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Autolysis-assisted production of fish protein hydrolysates with antioxidant properties from Pacific hake (*Merluccius productus*)

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Abstract

Fish protein hydrolysates (FPH) with antioxidative properties were prepared using Pacific hake fish with high endogenous proteolytic activity from *Kudoa paniformis* parasitic infection. Infection level of $\sim 10^7$ *K. paniformis* spores/g fish mince or higher yielded FPH with high antioxidant potential by autolysis and/or Validase® BNP or Flavourzyme® 500L. Autolyzing fish mince containing 30×10^6 spores/g for 1 h at 52 °C and pH 5.50 produced FPH (named E-1h) with Trolox equivalent antioxidant capacity (TEAC) in the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) radical assay of 262 ± 2 μmol/g and oxygen radical absorbing capacity (ORAC) of 225 ± 17 μmol Trolox equivalents/g freeze-dried sample. E-1h FPH also exhibited a marked concentration-dependent scavenging activity on 1,1-diphenyl-2-picrylhydrazyl radical. Antioxidant activity of E-1h FPH was higher ($p < 0.05$) than BHA and α-tocopherol in a linoleic acid peroxidation system over prolonged storage (~162 h). Antioxidative FPH from Pacific hake may be useful ingredients in food and nutraceutical applications.

Keywords: Pacific hake; Fish protein hydrolysate; Antioxidative activity; Proteolytic activity; Autolysis; *Kudoa*

1. Introduction

Dietary proteins have been found to play a significant role in improving human health beyond their well recognized nutritional value (Hartmann & Meisel, 2007). Fish protein hydrolysates (FPH) have been reported to possess antioxidative, antihypertensive, antimicrobial and immunomodulatory properties (Fujita & Yoshikawa, 1999; Shahidi, Han, & Synowiecki, 1995). In particular, protein hydrolysates with antioxidant properties have become a topic of great interest for pharmaceutical, health food, as well as food processing/preservation industries (Alasalvar, Shahidi, & Quantick, 2002; Hagen & Sandnes, 2004). The bioactive molecules in FPH responsible for these properties are peptides that are released upon hydrolysis of fish proteins, by the enzymes already present in fish mince (endogenous) and/or by different enzymes added at appropriate levels to the fish mince (exogenous). Antioxidant activity has been reported for protein hydrolysates prepared from various fish sources such as capelin, tuna, mackerel, yellowfin sole, Alaska pollack, Atlantic salmon, hoki, conger eel, and scad (Amarowicz & Shahidi, 1997; Berge, 2005; Jao & Ko, 2002; Je, Kim, & Kim, 2005; Je, Park, & Kim, 2005; Jun, Park, Jung, & Kim, 2004; Ranathunga, Rajapakse, & Kim, 2006; Thiansilakul, Benjakul, & Shahidi, 2007; Wu, Chen, & Shiau, 2003). Further, a brine solution containing salmon FPH injected into smoked salmon fish fillets was shown to reduce lipid oxidation measured as 2-thiobarbituric acid reactive substances (TBARS) during 6 weeks of cold storage (4 °C) and 8 months of frozen storage (−18 °C) (Hagen & Sandnes, 2004).

Pacific hake (*Merluccius productus*), also referred to as Pacific whiting, is a fish with low economic value because of the presence of a parasitic Myxosporea, *Kudoa paniformis*, and the "soft flesh" condition that results from autolysis during subsequent processing (Kabata & Whitaker,

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1985). Increased cathepsin L-like endogenous proteolytic activity of *K. paniformis* infected Pacific hake muscle (An, Seymour, Wu, & Morrissey, 1994) could be an asset, rather than a disadvantage, in making FPH without the need for adding commercial enzymes. Optimum conditions for the proteolytic activity of Pacific hake fish fillet mince were pH 5.25–5.50 and 52–55 °C (An et al., 1994; Samaranayaka, Ho, & Li-Chan, 2006). *K. paniformis* spore count was significantly ($p < 0.05$) correlated with endogenous proteolytic activity of fish mince, and Pacific hake with *K. paniformis* spore counts greater than 1×10^7 per gram fish mince possessed high proteolytic activity (Samaranayaka et al., 2006).

Benjakul and Morrissey (1997) showed the feasibility of preparing FPH with good nitrogen recovery and comparable amino acid composition to fish muscle by hydrolyzing Pacific whiting solid wastes using the commercial enzyme Alcalase 2.4L. Further, endogenous fish enzymes were successfully used in preparing fish sauce with various biochemical properties from Pacific whiting and its surimi byproducts (Tungkawachara, Park, & Choi, 2003), as well as from other fish sources such as capelin (Shahidi et al., 1995). However, the endogenous protease activity was attributed predominantly to digestive enzymes of the viscera (Shahidi et al., 1995). To date, there has not been any investigation on producing FPH by autolysis of Pacific hake fish muscle, nor on the possible influence of *Kudoa* parasitization on production and antioxidant properties of the FPH.

The overall objective of this study was to investigate the potential of using endogenous enzymes present in Pacific hake fish muscle to produce FPH with antioxidative properties. Firstly, the antioxidant properties of the FPH produced by autolysis as a function of hydrolysis time were studied, and compared to FPH produced by addition of food-grade commercial enzymes, Flavourzyme® 500L and Validase® bacterial neutral protease (BNP). Secondly, the effect of *K. paniformis* infection level of fish mince on autolysis-assisted production of FPH with antioxidant properties was investigated. Antioxidant activity of FPH was assessed using *in vitro* chemical assays and model systems, compared to the activities of two common synthetic food antioxidants, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), and the natural antioxidant, α-tocopherol. The development of FPH ingredients with antioxidative properties for food and nutraceutical applications could be an ideal approach to utilize the huge stock of Pacific hake caught in waters near the Pacific coast.

