Comparative study on antioxidant activity of hydrolysates from splendid squid (*Loligo formosana*) gelatin and protein isolate prepared using protease from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*)

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Abstract Antioxidative activities of splendid squid gelatin hydrolysate (SGH) and squid mantle protein isolate hydrolysate (SMPIH) with different DHs (10–50 %) prepared using crude protease from hepatopancreas of Pacific white shrimp were comparatively studied. At the same DH, SGH had higher FRAP and metal chelating activity and SMPIH showed higher ABTS radical scavenging activities. However, SMPIH had higher ABTS radical scavenging activity than SGH at all DHs tested. SMPIH showed higher DPPH radical scavenging activities than SGH when DHs were up to 30 % DH. However, the former had the lower activity as DHs were 40 and 50 %. ABTS radical scavenging activity of SGH and SMPIH with 50 % DH in gastrointestinal model system increased in the duodenal condition, while SGH showed higher activity in stomach condition than SMPIH. SGH and SMPIH (500, 1000 and 2000 mg/L) could retard lipid oxidation in a lecithin liposome system in a dose dependent manner. Based on Sephadex G-25 column chromatography, the fraction with ABTS radical scavenging activity had MW of 283 and 1381 Da, respectively. Therefore, both hydrolysates from

squid gelatin and mantle protein isolate could be used as the alternative natural antioxidants.

Keywords Splendid squid · Protein hydrolysate · Gelatin · Mantle · Crude protease · Pacific white shrimp · Hepatopancreas

Introduction

Protein hydrolysates and peptides from seafood have shown significant antioxidant activities and can be used as alternatives to synthetic antioxidants (Chi et al. 2015). Enzymatic hydrolysis is the most widely used technique for producing the peptides with bioactivities (Adjonu et al. 2013). Papain from plant, pepsin, chymotrypsin and trypsin from animal origins and Alcalase from microbial origins are the most common commercial proteases used for production of fish protein hydrolysate (Ovissipour et al. 2009). Nevertheless, a drawback of using commercial proteases is the high cost associated with purification process. In order to reduce the cost of commercial protease, crude proteases extracted from fish and shellfish viscera, the byproducts, can be a promising processing aid with the lower cost (Younes et al. 2014). Previously, crude protease extract from hepatopancreas of Pacific white shrimp (*Litopenaeus vannamei*) was successfully used to extract carotenoprotein from shells of Pacific white shrimp (Senphan et al. 2014). Additionally, it was used to hydrolyse seabass (*Lates calcarifer*) skin gelatin (Senphan and Benjakul 2014). Crude protease from hepatopancreas of Pacific white shrimp can serve as the potential cheap enzyme for production of protein hydrolysate, especially from marine animals such as squid. Those protein hydrolysates with antioxidative

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activity can be further used as natural food additive or for health benefit.

Gelatin hydrolysates with antioxidant activity have been produced from squid skin (Giménez et al. 2009; Mendis et al. 2005). Squid meat, especially mantle, could be used as an alternative raw material for protein hydrolysate. However, squid mantle is tough in texture and hard to grind before hydrolysis. The use of acid- and alkaline-aided solubilisation of proteins to produce protein isolate can be a means to dissociate proteins with simultaneous removal of lipids and phospholipids in the muscle (Yarnpakdee et al. 2014). Both squid mantle protein isolate and skin gelatin might be susceptible to hydrolysis by crude protease from Pacific white shrimp differently and the resulting protein hydrolysates plausibly had different antioxidative activities. Nevertheless, no information on antioxidative activity of hydrolysate from proteins of splendid squid skin and mantle exists. This study aimed to compare antioxidative activities of protein hydrolysate from skin gelatin and protein isolate from mantle of splendid squid with various DHs.

