of 0.1 N NaOH for 1 h. After centrifugation at 10,000 × *g* for 15 min, the pellet was rinsed with tap water and immersed in 6 volumes of 0.5% tartaric acid. The mixture was then stirred for 1 h and centrifuged again at 10,000 × *g* for 15 min. After washing the precipitate with tap water, the pH of swollen starfish was changed to 6.0 with tartaric acid and the starfish was homogenized using a sonicator (Model-GE 750; Sonics & Materials, Newtown, CT, USA) for 30 min. The mixture was then heated at 80°C for 3 h. After heating, the solution was centrifuged at 10,000 × *g* for 1 h, and the supernatant was collected and lyophilized.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) SDS-PAGE was conducted according to the method of Ahmad *et al.* (11) with minor modifications. Ten milliliters of 5% SDS solution was added to 400 mg of SFG and continuously mixed at 20°C for 12 h. After centrifugation at 3,000 × *g* for 5 min, the supernatant was blended with sample buffer (1 M Tris-HCl, 10% SDS, 50% glycerol, 5% dithiothreitol, 8 M urea, and 1% bromophenol blue) and loaded onto a polyacrylamide gel consisting of a 5% stacking gel and 12.5% separating gel. The electrophoresis was carried out at 20 mA per gel, and the gel was stained using Coomassie Brilliant Blue R-250 after electrophoresis.

Preparation of the SFG films containing vanillin The SFG (5 g), vanillin (0.03, 0.05, 0.07, or 0.1 g), and emulsifier (Tween 80, 25% of vanillin, w/w) were added to 100 mL of distilled water, blended at room temperature for 30 min, and processed with a homogenizer (Ultra-Turrax T25, IKA, Staufan, Germany) at 12,000 rpm for 5 min. The mixture was then sonicated (Model-GE 750; Sonics & Materials) for 8 min, degassed under vacuum for 5 min, and then heated at 40°C for 30 min. After heating, sorbitol (1.5 g) as a plasticizer was added into the cooled SFG solution and stirred for 30 min. The solution was passed through a cheese cloth and the filtrate (80 mL) was poured into a Teflon-coated plate to cast the films.

Physical properties of the SFG film The tensile strength (TS) and elongation at break (E) of the SFG films were evaluated using an Instron (M250-2.5 CT; The Testometric Company Ltd., Lancashire, UK) according to the ASTM method D882-91. After conditioning at a constant temperature of 25°C and 50% relative humidity (RH) for 48 h, the SFG films were cut into uniform size (2.54 × 10 cm^2). The TS and E were determined for five independent replicates of each film.

Water vapor permeability (WVP) The WVP of the SFG film was evaluated according to the ASTM Method E96-95 with slightly modifications. Distilled water (18 mL) was poured into a polymethyl-acrylate cup, and the top of each cup was covered by an SFG film (2 × 2 cm^2). Reductions in the weight of the cup were measured in a chamber at 25°C and 50% RH every hour. Three independent replicates were evaluated for each film.

Determination of Hunter b value and opacity The Hunter b value of the SFG films was measured using a colorimeter (CR-400; Minolta, Tokyo, Japan). Three measurements were conducted for each sample and the color value was expressed using a Hunter Lab system. In addition, the opacity of the SFG films was determined by measuring the absorbance of the SFG films at 600 nm (18). The absorbance was obtained using a spectrophotometer (UV-2450; Shimadzu Corporation, Kyoto, Japan). The film opacity was calculated using the following equation.

$$\text{Opacity} = Abs_{600}/x$$

where Abs_{600} is the absorbance value at 600 nm and x is film thickness (mm).

Thermo-gravimetric analysis (TGA) Thermo-gravimetric analysis of the SFG films was carried out using a Mettler Toledo DSC 1 (Mettler Toledo, Schwerzenbach, Switzerland). The SFG film samples were heated from 25 to 600°C at a heating rate of 10°C/min under nitrogen atmosphere.

Scanning electron microscopy (SEM) The microstructure of the surface of the SFG films containing vanillin was observed using a scanning electron microscope (LYRA3 XMU, Tescan, Brno, Czech Republic) with an accelerating voltage of 30 kV. All SFG film samples were mounted on metal holders and then coated with a platinum layer under vacuum before analysis.

Antimicrobial activity of the SFG film containing vanillin A disc diffusion test was conducted to measure the antimicrobial activity of the SFG films with added vanillin according to the method described by Song *et al.* (3). *Listeria monocytogenes* (ATCC 19115) was cultured in brain heart infusion at 37°C for 24 h, and Oxford medium base was used as the culture medium. The inoculum (0.1 mL) was placed onto selective medium, and the SFG film discs with a diameter of 10 mm were put onto the inoculated medium and cultured at 37°C for 24 h. The diameter of the inhibition zone was evaluated using a digimatic caliper (Model 500-181-20; Mitutoyo Co., Kawasaki, Japan).

