CHAPTER 8

ANTIOXIDATIVE ACTIVITY AND PROPERTIES OF FISH SKIN GELATIN FILMS INCORPORATED WITH BHT AND α-TOCOPHEROL

8.1 Abstract

Antioxidative activity and properties of bigeye snapper and brownstripe red snapper skin gelatin based films incorporated with BHT or α-tocopherol were investigated. Changes in FTIR spectra of fish skin gelatin films were observed when BHT or α-tocopherol at a level of 200 ppm was incorporated, suggesting some interaction occurred between gelatin molecules and the antioxidants added. Incorporation of BHT generally increased tensile strength (TS) and elongation at break (EAB) of film from bigeye snapper skin gelatin, while decreased EAB of film from brownstripe red snapper skin gelatin ($P<0.05$). Addition of α-tocopherol (200 ppm) decreased TS and EAB of film from brownstripe red snapper skin gelatin, and lowered EAB of film from bigeye snapper skin gelatin ($P<0.05$). Both BHT and α-tocopherol decreased water vapor permeability (WVP) of resulting film from skin gelatin of both species ($P<0.05$) but affected the transparency differently, depending upon gelatin source. During storage, all films had the increase in TS with the coincidental decrease in EAB ($P<0.05$). Antioxidative activity of fish skin gelatin films incorporated with BHT or α-tocopherol increased markedly with increasing storage time as indicated by the increase in DPPH radical scavenging activity ($P<0.05$). Films without and with BHT or α-tocopherol incorporated showed the preventive effect on lard oxidation as evidenced by the retardation of TBARS and PV formation.

8.2 Introduction

Chemical changes of food composition during transportation and storage lower the product quality. Generally, packaging is needed to maintain the food quality. The protection of foods by packaging has traditionally been based on provision of an inert barrier to the

outside environment (Rooney and Yam, 2004). Recently, there has been an increasing attention on active packaging, which is a group of packaging technologies performing some role in the preservation of the food other than providing an inert barrier (Labuza and Breene, 1989; Brody, Strupinsky and Kline, 2001). Antioxidant releasing packaging is a kind of food preservation systems, in which an antioxidant or a mixture of antioxidants is incorporated into the package. Antioxidant released in a controlled manner to the food contributes to the shelf-life extension. Since the oxidation is commonly initiated at the food surface, antioxidant-releasing packaging is a promising means to protect the food surface from rancidity. The slow release mechanism also provides a continuous replenishment of antioxidant to the food (Rooney *et al.*, 2004). Therefore, the small amount of additive is required (Guilbert *et al.*, 1996). Miltz *et al.* (1998) reported that oat meal cereals packed in high-level (0.32%) BHT-impregnated HDPE had an extended shelf-life, compared with those packed in low-level (0.022%) BHT impregnated HDPE and HDPE film without BHT. The storage stability of vegetable oil increased with antioxidant-impregnated plastic film (Sharma *et al.*, 1990). Recently, there has been an increasing interest in the application of natural antioxidants such as vitamin E, sesamol and carnosic acid in the food systems. Vitamin E (α-tocopherol) is a non toxic agent that has positive public perception, broad regulatory approvals and friendly appeal to the consumer (Rooney *et al.*, 2004). Incorporation of α-tocopherol at the high concentrations into LDPE film inhibited oxidation of linoleic acid emulsion stored in contact with the film (Wessling *et al.*, 2000). However, antioxidant-releasing packagings from biopolymer, particularly from fish protein, have not been investigated. Recently, fish gelatin based film has been successfully prepared (Jongjareonrak *et al.*, 2006). Therefore, the objective of this investigation was to prepare and characterize fish skin gelatin based films incorporated with BHT or α-tocopherol. The antioxidative effect of fish skin gelatin films impregnated with BHT or α-tocopherol against the oxidation of lard was also elucidated.

8.3 Materials and Methods

Chemicals

Butylated-hydroxy-toluene (BHT) and glycerol were purchased from Merck (Darmstadt, Germany). α-tocopherol, DPPH (2,2-diphenyl-1-picrylhydrazyl) and thiobarbituric acid were obtained from Sigma Chemical Co. (St. Louis, Mo., USA).

Fish skin preparation

Bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) with an average total length of 22-25 cm were caught from Songkhla coast along the Gulf of Thailand, stored in ice and off-loaded after 24-36 h of capture. Upon the arrival to the dock in Songkhla, fish were stored in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Fish were washed with a tap water. Skins were then removed, descaled and cut into small pieces (0.5x0.5 cm). Prepared skins were kept on ice until used for gelatin extraction.

Extraction of fish skin gelatin

Gelatin was extracted from fish skin according to the method of Jongjareonrak *et al*. (2006). Skins were soaked in 0.2 M NaOH with a skin/solution ratio of 1:10 (w/v) at 4°C with a gentle stirring. The solution was changed every 30 min for 3 times to remove noncollagenous proteins and pigments. Alkaline treated skins were then washed with tap water until neutral or faintly basic pHs (pH 7-7.5) of wash water were obtained. The skins were then soaked in 0.05 M acetic acid with a skin/solution ratio of 1:10 (w/v) for 3 h at room temperature (25°C) with a gentle stirring to swell the collagenous material in fish skin matrix. Acid treated skins were washed as previously described. The swollen fish skins were soaked in distilled water with a skin/water ratio of 1:10 (w/v) at 45°C for 12 h with a continuous stirring to extract gelatin from the skin matter. The mixture was then filtered using two layers

of cheese cloth. The resultant filtrate was freeze-dried using a freeze dryer (Model Dura-Top™ μP/Dura Dry™ μP, FTS® System, Inc., Stone Ridge, New York, USA) and the dry matter was referred to as "gelatin powder".

