International Food Research Journal 22(1): 246-253(2015)
Journal homepage: *http://www.ifrj.upm.edu.my*

Effect of phenolic compounds and bark/wood extracts oxidised by laccase on properties of cuttlefish *(Sepia pharaonis)* skin gelatin gel

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Article history

Received: 21 April 2014
Received in revised form: 17 June 2014
Accepted: 30 June 2014

Keywords

cuttlefish
gelatin
cross-linking
laccase
phenolic compounds
wood
bark

Abstract

Effects of different phenolic compounds (catechin, ferulic acid, tannic acid and gallic acid) or the extracts including ethanolic kiam wood extract (EKWE) and ethanolic cashew bark extract (ECBE) oxidised by laccase (20 U/mL) on the properties of gelatin from cuttlefish *(Sepia pharaonis)* skin were investigated. Gel incorporated with all oxidized phenolic compounds or extracts showed the increases in gel strength, compared with the control ($P<0.05$). Gel added with oxidised gallic acid showed the highest gel strength, followed by that containing oxidized catechin ($P<0.05$). Both oxidised gallic acid and catechin showed the cross-linking activity as evidenced by the decreases in free amino group contents of gelatin. Those oxidised compounds were able to stabilise the gel matrix via non-disulphide covalent bonds more effectively than others and the gel network had the thick strands with small voids. However, the addition of oxidised phenolic compounds or extracts resulted in the lower lightness but increased yellowness, compared with the control gel. Thus the use of gallic acid or catechin oxidised by laccase could be a means to improve the gel strength of gelatin from cuttlefish skin.

Introduction

Cuttlefish has become an important fishery product in Thailand and is mainly exported worldwide. During processing, skin is generated as a byproduct with low market value and is mainly used as animal feed (Aewsiri *et al*., 2009). The extraction of gelatin from cuttlefish skin could increase its profitability. Aewsiri et al. (2009) used H_2O_2 as a bleaching agent for pretreatment of cuttlefish skin prior to gelatin extraction and found that such a pretreatment had the influence on yield and properties of resulting gelatin. Generally, gelatin from fish resources has poorer gel strength, compared with mammalian gelatin, due to its lower imino acid content (Grossman and Bergman, 1992). Bloom strength of fish gelatin has been improved by chemical modification (glutaraldehyde, genipin, carbodiimides and phenolic compounds) (Chiou *et al*., 2006; Strauss and Gibon, 2004) or enzyme modification (transglutaminase) (Gomez-Guillen *et al*., 2001; Kolodziejska *et al.*, 2004).

Phenolic compounds are abundant in plants and can interact with proteins through non-covalent and covalent interaction (Rawel *et al.*, 2000). To enhance the cross-linking ability of phenolic compounds, the oxidation of phenolics, in which the quinones are formed, has been implemented (Balange and Benjakul, 2009). Oxidation of phenolics can be carried out via oxygenation under alkaline condition (Balange and Benjakul, 2009) or via enzymatic reaction. Laccase (E.C.1.10.3.2, *p*-benzenediol:oxygen oxidoreductase) is an oxidoreductase able to catalyse the oxidation of various aromatic compounds (particularly phenols) with the concomitant reduction of oxygen to water (Duran *et al*., 2000). A phenolic substrate is subjected to a one-electron oxidation, giving rise to an aryloxyradical. This active species can be converted to a quinone in the second stage of the oxidation. The quinone and free radical product undergo non-enzymatic coupling reactions, leading to polymerisation (Duran and Esposito, 2000). Laccase has also been reported to catalyse polymerisation of certain peptides and proteins (Mattinen *et al*., 2005; 2006)

Apart from using commercial phenolic compounds, the plant extracts, especially from wood or barks, can serve as the potential phenolic compounds. The use of the extracts from cashew bark or kiam wood containing phenolic compounds oxidised under strong alkaline condition could improve the gel strength of gelatin from cuttlefish (Temdee and Benjakul, 2014). However, no information on the use of phenolic compounds or plants extracts oxidised by laccase has been reported. Therefore, this study aimed to investigate the effect of different phenolic compounds and bark/wood

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extracts oxidised by laccase on gel properties of cuttlefish skin gelatin.

