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Influence of molecular weight on intracellular antioxidant activity of invasive silver carp (*Hypophthalmichthys molitrix*) protein hydrolysates

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ABSTRACT

Protein hydrolysates from underutilized silver carp (SPH) were prepared using Flavourzyme (F-15 to F-60) and Alcalase (A-15 to A-60) at 15, 30, 45 and 60 min, respectively. SPH F-30 and A-60 showed promising chemical-based antioxidant activity and were further fractionated according to size to evaluate caco-2 cell based antioxidant activity. F-30 and A-60 peptide fractions with <3 kDa (F-30 < 3, A-60 < 3) showed higher cell-based antioxidant activity under stressed and non-stressed conditions. Further, IC_{50} values of F-30 < 3 (1–3 mg/mL) was lower than A-60 < 3 fractions (4 to 12 mg/mL), indicating higher cellular antioxidant activity of F-30 < 3 compared to A-60 < 3 under all conditions. The presence of active peptides with desired amino acid sequence in F-30 < 3 compared to A-60 < 3 may have contributed to its higher cellular antioxidant activity. Overall, SPH exhibited antioxidant capacity, hence using an underutilized, invasive fish for environmental and economic gain in the form of promising functional ingredients.

1. Introduction

Silver carp (*Hypophthalmichthys molitrix*), is an invasive fish species that has established itself throughout much of the Mississippi River basin in the Midwestern United States (Chick & Pegg, 2001). Their continuing spread is of great concern as it has potential to become established in wider variety of novel habitats than previously thought (Coulter, Keller, Amberg, Bailey, & Goforth, 2013). Efforts are thus being made to decrease its abundance and minimize invasive pressure on the Great Lakes basin through recreational and commercial harvest (Rogowski et al., 2005). Most of the harvested silver carp are used in fertilizers and livestock feeds, or are simply discarded. However, these species are highly nutritious and widely consumed in several Asian, European and African countries (Kolar et al., 2005).

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Abbreviations: SPH, silver carp protein hydrolysates; SPH-A, silver carp protein hydrolysates prepared using Alcalase; SPH-F, Silver carp protein hydrolysates prepared using Flavourzyme; DPPH, 1,1-diphenyl-2-picrylhydrazyl; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; TNBS, 2,4,6-trinitrobenzenesulphonic acid; AAPH, 2,2-azobios-(2-amidinopropane) dihydrochloride; DCFH-DA, 2,7-dichlorodihydrofluorescein diacetate

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An alternative for using harvested silver carp is to recover and modify the protein-rich meat into different value-added alternatives suitable for use in a broad range of food products.

Fish protein hydrolysates (FPH) are classified as by-products and defined as the soluble products of proteolytic reactions. It is well recognized that FPH from various sources have improved functional and bioactive properties (Gómez-Guillén, Giménez, López-Caballero, & Montero, 2011). The role of food-derived bioactive peptides, in particular those derived from marine processing waste are thoroughly reviewed by Harnedy and FitzGerald (2012). In particular, antioxidant properties of FPH in species such as tilapia (Foh, Amadou, Foh, Kamara, & Xia, 2010; Raghavan, Kristinsson, & Leeuwenburgh, 2008), Pacific hake (Cheung, Cheung, Tan, & Li-Chan, 2012; Samaranayaka & Li-Chan, 2008a, 2008b), herring (Sannaveerappa, Westlund, Sandberg, & Undeland, 2007), tuna (Je, Qian, Byun, & Kim, 2007; Je, Qian, Lee, Byun, & Kim, 2008; Nalinanon, Benjakul, Kishimura, & Shahidi, 2011), Alaskan pollack (Jia et al., 2010) is well documented. In addition, a couple of antioxidant activity studies on silver carp protein hydrolysates (SPH) from China, showed promising radical scavenging and lipid peroxidation activities (Dong et al., 2008; Zhong, Ma, Lin, & Luo, 2011). However, intracellular antioxidant activity, as well as the effect of free amino acid composition and peptide molecular weight have not been fully explored from invasive silver carp SPH harvested in the U.S.

The main objective of this study was to generate bioactive peptides from protein hydrolysates obtained from invasive silver carp, and evaluate their intracellular antioxidant activity. In this study, SPH derived from two different commercial enzyme (Flavourzyme™ and Alcalase™) treatments and different hydrolysis times were used. Hydrolysates with high chemical based antioxidant activity were tested using human heterogeneous epithelial colorectal adenocarcinoma cells (caco-2 cells) as a model under oxidative stress and non-stress conditions. In addition, the effect of peptide molecular weight and peptide sequence on *in vitro* antioxidant activity was also evaluated using a cell-based assay.