Preparation of fish skin gelatin films

Gelatin films were prepared according to the method of Jongjareonrak *et al.* (2006). Gelatin powder was mixed with distilled water to obtain the film-forming solution (FFS) with the protein concentration of 3% (w/v). Glycerol at the concentration of 50% of protein was added into FFS as the plasticizer. To reduce the degradation of bigeye snapper gelatin caused by heat-activated proteinase, 10 mM EDTA was added into FFS. The FFS of fish skin gelatins from both species were incubated at 70°C for 30 min in a water bath with an occasional stirring for total dissolution. The solution was then homogenized using a homogenizer (Model PT-MR 2100, POLYTRON®, KINEMATICA AG, Littau-Lucerne, Switzerland) at 11,000 rpm for 1 min. Thereafter, FFS (4±0.01 g) was cast onto a rimmed silicone resin plate (50 x 50 mm) and dried with a ventilated oven (Environmental chamber; Model KBF 115, WTB BINDER, Tuttlingen, Germany) at 25±0.5°C and 50±5% relative humidity (RH) for 24 h. Dried films obtained were manually peeled off. The films were conditioned for 48 h at 25±0.5°C and 50±5% RH prior to analyses.

To study the effects of BHT and α-tocopherol on film properties and antioxidative activity of incorporated film, BHT and α-tocopherol were dissolved in methanol and added into FFS to obtain the final concentration of 200 ppm. FFS was then stirred gradually for 10 min, cast and dried as previously described.

Films incorporated without and with BHT or α-tocopherol were subjected to the determinations of properties and antioxidative activity during storage in a humidity control chamber (50%RH) at 28°C for 0, 3 and 6 weeks.

FTIR analysis

Prior to analysis, films were conditioned in a desiccator containing silica gel for 7 days at room temperature to obtain the most dehydrated films. The spectra of films without and with BHT or α-tocopherol were recorded using a Fourier Transform Infrared (FTIR) spectrometer (Model Equinox 55, Bruker Co., Ettlingen, Germany) at room temperature. Light source of transmittance was in the range of 400-4000 cm^{-1}. The spectra obtained were used to determine possible interactions of functional groups between gelatin molecules with BHT or α-tocopherol.

Mechanical properties

TS and EAB of films were determined using a material testing systems (Model LR30K, LLOYD INSTRUMENTS Ltd., Segensworth Fareham, England). Ten samples were measured for each treatment.

Water vapor permeability

WVP of films was measured using a modified ASTM method (1989) as described by Shiku *et al.* (2004). Films were sealed onto a glass permeation cup containing silica gel (0% RH) with silicone vacuum grease and an O-ring to hold the film in place. The cups were then placed in a desiccator saturated with water vapor at 30°C. The cups were weighed at 1 h intervals over a 7 h period and WVP of films was calculated as follows (McHugh *et al.*, 1993):

$$WVP = wxA^{-1}t^{-1}(P_2-P_1)^{-1}$$

where w is the weight gain of the cup (g), *x* is the film thickness (m), A is the area of exposed film (m^2), t is the time of gain (s), and $(P_2-P_1)^{-1}$ is the vapor pressure differential across the film (Pa).

WVP was expressed as $g.m^{-1}s^{-1}Pa^{-1}$. A total of five samples were determined for each treatment.

Light transmission

The barrier properties of films against ultraviolet (UV) and visible light were measured at selected wavelengths between 200 and 800 nm, using a UV-Visible Recording spectrophotometer (Model UV-1601, Shimadzu Co., Kyoto, Japan) according to the method of Fang *et al*. (2002). The transparency of films was calculated by the following equation (Han and Floros, 1997):

transparency = -logT600/x

where T600 is the transmittance at 600 nm and x is the film thickness (mm).

Color

Color of films was measured in the L*, a*, b* mode of CIE using a colorimeter (Model ColorFlex, HunterLab Reston, VA, USA). Color measurement was carried out in ten replicates for each treatment.

Effect of fish skin gelatin film incorporated with BHT and α-tocopherol on the retardation of lard oxidation

Lard purchased from the market in Hat Yai, was rendered in a pan at 200-210°C, filtered using two layers of cheesecloth and cooled to room temperature. Lard was transferred to the cylindrical plastic cup with a diameter of 20 mm, covered with the skin gelatin films incorporated without and with antioxidants, and sealed with an O-ring. The samples were stored in a humidity control chamber (50% RH) at 28°C. The samples were taken after 0, 1, 2, 3 and 4 weeks for the analysis of peroxide value (PV) and thiobarbituric acid reactive substances (TBARS). Samples without film covering were used as the control.

Determination of thiobarbituric acid reactive substances

TBARS value of lard covered without and with film containing antioxidants was determined according to the method of Buege and Aust (1978). Lard (0.1 g) was mixed

with 5 ml of TBA solution (0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of hydrochloric acid were mixed thoroughly in 100 ml of distilled water). The mixture was heated for 10 min in a boiling water bath (95-100°C) to develop pink color, cooled with tap water and centrifuged at 7,500 rpm for 10 min. Absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using malonaldehyde bis(dimethyl acetal) (MDA) at concentrations ranging from 0 to 1000 μM. TBARS value in each sample was expressed as mg MDA/kg sample using standard curve.

