

Preventive effect of Nile tilapia hydrolysate against oxidative damage of HepG2 cells and DNA mediated by H₂O₂ and AAPH

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Abstract Antioxidant activities of protein hydrolysate prepared from Nile tilapia protein isolate using Alcalase (HA), Alcalase followed by papain (HAPa) and their Sephadex G-25 fractions (FHA and FHAPa) were investigated in both chemical and cellular based models. Amongst all samples, FHAPa showed the highest chemical antioxidant activities, however it had no metal chelation activity. Cellular antioxidant ability of HA, HAPa and their fractions against H₂O₂ and AAPH induced oxidative damage of HepG2 cell and DNA were tested. When cells were pretreated with all hydrolysates or fractions at different concentrations (0.5–2 mg/mL) in the absence and presence of 50 μM Trolox, cell viability was in the range of 91.10–111.40 %. However, no difference in cell viability was observed among samples having various concentrations ($P>0.05$). Cell reactive oxygen species (ROS) generation as mediated by H₂O₂ and AAPH decreased with treatment of hydrolysates or their fractions, especially in combination with 50 μM Trolox. FHAPa effectively inhibited H₂O₂ and peroxy radical induced DNA scission in a dose dependent manner. Therefore, Nile tilapia protein hydrolysates could serve as a functional food ingredient.

Keywords Nile tilapia protein hydrolysate · Antioxidant activity · HepG2 cell · DNA damage · H₂O₂

Introduction

Generally, oxygen consumption inherent in cell growth leads to the generation of a series of reactive oxygen species (ROS). The formations of ROS including superoxide radical (O₂^{•-}), hydroxyl radical (OH[•]), hydrogen peroxide (H₂O₂), etc. are inevitable consequence in aerobic organisms during respiration. Some of them are essential for biological functions involved in cell mechanism, proliferation, apoptosis and signal transduction (Owuor and Kong 2002). Nevertheless, the excessive production of ROS can attack biological macromolecules such as protein, lipid and DNA, etc., leading to cell or tissue injury. Under normal condition, ROS are effectively eliminated by the antioxidant defense system, such as antioxidant enzymatic and non-enzymatic factors in human body (Kim et al. 2007). However, under pathological conditions, the imbalance between the generation and the elimination of ROS was contributed to oxidative stress on cell, resulting in many disorders. Oxidative damage has been associated with numerous chronic diseases including diabetes, cancer, neurodegenerative and coronary heart disease (Chai et al. 2013).

In recent years, a large number of studies has been conducted to find potent radical scavengers to protect against oxidative stress (García-Nebot et al. 2014; Himaya et al. 2012). Numerous antioxidant compounds, particularly fish peptide, have been widely studied. Potent antioxidant peptides have been isolated from protein hydrolysates from the muscle of yellow stripe trevally (*Selaroides leptolepis*) (Klompong et al. 2009), cod (*Gadus morhua*) (Halldorsdottir et al. 2014) and spotless smooth hound (*Mustelus griseus*) (Wang et al. 2014), etc. Protein, peptides and free amino acids released during enzymatic hydrolysis are capable of modulating

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specific biological function. Different modes of actions, namely the donation of electrons/hydrogen atom, direct scavenging of free radicals and sequestration of pro-oxidative metal-ions, have been observed in various protein hydrolysates (Samaranayaka and Li-Chan 2011). The activity is closely related to the amino acid composition and sequence, size and configuration of peptides (Samaranayaka and Li-Chan 2011). Additionally, protein hydrolysates possessing antioxidant activity have been governed by the type of proteases used for step-wise hydrolysis. Recently, the application of Alcalase in combination with papain for hydrolysis of Nile tilapia protein isolate (PI) gave a hydrolysate with antioxidant properties and reduced bitterness (Yarnpakdee et al. 2014). Although protein hydrolysates derived from PI have been widely reported to exhibit in vitro antioxidant activity, a little information regarding the antioxidant ability on cell-based models, especially those induced by different types of free radicals, have been reported. Thus, the objective of this study was to investigate antioxidant properties of Nile tilapia protein hydrolysates prepared using Alcalase (HA), Alcalase/papain (HAPa) and their Sephadex G-25 fractions in both chemical and cellular antioxidant based models.

Material and methods

Chemical/enzymes

Alcalase 2.4 L (E.C. 3.4.21.62) (2.4 AU/g) was provided by Novozymes (Bagsvaerd, Denmark). Papain (E.C. 3.4.22.2) (≥ 3 AU/mg), 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,4,6-trinitrobenzenesulfonic acid (TNBS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), 2,2'-azobis (2-methylpropionamide) (AAPH) and hydrogen peroxide (H_2O_2) were purchased from Sigma (St. Louis, MO, USA). Sephadex G-25 was procured from GE-Healthcare (Uppsala, Sweden). The HepG-2 human hepatoma cell line was obtained from American Type Culture Collection (ATCC 8065, Rockville, MD, USA). Cell culture medium and all the other materials required for culturing were obtained from Invitrogen (Carlsbad, CA, USA). Plasmid DNA (pUC 18) was purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). All chemicals were of analytical grade.

Fish sample collection

Fresh Nile tilapia (*Oreochromis niloticus*) with a weight of 0.8–1.0 kg/fish were purchased from a local market in Hat Yai, Songkhla, Thailand. Fish were stored in ice and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon arrival, fish flesh was separated manually and minced using a Moulinex AY46 blender (Group SEB, Lyon, France) in a walk-in-cold room

(4 °C). The mince was placed in polyethylene bags and stored in ice until used.

Preparation of Nile tilapia protein isolate (PI)

PI from pre-washed mince was prepared following the method of Yarnpakdee et al. (2014). Washed mince was homogenised with five volumes of cold distilled water (2–4 °C) using an IKA Labortechnik homogeniser (Selangor, Malaysia) at a speed of 11,000 rpm for 1 min. The homogenate was adjusted to pH 11 and placed on ice for 60 min with a continuous stirring. The mixture was then centrifuged at $5,000\times g$ for 10 min at 4 °C using an Avianti J-E centrifuge (Beckman Coulter, Inc., Fullerton, CA, USA). The alkaline soluble fraction obtained, referred to as 'PI solution', was used as substrate for hydrolysis.