Materials and Methods

Chemical and laccase

Ethanol,*p*-dimethylaminobenzaldehyde, trichoroacetic acid, Folin-Ciocalteu's phenol reagent, acetic acid, hydrogen peroxide (H_2O_2) and tris (hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). 2-Mercaptoethanol (βME), bovine serum albumin, ferulic acid, tannic acid, catechin, 2,4,6-trinitrobenzenesulfonic acid (TNBS) and protein maker were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Sodium dodecyl sulfate (SDS), Coomassie Brilliant Blue G250 and *N*,*N*,*N'*,*N'*-tetramethyl ethylene diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Gallic acid and laccase from *Trametes versicolor* (EC 1.10.3.2) (23.1U/mg) were purchased from Fluka Chemicals (Kassel Germany).

Collection and preparation of cuttlefish skin

Dorsal skin of cuttlefish *(Sepia pharaonis)* was obtained from a dock in Songkhla province. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1×1 cm), placed in polyethylene bags and stored at −20°C until use. Storage time was not longer than 2 months. Prior to extraction, the frozen skin was thawed using running water until the core temperature reached 10°C

Extraction of gelatin

The skin was treated with 0.4M NaOH in combination with 0.75% H_2O_2 using the skin/solution ratio of 1:10 (w/v). The mixture was stirred for 12 h with the changes of solutions every 3 h at 4ºC. The pretreated skin was then soaked in 10% H_2O_2 for 48 h using a pretreated skin/solution ratio of 1:10 (w/v). The mixture was stirred continuously at 4°C. All samples were washed using tap water until neutral pH of wash water was obtained.

The prepared skins were mixed with 5 volumes of warm (50°C) water. During extraction of 18 h at 50°C, the mixture was stirred continuously using a stirrer equipped with a propeller (IKA® Laboratory equipment, Staufen, Germany). The extract was centrifuged at 8,000 x g for 30 min using a centrifuge (Avanti J-E, Beckman Coulter Inc., Newton, CT, USA) at room temperature (28-30ºC) to remove insoluble material. The supernatant was collected and freeze-dried (CoolSafe 55, ScanLafA/S, Lynge, Denmark).

Preparation of kiam wood and cashew bark extracts

Kiam *(Cotylelobium lanceotatum craih)* wood was obtained from a forest of Phattalung province, Thailand. The tree was about 15-20 years old. The tree was cut by using a sawing machine and the trunk was sun-dried for three months. Cashew *(Anacardium occidentale)* bark from the tree with 15-20 years old was obtained from a forest of Songkhla province, Thailand. The pieces of wood and bark with an average thickness of 1.5 cm were dried in hot air oven at 70°C for 8 h. Prepared wood and bark were ground using a portable grinding machine with a sieve size of 6 mm. The ground samples were further blended using a blender and finally sieved using a stainless steel sieve of 80 mesh size.

Ethanolic extracts from kiam wood and cashew bark powder were prepared as per the method of Santoso *et al*. (2004) with slight modifications. The powder (10 g) was homogenised with 150 mL of ethanol using a homogeniser at 10,000 rpm for 2 min using an IKA Labortechnik homogenizer (Selangor, Malaysia). The homogenate was stirred at room temperature (28-30 °C) using a magnetic stirrer for 12 h and centrifuged at 5000 x g for 10 min at 25°C and the supernatant was filtered through a Whatman filter paper No. 1. (Whatman International Ltd., Maidstone, England). The collected supernatants were evaporated to remove ethanol using a rotary evaporator (EYELA, N-100, Tokyo Rikakikai, Tokyo, Japan). The extract was then dried in a hot air oven at 70°C for 12 h. Dried extract was powdered using a mortar and pestle. Extract powders were referred to as ethanolic kiam wood extract (EKWE) and ethanolic cashew bark extract (ECBE). Both EKWE and ECBE were kept in a desiccator at room temperature until used. The storage time was not longer than 2 months.

Effect of different phenolic compounds and extracts oxidized by laccase on properties of gelatin gel

The gelatin solution (6.67%) was prepared as per the method of Nagarajan *et al*. (2013). To the solution, commercial phenolic compounds (catechin, ferulic acid, tannic acid and gallic acid) or the extracts, EKWE and ECBE, were added to obtain the concentration of 2% (w/w, based on gelatin). Thereafter, laccase (20 U/mL) was added and mixed well. The prepared solutions were placed in a temperature-controlled water bath (40°C) and subjected to oxygenation for 30 min by bubbling the solution with oxygen to convert the phenolic compounds to quinone. The mixtures

were further stirred for 1 h at room temperature (25-27°C). The obtained gelatin solution was then used for gel preparation and analyses. The control gelatin solution (6.67%) was also prepared without the addition of phenolic compounds or the extracts.