Determination of peroxide value (PV)

PV of lard was measured according to the method of IUPAC (1979). The sample (1-4 g) was mixed with 25 ml of acetic acid:chloroform mixture (3:2, v/v), followed by addition of saturated potassium iodide solution (1 ml). The reaction mixture was allowed to stand for 5 min in the dark. Distilled water (75 ml) was added to the mixture. The mixture was titrated with 0.01 M sodium thiosulphate and 1 ml of 1% starch solution was added as an indicator. PV was expressed as meq/kg sample using the following equation:

PV (meq/kg sample) = (a-b) x N x 100 / w

where a and b are the volume (ml) of sodium thiosulphate using for the blank and sample titration, respectively, N is the concentration of sodium thiosulphate (Normal), and w is a sample weight (g).

Antioxidative activity of fish skin gelatin films incorporated with BHT or α-tocopherol

Fish skin gelatin films incorporated with BHT or α-tocopherol was stored in the humidity control chamber (50% RH) at 28°C. Films were taken after 0, 3 and 6 weeks of storage for analyses. Films were solidified in a mortar using liquid nitrogen and ground with a pestle. Ground film (0.1 g) was mixed with 2 ml of methanol and stirred vigorously for 3 h and centrifuged at 5,500 rpm for 10 min. The supernatant obtained was determined for DPPH radical scavenging activity.

Determination of DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method of Yen and Hsieh (1995) with a slight modification. An aliquot of methanol extracts (500 μl) was added with 2 ml of 0.06 mM DPPH in methanol. The mixture was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the mixture was measured at 517 nm using spectrophotometer. The control was conducted in the same manner but methanol was used instead of DPPH solution. DPPH radical scavenging activity was calculated as follows (Singh and Ragini, 2004):

Radical scavenging activity (%) = $(1 - (A_{sample}/A_{control})) \times 100$

where A_{sample} is the absorbance of sample and $A_{control}$ is the absorbance of the control.

Statistical analysis

Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple-range test (Steel and Torrie, 1980). Analysis was performed using a SPSS package (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL).

8.4 Results and Discussion

FTIR spectra of fish skin gelatin film incorporated with BHT or α-tocopherol

FTIR spectra of bigeye snapper and brownstripe red snapper skin gelatin films with and without BHT or α-tocopherol incorporated are depicted in Figure 22. The spectra of gelatin films from both species had the similar patterns with a major peak at 1630 cm^{-1}, suggesting the stretching of C=O bonds. A broad peak was also observed at 3300 cm^{-1}, indicating the stretching of the O-H and N-H bonds. In addition, absorption peaks at 2845 cm^{-1} and 2914 cm^{-1} (C-H stretch), 1570-1515 cm^{-1} ($-NH_2$), and 1400 cm^{-1} (COO^-) were generally found from the FTIR spectra of gelatin films without antioxidants. Nevertheless, bigeye snapper and brownstripe red snapper skin gelatin films showed some slight differences

in FTIR spectral pattern. This suggested that there might be some variations between skin gelatins from different fish species in term of amino acid compositions, sequence and the active groups responsible for film formation. From the results, bigeye snapper skin gelatin films incorporated with BHT or α-tocopherol generally showed the similar pattern to the control (without BHT or α-tocopherol) (Figure 22A). However, the peak at 1545 cm^{-1} disappeared when BHT or α-tocopherol was incorporated, indicating some interaction between amine groups of gelatin and the active groups of BHT or α-tocopherol. Additionally, the peak around 660 cm^{-1} was found in the presence of BHT or α-tocopherol. For brownstripe red snapper skin gelatin films, the spectra of films incorporated with BHT or α-tocopherol had the same pattern with that of the control film (without BHT or α-tocopherol) (Figure 22B). Similarly, peak at 660 cm^{-1} was also noticeable in film from brownstripe red snapper skin gelatin when BHT or α-tocopherol was incorporated. These results indicated that some interactions between active groups of BHT or α-tocopherol and the functional groups of gelatin occurred.

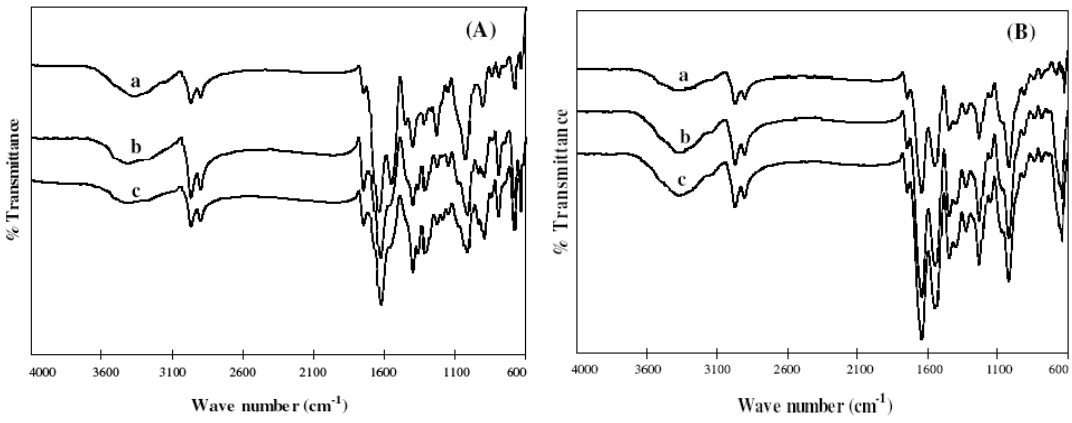

Figure 22. FTIR spectra of bigeye snapper skin gelatin film (A) and brownstripe red snapper skin gelatin film (B) incorporated without and with BHT or α-tocopherol. Film without BHT or α-tocopherol incorporated (a), film with BHT incorporated (b), film with α-tocopherol incorporated (c).