Production of Nile tilapia protein hydrolysates

PI solution was mixed with distilled water to obtain a final protein concentration of 2 % (w/v) as determined by the Biuret method (Robinson and Hogden 1940). The hydrolysis was conducted for 1 h using Alcalase at a level of 5.54 % (w/w) (pH 8.0, 50 °C) to obtain the degree of hydrolysis (DH) of 40 % as described by Benjakul and Morrissey (1997). After 1 h of hydrolysis, the reaction was terminated by placing the mixture in boiling water for 10 min. One half of the resulting hydrolysate was subjected to centrifugation at $2,000\times g$ at 4 °C for 10 min. The supernatant was lyophilised using a Scanvac Model Coolsafe 55 freeze dryer (Coolsafe, Lyngø, Denmark). Another portion was further hydrolysed using papain at the same amount used in the first step. Reaction was conducted for 1 h at pH 7.0 and 40 °C and the mixture was submerged in boiling water for 10 min to terminate the enzyme. A DH of 53 % was obtained. Thereafter, the mixture was subjected to centrifugation and lyophilisation as mentioned previously. The resulting hydrolysates using Alcalase and Alcalase/papain were named as 'HA' and 'HAPa', respectively.

To fractionate both hydrolysates, HA and HAPa were further separated using Sephadex G-25 gel filtration chromatography as described by Yarnpakdee et al. (2014). Sample (50 mg/mL) was loaded onto a SephadexG-25 column (1.6 \times 63.5 cm) and the elution was performed using distilled water at a flow rate of 0.5 mL/min. The 3 mL-fractions were collected and their absorbance was monitored at 220 and 280 nm. All fractions were determined for their ABTS radical scavenging activity. Active fractions with the highest ABTS radical scavenging activity were pooled and lyophilised. Pooled fractions obtained from HA and HAPa were referred to as 'FHA' and 'FHAPa', respectively. All samples were kept in -20 °C until further analysis.

Chemical antioxidant activities

Seven chemically based antioxidant assays were used for measurement of in vitro antioxidant properties of all hydrolysates and their fractions. The DPPH and ABTS radical scavenging activities as well as metal chelating activity were measured as per the method of Binsan et al. (2008). Ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain (1996), whilst hydrogen peroxide (H₂O₂) and singlet oxygen scavenging activities were assayed according to the method of Kittiphattanabawon et al. (2012). For all assays, the sample at a concentration of 10 mg/mL was used. Spectrophotometric measurements were carried out using a UV-160 spectrophotometer (Shimadzu, Kyoto, Japan).

The oxygen radical absorbance capacity (ORAC) assay was performed according to Halldorsdottir et al. (2014). The samples with the concentration range of 0.3–0.7 mg/mL were used in the assay and the measurement of area under the fluorescence decay curve was conducted using POLARstar Optima microplate reader (BMG Labtech, Offenburg, Germany).

Cellular antioxidant activities

Cell culture

HepG2 cells (ATCC 8065, American Type Culture Collection, Rockville, MD, USA) were maintained in Minimum Essential α (MEM α) and supplemented with 10 % (v/v) heat inactivated fetal bovine serum (FBS), penicillin (50 units/mL) and streptomycin (50 μ g/mL). Cells were incubated at 37 °C in a fully humidified environment under 5 % CO₂ and HepG2 cells at passage 80–100 were used for the experiments. Cell culture medium was replaced every other day, and cells were subcultured at 3–5 days intervals before reaching 90 % confluence.

Cytotoxicity effect of H₂O₂ or AAPH on HepG2 cells

Cells were seeded at 6.0×10^4 cells per well on black 96-well plates (BD Falcon™, Franklin Lakes, NJ, USA) and incubated in a humidified incubator containing 5 % CO₂ at 37 °C, for 24 h. Then, the cells were treated with H₂O₂ or AAPH at various concentrations (200–1000 μ M; 100 μ L) and incubated for another 24 h. After incubation, the culture medium was removed and the cell viability was evaluated by exposure to 100 μ L of 10 % PrestoBlue® (Invitrogen) solution in MEM α at 37 °C in dark for 1 h. Thereafter, the fluorescence was measured at excitation wavelength (λ_{ex}) of 570 nm and emission wavelength (λ_{em}) of 610 nm using a POLARstar OPTIMA microplate reader. Control cells were prepared in the same manner without oxidative stressors (H₂O₂ or AAPH)

addition. The result was expressed as percentage of viable cells compared to the control culture as follows:

$$\text{Cell viability (\%)} = \left(\frac{\text{Mean fluorescence of treated cells}}{\text{Mean fluorescence of control cells}} \right) \times 100$$

The oxidative stressors at a level rendered a ~50 % cell viability were selected for further studies

Protective effect of HA, HAPa and their Sephadex G-25 fractions against oxidative stress on HepG2 cell

Cell viability determination Cells were placed in a 96-well plate (6.0×10^4 cells/well) and incubated in a humidified incubator containing 5 % CO₂ at 37 °C, for 24 h. Then, cells were pretreated with various hydrolysates and fractions (HA, FHA, HAPa and FHAPa) at different concentrations (0.5, 1 and 2 mg/mL; 100 μ L) in the presence and absence of 50 μ M Trolox and further incubated for 24 h. In the first set, the cells were subjected to determination of the viability using Prestoblu[®] assay as described above. For the second set, the culture medium was removed and replaced with a fresh medium containing 100 μ L of 800 μ M H₂O₂ or 800 μ M AAPH to give the oxidative stress. Subsequently, the cells were further incubated at 37 °C in dark for another 24 h. The control cells were cultured without any treatment. A commercial antioxidant, 50 μ M of Trolox, was used as positive control. After incubation, cell viability was assessed and the results were expressed as a percentage of viable cells, compared to the control culture (without any treatment).

Cell ROS determination by DCFH-DA Intracellular formation of ROS was assessed using an oxidation sensitive dye DCFH-DA as the substrate according to the method of Halldorsdottir et al. (2014) with some modification. HepG2 cells (6×10^4 cells/well) seeded in black 96-well plates were loaded with 100 μ L DCFH-DA (1 μ M in Hank's Balanced Salt Solution, HBSS) and incubated in the dark for 30 min. Cells were then treated with various hydrolysates and fractions at different concentrations (0.5, 1 and 2 mg/mL; 100 μ L) in the presence and absence 50 μ M Trolox and incubated for another 1 h. After removal of the test compounds, 100 μ L of 800 μ M H₂O₂ or 800 μ M AAPH in HBSS were added. The formation of 2',7'-dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of ROS was read every 10 min for 90 min at λ_{ex} of 485 nm and the λ_{em} of 535 nm using a POLARstar OPTIMA microplate reader. Negative control wells consisted of cells connect to the DCFH-DA probe and oxidative stressors (H₂O₂ or AAPH). The % cellular ROS was expressed as the percentage of relative fluorescence intensity of negative control cell.