2. Materials and methods

2.1. Materials

Validase® BNP (bacterial neutral protease from *Bacillus subtilis*, 2000 NPU/g) in liquid form was a gift from Valley Research Inc., South Bend, IN. Flavourzyme® 500L (protease from *Aspergillus oryzae*, 500 LAPU/g, product of Novozymes®) was kindly donated by Brenntag Canada Inc., Langley, BC. Trypsin (T7409), L-leucine (L8912), 2,4,6-trinitrobenzenesulphonic acid (TNBS) (92822), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (11557), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (238813), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (D9132), potassium ferricyanide (455946), ferrozine (P9762), ferrous chloride (220299), fluorescein (F6377), 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH) (440914), linoleic acid (L1376), BHT (W218405), and α-tocopherol (T4389) were purchased from Sigma-Aldrich Canada Ltd., Oakville, ON. BHA (101159) was purchased from ICN Biomedicals Inc., Aurora, Ohio.

2.2. Fish samples and preparation of fish mince

Pacific hake were harvested off the coast of Vancouver Island, Canada (48.5°N 124.7–125.5°W) between April 2004 and May 2005. Whole fish were transported on ice to the University of British Columbia Food Science Laboratory within two days of capture, individually packed in polyethylene bags, labeled and stored frozen (−25 °C) until used. Based on the *K. paniformis* spore counts determined as described in the next section, fillets from 34 selected fish were used to prepare fish mince batches with infection level of 0 (Inf-0), 1 (Inf-1), 2 (Inf-2), 7 (Inf-7), 9 (Inf-9), 20 (Inf-20), 30 (Inf-30), and 100 (Inf-100) $\times 10^6$ spores/g mince. Weights of fish used ranged between 434 and 1946 g.

Fish were thawed in a cold room (4 °C) for 16 h, filleted, de-skinned, and then ground twice using a grinder (BEEM Gigant, Butcher & Packer Supply Company, Detroit, MI) with a 4 mm screen. From each batch of fish mince, a sample (∼100 g) was taken for *K. paniformis* spore counting and proximate analysis, and the rest of the mince was portioned (∼100 g) into polyethylene bags, vacuum packed and stored frozen (−35 °C) for further analyses.

2.3. K. paniformis spore counts

The method of Dawson-Coates et al. (2003), as modified by Samaranayaka et al. (2006), was used for isolating and counting *K. paniformis* spores in the batches of fish mince prepared. Duplicate analyses were performed for each mince sample, and the results were expressed as number of *K. paniformis* spores per g of sample.

2.4. Proximate analysis

Proximate composition of Pacific hake fish fillet mince was determined by analysis of Inf-20 batch. Moisture and ash contents were analyzed using standard methods of the Association of Official Analytical Chemists (AOAC) 930.15 and AOAC 942.05, respectively (AOAC, 1995). Lipid content was determined by the method of Bligh and Dyer (1959), and crude protein was determined from nitrogen content (N × 6.25) measured by a combustion method AOAC 992.15 (AOAC, 1995) using a Leco instrument

(Leco Crop., St. Joseph, MI) calibrated with ethylenediaminetetraacetic acid (EDTA) (9.58%N).

2.5. Preparation of FPH

In the first part of the study, several FPH were prepared using fish mince from Inf-20 batch, by incubation at pH 5.50 and 52 °C as shown in Fig. 1. Autolysis of the fish mince slurry for 1–6 h was used to prepare E-1h, E-2h, E-3h, E-4h, and E-6h FPH. Food grade commercial enzymes Validase® BNP (V) and Flavourzyme® 500L (F) were used to make V-2h, F-2h, E + V-2h, and E + F-2h FPH. Two types of control FPH were also prepared. The E-0h FPH was a control to measure the extent of autolysis during the 10–12 min incubation in the 55 °C water bath required to bring the slurry temperature to 52 °C before starting the actual hydrolysis process by adjusting pH to 5.50. The second type of control (C-2h) was prepared by inactivating endogenous proteases prior to incubation by heating fish mince slurries in a boiling water bath (>90 °C) for 15 min. Another set of FPH was prepared following a similar procedure shown in Fig. 1, using Inf-30 fish mince and autolysis or Validase® BNP, and also including E-5h, C-0h, C-1h, V-1h, and E + V-1h FPH.

In the second part of the study, C-0h, E-1h, and E-6h FPH were made from each of the eight fish mince batches in order to assess the effect of fish mince *K. paniformis* infection level on production of FPH with antioxidative properties.

After incubation for the indicated time of autolysis with endogenous enzyme and/or hydrolysis with Validase or Flavourzyme, 100 ml aliquots of the resulting FPH and control slurries were heated in a boiling water bath (>90 °C) for 15 min, centrifuged at 17,000*g* for 20 min, and filtered through 2 layers of cheese cloth. The filtrates were adjusted to pH 7.0 and freeze dried. Freeze dried FPH samples were homogenized using a pestle and a mortar, then stored in sealed vials at −18 °C until used for further analysis. Yields of FPH produced were calculated using the following equation.

$$\text{FPH yield}(\%, \text{dry basis}) = \frac{\text{Weight of freeze dried FPH(g)}}{\text{Dry matter weight of fish mince used(g)}} \times 100$$

2.6. Measurement of the extent of hydrolysis

The extent of hydrolysis was estimated by measuring the content of free amino groups using the TNBS method (Adler-Nissen, 1979) as described by Samaranayaka et al. (2006) and expressed as L-leucine equivalents (mmol)/g protein of the fish mince slurry after hydrolysis, based on a standard curve constructed with 0–1.6 mM L-leucine.