Changes in properties of fish skin gelatin films incorporated with BHT or α-tocopherol during storage

Mechanical properties

Tensile strength (TS) of fish skin gelatin based films from both species incorporated with BHT or α-tocopherol at the concentration of 200 ppm is shown in Table 20. Generally, TS of bigeye snapper skin gelatin films increased when BHT was incorporated but that of brownstripe red snapper skin gelatin films decreased with the addition of α-tocopherol in comparison with the control (without antioxidant) ($P<0.05$). The increase in TS of films added with BHT might be due to the interaction between BHT and fish skin gelatin in the fashion, which strengthened the film network. Hydroxyl group of BHT possibly acted as hydrogen donor and hydrogen bonds could be formed between BHT and gelatin molecules. For films added with α-tocopherol, a slight decrease in TS could be probably caused by incompatibility of α-tocopherol and gelatin molecules, resulting in the lessen integrity of film structure. α-tocopherol is hydrophobic in nature (Nawar, 1996) and could not be dissolved completely in film forming solution. TS of all films generally increased as the storage time increased ($P<0.05$). Among all films, those from both species added with α-tocopherol had a greater increase in TS after 6 weeks of storage, when compared with those added with BHT and without antioxidant added, respectively. The increase in TS of films after storage might be owing to the renaturation of gelatin chains into triple helix structure (Arvanitoyannis *et al.*, 1997; Sobral *et al.*, 2001b). Additionally, the greater aggregation of gelatin molecules might be obtained in films containing α-tocopherol.

Elongation at break (EAB) of fish skin gelatin films incorporated with BHT or α-tocopherol at the concentration of 200 ppm is shown in Table 20. In general, EAB of skin gelatin films from both species decreased when BHT or α-tocopherol was added except bigeye snapper gelatin films added with BHT, which possessed the increased EAB. The addition of BHT or α-tocopherol to the film might alter gelatin film structure and decreased the movement of the macromolecules in the film matrix, leading in the decrease of EAB. For bigeye snapper film added with BHT, the increase in EAB was possibly due to the increase in gelatin chain

length induced by BHT as well as the compatibility of BHT in the films matrix. However, EAB of all films decreased with storage time increased ($P<0.05$). This might be due to the recrystallinity of gelatin in the film matrix during storage (Arvanitoyannis *et al.*, 1997), leading to the rigidity of film as evidenced by the decrease in EAB of the films. In addition, BHT and α-tocopherol possessing the longer hydrocarbon chain has the lower chain mobility, compared with glycerol (Ayranci and Tunc, 2001). Thus, the formation of film network structure with less movement of the macromolecules could be obtained in film incorporated with BHT or α-tocopherol, especially with increasing storage time.

Table 20. Mechanical properties and WVP of bigeye snapper and brownstripe red snapper skin gelatin films incorporated without and with BHT or α-tocopherol during storage

Source of gelatin	Antioxidant type	Week	TS[1]	EAB[1]	WVP[2]
bigeye snapper skin	w/o	0	42.63±4.75^{cB*}	23.56±2.46^{aB}	1.71±0.13^{aA}
		3	50.46±3.84^{bB}	20.40±1.07^{bB}	1.21±0.03^{bB}
		6	58.63±5.01^{aC}	13.07±0.49^{cA}	1.11±0.02^{bA}
	BHT	0	50.47±4.05^{cA}	30.90±2.67^{aA}	1.43±0.12^{aB}
		3	57.07±4.67^{bA}	22.46±1.84^{bA}	1.37±0.08^{aA}
		6	63.97±4.67^{aAB}	11.55±0.50^{cB}	1.08±0.02^{bA}
	α-tocopherol	0	40.64±5.66^{cB}	17.05±2.26^{aC}	1.62±0.05^{aAB}
		3	55.45±5.63^{bAB}	11.89±1.41^{bC}	1.17±0.08^{bB}
		6	68.45±5.02^{aA}	8.74±0.87^{cC}	0.93±0.03^{cB}
brownstripe red snapper skin	w/o	0	56.20±1.58^{cA}	26.26±1.98^{aA}	1.93±0.09^{aA}
		3	62.13±1.54^{bAB}	14.57±0.98^{bA}	1.48±0.02^{bA}
		6	68.21±2.04^{aC}	11.46±0.87^{cA}	1.19±0.03^{cA}
	BHT	0	58.35±4.31^{bA}	13.89±1.34^{aB}	1.16±0.08^{aB}
		3	69.40±1.97^{aA}	11.22±0.82^{bB}	1.04±0.08^{abB}
		6	73.28±4.69^{aB}	9.74±0.78^{cB}	1.00±0.01^{bC}
	α-tocopherol	0	48.24±2.90^{cB}	13.23±0.82^{aB}	1.21±0.06^{aB}
		3	66.31±6.46^{bB}	10.98±0.84^{bB}	1.08±0.02^{bB}
		6	78.58±3.59^{aA}	7.95±0.89^{cC}	1.06±0.03^{bB}

[1] Values are given as mean ± SD from ten determinations. [2] Values are given as mean ± SD from five determinations. * Different letters in the same column under the same gelatin source indicate significant differences ($P<0.05$). Different capital letters in the same column under the same storage time and gelatin source indicate significant differences ($P<0.05$). w/o: Films without BHT or α-tocopherol incorporated.

