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Natural peptides with antioxidant activity from Atlantic cod and Atlantic salmon residual material

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Summary. Water-soluble peptides/proteins with molecular weight below 10 kDa were isolated from residual material of cod (liver, skin, and cod mix i.e. skin, frames, and viscera), and salmon (skin, and salmon mix i.e. skin, frames, and viscera) by cut-off filtration. Peptide motifs with reported bioactivity were identified in all samples by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) (Orbitrap), bioinformatics, and database search. Peptides with potential type 2 diabetes, cardio system, immunomodulation, prolyl endopeptidase (PEP), and antioxidant activity were detected. The potential antioxidant activity in the samples was confirmed by two antioxidant assays, namely hydroxyl radical scavenging activity (HRSA), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical cation decolorization assay. In these assays the salmon samples were found to possess higher antioxidant activity than the cod samples. All samples except the cod skin were found to have higher antioxidant activity than alanine-histidine (AH), a dipeptide with known antioxidant activity.
Industrial relevance. Residual material from fisheries and aquaculture makes up large quantities of material. Although previously regarded as waste this material has valuable components that are of interest for the biotech industry. The fractionation process utilized in this work offers the possibility for simple isolation of interesting peptides with antioxidant activity. This method should be of interest for the food industry and biotech industry for product development.
Keywords. bioactive peptides; antioxidant activity; residual material; Atlantic cod; Atlantic salmon

INTRODUCTION

The world fishery catches for all types of fish in 2011 were 90 400 000 tons.[1] Depending on the fish species, between 25 and 50% of the catch is not used for human consumption and ends up as residual material (residual material in this context includes trimming, fins, frames, heads, skin and viscera).[2,3] Taking into account these quantities, it is essential for the fishery sector to find valuable utilisation of these resources.[2] Up until recently, large quantities of these raw materials were sent to fish meal plants for processing into animal feed, while the rest was simply discarded.

Inspired by the recent increased interest in bioprospecting, and in particular marine bioprospecting, the hunt for bioactive components in residual material from the fishery industry has gained momentum. Recent studies have shown that residual material from fish contains peptides (< 20 amino acid residues) with biological activity.[4-7] Thus, there is a great potential to utilize this waste as starting material for generating valuable products. Fish derived bioactive peptides are proven to be a source of health enhancing components that potentially can be released during gastrointestinal digestion or food processing.[8] These peptides have various biological actions including antioxidant, cardiovascular (antithrombotic, hypocholesterolemic and antihypertensive), immunomodulatory, antimicrobial and opiate-like activities.[6-11] Many of these peptides are also multifunctional, being able to exert more than one effect. Since a large fraction of these bioactive peptides are small, numerous research groups and companies around the world have focused their attention on converting the remaining proteins from residual material into bioactive peptides by enzymatic treatment (i.e. hydrolysates).[7,10,12-15]

Antioxidants are molecules that protect biological targets against oxidative damage,[16] and are a major part of the individual defence mechanism. A range of the peptides present in residual material from fish possess bioactive motifs with antioxidant activity.[6,17,18] Antioxidant activity can be directly tested by *in vitro* assays,[19] such as oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) scavenging, hydroxyl radical scavenging activity (HRSA), and 2,2'-azinobis-(3-ethylbenzothiazole-6-sulponate radical cation (ABTS$^{\bullet+}$) decolourization assay. In this study, the two latter mentioned methods were used in order to test for antioxidant activity. Bioactive motifs can be located within a peptide/protein, which may lead to the biological activity being hampered or reduced due to steric hindrance. It is therefore important to test the actual activity by *in vitro* assays in order to verify the biological activity after peptides/motifs identification by LC-MS/MS.

In the present study, bioactive peptides with molecular weight below 10 KDa in residual material from Atlantic cod (*Gadus morhua*) (Figure 1a) and Atlantic salmon (*Salmo salar*) (Figure 1b) have been identified by liquid chromatography-mass

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spectrometry/mass spectrometry (LC-MS/MS) analysis combined with bioinformatics and database match towards an in-house database (BioPepDB).[6] In the LC-MS/MS analysis a range of peptides with proven antioxidant activity motifs were identified. The antioxidant activity of the samples were tested using two independent *in vitro* assays. The activity of these peptides/motifs was also compared to synthetic di-peptides (i.e. AH, VW, VK) where the former di-peptide has proven antioxidant activity.[20]

Figure 1. a) Atlantic cod (*Gadus morhua*); b) Atlantic salmon parr (*Salmo salar*).

MATERIALS AND METHODS

Sample collection. Atlantic cod was obtained from the fish market in Stavanger, Norway, and Atlantic salmon parr (approximately 500 g) was obtained from EWOS Innovation in Dirdal, Norway. The following types of material was utilized from the fishes: Cod liver (from three individuals), cod skin (from nine individuals) and cod residual material (i.e. the fish remains when the filet is taken out) (from nine individuals), salmon skin (three individuals), and salmon residual material (from three individuals).

Sample preparation. A summary of the samples is reported in Table 1. Samples were homogenized in PBS buffer (50 mM, pH 6, 1:1 (w/v), 4 ^{o}C) and centrifuged for 20 min (12 000 × g, 4 ^{o}C). The supernatant was then divided into 0.5 mL aliquots and filtered through a 10 kDa cut-off filter (Microcon Centrifugal Filter Devices, Millipore, 10 000 × g, 30 min, 4 ^{o}C) (Figure 2).

Table 1. Type and number of cod and salmon samples.