Materials and methods

Chemicals

2,4,6-trinitrobenzenesulphonic acid (TNBS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis (3-thylbenzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tripyridyltriazine (TPTZ), ethylenediamine tetraacetic acid (EDTA), 6-hydroxy-2,5,7,8-tetra-methyl-chroman-2-carboxylic acid (Trolox), 3-(2-Pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulphonic acid monosodium salt hydrate (ferrozine), pepsin from porcine gastric mucosa (EC 3.4.23.1), pancreatin from porcine pancreas were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Folin-Ciocalteu's phenol reagent, trichloroacetic acid (TCA), potassium persulphate, sodium sulphite and ferrous chloride were obtained from Merck (Darmstadt, Germany). Sephadex G-25 was purchased from GE Healthcare UK Limited (Little Chalfont, Buckinghamshire, UK). All chemicals used were of analytical grade.

Preparation of crude protease extract from hepatopancreas of Pacific white shrimp (CPE)

Hepatopancreases of Pacific white shrimp (*L. vannamei*) were obtained from the Sea Wealth Frozen Food Co., Ltd., Songkhla province, Thailand. Hepatopancreases were the leftover from the hepatopancreas-free whole shrimp, constituting around 6.5–7.5 % of total weight. Samples were placed in polyethylene bag and stored in ice with an ice/

sample ratio of 2:1 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, Thailand within 2 h. Samples were kept at -18°C until use, but no longer than 1 month.

Crude protease extract (CPE) from hepatopancreases was prepared as per the method of Senphan et al. (2014). Hepatopancreases of Pacific white shrimp were powdered in liquid nitrogen and defatted using the cold acetone (-20°C). To prepare the crude extract, acetone powder was suspended in 10 mM Tris-HCl, pH 8.0 containing 1 mM CaCl_2 at a ratio of 1:50 (w/v) and stirred continuously at 4°C for 3 h. The suspension was centrifuged for 10 min at 4°C at $10,000\times g$ to remove the tissue debris using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Fullerton, CA, USA). The supernatant was lyophilised using a SCANVAC CoolSafeTM freeze-dryer (CoolSafe 55, ScanLaf A/S, Lyngø, Denmark).

One unit of activity was defined as that releasing 1 μmol of tyrosine per min ($\mu\text{mol Tyr/min}$) assayed at pH 8 and 60°C using casein as the substrate. Before use, the lyophilised CPE (10 g) was dissolved with 50 mL of cold distilled water (4°C).

Preparation of splendid squid mantle protein isolate (SMPI)

Fresh splendid squids (*Loligo formosana*) with the size of 270–300 g/squid (5 kg) were purchased from a local market in Hat Yai, Songkhla, Thailand. The elapsed time after the capture until being processed was 24–36 h. The samples were transported in ice using a squid/ice ratio of 1:2 (w/w) to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 30 min. Squid were cleaned, deskin and eviscerated. The mantles were then washed with iced tap water ($0-2^{\circ}\text{C}$) two times. The mantles were minced to uniformity using a blender. To prepare SMPI, the mince was homogenised with five volumes of cold distilled water ($2-4^{\circ}\text{C}$) at a speed of 11,000 rpm for 1 min using IKA Labortechnik homogeniser (Model T25 basic, Selangor, Malaysia). The homogenate was adjusted to pH 11.5 using 2 and 0.5 N NaOH and placed on ice for 60 min with a continuous stirring. The mixture was then centrifuged at $10,000\times g$ for 10 min at 4°C . The alkaline soluble fraction was collected and adjusted to pH 5.5 using 2 and 0.5 N HCl to precipitate the proteins. The mixture was then centrifuged at $10,000\times g$ for 10 min at 4°C . The pellet obtained was referred to as 'SMPI'.

Preparation of gelatin from splendid squid skin (SG)

The outer skin of fresh splendid squid was obtained from Sea Wealth Frozen Food Co., Ltd., Songkhla, Thailand and

stored in ice using a skin/ice ratio of 1:2 (w/w). Upon arrival, the skin was cleaned and washed with iced tap water, then cut into small pieces ($0.5 \times 0.5 \text{ cm}^2$), placed in polyethylene bags and stored at -20°C until use. The storage time was no longer than 2 months. Gelatin was prepared as per the method of Nagarajan et al. (2012).