From the results, similar changes in TS and EAB as affected by the incorporation of BHT or α-tocopherol as well as storage time were observed between bigeye snapper and brownstripe red snapper skin gelatin films. Nevertheless, TS and EAB of bigeye snapper skin gelatin films were generally lower than those of brownstripe red snapper skin film under the same condition examined. This was possibly owing to the differences in protein extension, amino acid composition, as well as sequence of amino acid residues (Kroachta, 1997). Jongjareonrak *et al.* (2006) also reported that film from brownstripe red snapper skin gelatin had the greater TS and EAB than film from bigeye snapper skin gelatin.

Water Vapor permeability

Water vapor permeability (WVP) of fish skin gelatin films of both species incorporated with BHT or α-tocopherol is shown in Table 20. WVP of films generally decreased when BHT or α-tocopherol was incorporated. This was possibly caused by the increasing hydrophobicity of the films matrix in the presence of BHT or α-tocopherol. The greater decrease in WVP was noticeable in films from both species added with BHT, compared with those incorporated with α-tocopherol. The differences in size and the hydrophobicity between BHT and α-tocopherol might result in the different WVP of the films. BHT and α-tocopherol are both insoluble in water (Nawar, 1996). BHT has a smaller molecule and could be dispersed in the gelatin film matrix more regularly than α-tocopherol with the longer aliphatic chain. When the storage time increased, WVP of all films from both fish species continuously decreased ($P<0.05$) (Table 20). This might be caused by the renaturation of gelatin molecules as well as more pronounced cross-linking between gelatin or between antioxidants and gelatin during extended storage (Arvanitoyannis *et al.*, 1997). Consequently, the network structure of films became denser and less permeable.

Light transmission and film transparency

Transmission of UV and visible light at selected wavelength in the range of 200-800 nm to fish skin gelatin from both species incorporated without and with antioxidants

is presented in Table 21. Generally, fish skin gelatin films exhibited the low light transmission in the UV range (200-280 nm). However, skin gelatin films of both species incorporated with BHT or α-tocopherol showed slightly greater light transmission at 280 nm, compared with the control films (without BHT or α-tocopherol). BHT or α-tocopherol might interact with the sensitive chromophores (aromatic amino acid) responsible for the absorption of light below 300 nm of gelatin films (Li *et al*., 2004). BHT incorporated films generally exhibited the lower light transmission in the visible range (350-800 nm) than the control film, while those added with α-tocopherol showed slightly greater light transmission at all wavelength tested. The differences in light transmission of fish skin gelatin films added with BHT and α-tocopherol could be caused by the differences in interaction of BHT or α-tocopherol with gelatin, which determined the light transmission properties of the films. From the results, it was observed that light transmission of fish skin gelatin films slightly decreased as storage time increased. Regardless of antioxidant addition and type, brownstripe red snapper skin gelatin films generally showed a lower light transmission in the UV range but had a greater transmission in the visible range, compared with those from bigeye snapper skin. Jonjareonrak *et al*. (2005) reported that transmission of the fish skin gelatin film was influenced by types of fatty acid and their sucrose esters.

The transparency of fish skin gelatin films incorporated with BHT or α-tocopherol is shown in Table 21. Film from bigeye snapper skin gelatin added with α-tocopherol were more transparent than the control film and that incorporated with BHT ($P < 0.05$), respectively, as indicated by the lower value. However, no differences in transparency were observed between films from brownstripe red snapper without and with BHT or α-tocopherol ($P > 0.05$). The differences in interaction between BHT or α-tocopherol and gelatin possibly caused the differences in film transparency. During the storage, no differences in transparency of films from bigeye snapper skin was observed ($P > 0.05$). On the other hand, film from brownstripe red snapper became more transparent (lesser value), especially after 3 weeks of storage ($P < 0.05$). Regardless of antioxidant addition, brownstripe red snapper skin gelatin film was generally more transparent than bigeye snapper skin gelatin film ($P < 0.05$).

Table 21. Light transmission (%T) and transparency of bigeye snapper and brownstripe red snapper skin gelatin films incorporated without and with BHT or α-tocopherol during storage

