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RESEARCH PAPER

Improvement of Properties of Sardine Myofibrillar Protein Films Using Squid Ink Tyrosinasein Combination with Tannic Acid

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Abstract

The effect of squid ink tyrosinase (SIT) and tannic acid (TA) on the properties of myofibrillar protein films from washed sardine mince was studied. The film made from film forming solution added with SIT (5 k units L-1) and TA (1 g L-1) had the highest tensile strength (TS) (9.49 ± 0.11 MPa) but lowest elongation at break (EAB) (25.91 ± 1.81%) ($P<0.05$). The addition of mixture of SIT and TA reduced the water vapour permeability (WVP) of films ($P<0.05$).Fourier transform infrared (FTIR)spectra of films containing both SIT and TA showed a shift in the amide-III band to the lower wavenumberof 1230 cm-1. The decrease in myosin heavy chain (MHC) band intensity with the presence of high-molecular-weight aggregates in the film added with the mixture of SIT and TAwas observed, indicating the increased cross-linking. The films added with the mixture of SIT and TA were less transparent, compared to the control film. The microstructure studyrevealedthat the surface of films added with only SIT or the mixture of SIT and TA became slightly rougher. Thus, the use of SIT in conjunction with TA could improve mechanical property and water barrier property of resulting film.

Keywords: Ardine, myofibrillar protein, squid ink tyrosinase, tannic acid, cross-linking.

Introduction

Biodegradable packaging has gained more attention recently since it is environment friendly. Biopolymers can be potential alternatives to non-biodegradable synthetic polymers. Biodegradable/edible packagings can be prepared from various biopolymers such as polysaccharides, proteins, lipids etc. Among the various sources, proteins have been extensively used for the preparation of biodegradable films because of its good film-forming ability (Wihodo & Moraru, 2013; Azeredo & Waldron, 2016)). Films have been prepared using myofibrillar proteins from different fish species such as round scad (Decapterus maruadsi) muscle (Artharn *et al.* 2007), red tilapia (Oreochromis niloticus) muscle (Tongnuanchan *et al.*, 2011) and bigeye snapper (Priacanthus tayenus) surimi (Chinabhark *et al*., 2007). However, myofibrillar protein films still have poor mechanical and water vapour barrier properties, compared to their synthetic counterparts (Cuq, 2002; Venugopal, 2009).

Various methods including chemical and enzymatic reactions, irradiation treatments or incorporation of hydrophobic material or synthetic polymers have been implemented to improve the properties of protein based films.The addition of tannic acid, caffeic acid and ferulic acid increased the tensile strength of porcine plasma protein-based films (Nuthong, Benjakul & Prodpran, 2009). Mechanical properties of films from myofibrillar proteins of bigeye snapper (Priacanthustayenus) were improved by the addition of phenolic compounds such as caffeic acid, catechin, ferullic acid and tannic acid (Prodpran, Benjakul & Phatcharat, 2012). Prior enzymatic cross-linking of bovine gelatin via horseradish peroxidase, glucose oxidase and glucose improved the microstructure and mechanical properties of target film (Han & Zhao, 2016).

Tyrosinase from squid melanin-free ink in combination with tannic acid improved the gel properties of sardine surimi (Vate & Benjakul, 2016) via increasing the protein cross-linking in heat induced aggregation of natural actomyosin (Vate & Benjakul, 2016a). Protein cross-linking induced by tyrosinase from melanin-free ink in the presence of appropriate phenolic compound might improve the properties of fish myofibrillar protein films. Nevertheless, no information on the use of this approach for improvement of fish myofibrillar protein films exists. Therefore, the aim of this study was to investigate the effect of mixture of squid ink

tyrosinase and tannic acid on the properties of myofibrillar protein film from washed sardine mince.

Materials and Methods

Chemicals

Tannic acid, glycerol, sodium chloride (NaCl),Brij 35, L-DOPA (3,4-Dihydroxy-L-phenylalanine),β-mercaptoethanoland wide range molecular weight protein markers were purchased from Sigma (St. Louis, MO, USA). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Merck (Darmstadt, Germany).Bovine serum albumin (BSA) was purchased from Fluka (Buchs, Switzerland).Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, N,N,N,N-tetramethyl ethylene diamine (TEMED) and all chemicals for electrophoresis were procured from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals mentioned above were of analytical grade.