Analyses

Determination of gel strength

Gel strength of gelatin after setting at 10°C for 16-18 h (3 cm diameter and 2.5 cm height) was determined using a Model TA-XT2 Texture Analyser (Stable Micro System, Surrey, UK) with a load cell of 5 kN and equipped with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger following the method of Nagarajan *et al*. (2013). The maximum force (in grams), when the penetration distance of 4 mm was obtained, was recorded. The speed of the plunger was 0.5 mm/s.

Determination of colour

Colour of gel samples was determined using a colourimeter (ColourFlex, HunterLab Reston, VA, USA). CIE L^* (lightness), a^*(+) (redness) or a^*(-) (greenness) and b^* (+) (yellowness) or b^* (-) blueness) values were measured.

Determination of free amino group contents

Free amino group content was determined according to the method of Benjakul and Morrissey (1997). Prior to analysis, gelatin gel was incubated at 60°C for 30 min for liquefaction. Diluted samples (125 µL) were mixed thoroughly with 2.0 mL of 0.2 M phosphate buffer, pH 8.2, followed by the addition of 1.0 mL of 0.01% TNBS solution. The mixture was then placed in a water bath at 50°C for 30 min in dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixture was cooled to room temperature for 15 min. The blank was performed in the same manner except deionized water was used instead of TNSB solution. The absorbance was measured at 420 nm and free amino group content were calculated after blank subtraction and expressed in terms of L-leucine.

Determination of solubility

Solubility of protein in gelatin gel was determined as described by Benjakul . (2001). Finely chopped gel sample (1 g) was solubilised with various solvents including 20 mM Tris–HCl, pH 8.0 containing 1% SDS (S_1); 20 mM Tris–HCl, pH 8.0 containing 1% SDS and 8 M urea (S_2) and 20 mM Tris–HCl, pH 8.0 containing 1% SDS, 2% β-mercaptoethanol and 8 M urea (S_3). The mixture was homogenised for 1 min, boiled for 2 min and stirred for 4 h at room temperature using a magnetic stirrer (IKA-Werke, Staufen, Germany). The mixture was centrifuged at 10,000×g for 30 min at 25°C using a centrifuge (Sorvall Model RCB plus, Newtown, CT, USA). Two mL of 50% (w/v) cold trichloroacetic acid (TCA) were added to 10 mL of supernatant. The mixture was kept at 4°C for 18 h prior to centrifugation at 10,000×g for 20 min. The precipitate was washed with 10% (w/v) TCA, followed by solubilising in 0.5M NaOH. Protein concentration was determined by the Biuret method (Robinson and Hodgen, 1940). Solubility of gel samples was expressed as the percentage of total protein in gelatin. To completely solubilise the total proteins, gels were solubilised directly in 0.5M NaOH.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of gelatin gels were analysed by SDS-PAGE according to the method of Laemmli (1970). The gelatin gels were dissolved in 5% SDS solution. The mixture was then homogenised using a homogeniser (IKA Labortechnik, Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was incubated at 85°C for 1 h to dissolve total proteins. The samples were centrifuged at 8,500 ×g for 5 min to remove undissolved debris. Solubilised samples were mixed at 1:1(v/v) ratio with the sample buffer (0.5M Tris-HCL, pH 6.8 containing 4% SDS, 20% glycerol and 10% βME). The samples were loaded onto the polyacrylamide gel made of 7.5% running gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.05% (w/v) Coomassie Brilliant Blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% methanol (v/v) and 10% (v/v) acetic acid, followed by 5% methanol (v/v) and 7.5% (v/v) acetic acid.

Scanning electron microscopy (SEM)

The microstructure of gelatin gels was observed using SEM. Gels containing oxidised phenolic compounds or wood/bark extracts and the control gel (without oxidised phenolic compounds or wood/bark extracts) with a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2M phosphate buffer (pH 7.2). The samples were then rinsed for 1 h in distilled water before being dehydrated in ethanol with serial concentrations of 50%, 70%, 80%, 90% and 100% (v/v). Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The

Table 1. Colour and free amino group content of cuttlefish skin gelatin gel incorporated with different phenolic compounds or extracts oxidised by laccase at a level of 2% (w/w of gelatin)