Production of hydrolysates from SG and SMPI

SG and SMPI were added with distilled water to obtain a final protein concentration of 2 % (w/v) as determined by the Kjeldahl method (AOAC 2000). The pH of the mixtures were adjusted to 8 using 2 and 0.5 M NaOH. All mixtures were pre-incubated at 60°C for 15 min. The enzymatic hydrolysis was started by adding CPE at amounts yielding the DHs of 10, 20, 30, 40 and 50 % DH into the mixtures as described by Benjakul and Morrissey (1997). After hydrolysis for the designated time, the reaction was terminated by heating the mixtures at 90°C for 10 min. The hydrolysates from SG and SMPI were then centrifuged at $8000 \times g$ for 10 min. The supernatants were lyophilised. The resulting hydrolysates from SG and SMPI were referred to as 'SGH' and 'SMPIH', respectively. All hydrolysates were determined for degree of hydrolysis (DH) using 2,4,6-trinitrobenzenesulfonic acid (TNBS) method as described by Benjakul and Morrissey (1997).

Determination of antioxidative activities using various in vitro assays

DPPH radical scavenging activity

DPPH radical scavenging activity of SGH and SMPIH with different DHs was measured according to the method of Wu et al. (2003). A standard curve was prepared using Trolox in the range of 10–60 μM . The activity was expressed as μmol Trolox equivalents (TE)/g sample.

ABTS radical scavenging activity

ABTS radical scavenging activity of all hydrolysates was determined as described by Arnao et al. (2001). A standard curve of Trolox ranging from 50 to 600 μM was prepared. The ABTS radical scavenging activity of the hydrolysates was expressed as μmol TE/g sample.

Ferric reducing antioxidant power (FRAP)

FRAP of hydrolysates was determined as described by Benzie and Strain (1996). FRAP was expressed as μmol TE/g sample. The standard curve was prepared using Trolox ranging from 50 to 600 μM .

Chelating activity towards Fe^{2+}

The chelation of Fe^{2+} was determined using the method of Boyer and McCleary (1987), with a slight modification. The Fe^{2+} chelating activity was expressed as EDTA equivalents (EE)/g sample. A standard curve of 0–50 μM EDTA was prepared.

Determination of antioxidative activity in lecithin liposome model system

The antioxidative activities of SGH and SMPIH with 50 % DH were determined in lecithin liposome system, following the method of Frankel et al. (1997) with slight modifications as described by Thiansilakul et al. (2007). The system containing 100 mg/L Trolox was also prepared. The system oxidation was monitored during 60 h of incubation by determining of thiobarbituric acid reactive substances (TBARS) and conjugated dienes (CD) as described by Buege and Aust (1978) and Frankel et al. (1997), respectively.

Stability of the hydrolysates in gastrointestinal tract model system

Simulated gastrointestinal digestion using an in vitro pepsin–pancreatin hydrolysis was carried out according to the method of You et al. (2010) with slight modification. SGH and SMPIH were dissolved in distilled water to obtain a concentration of 25 mg/mL (15 mL). The solution was adjusted to pH 2.0 with 2 M HCl and pepsin dissolved in 0.1 M HCl was added to obtain the final concentration of 40 g pepsin/kg hydrolysate. The mixture was incubated with continuous shaking (Mettler Model SV 1422, Schwabach, Germany) for 1 h at 37°C . Thereafter, the pH of the reaction mixture was adjusted to 5.3 with 1 M NaOH before addition of 20 g pancreatin/kg hydrolysate. Subsequently, the pH of mixture was adjusted to 7.5 with 1 M NaOH. The mixture was further incubated with continuous shaking for 3 h at 37°C . To terminate the digestion, the solution was submerged in boiling water for 10 min. During digestion, the mixture was randomly taken at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210 and 240 min for determination of ABTS radical scavenging activity.

Fractionation of antioxidative peptides from hydrolysate of squid gelatin and mantle protein isolate

The 2 mL (80 mg/mL) of SGH and SMPIH (50 % DH) was loaded onto a Sephadex G-25 column ($1.6 \times 70 \text{ cm}$). The elution was carried out with deionised water at a flow rate of 0.5 mL/min. The 3 mL fractions were collected and

their absorbance was read at 220 nm. The fractions were subjected to analysis for ABTS radical scavenging activity. The molecular weight distribution of antioxidative peptides was determined from the retention time of selected fraction, in comparison with those of protein standards including inulin B (3495.89 Da), vitamin B12 (1355.5 Da), glutathione (307.32 Da), Gly-Tyr (238.25 Da) and tyr (181.2 Da). Blue dextran (2000 kDa) was used to measure the void volume of the column.

