CHAPTER III
MATERIALS AND METHODS

3.1 Materials and chemicals

Table 3.1 Main materials

Material	Supplier (country)
Bacterial cellulose pellicles (size 1 cm × 1 cm × 1 cm)	The laboratory of Pramote Thammarate at the Institute of Food Research and Product Development, Kasetsart University, Bangkok
Sodium alginate	Acros, Belgium
Gelatin from porcine skin type B (pI 5.2, 180 g bloom)	Fluka, Norway
Tannic acid (food grade)	Sigma-Aldrich, USA
Mangosteen ethanolic extract with tween-80	Bungon Kietthanakorn at Thai-China Flavours and Fragrances Co., Ltd., Phra Nakhon Si Ayutthaya, Thailand.
Glycerol (99.5% v/v)	Ajax Fine Chem Pty Ltd., Australia
D-sorbitol powder	Sigma-Aldrich, USA

Table 3.2 Minor chemicals

Chemical	Supplier (country)
Calcium chloride dehydrate	Ajax Fine Chem Pty Ltd., Australia
Absolute ethanol	QRec, New Zealand
Sodium hydroxide	Ranken, India
α-mangostin	ChromaDex, USA
Gallic acid	Sigma-Aldrich, China
Folin-Ciocalteu reagent	Sigma-Aldrich, Switzerland

Sodium acetate	Ajax Fine Chem Pty Ltd., Australia
Glacial acetic acid	QRec, New Zealand
Sodium acetate trihydrate	Ajax Fine Chem Pty Ltd., Australia
Sodium chloride	Ajax Fine Chem Pty Ltd., Australia
Potassium chloride	Ajax Fine Chem Pty Ltd., Australia
Di-sodium hydrogen phosphate	Ajax Fine Chem Pty Ltd., Australia
Potassium di-hydrogen phosphate	Ajax Fine Chem Pty Ltd., Australia
Tween-80	Ranken, India

3.2 Equipment

- Petri plate and mold
- Homogenizer (Phillip Cusina)
- Micrometer thickness gage (Mitutoyo, Japan)
- Fourier transform infrared (FT-IR) spectrometer (Perkin Elmer Spectrum One Massachusetts, USA)
- Universal testing machine (Hounsfield H 10 KM, Redhill, England)
- Scanning electron microscopy, SEM (JOEL JSM-5410LV, Tokyo, Japan)
- UV-visible spectrophotometer (Shimadzu UV-2550, Tokyo, Japan)
- Oxygen permeation tester (Illinois Instruments, Model 8000, Johnsburg, IL)
- Water vapor permeation tester (Lyssy L80-4000)
- X-ray diffraction, XRD (Bruker AXS Model D8 Discover,USA)
- Autoclave (Model Tomy Autoclave SS-325, Nerima-ku, Tokyo, Japan)

3.3 Preparation of BC slurry

The BC pellicles was treated with 1.0% (w/v) NaOH at room temperature (about 30 °C) for 24 h to remove bacterial cells and rinsed with deionized water (DI) until the pH was 7.0. Then the purified BC pellicles were crushed and homogenized to form BC slurry by using a homogenizer at ambient temperature.

3.4 Preparation of sodium alginate solution

Sodium alginate of 2% (w/v) was dissolved in distilled water with constant stirring at room temperature to form gel-like solution.

3.5 Preparation of gelatin solution

Gelatin powder from porcine skin (15 g) was hydrated with distilled water at room temperature and heated up to 50±5 °C with constant stirring until completely dissolved to obtain 15% (w/v) gelatin solution.

3.6 Antimicrobial agent: Mangosteens from the crude ethanol extract of *G. mangostana* in form of solution containing Tween-80

3.6.1 Quantitative determination of bioactive compounds in the mangosteen ethanolic extract

3.6.1.1 Determination of content of total phenolic compounds

The total phenolic compounds content was evaluated using modified Folin-Ciocalteu procedure (Pothitirat et al., 2009). A standard solution was prepared by dissolving 12.50 mg of gallic acid in 100 ml of absolute ethanol in volumetric flask. Various concentrations of the standard solution were provided at the final concentrations of 0.24, 0.49, 0.98, 1.95, 3.91, 7.81, 15.63, 31.25, 62.50 and 125.00

μg/ml. The mangosteen ethanolic extract sample or the standard solution of 0.4 mL was mixed with 1.6 mL of sodium bicarbonate solution (7.5% w/v) and then was added with 1.0 mL of the Folin-Ciocalteu reagent (previously diluted at 1:10 with deionized water). The mixture was shaken for well-mixing and incubated at room temperature for 30 minutes. The content of total phenolic compounds was calculated and the mean values (n=3) were recorded in grams of gallic acid equivalents (GAE)/100g of the extract by using a UV–visible spectrophotometer (Shimadzu UV-2550, Tokyo, Japan). The absorbance of the mixture and standard solution were measured at 765 nm wavelength.