Preparation of Squid Ink Tyrosinase

Preparation of Melanin-Free Ink

Squids of mantle size 18-20 cm were purchased from a local market in Hat Yai, Thailand, after 24 h of capture, stored in ice using a squid/ice ratio of 1:2 (w/w), and transported to the Department of Food Technology, Prince of Songkla University, Thailand within 30 min. Upon arrival, ink sac was separated from the squid by cutting the ink duct and ink was squeezed out from the ink sac. Melanin-free ink was prepared according to the method of Vate and Benjakul (2013).

Fractionation of Tyrosinase

Tyrosinase from MFI was fractionated as per the method of Simpson, Marshall and Otwell (1987) with a slight modification. MFI (50 mL) was mixed with 50 mL of 0.05 M sodium phosphate buffer (pH 7.2), containing 1.0 M NaCl and 0.2% Brij 35. The mixture was stirred continuously at 4°C for 30 min. Thereafter, the solid ammonium sulphate (36.1 g) was added into the mixture (100 mL) gradually with gentle stirring until complete solubilization, in which 60% saturation was obtained. The mixture was allowed to stand at 4°C for 30 min. The precipitate was collected by centrifugation at 12,500×g at 4°C for 30 min. The pellet obtained was dissolved in a minimum volume of 0.05 mM sodium phosphate buffer (pH 7.2) and dialyzed with 15 volumes of the same buffer with three changes overnight. The fraction containing tyrosinase referred to as 'squid ink tyrosinase, SIT' was kept at -40°C until used.

Measurement of Tyrosinase Activity

Tyrosinase activity was assayed using L-DOPA as a substrate according to the method of Simpson *et al*. (1987) with a slight modification. Reaction mixtures consisted of 600 µl of 15 mM L-DOPA in deionised water, 400 µl of 0.05 M phosphate buffer (pH 6.0) and 100 µl of deionised water. To initiate the reaction, 100 µL of SIT was added and the reaction was run for 3 min at room temperature. The formation of dopachrome was monitored by reading at 475 nm using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan). One unit of activity was defined as the enzyme causing an increase in the absorbance at 475 nm by 0.001/min. Enzyme and substrate blanks were prepared by excluding the substrate and enzyme, respectively, from the reaction mixture and deionised water was used instead.

Collection and Preparation of Fish

Sardines (*Sardinella albella*) with an average weight of 50-60 g were caught from Songkhla coast along the Gulf of Thailand. The fish, off-loaded approximately 12 h after capture, were placed 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 within 1 h. The fish were headed, gutted and washed with iced tap water. The flesh was separated manually from skin and bone and kept on ice prior to washing. The storage time was not longer than 24 h.

Preparation of Washed Mince

Washed mince was prepared according to the method of Toyohara *et al.* (1990) with slight modifications. Fish mince was homogenised with 5 volumes of cold 0.05 M NaCl (2-4°C) at a speed of 13,000 rpm for 2 min using an IKA Labortechnik homogeniser (Selangor, Malaysia). The washed mince was filtered through two layer of cheese-cloth. The washing process was repeated twice. Washed mince obtained was stored on ice until used for film preparation.

Preparation of Film-Forming Solution

The film-forming solution (FFS) from washed mince was prepared according to the method of Chinabhark, Benjakul and Prodpran (2007). The washed mince (200 g) was added with 3 volumes of distilled water and homogenised at 13,000 rpm for 1 min. The protein concentration of the mixture was fixed at 2% (w/v). Glycerol, used as a plasticiser, was added at 50% (w/w) of protein. The pH of the mixture was adjusted to 3 using 1M HCl to solubilise the protein. FFS was added with SIT (5 k units L-1) or TA (0.5 and 1 g L-1), referred to as SIT, TA-I and TA-II, respectively.

For TA/SIT mixture, TA solution was mixed with SIT to obtain the different TA and SIT working concentrations. SIT/TA mixtures included 5 k units L-1 SIT + 0.5 g L-1 TA or the mixtures of 5 k units

L-1 SIT + 1 g L-1TA. The mixtures were incubated at 25°C for 30 min. The reaction was terminated by boiling the mixture for 3 min. Thereafter, the obtained mixtures were cooled prior to addition into FFS. FFS obtained was named as SIT/TA-I and SIT/TA-II, respectively.

All FFS was degassed using the sonicating bath with 50/60 Hz (Elmasonic S 30 H, Singen, Germany) for 10 min. FFS obtained was filtered through a layer of cheese-cloth to remove undissolved debris. The filtrate was used for film casting.

