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Physicochemical, Antimicrobial, and Cytotoxic Characteristics of a Chitosan Film Cross-Linked by a Naturally Occurring Cross-Linking Agent, Aglycone Geniposidic Acid

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The purpose of this study was to evaluate the characteristics of a chitosan film cross-linked by a naturally occurring compound, aglycone geniposidic acid (aGSA). This newly developed aGSA-cross-linked chitosan film may be used as an edible film. The chitosan film without cross-linking (fresh) and the glutaraldehyde-cross-linked chitosan film were used as controls. The characteristics of test chitosan films evaluated were their degree of cross-linking, swelling ratio, mechanical properties, water vapor permeability, antimicrobial capability, cytotoxicity, and enzymatic degradability. It was found that cross-linking of chitosan films by aGSA (at a concentration up to 0.8 mM) significantly increased its ultimate tensile strength but reduced its strain at fracture and swelling ratio. There was no significant difference in the antimicrobial capability between the cross-linked chitosan films and their fresh counterpart. However, the aGSA-cross-linked chitosan film had a lower cytotoxicity, a slower degradation rate, and a relatively lower water vapor permeability as compared to the glutaraldehyde-cross-linked film. These results suggested that the aGSA-cross-linked chitosan film may be a promising material as an edible film.

KEYWORDS: Chitosan; glutaraldehyde; aglycone geniposidic acid; edible film

INTRODUCTION

Edible films have been widely used to cover food surfaces, separate different components, or act as casings, pouches, or wraps (*1*). The advantages of edible films include extending the shelf life and the quality of food by forming barriers to oxygen, aroma, oil, or moisture, carrying functional ingredients such as antioxidants or antimicrobials, and improving appearance and handling characteristics (*2*). Chitosan, derived from the deacetylation of chitin, can be formed into fibers, films, beads, or nanoparticles for different applications (*3*−*6*). As compared with other biobased food-packaging materials, chitosan has the advantage of being able to incorporate functional substances such as minerals or vitamins and possesses antibacterial activity (*7*−*9*). Therefore, chitosan films have been used as a packaging material for the quality preservation of a variety of food (*10*−*13*).

The water vapor permeability of chitosan films may be moderated by chemical modification with a cross-linking agent (*11*, *12*, *14*−*16*). However, currently available cross-linking agents such as glutaraldehyde and diepoxy compounds are all synthetic compounds and highly cytotoxic (*17*, *18*).

In this study, a naturally occurring cross-linking agent, aglycone geniposidic acid (aGSA), was produced from the fruits of *Gardenia jasminoides* ELLIS using an enzyme-immobilized method and identified by HPLC and NMR spectral analyses. The obtained aGSA was used to cross-link chitosan for the development of edible films. The chemical characteristics, mechanical properties, water vapor permeability, antibacterial capability, cytotoxicity, and enzymatic degradability of the aGSA-cross-linked chitosan film were investigated. Fresh and glutaraldehyde-cross-linked chitosan films were used as controls.

MATERIALS AND METHODS

Materials. Dried fruits of *G. jasminoides* ELLIS were acquired from a local herbal-medicine store. Chitosan (MW $\approx 2.5 \times 10^5$) with a degree of deacetylation of approximately 85% was purchased from Fluka Chemical Co. (Switzerland). Cellulase (1.92 units/mg) and lysozyme (1000 units/mg) were obtained from Sigma Chemical Co. All other reagents and solvents were reagent grade.

Production of aGSA. Geniposide (GS) was isolated from dried fruits of *G. jasminoides* ELLIS using a method reported by Paik et al. (*19*, *20*). ^{13}C NMR (500 MHz, D_2O): δ 172.9 (−CO_2−), 155.2 (C-3), 144.0 (C-8), 131.8 (C-7), 114.4 (C-4), 101.5 (C−G1), 99.8 (C-1), 78.9 (C−G5), 78.3 (C−G3), 75.4 (C−G2), 72.2 (C−G4), 63.3 (C−G6), 62.4 (C-10), 54.5 (−OCH_3), 48.4 (C-9), 40.7 (C-6), 36.9 (C-5). The isolated GS (0.5 mmol) was added to 1 mL of NaOH aqueous solution (1 mmol) at 60 °C and the resulting solution stirred for 1 h. After being reacted

