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RESEARCH ARTICLE

Extraction of crude collagen from *Pterygoplichthys pardalis* skin and determination of functional properties of its hydrolysates

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ABSTRACT

Collagen has a great demand and fish skin is a safe alternative source of collagen. Pterygoplichthys pardalis is an endemic, freshwater fish with no economic benefit. The study was conducted to extract crude collagen from the skin of P. pardalis using a simple method and identify the functional properties of its hydrolysates. Acid and pepsin soluble collagens were extracted from skin using citric acid (CA) and ethylene diamine tetraacetic acid (EDTA). Selected crude collagens were hydrolysed using pepsin, protease and trypsin enzymes (1:100). Extracted crude collagens and best hydrolysates were selected using 8 and 15% SDS-PAGE. Hydrolysates were analysed for antioxidant, Fe(II) chelating and antibacterial activities. CA treatment showed significantly ($P<0.05$) higher yield than EDTA treatment. Antioxidant properties, metal chelation and antibacterial activities were not significantly ($P>0.05$) *different. Hydrolysates showed significantly ($P<0.05$) higher functional properties than crude collagen in both treatments. These results conclude that collagen hydrolysates from P. pardalis have good antioxidant, metal chelating and antibacterial properties.*

Keywords*: Fish collagen, enzyme hydrolysis, antioxidant, metal chelating, antibacterial*

INTRODUCTION

Fish proteins play an important role in daily diets and act as a major source of high-quality proteins (Jabeen and Chaudhry, 2011). They have higher protein content than most land-animal meats and are highly digestible, rich in several peptides and essential amino acids (Khalili *et al.*, 2018). The collagen family is one of the predominant groups of proteins of animal origin and it constitutes approximately 30% of total animal proteins (Zhang *et al.*, 2009). There are different types of collagen which are classified according to their structure (Schmidt *et al.*, 2016) named as type I – XXVII. Type I collagen is a right handed super helical rod consisting of 3 polypeptide chains (Nazeer *et al.*, 2014) as α_1, α_2 and β (Schmidt *et al.*, 2016).

Collagens have a wide range of industrial applications such as food, pharmaceuticals and cosmetics due to their unique characteristics of

biodegradability and weak antigenicity (Kołodziejska *et al.*, 1999). They are also utilised in leather and film industries (Kittiphattanabawon *et al.*, 2005). It has high protein content and functional properties such as water absorption capacity, gel formation and the ability to form and stabilise emulsions, so, it promotes the collagens to have a great demand in the food industry especially as sausage casings (Zhang *et al.*, 2009; Schmidt *et al.*, 2016). Collagens are used as a vehicle for drugs, proteins, genes as well as a substitute for human skin, blood vessels and ligaments in pharmaceutical industry (Schmidt *et al.*, 2016). Furthermore, collagens have properties such as good inertness, structural stability and good biocompatibility. Hence, collagen films are used as promising carriers for anticancer drug delivery system and ophthalmic drug delivery system in biomedical applications (Hema *et al.*, 2013). Apart from these, collagen type I, II and III are the most abundant collagen types in biomedical applications, because they are used as plastic material widely in medicine and cosmetology (Mocan *et al.*, 2011).

Mostly, collagens are obtained from skins and bones of land-based animals (pig, cattle and poultry by-products) (Kittiphattanabawon *et al.*, 2005). Consumers are seriously concerned regarding those products due to zoonotic disease outbreaks, religious matters and some social reasons (Silvipriya *et al.*, 2015). Bovine and porcine collagens have the risk of causing mad cow disease (Bovine Spongiform Encephalopathy: BSE) (Schmidt *et al.*, 2016), Transmissible Spongiform Encephalopathy (TSE) (Li *et al.*, 2004) and Foot and Mouth Disease (FMD) (Nazeer *et al.*, 2014). Moreover, collagens extracted from bovines are rejected by Hindus and Sikhs and concurrently, porcine collagens are strictly not accepted by Muslims and Jews due to their religious constraints (Shen *et al.*, 2007).

As a consequence, many researche has been conducted to find safe alternative sources for collagens. Specially fish skins including marine and freshwater fish species as well as fish collagens which have different chemical and physical properties compared to mammalian collagens have been studied (Zhang *et al.*, 2009). Collagens obtained from skins of several marine fish species have been isolated by Kittiphattanabawon *et al.* (2005). Conversely, collagen studies were rarely reported from freshwater fish except for Nile perch (*Lates niloticus*) (Muyonga *et al.*, 2004), Grass carp (*Ctenopharyngodonidella*) (Zhang *et al.*, 2007) and Channel catfish (*Ictalurus punctaus*) (Liu *et al.*, 2007).

P. pardalis is a freshwater species (Hubilla *et al.*, 2008) belonging to family Loricariidae and is a native of South America (Jumawan *et al.*, 2014). This species was introduced to Sri Lanka in 1996 as an ornamental fish (Marambe *et al.*, 2011). These fish escaped to the natural ecosystems due to negligence and then became a threat to endemic fish and inland aquaculture in Sri Lanka (Wijethunga and Epa, 2008). At present, it has invaded many freshwater systems of the country along with another *P. disjuctivus* (Marambe *et al.*, 2011; Wijethunga and Epa, 2008). Furthermore, *P. pardalis* disrupts the food chains in the aquatic ecosystems by overgrasing of benthic algae (Mogalekar *et al.*, 2017). These factors have

accelerated them becoming an Invasive Alien Species (IAS) (MOE, 2012). *P. pardalis* species is not regarded as a commercially important fish because of their hard bony armour, low flesh yield, propensity to compete for food resources and their potential to bio-accumulate heavy metals in polluted environments (Jumawan *et al.*, 2014). However, a few research has been carried out for the development of value-added products by using this species (Hossain *et al.*, 2018).