Protective effect on DNA oxidation induced by H₂O₂ and AAPH

The ability of HA, HAPa and their fractions to protect DNA damage from ROS was assessed as per the method of Kittiphattanabawon et al. (2013) with a minor modification. A reaction was conducted in an Eppendorf tube at a total volume of 10 μ L. Supercoiled plasmid DNA (pUC 18) (0.125 μ g/ μ L, 4 μ L) dissolved in 10 mM Tris–HCl containing 1 mM EDTA (pH 8.0) was mixed with 2 μ L of different hydrolysates or fractions to obtain final concentrations of 0.5, 1.0 and 2.0 mg/mL. To initiate the oxidation reaction, 4 mL of 30 mM AAPH or 30 mM H₂O₂ were added. The mixture was incubated at 37 °C for 1 h in the dark. The controls were prepared in the same manner by using distilled water instead of oxidants. After incubation, 2 mL of the loading dye (0.25 % bromophenol blue, 50 % glycerol) were added to the reaction mixture. The mixture (12 mL) was loaded onto 1 % agarose gel, and the DNA bands were stained with ethidium bromide. Electrophoresis was conducted at 100 V for 50 min using a horizontal gel electrophoresis system (Sub cell[®] model 192 cell, Biorad, Hercules, CA, USA) equipped with PowerPac[™] basic power supply (Biorad, Hercules, CA, USA). The DNA bands were visualised under transillumination of UV light using Gel Doc[™] 2000 Gel Documentation System (Biorad, Hercules, CA, USA). The retention of supercoiled DNA strand (%) was calculated using following equation:

$$\text{Retention (\%)} = \left(\frac{\text{Intensity of supercoil DNA of sample}}{\text{Intensity of supercoil DNA of control}} \right) \times 100$$

Statistical analysis

Experiments were run in triplicate using three lots of samples. Data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range tests (Steel and Torrie 1980). Statistical analysis was performed using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

Results and discussions

Chemical antioxidant activity of HA, HAPa and their Sephadex G-25 fractions

Chemically based antioxidant activities of different hydrolysates and their fractions (HA, FHA, HAPa and FHAPa) are shown in Table 1.

DPPH and ABTS radical scavenging activities

DPPH scavenging activities ranged from 4.59 to 15.89 μ mol TE/g solid, whilst ABTS radical scavenging activity varied from 106.33 to 322.79 μ mol TE/g solid. In general, crude protein hydrolysates (HA or HAPa) had lower DPPH and ABTS radical scavenging activities in comparison with their corresponding fractions (FHA or FHAPa). The differences in scavenging capacity for DPPH and ABTS radicals were likely related with the different peptides in each sample. This might be governed by the specificity of the enzyme hydrolysis conditions and degree of hydrolysis when different processes were used. Peptides containing more hydrophobic side chain were found to be more accessible by DPPH[•] (Zhu et al. 2008). The shorter peptides, including di- or tri- peptides, that are more hydrophilic were found to readily react with water soluble ABTS^{•+} but not with lipid soluble DPPH[•] (Zhu et al. 2008). The activities of both HAPa and FHAPa were much higher than those of HA and FHA for both assay tested. The result suggested that the application of two-step hydrolysis using Alcalase and papain might liberate peptides with higher antioxidant activity. Amongst all samples, FHAPa showed the highest DPPH and ABTS radical scavenging activities ($P < 0.05$). After gel filtration, peptides with high radical scavenging activity were fractionated and concentrated.

ORAC

All hydrolysates and fractions showed ORAC in the ranges of 369.17–378.16 μ mol TE/ g solid. After fractionation, higher ORAC was obtained. It was noted that FHA had higher ORAC than FHAPa ($P < 0.05$). FHA might contain a larger proportion of peptides possessing a higher capacity in scavenging peroxy radical than FHAPa. Peroxy radicals, which are formed by direct reaction of triplet oxygen with alkyl radicals during the propagation step in fatty acid oxidation, produce hydroperoxides by abstracting hydrogen from other molecules (Chandrasekara and Shahidi 2011). Samaranayaka et al. (2010) reported that ORAC of both crude and fractionated Pacific hake (*Merluccius productus*) protein hydrolysate was found in the range of 225–330 μ mol TE/ g sample. Theodore et al. (2008) also reported that ORAC of protein hydrolysate prepared from catfish isolates ranged from 2 to 4 μ mol TE/g protein. Peptides in Nile tilapia protein hydrolysate could therefore serve as the powerful antioxidant for donating a hydrogen atom to peroxy radical.

FRAP

Table 1 shows FRAP of various hydrolysates and their fractions. A higher FRAP was noticeable in HAPa (13.56 μ mol TE/g solid), compared with that of HA (10.13 μ mol TE/g

Table 1 Antioxidant activity of Nile tilapia protein hydrolysates and Sephadex G-25 fractions prepared using different enzymes

Chemical antioxidant assays [†]	HA	FHA	HAPa	FHAPa
DPPH radical scavenging ($\mu\text{mol TE/g solid}$)	4.59 \pm 0.42 ^{*, d}	9.05 \pm 0.39 ^c	11.46 \pm 0.41 ^b	15.89 \pm 0.17 ^a
ABTS radical scavenging ($\mu\text{mol TE/g solid}$)	106.33 \pm 3.35 ^d	268.93 \pm 7.41 ^b	147.51 \pm 8.29 ^c	322.79 \pm 4.44 ^a
ORAC ($\mu\text{mol TE/g solid}$)	369.17 \pm 26.51 ^c	949.07 \pm 38.98 ^a	378.16 \pm 17.64 ^c	789.20 \pm 24.35 ^b
FRAP ($\mu\text{mol TE/g solid}$)	10.13 \pm 0.41 ^c	10.78 \pm 2.70 ^c	13.56 \pm 0.02 ^b	16.84 \pm 0.10 ^a
Metal chelating activity ($\mu\text{mol EE/g solid}$)	1.36 \pm 0.29 ^b	ND ^{**}	5.70 \pm 0.53 ^a	ND
Singlet oxygen scavenging activity ($\mu\text{mol TE/g solid}$)	686.02 \pm 1.50 ^d	1413.88 \pm 42.48 ^a	779.34 \pm 86.86 ^c	1642.72 \pm 61.59 ^a
H ₂ O ₂ scavenging activity ($\mu\text{mol TE/g solid}$)	174.60 \pm 17.57 ^d	350.10 \pm 2.29 ^c	507.93 \pm 10.27 ^b	618.48 \pm 5.14 ^a