Statistical analysis

All the experiments were done using three different lots of samples and the mean $\pm$ standard deviation was reported. The one-way ANOVA and the Duncan's multiple-range test was used to evaluate significant differences ($P < 0.05$) between the means for each sample using SPSS software (Version 16.0, Chicago, IL, USA).

Results and discussion

Effect of CPE concentration and hydrolysis time toward hydrolysis of SG and SMPI

DHs of hydrolysates from squid skin gelatin (SGH) and squid mantle protein isolate (SMPIH) using CPE at levels of 5, 10 and 15 units/g protein as a function of time are shown in Fig. 1a. The DHs of hydrolysates increased with increasing hydrolysis time and enzyme concentrations for both proteinaceous substrates ($P < 0.05$). The sharp increase in DH of SGH was obtained within the first 15 min, followed by the gradual increase in DH up to the end of hydrolysis (180 min). For SMPIH, the marked increase in DH was noticeable up to 60 min ($P < 0.05$). Subsequently, the rate of increase was lowered ($P < 0.05$). An initial rapid phase indicated that a large number of peptide bonds were cleaved, and the following slower rate of hydrolysis might be hypothetically attributed to a decrease in the concentration of peptide bonds available for hydrolysis, the decrease in enzyme activity or a product inhibition (Intarasirisawat et al. 2012). At the same level of enzyme and hydrolysis time, higher DHs were observed in SMPIH (Fig. 1b), compared with those found in SGH. The result suggested that squid mantle protein isolate was more susceptible to hydrolysis by CPE. Protein isolate prepared using the alkaline pH-shift method more likely contained the dissociated protein substrate with less compactness. Moreover, lipids and connective tissues were removed, thereby providing higher proportion of muscle proteins served as substrate for CPE (Theodore et al. 2008). The lower DH of SGH could be due to the presence of proline and/or hydroxyproline, which are generally resistant to degradation by digestive enzymes.

Fig. 1 Degree of hydrolysis of protein hydrolysates from squid skin gelatin and squid mantle protein isolate during hydrolysis with crude proteases extract of hepatopancreas from Pacific white shrimp (CPE) at different concentrations [5 units (*triangle*), 10 unit (*diamond*) and 15 units (*square*)] (a). Relationship between DH and $\log_{10}$ enzyme concentration of CPE in SG (*square*) and SMPI (*triangle*). Hydrolysis was conducted at pH 8 and 60 °C for 30 min (b). DH, degree of hydrolysis. SG, Gelatin from splendid squid skin. SGH, hydrolysate from squid skin gelatin. SMPI, protein isolate from splendid squid mantle. SMPIH, hydrolysate from squid mantle protein isolate. Bars represent the standard deviation ($n = 3$)

Gelatin with a glycine content of around 33 % might not be a preferable substrate for proteases (Benjakul et al. 2014).

Six levels of CPE (5, 10, 15, 20, 25 and 30 units/g protein) were added to hydrolyse SG and SMPI for 30 min. When $\log_{10}$ of enzyme concentration and DH were plotted, a linear relationship was obtained (Fig. 1b). From the equations, the amounts of CPE required to hydrolyse SG and SMPI to obtain designated degrees of hydrolysis (10, 20, 30, 40 and 50 %) were calculated.