Oxidized phenolic compound/extracts	Colour			Free amino group content (mmole/ g sample)
	L*	a*	b*	
Control	64.31 ±0.67$^{\dagger a\dagger\dagger}$	-6.39±0.37^{f}	9.18±0.68^{f}	2.59±0.08^{a}
ECBE	47.27 ±0.71^{e}	9.27 ±0.43^{a}	12.13±0.47^{d}	2.37±0.21^{a}
EKWE	51.46 ±0.51^{d}	4.15±0.43^{c}	19.75±1.05^{c}	2.23±0.23^{a}
Catechin	53.65±0.49^{d}	6.01±0.64^{b}	35.41±0.93^{a}	1.92±0.47^{b}
Ferulic acid	60.81 ±0.63^{b}	-5.79±0.11^{e}	11.39.±0.85^{e}	2.42±0.41^{a}
Gallic acid	44.91± 0.63^{f}	-17.09±0.79^{f}	22.16±0.11^{b}	1.67±0.11^{c}
Tannic acid	57.14±0.27^{c}	0.39±0.21^{d}	22.02±0.79^{b}	2.18±0.81^{a}

†Mean±SD (n=3)

††Different superscripts in the same column indicate significant differences ($p<0.05$)

specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 10 kV.

Statistical analysis

Experiments were in triplicate using three lots of samples. All data were subjected to analysis of variance and differences between means were evaluated by Duncan's multiple range test (Steel and Torrie, 1980). The SPSS statistical program (Version 10.0) (SPSS Inc., Chicago, IL) was used for data analysis.

Results and Discussion

Gel strength

Gel strength of cuttlefish skin gelatin gel incorporated with different phenolic compounds or extracts oxidized by laccase is depicted in Figure 1. Gels incorporated with oxidised phenolic compounds or extracts had the increases in gel strength when compared with the control (without addition of laccase and phenolic compounds/extracts) ($p<0.05$). Laccase more likely induced the polymerisation of gelatin molecules prior to gel setting. Laccase has been reported to induce protein cross-linking (Mattinen *et al*., 2005). Gelatin gel incorporated with oxidised gallic acid had the highest gel strength (211.45 g) ($p<0.05$), followed by that added with oxidised catechin. No differences in gel strength were noticeable between gels incorporated with oxidized ferulic acid, tannic acid and EKWE ($p<0.05$). Oxidized ECBE exhibited the lowest gel strengthening effect on gelatin but yielded the resulting gel with the increased strength, compared with the control ($p<0.05$). Laccase was able to

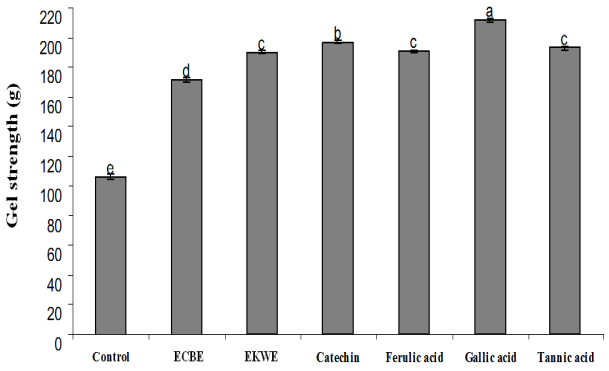

Figure 1. Gel strength of gelatin from cuttlefish skin incorporated with different phenolic compounds or extracts oxidised by laccase at a level of 2% (w/w of gelatin). Bars represent the standard deviation (n=3). Different letters on the bars denote the significant differences ($p<0.05$)

oxidise a broader range of substrates to quinones. Laccase typically has the wide substrate specificity (Selinheimo, 2008). In addition to mono and polyphenols, laccase is capable of oxidising various aromatic compounds, such as substituted phenols, diamines, aromatic amines and thiols, and even some inorganic compounds such as iodine (Flurkey, 2003; Baldrian, 2006). Quinones are particularly susceptible to nucleophilic attack by free sulfhydryl and amino groups of amino acid side-chains, resulting in formation of tyrosine-cysteine and tyrosine-lysine cross-links in the protein structures (Marumo and Waite, 1986). Lantto *et al*. (2005) reported that laccase improved gel property of chicken-breast myofibril proteins. In laccase-catalysed oxidation reactions, high reactivity of the produced radicals can lead to polymerisation of substrates. Radical-mediated oxidation of free sulfhydryl groups of proteins has been found to be accelerated in laccase

Table 2. Protein solubility (%) of cuttlefish skin gelatin gel incorporated with different phenolic compounds or extracts oxidised by laccase at a level of 2% (w/w of gelatin)