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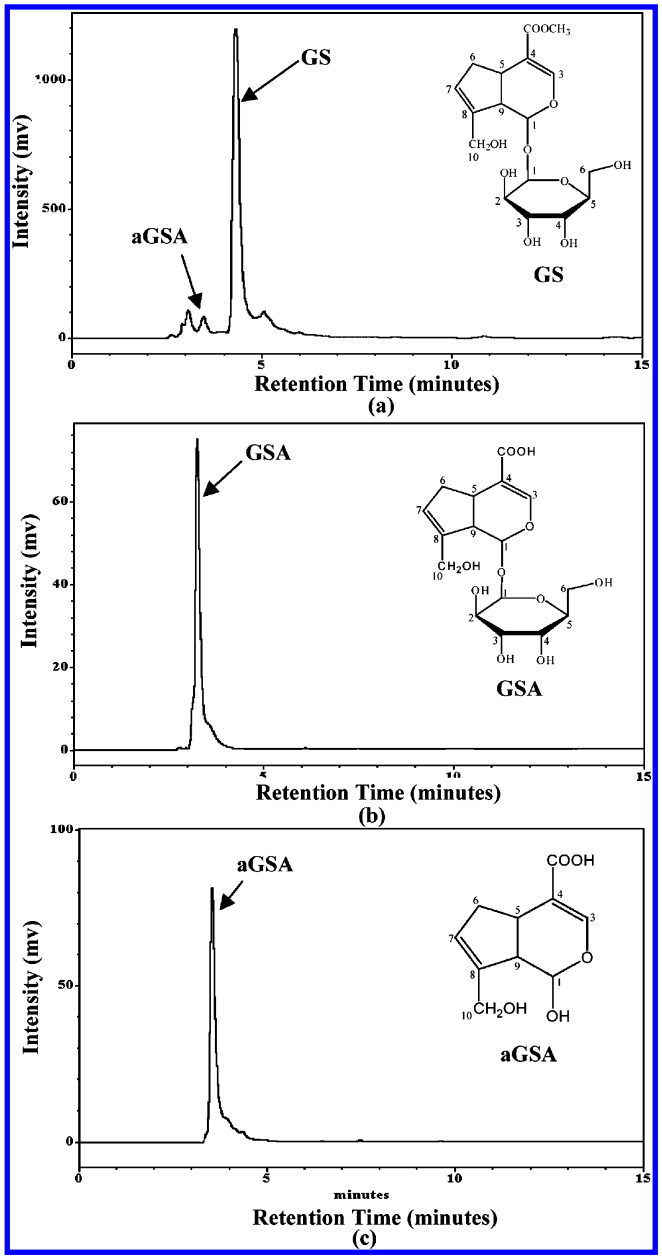

Figure 1. HPLC chromatograms of (**a**) GS isolated from dried fruits of *G. jasminoides* ELLIS, (**b**) GSA, and (**c**) aGSA.

with the alkaline solution, GS was converted to geniposidic acid (GSA). Subsequently, the obtained GSA was hydrolyzed to aGSA using an enzyme (cellulase)-immobilized method as described below. A free form of the enzyme was used as a control.

To prepare the immobilized enzyme, sodium alginate (800 mg) and cellulase (120 units) were dissolved in 40 mL of distilled water and the resulting solution stirred thoroughly. The mixed alginate/cellulase was then added dropwise to a 1.0% (by w/v) $CaCl_2$ aqueous solution under continuous stirring for the formation of gel beads. The gel beads immobilized with cellulase were washed with 200 mL of acetate buffer (0.1 M, pH 4.5). The prepared gel beads were then added to the GSA solution (pH 4.0), and the enzymatic reaction was carried out at 50 °C for up to 18 h. The obtained crude aGSA (5 mL, ~0.2 mol) was extracted by 50 mL of ethyl acetate and subsequently purified using a silica gel column. The column (40 × 3 cm) was eluted with chloroform/acetone gradients of 200 mL/0 mL, 70 mL/30 mL, and 100 mL/0 mL. The concentration of aGSA was measured by high-performance liquid chromatography (HPLC). The mobile phase was acetonitrile−water−perchloric acid (17:83:0.1 by volume) at a flow rate of 1.0 mL/min. ^{1}H NMR (500 MHz, CD_3COCD_3): δ 7.48 (s, 1, H-3), 5.79 (t, 1, H-7), 4.85 (d, 1, J = 8 Hz, H-1), 4.25 (dd, 2, J = 1.5, 1.5 Hz, H-10), 3.10