[†] Values are given as mean \pm SD

* Different superscripts in the same row indicate the significant differences ($P < 0.05$)

** Not detectable

HA Hydrolysate prepared using Alcalase

FHA Sephadex G-25 fraction of HA

HAPa Hydrolysate prepared using Alcalase, followed by papain

FHAPa Sephadex G-25 fraction of HAPa

solid) ($P < 0.05$). FHAPa (16.84 $\mu\text{mol TE/g solid}$) also showed higher FRAP than FHA (10.78 $\mu\text{mol TE/g solid}$). Further cleavage of peptides in two-step hydrolysis process by papain led to an enhanced reducing ability, plausibly due to the increases in the exposure of some amino acids, such as leucine, lysine, methionine or isoleucine at terminal of released peptides. You et al. (2011) reported that loach peptides having a stronger reducing power contained histidine, methionine, tryptophan, lysine, and tyrosine in their sequences. Rapeseed fraction with the strongest reducing power had abundant hydrophobic amino acids, which were considered to contribute to enhancing the reducing power of peptides (Zhang et al. 2008). After fractionation, FRAP of HAPa sample increased as evidenced by higher FRAP for FHAPa. However, there was no difference in FRAP between HA and FHA ($P > 0.05$).

Metal chelating activity

The metal chelating activity of HA and HAPa was 1.36 and 5.70 $\mu\text{mol TE/g solid}$, respectively, whilst FHA and FHAPa had no metal chelating activity. The result indicated that the further hydrolysis using papain resulted in enhanced metal chelating capacity. This might be attributed to more exposure of effective sites capable of chelating ferrous ion. Nevertheless, some peptides or free amino acids possessing metal chelating activity were possibly removed during fractionation. Carboxyl and amino groups of acidic (Glx, Asx) and basic (Lys, His, Arg) amino acids were thought to play an important role in chelating metal ions (Saiga et al., 2003). In particular, His is known to be ion chelator (Chen et al., 1996). Ferrous ion (Fe^{2+}) is a pro-oxidant and can interact with hydrogen peroxide in a Fenton reaction to produce reactive oxygen species and hydroxyl (OH^\cdot) free radicals, leading to

the initiation and/or acceleration of lipid oxidation. Yarnpakdee et al. (2014) demonstrated that the peptide from Nile tilapia protein hydrolysates with MW of 513 Da exhibited the strongest ABTS radical scavenging activity, whereas those with MW 1484 Da possessed the highest metal chelating activity. Thus, varying peptides in Nile tilapia protein hydrolysates, especially in preparation using different enzymes, showed different metal chelating activity.

Singlet oxygen scavenging activity

The greatest singlet oxygen radical scavenging activity was observed for FHAPa, followed by FHA, HAPa and HA, respectively. The result demonstrated that HAPa and its fraction were stronger in scavenging singlet oxygen than did HA. Active peptides possessing singlet oxygen scavenging activity might be more generated during the second hydrolysis mediated by papain. Singlet oxygen, which is a highly reactive, electrophilic and non-radical molecule, can be formed by the reaction between photosensitisers and triplet oxygen in the presence of light (Kittiphattanabawon et al. 2012). Singlet oxygen can directly react with electron-rich double bonds of unsaturated fatty acids without the formation of free-radical intermediates (Choe and Min 2005). This was correlated well with Kittiphattanabawon et al. (2012) who reported that peptides with the shorter chain length from blacktip shark skin were able to trap or bind singlet oxygen to a higher extent. In general, the higher activity was noticeable in Sephadex G-25 fractions, compared with that found in hydrolysates. The result suggested that peptides with singlet oxygen scavenging activity were more concentrated in the fraction obtained.

H₂O₂ scavenging activity

H₂O₂ scavenging activity of all hydrolysates and their fractions is presented in Table 1. H₂O₂ is a reactive non radical, which can permeate biological membranes and be converted to more reactive species such as hydroxyl radical and singlet oxygen (Choe and Min 2005). Hydrogen peroxide is the precursor for the generation of hydroxyl radical, which is a strong initiator of lipid oxidation (Choe and Min 2005). HAPa exhibited higher ability for scavenging H₂O₂ than HA ($P<0.05$). This might be governed by the differences in peptide produced by enzyme used. Guo et al. (2009) reported that dipeptide derived from royal jelly protein by Protease N containing tyrosine residue at its C-terminus was associated with strong H₂O₂ scavenging activity. Both fractions, FHA and FHAPa, had a higher H₂O₂ scavenging activity than the corresponding hydrolysates ($P<0.05$). Similar results were observed to those of DPPH and ABTS radical scavenging activities.

From the above measurements, HAPa showed higher antioxidant activities than those of HA. This result indicated that the second step hydrolysis could release bioactive peptides from the hydrolysates obtained from the first step. Changes in size, amount, exposure of the terminal amino groups of the products obtained, and composition of free amino acids or small peptides determine antioxidative activity (Yarnpakdee et al. 2014). The small peptides generally showed a higher antioxidant activity (Qian et al. 2008). In addition, their antioxidant activities, except for metal chelating activity, were enhanced after fractionation. Therefore, HA and HAPa contained various antioxidant peptides with various modes of action. The partial purification using gel filtration could be an effective means to concentrate antioxidant peptides.

Cellular antioxidant activity of hydrolysates and Sephadex G-25 fractions of HepG2 cell

Effect of oxidative stressors on cytotoxicity of HepG2 cell

Effect of H₂O₂ and AAPH on HepG2 cell viability is depicted in Fig. 1. When the cells were exposed to oxidative stressors, the viability of cells was decreased ($P<0.05$). With increasing levels of H₂O₂ or AAPH, the cell viability was significantly lowered. Cell viability was 49.1 and 14.2 % after exposure to 1000 μ M H₂O₂ and 1000 μ M AAPH, respectively. The result suggested that HepG2 cells were sensitive to H₂O₂ and peroxy radical, especially when the level was higher than 200 μ M ($P<0.05$). In general, ROS can cause oxidative stress and damage of biomolecules in the cell, leading to cell death and serious chronic diseases (Halldorsdottir et al. 2014).