Film Casting and Drying

To prepare the film, FFS (4 g) was cast onto a rimmed silicone resin plate (50 × 50 mm^2) and air-blown for 12h at room temperature prior to further drying at 25°C and 50±5% relative humidity (RH) for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and used for analyses.

Determination of Film Properties

Film Thickness

The thickness of film was measured using a micrometer (°°°otech, Model GT-313-A, Gotech testing machines Inc, Taichung city, Taiwan). Five random locations around each film samples were used for average thickness determination.

Mechanical Properties

Prior to testing, films were conditioned for 48 h at 25°C and 50±5% RH. Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata *et al.* (2000) with a slight modification using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK) equipped with tensile load cell of 100 N. Ten samples (2 × 5 cm^2) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

Water Vapour Permeability (WVP)

WVP was measured following a modified ASTM method (American Society for Testing and Materials, 1989). The film was sealed on an aluminium permeation cup containing dried silica gel (0% RH) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30°C. The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$WVP\ (g\ m{-}1\ s{-}1\ Pa{-}1) = w\ l\ A\ {-}1\ t{-}1\ (P2{-}P1)\ {-}1$$

where w is the weight gain of the cup (g); l is the film thickness (m); A is the exposed area of film (m2); t is the time of gain (s); (P2–P1) isthe vapour pressure difference across the film (Pa).

Colour

Colour of films was determined using a CIE colourimeter (Hunter associates laboratory, Inc., VA, USA), using D65 (day light) and a measure cell with opening of 30 mm. The colour of the films was expressed as L* (lightness), a* (redness) andb*(yellowness/greenness).The difference of colour (ΔE*) was calculated as follows:

$$〖ΔE〗\ ^*=√(\ 〖(\ 〖ΔL〗\ ^*)〗\ ^2+\ 〖(\ 〖Δa〗\ ^*)〗\ ^2+\ 〖(\ 〖Δb〗\ ^*)〗\ ^2\ )$$

where ΔL*, Δa* and Δb* are the differences between the colour parameters of the samples and those of the white standard.

Light Transmittance and Transparency Value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) as described by Shiku, Hamaguchi, Benjakul, Visessanguan and Tanaka (2004). The transparency value of film was calculated using the following equation (Han & Floros, 1997):

$$Transparency\ value = -logT600/x$$

where T600is the fractional transmittance at 600 nm and x is the film thickness (mm). The greater transparency value represents the lower transparency of film.

Protein Pattern

Protein patterns of films was determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). To solubilise the films prior to SDS-PAGE analysis, films were mixed with 5% SDS and heated at 85°C for 1h. Proteins (15 µg) determined by the Biuret method (Robinson & Hodgen, 1940) were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-PROTEAN II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 12 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h. Molecular weights of proteins were estimated from protein markers.

Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) Spectroscopy

Films were scanned with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at room temperature as described by Nuthong *et al.* (2009). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 400-4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and the ratio was taken against a background spectrum recorded from the clean empty cell at 25°C.

Scanning Electron Microscopy (SEM)

Morphology of surface and cross-section of film samples was visualised using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, Netherlands). For cross-section, samples were fractured under liquid nitrogen prior to morphology visualisation. Then, the samples were mounted on bronze stub and sputtered with gold (Sputter coater SPI-Module, PA, USA) in order to make the sample conductive. The photographs were taken at an acceleration voltage of 15 kV.

Statistical Analysis

All experiments were run in triplicate using three different lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by the Duncan's multiple range tests (Steel and Torrie, 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 17.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and Discussion