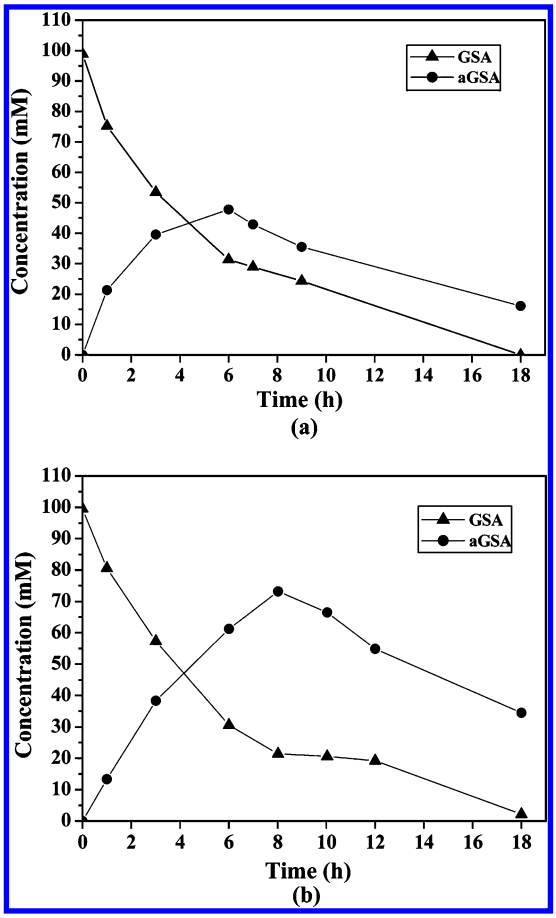

Figure 2. Time courses of hydrolysis of GSA using (**a**) a free form of cellulase and (**b**) an immobilized cellulase to obtain aGSA.

(m, 1, H-5), 2.81 (m, 2, H-6), 2.51 (m, 1, H-9). ^{13}C NMR (500 MHz, CD_3COCD_3): δ 168.73 (−CO_2−), 153.33 (C-3), 145.79 (C-8), 127.31 (C-7), 111.40 (C-4), 97.21 (C-1), 61.36 (C-10), 48.27 (C-9), 39.53 (C-6), 37.28 (C-5).

Preparation of Test Chitosan Films. The chitosan films used in the study were fabricated by means of a casting/solvent evaporation technique. Chitosan solution (1.5% w/v) was prepared by dissolving chitosan powder (3 g) in 200 mL of deionized water containing 1.0% (v/v) acetic acid at room temperature. The prepared chitosan solution was sonicated for 5 min and allowed to stand overnight to remove the trapped air bubbles. The air-bubble-free chitosan solution was poured into a glass disk in a dust-free environment and dried in air. The dried chitosan films were neutralized by an aqueous NaOH solution (1 N) and then thoroughly washed with phosphate-buffered saline (PBS). Aqueous glutaraldehyde and aGSA solutions (with a concentration of 0.4−6.4 mM) were prepared. Subsequently, the prepared chitosan films were immersed in the aqueous glutaraldehyde (glutaraldehyde-cross-linked chitosan film) or aGSA (aGSA-cross-linked chitosan film) solution for cross-linking. After 6 h, the cross-linked chitosan films were thoroughly washed with deionized water to remove the excess glutaraldehyde or aGSA and dried in air.

Degree of Cross-Linking. The degree of cross-linking of the chitosan film, using the fixation index determined by the ninhydrin assay, was defined as the percentage of free amino groups in the test chitosan film reacted with glutaraldehyde or aGSA after reaction. In the ninhydrin assay, the test sample first was lyophilized for 24 h and then weighed. Subsequently, the lyophilized sample was heated with a ninhydrin solution for 20 min. After heating with ninhydrin, the optical absorbance of the solution (at 570 nm) was recorded with a spectrophometer (model UV-150-02, Shimadzu Corp., Kyoto, Japan) using glucosamine at various known concentrations as a standard.

Swelling Ratio. The swelling ratios of the fresh chitosan film (the film without cross-linking) and the glutaraldehyde- and aGSA-cross-linked chitosan films were determined by soaking each test film (200