3.6.1.2 Determination of content of mangostin content

The analytical condition for the determination of mangostin content was modified from the method previously reported by Pothitirat and Gritsanapan (2008). A stock solution of α-mangostin standard was prepared by dissolving 1.88 mg of α-mangostin in 100 ml of absolute ethanol in a volumetric flask. Afterwards, various concentrations of the standard solution were prepared to provide the final concentrations at 14.69, 29.38, 58.75, 117.50, 235.00, 470.00, 940.00 and 1880.00 μg/ml. The absorbance of all samples and the standard solutions were measured at 320 nm by UV–visible spectrophotometer (Shimadzu UV-2550, Tokyo, Japan). The mangostin content was calculated and the mean values (n=3) were recorded in grams of α-mangostin equivalents (AME)/100g of the extract.

3.6.2 Testing of antimicrobial activity of tannic acid and mangosteen ethanolic extract

3.6.2.1 Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the extract to inhibit the growth of microorganism (no visible growth). The microorganisms used in this test were as follow: gram negative bacteria

including *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* (*S. typhimurium*), gram positive bacteria including *Staphylococcus aureus* (*S. aureus*) and *Listeria monocytogenes* (*L. monocytogenes*), and fungi as *Aspergillus niger* (*A. niger*).

3.6.2.1.1 The minimum inhibitory concentration (MIC) of tannic acid

The minimum inhibitory concentration (MIC) values for the bacteria were determined by twofold serial micro-dilution assay (Wikler, 2006; Pothitirat et al., 2009). The aqueous tannic acid extract was dissolved in sterile DI water at the concentration of 30.00 mg/ml. The solutions were prepared by the serial doubling dilution with the final concentrations of 30.00, 15.00, 7.50, 3.75, 1.88, 0.94, 0.47, 0.23 and 0.12 mg/ml, respectively using medium as solvent. Then, 1 ml of the diluted extract solutions was transferred to the test tubes containing 1 ml of $1x10^8$ colony forming units (CFU) of tested microorganism. In addition, the control tube contained only microorganism and the medium. After 24 h incubation at 37 °C, the lowest concentration of the extract that showed no visible growth (turbidity = 0) was recorded as MIC. Each experiment was done three duplicate.

3.6.2.1.2 The minimum inhibitory concentration (MIC) of the mangosteen ethanolic extract

Mangosteens from the crude ethanol extract were testified for the antimicrobial activities in the similar procedure. The mangosteen solution containing tween-80 was initially prepared at 5.84 mg/ml DMSO. The diluted solutions were prepared by the serial doubling dilution using medium so as to reduce the effect of DMSO on viability of the microorganism. Then the prepared solutions were tested following the same procedure in Topic 3.6.2.1.1.

3.6.2.2 Determination of the minimum bactericidal concentration (MBC)

The minimum bactericidal concentration (MBC) was the lowest concentration of the extract required to kill a particular bacterium. The broth from inhibitory concentration was sub-cultured to agar media without antibiotic and incubated for 24 h at 37 °C. The lowest concentration of the extract that showed no viable cell in the agar media was recorded as MBC. Each experiment was done three duplicate.

3.7 Preparation of BAG films

3.7.1 Preparation of BAG films with/without plasticizers

The BC slurry was mixed with the alginate and gelatin solutions at different ratios to form bacterial cellulose-alginate-gelatin (BAG) blend mixtures. Glycerol and/or sorbitol were added as a plasticizer at a certain concentration ratio (0, 1, 2, 3 g per 10 g gelatin solution). MBAG refers to BAG containing plasticizer. The mixture was thoroughly stirred at 50±5 °C until the homogeneous mixture was formed and subsequently it was poured into polystyrene petri plates and incubated at room temperature for 1 day to form films with an average thickness of 50±10 μm. After that it was cross-linked by an aqueous solution of 1% (w/v) $CaCl_2$ for 1 h and rinsed with distilled water to remove the excess chlorides. The cross-linked gel was air-dried at room temperature and stored in plastic before use.