Effect of SIT/TA Mixtures On Properties of Sardine Myofibrillar Protein Films

Thickness and Mechanical Properties

Thickness and mechanical properties of films prepared from washed sardine minceadded without and with SIT, TA or the SIT/TA mixtures are given in Table 1. The film added with only SIT (5 k units L-1 FFS) had the highest thickness amongst all film samples ($P<0.05$). The presence of SIT might reducethe interaction between protein molecules in the film in which the compact network was not formed. However, films added with only TA or SIT/TA-II had the lowest thickness ($P<0.05$). TS of film containing only SIT was similar to that of control film (film without SIT, TA or SIT/TA mixture addition) but was lower than that added with only TA ($P<0.05$). This was more likely due to the lower activity of SIT in FFS at pH3. The optimum pH of tyrosinase was around 6 (Simpson *et al.*, 1987). The addition of TA increased TS of the film in a dose dependent manner ($P<0.05$). TA was reported to exhibit multidentate mechanism, in which phenolic compounds could react with several protein sites and led to the augmented cross-links between proteins (Haslam, 1989). Amongst different phenolic compounds (caffeic acid, catechin, ferullic acid and tannic acid), tannic acid yielded the film from myofibrillar proteins of bigeye snapper (Priacanthus tayenus) with the highest TS ($P<0.05$) (Prodpran *et al.*, 2012). TS of the film increased with the addition of SIT/TA mixtures and the highest TS was found for the film added with the SIT/TA-II. Oxidation ofTA by SIT couldtake place and the resulting quinones could induce the cross-linking of proteins in washed sardine mince. The quinones formed via the oxidation of TA by SIT mostly increased the cross-linking between the actomyosin molecules during heat induced aggregation (Vate & Benjakul, 2016a). These diquinones formed during diphenolase activity of tyrosinase are very reactive and can react with various amino acid side chains such as sulfhydryls, amines, amides, indoles and other tyrosinescommonly present in proteins (Selinheimo *et al.*, 2008).

EAB of films decreased with the addition of TA or SIT/TA mixtures. In general, the increased TS of film was concomitant with the lowered EAB. The higher EAB reflected the higher extensibility of films. Extensibility of films was reduced by the addition of TA or SIT/TA mixture. This was plausibly due to the increased cross-linking of proteins, particularly in the presence of SIT and TA. EAB of film was decreased when the strong bonds were introduced in film matrix (Tongnuanchan, Benjakul & Prodpran, 2011). The result suggested that the films added with SIT/TA mixture had stronger bonds, compared to those added with only SIT and only TA. The addition of SIT/TA-II yielded the film withhigher protein cross-linking than SIT/TA-I as evidenced by the lower EAB and higher TS of the former. Quinone or diquinone, intermediate from oxidation of TA by SIT more likely induced the cross-linking of proteins in the network along with the formation of non-disulphide covalent bonds (Vate & Benjakul, 2016). Hence the addition of mixture of SIT and TA, especially in the presence of higher level of TA, increased TS but decreased the extensibility as indicated by lower EAB values.

Water Vapour Permeability (WVP)

WVP of films prepared from washed sardine mince as affected by the addition of SIT, TA and their mixtures is shownin Table 1. The control filmhad the highest WVP ($P<0.05$). This suggested that these films had lower barrier towardwatervapour

permeability.The film added with only SIT had higher WVP than those incorporated with only TA and the mixtures of SIT and TA (P<0.05). This indicated that there was lower protein interaction in the film added with SIT, compared with those added with TA orthe SIT/TA mixture. Both the film samples with only TA (0.5 g and 1 g L-1 FFS) had the similar WVP (P>0.05). Thus, TA addition could improve water vapour barrier property more effectively than SIT in this study. The films added with mixture of SIT and TA had the lower WVP values (P<0.05). These results were in agreement with the increased TS of resulting films (Table 1). Increased compactness of the film structure by high protein cross-linking more likely reduced the permeability of moisture vapour through the films. Despite having good mechanical properties, protein films generally possess poor moisture barrier properties because of their hydrophilic nature (Guilbert, 1986; Kester & Fennema 1986). Fish muscle is hydrophilic, associated with polar amino acids. Paschoalick, Garcia, Sobral, and Habitante (2003) reported that the muscle protein of Nile tilapia had polar ionic amino acids (aspartic acid, glutamic acid, arginine and lysine) at high content. The cross-linked proteins mediated by TA and SIT/TA mixture could reduce the hydrophilic moieties in the matrix of film, resulting in decreased film hydrophilicity and thus decreasing WVP of the film.

Colour, Light Transmittance and Transparency Value

L*, a* and b* and transparency values of protein films from washed sardine mince added without and with SIT, TA and their mixtures are presented in Table 2. The control film and film containing 0.5g TA L-1 FFS had higher L* (lightness) values, compared to other films (P<0.05). There was a coincidental increase in a*-value (redness) in the sample added with the SIT/TA mixture. The b*-value (yellowness) was highest for the films added with SIT. This might be due to the indigenous pigment of SIT. In general, films added with TA at both levels had the lower b*-value than those containing SIT (P<0.05). Decreased L* values were in accordance with the increases in a*-values and b*-values of myofibrillar protein films incorporated with phenolic compounds (Prodpran *et al.*, 2012).