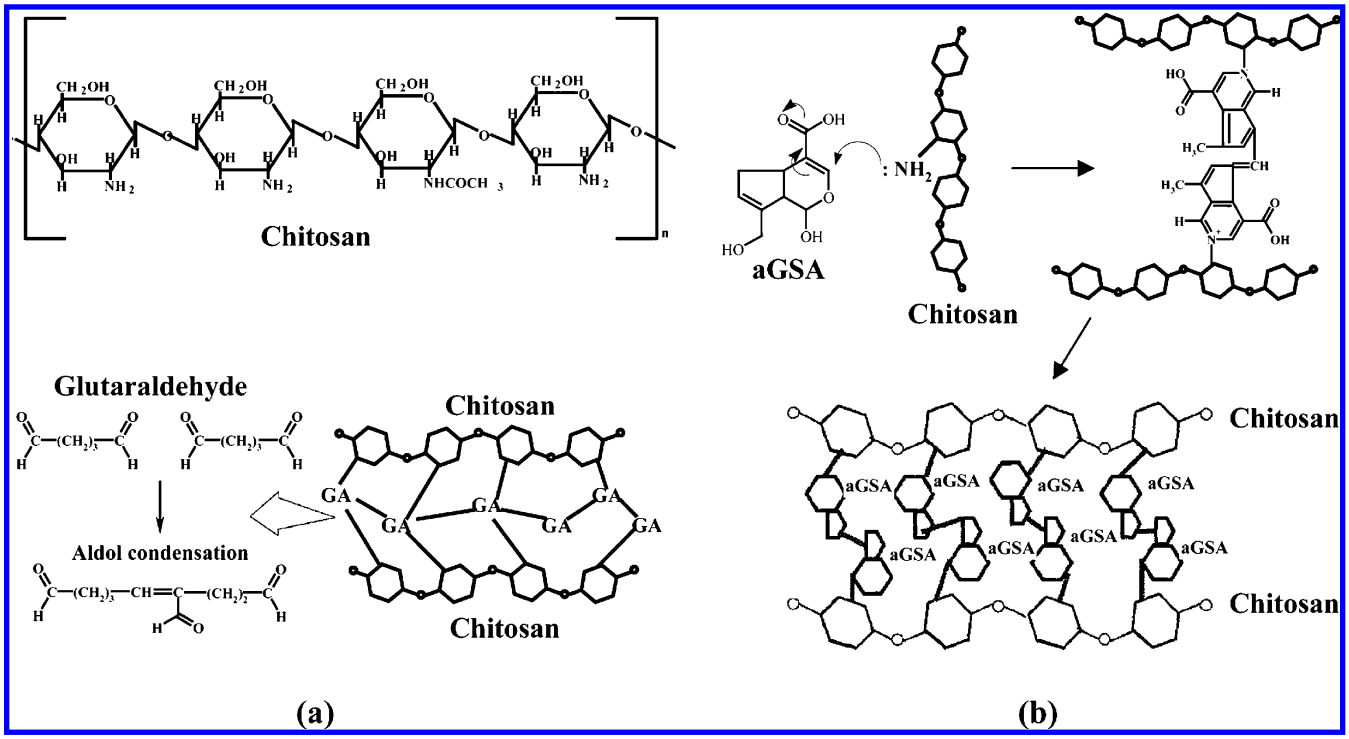

Figure 3. Presumed cross-linking structures of (**a**) glutaraldehyde-cross-linked chitosan and (**b**) aGSA-cross-linked chitosan.

mg) in deionized water at room temperature. Subsequently, the swollen test films were taken out, blotted with a filter paper, and then weighed immediately (W_e). The swelling ratio of each test film in the medium was calculated as follows:

$$E_{sw}\,(\%) = [(W_e - W_0)/W_0] \times 100$$

where E_{sw} is the swelling ratio of the test film at equilibrium and W_0 is the weight of the dried test film.

Mechanical Properties. Stress−strain curves of each test film (in a dumbbell shape) were determined by uniaxial measurements using an Instron material testing machine (Mini 44, Canton, MA) at a constant speed of 50 mm/min. The strain at fracture was taken as the percent strain at the point of fracture, while the ultimate tensile strength was taken as the force at which fracture occurred divided by the initial cross-sectional area.

Enzymatic Degradability. Test films were immersed in a 1000 units/mL lysozyme solution (pH 7.4) and incubated at 37 °C for up to 8 weeks. After degradation, the formation of oligomers containing *N*-glucosamine units, due to the cleaved β-glycosidic bonds of chitosan, induced an increment in the content of free amino groups in the incubation medium which can be determined by the ninhydrin assay. The degradation of each studied group was examined by analyzing the increased *N*-glucosamine units in the incubation medium (*21*). The medium was heated with a ninhydrin solution for 20 min. After heating with ninhydrin, the optical absorbance of the medium (at 570 nm) was recorded with a spectrophometer (model UV-150-02, Shimadzu Corp.) using glucosamine at various known concentrations as a standard to determine the amount of *N*-glucosamine degraded from chitosan.

Water Vapor Permeability. The water vapor permeability of each test film was determined using the ASTM method (E96-14) in a controlled chamber conditioned at 25 °C with a 50% relative humidity. A cylinder (3 cm in diameter and 5 cm in height) filled with 10 g of deionized water was placed in the closed chamber. The test film was fixed on the opening of the cylinder. Evaporation of water through the test film was monitored by measuring the weight of water remaining in the cylinder.