Oxidised phenolic compound/extracts	S_1	S_2	S_3
Control	69.02±0.98$^{\dagger a\dagger\dagger}$	95.11±1.21^{a}	98.15±1.52^{a}
ECBE	68.39±0.77^{a}	94.37±0.42^{a}	97.97 ±0.94^{a}
EKWE	68.79 ±1.46^{a}	93.15±0.44^{ab}	95.95±1.11^{ab}
Catechin	64.27 ±0.51^{b}	91.172±0.71^{b}	93.43±1.75^{b}
Ferulic acid	66.92 ±1.82^{ab}	93.97±1.53^{ab}	96.17 ±0.49^{ab}
Gallic acid	64.45±0.93^{b}	91.16±0.97^{b}	94.01±0.97^{b}
Tannic acid	66.14±2.55^{ab}	92.21±1.15 ^{ab}	96.22±1.43^{ab}

†Mean±SD (n=3)
††Different superscripts in the same column indicate significant differences ($p<0.05$).
S1: 20 mM Tris, pH 8.0 was containing 1% (w/v) SDS
S2: 20 mM Tris, pH 8.0 containing 1% (w/v) SDS and 8 M urea
S3: 20 mM Tris, pH 8.0 containing 1% (w/v) SDS, 8 M urea, and 2 % (v/v) β-mercaptoethanol.

catalysed reactions in the presence of exogenous phenolic acid. Therefore, laccase could be used to convert the phenolic compounds to quinone, which functioned as the protein cross-linkers in the gelatin gel matrix.

Colour

Colour of gelatin gel containing different phenolic compounds or extracts oxidised by laccase is shown in Table 1. L^*-value of gel decreased with the addition of oxidised phenolic compounds or extracts to different degrees, depending on the types of compounds used. The increases in both a^*-value and b^*-values of gelatin gels were observed when being incorporated with oxidised phenolic compounds or extracts ($p<0.05$). The highest a^*-value was found in gel added with oxidised ECBE, whilst the highest b^*-value was observed in gel added with oxidised catechin ($p<0.05$). In general, the differences in colour amongst the samples were most likely attributed to the varying pigments in the extracts or different typical colour of phenolic compounds used. The darker colour was produced when the oxidation of phenolic compare took place. As a result, gelatin gel turned to be darker or reddish/yellowish when the oxidised phenolic compounds or extracts were added. Phenolic compounds were reported to contribute to discolouration in cheese products (O'Connell and Fox, 2001).

Free amino group content

Free amino group content of cuttlefish skin gelatin gel incorporated with various phenolic compounds or extracts oxidised by laccase is shown in Table 1. Free amino group content in the gelatin gel samples varied, depending on the oxidised phenolic compounds or extracts added. Gelatin incorporated with the oxidised catechin and gallic acid had the lower free amino group content than the control gelatin (without laccase or phenolic compounds/extracts) ($p<0.05$). Laccase is known to readily oxidise both para-, meta- and ortho-diphenols (Selinheimo, 2008). It indicated that nucleophilic amino groups might interact with electrophilic quinone, an oxidised form of phenolic compounds, induced by laccase. Nevertheless, the non-significant decrease in free amino group content was found in the samples added with oxidised ECBE, EKWE, ferulic acid and tannic acid ($P>0.05$). Thus, the cross-linking activity varied with the types of compounds. Furthermore, the different rate of conversion of phenolics to quinones induced by laccase was presumed. The results suggested that the oxidised gallic acid showed the highest cross-linking activity as indicated by the lowest free amino group content in the gelatin gel ($p<0.05$). The higher cross-links found in gelatin added with oxidised gallic acid, as indicated by the higher decrease in free amino group content, were in agreement with the highest gel strength (Figure 1). Gallic acid is a trihydroxybenzoic acid and contains totally 4 hydroxyl groups. Catechin consists of two benzene rings (called the A- and B-rings) and a dihydropyran heterocycle (the C-ring) and it contains 5 hydroxyl groups in structure. Those phenolic compounds are the potential antioxidants (Maqsood *et al*., 2013). When the oxidation took place, as induced by laccase, those hydroxyl groups could be converted to quinones, which further interacted with side chains, especially amino groups, of gelatin molecules. Those compounds with more than one reactive site were able to form cross-links between