Light transmittance in both UV and visible ranges of protein films from sardine washed mince with different treatments is given in Table 3. Light transmittance decreased as SIT was added. Only slight decrease of transmittance was found in films added with TA at both levels. Nevertheless, the decrease in transmittance of films was also noticeable in the wavelength of 350-600 nm when the SIT/TA mixtures were incorporated. Light transmittance in the UV range (200 and 280 nm) was lower for all the films. Protein-based films had good UV barrier properties owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi, Wu, Yin & Tanaka, 2007). The enhanced cross-linking of the protein molecules in the film matrix might also lower the light transmittance throughout the films. This indicated that the films added with SIT/TA mixture, especially in the presence of 1 g L-1 TA, were more compact and were able to prevent the light

Table 1. Thickness, mechanical properties and water vapour permeability of films from washed sardine mince added with SIT, TA or SIT/TA mixtures

Samples	TS (MPa)	EAB (%)	WVP ($\times 10^{-11}$ g m^{-1} s^{-1} Pa^{-1})	Thickness (mm)
Control	7.12 ± 0.20 ^{e}	38.17 ± 2.75 ^{a}	2.98 ± 0.07^{a}	0.056 ± 0.004^{b}
SIT	7.15 ± 0.19 ^{e}	37.75 ± 3.01 ^{ab}	2.69 ± 0.04^{b}	0.061 ± 0.004^{a}
TA-I	7.66 ± 0.14 ^{d}	34.27 ± 2.58^{bc}	2.60 ± 0.06^{c}	0.049 ± 0.002^{c}
TA-II	7.83 ± 0.10 ^{c}	32.46 ± 1.92 ^{c}	2.56 ± 0.09^{cd}	0.055 ± 0.004^{b}
SIT/TA-I	8.44 ± 0.44 ^{b}	27.18 ± 1.27 ^{d}	2.39 ± 0.08^{e}	0.054 ± 0.003^{b}
SIT/TA-II	9.49 ± 0.11^{a}	25.91 ± 1.81 ^{e}	2.21 ± 0.02^{f}	0.049 ± 0.003^{c}

Values indicate mean ± SD (n = 3). Different superscript lowercase letters in the same column indicate the significant differences (P<0.05). SIT: 5 k units L^{-1} SIT; TA-I: 0.5 g L^{-1} TA; TA-II: 1 g L^{-1} TA; SIT/TA-I: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; SIT/TA-II: 5 k units L^{-1} SIT+ 1 g L^{-1} TA

Table 2. Colour and transparency value of the films from washed sardine mince added with SIT, TA or SIT/TA mixtures

Samples	L*	a*	b*	Transparency value
Control	88.49 ± 0.05 ^{a}	-2.02 ± 0.04 ^{e}	7.27 ± 0.16^{c}	1.97 ± 0.23^{c}
SIT	86.52 ± 0.27 ^{c}	-1.72 ± 0.09 ^{d}	8.72 ± 0.45^{a}	2.69 ± 0.51^{a}
TA-I	88.45 ± 0.06 ^{a}	-1.58 ± 0.03 ^{c}	5.71 ± 0.10^{e}	2.32 ± 0.20^{b}
TA-II	87.81 ± 0.08 ^{b}	-1.44 ± 0.05 ^{b}	6.06 ± 0.12^{d}	2.16 ± 0.20^{bc}
SIT/TA-I	85.86 ± 0.07 ^{d}	-1.37 ± 0.03 ^{a}	8.07 ± 0.15^{b}	2.29 ± 0.15^{b}
SIT/TA-II	85.55 ± 0.16 ^{e}	-1.36 ± 0.04 ^{a}	8.80 ± 0.18^{a}	2.39 ± 0.18^{b}

Values indicate mean ± SD (n = 3). Different superscript lowercase letters in the same column indicate the significant differences (P<0.05). SIT: 5 k units L^{-1} SIT; TA-I: 0.5 g L^{-1} TA; TA-II: 1 g L^{-1} TA; SIT/TA-I: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; SIT/TA-II: 5 k units L^{-1} SIT+ 1 g L^{-1} TA

to pass through the film. As a result, it could lower the oxidation of lipids in food induced by light. When considering the transparency value, the control film had the lowest transparency value, compared to others ($P<0.05$). The lower transparency value indicated that the film was more transparent. The film added with SIT (5 k units L-1) had the highest transparency value ($P<0.05$). This result was coincidental with the lower light transmittance of the film (Table 3). There were no differences in transparency values amongst all films added with TA, regardless of SIT addition. The result suggested that the incorporation of SIT, TA or their mixtures affected the transparency of resulting films to varying degrees.