Antibacterial Capability. The antibacterial capability of test films was studied per a method described by Grzybowski et al. (*22*). Test films were sterilized in a graded series of ethanol solutions with a gradual increase in concentration from 20% to 75% over a period of 4 h and subsequently rinsed in sterilized PBS. Test samples (16 mm in diameter) cut from the sterilized films were placed at the bottom of each well in a 24-well plate. Subsequently, a 50 μL bacterial broth (*Escherichia coli* or *Staphylococcus aureus*) was seeded onto each test film (10^5 colony-forming units (CFUs)/mL). The bacterial broth cultured in the well without any test film was used as a control. Subsequently, the 24-well plate was placed in a moisture incubator at 37 °C. After 4 h of incubation, each test film was transferred to a test tube containing 1 mL of PBS and sonicated for 75 s. A 50 μL sample of the solution was then taken from each test tube, seeded on an agar plate containing nutrient broth, and incubated at 37 °C for 24 h. Finally, the CFUs in each agar plate were counted.

Cytotoxicity Study. The cytotoxicity of the test films was evaluated using an in vitro cell-culture assay. Each test film (2 × 1 cm) cut from the sterilized films was glued to the center of a cultural dish (60 mm in diameter) using a sterilized collagen solution. Subsequently, human foreskin fibroblasts (HFFs) at 1×10^5 cells/well were seeded evenly onto the surface of each test sample in 1 mL of Dulbecco's modified Eagle's medium (DMEM; Gibco 430-2800EG, Grand Island, NY) with 10% fetal calf serum (FCS; Hyclone Laboratories, Logan, UT). The cell cultures were maintained in a humidified incubator at 37 °C with 5% CO_2 in air. After 1, 2, or 4 days, the media were removed, and the cells cultured on the surface of each test film were photographed using an inverted light microscope (Olympus Optical Co., Ltd., IX70, Tokyo, Japan).

Statistical Analysis. Statistical analysis for the determination of differences in the measured properties between groups was accomplished using one-way analysis of variance and determination of confidence intervals, performed with a computer statistical program (Statistical Analysis System, Version 6.08, SAS Institute Inc., Cary, NC). All data are presented as a mean value with its standard deviation indicated (mean ± SD).

RESULTS AND DISCUSSION

Production of aGSA. Figure 1a presents an HPLC chromatogram of GS isolated from dried fruits of *G. jasminoides* ELLIS. The obtained GS was converted to GSA after being treated with an alkaline solution (**Figure 1b**). Parts **a** and **b** of **Figure 2** show the time courses of hydrolysis of GSA using a free form of cellulase and an immobilized cellulase, respectively. As indicated, the concentrations of aGSA obtained by both

Table 1. Degree of Cross-Linking, Swelling Ratio, Ultimate Tensile Strength, and Strain at Fracture of Chitosan Films without Cross-Linking (Fresh) and Those Cross-Linked with GA or aGSA at Various Known Concentrations ($n = 5$)

test sample	fresh	0.4 mM	0.8 mM	1.6 mM	3.2 mM	6.4 mM
			Degree of Cross-Linking (%)			
GA	0.0 ± 0	7.3 ± 0.6	7.4 ± 2.7	14.0 ± 3.0	34.1 ± 3.1	63.4 ± 1.0
aGSA	0.0 ± 0	17.0 ± 2.7	17.5 ± 1.9	25.1 ± 2.5	42.1 ± 2.9	70.8 ± 1.7
			Swelling Ratio (%)			
GA	101.4 ± 7.7	92.1 ± 2.5	84.3 ± 4.2	74.8 ± 4.3	65.7 ± 3.7	57.2 ± 4.5
aGSA	101.4 ± 7.7	71.2 ± 7.5	68.2 ± 6.2	65.6 ± 5.0	56.7 ± 5.0	51.1 ± 3.0
			Ultimate Tensile Strength (MPa)			
GA	18.2 ± 1.1	22.1 ± 0.8	24.9 ± 1.1	26.9 ± 2.1	18.9 ± 1.1	9.5 ± 1.0
AGSA	18.2 ± 1.1	24.4 ± 1.0	28.5 ± 1.8	21.1 ± 1.5	13.8 ± 1.4	7.1 ± 2.0
			Strain at Fracture (%)			
GA	25.9 ± 0.5	21.5 ± 0.9	19.8 ± 1.1	16.7 ± 0.9	9.1 ± 0.7	7.5 ± 0.9
aGSA	25.9 ± 0.5	19.5 ± 1.3	16.9 ± 0.5	10.5 ± 1.2	7.8 ± 1.0	5.9 ± 0.6

methods decreased significantly after 8 h of hydrolysis. Therefore, an 8 h duration was chosen for the hydrolysis of GSA by cellulase to obtain aGSA in the study. It was found that the amount of aGSA obtained using the immobilized cellulase was greater than that of the free form of cellulase. This can be attributed to the fact that the obtained aGSA can react directly with the free amino groups of the free form of cellulase (a protein-type enzyme), and thus, the activity of cellulase is decreased significantly. The decrease in the activity of cellulase may be limited by encapsulating the enzyme in the alginate beads. Finally, the obtained aGSA was purified using a silica gel column (**Figure 1c**). As shown in the HPLC chromatograms **(Figure 1)**, the retention times of GS, GSA, and aGSA were 4.3, 3.2, and 3.5 min, respectively.