Packaging of crab sticks with the SFG films Crab stick samples were cut into pieces that were approximately 1.5 × 4 × 1 cm (10 g) using a sterile stainless steel knife. Prior to inoculation, each side of crab stick was sterilized under UV light for 10 min. *L. monocytogenes* (ATCC 19115) was incubated at 37°C in brain heart infusion until 10^6 CFU/mL was reached. The inoculum of *L. monocytogenes* (1 mL) was spread on each side of the crab stick samples with a sterile glass spreader and dried for 20 min. The inoculated crab stick samples were then wrapped with the SFG or SFG-vanillin film. Crab sticks that were packed in polyethylene terephthalate without the film were used as control samples, and all samples were kept at 4±1°C for 9 days.

Microbiological analysis To determine the microbial count of crab sticks during storage, the crab stick samples were put into sterile sample bags with 0.1% peptone water (90 mL). The samples were homogenized for 3 min with a stomacher (MIX 2; AES Laboratoire, Combourg, France) and diluted with 0.1% peptone water. Each diluted solution was then spread on Oxford medium and cultured at 37°C for 48 h. Microbial counts were the averages of 3 replications and represented as log colony forming units (CFU)/g.

Statistical analysis To conduct analysis of variance and Duncan's multiple range tests (p<0.05), SAS program version 9.4 (SAS Institute, Inc., Cary, NC, USA) was used. All data are indicated as the mean ± standard deviation. All tests were replicated 3 times, but measurements of the physical and optical properties of the films were conducted 5 times.

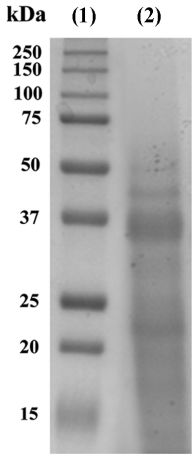

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of SFG. (1) Molecular weight marker proteins, (2) SFG

Results and Discussion

SDS-PAGE profile of SFG The protein pattern extracted from starfish was analyzed by SDS-PAGE (Fig. 1). Major SFG bands were observed at 37 and 22 kDa. In contrast, Park *et al.* (19) reported that pepsin-soluble starfish collagen had a major protein band below 96 kDa. This difference could be explained by the high SFG extraction temperature (80°C) in this study, which made probable denaturation and cleavage of protein molecules due to heat-labile characteristic of the protein (17).

Physical properties of the SFG films containing vanillin To prepare SFG films with antimicrobial activities and flavor, vanillin was incorporated into the SFG film-forming solution. The physical properties of the films are indicated in Table 1. The physical properties of the SFG films were affected by the concentrations of added vanillin. As the vanillin concentration was increased from 0.03 to 0.1%, the TS of the SFG films increased, whereas the E value decreased. The SFG film containing 0.1% vanillin had the highest TS (32.99 MPa), while the SFG film without vanillin had the lowest TS (19.61 MPa). Generally, the addition of a cross-linking agent increases the TS. Strong cross-links between the protein molecules are formed by the addition of such an agent, resulting in an increase in TS (20). Sangsuwan *et al.* (21) reported that the TS of chitosan-methyl cellulose-based films increased and the E value decreased when vanillin was incorporated. The increase in TS might be due to the structure of vanillin. Vanillin has an aromatic benzene ring, which could make the film more rigid and can also lead to a decrease in the degree of mobility of the protein molecules (21). Our results were consistent with those of Ravindra *et al.* (16), where the TS of the chitosan films increased and the E value decreased with increasing concentrations of vanillin. The strong hydrogen bonds in the chitosan film containing vanillin were formed by the addition of vanillin, and therefore chitosan films became more compact and less elastic (16). The WVP of the SFG films containing vanillin was low compared with that of the control film without vanillin. Hernández-Muñoz *et al.* (22) also reported a reduction in WVP for glutenin-rich films containing cross-linking agents such as formaldehyde, glyoxal, and glutaraldehyde. Increased network formation with low molecular weight aldehyde groups could be responsible for the decrease in the free volume of the film matrix and water vapor transmittance. As a result, the water vapor barrier property of the SFG film was improved with the addition of vanillin.