Characteristics of the collagens depend on the raw material used to extract (Wang *et al.*, 2014) and the extraction methods which successively determine its application (Kaewdang *et al.*, 2014). Most commonly used extraction methods are extractions using saline solutions (Yang and Shu, 2014), acid solutions (Wang *et al.*, 2008), acid solutions with added enzymes (Woo *et al.*, 2008) as variations in collagen types (Brodsky, 1982) and solubility of collagens in those solutions (Schmidt *et al.*, 2016). Among those extraction methods, extraction by saline solutions is less commonly used due to lower yields and some limitations (Yang and Shu, 2014). Organic acids (acetic acid, citric acid and lactic acid) and inorganic acids (hydrochloric acid) are used to perform the acidic extraction. However, organic acids are more efficient than inorganic acids (Yousefi *et al.*, 2017; Wang *et al.*, 2008). Because, organic acids are capable of solubilising non-cross linked collagens and also of breaking some of the inter-strand cross-links in collagens which leads to a higher solubility of the collagen during the extraction process (Liu *et al.*, 2010). The utility of acidic extraction is limited due to some reasons as time required to solubilise collagen from tissues with high protein loss and partial degradation of the collagen (Wohllebe and Carmichael, 1978). Although enzymatic extraction can be more expensive there are some advantages such as specificity, degree of control of hydrolysis (Schmidt *et al.*, 2016), generate less waste and may reduce the processing time (Ran and Wang, 2014).

When fish skin is considered as wastage, it represents 30–55% of the total body weight of a fish (Sotelo *et al.*, 2016). Many collagen proteins present in fish skin and fish collagen hydrolysates extracted from fish skins have many functional properties such as anti-oxidative properties, antimicrobial properties and metal chelating properties which are important in food industries (Jiang *et al.*, 2014). Therefore, these findings can be used in functional foods and medication as commercial applications similar to fish collagens. Thus, the aim of this research was to develop a simple, non-toxic and cheap method to extract crude collagen from *P. pardalis* fish skin and to check the functional properties of its hydrolysates for possible commercial applications.

MATERIALS AND METHODS

Fish sample collection

Mature females of *P. pardalis* were collected from Victoria reservoir at Digana, Central province, Sri Lanka. Collected samples were transported to the Protein Chemistry Laboratory, Uva Wellassa University. The mixture was kept to shake in an open-air shaker (Model: OS-2000) with 150 rpm min^{-1} for 24 h. Then, the

supernatant was obtained after centrifugation at 3000 x g for 30 min in 4 °C using a centrifuge machine (Thermo Scientific, SORVALL ST 40R). The residue was again extracted with 0.5 M acetic acid as above and with the combined supernatant.

The collagen was precipitated by adding NaCl to a final concentration of 2.6 M of the obtained supernatant and it was kept to stir for 24 h using a magnetic stir. The resultant precipitate was collected by centrifuging at 4500 x g for 30 min at 4 °C using a centrifuge machine (Thermo Scientific, SORVALL ST 40R). Then, the precipitate was dissolved in a minimum volume of 0.5 M acetic acid and dialysed against 50 volumes of 0.1 M acetic acid for 24 h, followed by dialysis in the same volume of distilled water for another 24 h. The dialysate was lyophilised in a freeze drier (ilShinBionBase, FD 5512, Korea) and was referred to as "acid soluble collagens (ASC)". The residue obtained after ASC was used for pepsin soluble collagen extraction. The residue was soaked in 0.5 M acetic acid at a sample/solution ratio 1:15 (w/v) and it was homogenised for 1 min using a homogeniser (Model: D-500). Thereafter, pepsin enzyme from porcine gastric mucosa (Sigma-Aldrich, USA) was added to the solution with a sample: enzyme ratio 1:100 (w/w). The mixtures were continuously shacked for 48 h using an open-air shaker (Model: OS-2000) with 150 rpm min^{-1} for 48 h. After that, the supernatant was obtained centrifuging at 3000 x g for 30 min at 4 °C using a centrifuge machine (Thermo Scientific, SORVALL ST 40R). The obtained supernatant was subjected for precipitation and precipitate was dialysed in the same manner as those for acid soluble collagen extraction previously described. The dialysate was lyophilised in a freeze drier (ilShinBionBase, FD 5512, Korea) and was referred to as "Pepsin Soluble Collagen (PSC)".

Hydrolysis of extracted crude collagens

Preparation of enzymatic hydrolysates was carried out using extracted skin collagen of *P. pardalis* by referring the method described in Abeyrathne *et al*. (2014). Three enzymes were used as pepsin from porcine gastric mucosa (Sigma-Aldrich, USA), trypsin from bovine pancreas (Sigma-Aldrich, USA) and protease from *Bacillus licheniformis* (Sigma-Aldrich, USA). Concentration of 20 mg mL^{-1} of solution was made using lyophilised skin collagen dissolving in distilled water. pH was adjusted according to the optimum conditions for each enzyme at 37 °C as shown in the following Table 1.

Table 1: Optimum pH and temperature conditions from enzymatic hydrolysis of extracted skin collagens.

Enzyme	Optimum pH	Temperature (°C)
Pepsin from porcine gastric mucosa	2	37
Trypsin from bovine pancreas	8	37
Protease from *Bacillus licheniformis*	10	37

Each enzyme was added to the lyophilised skin collagen solution separately with enzyme to substrate ratio of 1:100 (v/v) and incubated at 37 °C for 0, 3, 6, 9, 12 and 24 h (0 h incubation refers immediately after addition of enzyme to the sample). After each incubation period, the sample solution was heated using a heat block (Model: DB-006E) for 15 min at 100 °C to inactivate added enzymes. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and visual observation were used to analyse the level of hydrolysis after treating lyophilised collagen with the enzymes. Then, the best hydrolysate was selected.