3.7.2 Tannic acid cross-linked MBAG films

The selected MBAG films were modified by supplement of tannic acid as crosslinking agent for gelatin. The prepared BC slurry was mixed with the alginate and gelatin solutions. Then glycerol at suitable content was added to the mixture to form the blend mixture for the fabrication of MBAG. The mixture was thoroughly

stirred at 50±5 °C until the homogeneous mixture was formed, and subsequently tannic acid was added at a certain amount ratio (0, 5, 10, 15, 20 mg per g gelatin solution). During the tannic acid addition, the pH of the blend mixture was adjusted to 7.0 with 1 N NaOH solution in order to prevent the formation of gel. The film forming solution was maintained at 50±5 °C until the homogeneous mixture was formed, and afterward it was poured into polystyrene petri plates and incubated at room temperature for 1 day to form films with an average thickness of 50±10 μm. After that it was cross-linked by an aqueous solution of 1% (w/v) $CaCl_2$ for 1 h and rinsed with distilled water to remove the excess chlorides and tannic acid. The cross-linked film was air-dried at room temperature and stored in plastic before use. MBAGT refers to MBAG containing tannic acid.

3.7.3 Supplement of mangosteen ethanolic extract as antimicrobial agent

The functional antimicrobial properties of the selected MBAGT film was improved by incorporating manosteen extract. Mangosteen extract was obtained from the crude ethanolic extract of *G. mangostana* in form of solution containing tween-80. The blend mixture for the fabrication of MBAGT was added with the mangosteen extract at concentration ratio of 0, 1, 5, 10% v/v (0, 5.93, 11.72 and 22.80 mg/ml). The mixture was thoroughly stirred at 50±5 °C until the homogeneous mixture was formed, and then it was poured into polystyrene petri plates and incubated at room temperature for 1.5 days to form films with an average thickness of 50±10 μm. After that it was cross-linked by an aqueous solution of 1% (w/v) $CaCl_2$ for 1 h and rinsed with distilled water to remove the excess chlorides and tannic acid. The cross-linked film was air-dried at room temperature and stored in plastic. MBAGTM refers to MBAGT containing the mangosteen extract.

3.8 Characterization of the films

The films were characterized by micrometer thickness gage for investigating thickness, by Fourier transform infrared (FT-IR) spectrometer for identifying the chemical structure, by Swelling tester for estimating water absorption capacity, by universal testing machine for determining Tensile strength and elongation at break, by Scanning electron micrographs (SEM) for investigating morphology, by Oxygen permeation tester for estimating oxygen gas transmission rate (OTR), by Water vapor permeation tester for estimating water vapor transmission rate (WVTR), and by X-ray diffraction(XRD) for estimating %crystallinity.

Moreover, biological characteristic of the films were investigated by antimicrobial activity: antibacterial assay and antifungal assay (disc diffusion method), and testing of amount bacteria in chicken sample.

3.8.1 Film thickness

Film thickness was measured with a micrometer thickness gage (Mitutoyo, Japan) MYL 314 with a sensitivity of 0.01 mm. The film thickness was the average value determined from 10 measurements of each sample.

3.8.2 Transparency

The transparency of the films was determined as previously described by Han and Floros (1997). The film samples were cut into rectangles and placed on the internal side of cuvette. The transmittance of films was determined at 600 nm using a UV–visible spectrophotometer (Shimadzu UV-2550, Tokyo, Japan). The transparency of the films was calculated as follows:

$$Transparency = log\,(T_{600}/x) \quad (1)$$

Where T_{600} is the transmittance at 600 nm and x is the film thickness (mm)

3.8.3 Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectroscopy was used to identify the chemical structure of the films which it is primary used to identify the chemical structure of sample. The FT-IR spectra of the films were measured at wave numbers ranging from 4000 to 400 cm^{-1} by a Perkin Elmer (Spectrum One, Massachusetts, USA) at Scientific and technological research equipment centre, Chulalongkorn University.