2. Materials and methods

2.1. Materials

Fresh silver carp (*Hypophthalmichthys molitrix*) were harvested from the Wabash River (Lafayette, IN, USA). The fish were transported on ice within 24 h to the Purdue University Food Science Department where they were beheaded, eviscerated, filleted and immediately frozen at –20 °C for later use. The enzymes Alcalase™ 2.4 L (EC 3.4.21.14, P-4860, 2.4 U/G) and Flavourzyme™ (EC 232-752-2, P-6110, 500 U/G), L-leucine, and 2,4,6-trinitrobenzenesulphonic acid (TNBS), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium pyruvate 100 mM, penicillin–streptomycin (100×), fetal bovine serum, trypsin EDTA (ethylenediaminetetraacetic acid 0.25%), 96 well culture plates and culture dishes were purchased from VWR (Radnor, PA, USA). Dulbecco's modified eagle medium (DMEM)/high w/L-glutamine/ with sodium pyruvate (1000 mL), phosphate buffered saline (1×, 500 mL), 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH), 2,7-dichlorodihydrofluorescein diacetate and DMEM/high modified no phenol red were purchased from Fisher Scientific (Pittsburgh, PA, USA). Caco-2 BBe (brush border expressing) line was obtained from American Type Culture Collection.

2.2. Methods

2.2.1. Preparation of SPH

SPH were prepared according to Liceaga-Gesualdo & Li-Chan (1999) with some modifications. Fillets (500 g) were thawed at 4 °C overnight, skin was removed and rinsed once using cold distilled water. The fillets were minced using a meat grinder (Cabela's, Hammond, IN, USA) and homogenized in a blender with two volumes of water (w/v). The slurry was hydrolyzed using Alcalase (0.072 U/g of mince protein) or Flavourzyme (15 U/g of mince protein), respectively, under optimum temperature (50 °C) at different incubation times (15, 30, 45 and 60 min). Enzyme choice was made to observe the effect of endopeptidase and exopeptidase activity on the antioxidant activity of protein hydrolysates. The pH of the slurry was measured to ensure it was within optimum pH range of the enzymes (6.5–8.5 and 5.5–7.5, respectively). After incubation, samples were immediately pasteurized (90 °C, 15 min) and centrifuged at 16,300 × *g* for 15 min. The supernatant was freeze-dried to yield SPH using Alcalase (SPH-A) and SPH using Flavourzyme (SPH-F). SPH-A and SPH-F samples from different hydrolysis times (15, 30, 45 and 60 min) were denoted by A-15, A-30, A-45, A-60 and F-15, F-30, F-45, F-60, respectively. For control sample, mince slurry was pasteurized immediately without any enzyme addition and incubation. Freeze dried samples were stored at –20 ± 3 °C until used.

Different chemical based antioxidant assays such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity (Bougatef et al., 2009), ferric ion reducing antioxidant capacity (Samaranayaka & Li-Chan, 2008a, 2008b) and lipid peroxidation inhibition (Je et al., 2007) were performed on SPH. Samples with highest antioxidant activity were further selected for ultrafiltration and cell-based studies, as will be described later.

2.2.2. Size-exclusion chromatography and ultrafiltration

F-30 and A-60 samples were fractionated according to molecular weight into two fractions: <10 to >3 kDa (F-30 > 3 and A-60 > 3, respectively) and <3 kDa (F-30 < 3 and A-60 < 3, respectively). The A-60 and F-30 peptide fractions with molecular weight <3 kDa were fractionated using low pressure size exclusion chromatography using a sephadex G25 fine column (Pharmacia Biotec Inc, Piscataway, NJ, USA). Column dimensions were 1.7 cm (diameter) × 90 cm (height) with a void volume of 109 mL. Sample (2.5 mg/mL) was dissolved in 5 mM phosphate buffer (pH 7) and filtered through 25 mm syringe filter with 0.2 μm membrane (Fisher Scientific, Pittsburgh, PA, USA). Purified solution (5 mL) was injected into column and eluted using distilled water with a flow rate of 1.5 mL/min. Peptide fractions were collected every 4 min using Bio-Rad model 2110 fraction collector (Bio-Rad, Hercules, CA, USA) and analyzed for molecular weight profile using HPLC. Sample (20 μL) was injected to a Waters e2695 Separation Module HPLC system (Waters Corporation, Milford, MA, USA) equipped with an auto injector and UV/Vis detector. Sepax zenix SEC-100, 3 μm, 100 A°, 4.6 × 300 mm^2 column and a guard column (2.1 × 50 mm^2) was

used to separate samples according to size at 215 nm absorbance. Phosphate buffer (150 mM, pH 7.0) was used as solvent with a flow rate of 0.35 mL/min and separation was performed under ambient temperature (23 °C). Molecular profile of the samples was obtained using a calibration curve generated with different protein standards: carbonic anhydrase (29 kDa), aprotinin (6.5 kDa), and cytidine (0.243 kDa). Sample tubes for peptide fractions <3 kDa of F-30 and A-60 were collected.

Peptide fractions ranging from <10 to >3 kDa of F-30 and A-60 samples were fractionated using ultrafiltration. Two different filters with molecular weight cut off of 10 and 3 kDa were used as outlined by Centricon® centrifugal filter device user manual. The fractionated samples with molecular weight ranging from <10 to >3 kDa were freeze-dried and their molecular weight profile was confirmed using HPLC as outlined earlier. All the molecular weight fractions of F-30 and A-60 samples were freeze-dried and stored at –20 °C until used.