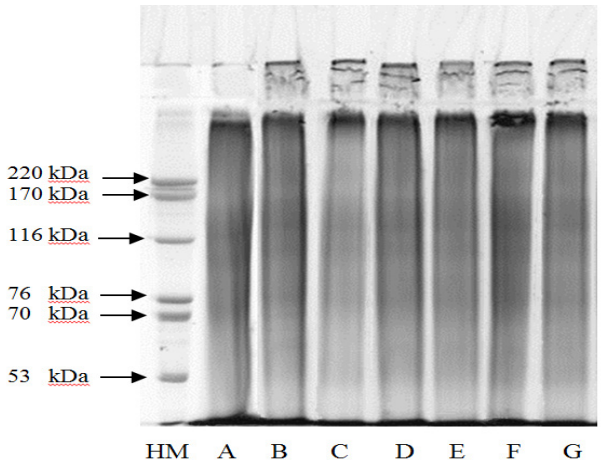

Figure 2. SDS-PAGE patterns of gelatin from cuttlefish skin incorporated with different phenolic compounds or extracts oxidised by laccase at a level of 2% (w/w of gelatin). (HM: high molecular weight marker; A: control; B: ECBE; C: EKWE; D: catechin; E: ferulic acid; F: gallic acid and G: tannic acid

proteins effectively via multidentate mechanism. In general, a much lower phenolic compound / protein molar ratio is required for the multimeric compounds (Haslam, 1989).

Solubility

Protein solubility of gelatin gels incorporated without and with phenolic compounds or extracts oxidised by laccase in various solvents is presented in Table 2. Protein solubility of gelatin gel incorporated with oxidised phenolic compounds or extracts varied in all solvents used ($p<0.05$). For solubility in S_1, the lowest solubility was found in the samples added with oxidised catechin and gallic acid ($P<0.05$). SDS is capable of destroying hydrogen and some hydrophobic interactions (Prodpran *et al*., 2007). The result indicated that both oxidised catechin and gallic acid could induce the interaction of gelatin chain via hydrogen bonds more effectively than other compounds or extracts. It was noted that the marked increases in solubility were observed in S_2 (20 mM Tris, pH 8.0 containing 1% (w/v) SDS and 8 M urea), in comparison with that observed in S_1. The result indicated the presence of hydrophobic interaction and hydrogen bond in gelatin gels matrix. The lower solubility in S_2 was also found in the gel samples incorporated with the oxidised catechin or gallic acid ($P<0.05$). SDS and urea have been known to destroy hydrogen bond and hydrophobic interaction (Prodpran *et al.*, 2007). When S_3 was used for solubilisation, only slight increase in solubility was noticeable, suggesting the negligible disulphide bonds involved in cross-linking. Gelatin from cuttlefish skin had the negligible content of cysteine (Hoque *et al*., 2010). However, the lowest solubility in S_3 was also found

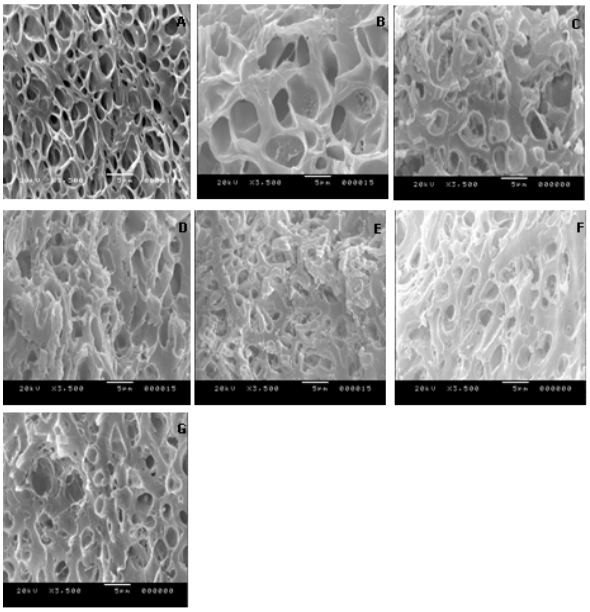

Figure 3. Electron microscopic images of cuttlefish skin gelatin gel incorporated with different phenolic compounds or extracts oxidised by laccase at a level of 2% (w/w) A: control; B: ECBE; C: EKWE; D: catechin; E: ferulic acid; F: gallic acid and G: tannic acid. Magnification: 3500x

in gelatin gel added with oxidised gallic acid or catechin. For the samples added with other oxidised phenolic compounds or extracts, the non-significant lower solubility was obtained in comparison with that of control ($p<0.05$). βME is the reducing agent, which can destroy disulphide bond (Prodpran *et al*., 2007). The lower solubility in S3 of gelatin gel incorporated with oxidised gallic acid or catechein was coincidental with the increases in gel strength (Figure 1). The result suggested that non-disulphide covalent bonds were more involved in gelatin gel network when oxidised catechin or gallic acid was incorporated. The result reconfirmed that quinone formed, induced by laccase, most likely acted as the potential cross-linker, in which the strong gel matrix could be developed.