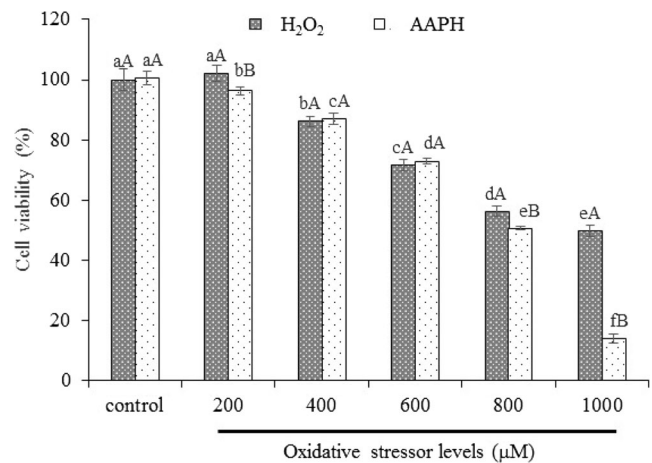


Fig. 1 Dose-dependent toxic effects of H₂O₂ and AAPH on HepG2 cell viability. HepG2 cells were exposed to oxidative stressors with different concentrations (200–1000 μ M) for 24 h. Bars represent the standard deviation ($n=2$). Different letters within the same oxidative stressors indicate the significant differences ($P<0.05$). Different capital letters within the same concentration indicate the significant difference ($P<0.05$)

Zhang et al. (2012) reported that the use of H₂O₂ (50–200 μ M) exhibited a dose dependent decrease in PC12 cell viability. Wiriyaphan et al. (2012) found that HepG2 cell had a 44 % of cell death when exposed to tert-butyl hydroperoxide at a concentration of 500 μ M. Elisia and Kitts (2008) noted that the exposure of Caco2 cell to 15 mM AAPH led to cell apoptosis. However, no difference in viability between cells exposed to H₂O₂ and AAPH at levels below 600 μ M was noticeable ($P>0.05$). A damaging effect was found when HepG2 cells were challenged with AAPH than H₂O₂ at levels of 800–1000 μ M ($P<0.05$). The result indicated that peroxy radical acted as a stronger oxidative stressor in HepG2 cell, compared with H₂O₂. It is well known that H₂O₂ itself is not highly reactive, however it forms a highly reactive hydroxyl radical (OH[•]) in the presence of transition metal ions, such as Fe²⁺ and Cu⁺. Wijeratne et al. (2005) reported that the loss in viability of Caco2 cells exposed to 10 mM H₂O₂ was caused by the changes in cell membrane permeability. This could lead to the increased entry of toxins to cells. Although hydroxyl radicals have been proven as highly damaging free radical in cells, they have short half-life (10⁻⁹ s) (Kim et al. 2011). Since peroxy radicals have a long half-life (10⁻² s), they show a greater affinity to diffuse into cells. This leads to more macromolecular damage. Poli et al. (2004) reported that peroxy radical induced lipid peroxidation contributed to the adverse changes of biomembrane composition. Thus, it was postulated that HepG2 cells were more highly sensitive to peroxy radical than H₂O₂. Based on cell viability test, the level of 800 μ M H₂O₂ or 800 μ M AAPH, causing ~50 % cell viability, was selected for study on the role of hydrolysates and fractions in prevention of cells toward oxidative stress.

Protective effect of hydrolysates and fractions on oxidative damage of HepG2 cell

Cell viability The effect of different hydrolysates and fractions in the absence and presence of Trolox on viability of HepG2 cells was assessed as shown in Table 2. The viability of cells treated with various hydrolysates and fractions was slightly higher than that observed for non-treated control cells, regardless of Trolox incorporation. However, no difference

was observed for the concentration range of 0.5–2.0 mg/mL. All samples with concentrations selected for this study were non-cytotoxic on HepG 2 cells (data not shown). The slightly higher cell viability in the presence of hydrolysates or fractions might be attributed to nutrient balance for cell survival. Kim et al. (2007) found that the addition of soy protein hydrolysate on Chinese hamster ovary cells resulted in the increased cell intensity and cell growth promotion. Short chain proteins, peptides or amino acids generated during

Table 2 The viability of HepG2 cell treated with Nile tilapia protein hydrolysates and Sephadex G-25 fractions in the absence and presence of oxidative stressors

Samples	Concentrations (mg/ml)	Cell viability (%)		
		without oxidative stressors	H ₂ O ₂	AAPH
HA	0.5	105.94±0.33 ^{*,†,defghij}	98.02±3.24 ^{cde}	93.05±2.17 ^{bcd}
	1	103.38±3.55 ^{defghi}	94.80±2.82 ^{def}	92.68±0.77 ^{bcdefgh}
	2	102.31±4.91 ^{fghijkl}	92.61±5.00 ^{ef}	89.63±2.95 ^{gh}
FHA	0.5	107.42±1.34 ^{defghij}	93.06±1.31 ^{ef}	89.49±2.83 ^h
	1	111.18±0.99 ^{bcde}	96.93±5.22 ^{def}	93.59±2.25 ^{bcd}
	2	111.04±2.70 ^{bcde}	97.36±4.15 ^{cdef}	94.34±1.39 ^{bc}
T+HA	0.5	100.09±6.05 ^{def}	95.65±1.04 ^{def}	93.42±1.38 ^{bcd}
	1	102.65±3.34 ^{efghij}	95.55±3.46 ^{def}	94.77±1.69 ^b
	2	102.24±3.65 ^{hijkl}	95.38±3.29 ^{def}	89.88±3.21 ^{fgh}
T+FHA	0.5	105.44±2.85 ^{fghijk}	94.74±5.35 ^{def}	90.86±3.57 ^{defgh}
	1	111.58±2.70 ^{bcd}	95.94±4.94 ^{def}	93.15±1.56 ^{bcd}
	2	108.21±6.67 ^{dfgh}	95.18±3.42 ^{def}	93.63±3.06 ^b
HAPa	0.5	107.42±1.27 ^{ijk}	95.84±1.27 ^{def}	89.59±0.89 ^{gh}
	1	107.79±1.61 ^{ghijkl}	97.14±2.86 ^{def}	90.73±2.51 ^{efgh}
	2	104.77±2.40 ^{efghijk}	91.58±3.48 ^f	91.10±1.83 ^{cdefgh}
FHAPa	0.5	108.81±1.73 ^{defg}	99.98±4.17 ^{cde}	91.49±1.29 ^{bcdefgh}
	1	116.03±0.67 ^{ac}	107.64±1.98 ^{ab}	91.74±0.92 ^{bcdefgh}
	2	117.08±1.11 ^a	111.40±1.96 ^a	93.03±1.12 ^{bcd}
T+HAPa	0.5	103.09±0.78 ^{ijkl}	102.97±4.13 ^{bc}	89.91±2.86 ^{cd}
	1	105.98±0.11 ^{ijkl}	96.70±3.39 ^{def}	94.19±1.43 ^{bcd}
	2	108.96±0.20 ^{kl}	96.92±5.21 ^{def}	92.86±1.19 ^{bd}
T+FHAPa	0.5	109.36±0.27 ^{de}	98.06±4.99 ^{cd}	93.00±1.56 ^{bcd}
	1	116.34±1.74 ^{ab}	106.63±2.77 ^{ab}	94.34±1.31 ^{bc}
	2	117.98±0.05 ^a	109.64±1.97 ^a	94.29±1.31 ^{bc}
T		105.43±4.39 ^{fghijk}	93.05±2.00 ^{ef}	91.57±1.28 ^{bcdefgh}
Control	–	100.00±0.00 ^l	100.00±1.97 ^{cd}	100.00±0.95 ^a
Control damage	–		58.08±1.65 ^e	48.14±0.52 ⁱ