2.2.3. *LC-MS/MS (ESI)*

Peptides were separated on a nanoLC system (1100 Series LC, Agilent Technologies, Santa Clara, CA, USA) loaded on the Agilent 300SB-C18 enrichment column for concentration and the enrichment column was switched into the nano-flow path after 5 min. Peptides were separated with the C18 reversed phase ZORBAX 300SB-C18 analytical column (0.75 μm × 150 mm, 3.5 μm) from Agilent. Column was connected to the emission tip from New Objective and coupled to the nano-electrospray ionization (ESI) source of the high resolution hybrid ion trap mass spectrometer LTQ-Orbitrap LX (Thermo Scientific, LLC). Peptides were eluted from the column using the acetonitrile/0.1% formic acid (mobile phase B) linear gradient. For the first 5 min, the column was equilibrated with 95% H_2O/0.1% formic acid (mobile phase A) followed by the linear gradient of 5% B to 40% B in 50 min at 0.3 μL/min, then from 40% B to 100% B in additional 10 min. Column was washed with 100% of ACN/0.1%FA and equilibrated with 95% of H2O/0.1%FA before next sample was injected. A blank injection was run between samples to avoid carryover.

The LTQ-orbitrap mass spectrometer was operated in the data-dependent positive acquisition mode in which each full MS scan (30.000 resolution) was followed by six MS/MS scans where the six most abundant molecular ions were selected and fragmented by collision induced dissociation (CID) using a normalized collision energy of 35%. Raw data were collected by Xcalibur (v 2.0.7).

Database search analysis was done using Spectrum Mill A.03.02.060 software package (Agilent Technologies, Santa Clara, CA, USA). The search was performed against NCBI database, species: Fish Reptiles. The search parameters were precursor mass tolerance 0.05 Da, fragment mass tolerance 0.7 Da, no enzyme was specified. For peptide identification only peptides with a spectrum mill score of 5 or higher and spectrum mill scored peak intensity (SPI) of 70 or higher were considered positive.

2.2.4. *Caco-2 BBe cell culture*

Intracellular antioxidant assay of F-30, A-60 and their fractions was done using the caco-2 BBe cell line. In this experiment, caco-2 BBe cells (passage: 65–69) were used as mammalian cell model but not to simulate highly differentiated intestinal cell model. Caco-2 BBe cell lines were incubated in DMEM, supplemented with 10% (v/v) fetal bovine serum, penicillin (100 unit/mL), streptomycin (100 μg/mL) and sodium pyruvate (0.11 g/L). Cells were incubated in a fully humidified environment under 5% CO_2 at 37 °C and were sub-cultured at 2–3 day interval before reaching 70–80% confluence.

2.2.5. *Cell based antioxidant activity*

Cell based antioxidant activity assay was performed as outlined by Kim, Kim, Wiacek, Chen, & Kim (2012). Caco-2 cells were seeded in 96-well plates at a density of 20,000 cells/cm^2 and incubated for 24 h under 5% CO_2 at 37 °C. Sample solutions with different concentrations (0.625, 1.25 and 5 mg of protein/mL) were prepared by diluting in DMEM/high modified phenol red free and were added to cells. In one set of treatment 0.1 mM of 2,2-azobis-(2-amidinopropane) dihydrochloride (AAPH) was added which serves as an oxidative stress (stressed) condition and another set without addition of AAPH serves as normal physiological (non-stressed) condition. Cells with the sample/sample + AAPH were incubated for 1 h at 37 °C, and probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) to make final concentration of 20 μM was added. After addition of probe, samples were incubated in the dark for 30 min at 37 °C and fluorescence signal ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 538 nm) was measured at different time intervals while keeping the cells in dark. The increase in fluorescence implies increase in cellular oxidation. A control sample with DMEM/high modified no phenol instead of sample was used as a negative control. A positive control (vitamin C) at different concentrations (40, 20 and 10 μM) was also analyzed. Both the controls were tested at stressed and non-stressed conditions with and without AAPH respectively. The percentage cellular oxidation in the presence of a sample was calculated by considering cellular oxidation of negative control as 100% at a particular incubation time.

In order to measure the effect of incubation on decrease of cellular oxidation, IC_{50} values of the sample (minimum concentration of the sample required to inhibit 50% of cellular oxidation) were determined at 30 min and 120 min of incubation. IC_{50} values of the samples were calculated using graph between concentration of the sample and percentage of cellular oxidation in the presence of the sample.

2.3. *Statistical analysis*

Analysis of variance (ANOVA) using a general linear model with Tukey's pairwise comparison of means ($P < 0.05$) was used to determine the statistical significance of observed difference among means. The statistical software program MINITAB® Version 16.0 (Minitab Inc, State College, PA, USA) was used.

3. Results and discussions

In order to test antioxidant properties of SPH, different chemical based antioxidant assays were performed owing to the

multifaceted nature of antioxidants. Based on chemical based assay results, among SPH-F and SPH-A samples, F-30 and A-60 hydrolysate samples were selected to study further.