2.7. Measurement of antioxidative activity of FPH

2.7.1. DPPH radical scavenging capacity assay

The method described by Kitts, Wijewickreme, and Hu (2000) was used with slight modifications in order to assess the DPPH radical scavenging activity of FPH. DPPH solution (1.8 ml, 0.1 mM in 80% ethanol) was mixed with FPH

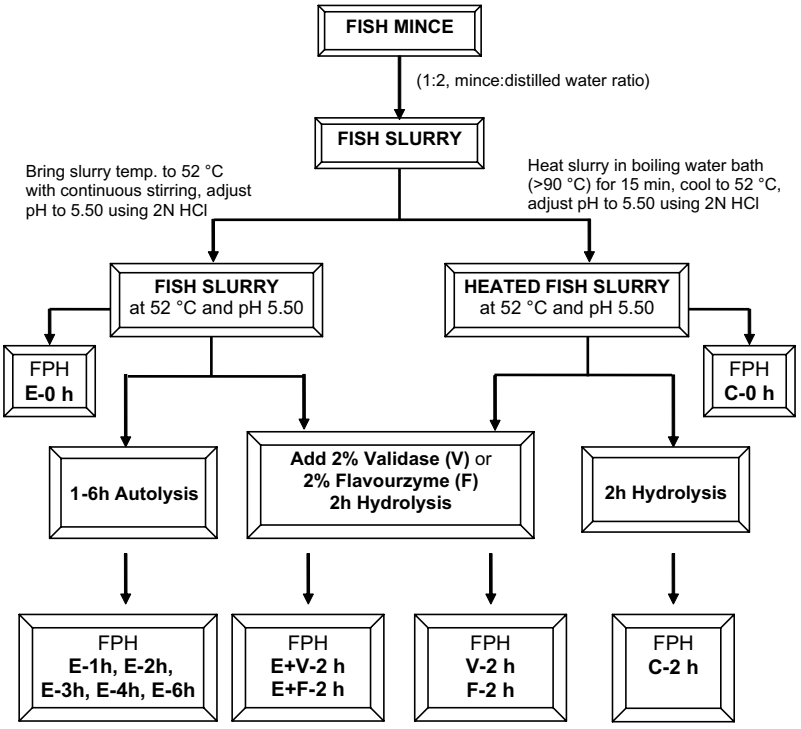

Fig. 1. Process of fish protein hydrolysate (FPH) production with no (C), endogenous (E), Validase® BNP only (V) or with endogenous (E + V), Flavourzyme® 500L only (F) or with endogenous (E + F) enzyme treatments.

solution (0.20 ml, at 3 mg/ml final assay concentration in 50% ethanol). Absorbance (Abs.) of the solution was read at 517 nm after 30 min of incubation at room temperature. For the assay control, 0.20 ml of 50% ethanol was used in the assay instead of the FPH solution. Sample controls were also made for each FPH by mixing 0.20 ml FPH solution with 1.8 ml of 80% ethanol. Radical scavenging capacity of FPH was calculated as follows:

$$\text{DPPH radical scavenging capacity}(\%) = \left(1 - \frac{(\text{Abs. of sample} - \text{Abs. of sample control})}{\text{Abs. of assay control}}\right) \times 100$$

2.7.2. Ferric ion reducing antioxidant capacity

The method described by Oyaizu (1988) was used to measure the ferric ion reducing capacity of FPH. A 2.0 ml aliquot of FPH stock solution in phosphate buffer (PB) (0.2 M, pH 6.6) was mixed with 2.0 ml of the same buffer and 2.0 ml of 1% potassium ferricyanide to yield final FPH concentration of 3 mg/ml. After incubation at 50 °C for 20 min, 2.0 ml of 10% trichloroacetic acid was added, and a 2.0 ml aliquot was mixed with 2.0 ml of distilled water and 0.4 ml of 0.1% ferric chloride. Absorbance at 700 nm after 10 min was measured as an indication of reducing power.

2.7.3. ABTS assay

A modified method of Re et al. (1999) was used to evaluate the ABTS radical scavenging capacity of FPH. A stock solution of ABTS radicals was prepared by mixing 5.0 ml of 7 mM ABTS solution with 88 μl of 140 mM potassium persulfate, and keeping in the dark at room temperature for 16 h. An aliquot of stock solution was diluted with PB (5 mM, pH 7.4) containing 0.15 M NaCl in order to prepare the working solution of ABTS radicals with absorbance at 734 nm of 0.70 ± 0.02. A 65 μl aliquot of FPH dissolved in the same phosphate buffer (66.67 μg/ml final assay concentration) or only buffer (for the control) was mixed with 910 μl of ABTS radical working solution, incubated for 8 min at room temperature in the dark, and then absorbance was measured at 734 nm. The % reduction of $ABTS^{\cdot+}$ to ABTS was calculated according to the following equation:

$$\text{ABTS radical scavenging capacity}(\%) = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

Further, the Trolox equivalent antioxidant capacity (TEAC), which is the concentration of sample giving the same % inhibition of absorbance at 734 nm of ABTS radical cation as 1 mM Trolox, was determined by assessing ABTS radical scavenging capacities of FPH solutions at four different final assay concentrations (6.67, 16.67, 33.33, and 66.67 μg/ml) and by using a standard curve prepared with 0–20 μM Trolox final assay concentrations. TEAC values for BHT and BHA were obtained using a similar procedure with the appropriate sample concentrations.