Antioxidative activities of squid protein hydrolysates with different DHs produced by CPE

DPPH radical scavenging activity

DPPH radical-scavenging activities of SGH and SMPIH with different DHs are given in Fig. 2a. As the DH

Fig. 2 DPPH radical scavenging activity (a), ABTS radical scavenging activity (b), FRAP (c) and metal chelating activity (d) of SGH and SMPH with different degrees of hydrolysis (DH). SGH, hydrolysate from squid skin gelatin. SMPH, hydrolysate from squid

increased, DPPH radical-scavenging activity of hydrolysates increased ($P < 0.05$). However, no differences were observed for hydrolysates with 10 and 20 % DH ($P > 0.05$), regardless of the substrate. Furthermore, SMPH with 30 and 40 % DH had no differences in DPPH radical scavenging activity ($P > 0.05$). At the DH less than 40 %, SMPH showed higher activity than SGH ($P < 0.05$). Conversely, SGH with 40 and 50 % DH had higher DPPH radical-scavenging activities than SMPH ($P < 0.05$). Hydrolysates with 50 % DH showed the highest activity, compared with other DHs ($P < 0.05$). You et al. (2009) reported that DPPH radical scavenging activity of Loach protein hydrolysate prepared using Alcalase and papain enzyme increased with increasing DH from 18 to 23 %. However, DPPH radical scavenging activity of hydrolysates decreased as DH increased from 23 to 33 % ($P < 0.05$). DPPH radical scavenging activity of protein hydrolysate from defatted skipjack (*Katsuwonus pelamis*) roe, hydrolysed by Alcalase decreased as DH increased up to 50 % ($P < 0.05$) (Intarasirisawat et al. 2012). During hydrolysis, a wide variety of smaller peptides and free amino acids are generated, depending on

mantle protein isolate. Bars represent the standard deviation ($n = 3$). Different capital and small letters on the bars within the same protein substrate indicate the significant differences ($P < 0.05$)

enzyme specificity. Changes in size, composition of free amino acids and amino acid sequence of small peptides affect the antioxidative activity (Klompong et al. 2007). At 50 % DH, peptides in SGH plausibly showed higher ability in providing proton to DPPH radical, in which DPPH-H could be formed to a higher extent.

ABTS radical scavenging activity

As shown in Fig. 2b, both SGH and SMPH at all DHs were able to quench ABTS radical. ABTS radical scavenging activities of hydrolysate increased drastically with increasing DH up to 50 % for SGH and up to 30 % for SMPH ($P < 0.05$). At the same DH, SMPH had higher ability to scavenge the radicals ($P < 0.05$). The amino acid sequence and composition in peptides, chain length and also free amino acids produced might affect the antioxidant activity and determined the scavenging activity of hydrolysates (Intarasirisawat et al. 2012; Khantaphant and Benjakul 2008). With increasing DH up to 30 %, the seabass skin hydrolysates using Alcalase showed the increases in ABTS radical scavenging activity ($P < 0.05$) and there

were no differences between hydrolysate with DHs of 30 and 40 % (Senphan and Benjakul 2014). Raghavan et al. (2008) prepared hydrolysates from tilapia (*Oreochromis niloticus*) protein isolate using five proteases including Amano A₂, Amano N, Flavourzyme, Neutrase, and Cryotin-F. ABTS radical scavenging activity of the hydrolysates increased with an increase in % DH. The result indicated that hydrolysates produced from SGH and SMPIH contained peptides which were able to act as hydrogen donors and could react with the radicals, thereby converting them to more stable products and terminate the radical chain reaction.

Ferric reducing antioxidant power (FRAP)

The reducing power of SGH and SMPIH with different DHs is given in Fig. 2c. At the same DHs, SGH showed higher activity than SMPIH ($P < 0.05$). The reducing power of hydrolysates increased when DH increased ($P < 0.05$). Nevertheless, no differences in activity were observed between hydrolysate with 10 and 20 % DH, regardless of the substrate. Also there were no differences between SMPIH with DHs of 20, 30 and 40 % ($P > 0.05$). The greater reducing power, especially at high DH, indicated that hydrolysates could donate the electron to the free radical, leading to the prevention or retardation of propagation (Khantaphant and Benjakul 2008). The result of the study was in agreement with gelatin hydrolysate from brownstripe red snapper skin (Khantaphant and Benjakul 2008). However, Klompong et al. (2007) reported that reducing power of hydrolysates from yellow stripe trevally prepared using Alcalase decreased when DH increased from 5 to 25 %.