Checking the functional properties of produced hydrolysates

Measuring of antioxidant properties

Antioxidant activity of the extracted peptides was measured by Thiobarbituric Acid Reactive Substances (TBARS) method (Abeyrathne *et al.*, 2014) and Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity (Jiang *et al.*, 2014).

DPPH–radical scavenging activity assay

The DPPH scavenging activity of collagen hydrolysates from each enzyme was measured by referring Jiang *et al.* (2014) with slight modifications. 750 µL of hydrolysates were added to 300 µL of DPPH solution (0.1 mM in methanol). The mixture was incubated at room temperature for 30 min in dark environment. Then, absorbance was measured at 518 nm using UV spectrophotometer (Model: SPECTROPHOTOMETER UV-2005).

$$\text{Scavenging activity of DPPH (\%)} = [1–(A_s–A_1)/A_o] \times 100$$

A_s – Absorbance of the samples
A_o – Absorbance of the pure DPPH
A_1 – Absorbance of the sample added to methanol

Thiobarbituric acid reactive substance assay (TBARS Assay)

TBARS assay was carried out by referring the method described in Abeyrathne *et al.* (2014) with some modifications. Oil in water emulsion was prepared by homogenising 1 g of pure corn oil (locally purchased) and 100 µL of Tween 20 with 100 mL of distilled water using a homogeniser (Model: D-500) for 2 min. Thereafter, oil in water emulsion was incubated at 37 °C for 20 min. 8 mL of oil emulsion, 1 mL of distilled water and 1 mL of hydrolysed collagen sample from each enzyme were mixed in 15 mL falcon tube to prepare the samples for lipid oxidation assay and it was incubated at 37 °C for 16 h in a water bath (Model: YCW-010E). At the end of the incubation, 1 mL of sample was transferred to a 50 mL falcon tube consisted of 2 mL of 20 mM 15% of TBA/TCA acid solution and 50 µL of 10% butylated hydroxyanisole (SIGMA-ALDRICH, USA) in 90% ethanol. The mixture was vortex mixed using vortex machine (Model: ZX3) and incubated at 90 °C in a water bath (YCW-010E) for 15 min to develop the colour. Then, sample was cooled in ice bath for 10 min and it was centrifuged at 3000 x

g for 15 min in 5 °C using centrifuge machine (Model: ST 40R). The absorbance of the supernatant was measured at 532 nm against a blank using UV spectrophotometer (Model: SPECTROPHOTOMETER UV-2005). Blank was prepared with 1 mL of distilled water and 2 mL of TBA/TCA solution. The amount of TBAR expressed as mg of malondialdehyde per liter of the emulsion.

Measuring of antibacterial property

Locally isolated bacteria cultures were inoculated separately on nutrient agar plates and incubated at 37 °C for 48 h according to Abbaz *et al*. (2016). Hydrolysed collagen samples were prepared to check the antimicrobial property using Agar well diffusion method (1250, 2500, 5000, 10,000 and 20,000 ppm – Concentrations of hydrolysates used).

Measuring of Fe^{2+}chelation activity

Iron chelating activity was measured by using the method described in Abeyrathne *et al*. (2014). 100 µL of hydrolysates, 900 µL of distilled water and 1 mL of 100 ppm $FeSO_4$ were vortex mixed in a 50 mL falcon tube using a vortex machine (Model: ZX3). It was incubated for 15 min at room temperature. Then, 900 µL of 11.3% TCA were added and centrifuged at 2500 x g for 10 min using a centrifuge machine (Model: ST-40R). after that, 1 mL of supernatant was transferred to a culture tube. 1 mL of distilled water, 800 µL of 10% ammonium acetate, 200 µL of ferroin colour indicator (diluted ferroin colour indicator in ratio of 1:1) and vortex mixed using a vortex machine (Model: ZX3). It was incubated at room temperature for 5 min. Absorbance was measured at 562 nm. Fe^{2+} chelating activity was calculated by using the following equation,

$$Fe^{2+}\text{chelating activity (\%)} = [1–(A_1/A_0] \times 100$$

A_1 – Absorbance of the samples
A_0 – Absorbance of the blank

Statistical analysis

One-way ANOVA was used to analyse the data in functional properties analysis and statistical significance was considered at $P<0.05$. Three replicates were used for each trial. Data were analysed using Minitab 17.0 version statistical software package and Microsoft office excels software package. All the data were expressed as means with standard deviation for minimum three independent measurements.

RESULTS AND DISCUSSION

Yield analysis of the extracted crude collagens

Decalcification is a process of removal of calcium-deficient hydroxyapatite ($Ca_5(PO_4)_3OH$) like calcium salts from the scales of the fish (Prasad and Donoghue, 2013). *P. pardalis* skin is composed with hard bony armor as an outer

coverage. It is merely attached to their fish skin. Manual removal of bony plates is difficult and could damage to their skin. Therefore, decalcification process was undertaken in the pre-treatment process by using two methods using ethylene diamine tetraacetic acid (EDTA) and three concentrations of citric acid solutions (Wang and Regenstein, 2009). After decalcification, acid soluble collagens (ASC) and pepsin soluble collagens (PSC) were extracted from the *P. pardalis* skin and collagen samples were followed for SDS PAGE with standard collagen. Similar band patterns were observed from samples with the standard collagen confirming the extraction is crude collagen (Plate 1). Yield of ASC and PSC extracts with 1.2, 2.2 and 3.2 g L^{-1} citric acid treatments showed higher yield compared to yield of ASC and PSC extracts with 0.1, 0.2 and 0.3 mol L^{-1} EDTA treatments (Figure 1). According to previous studies, collagen yield with EDTA treatment had higher yield than citric acid treatment in gelatin extraction from silver carp scales. However, results obtained in this study did not match with previous studies (Wang and Regenstein, 2009). This may be due to the low purity of extracted crude collagens with citric acid treatments. Therefore, further studies are needed for the evaluation of type of impurities present in the extracted crude collagens.