Degree of Cross-Linking. Chitosan, containing hydroxyl and amino groups, is readily hydrated in water. To reduce its degree of hydration, chemical modification or cross-linking of chitosan is needed. In the study, glutaraldehyde, a commonly used cross-linking agent, and aGSA were used to cross-link chitosan films. After cross-linking, the glutaraldehyde-cross-linked chitosan film turned yellow, while the aGSA-cross-linked film became brown. The mechanism of cross-linking of chitosan with glutaraldehyde was discussed in detail previously (*21*). The bifunctional glutaraldehyde reacts with the free amino groups on chitosan to form Schiff bases (−C═N− linkage). Furthermore, glutaraldehyde may undergo an aldol condensation to polymerize in an aqueous environment. With the polymerization of glutaraldehyde molecules, a network cross-linking structure can be created between chitosan molecules.

It is known that aGSA can spontaneously react with the free amino groups of amino acids or proteins to form red pigments (*23*, *24*). These red pigments have been used in the fabrication of food dyes. The probable mechanism for the formation of red pigments is due to a nucleophilic attack by the primary amino group of amino acids on the third carbon of aGSA. This followed by the opening of the aGSA ring formed an intermediate aldehyde group. The resulting aldehyde group is subsequently attacked by the attached secondary amino group. Dimerization occurs at the second stage, perhaps by radical reaction, to form a heterocyclic cross-linking structure (*24*, *25*). Accordingly, both glutaraldehyde and aGSA can intramolecularly and intermolecularly cross-link chitosan **(Figure 3)**.

As shown in **Table 1**, the degrees of cross-linking (the fixation indices) of both the glutaraldehyde- and aGSA-cross-linked chitosan films increased with increasing concentration of the cross-linking agent. The fixation index of the aGSA-cross-linked film, at the same concentration, was significantly greater than that of its glutaraldehyde-fixed counterpart ($p < 0.05$).

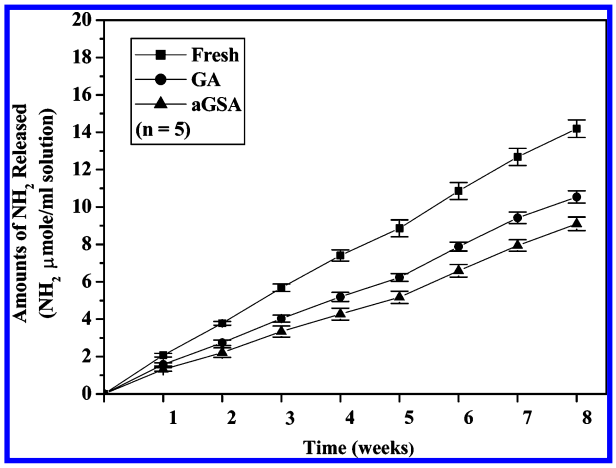

Figure 4. Degradation profiles of the fresh chitosan film and GA- and aGSA-cross-linked chitosan films with time incubated in a lysozyme solution.

Table 2. Number of CFUs of *E. coli* or *S. aureus* Growing on Fresh and GA- and aGSA-Cross-Linked Chitosan Films ($n = 5$)[a]

	no. of CFUs	
test sample	*E. coli* (×10^5)	*S. aureus* (×10^5)
control	576 ± 75	258 ± 34
fresh	108 ± 15	49 ± 12
GA	134 ± 56	58 ± 16
aGSA	125 ± 39	63 ± 19

[a] The bacterial broth cultured in the well without any test film was used as a control.

Swelling Ratio. Both the glutaraldehyde- and aGSA-cross-linked chitosan films showed a significant decrease in the swelling ratio as compared to their fresh counterpart ($p < 0.05$, **Table 1**). With increasing degree of cross-linking, the swelling ratios of both studied groups decreased. The swelling ratio of the aGSA-cross-linked chitosan film, due to a higher degree of cross-linking, was relatively lower than that of its glutaraldehyde-cross-linked counterpart.