Protein Pattern of Films

Protein patterns of films prepared from washed sardine mince incorporated with SIT, TA or their mixtures, under reducing conditions are shown in Figure 1. Similar protein patterns were observed among all film samples. Some cross-links between stacking gel and separating gel were found in the control films, suggesting thecross-linking of proteins in film via covalent bonds. Increase in polymers found in the stacking gel was found in the film samples added with SIT, TA and the mixture of SIT and TA. This suggested that MHC was more susceptible to crosslinking induced by the quinones formed via the oxidation of TA by SIT. The formation of cross-link of proteins was more likely stabilised by non-disulphide covalent bond, especially during film drying process. This was in accordance with increased TS of those samples (Table 1). In general, non-disulphide covalent bond, disulphide bond as well as other weak bonds most likely contributed to the film strengthening.The addition of phenolic compounds, especially oxidised form, contributed to the improved mechanical property of fish myosin protein based films (Prodpran *et al.*, 2012).

Table 3. Light transmittance values of the films from washed sardine mince added with SIT, TA or SIT/TA mixtures

Samples	Light transmittance (%)							
	200	280	350	400	500	600	700	800
Control	0.01	0.02	47.95	62.03	74.24	77.96	79.74	81.03
SIT	0.01	0.08	38.72	51.42	64.01	68.54	71.06	72.97
TA-I	0.01	0.01	50.58	62.43	73.02	76.77	78.92	80.43
TA-II	0.01	0.01	48.16	60.94	72.31	76.08	78.27	79.70
SIT/TA-I	0.01	0.01	44.10	58.01	70.99	75.44	78.06	79.92
SIT/TA-II	0.01	0.01	41.59	56.85	71.61	76.54	79.46	81.48

SIT: 5 k units L^{-1} SIT; TA-I: 0.5 g L^{-1} TA; TA-II: 1 g L^{-1} TA; SIT/TA-I: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; SIT/TA-II: 5 k units L^{-1} SIT+ 1 g L^{-1} TA

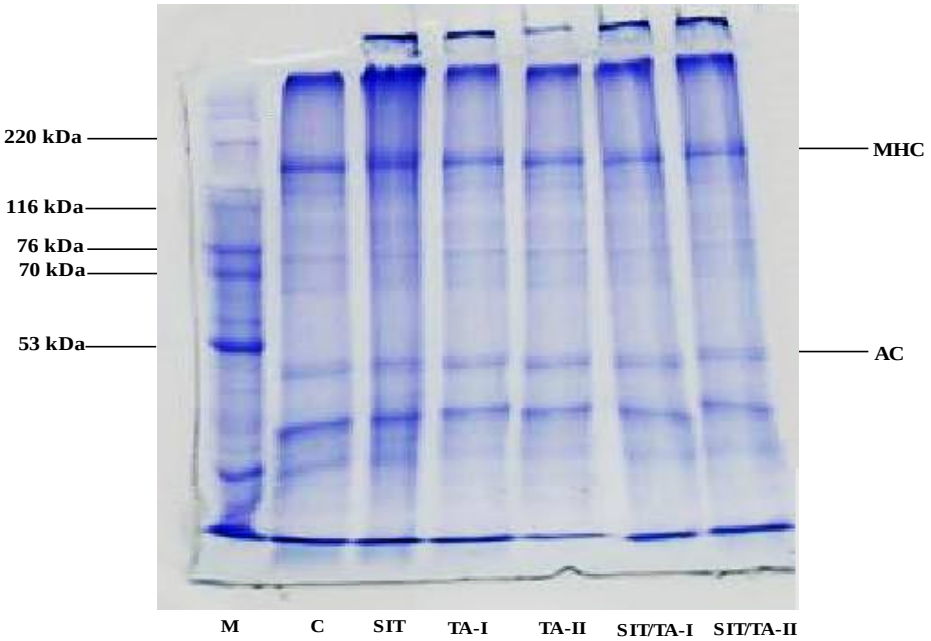

Figure 1. SDS–PAGE patterns of proteins of films from washed sardine mince without and with SIT, TA or SIT/TA mixtures at different concentrations. M, high molecular weight protein markers; C, control; MHC, myosin heavy chain; AC, actin; SIT, 5 k units L^{-1} SIT; TA-I, 0.5 g L^{-1} TA; TA-II, 1 g L^{-1} TA; SIT/TA-I, 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; SIT/TA-II, 5 k units L^{-1} SIT+ 1 g L^{-1} TA.

Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR spectra of films from sardine washed mince with various treatments in comparison with control film are illustrated in Figure 2. All films exhibited the major bandssuch as amide-I at around 1646 cm-1representing C=O stretching/hydrogen bonding coupled with COO andamide-II at 1539 cm-1, which is arising from bending vibration of N-H groups and stretching vibrations of C-N groups (Pal & Suresh, 2017; Aewsiri, Benjakul, &Visessanguan, 2009; Muyonga, Cole, & Duodu, 2004). Amide-III band representing the vibrations in plane of C- N and N-H groups of bound amide or vibrations of CH2 groups of glycine, shifted from around 1234 cm-1 in the control film to 1230 cm-1 in film added with SIT/TA-II. Amide III is arisen from N-H bend couple with C-N stretch (β sheet protein) (Pal & Suresh, 2017). This indicated protein interaction between C-N and N-H groups induced by the mixture of SIT and TA.The band situated at the wavenumber of around 1038 cm^{-1} was found in all film samples, corresponding to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo & Sobral, 2007; Hoque, Benjakul, & Prodpran, 2011).Amide-A and amide-B bands were observed at the wavenumber of 3275 cm-1and 2930 cm-1,respectively. The amide-A band represents the NH-stretching coupled with hydrogen bonding and amide-B band represents the asymmetric stretching vibration of CH as well as NH_3^+ (Ahmad &Benjakul, 2011; Muyonga *et al.*, 2004).

Scanning Electron Microscopy

Scanning electron microscopic images of surface and freeze-fractured cross-section of films from washed sardine mince without and with SIT, TA or SIT/TA mixture are depicted in Figure 3. The surface of the control film and film added with TA at 0.5 g L-1 FFS was smoother, compared to those of other film samples. The surface of the film added with SIT at 5 k unitsL-1FFS had rough surface. This was more likely due to the poorer cross-linking of the protein molecules owing to the lower activity of SIT at pH 3. The surfaces of other films were smoother, owing to the presence of ordered and homogeneous network. The freeze-fractured cross-section of the control film or those added with only TA or SIT showed the larger cracks, compared to those of films added with the SIT/TA mixture. The compact network of those films