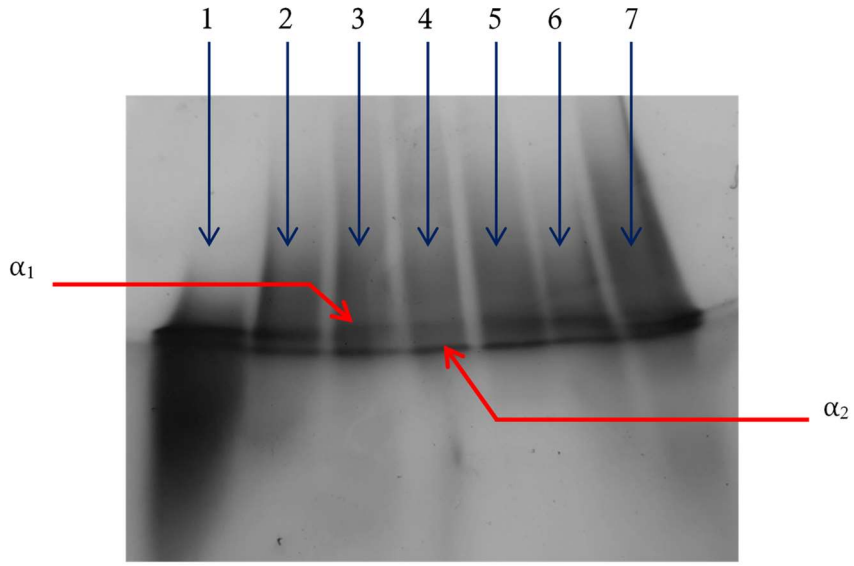

Plate 1: 8% SDS-PAGE of *P. pardalis* skin collagen. Lane 1 = standard collagen; Lane 2 = acid soluble collagen (ASC) (citric acid 1.2 g L^{-1}); Lane 3 = acid soluble collagen (ASC) (citric acid 2.2 g L^{-1}); Lane 4 = acid soluble collagen (ASC) (citric acid 3.2 g L^{-1}); Lane 5 = pepsin soluble collagen (PSC) (citric acid 1.2 g L^{-1}); Lane 6 = pepsin soluble collagen (PSC) (citric acid 2.2 g L^{-1}); Lane 7 = pepsin soluble collagen (PSC) (citric acid 3.2 g L^{-1}).

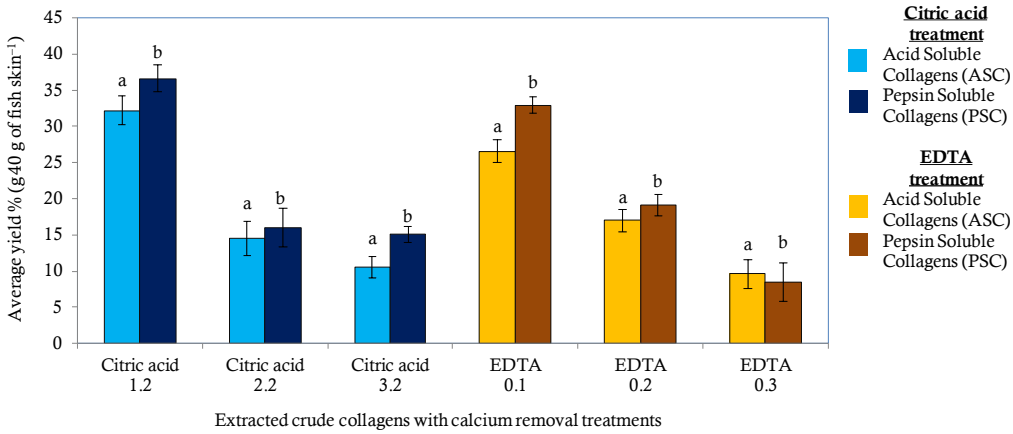

Figure 1: Graphical representation of extracted crude collagen yield under two different calcium removal treatments.

The yield of extracted crude collagens was significantly (P<0.05) decreased when increasing the concentrations of citric acid and EDTA and the highest yield was obtained for both ASC and PSC from citric acid 1.2 g L^{-1} (ASC: 32.19±1.95% & PSC: 36.63±1.83%) and EDTA 0.1 mol L^{-1} (ASC: 26.52±1.56% & PSC: 32.93±1.1%). The results may correlate to collagen loss during the decalcification process. The highest yields obtained from the collagen extractions from ACS and PSC were selected to prepare the collagen hydrolysates in this study. Therefore, EDTA is not a food grade chemical: citric acid treatment for decalcification process is a good substitute for EDTA as a cost-effective method. Thus, collagen extraction with decalcification using citric acid treatment from *P. pardalis* skin is good source and good non-toxic method in the food industry.

Determination of the best collagen hydrolysates

Enzymatic hydrolysis of protein is a process of decomposition of protein into individual amino acids and/or peptides that occur with the participation of enzymes (Eckert, 2013). This is one of the hydrolysis processes used to produce fish protein hydrolysates (FPHs). The degree of hydrolysis is an important factor and it determines the terminal peptides and bioactivity (Barzideh *et al.,* 2014). In this study, collagen was hydrolysed using pepsin, trypsin and protease enzymes at 37 °C for 0, 3, 6, 9, 12 and 24 h and the best time lag for the prepared collagen hydrolysates was selected from each enzyme treatment using the physical appearance of the produced collagen hydrolysates and 15% SDS-PAGE gel electrophoresis visual observations (Table 1).

Physical appearance of the collagen hydrolysates was observed visually. All the collagen hydrolysates were white in colour and cotton wool structure prior to hydrolysis. According to 15% SDS-PAGE, image showed different collagen

hydrolysates produced from all three enzymes, there were no any band patterns in all lanes containing hydrolysates. Abeyrathne *et al*. (2014) revealed that peptides with molecular weight <2 k Da did not show bands in 15% SDS-PAGE. According to that, pepsin, trypsin and protease enzymes can produce peptides with molecular weight <2 k Da from collagen even in 0-hour incubation time.