Mechanical Properties. It was found that the values of the ultimate tensile strength of the cross-linked films increased significantly over that of the fresh counterpart and with increasing concentration of glutaraldehyde or aGSA up to 0.8 mM ($p < 0.05$, **Table 1**). With a further increase of the

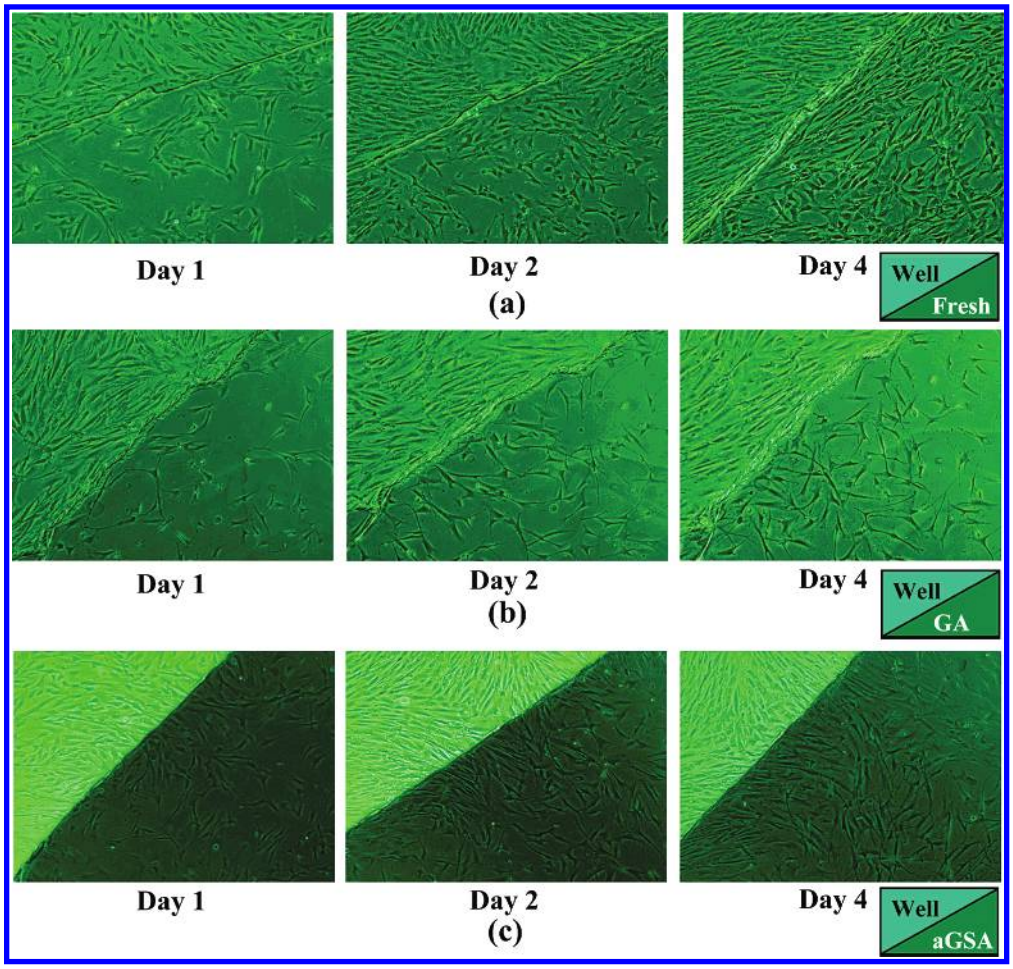

Figure 5. Photomicrographs (original magnification 40×; reproduced here at 80% of original size) of HFFs cultured on the surface and its vicinity of the (**a**) fresh chitosan film, (**b**) GA-cross-linked chitosan film, and (**c**) aGSA-cross-linked chitosan film after 1, 2, or 4 days of cell culture. The test sample was placed on the right bottom corner of the cell-culture well in each photomicrograph.

concentration of the cross-linking agent, the values of the ultimate tensile strength of the cross-linked films decreased significantly ($p < 0.05$). The amino and hydroxyl groups present in each repeat unit of the *N*-glucosamine on chitosan can readily lead to intermolecular hydrogen bonds between chitosan molecules. A high degree of cross-linking may disrupt the intermolecular hydrogen bonds and reduce the crystallinity of the chitosan film. As a result, a decrease in the value of the ultimate tensile strength was observed for the chitosan film cross-linked with a relatively high concentration of glutaraldehyde or aGSA. The values of the strain at fracture for the cross-linked chitosan films decreased significantly with increasing concentration of glutaraldehyde or aGSA ($p < 0.05$, **Table 1**). This is because cross-linking of chitosan films led to a decrease in their elongation (**Figure 3**).