† Value are given as mean ± SD

* Different superscripts in the same column indicate the significant differences ($P<0.05$).

HA Hydrolysate prepared using Alcalase

FHA Sephadex G-25 fraction of HA

HAPa Hydrolysate prepared using Alcalase, followed by papain

FHAPa Sephadex G-25 fraction of HAPa

T Trolox

Control Without antioxidant, hydrolysates or fractions

Control damage Including oxidative stressor (without antioxidant, hydrolysates or fractions)

hydrolysis might be required for cell metabolism. Zhang et al. (2012) reported that the protective ability of WPH on cell death was increased with increasing peptide concentration (50–200 µg/ml). Samaranyaka et al. (2010) also reported that fish protein hydrolysate derived from Pacific white hake showed no toxicity to human hepatocellular liver carcinoma cells when treated at concentrations up to 1 mg/mL.

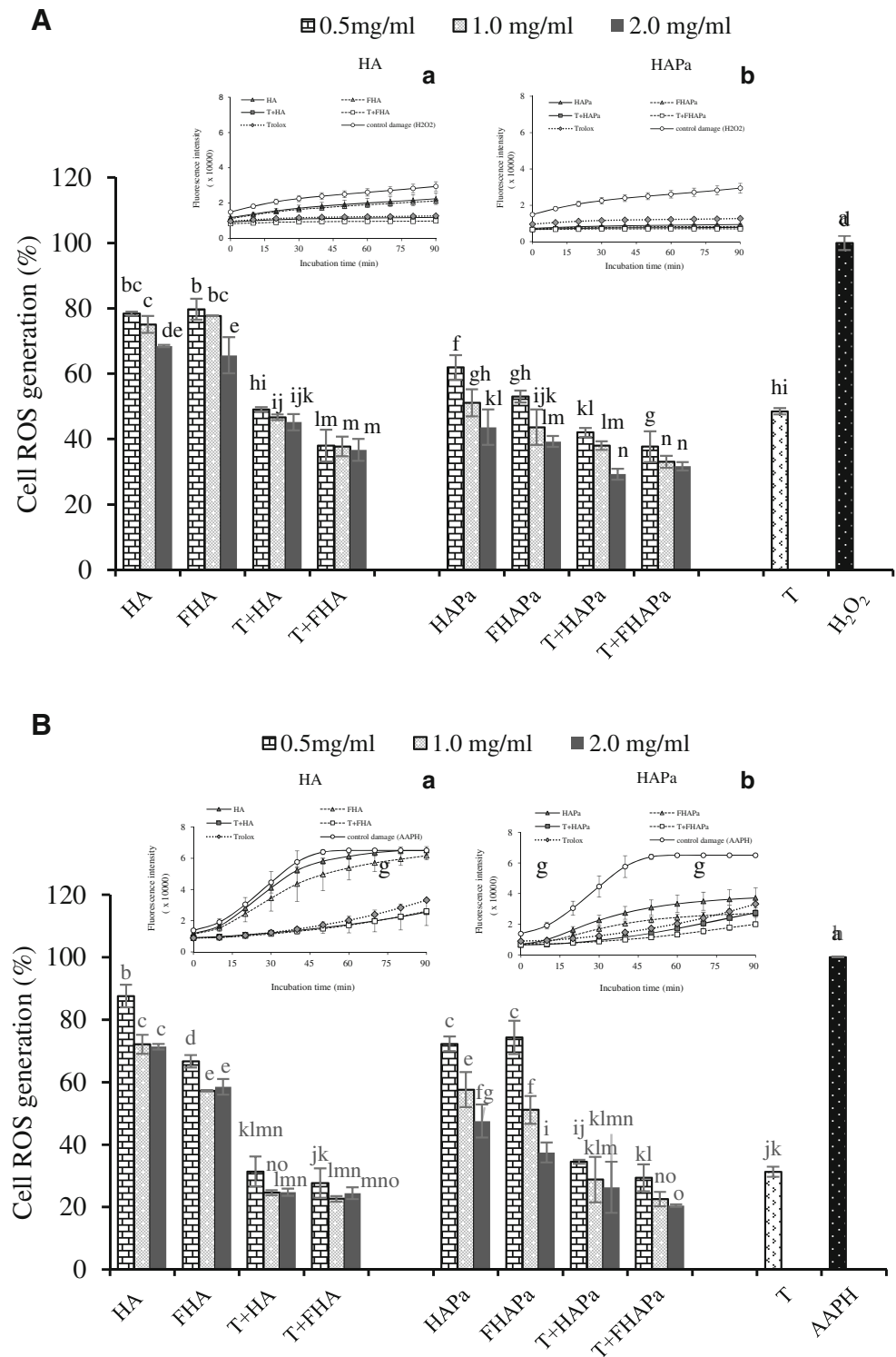
When the cells were subsequently exposed to H₂O₂ and AAPH, the protective ability of hydrolysates and fractions in the absence and presence of Trolox is presented in Table 2. The lowest cell viability (58.1 and 46.2 %) was observed when cells were only treated with H₂O₂ and AAPH, respectively. Pretreated cells in the presence of different hydrolysates or fractions significantly elevated the cell viability to a range of 92.6–111.4 % and 89.6–94.8 % for H₂O₂ and AAPH induced oxidative stress cells, respectively. The result was similar to cells incorporated with Trolox (91.6–93.0 %). This indicated that Nile tilapia protein hydrolysate exerted a protective effect against free-radical induced cytotoxicity of HepG2 cell. The difference in protection mechanism might be related to peptides, which reacted with free radicals in different manners. It was noted that the concentrations tested did not have an impact on cell viability. The concentration used was probably excessive to overcome H₂O₂ or AAPH induced cell death. Furthermore, there was no difference in cell viability, irrespective of Trolox incorporation. Kim et al. (2007) reported that MRC-5 cell viability increased with increasing concentrations of antioxidant peptide from hoki frame protein hydrolysate. The viability of cell exposed to *t*-BHP-induced cytotoxicity increased up to 91.1 % when hydrolysate at a concentration of 55.5 µM was used. Mendis et al. (2005) reported that the cell viability of antioxidant peptide purified from jumbo squid skin treated *t*-BHP induced oxidative stress human lung fibroblast cells was increased in a dose dependent manner (25–100 µM). Chai et al. (2013) reported that the survival rate of neuroblastoma cells increased (67.2–82.3 %) when the cells were treated with lanternfish hydrolysate (0.10 to 1.44 mg/ml) and H₂O₂ (400 µM) for 24 h. Although there was no significant correlation between results obtained from chemical (Table 1) and cell antioxidant based assays, the results confirmed the antioxidant activity of Nile tilapia protein hydrolysate.