3.1. *Fractionation of F-30 and A-60 samples according to size*

According to the standard curve, peptide fractions with molecular weight <3 kDa eluted after 8.63 min. The molecular profile of peptide fractions F-30 < 3 and A-60 < 3 showed that more than 80% of the sample was eluted after 8.63 min, confirming the purity of peptides <3 kDa (Fig. 1(A) and (B)). Protein contents of the freeze dried peptides <3 kDa fractionated from F-30 and A-60 samples were 84.2 and 82.9%, respectively.

Peptide fractions greater than 3 kDa were separated by ultrafiltration with molecular weight cut-offs of 10 and 3 kDa. The molecular weight profile of these peptide fractions collected was tested using a Sepax zenix column, where more than 70% of the sample was eluted before 8.63 min, showing that peptides >3 kDa were fractionated (Fig. 1(C) and (D)).

3.2. *Peptide sequencing of F-30 < 3 and A-60 < 3 samples*

The amino acid sequence of peptides which match with the common characteristics of most antioxidant peptides in the literature were identified. It is well recognized that hydrophobic amino acid at N-terminal has shown to increase the antioxidant activity of peptides (Elias, Kellerby, & Decker, 2008; Park, Jung, Nam, Shahidi, & Kim, 2001) and more than half of the antioxidant peptides identified have hydrophobic amino acids at N-terminal (Power, Jakeman, & FitzGerald, 2013). The higher electronic property of C-terminal amino acids is also an important prediction of antioxidant activity. Most of the antioxidant peptide's C-terminal amino acids are often occu-

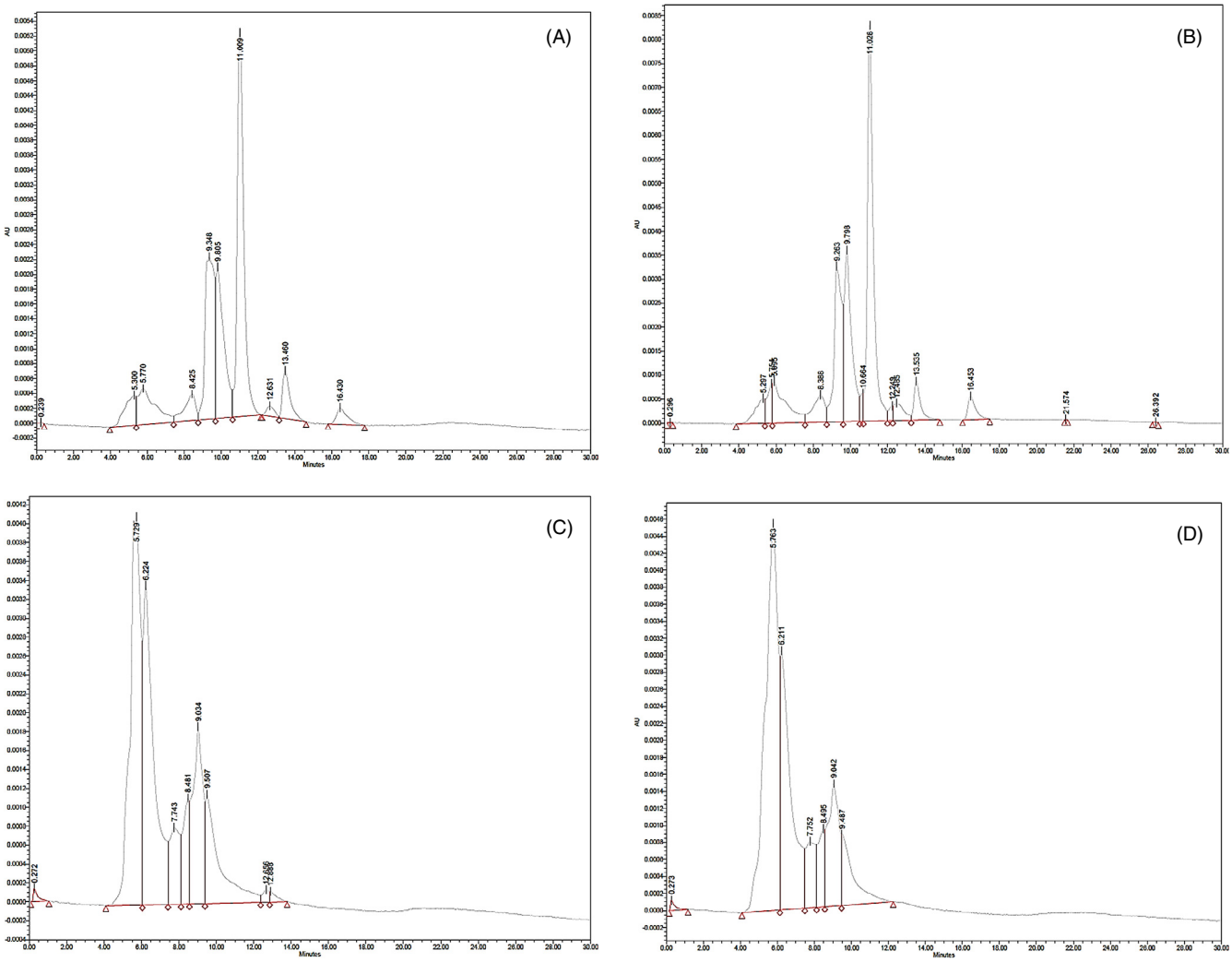

Fig. 1 – Molecular weight profile of (A) <3 kDa peptide fractions of A-60 (A-60 < 3), (B) <3 kDa peptide fraction of F-30 (F-30 < 3), (C) >3 kDa peptide fractions of A-60 (A-60 > 3) and (D) >3 kDa fraction of F-30 (F-30 > 3) ran on Waters e2695 Separation Module HPLC system equipped with UV/Vis detector and Sepax zenix SEC-100, 3 μm, 100 A°, 4.6 × 300 mm² column. Phosphate buffer (150 mM, pH 7.0) was used as solvent phase at 0.35 mL/min flow rate. The retention time of 3 kDa peptide occurs at 8.63 min.