2.7.4. Oxygen radical absorbing capacity (ORAC) assay

ORAC of selected FPH was measured using the method described by Kitts and Hu (2005). Briefly, the samples and Trolox antioxidant standard in PB (50 mM, pH 7.0) were incubated with 60 nM fluorescein at 37 °C for 15 min in a 96-well plate (FluoroNunc™ Fluorescent microplate, VWR International, Ltd., Mississauga, ON). After addition of the peroxyl radical initiator, AAPH, the fluorescence using excitation wavelength of 485 nm and emission wavelength of 527 nm was continuously recorded for 60 min (Fluoroskan Ascent FL, Thermo Fisher Scientific, Inc., Milford, MA). Data transformation and interpretation was performed according to the method described by Valos, Mez-Cordoveäz, and Bartolomeä (2004). The ORAC value was expressed as μmol Trolox equivalents/g sample.

2.7.5. Metal ion chelating activity

The Fe^{2+}chelating activity of FPH at 5 mg/ml assay concentration was measured by the method of Decker and Welch (1990). The Fe^{2+} chelating effect of FPH was calculated as:

$$Fe^{2+}\text{ion chelating ability}(\%) = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right) \times 100$$

2.7.6. Lipid peroxidation in a linoleic acid model system

The method described by Osawa and Namiki (1985) was used to measure the inhibition of lipid peroxidation in a linoleic acid/ethanol/water emulsion system by selected FPH samples, BHA, BHT, and α-tocopherol at final assay concentration of 0.2 mg/ml. For FPH, a 5 mg sample was first dissolved in 10.0 ml of PB (50 mM, pH 7.0) and added to a solution of 0.13 ml of linoleic acid and 10.0 ml of absolute ethanol in 250 ml conical flask. BHA, BHT and α-tocopherol were first dissolved in 10.0 ml absolute ethanol and then mixed with 0.13 ml linoleic acid and 10.0 ml of the same PB. The total volume of each flask was adjusted to 25 ml with distilled, deionized water. A control reaction mixture was also prepared using the same procedure, but without adding any FPH sample or commercial antioxidant to the emulsion system. Contents of the flasks were mixed well, and the flasks were sealed and were incubated at 40 °C in a shaking incubator (in the dark) for seven days. Extent of lipid peroxidation was measured by the ferric thiocyanate method (Mitsuda, Yasumato, & Iwami, 1966) using 0.1 ml aliquots taken in duplicate from each flask at 18 h, 42 h, 90 h, and 162 h of incubation. Reduction of absorbance at 500 nm of emulsion systems containing FPH or commercial antioxidants compared to the control emulsion in the assay indicates its antioxidant potential.

2.8. Statistical analysis

All analyses were performed in triplicate. Statistical analyses were performed using the SPSS statistical program

(SPSS 10.0, SPSS Inc., Chicago, IL). Tukey's test was used to evaluate differences among mean values for treatments at $p < 0.05$.

3. Results and discussion

3.1. Fish mince proximate composition

The proximate composition of Inf-20 fish mince was: 82.99 ± 0.05% moisture, 15.16 ± 0.06% crude protein, 1.20 ± 0.03% ash, and 1.78 ± 0.05% crude lipids. Similar values (84.62 ± 0.28% moisture, 14.16 ± 0.61% crude protein, 1.00 ± 0.10% ash, and 0.39 ± 0.11% crude lipid) have been reported previously for muscles of Pacific whiting caught around Oregon, WA (Benjakul & Morrissey, 1997).

3.2. Antioxidant activity of FPH produced by autolysis and/or exogenous enzyme hydrolysis

The main purpose of this first study using Inf-20 and Inf-30 fish mince was to assess whether antioxidative FPH can be prepared using parasitized Pacific hake fish fillet mince, particularly to investigate whether addition of exogenous enzymes is necessary to produce FPH with high antioxidant potential compared to the FPH made through autolysis. Two batches of fish mince were used to assess consistency of the results obtained, and the effects of incubation time on the yields, extent of hydrolysis and antioxidant properties were monitored. Validase® BNP and Flavourzyme® 500L were selected as exogenous enzymes since the optimal temperature and effective pH ranges of these two commercial enzymes, i.e., pH 5.5–8.0 and 50–55 °C for Validase® BNP (Valley Research Inc., 2004), and pH 5.0–7.0 and 50 °C for Flavourzyme® 500L (Novozymes®, 2004) are close to the optimal temperature and pH ranges reported for cathepsin L-like endogenous proteases present in Pacific hake, i.e., pH 5.25–5.50 and 52–55 °C (Samaranayaka et al., 2006). Therefore, the same pH and temperature conditions could be used for making FPH by autolysis or exogenous enzymes (Fig. 1). Validase® BNP is an endopeptidase whereas Flavourzyme® 500L is a mixture of endo- and exopeptidases (Valley Research Inc., 2004; Novozymes®, 2004). Cathepsin L, the most active endogenous enzyme in Pacific hake fish muscle at pH 5.5 and 55 °C, is also an endopeptidase (Kang & Lanier, 2000). Protein hydrolysates made using these different enzymes most likely possess peptides of differing lengths and amino acid sequences that may determine their antioxidant capacities.