Color and opacity The color and opacity of the SFG films containing vanillin are shown in Table 1. The addition of vanillin significantly affected the color and opacity of the films. The Hunter b value of the SFG films increased from 31.34 to 43.68 as vanillin concentration was increased. The increase in the b value of the films indicated that the color of the SFG films became more yellow. The change in the color

Table 1. Physical and optical properties of the SFG films containing various amounts of vanillin

Vanillin (%)	Tensile Strength (MPa)	Elongation at break (%)	WVP (x10^{-9} g m/m^2 s Pa)	Hunter b	Opacity
0	19.61±0.48$^{d,1)}$	30.50±0.49^{a}	2.37±0.18^{a}	31.34±0.08^{e}	1.94±0.07^{d}
0.03	21.96±0.77^{cd}	10.02±0.51^{b}	2.20±0.14^{ab}	33.36±0.14^{d}	2.02±0.08^{d}
0.05	24.05±0.68^{c}	9.10±0.01^{b}	2.05±0.17^{ab}	35.97±0.03^{c}	2.21±0.03^{c}
0.07	27.80±0.93^{b}	5.47±0.28^{c}	1.98±0.08^{b}	38.54±0.20^{b}	2.45±0.01^{b}
0.1	32.99±0.87^{a}	3.71±0.69^{d}	1.85±0.08^{b}	43.68±0.09^{a}	3.67±0.07^{a}

[1)]Values are mean±SD (n=5). Any means in the same column followed by different letters are significantly (p<0.05) by Duncan's multiple range test.

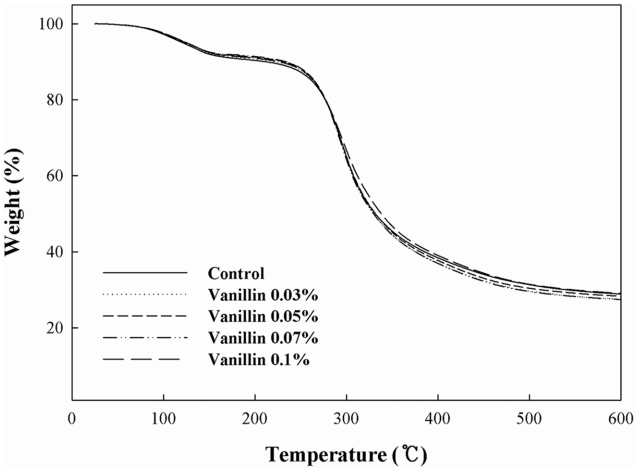

Fig. 2. Thermo-gravimetric curves of SFG films containing various amounts of vanillin.

of the SFG films containing vanillin could be explained by the yellow component of vanillin (23). Additionally, as the amount of vanillin increased, the opacity of the SFG films increased. Similarly, Sangsuwan *et al.* (21) reported significant increases in the b value and opacity of the chitosan-methyl cellulose-based film containing vanillin. Gómez-Estaca *et al.* (24) also indicated that sole and catfish gelatin films incorporated with borage extract possessed high opacity values compared with the films without borage extract. Therefore, our results suggest that the addition of vanillin to the SFG films can enhance light barrier properties of the films.

Thermal properties of SFG films containing vanillin The thermal stabilities of SFG films with different amounts of vanillin were determined using TGA and their corresponding results are shown in Fig. 2. The weight losses of all SFG films containing vanillin involved two major steps. In the initial weight loss step, the weight loss was mainly due to the loss of water molecules (11). The second weight loss was related to the decomposition of protein and sorbitol in the SFG films. Overall, the thermal stability of the SFG films was not affected much by the addition of vanillin. However, it should be noted that there was slight change in the residual mass value of the SFG films at 600°C by the addition of vanillin.

Table 2. Antimicrobial activity of the SFG films containing various amounts of vanillin against *L. monocytogenes*

Vanillin (%)	Inhibition zone (mm)
	L. monocytogenes
0	0
0.03	17.24±0.73$^{d,1)}$
0.05	19.90±0.74^{c}
0.07	22.41±0.62^{b}
0.1	25.24±0.65^{a}

[1)]Values are mean±SD (n=3). Any means in the same column followed by different letters are significantly (p<0.05) different by Duncan's multiple range test.

Scanning electron microscopy (SEM) analysis The surface SEM images of the SFG films containing different amounts of vanillin were observed (Fig. 3). The surface of the SFG films was not affected by the concentration of added vanillin, and the films had homogeneous and smooth surfaces without cracks. Ravindra *et al.* (16) reported similarly that the surface of the chitosan films with vanillin was homogeneous and smooth because of complete dispersion between chitosan and vanillin. Therefore, these results indicate that vanillin had good miscibility with SFG, contributing to the higher TS and lower WVP values of the SFG films.