pied by Trp, Leu, Glu, Ile, Met, Val, and Tyr (Li, Li, He, & Qian, 2011; Power et al., 2013). According to Li et al. (2011) the second amino from the C-terminal is a major contributor to antioxidant activity and basic or acidic amino acids (Asp, Glu, His, Arg, Lys) or hydrophilic amino acids (Ser, Thr) are preferred. Most of the potent antioxidants derived from casein and β-lactoglobulin typically have Glu, Agr, Asp, Pro and Leu in this position. Based on this information, peptides with the characteristics stated earlier were pooled from both F-30 < 3 and A-60 < 3 samples.

Apart from N- and C-terminal amino acids, the presence of antioxidant amino acids such as Tyr, Met, His, Cys and Trp in the sequence can contribute to the antioxidant activity of the peptide (You, Zhao, Cui, Zhao, & Yang, 2009). The total numbers of peptides with desired N- and C-terminal amino acids and with three of the earlier antioxidant amino acids from F-30 < 3 and A-60 < 3 samples were 7 and 4, respectively. In addition, two of the peptides from F-30 < 3: (V)NCSQNLMNYSQHTKIS(I) and (F)YMGKSHCHIFHSIMS(I) had a combination of Tyr, Met, Cys, and His, showing high potential to contribute to antioxidant activity. Interestingly, the total number of peptides with sulphur containing amino acids such as Cys and Met was higher in F-30 < 3 (49 peptides) compared to A-60 < 3 (36 peptides).

3.3. *Inhibition of cellular oxidation of different molecular weight peptides under stress and non-stress conditions*

Inhibition of intracellular oxidation in the presence of different molecular weight peptide fractions of F-30 and A-60 samples at different concentrations under both oxidation stress (AAPH, 0.1 mM, 1 h at 37 °C) and non-stress (no AAPH added) conditions in a caco-2 human intestinal cell model was evaluated. This model was chosen based on its common use as an intestinal cell model (Albersen et al., 2013; Thompson, Al Mutairi, Sharp, Elliott, & Fairweather-Tait, 2010) and the exposure of the gut to oxidants and reactive oxygen species (ROS) derived from the diet and/or generated from digestion and normal metabolic function. Addition of AAPH, an oxidative stress inducer, results in diminishing of antioxidant reserves of the cell and increase oxidative damage (Razinger, Drinovec, & Zrimec, 2010). The pronounced increase in cellular oxidation due to AAPH is evident in negative control samples under stress when compared to that in non-stress conditions.

Dose-dependent inhibition of cellular oxidation of different fractions of F-30 and A-60 samples as well as vitamin C under non-stressed (no AAPH added) and stressed (AAPH, 0.1 mM, 1 h at 37 °C) conditions is shown in Figs. 2 and 3. The positive control (vitamin C) failed to show dose dependency in inhibiting cellular oxidation. Comparable results were observed by Intra and Kuo (2007) at similar concentration of vitamin C in human caco-2 cell model. One possible reason is that many antioxidants such as vitamins can have prooxidant effect besides antioxidant activity, especially at higher doses and prolonged incubation (Brown, Morrice, & Duthie, 1997; Intra & Kuo, 2007; Versari et al., 2006).

In this study, under both conditions, with the increase in concentration from 0.625 to 5 mg/mL there was an increase in inhibition of cellular oxidation in the presence of F-30, F-30 < 3, A-60 and A-60 < 3 samples. However, dose-dependent inhibition in cellular oxidation was not demonstrated by peptide fractions greater than 3 kDa samples (F-30 > 3 and A-60 > 3) under the non-stress condition. Interestingly, in the stress condition there was a significant increase ($P < 0.05$) in cellular oxidation inhibition by F-30 > 3 at its highest concentration (5 mg/mL) compared to lower concentrations (0.625 and 1.25 mg/mL). The reason for this improvement in its antioxidant activity is unknown and further evaluation is required. The results earlier emphasize that the antioxidant activity of peptides is highly dependent on molecular weight and this observation is supported by many studies (Ahn, Je, & Cho, 2012; Je, Park, & Kim, 2005; Lee, Jeon, & Byun, 2011). Overall, IC_{50} values of F-30 fractions were lower than the A-60 fractions, indicating higher cellular antioxidant activity of F-30 fractions under both conditions (Table 1). This is in accordance with the higher chemical-based radical scavenging activity of F-30 compared to the A-60.