Source of gelatin	Antioxidant type	Week	Wavelength (nm)								Trans-parency[1]
			200	280	350	400	500	600	700	800	
bigeye snapper skin	w/o	0	0.10	26.10	68.70	73.25	78.15	80.25	81.75	83.30	2.30±0.04^{aB*}
		3	0.10	26.00	68.45	73.20	78.05	80.00	81.60	83.20	2.33±0.04^{aB}
		6	0.10	25.85	68.20	73.10	78.10	80.10	81.50	83.20	2.31±0.06^{aB}
	BHT	0	0.10	27.15	65.05	71.05	76.55	78.65	79.85	81.05	2.50±0.02^{bA}
		3	0.10	27.05	64.80	70.95	76.50	78.55	79.70	80.90	2.52±0.06^{abA}
		6	0.10	27.05	64.60	70.95	76.40	78.40	79.50	80.80	2.54±0.02^{aA}
	α-tocopherol	0	0.10	28.85	70.40	75.25	79.10	80.95	81.90	83.95	2.20±0.04^{aC}
		3	0.10	28.25	70.35	75.25	79.00	80.75	81.80	83.80	2.23±0.03^{aC}
		6	0.10	27.85	70.30	75.25	78.80	80.85	81.70	83.80	2.22±0.02^{aC}
brownstripe red snapper skin	w/o	0	0.10	13.15	60.25	72.05	81.25	84.05	85.15	85.85	1.85±0.01^{aA}
		3	0.10	12.65	60.00	71.85	81.00	83.80	85.05	85.70	1.84±0.01^{aA}
		6	0.10	11.65	59.80	71.35	80.80	83.70	84.90	85.50	1.59±0.02 ^{bA}
	BHT	0	0.10	13.45	58.60	70.60	81.05	84.10	85.05	85.55	1.88±0.04^{aA}
		3	0.10	13.40	58.40	70.55	80.90	83.85	84.95	85.35	1.87±0.05^{aA}
		6	0.10	13.30	58.10	70.40	80.80	83.60	84.70	85.25	1.59±0.03^{aA}
	α-tocopherol	0	0.00	19.00	64.25	75.15	83.85	86.15	87.15	87.70	1.89±0.05^{aA}
		3	0.00	18.50	63.75	75.00	83.75	86.10	87.00	87.70	1.91±0.08^{aA}
		6	0.00	18.00	63.65	75.00	83.70	86.00	87.00	87.50	1.60±0.04^{bA}

[1] Values are given as mean ± SD from triplicate determinations. * Different letters in the same column under the same gelatin source indicate significant differences ($P<0.05$). Different capital letters in the same column under the same storage time and gelatin source indicate significant differences ($P<0.05$). w/o: Films without BHT or α-tocopherol incorporated.

Color

Color of fish skin gelatin films from both species incorporated with BHT or α-tocopherol is shown in Table 22. In general, no marked change in L* (lightness) and a*(-a* = greenness, +a* = redness) of films was observed with the addition of BHT or α-tocopherol ($P>0.05$). Nevertheless, b* values (-b* = blueness, +b* = yellowness) of skin gelatin films from both species incorporated with α-tocopherol were lower than that of the control (without BHT or α-tocopherol) and BHT incorporated films ($P<0.05$). From the result, L* and a* values of the control films from both species were not changed during storage of 6 weeks ($P>0.05$). Nevertheless, slight increases in a* and b* values of α-tocopherol incorporated films of both species were observed with increasing storage time ($P<0.05$). When the color values of bigeye snapper and brownstripe red snapper skin gelatin films were compared, L* and a* values

of the former were generally greater, whereas b* value was lower. This could be associated with the different color characteristics of the gelatin from both fish skins.

Table 22. Color of of bigeye snapper and brownstripe red snapper skin gelatin films incorporated without and with BHT or α-tocopherol during storage

Source of gelatin	Antioxidant type	Week	L*[1]	a*[1]	b*[1]
bigeye snapper skin	w/o	0	89.39±0.27^{abB**}	-1.43±0.07^{aA}	2.64±0.17^{aA}
		3	89.38±0.27^{abB}	-1.40±0.09^{aA}	2.52±0.21^{aA}
		6	89.47±0.20^{aB}	-1.37±0.09^{aA}	2.45±0.29^{aA}
	BHT	0	89.89±0.28^{aA}	-1.50±0.13^{aA}	2.61±0.20^{aA}
		3	90.12±0.41^{aA}	-1.47±0.08^{aA}	2.60±0.18^{aA}
		6	90.12±0.40^{aA}	-1.40±0.07^{aA}	2.59±0.23^{aA}
	α-tocopherol	0	89.49±0.19^{aAB}	-1.46±0.12^{aA}	2.14±0.06^{bB}
		3	89.54±0.17^{aB}	-1.43±0.10^{aA}	2.24±0.12^{bB}
		6	89.54±0.21^{aB}	-1.32±0.12^{aA}	2.57±0.18^{aA}
brownstripe red snapper skin	w/o	0	88.36±0.20^{aA}	-1.82±0.09 ^{aA}	6.44±0.21^{aA}
		3	88.44±0.17^{aA}	-1.84±0.04^{aB}	6.40±0.18^{aA}
		6	88.44±0.20^{aA}	-1.81±0.12^{aB}	6.29±0.20^{aA}
	BHT	0	88.33±0.26^{aA}	-1.80±0.08^{bA}	6.63±0.31^{aA}
		3	88.39±0.26^{aA}	-1.72±0.06^{bA}	6.55±0.42^{aA}
		6	88.49±0.20^{aA}	-1.60±0.10^{aA}	6.41±0.38^{aA}
	α-tocopherol	0	88.61±0.26^{aA}	-1.82±0.08^{aA}	6.15±0.18^{bB}
		3	88.63±0.13^{aA}	-1.82±0.08^{aB}	6.26±0.27^{bA}
		6	88.65±0.15^{aA}	-1.69±0.18^{aAB}	6.64±0.26^{aA}

[1] Values are given as mean ± SD from ten determinations. ** Different letters in the same column under the same gelatin source indicate significant differences ($P<0.05$). Different capital letters in the same column under the same storage time and gelatin source indicate significant differences ($P<0.05$). w/o: Films without BHT or α-tocopherol incorporated.