Analysis of functional properties of collagen hydrolysates

Antioxidant activity of collagen hydrolysates

Oxidative stress involved in cardiovascular disease, cancer and other chronic diseases is a major cause for sudden death at present. Antioxidants are compounds that inhibit the oxidative processes and thereby prevent oxidative stress (Willcox *et al.*, 2004; Pham-Huy *et al.*, 2008). These antioxidants can be considered as important nutraceuticals of many health benefits (Droge, 2002; Valko *et al.*, 2007). Therefore, oxidation leads to a significant loss of nutritional value of food. Collagen hydrolysates can act as antioxidant for food and can prevent above consequences resulted in lipid oxidation.

Some antioxidant compounds are good radical scavengers while some others are effective lipid peroxidation inhibitors (Barzideh *et al.*, 2014). Therefore, different antioxidant assays are required to evaluate antioxidant capacity of collagen hydrolysates. In previous studies, collagen and gelatin hydrolysates from different sources showed antioxidant activities (Kim *et al.*, 2001; Mendis *et al.*, 2005). In the present study, the antioxidant activity of *P. pardalis* skin collagen hydrolysed with pepsin, trypsin and protease enzymes in 0 h incubation period at 37 °C was evaluated using DPPH free radical scavenging activity assay and Thio-Barbituric Acid Reactive Substances (TBARS) assay.

TBARS Assay

TBARS assay is widely used in scientific researches to quantify the level of oxidation by means of measuring malonaldehyde (MDA) (Oakes and Van der Kraak, 2003). The MDA is capable of cross-linking with amino acids to form amidine linkages and may also interact with other components of foods such as nucleosides, nucleic acids, amino acids of phospholipids and other aldehydes which are end products of lipid oxidation (Herath *et al.*, 2015). TBARS assay, which is reacting with thiobarbituric acid (TBA), is a common method to measure the occurrence of MDA (Hodges *et al.*, 1999). In TBARS assay MDA is a secondary end product of lipid peroxidation and reacts with TBA and form a pink pigment (Jardine *et al.*, 2002).

Considering the level of MDA (mg L^{-1}) produced from each collagen hydrolysed samples, it has shown a significant (P<0.05) difference with oil emulsion and collagen hydrolysed samples. Thus, oil emulsion showed 0.107±0.01 mg L^{-1} MDA formation. Extracted crude ASC from both calcium treatments were reduced that level into 0.000 mg L^{-1} (Figure 2).

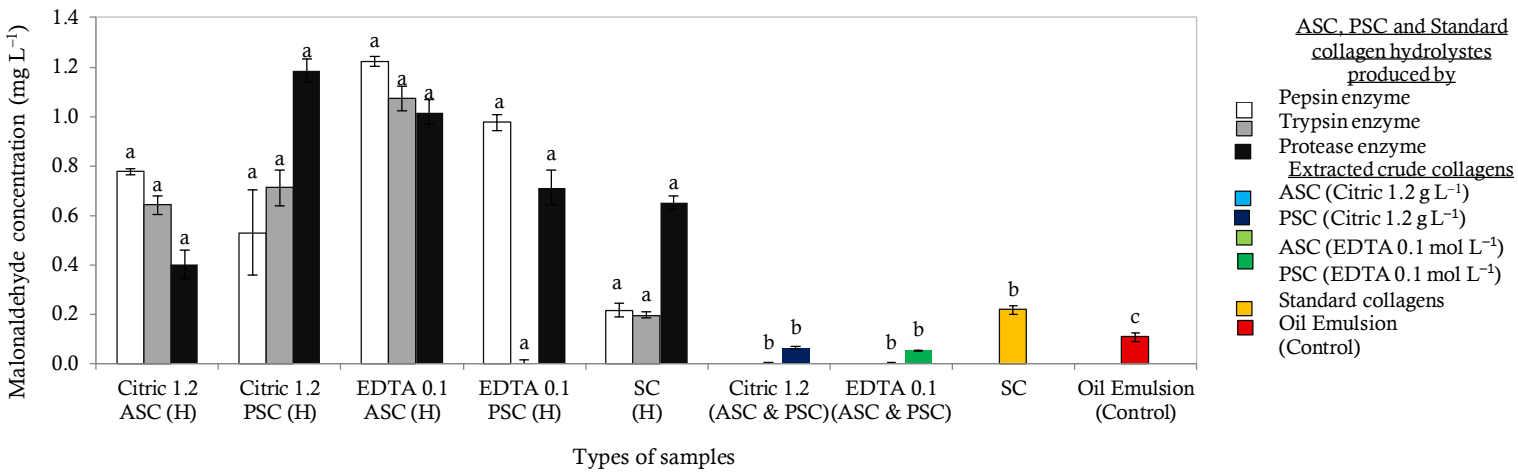

Figure 2: Produced melonaldehyde concentration of hydrolysateso obtained from different enzymes (citric acid 1.2 g L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC); EDTA 0.1 mol L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC), standard collagen hydrolysates, extracted crude collagens (citric acid 1.2 g L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC); EDTA 0.1 mol L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC), standard collagen and oil emulsion (control).

When comparing all collagen extractions, it did not show a significant (P>0.05) difference between treatments. Level of malondialdehyde produced from collagen hydrolysates produced by trypsin enzyme showed low concentration when compared to other collagen hydrolysates. The highest MDA formation can be observed from the collagen hydrolysates produced from protease enzymes. Extracted crude collagens showed significantly (P<0.05) lower MDA formation than the collagen hydrolysates produced from each enzyme. However, those hydrolysates can be used as good preservative to minimise the oxidation in food industry with further studies.