Table 1 – IC_{50} values (mg/mL) of F-30, F-30 < 3, A-60 and A-60 < 3 samples at 30 and 120 min incubation times under both stress and non-stress conditions.

Samples	Non-stress		Stress	
	30 min	120 min	30 min	120 min
F-30	4.20 Axy	5.31 Ax	3.08 Ay	4.54 Ax
F-30 < 3	3.02 Ax	2.62 Bx	1.97 Axy	1.19 By
A-60	5.87 Bx	8.88 Cy	4.74 Bx	4.72 Ax
A-60 < 3	8.77 Cx	5.05 Az	11.62 Cy	4.60 Az

Different letters (A, B, C) indicate significant difference ($P < 0.05$) in means within each column for each individual incubation time. Different letters (x, y, z) indicate significant difference ($P < 0.05$) among the means within each row for stress or non-stress condition, respectively. Samples are defined as F-30: Flavourzyme-hydrolyzed for 30 min; F-30 < 3: less than 3 kDa fraction of Flavourzyme-hydrolyzed for 30 min; A-60: Alcalase-hydrolyzed for 60 min; A-60 < 3: less than 3 kDa fraction Alcalase-hydrolyzed for 60 min.

A large part of the cellular oxidation occurs in mitochondria because they are constantly exposed to high ROS levels during cellular activity. Therefore an ideal antioxidant should be able to permeate through the cell, target mitochondria and should protect mitochondria against oxidative stress (Szeto, 2008). Some small peptides with active amino acid sequence have shown to penetrate the cell membrane freely and inhibit mitochondrial oxidation (Rocha et al., 2010; Szeto, 2008). At all concentrations of peptide fractions with molecular weight greater than 3 kDa (F-30 > 3 and A-60 > 3) there was a significant increase in cellular oxidation with increase incubation (Figs. 2and 3). Similarly, under non-stress conditions, the overall antioxidant activity of F-30 and A-60 samples decreased from incubation periods of 30 min (at 30 min: IC_{50} of F-30 = 4.20; IC_{50} of A-60 = 5.87) to 120 min (at 120 min: IC_{50} of F-30 = 5.31; IC_{50} of A-60 = 8.88). This observation may be attributed to their poor cell permeability and inability to inhibit mitochondrial oxidation. However, F-30 < 3 was able to inhibit cellular oxidation rapidly within 30 min of incubation at all concentrations and was also able to retain its activity throughout 120 min of incubation (Fig. 2). This observation may suggest the rapid uptake of F-30 < 3 into the cell interior and targeting mitochondrial oxidation. However, further confirmation by studying cellular and mitochondrial uptake of peptides should

be performed. Conversely, under both stress and non-stress conditions, A-60 < 3 samples showed very low antioxidant activity at 30 min but with further incubation (120 min), its antioxidant activity significantly improved ($P < 0.05$) as evident with the significant decrease ($P < 0.05$) in its IC_{50} value from 30 min to 120 min incubation. This delayed inhibition of cellular oxidation of A-60 < 3 can be attributed to many factors such as a delay in cellular permeation or delay in targeting cellular mitochondria. Since amino acid sequence of a peptide is a crucial factor that influences its cell permeability (Lee & Yamamoto, 1989; Szeto, 2008), peptides obtained from Alcalase hydrolysis of silver carp protein may not have an amino acid sequence "active" for rapid cellular uptake unlike the peptides derived from Flavourzyme (an endo- and exo-peptidase). Another reason may be due to the higher number of active peptides with desired amino acid sequence of F-30 < 3 which may have contributed to its higher cellular antioxidant activity during initial incubation times. However, with increased incubation time, A-60 < 3 peptide could have generated more potent antioxidant peptides with the action of membrane proteases.

In addition, the higher number peptides with sulphur containing amino acids (Cys and Met) present in the F-30 < 3 kDa fractions may also contribute to its higher antioxidant activity. The presence of sulphur containing amino acids such as cysteine and methionine can enhance glutathione synthesis by activating γ-glutamyl cysteine ligase enzyme (Kim et al., 2003). Glutathione, a major component of cellular antioxidant system, plays a crucial role in reducing ROS and free radicals in the cell. It is a substrate for glutathione transferases and peroxidases that detoxify xenobiotics and ROS in the cell (Fang, Yang, & Wu, 2002; Parcell, 2002). Therefore the presence of sulphur containing amino acids may increase the concentration of glutathione and reduce the vulnerability of the cell to oxidative stress (Atmaca, 2004).