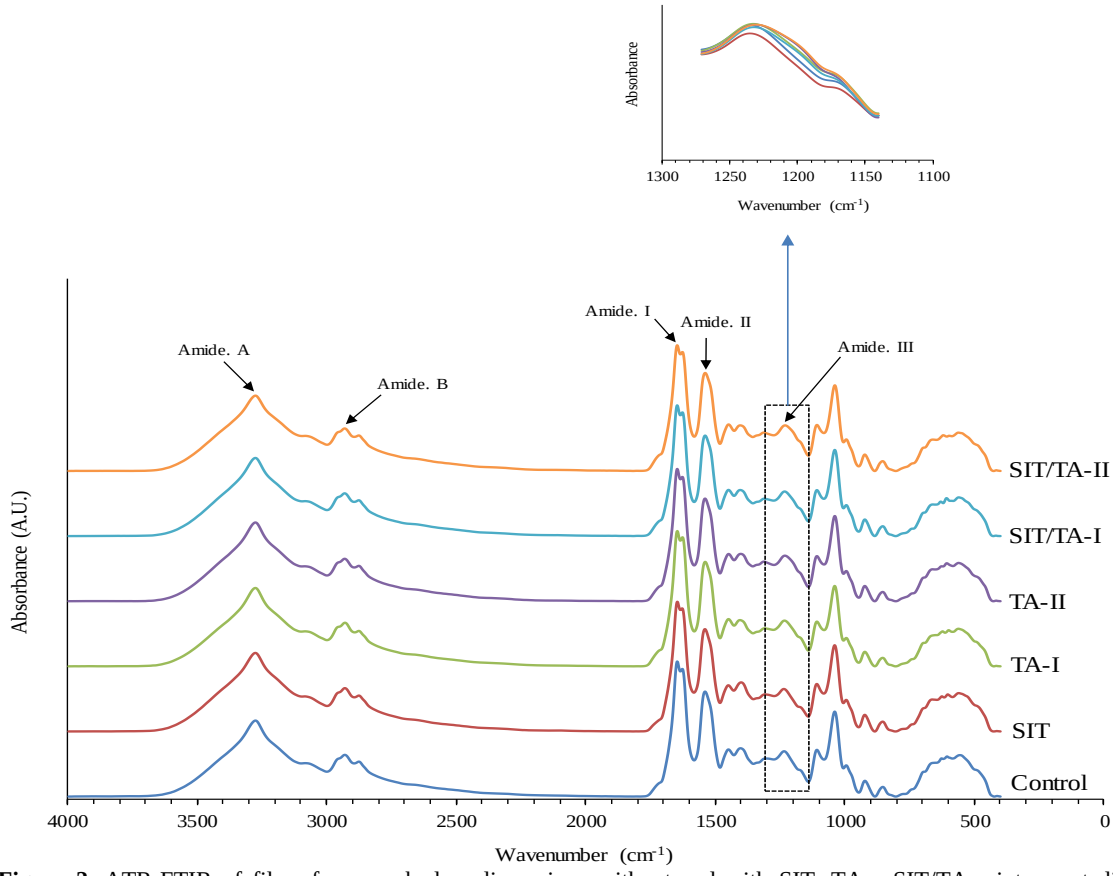

Figure 2. ATR-FTIR of films from washed sardine mince without and with SIT, TA orSIT/TA mixtures at different concentrations. SIT: 5 k units L^{-1} SIT; TA-I: 0.5 g L^{-1} TA; TA-II: 1 g L^{-1} TA; SIT/TA-I: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; SIT/TA-II: 5 k units L^{-1} SIT+ 1 g L^{-1} TA.

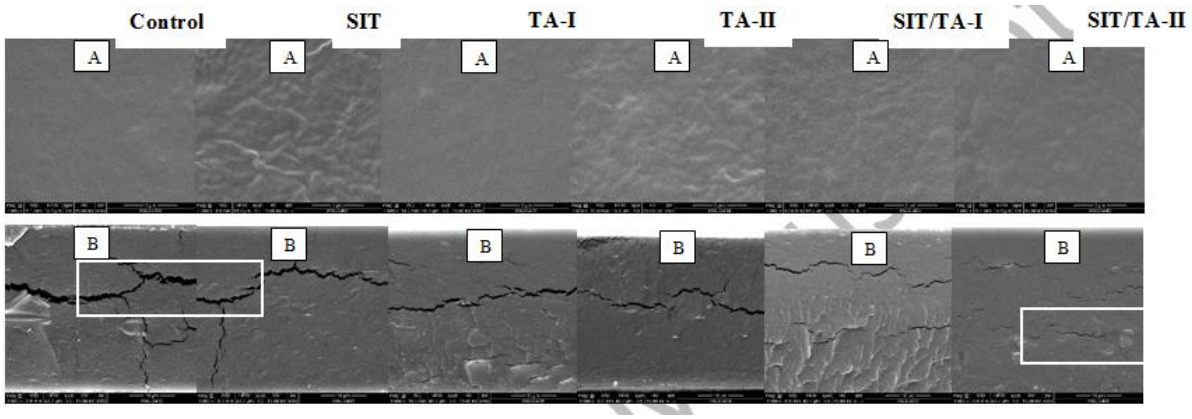

Figure 3. Scanning electron micrograph of films without and with SIT, TA orSIT/ TA mixtures at different concentrations. Surface (A) and freeze-fractured cross-section (B).SIT: 5 k units L^{-1} SIT; TA-I: 0.5 g L^{-1} TA; TA-II: 1 g L^{-1} TA; SIT/TA-I: 5 k units L^{-1} SIT+ 0.5 g L^{-1} TA; SIT/TA-II: 5 k units L^{-1} SIT+ 1 g L^{-1} TA. White frame indicates the crack in films.

added with SIT/TA mixtures was in agreement with higher TS and lowered WVP. The result suggested the higher protein cross-linking induced via quinones produced by oxidation of TA by SIT.

Conclusion

The combination of SIT at 5 k units L-1 and TA at 1 g L-1 FFS had the highest enhancing effect on tensile strength of protein films from washed sardine mince. The SIT/TA mixture also decreased WVP in the resulting films. Higher crosslinking associated with film strengthening was induced by the mixture of SIT and TA. Hence the myofibrillar protein films having poor mechanical properties can be improved by the addition of SIT/TA mixture. The application of the developed films as food packaging should be further studied.

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