Cell ROS generation The cellular ROS scavenging activities of different hydrolysates and their fractions in the absence and presence of Trolox against H₂O₂ and AAPH induced intracellular ROS generation are shown in Fig. 2. In general, the cells were labeled with DCFH-DA fluorescence probe for 30 min. When DCFH-DA diffuses through cell membrane, it is esterified into DCFH inside the cytosol and then is oxidised by intracellular ROS to form fluorescing DCF. As shown in Fig. 2A(a, b) and B(a,b), the fluorescence gradually increased as the time increased up to 90 min. The much higher

fluorescence intensity was noticeable in AAPH challenged system than did H₂O₂ containing counterpart, indicating that peroxy radical was more potent oxidising agent than H₂O₂. Peroxy radical generally has a higher stability, leading to a higher effectiveness toward cells. García-Nebot et al. (2014) reported that H₂O₂ had a poor activity on DCFH oxidation in such a short induction time. When pre-treated cells were incorporated with hydrolysates and fractions, the reduction of fluorescence intensity by either H₂O₂ or AAPH challenged systems was evoked, regardless of Trolox incorporation. The lower fluorescence intensity indicated the suppression of DCFH-DA oxidation, which corresponded to the reduction in intracellular ROS production.

For H₂O₂ induced system, the ROS generation in cell treated with various hydrolysates or their fractions was observed in the range of 31.7–78.4 %, compared to control damage cell (Fig. 3a). It was suggested that Nile tilapia hydrolysate had a cellular radical scavenging effect. The ROS reduction was observed in a dose-dependent manner ($P < 0.05$). In the absence of Trolox, the intracellular ROS production (53.0–78.3 %) was higher than that with Trolox (alone) (48.5 %) at a low dosage used (0.5 mg/mL). Both HAPa and FHAPa exhibited a lower ROS production, compared with HA and FHA. The difference in protective ability between HA and HAPa might be caused by the difference in the peptides. Lima et al. (2006) noted that the antioxidant efficacy on cell depends on both ability to penetrate the cell membrane and the antioxidant capacity in solution. Due to a higher DH obtained in HAPa, low MW peptides plausibly penetrated into lipid bilayer of HepG2 cell more effectively and reacted with free radical inside cells. Wiriyanphan et al. (2012) reported that the higher protective ability of refiner discharge threadfin bream hydrolysate derived by Alcalase against cytotoxicity was associated with lower MW peptides, resulting in a higher cell permeability. The further hydrolysis of HA by papain did not only produce shorter peptides but also enhance the exposure of the functional groups, which favoured antioxidant activity. The result was in accordance with a higher chemical antioxidant activity observed in HAPa comparing with HA (Table 1). Several studies have also documented that protein hydrolysates from fish are a good source of free radical scavenging peptides (Raghavan and Kristinsson 2008). Peptides with higher ratios of hydrophobic amino acids (e.g. tyrosine, tryptophan, phenylalanine, histidine, methionine or cysteine) are considered more effective (Ren et al. 2008). Aromatic residues, such as tryptophan (indolic group) or tyrosine (phenolic group), may stabilise ROS by means of direct electron transfer and resonance (Qian et al. 2008). Je et al. (2008) reported that the purified peptide isolated from bigeye tuna had a considerable radical scavenging effect in HT1080 cell at a concentration of 50 µg/mL. Amongst all samples, FHAPa exhibited the strongest cytoprotective ability as evidenced by the lowest cell

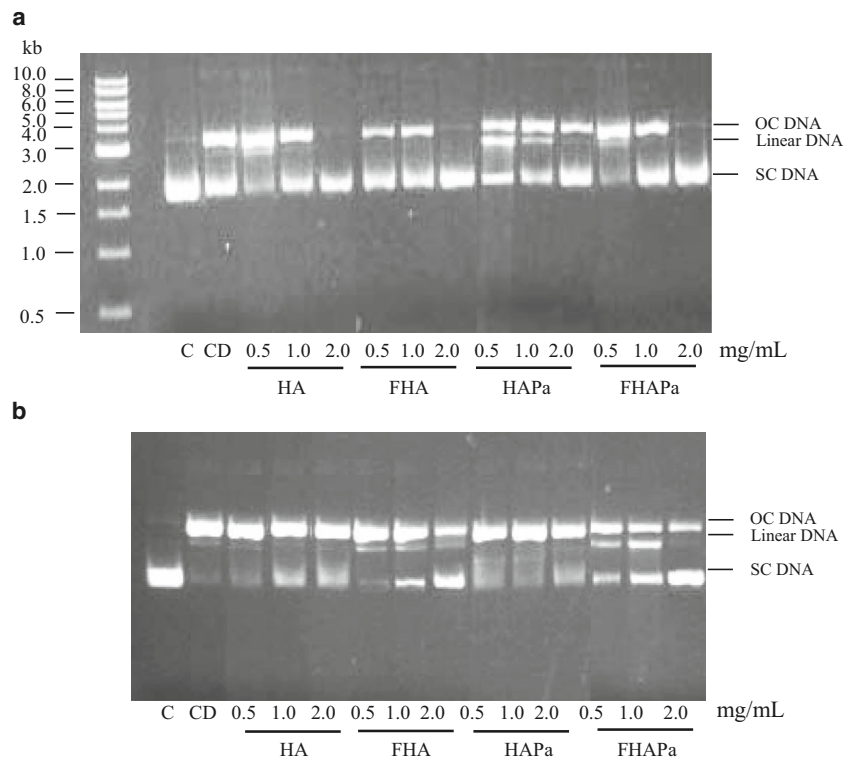
Fig. 2 The cellular radical scavenging activities of HA, HAPa and their fractions on HepG2 cell. The cells were labeled with 100 μ M fluorescence dye (DCFH-DA) and treated with various concentrations of different samples (0.5, 1.0 and 2.0 mg/mL) for 1 h prior to exposure to 800 μ M H₂O₂ (A) or 800 μ M AAPH (B). Fluorescence intensity (FI) of the oxidation of DCFH to DCF was monitored at $E_x=493$ nm and $E_m=527$ nm. The changes of FI as a function of time were tested using different samples at a level of 2 mg/mL. The results were calculated as the percentage of relative FI of H₂O₂ or AAPH. Values are expressed as the mean \pm S.D. ($n=2$). Different letters on the bar indicate the significant differences ($P<0.05$)