Protein patterns

Protein patterns of gelatin gels with and without the addition of different phenolic compounds or extracts oxidised by laccase are depicted in Figure 2. All cuttlefish gelatin gels incorporated with oxidised phenolic compounds or extracts contained the polymerised protein as appeared on the stacking gel. Those cross-links mainly contributed to the increases in gel strength of gelatin gel added with oxidised phenolic compounds or extracts. Quinone was able to induce the cross-linking of gelatin molecules, in which the large aggregate could be formed. This aggregate or polymerised gelatin more likely contributed to the increased gel strength. However,

negligible protein cross-links were found in the control gel (without oxidised phenolic compounds or extracts). Strauss and Gibson (2004) described the use of plant phenolics as cross-linkers in gelatin gels. Cao *et al*. (2007) also reported the polymerisation of protein molecules as a possible subsequent reaction of different proteins with phenolic substances.

Microstructure

The microstructures of gelatin gels from cuttlefish skin incorporated without and with phenolic compounds or extracts oxidised by laccase are illustrated in Figure 3. Similarly, all gelatin gels contained the pores with different sizes and uniformity. The control gel showed a uniform network with a thin strands (Figure 3 A). Gelatin gel incorporated with oxidised phenolic compounds or extracts varied in structure. Amongst all gels, that added with oxidised ECBE had the largest void with varying sizes (Figure 3B). This might be associated with the poorer gel. For gel added with oxidised gallic acid which showed the highest gel strength, the small voids were obtained but the strand was thick (Figure 3F). The structure would be resistant to the force applied as evidenced by the highest gel strength of this gel. Therefore, the oxidised phenolic compounds or extracts could function as the gelatin cross-linker, in which the oxidised forms were able to induce the cross-linking more effectively. As a consequence, the gel strength could be improved with the addition of either extracts or commercial phenolic compounds oxidised by laccase.

Conclusion

Catechin or gallic acid oxidised by laccase could be used as the gel strengthening agent for improving the property of gelatin from cuttlefish skin. However, the incorporation of those oxidized compounds led to some changes in colour of resulting gel.

Acknowledgements

Authors would like to express their sincere thanks to Seafood quality and safety excellent laboratory, Prince of Songkla University for the financial support. The National Research Council of Thailand and The Senior Research Scholar program were also acknowledged for the financial support.

References

Aewsiri, T., Benjakul, S. and Visessanguan, W. 2009. Functional properties of gelatin from cuttlefish *(Sepia pharaonis)* skin as affected by bleaching using hydrogen peroxide. Food Chemistry 115 (1): 243-249.

Balange, A. and Benjakul, S. 2009. Enhancement of gel strength of bigeye snapper *(Priacanthus tayenus)* surimi using oxidised phenolic compounds. Food Chemistry 113 (1): 61–70.

Baldrian, P. 2006. Fungal laccases occurrence and properties. FEMS Microbiology Reviews 30 (2): 215–242.

Benkajul, S. and Morrissey, M. T. 1997. Protein hydrolysates from Pacific whiting solid wastes. Journal of Agricultural and Food Chemistry 45 (9): 3423-3430.

Benjakul, S., Visessanguan, W., and Srivilai, C. 2001. Gel properties of bigeye snapper *(Priacanthus tayenus)* surimi as affected by setting and porcine plasma protein. Journal of Food Quality 24 (5): 453–471.

Cao, N., Fu, Y. and He, J. 2007. Mechanical properties of gelatin films cross-linked, respectively, by ferulic acid and tannin acid. Food Hydrocolloids 21 (4): 575–584.

Chiou, B. S., Avena-Bustillos, R. J., Shey, J., Yee, E., Bechtel, P. J., Imam, S. H., Glenn, G. M., and Orts, W. J. 2006. Rheological and mechanical properties of cross-linked fish gelatins. Polymer 47 (18): 6379–6386.

Duran, N. and Esposito, E. 2000. Potential applications of oxidative enzymes and phenoloxidase-like compounds in wastewater and soil treatment: a review. Applied Catalysis B: Environmental 28 (2): 83–99.

Flurkey, W. H. 2003. Laccase. In Whitaker, J. R., Voragen, A. G. J. and Wong, D. W. S. (Eds.). Handbook of food enzymology, p. 525–537. New York: Marcel Dekker.