As discussed above (**Table 1**), the chitosan films cross-linked with 0.8 mM glutaraldehyde or aGSA had the highest values of the ultimate tensile strength among their counterparts cross-linked at different concentrations. Therefore, these samples were chosen for the rest of the study.

Enzymatic Degradability. Figure 4 presents the degradability of each test film with time incubated in a lysozyme solution. It was observed that the increment in the free amino group content in the medium incubated with the fresh chitosan film was significantly greater than those of the media incubated with the glutaraldehyde- and aGSA-cross-linked chitosan films ($p < 0.05$). This indicated that the fresh chitosan film had the greatest degradability among all studied groups. Due to a higher degree of cross-linking, the degradability of the aGSA-cross-linked chitosan film was lower than that of its glutaraldehyde-cross-linked counterpart ($p < 0.05$). Additionally, the bulky heterocycle-cross-linking structure of the aGSA-cross-linked chitosan film may have a greater stereohindrance for the penetration of lysozyme than the network-cross-linking structure of the glutaraldehyde-cross-linked chitosan film (**Figure 3**). The structure of stereohindrance may prevent lysomyze from binding the *N*-acetylglucosamine residues on chitosan.

Water Vapor Permeability. The fresh chitosan film had a higher value of water vapor permeability (835 ± 69 g·m^{-2}·day^{-1}·atm^{-1}) than the glutaraldehyde-cross-linked (724 ± 38 g·m^{-2}·day^{-1}·atm^{-1}) and aGSA-cross-linked (684 ± 56 g·m^{-2}·day^{-1}·atm^{-1}) chitosan films ($p < 0.05$). It is known that the free hydroxyl and amino groups on chitosan are hydrophilic. The decrease in the water vapor permeability for the cross-linked chitosan films may possibly be due to the consumption of the free amino groups on chitosan after cross-linking. The water vapor permeability of the aGSA-cross-linked film was relatively lower than that of its glutaraldehyde-cross-linked counterpart, again due to its higher degree of cross-linking.

Antibacterial Capability. As compared with the control group, the numbers of colony-forming units (*E. coli* or *S. aureus*) observed on fresh and glutaraldehyde- and aGSA-cross-linked chitosan films were significantly inhibited ($p < 0.05$, **Table 2**). The antibacterial activity of chitosan against a broad spectrum of bacteria has been documented in the literature (*26*−*28*). It

was proposed that the interaction between the polycationic chitosan (NH_3^+ on glucosamine) and the negatively charged surface of bacteria may alter the permeability of the bacterial wall and lead to the leakage of intracellular electrolytes and proteins (*29*). However, the differences in the numbers of colony-forming units seen on all test films were insignificant ($p > 0.05$). These results suggested that cross-linking of chitosan films did not alter their antibacterial capability. This may be due to the fact that the cross-linking degrees of the glutaraldehyde- and aGSA-cross-linked chitosan films used in this part of the study were relatively low (<18%, with a concentration of cross-linking agent of 0.8 mM, **Table 1**).

Cytotoxicity Study. As shown in **Figure 5a**,**c**, after a 4 day culture, the surfaces of fresh and aGSA-cross-linked chitosan films and their vicinity were filled with HFF cells. However, only a few cells were observed on the surface of the glutaraldehyde-cross-linked film **(Figure 5b)**. These results suggested that the cytotoxicity of the aGSA-cross-linked chitosan film was less than that of its glutaraldehyde-cross-linked counterpart. It is known that glutaraldehyde is highly cytotoxic and may impair the biocompatibility of its cross-linked products (*30*).

Interestingly, the transparent chitosan biobased films became brown after being cross-linked with aGSA, without addition of any pigments. The aGSA-cross-linked chitosan film demonstrated a lower water vapor permeability, a superior antibacterial capability, and a lower cytotoxicity, as compared with those cross-linked with traditional cross-linking agents. These results suggested that the aGSA-cross-linked chitosan film may represent a promising and new type of edible films for packaging of foods.

Conclusion. There was no significant difference in the antimicrobial capability between the cross-linked chitosan films and their fresh counterpart. The aGSA-cross-linked chitosan film had a relatively lower water vapor permeability, a lower cytotoxicity, and a slower degradation rate than the glutaraldehyde-cross-linked film. These results suggested that the aGSA-cross-linked chitosan film may be a promising material as an edible film.

ABBREVIATIONS USED

GS, geniposide; GSA, geniposidic acid; aGSA, aglycone geniposidic acid; HFFs, human foreskin fibroblasts; PBS, phosphate-buffered saline.

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