ROS generation. It was postulated that the antioxidant peptides might be more concentrated in their fractions. When the samples (hydrolysates or fractions) and Trolox were combined, ROS generation was sharply decreased (29.2–45.2 %) and the percentage of cell ROS generation was lower than that found in a system incorporated with Trolox at all dosages

tested, suggesting a synergistic effect on cytoprotection. A similar pattern was observed in the system stimulated with AAPH (Fig. 2B). Nile tilapia protein hydrolysates were more effective in scavenging peroxy radical than did H₂O₂, as evidenced by the lower percentage of cell ROS generation obtained. FHAPa most effectively reduced ROS generation

Fig. 3 Agarose gel electrophoresis of DNA treated with H₂O₂ (a) and AAPH (b) in the absence and presence of HA and HAPa and their fractions at different concentrations (0.5, 1.0 and 2.0 mg/mL). C denote control (DNA alone); CD denote control damage (DNA + oxidative stressors); HA, FHA, HAPa and FHAPa denote oxidative DNA damage pre-treated with various hydrolysates or fractions (DNA + oxidative stressors + hydrolysates or fractions)



induced by H₂O₂ and AAPH, especially when used in combination with Trolox.

Protective ability of hydrolysates and their fractions against DNA damage

DNA is another sensitive biotarget for ROS-mediated oxidative damage, leading to mutagenicity. Protective effect of different hydrolysates and fractions against H₂O₂ and AAPH induced DNA damage is depicted in Fig. 3. The antioxidant ability of hydrolysates or fractions was assessed, based on their protection of supercoiled DNA strand from scission by oxidative stressor into the open circular or linear form. As shown in Fig. 3a and b, the supercoiled DNA was converted into the open circular DNA when exposed to H₂O₂ or AAPH. The supercoiled DNA band of sample treated with H₂O₂ was retained by 50.7 %, whilst those treated with AAPH was not detectable (lane CD). Peroxyl radical induced DNA breakage was more pronounced than that induced by H₂O₂, suggesting that peroxyl radical resulted in a higher damage in biological molecules. This was in accordance with the decrease cell viability of HepG2 cell treated with AAPH (Fig. 1). Oxidative stress in cells caused by ROS, particularly H₂O₂ and peroxyl radicals, generally leads to DNA damage. There are a wide variety of reports for DNA modifications caused by ROS, including strand scission, sister chromatid exchange, DNA-DNA and DNA-protein cross-links as well as base modification (Kittiphattanabawon et al. 2012). H₂O₂ has been

known to be a non-reactive radical, however it becomes highly reactive when reacts with transition metal, yielding the hydroxyl radical (HO[•]). Klompong et al. (2009) reported that OH[•] induced oxidative stress resulted in pUC18 DNA relaxation, whilst H₂O₂ (alone) treated DNA had no damage. Kim et al. (2007) reported that the supercoiled pBR322 DNA was completely changed to open circular form when exposed to Fe²⁺-H₂O₂ solution, whereas it decreased to 50 % when treated with H₂O₂ alone. However, hydroxyl radicals have a shorter half-life as compared to peroxyl radicals (Kittiphattanabawon et al. 2013). Therefore, the major oxidative damages in the cells were caused by peroxyl radical to a higher extent.

When DNA was incorporated with all hydrolysates and fractions at levels of 0.5–2 mg/mL, the retention of supercoiled DNA increased in a dose dependent manner for both H₂O₂ and AAPH induced systems. For DNA induced with AAPH, supercoiled DNA band intensity was retained by 24.2, 76.7, 22.9 and 92.6 % when HA, FHA, HAPa and FHAPa at a level of 2 mg/mL were present, respectively. It was noted that the use of all hydrolysates or fractions at 2 mg/mL could effectively protect DNA scission from H₂O₂ as evidenced by 100 % supercoiled DNA observed, except for HAPa, in which 89.5 % retention were found. The result indicated that Nile tilapia protein hydrolysates had a protective ability against DNA scission induced by H₂O₂ and peroxyl radical to different degrees. This was possibly due to the differences in ability to scavenge free radicals and H₂O₂ as

reported in Table 1. According to a result, HAPa showed higher protective effect, compared to HA, especially after fractionation. The difference in their capacity might be related to peptides in various hydrolysates. Surguladze et al. (2004) reported the scission of supercoiled DNA strand to nicked circular form caused by free radicals. The rate of nicking correlated with the iron content and was strongly inhibited by radical scavengers and chelators. Je et al. (2009) reported that the second step hydrolysates from tuna liver showed more active protection toward supercoiled DNA conversion than did the hydrolysates from the first step. Oxidative stress caused by ROS, such as H₂O₂ and peroxy radical, resulted in damaged DNA, which may possibly be implicated in mutagenesis and carcinogenesis. Therefore, hydrolysates, especially those prepared using Alcalase and papain contributed to inhibitory effect against DNA oxidation induced by H₂O₂ or peroxy radical.

Conclusion

Protein hydrolysates from Nile tilapia PI prepared using Alcalase and Alcalase together with papain exhibited a good antioxidant potential, in both chemical and cellular based assays. Hydrolysates and their fractions exerted a protective ability against H₂O₂ and peroxy radical induced oxidative damage on HepG2 cells and DNA via scavenging free radical and H₂O₂. Generally, HAPa, especially after fractionation, showed a stronger antioxidant activities than did HA. Therefore, protein hydrolysates from Nile tilapia prepared by two step hydrolysis using Alcalase and papain could yield peptides with high antioxidant activity and could serve as functional foods.

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