3.8.4 Water absorption capacity

Water absorption capacity (WAC) was determined by immersing the pre-weighted of dried BAG films in distilled water at room temperature until equilibration. After that the films were removed from the water and excess water at the surface of the films was blotted out with soft paper. The weights of the re-swollen films were measured. The procedure was repeated until no further weight change was observed. The water content was calculated using the following equation:

$$\%WAC = \frac{(W_s - W_d)}{W_d} \times 100 \quad (2)$$

Where W_s is the weight of re-swollen samples (g); W_d is the weight of dry samples (g). The WAC was the average value determined from 3 measurements.

3.8.5 Mechanical properties

All the films under the study in dry and re-swollen forms were tested for tensile strength and elongation at break. The film samples were cut into strip-shaped specimens of 20 mm width and 10 cm long (70 mm between the grips). The maximum tensile strength and break strain of the film samples were determined with a Hounsfield (London, UK) H10KM universal testing machine. The test conditions

followed ASTM D 882. The tensile strength and break strain were the average values determined from 5 specimens.

3.8.6 Scanning electron microscopy

Scanning electron microscopy (SEM) was examined the surface properties of the films by JOEL JSM-5410LV (Tokyo, Japan) at Scientific and technological research equipment centre, Chulalongkorn University. The BAG films in wet and re-swollen form films were frozen in liquid nitrogen, immediately snapped, vacuum-dried and then sputtered with gold and photographed in a Balzers-SCD 040 sputter coater (Balzers, Liechtenstein). The coated specimens were kept in dry place before experiment. The accelerating voltage was adjusted to 10 kV which is considered to be a suitable condition since too high energy can be burn the samples. The specimens were examined at magnification 200×, 10,000X and 3,500X for overview surface morphology, surface morphology and cross sectional morphology, respectively.

3.8.7 Oxygen permeability

Oxygen gas transmission rate (OTR) of the films with diameter over 13 cm was determined with the oxygen permeation tester: Illinois Instruments (Johnsburg, IL) Model 8000 at Thai packaging centre, Thailand Institute of Scientific and Technological Research. The test condition followed ASTM D 3985-05 oxygen gas transmission rate through plastic film and sheeting using a Coulometric Sensor. The determination of OTR was done at 23 °C and 0% relative humidity (%RH). The films were held in such a manner that it separate two side of test chamber. One side was exposed to a nitrogen atmosphere. Testing was completed when the concentration of oxygen in the nitrogen side was constant.

3.8.8 Water vapor permeability

Water vapor transmission rate (WVTR) of the films with diameter over 13 cm was determined with the water vapor permeation tester: Lyssy L80-4000 at Thai packaging centre, Thailand Institute of Scientific and Technological Research. The test conditions followed ASTM E 398-03 (Reapproved 2009) water vapor transmission rate of sheet materials using relative humidity measurement. The determination of WVTR was done at 38 °C and 90% relative humidity (%RH). The test specimen was sealed to the open mount of test dish containing a desiccant, and the assembly placed in a controlled atmosphere. Periodic weighting was performed to determine the rate of water vapor movement through the specimen into the desiccant.

3.8.9 X-ray diffraction

X-ray diffraction patterns of the BAG films were determined with X-ray diffractometer (Bruker AXS Model D8 Discover, USA) at Scientific and technological research equipment centre, Chulalongkorn University. The operation conditions were as follows: Cu Target, 40 kV Voltage, 40 mA Current, 5-40 degree angle, 0.02 degree increment and scan speed of 0.5 sec/step with VÅNTEC-1 Detector (Super Speed Detector). The degree of crystallinity (%) was calculated by Topas program using the following equation:

$$\textbf{Degree of crystallinity (\%)} = \frac{Crystalline\ area \times 100}{Total\ area\ (Crystalline\ area + Amorphous\ area)} \quad (3)$$

3.8.10 Antibacterial assay

The antibacterial test of the MBAGTM films were examined against gram negative bacteria including *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* (*S. typhimurium*) and gram positive bacteria including *Listeria monocytogenes* (*L. monocytogenes*) and *Staphylococcus aureus* (*S. aureus*) by disc diffusion method at

Microbiology Laboratory, Department of Microbiology, Faculty of Sciences, Chulalongkorn University.

The film samples were cut into 38 mm diameter discs and then placed on agar plates. These samples were sterilized by using UV irradiation for 20 min and were seeded with 0.1 ml of inoculums containing approximately 10^5-10^6 CFU/ml of tested bacteria. The plates were then incubated at 37 °C for 48 h. under aerobic conditions. Observations of the diameter of the inhibitory zone surrounding film discs were made. Experiments were done in triplicate.