Antimicrobial activity of SFG films containing vanillin The antimicrobial activities of the SFG films containing vanillin are shown in Table 2. The disc diffusion test was used to assess the antimicrobial activities of the films. The addition of vanillin into the films led to an inhibitory effect against the growth of *L. monocytogenes*. The inhibition zones of the SFG films containing vanillin increased from 17.24 to 25.24 mm with increasing vanillin concentrations, whereas the SFG

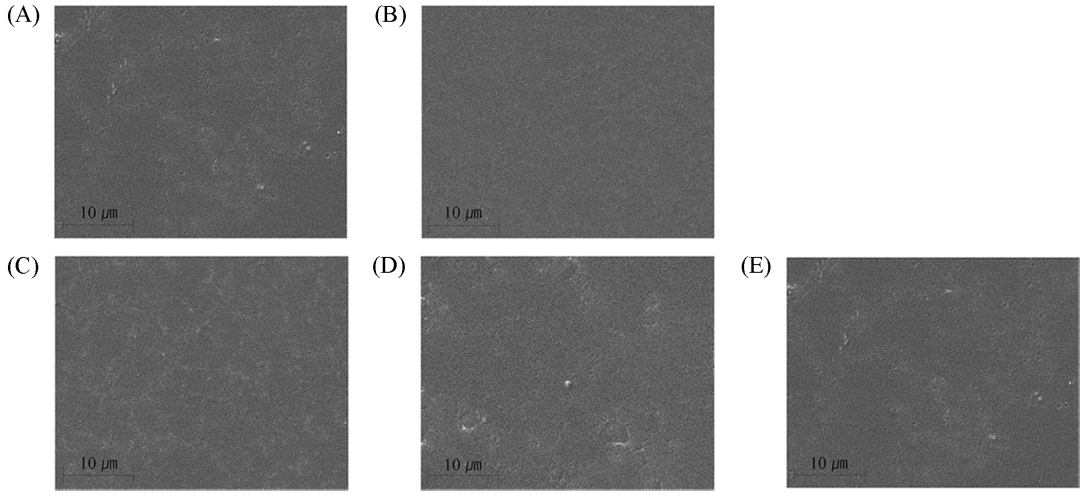

Fig. 3. SEM images of SFG films containing various amounts of vanillin. (A) Control, (B) 0.03 %, (C) 0.05 %, (D) 0.07 %, (E) 0.1 %

Table 3. Changes in the populations of *L. monocytogenes* in crab stick during storage at 4°C (unit: log CFU/g)

Sample	Storage time (day)			
	0	3	6	9
Control	6.02±0.16[a,1)]	6.95±0.07[a]	8.18±0.01[a]	8.91±0.12[a]
SFG film without vanillin	6.02±0.16[a]	6.75±0.18[a]	7.90±0.14[b]	8.59±0.16[b]
SFG film containing vanillin	6.02±0.16[a]	6.27±0.16[b]	6.83±0.06[c]	7.43±0.12[c]

[1)]Values are mean±SD (n=3). Any means in the same column (a-c) followed by different letters are significantly (p<0.05) different by Duncan's multiple range test.

films without vanillin had no inhibition zone. Similarly, Cava-Roda *et al.* (25) observed the antimicrobial activity of vanillin in *L. monocytogenes* populations in milk. Moon *et al.* (26) also found that 40 mM vanillin effectively inhibited the growth of *L. monocytogenes* in apple juice at low pH. The antimicrobial activity of vanillin is mainly attributed to its hydrophobicity. Because of this nature of vanillin, the cytoplasmic membrane of bacterial cells is disrupted through interactions between vanillin and protein molecules, resulting in the loss of respiratory rate in bacterial cells.

Microbiological analysis of crab sticks packed with SFG films The change in the population of *L. monocytogenes* on the crab sticks wrapped with SFG films containing 0.05% vanillin was determined during 9 days of storage (Table 3). The initial population of *L. monocytogenes* on the crab stick was 6.02 log CFU/g. The populations of *L. monocytogenes* in all samples continuously increased during 9 days of storage at 4°C, but the crab sticks packed with SFG films containing vanillin had a lower increase in the microbial population compared with the control sample. In particular, after 9 days, the population of *L. monocytogenes* on the control sample was 8.91 log CFU/g, whereas the microbial population on the crab stick wrapped with SFG films containing 0.05% vanillin was 7.43 log CFU/g, indicating a reduction of 1.48 log CFU/g. Rojas-Graü *et al.* (27) also reported that edible coatings of alginate-apple puree incorporated with vanillin significantly decreased the population of *L. innocua* in fresh-cut apples during storage compared with the control. Therefore, these results suggest that the packaging of crab sticks with SFG films containing vanillin could be an effective method to extend the shelf life of crab sticks by inhibiting the growth of pathogenic bacteria such as *L. monocytogenes*.

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Disclosure The authors declare no conflict of interest.

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