The yields, extent of hydrolysis measured as free amino group content, and results from different antioxidant assays of FPH are shown in Table 1. E + V-2h and E + F-2h, which were made by hydrolyzing fish proteins with both endogenous and exogenous enzymes, had the highest yields (64.2% and 63.8%, dry basis, respectively) as well as highest content of free amino groups indicating greater extent of hydrolysis. Yields of FPH made through autolysis ranged from 47.74% (E-1h) to 55.40% (E-2h) and did not show large increases after 1 h. Further, similar extents of autolysis were observed for FPH samples made using Inf-20 and Inf-30 fish mince. As expected, control

Table 1
Yield, extent of hydrolysis and antioxidant properties of fish protein hydrolysates (FPH) prepared by no (C), endogenous (E), Validase® BNP only (V) or with endogenous (E + V), Flavourzyme® 500L only (F) or with endogenous (E + F) enzyme treatments using Inf-20 and Inf-30 fish mince[a]

FPH	Yield (%)	α-Amino group content[b]		DPPH scavenging capacity[c] (%)		$ABTS^{\cdot+}$ scavenging capacity[d] (%)	Reducing power[c] (abs at 700 nm)	Fe^{2+} ion chelation capacity (%)[e]
	Inf-20	Inf-20	Inf-30	Inf-20	Inf-30	Inf-30	Inf-30	Inf-30
C-0h	NA[f]	0.216^{A}	0.252^{A}	NA	NA	28.2^{A}	NA	NA
C-1h	NA	0.225^{A}	0.297^{A}	NA	37.9^{E}	35.3^{B}	0.439^{D}	42.9^{F}
C-2h	14.8	0.234^{A}	0.314^{A}	1.61^{A}	36.3^{E}	41.8^{C}	0.545^{F}	46.4^{F}
E-0h	19.7	0.320^{A}	0.337^{A}	NA	51.4^{G}	46.9^{D}	$0.515^{E,F}$	$18.1^{C,D}$
E-1h	47.7	0.960^{B}	0.964^{B}	73.3^{E}	61.3^{I}	56.6^{E}	0.486^{E}	7.65^{A}
E-2h	55.4	1.25^{C}	1.13^{C}	60.4^{D}	41.5^{F}	61.9^{G}	$0.523^{E,F}$	$15.0^{B,C}$
E-3h	51.0	1.50^{D}	1.44^{D}	59.3^{D}	33.0^{D}	62.0^{G}	0.399^{C}	$18.0^{C,D}$
E-4h	48.2	1.36^{C}	1.41^{D}	41.0^{C}	23.7^{B}	$57.6^{E,F}$	0.323^{B}	23.8^{E}
E-5h	50.2	NA	1.54^{D}	NA	22.5^{B}	$58.4^{E,F,G}$	0.356^{B}	$14.8^{B,C}$
E-6h	52.1	1.75^{E}	1.83^{E}	12.2^{B}	26.4^{C}	$59.0^{E,F,G}$	0.353^{B}	6.65^{A}
E + V-1h	NA	2.71^{G}	$1.86^{E,F}$	NA	15.8^{A}	$61.1^{F,G}$	0.227^{A}	19.8^{D}
E + V-2h	64.2	3.25^{I}	2.38^{G}	18.8^{B}	16.6^{A}	$59.2^{E,F,G}$	0.257^{A}	$21.2^{D,E}$
V-1h	NA	1.52^{D}	1.20^{C}	NA	57.0^{H}	$57.2^{E,F}$	0.603^{G}	12.9^{B}
V-2h	41.5	1.91^{F}	1.98^{F}	58.1^{D}	27.0^{C}	$45.1^{C,D}$	$0.415^{C,D}$	19.2^{D}
E + F-2h	63.8	3.10^{H}		20.7^{B}				
F-2h	49.9	2.03^{F}		56.8^{D}				

[a] Average results from triplicate analysis. Values within a column bearing different superscript letters (A–I) are significantly different at $p < 0.05$.
[b] mM L-Leucine equivalents/g fish protein, measured using TNBS method.
[c] At 3 mg/ml assay concentration of FPH.
[d] At 66.67 μg/ml assay concentration of FPH.
[e] At 5 mg/ml assay concentration of FPH.
[f] NA = Not analyzed.

samples of C-0h, C-1h, C-2h and E-0h had undergone the lowest extent of hydrolysis indicating the inactivation of endogenous proteases during initial heating of these samples above 90 °C for 15 min.

For both FPH made using Inf-20 and Inf-30 fish mince, E-1h FPH possessed the highest ($p < 0.05$) DPPH radical scavenging power (Table 1). Autolysis beyond 1 h reduced the DPPH radical scavenging ability of FPH. Furthermore, FPH with high extent of hydrolysis, such as E + V-2h and E + F-2h, also possessed low DPPH radical scavenging power (Table 1). Decrease in antioxidant capacity with higher extent of hydrolysis was previously reported for FPH from mackerel fish fillet mince, in which both peptide content of FPH and the antioxidant potential in a linoleic acid autoxidation system were decreased with hydrolysis beyond 10 h using Protease N (Wu et al., 2003). Further, extensive hydrolysis (i.e., 67% degree of hydrolysis) of yellowfin sole frame protein with mackerel intestine crude enzyme also resulted in decrease of antioxidative activity of FPH in a linoleic acid peroxidation system (Jun et al., 2004), while DPPH radical scavenging ability of tuna cooking juice hydrolyzed with Protease XXIII also decreased beyond 2.5 h of hydrolysis (Jao & Ko, 2002).