Oxidation of lard during storage as affected by gelatin film incorporated with BHT or α-tocopherol

Changes in TBARS

TBARS values of lard covered with fish skin gelatin films with and without BHT or α-tocopherol at a level of 200 ppm during storage at 28°C and 50% RH are depicted in Figure 23. In general, TBARS values of all treatments increased continuously up to 28 days

of storage ($P<0.05$). However, the increase in TBARS value of lard covered with fish skin gelatin films showed the much lower rate, compared with uncovered sample. Commonly, TBARS assay is a method used to determine the lipid peroxidation (Jardine *et al.*, 2002).

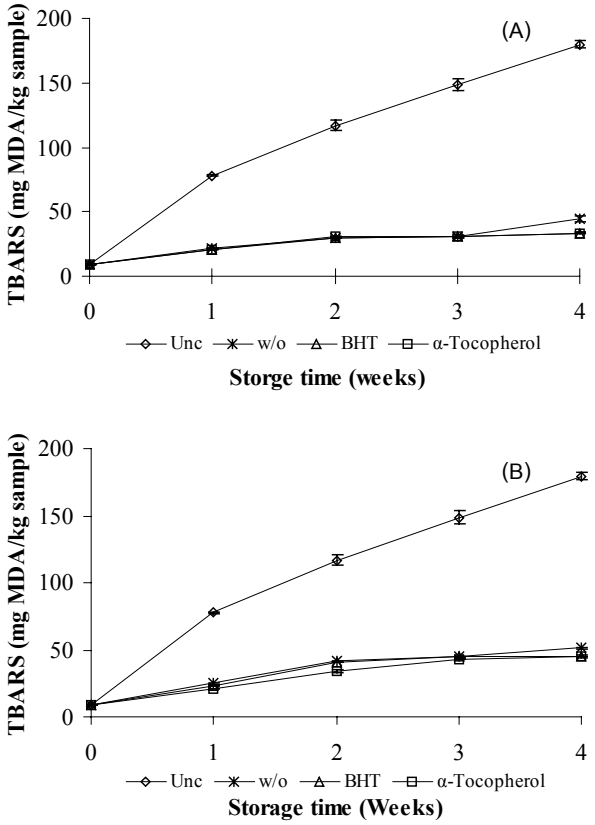

Figure 23. TBARS of lard covered with bigeye snapper skin gelatin film (A) and brownstripe red snapper skin gelatin film (B) incorporated without and with BHT or α-tocopherol during storage. Lard without film covered (Unc), lard covered with film without BHT or α-tocopherol incorporated (w/o), lard covered with film with BHT incorporated (BHT), lard covered with film with α-tocopherol incorporated (α-Tocopherol). Bars represent the standard deviation from triplicate determinations.

Regardless of antioxidant addition, TBARS value of lard covered with fish skin gelatin films from both bigeye snapper and brownstripe red snapper slightly increased during

storage. Fish skin gelatin films might function as a barrier to oxygen permeability at the lard surface. Therefore, only small amount of oxygen could penetrate to the lard, leading to the lower oxidation rate. From the result, no differences in TBARS were observed among lard covered with gelatin films added with and without BHT or α-tocopherol. Interaction between antioxidant and film matrix via some bondings possibly resulted in the lowered release of those antioxidants to the lard. As a consequence, no differences in TBARS value were found between samples covered with film with and without antioxidants. It was reported that the incorporation of hydrophobic compounds into films caused the negative effects on oxygen transmission rate (Wessling *et al*., 2000). When the TBARS values of lard covered with bigeye snapper and brownstripe red snapper skin gelatin films were compared, lard covered with the latter films had a slightly greater TBARS values at the same storage time determined. This was possibly associated with the higher oxygen transmission rate of brownstripe red snapper skin gelatin films, compared with bigeye snapper skin gelatin films.

Changes in peroxide values

Peroxide value was also used to monitor the oxidation of lard covered with bigeye snapper or brownstripe red snapper skin gelatin films incorporated without and with BHT or α-tocopherol at 200 ppm (Figure 24). PV of lard without film covering increased continuously within the first 3 weeks. Thereafter, the rate of increase was lower. The decomposition of peroxide to secondary products might take place to some extent, resulting in the lower peroxide already formed (Boselli *et al*., 2005). Among all treatments, lard covered with fish skin gelatin film with and without BHT or α-tocopherol incorporated showed no changes in PV within the first 2 weeks. Slight increase in PV was noticeable at week 3, but constant PV was obtained during 3-4 weeks of storage ($P>0.05$). From the result, films incorporated with antioxidants exhibited no differences in preventing the oxidation of lard when compared with the control film as shown by the similar PV as well as TBARS values. Thus gelatin film showed the effective preventive effect towards lipid oxidation of lard, but antioxidants incorporated exhibited negligible antioxidant effect on lard.