Metal chelating activity

Metal chelating activity of both hydrolysates produced using CPE are given in Fig. 2d. No changes in chelating activity of SMPIH were found with increasing DH ($P > 0.05$). Metal chelating activity decreased with increasing DH up to 30 % ($P < 0.05$). Thereafter, the activity increased when DH increased from 30 to 50 % and the highest activity was observed in SGH with the DH of 50 % ($P < 0.05$). Dong et al. (2008) reported that peptide cleavages led to an enhanced Fe^{2+} binding capacity due to an increased concentration of carboxylic groups and amino groups in branches of the acidic and basic amino acids. SGH showed higher activity than those from SMPIH at any designated DH ($P < 0.05$). The result of the study indicated that peptides in both hydrolysates could chelate the pro-oxidants and could act as the secondary antioxidant, leading to the retardation of lipid oxidation. Senphan and Benjakul (2014) reported that no changes in metal

chelating activity of hydrolysates from seabass skin prepared using protease from Pacific white shrimp hepatopancreas and Alcalase were obtained as DH increased from 10 to 40 %.

Based on different assays, antioxidant activity of hydrolysate from both substrates with the DH of 50 % showed the higher antioxidative activities than the others. Therefore, the hydrolysates with 50 % DH were selected for further study.

Antioxidative activity of hydrolysates in a lecithin liposome model system

Generally, the oxidation in liposome system, especially for the control (without squid protein hydrolysate) increased as the incubation time increased ($P < 0.05$). The sharp increase was obtained after 6 h of incubation for the control. However, the incorporation of SGH and SMPIH (500, 1000 and 2000 mg/L) affected the oxidation of the lecithin liposome system differently as observed by varying conjugated dienes (CD) (Fig. 3a) and TBARS (Fig. 3b). The marked increases in CD of samples added with squid protein hydrolysates and trolox occurred after 12 h ($P < 0.05$). Thereafter, a decrease in CD was found during 18–36 h. The rate of changes depended on the hydrolysates

Fig. 3 Changes in conjugated diene (a) and thiobarbituric acid reactive substances (TBARS) value (b) in the lecithin liposome system in the presence of SGH and SMPIH at different levels. SGH, hydrolysate from squid skin gelatin. SMPIH, hydrolysate from squid mantle protein isolate. Bars represent the standard deviation ($n = 3$)

and their concentration. CD formation occurs during the early stages of lipid oxidation (Frankel et al. 1997). The decrease or reaching a plateau of CD was generally accompanied by an increase in TBARS (Senphan and Benjakul 2014). Amongst the systems containing hydrolysates, those containing 1000 ppm SMPIH showed the longer delay time for oxidation. Also, no marked difference in CD between the systems added with SGH and SMPIH at 1000 ppm during the incubation of 60 h. The results indicated that these hydrolysates could retard the formation of CD. Similar pattern of CD formation was observed in liposome containing hydrolysates from round scad (*Decapterus maruadsi*) muscle (Thiansilakul et al. 2007) and protein hydrolysates from the muscle of brownstripe red snapper (*Lutjanus vitta*) (Khantaphant et al. 2011).

TBARS in most samples increased within the first 12–36 h ($P < 0.05$). The rate of the increase varied with the hydrolysate and concentration used. After the incubation of 36 h, a decrease or plateau was observed, suggesting the loss of the secondary lipid oxidation products (Stahnke 1995). During 48–60 h, the system containing 2000 ppm SGH showed the lowest TBARS value. The antioxidative activity of SGH and SMPIH could be attributed to the interaction of the polar portion of protein hydrolysates with the polar domains of lecithin liposomes, thus facilitating the electron transfer from peptide to the radicals. Their chelating abilities on Cu^{2+} , which is known as the catalyst for lipid peroxidation, could retard the oxidation of the system (Thiansilakul et al. 2007).