Studies have shown that the efficiency of dietary cysteine in the form of peptide imparted enhanced glutathione synthesis in spleen cells when compared to free form (Bounous, Batist, & Gold, 1989). The administration of protein with higher sulphur containing amino acids induced greater subnormal glutathione levels when compared to proteins with lower sulphur

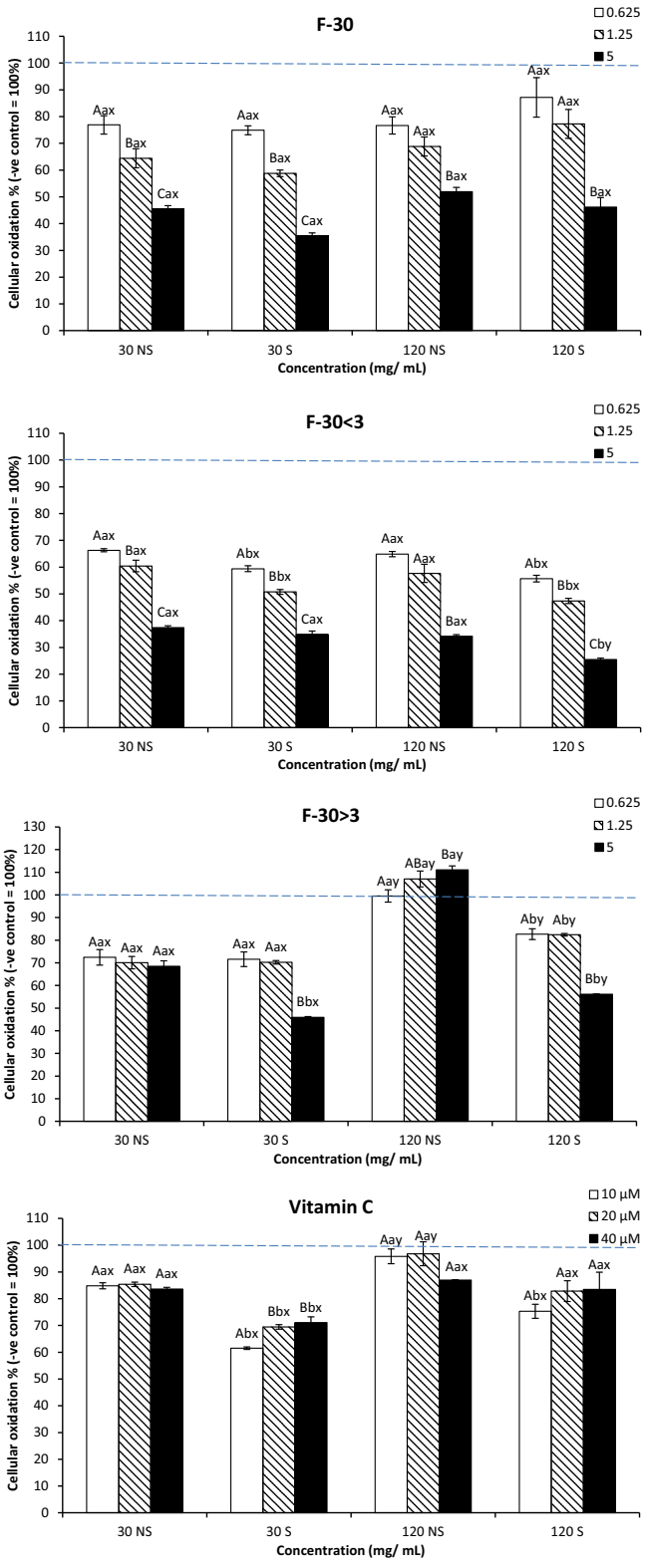

Fig. 2 – Percentage intracellular oxidation measured in caco-2 BBe cells in the presence of F-30, its fractions (F-30 < 3 and F-30 > 3) and positive control (vitamin C) measure at different incubation times under both non-stressed (no AAPH added) and stressed (AAPH, 0.1 mM, 1 h at 37 °C). Intracellular oxidation of negative control (no sample added) at a given condition was used as a reference (100% oxidation). Values are mean ± SE, $n = 3$.

30 NS and 30 S = after 30 min incubation under non-stressed (NS) and stressed (S) conditions; 120 NS and 120 S = after 120 min incubation under NS and S conditions.

Different upper case letters (A, B, C) indicate significant difference among the means at different concentrations within a condition (30 NS, 30 S, 120 NS and 120 S). Different lower case letters (a, b, c) represent significant difference among the means of stressed and non-stressed conditions under similar concentration and incubation time. Different lower case letters (x, y) represent significant difference among the means at different incubation times under similar concentration and condition (stressed and non-stressed). Assay was conducted in duplicate and analysis was done in triplicate.

containing amino acids (Bounous et al., 1989; Bounous & Gold, 1991). Cysteine-containing peptides such as γ-glutamylcysteine and cysteinylglycine have been shown to increase glutathione synthesis in many different cell systems and also peripheral body areas (Anderson & Meister, 1989; Borgström, Kågedal, & Paulsen, 1986; Dringen, Hamprecht, & Bröer, 1998). Further biomarker studies will be required to confirm the observations in our study.