Peptide solubility in a non-polar or an emulsion system can be decreased either by reducing the peptide chain length or by reducing the content of hydrophobic amino acids present in the peptide sequences (Saiga, Tanabe, & Nishimura, 2003). On the other hand, decrease of antioxidative activity of FPH with increasing hydrolysis time may also be due to breakdown of antioxidative peptide sequences formed during early stages of the hydrolysis process. Antioxidative peptides identified from fish sources in literature were reported to have molecular weights between 500 and 1500 Da (Je, Kim et al., 2005; Je, Park et al., 2005; Jun et al., 2004; Ranathunga et al., 2006; Wu et al., 2003). Many antioxidative peptides identified include hydrophobic amino acid residues valine, or leucine at the N-terminus of the peptides and proline, histidine, or tyrosine in the sequences (Chen, Muramoto, & Yamauchi, 1995; Uchida & Kawakishi, 1992).

All FPH made through 1–6 h autolysis showed high antioxidant potential in the ABTS assay at 66.67 μg/ml final assay concentration (Table 1). Compared to the DPPH assay which was conducted in ethanolic solution, ABTS assay was performed in an aqueous medium, and an increase in peptide solubility in aqueous medium with hydrolysis can be one reason for the increase in radical scavenging ability of FPH such as E-6h in the ABTS assay. Considering FPH made with Inf-30 fish mince using Validase® BNP only, antioxidant activity measured as DPPH and ABTS radical scavenging ability as well as the reducing power decreased significantly ($p < 0.05$) during hydrolysis from 1 to 2 h (i.e., V-1h to V-2h) (Table 1). When considering FPH made through autolysis, no significant differences ($p > 0.05$) were observed for ferric ion reducing capacity of E-0h, E-1h, and E-2h FPH. However, the reducing power did decrease further when the autolysis time was extended up to 6 h (Table 1). V-1h FPH possessed the highest ($p < 0.05$) reducing potential out of all FPH tested. According to these results, FPH samples including E-1h, E-2h, and V-1h possibly contained peptides which functioned as electron donors and could react with free radicals in polar as well as non-polar systems to form more stable products and terminate radical chain reactions.

Some proteins and peptides can chelate metal ions like Fe^{2+}, which can catalyze the generation of reactive oxygen species that accelerates lipid oxidation (Sarkar, 1987). Carboxyl and amino groups in the side chains of acidic and basic amino acids are thought to play an important role in chelating metal ions (Saiga et al., 2003). At 5 mg/ml assay concentration, the control FPH (C-1h and C-2h) possessed the highest ($p < 0.05$) Fe^{2+} ion chelating ability; FPH made through autolysis did not have high ion chelating ability at the concentrations tested, with the highest ($p < 0.05$) chelating power of only 24% observed for E-4h (Table 1).

The results from this study suggest that antioxidative FPH can be produced using Pacific hake fish fillet mince. Further, it was clear that addition of commercial proteases of Validase® BNP and Flavourzyme® 500L at 2% level, with or without endogenous muscle proteases, did not contribute in producing FPH with higher antioxidative potential compared to E-1h.

3.3. Antioxidative activity of FPH produced from fish mince with varying infection level

About 1000 fish samples received in our laboratory during the period from April 2004 to May 2005 were analyzed for *Kudoa* infection level. The majority of these fish samples were infected with *K. paniformis*, with average infection level of 5–20 fish captured per week in the range of 10^6–10^7 spores/g mince, and up to 10^8 in some weeks. For the purpose of assessing the effect of fish mince infection level (i.e., endogenous proteolytic activity) on production of FPH with antioxidant activity, fish with different infection levels were selected in order to prepare fish fillet mince batches with *K. paniformis* infection levels between 0 and 100×10^6 spores per g mince.

3.3.1. Extent of hydrolysis and yields of FPH

Extents of hydrolysis and yields of C-0h, E-1h, and E-6h FPH made from each of the eight fish mince batches varying in infection level are shown in Figs. 2a and b, respectively. Increasing infection level up to $\sim 10^7$ *K. paniformis* spores per gram mince was accompanied by large increases in free amino group content of E-1h and E-6h FPH during autolysis, with more moderate increases at infection levels beyond that (Fig. 2a). These results are consistent with those reported by Samaranayaka et al. (2006) that a strong linear relationship ($r = 0.957$, $p = 0.000$) was observed between spore counts below 10×10^6 per gram fish mince and the endogenous proteolytic activity; beyond that infection level, proteolytic activity remained high but did not change further ($p > 0.05$) with the infection level up to 100×10^6 spores per gram fish mince. Nevertheless, the

present study shows highest ($p < 0.05$) extent of autolysis in E-6h FPH that was prepared from fish mince Inf-100 with the highest infection level (Fig. 2a).

Yields of FPH also increased with fish mince infection level, with lowest yields for C-0h FPH (Fig. 2b). It was interesting to note that C-0h FPH yields were higher for fish mince batches Inf-30 and Inf-100 compared to other C-0h FPH from fish mince with lower infection levels, indicating possible autolysis of fish mince during storage or handling of samples prior to the actual hydrolysis process, due to high protease levels present in the heavily parasitized fish mince. For all fish mince batches, even though the free amino group contents were significantly higher for E-6h FPH than E-1h FPH (Fig. 2a), there were only small differences in yields (Fig. 2b). This may be due to proteolysis of peptides already present in the solution phase rather than of the less soluble intact fish muscle proteins during autolysis beyond 1 h. This was also confirmed by having comparatively similar total amino acid contents for E-1h (604 mg/g sample) and E-6h (555 mg/g sample) FPH made from Inf-30 fish mince (based on amino acid analysis; data not shown).