Changes in antioxidative activity of hydrolysates in gastrointestinal tract model system (GIMS)

Antioxidative activity of SGH and SMPIH produced by CPE with 50 % DH in gastrointestinal tract model system was monitored by measurement of ABTS radical

Fig. 4 Antioxidative activities of SGH and SMPIH with 50 % DH in gastrointestinal tract model system. SGH, hydrolysate from squid skin gelatin. SMPIH, hydrolysate from squid mantle protein isolate. Bars represent the standard deviation ($n = 3$)

scavenging activity (Fig. 4). The ABTS radical scavenging activity of SGH and SMPIH decreased slightly under the stomach condition ($P < 0.05$) and reached a minimum value of 253.77 and 84.22 $\mu\text{mol TE/g}$ sample after 1 h digestion by pepsin, respectively. ABTS radical scavenging activity of SGH was higher than that of SMPIH as treated by pepsin within 1 h ($P < 0.05$). Peptides might be further cleaved by pepsin under acidic condition, leading to the loss in ABTS radical scavenging activity of some peptides. Exposure of hydrophobic domains of peptide after pepsin treatment made them less likely to react with water-soluble ABTS radical. Subsequently the activity of both hydrolysates drastically increased under the duodenal condition ($P < 0.05$) and no changes in ABTS radical scavenging activity were observed during 80–240 min of incubation time ($P > 0.05$). The increase in hydrophilicity of the hydrolysates after digestion by pancreatin favors their trapping with ABTS radicals (You et al. 2010). In simulated gastrointestinal tract, the increases in antioxidative activities have been reported for hydrolysates from skin of seabass (*Lates calcarifer*) (Senphan and Benjakul 2014), the hydrolysate from brownstripe red snapper (*Lutjanus vitta*) muscle (Khantaphant et al. 2011) and protein hydrolysates prepared from ornate threadfin bream (*Nemipterus hexodon*) muscle (Nalinanon et al. 2011). The result demonstrated that during the simulated gastrointestinal digestion, some peptides with higher antioxidant activities were produced from SGH and SMPIH.

Molecular weight distribution

Elution profiles of both SGH and SMPIH with 50 % DH after being subjected to Sephadex G-25 gel filtration chromatography are depicted in Fig. 5. A_{220} was used to monitor peptides in SGH (Fig. 5a) and SMPIH (Fig. 5b). SGH and SMPIH had the peptides with wide range of MW. For SGH, the fraction containing peptides with a MW of 283 Da showed the highest ABTS radical-scavenging activity, followed by the peptide with MW of 1185 Da with the activity of 374.6 and 371.4 $\mu\text{mol TE/g}$ protein, respectively. Antioxidative peptides of gelatin hydrolysate from tilapia skin prepared using properase E and multifect neutral contained antioxidative peptides with the MW of 317.33 and 645.21 Da (Zhang et al. 2012). Amongst the fractions of SMPIH, the fractions containing peptides with MW of 1381 and 405 Da with the activity of 269.28 and 259.14 $\mu\text{mol TE/g}$ protein had the high ABTS radical scavenging activities. Nalinanon et al. (2011) reported that protein hydrolysates prepared from ornate threadfin bream (*Nemipterus hexodon*) muscle, using skipjack tuna pepsin (20 % DH) contained antioxidative peptides with a MW of approximately 1.3 kDa. The result suggested that both hydrolysates most likely contained the certain peptides that

Fig. 5 Separation of antioxidant peptides in the hydrolysate from squid skin gelatin (a) and squid mantle protein isolate (b) by Sephadex G-25 column

could act as electron donors or could scavenge free radicals to terminate the radical chain reaction.

Conclusion

Antioxidant activity of hydrolysate from splendid squid skin gelatin and squid mantle protein isolate prepared using crude proteases extract of hepatopancreas from Pacific white shrimp varied with DH. Squid mantle protein isolate was more preferable for hydrolysis by CPE as indicated by the higher DH. Antioxidative activity of both hydrolysates was enhanced in the gastrointestinal tract model system, particularly duodenal condition. The peptides with MW of 283 Da in SGH and 1185 Da in SMPH were the potential antioxidant peptides in the hydrolysates. Therefore, both squid gelatin and mantle protein isolate could be used to produce antioxidative peptides and are potential source of natural antioxidants to prevent lipid oxidation.

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