DPPH radical scavenging activity

DPPH scavenging assay is a standard chemical assay that used for analysis of antioxidant activities of biological compounds. Free radicals induce oxidative damage to the biomolecules and it causes to atherosclerosis, aging, cancer and some other diseases. Antioxidants, which scavenge free radicals act important role in preventing these free radical induced diseases. DPPH is a stable free radical which contains a free electron in one of the atoms in its nitrogen bridge (Eklund *et al.*, 2005). The principle of the assay is measuring the scavenging of free radical under presence of an antioxidant by spectroscopic techniques (Alma *et al.*, 2003).

DPPH radical scavenging activity assay is a method for measuring the capacity of an antioxidant to reduce free radicals and degree of color changes is changing with the antioxidant activity of the sample (Xie *et al.*, 2008). Here, DPPH radical scavenging was measured for the collagen hydrolysates which were produced by pepsin, trypsin and protease enzyme under zero-hour incubation time at 37 °C.

According to the Figure 3, comparing with the ascorbic acid (control) it shows a significant (P<0.05) difference with the extracted samples. Considering extracted samples, all collagen hydrolysates were having over 65% scavenging activity. When DPPH encounters a proton-donating substance (H^+), the radical is scavenged by changing color from purple to yellow and the absorbance is reduced (Liu *et al.*, 2010). In our results, the collagen hydrolysates reduced the DPPH radical to a yellow-colored compound from purple. Therefore, peptides derived from *P. pardalis* skin collagen hydrolysis can be used as an antioxidant compound by its free radical scavenging activity.

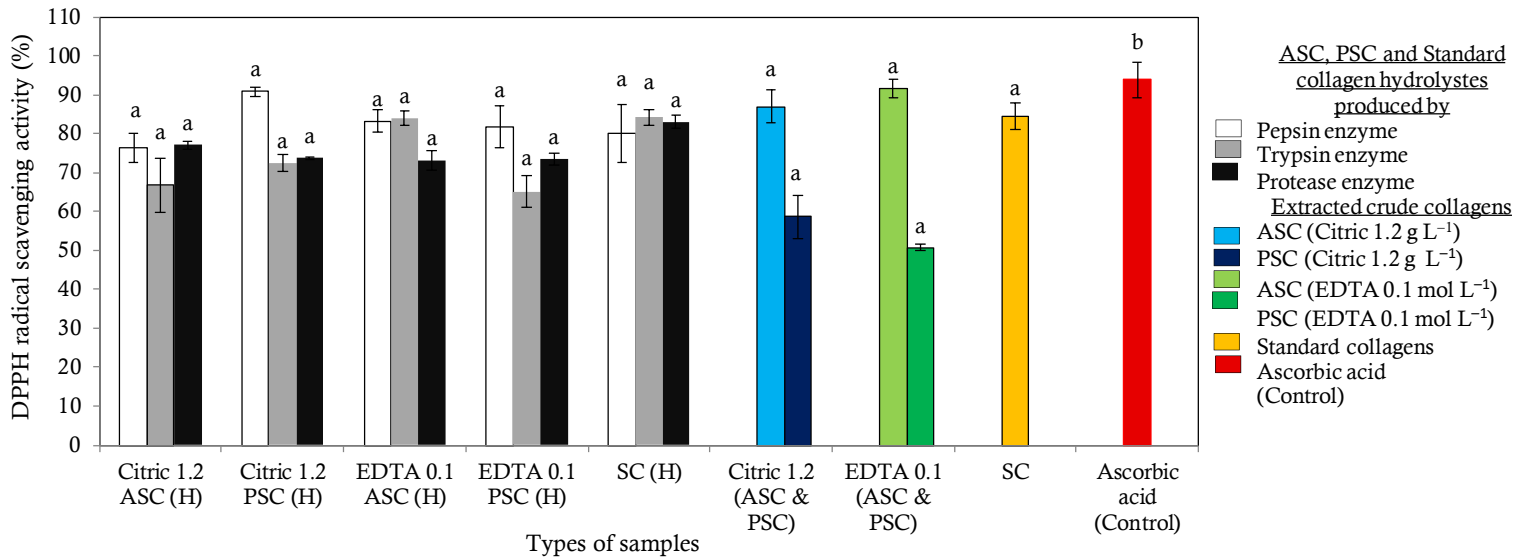

Figure 3: DPPH radical scavenging activity of collagen hydrolysates produced by pepsin, Trypsin and protease enzymes (citric acid 1.2 g L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC); EDTA 0.1 mol L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC), standard collagen hydrolysates, extracted crude collagens (citric acid 1.2 g L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC); EDTA 0.1 mol L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC), standard collagen with the ascorbic acid as the positive control.

Iron chelation activity

Food usually contains several types of transition metals including iron, copper and manganese. These transitional metals aid oxidation processes directly or indirectly causing undesirable consequences (Schaich, 1980). Therefore, metal chelation activity is important in food and pharmaceutical industry due to they are considering as carriers of micronutrients, facilitating their absorption (Clement, 2000). Peptides, which have chelating activity, are useful for increasing the bioavailability of minerals and to prevent the oxidation of food (Eckert *et al.*, 2013).

In living organisms, Fe^{2+} is oxidised into Fe^{3+} by reducing oxygen (O_2) in to superoxide ion which is able to form highly reactive $^{\bullet}OH$ free radical (Wettasinghe and Shahidi, 2002). If any substance present with iron chelation properties, it chelates the iron, avoiding formation of destructive free radicals. Therefore, Fe^{2+} chelation activity of collagen hydrolysates which were produced by pepsin, trypsin and protease enzymes under 0 h incubation time at 37 °C were measured.