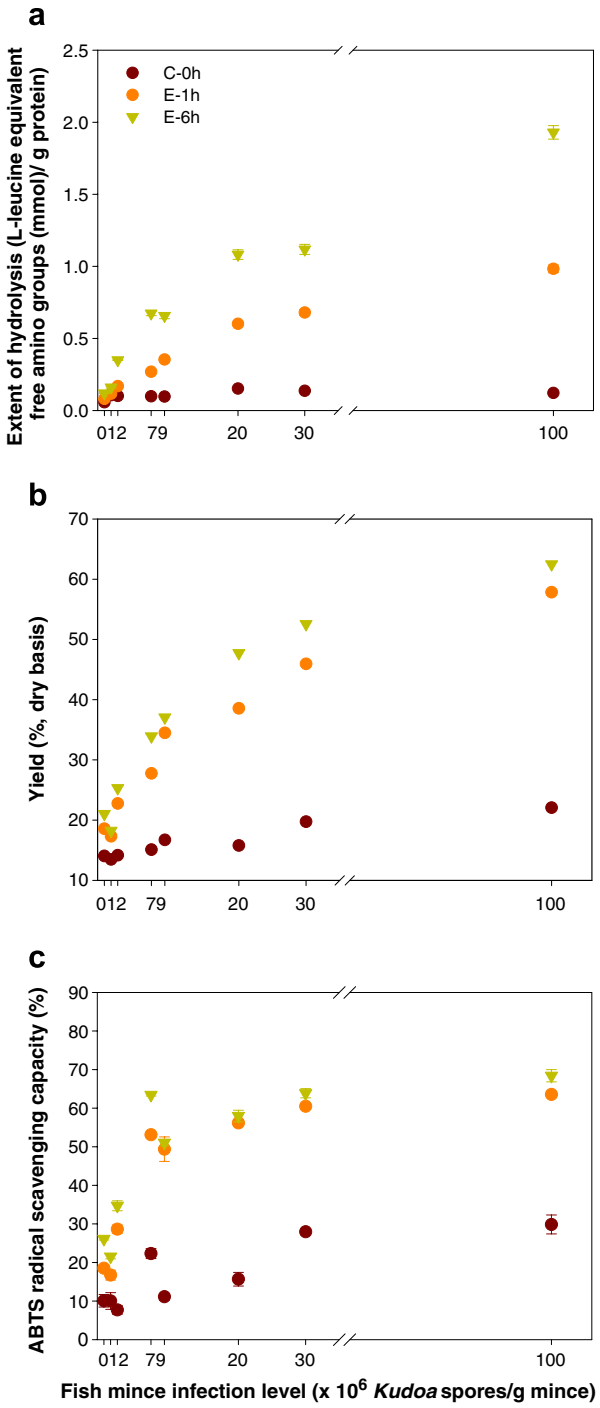

Fig. 2. Extent of hydrolysis (a), yield (b), and ABTS radical scavenging capacity (at 66.67 μg/ml assay concentration) (c) of fish protein hydrolysates (FPH) made by autolysis of fish mince batches with different *Kudoa paniformis* infection levels. Values and error bars in figures (a) and (c) are the mean and standard deviation from triplicate analyses.

3.3.2. ABTS radical scavenging capacity

Similar to the yields and extents of hydrolysis, control FPH (C-0h) possessed the lowest ABTS radical scavenging ability (Fig. 2c). E-1h and E-6h FPH made with fish mince batches Inf-7, Inf-9, Inf-20, Inf-30, and Inf-100 could reduce more than 50% of the ABTS radicals in the assay media at 66.67 μg/ml sample concentration. For these fish mince, little or no difference in ABTS radical scavenging activity resulted by autolyzing up to 6 h compared to 1 h autolysis, confirming that 1 h autolysis at 52 °C and pH 5.50 is sufficient in making antioxidative FPH from parasitized Pacific hake. The results from Section 3.2 also indicated that E-1h FPH possessed significantly ($p < 0.05$) higher DPPH radical scavenging ability compared to other FPH made through autolysis. However, it is important to note that E-1h FPH displaying >50% radical scavenging power at 66.67 μg/ml sample concentration in the ABTS assay was only obtained from fish mince infected by at least $\sim 10^7$ *K. paniformis* spores per g fish mince (Fig. 2c).

3.3.3. Linoleic acid peroxidation system

A linoleic acid peroxidation system was also used to assess antioxidant activities of E-1h FPH made with fish mince batches having different *K. paniformis* infection levels. In this study, $12.0 \pm 6.6\%$, $55.0 \pm 5.1\%$, $81.9 \pm 4.0\%$, and $85.7 \pm 1.8\%$ inhibition of lipid peroxidation in the system was observed after three days of storage with E-1h FPH (at 0.2 mg/ml assay concentration) made with Inf-0, Inf-2, Inf-30, and Inf-100 fish mince, respectively. Therefore, similar to the ABTS assay results (Fig. 2c), inhibition of lipid peroxidation by E-1h FPH produced from Inf-0 and Inf-2 Pacific hake fish mince was significantly ($p < 0.05$) lower than E-1h FPH from Inf-30 and Inf-100 fish mince.