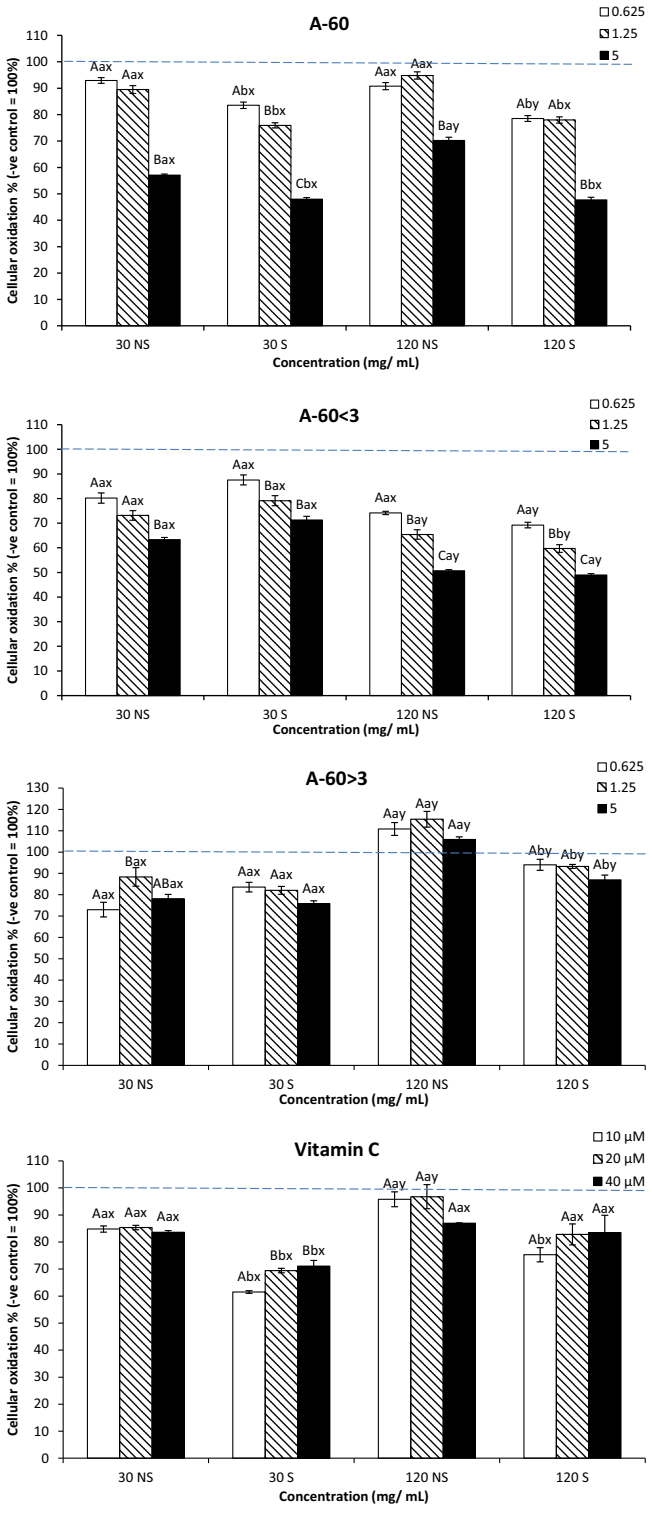

Fig. 3 – Percentage intracellular oxidation measured in caco-2 BBe cells in the presence of A-60, its fractions (A-60 < 3 and A-60 > 3) and positive control (vitamin C) measure at different incubation times under both non-stressed (no AAPH added) and stressed (AAPH, 0.1 mM, 1 h at 37 °C). Intracellular oxidation of negative control (no sample added) at a given condition was used as a reference (100% oxidation). Values are mean ± SE, *n* = 3.

30 NS and 30 S = after 30 min incubation under non-stressed and stressed conditions; 120 NS and 120 S = after 120 min incubation under non-stressed and stressed conditions.

Different upper case letters (A, B, C) indicate significant difference among the means at different concentrations within a condition (30 NS, 30 S, 120 NS and 120 S). Different lower case letters (a, b, c) represent significant difference among the means of stressed and non-stressed conditions under similar concentration and incubation time. Different lower case letters (x, y, z) represent significant difference among the means at different incubation times under similar concentration and condition (stressed and non-stressed). Assay was conducted in duplicate and analysis was done in triplicate.

Overall, at all incubation conditions, F-30 < 3 showed higher antioxidant activity which is evident by the lower IC_{50} values of F-30 < 3 (Table 1). Moreover, under the stress condition, F-30 < 3 was able to improve cellular antioxidant activity compared to the non-stress condition. These preliminary findings suggest that F-30 < 3 peptide fractions may have potential to protect mitochondria against oxidative damage even under stress conditions. However, further studies to test its stability toward digestion and *in vivo* studies are needed for its successful nutraceutical application. In addition, further purification of the most potent antioxidant peptide from the fraction and studying its amino acid sequence will help understand the mechanism of its antioxidant activity.

4. Conclusion

Intracellular antioxidant activity of different molecular weight fractions of F-30 and A-60 samples showed that antioxidant activity is highly dependent on size of the peptide fraction. The molecular weight fractions <3 kDa (F-30 < 3 and A-60 < 3) displayed higher antioxidant activity under both stress and non-stress conditions suggesting these fragments may hold the most promise as a value-added functional food ingredient or dietary supplement. Further investigating the influence of amino acid sequence of the peptide fractions on their cellular uptake and antioxidant machinery in the cell is essential to clearly understand their antioxidant mechanism. Nevertheless, this study has allowed us to open possibilities to use an underutilized, invasive fresh water fish for environmental and economic gain in the form of potential bioactive ingredients.

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