According to Figure 4, collagen hydrolysates produced by pepsin, trypsin and protease enzymes showed significantly (P<0.05) higher Fe^{2+} chelation ability compared to standard collagen hydrolysates and extracted crude collagens. However, collagen hydrolysates did not show any significant (P>0.05) difference among them. Chelating of metal ions can be a prevention mechanism against free radical reactions (Lopes *et al.*, 1999). In this study, collagen hydrolysates showed higher Fe^{2+} chelating activity and therefore, they can be used as an antioxidant for foods with further studies.

Antimicrobial properties

Total plate count method

Total plate count (TPC) method was used for this assay. This assay was conducted by measuring the inhibition zones after 48 h. The results obtained has graphically represented in Figure 5 and 6. According to the results, all the collagen hydrolysates parallel to the standard collagen hydrolysates showed significantly (P<0.05) higher antimicrobial activity compared with the extracted crude collagens. Collagen hydrolysates derived from all three enzymes (pepsin, trypsin and protease) extracted crude collagen had exhibited inhibition zones even at the smallest concentrations (1250 ppm) used.

There was no significant (P>0.05) difference among the produced collagen hydrolysates in all the concentration series. As per the results, pepsin treated collagen hydrolysates had been demonstrated best antimicrobial activity in both highest and least concentrations. The highest inhibition (5.70 mm) was observed in the highest concentration (20,000 ppm) of collagen hydrolysates produced using pepsin enzyme (EDTA 0.1 mol L^{-1}, pepsin soluble collagen – PSC).

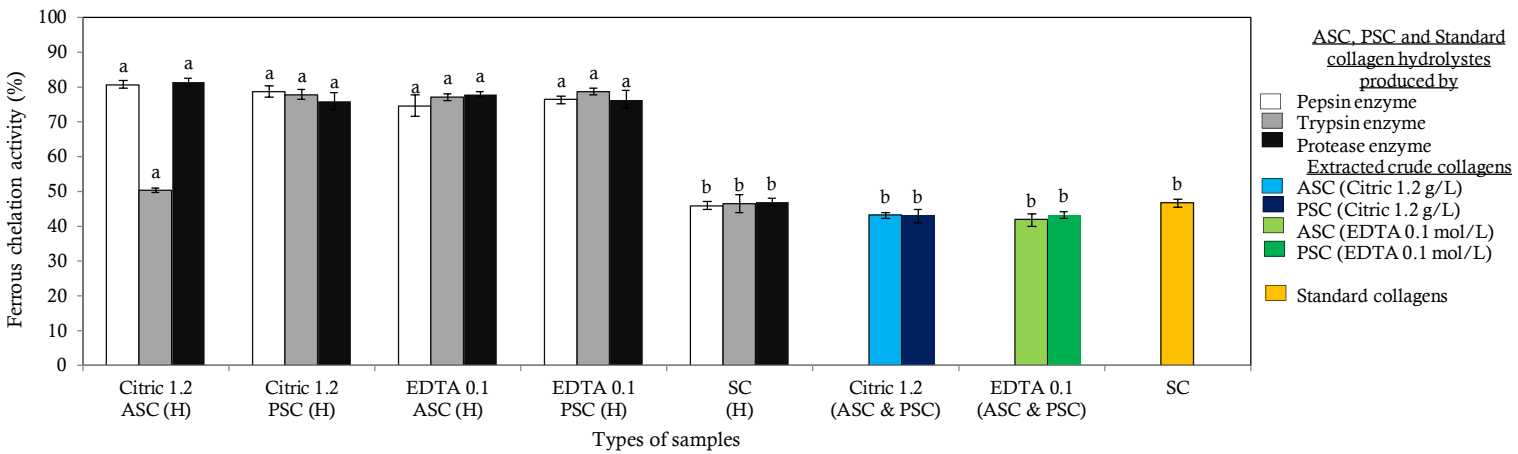

Figure 4: Fe^{2+} chelation activity of collagen hydrolysates produced by pepsin, trypsin and protease enzymes (citric acid 1.2 g L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC); EDTA 0.1 mol L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC), standard collagen hydrolysates, extracted crude collagens (citric acid 1.2 g L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC); EDTA 0.1 mol L^{-1}: acid soluble collagen (ASC) and pepsin soluble collagens (PSC) & standard collagen.