3.8.11 Antifungal assay

The antifungal test of the films was examined against *Aspergillus niger* (*A. niger*) by disc diffusion method at Microbiology Laboratory, Department of Microbiology, Faculty of Sciences, Chulalongkorn University. The film samples were punch into round-shaped sample of 38 mm diameter according to the method described by AATCC TM 39-1989 (Anti-bacterial Activity Assessment of Textile Materials: parallel Streak Method). The samples used for the antifungal assay were sterilized by using UV irradiation for 20 min in each side. The test of *A. niger* was performed in the AGAR plate for a week of incubation at 30 °C.

3.9 Release of bioactive compounds from the MBAGTM films

3.9.1 Preparation of acetate buffer

Acetate buffer pH 5.6 was used to simulate the suitable pH condition for common meat just after death that pH range about 5.8-5.4 (Tarté, 2009) and human skin that pH about 5.5 (Suwantong et al., 2007). For 100 ml of acetate buffer solution preparation, 0.1 M acetic acid (5.8 ml made to 1000 ml) and 0.1 M sodium acetate trihydrate (13.6 g/l) were mixed in the proportion during 4.8 ml of 0.1 M acetic acid solution and 45.2 ml of 0.1 M sodium acetate. Then the solution was adjusted the final volume to 100 ml with deionized water and adjusted the final pH using a sensitive pH meter to obtain acetate buffer at pH 5.6.

3.9.2 Preparation of phosphate buffer saline (PBS)

Phosphate buffer saline pH 7.4 was used to simulate the suitable pH condition for growing of bacteria (Tarté, 2009). For 1000 ml of phosphate buffer solution preparation, 2.7 mM potassium chloride (0.20 g), 10 mM di-sodium hydrogen phosphate (1.42 g), 1.76 mM potassium di-hydrogen phosphate (0.24 g) were dissolved in 1 liter of deionized water. Then the solution was adjusted the pH before use with 137 mM sodium chloride (8.0145 g) to obtain PBS buffer at pH 7.4.

3.9.3 Actual bioactive compound content

Actual amount of bioactive compound content in the MBAGTM films were determined. Each specimen (circular disc at 3.0 cm in diameter) was immersed in the absolute ethanol 10 ml at room temperature (30 °C) and stirred at 100 rpm for 72 h. After that, the solution was collected and the actual amount of bioactive compound content was measured by Shimadzu UV-2550 UV-visible spectrophotometer at the wavelength of 765 nm for phenolic compound and 320 nm for mangostins following the analytical methods which were previously described in Topic 3.8.

3.9.4 Bioactive compounds release assay

3.9.4.1 Release in acetate buffer

The release characteristics in acetate buffer at pH 5.6 of bioactive compounds from the MBAGTM films were investigated by total immersion (Suwantong et al., 2007). The releasing medium is composed of 96.5%v/v acetate buffer with 0.5%v/v Tween 80 and 3%v/v ethanol. Each specimen (circular disc at 3.0 cm in diameter) was immersed in 30 ml of the medium at the room temperature ($\approx$ 30 °C). At a specified immersion period ranging from 0 to 72 h (4320 min), either 0.7 ml of a sample solution was withdrawn from medium. The amounts of bioactive compounds in the sample solutions were determined using the UV-visible spectrophotometer at the wavelength of 765 nm for phenolic compounds and at wavelength of 320 nm for mangostins.

3.9.4.2 Release in phosphate buffer saline (PBS)

The release characteristics in PBS at pH 7.4 of bioactive compounds from the MBAGTM films were investigated by total immersion (Suwantong et al., 2007). The releasing medium is composed of 96.5%v/v PBS with 0.5%v/v Tween 80 and 3%v/v ethanol. Each specimen (circular disc at 3.0 cm in diameter) was immersed in 30 ml of the medium at the room temperature approximate 30 °C. At a specified immersion period ranging from 0 to 72 h (4320 min), either 0.7 ml of a sample solution was withdrawn from medium. The amounts of bioactive compounds in the sample solutions were determined using the UV-visible spectrophotometer at the wavelength of 765 nm for phenolic compounds and at wavelength of 320 nm for mangostins.