3.4. Comparison of E-1h FPH to commercial antioxidants

E-1h FPH made from Inf-30 fish mince was assessed further for its antioxidant potential in comparison with some commercial antioxidants. TEAC value of E-1h FPH evaluated using the ABTS radical cation decolorization method was 262 ± 2 μmol/g freeze dried sample, compared to

14.29 and 2.27 mmol/g for BHA and BHT, respectively. Total antioxidant capacity measured as ORAC value for E-1h FPH was 225 ± 17 μmol Trolox equivalents/g freeze dried sample. This indicates higher total antioxidant activity measured as Trolox equivalents for Pacific hake E-1h FPH compared to ORAC values reported for different fruits such as strawberries, blueberries and raspberries (approx. 150, 200, and 100 μmol Trolox equivalents/g dry matter, respectively) (Prior et al., 1998; Wang & Lin, 2000). These fruits contain phenolic compounds which have been reported to function as antioxidants in improving human health (Margetts & Buttriss, 2003). Therefore, FPH such as E-1h made using Pacific hake could also potentially possess significant health benefits through the antioxidant function attributed to its constituent peptides and/or amino acids.

E-1h FPH inhibited lipid peroxidation in a linoleic acid model system by 73% and 90.8% at 42 and 162 h of incubation, respectively (Fig. 3). It is interesting to note that the antioxidant activity of E-1h FPH was comparable to that of BHA until 92 h of incubation, and higher ($p < 0.05$) than BHA when the incubation period was extended up to 162 h. Moreover, antioxidant potential of E-1h FPH in this linoleic acid/ethanol/water model system was higher ($p < 0.05$) than α-tocopherol, a well known lipid soluble natural antioxidant, during 92–162 days of storage at 40 °C. Inhibition of lipid peroxidation by BHT was, however, higher ($p < 0.05$) than that of E-1h FPH throughout the incubation period (Fig. 3).

A reduction of antioxidant activity of BHA was observed at 162 h of storage, while antioxidant capacity of α-tocopherol also decreased over storage time (Fig. 3). In contrast, comparatively high antioxidant activity was observed for Pacific hake E-1h FPH even at 162 h of storage. Therefore, E-1h FPH may have potential to be used as an antioxidant in oil-in-water emulsion type food systems to inhibit lipid peroxidation for a longer time period than that of BHA and α-tocopherol. Similar results were reported for DPPH radical scavenging capacity of a porcine myofibrillar protein hydrolysate which continued to act as an effective radical scavenger in a non-polar system even after 1 h of incubation with DPPH radicals, whereas the scavenging effect of α-tocopherol had reduced gradually over 1 h (Saiga et al., 2003). Peptides from the hydrolysates of yellowfin sole (Jun et al., 2004), Alaska Pollack (Je et al., 2005), conger eel (Ranathunga et al., 2006), and capelin (Shahidi & Amarowicz, 1996) also showed inhibitory activity in the linoleic acid model system. Shahidi and Amarowicz (1996) also reported the concentration dependency of antioxidative potential of harp seal protein hydrolysates in a β-carotene/linoleate model system, with a prooxidative effect at 2 mg/ml assay concentration, compared to a weak antioxidant effect ($p < 0.05$) at 0.2 or 0.4 mg/ml concentration after 45, 105–120, and 30–120 min incubation. Antioxidative activity of these protein hydrolysates could be due to the ability of peptides to interfere with the propagation cycle of lipid peroxidation, thereby slowing radical mediated linoleic acid oxidation.

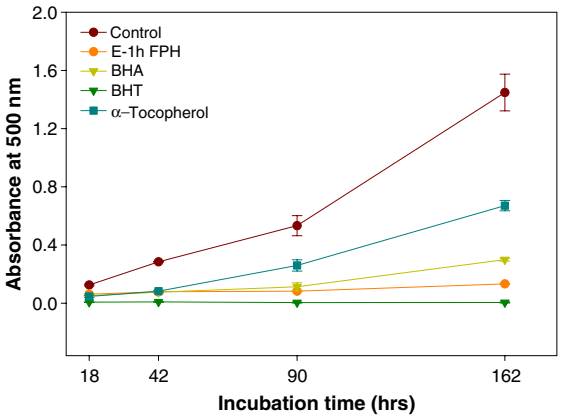

Fig. 3. Inhibition of lipid peroxidation by E-1h fish protein hydrolysate (FPH), BHA, BHT, and α-tocopherol at 0.02% sample concentration in a linoleic acid auto-oxidation system incubated at 40 °C for seven days. Values and error bars are the mean and standard deviation from triplicate analyses.

4. Conclusions

The present study clearly demonstrates that fish protein hydrolysates with antioxidant properties may be prepared using parasitized Pacific hake fish fillet mince through one hour autolysis at 52 °C and pH 5.50. The infection level of fish mince should be $\sim 10^7$ *K. paniformis* spores per gram fish mince or higher in order to obtain E-1h FPH with high antioxidant potential through autolysis. Since Pacific hake are commonly infected with *K. paniformis* at these high levels, the muscle from these fish possesses sufficiently high endogenous proteolytic activity for autolytic production of antioxidative FPH. Alternatively, FPH with high antioxidant potential may be produced from Pacific hake without considering the *Kudoa* infection level by conducting one hour hydrolysis with addition of 2% commercial enzymes such as Validase® BNP or Flavourzyme® 500L, after first inactivating the endogenous enzymes.

The potent antioxidant activity of E-1h FPH compared to BHA and α-tocopherol, particularly in inhibiting lipid peroxidation in a linoleic acid model system over prolonged storage, warrants further basic research to characterize the peptides that are responsible for these antioxidant properties, as well as applied research to investigate applications of the FPH as food and nutraceutical ingredients. This research is currently underway in our laboratory.

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