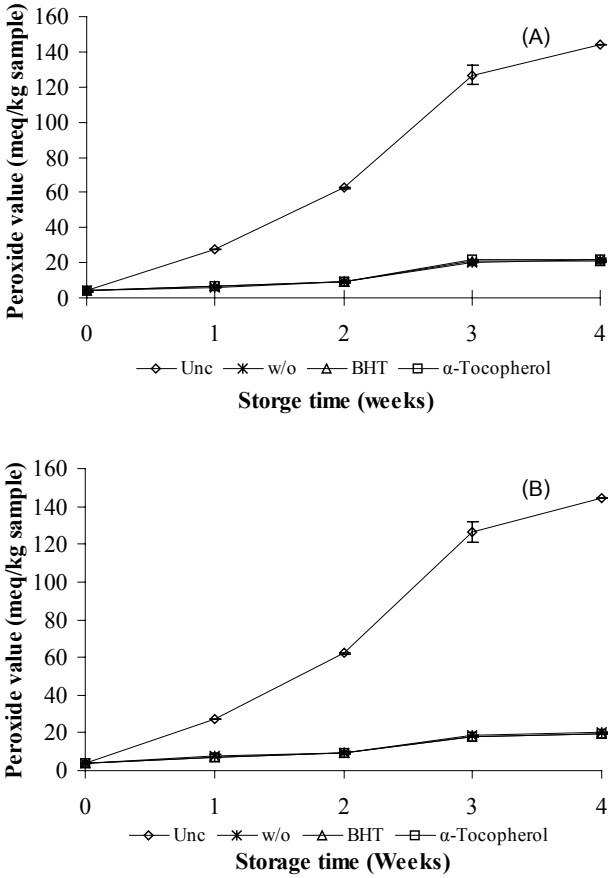

Figure 24. Peroxide values of lard covered with bigeye snapper skin gelatin film (A) and brownstripe red snapper skin gelatin film (B) incorporated without and with BHT or α-tocopherol during storage. Lard without film covered (Unc), lard covered with film without BHT or α-tocopherol incorporated (w/o), lard covered with film with BHT incorporated (BHT), lard covered with film with α-tocopherol incorporated (α-Tocopherol). Bars represent the standard deviation from triplicate determinations.

Changes in antioxidative activity of fish skin gelatin films incorporated with BHT or α-tocopherol during storage

Antioxidative activity of BHT and α-tocopherol incorporated in fish skin gelatin films of both species at the concentration of 200 ppm during storage was monitored

(Figure 25). It was found that the methanol extract of films from both species showed the lower DPPH radical scavenging activity than BHT or α-tocopherol in the free form at the same amount added into the FFS (data not shown). Hydroxyl group of BHT and α-tocopherol generally reacted as hydrogen donor (Nawar, 1996). Therefore, interactions between the antioxidants and gelatin film matrix might be formed via hydrogen bonding, particularly during film formation. As a result, a small amount of antioxidants could be extracted by methanol. However, DPPH radical scavenging activity of the methanol extracts from fish skin gelatin films of both species increased with increasing storage time ($P<0.05$). The renaturation of gelatin chains into the helix coil structure during storage (Arvanitoyannis *et al.*, 1997) might cause the more release of antioxidant from the film matrix. Consequently, a greater amount of BHT or α-tocopherol could be extracted from the film when the storage time increased. From the result, slightly greater amount of antioxidants could be extracted from brownstripe red snapper skin gelatin films after 3 weeks of storage, compared with the film from bigeye snapper. Wessling *et al.* (2000) reported that BHT-impregnated HDPE film was free from BHT and 19% of the original amount incorporated to the film remained in the cereal after 6 weeks. This suggested that there was no interaction between BHT and HDPE film. Due to the greater reactivity of proteins in interacting with antioxidants added, a greater amount of antioxidants should be incorporated to obtain the films with an excessive amount of antioxidants in the free form. The releasing characteristic of antioxidants incorporated as well as properties of the films should be further investigated.

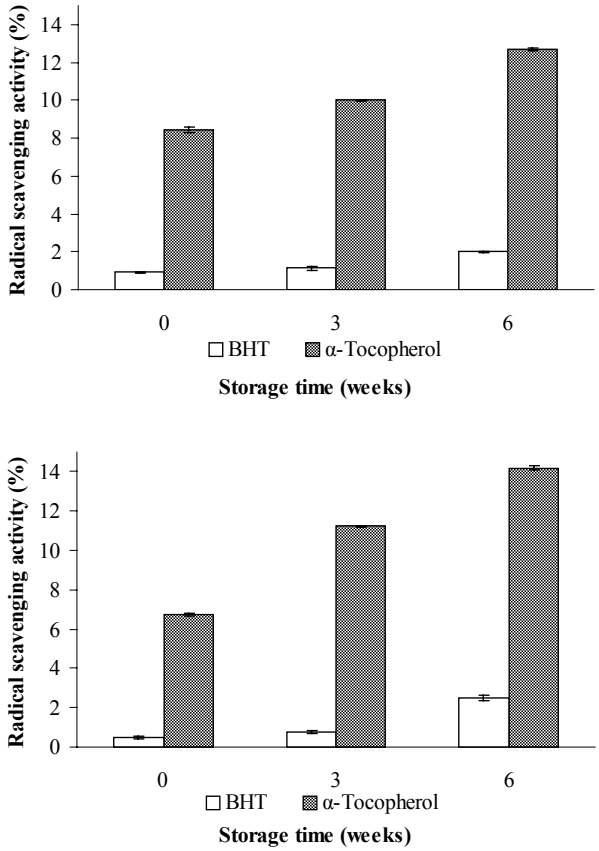

Figure 25. DPPH radical scavenging activity of bigeye snapper skin gelatin film (A) and brownstripe red snapper skin gelatin film (B) incorporated without and with BHT or α-tocopherol during storage. Bars represent the standard deviation from triplicate determinations.

8.5 Conclusion

Mechanical properties and color of fish skin gelatin films of both species were generally affected by the incorporation of BHT or α-tocopherol as well as storage time. WVP of films decreased, when both BHT and α-tocopherol were incorporated. Films were more transparent with the incorporation of α-tocopherol and increasing storage time. Oxidation of lard was effectively retarded when covering with fish skin gelatin films of both species regardless of antioxidant incorporation.