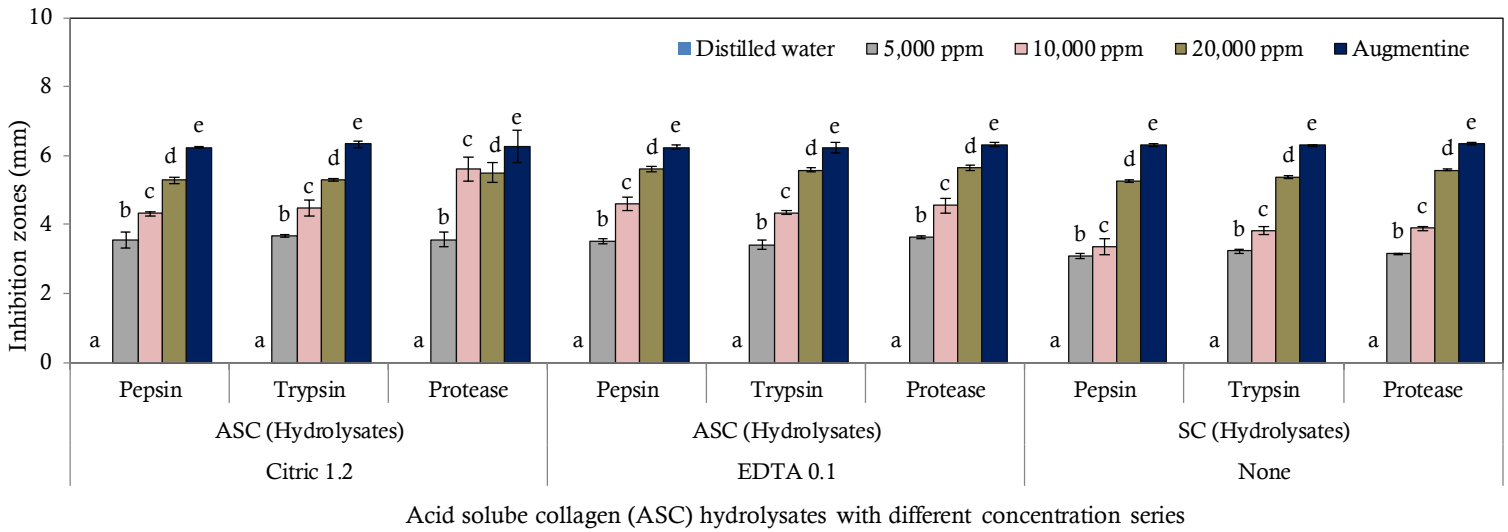

Figure 5: Antimicrobial property of acid soluble collagen (ASC) hydrolysates produced by pepsin, trypsin and protease enzymes (citric acid 1.2 g L^{-1}; EDTA 0.1 mol L^{-1}, standard collagen hydrolysates, with the augmentin as the positive control & distilled water as the negative control. $^{a, b, c, d}$ Means with different superscript differ significantly (P<0.05).

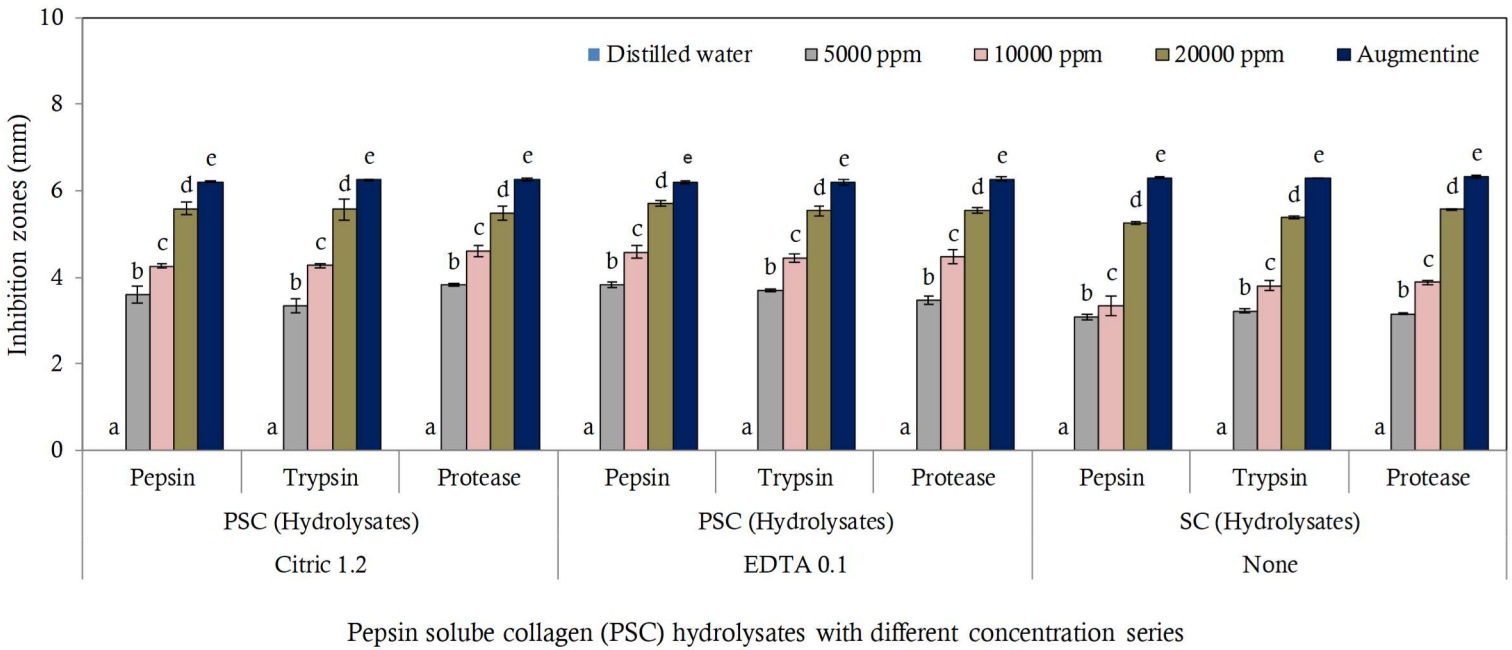

Figure 6: Antimicrobial property of pepsin soluble collagen (PSC) hydrolysates produced by pepsin, trypsin and protease enzymes (Citric acid 1.2 g L^{-1}; EDTA 0.1 mol L^{-1}, standard collagen hydrolysates, with the augmentin as the positive control and distilled water as the negative control. $^{a, b, c, d}$ Means with different superscript differ significantly (P<0.05).

Published information regarding antimicrobial properties from collagen and gelatin hydrolysates is scarce. But, there was antimicrobial activity in peptides derived from tuna and squid skin gelatin which were revealed by Gómez-Guillén *et al*. (2010). Therefore, further studies are needed to confirm antimicrobial activity of collagen hydrolysates *P. pardalis* skin.

CONCLUSIONS

P. pardalis skin is a good source for collagen extraction for both acid soluble collagens and pepsin soluble collagens. Citric acid is a suitable chemical for the decalcification process of *P. pardalis* hard bony armor. All the collagen hydrolysates produced by enzymatic hydrolysis (pepsin, trypsin and protease enzymes) of *P. pardalis* skin, contained strong antibacterial properties and metal chelation properties and antioxidant properties as favorable bioactivities. Therefore, collagen hydrolysates produced by pepsin, trypsin and protease enzymes from *P. pardalis* skin; decalcification with citric acid treatment can be used as an effective free radical scavenger, metal chelator and antibacterial agent in food industry with further studies.

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