Gomez-Guillen, M. C., Sarabia, A. I., Solas, M. T. and Montero, P. 2001. Effect of microbial transglutaminase on the functional properties of megrim *(Lepidorhombus boscii)* skin gelatin. Journal of the Science of Food and Agriculture 81 (7): 665–673.

Grossman, S. and Bergman, M. 1992. Process for the production of gelatin from fish skins. US patent 5,093,474.

Haslam, E. 1989. Vegetable tannins revisited. In Phillipson, J. D., Ayres, D. C. and Baxter, H. (Eds). Plant polyphenols, p. 230. Cambridge: Cambridge University Press.

Hoque, M. S., Benjakul, S. and Prodpran, T. 2010. Effect of heat treatment of film forming solution on the properties of film from cuttlefish *(Sepia pharaonis)* skin gelatin. Journal of Food Engineering 96 (1): 66-73.

Ko1odziejska, I., Kaczorowski, K., Piotrowska, B. and Sadowska, M. 2004. Modification of the properties of gelatin from skins of Baltic cod *(Gadus morhua)* with transglutaminase. Food Chemistry 86 (2): 203–209.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227 (5259): 680–685.

Lantto, R., Puolanne, E., Kalkkinen, N., Buchert, J. and Autio, K. 2005. Enzyme aided modification of chicken-breast myofibril proteins: effect of laccase and transglutaminase on gelation and thermal stability. Journal of Agricultural and Food Chemistry 53 (23):

9231–9237.
Maqsood, S., Benjakul, S. and Shahidi, F. 2013. Emerging role of phenolic compounds as natural food additives in fish and fish products. Critical Reviews in Food Science and Nutrition 53 (2): 162-179.
Marumo, K. and Waite, J.H. 1986. Optimization of hydroxylation of tyrosine and tyrosine-containing peptides by mushroom tyrosinase. Biochimica et Biophysica Acta (BBA)-Protein and Molecular Enzymology 872 (1-2): 98–103.
Mattinen, M. L., Hellmann, M., Permi, P., Autio, K., Kalkkinen, N. and Buchert, J. 2006. Effect of protein structure on laccase-catalyzed protein oligomerization. Journal of Agricultural and Food Chemistry 54 (23): 8883–8890.
Mattinen, M. L., Kruus, K., Buchert, J., Nielsen, J. H., Andersen, H. J. and Steffensen, C. L. 2005. Laccase-catalysed polymerization of tyrosine-containing peptides. FEBS Journal 272 (14): 3640–3650.
Nagarajan, M., Benjakul, S., Prodpran, T. and Songtipya, P. 2013. Effects of bleaching on characteristics and gelling property of gelatin from splendid squid *(Loligo formosana)* skin. Food Hydrocolloids 32 (2): 447-452.
O'connell, J., and Fox, P. 2001. Significance and applications of phenolic compounds in the production and quality of milk and dairy products: a review. International Dairy Journal 11 (3): 103-120.
Prodpran, T., Benjakul, S. and Artharn, A. 2007. Properties and microstructure of protein-based film from round scad *(Decapterus maruadsi)* muscle as affected by palm oil and chitosan incorporation. International Journal of Biological Macromolecules 41 (5): 605–614.
Rawel, H. M.; Kroll, J. and Riese, B. 2000. Reactions of chlorogenic acid with lysozyme: physicochemical characterization and proteolytic digestion of the derivatives. Journal of Food Science 65 (6): 1091-1098.
Robinson, H.W. and Hodgen, C.G. 1940. The biuret reaction in the determination of serum protein. I. A study of the condition necessary for the production of the stable colour which beans a quantitative relationship to the protein concentration. The Journal of Biological Chemistry 135 (2): 707–725.
Santoso. J., Yoshie-Stark. Y. and Suzuki, T. 2004. Anti-oxidant activity of methanol extracts from Indonesian seaweeds in an oil emulsion model. Fisheries Science 70 (1): 183–188.
Selinheimo, E. 2008. Tyrosinase and Laccase as Novel Crosslinking Tools for Food Biopolymers. Helsinki: VTT Publications 693.
Steel, R.G.D. and Torrie, J. H. 1980. Principles and procedures of statistics: A biometrical Approach. 2nd edn. New York: McGraw-Hill.
Strauss, G., and Gibson, S. M. 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. Food Hydrocolloids 18 (1): 81–89.
Temdee, W. and Benjakul, S. 2014. Effect of oxidized kiam wood and cashew bark extracts on gel properties of gelatin from cuttlefish skin. Food Bioscience (In revision).