Atlantic cod (*Gadus morhua*)		Atlantic salmon (*Salmo salar*)	
Sample type (number of individuals)	Number of samples	Sample type (number of individuals)	Number of samples
Liver (3)	3	Mix (3)	7
Mix (9)	7	Skin (3)	5
Skin (9)	5		

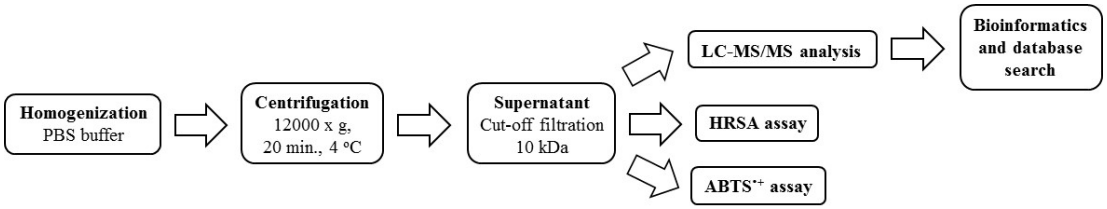

Figure 2. Flowchart showing the experimental design.

LC-MS/MS analysis. LC-MS/MS analysis were performed as previously reported from our laboratories,[21,22] with some modifications. The nanoflow liquid chromatography-mass spectrometer/mass spectrometer (LC-MS/MS) analysis were conducted using a Dionex Ultimate 3000 HPLC set up with a 300 µm i.d. × 0.5 cm Acclaim PepMap300 C_{18} trap column (Dionex) and a 75 µm i.d. × 15 cm Acclaim PepMap100 C_{18} analytical column (Dionex). The HPLC was coupled to an LTQ-Orbitrap (Thermo Scientific). The samples were loaded (5 µL) onto the trap column using 0.1% formic acid (VWR) in water (MilliQ, Elga) at a flow rate of 2 µL/min. The mobile phases for the analytical separation consisted of 0.1% formic acid in acetonitrile/water (2.5/97.5) (A) and 0.1% formic acid in acetonitrile/ water (80/20) (B), and was pumped with a flow of 300 nL/min. The peptides were separated on the analytical column using a linear gradient from 5% B to 60% B in 165 min after a 10 min delay post-injection. The gradient was then run to 100% B in 10 min, and held there for 30 min to wash the columns. A total run-time of 256 min was used, including the washing step and 30 min re-equilibration of the column. A PicoTip emitter (SilicaTip, New Objective) with 10 µm tip and without coating was used as an ESI interface. The electrospray voltage was set to 1 kV and no sheath gas was used. The mass spectrometer was used in positive mode. Full scans were performed in the Orbitrap in the *m/z* range from 200 to 2000, and data dependent MS/MS scans performed in the linear ion trap for the five most abundant masses with $z \geq 2$ and intensity $\geq$ 10 000 counts. Dynamic exclusion was used with 3 min exclusion after fragmentation of a given *m/z* value four times. Collision induced dissociation (CID) was used with collision energy of 35%, and with activation Q setting of 0.400 and activation time 30 ms for MS^2. The mass spectrometer was tuned daily and calibrated weekly using the calibration solution recommended by Thermo Scientific.

Bioinformatics. Bioinformatic analysis were performed as previously done in our laboratories,[21,22] with some modifications. The raw data files from the LC-MS/MS analysis were analysed using Proteome Discoverer 1.3 (Thermo Fisher Scientific Inc.) utilizing the Sequest search algorithm against the *Gadus morhua* translated EST database (downloaded from e!Ensemble 1st November 2012, containing 77408 sequences) for the Atlantic cod samples and the Teleost database (Tax. ID 32443, downloaded from NCBI on the 28/10/2013) for the Atlantic salmon samples. The precursor- and fragment mass tolerance was set at 10 ppm and 0.8 Da respectively. Oxidation of methionine (M) was set as a dynamic modification.

High and medium significant peptide confidence filter was set in Proteome Discoverer, with the following combination of charge (z) and X correlation: high significance: 1.9 (z = 2), 2.3 (z = 3) and 2.6 (z ≥ 4); medium significance 0.8 (z = 2), 1 (z = 3) and 1.2 (z ≥ 4). Furthermore, a decoy database search was performed with target false discovery rate, strict and relaxed parameters set to 0.01 and 0.05, respectively.

Bioactive peptide database search. The peptides identified by bioinformatics were search against the in-house database, BioPepDB, using an in-house Perl algorithm. The BioPepDB was organized as a text file, where the sequences were delimited by a new line. The algorithm compared the motifs (i.e. peptide sequences) to a fasta database, it counted the number of hits and reported the position of the motif for the relevant sequence. The algorithm was run from the command line and the output was a text file summarizing the findings.

The BioPepDB[6] contained 478 peptide motifs and was made using literature references up to April 2014. The database contained the following information: letter code, amino acid sequence, main activity, bibliographic reference, bioactive peptide source and related/studied species.

Hydroxyl radical scavenging activity (HRSA) assay. A series of sample dilutions (70 μL of samples with concentration 1.0, 0.5, 0.25, 0.125 mg/mL) and controls (70 μL), buffer $[Fe(phen)_3]^{2+}$ (30 μL of a 3.75 mM solution) and H_2O_2 (10 μL of a 42.33 mM solution) were added to a 96 well plate and incubated in the dark at 37 °C for 1 h. The optical density (OD) was then measured at 540 nm. Reactivity was expressed as % of HRSA (Equation 1, where AbsS = Absorbance sample; AbsNC = Absorbance negative control; AbsBC = Absorbance blank control). Three synthetic di-peptides (purchased from AnaSpec); alanine-histidine (AH) (1.0 mg/mL, 70 μL), with previously reported antioxidant activity,[20] and valine-tryptophan (VW) (1.0 mg/mL, 70 μL), and valine-lysine (VK) (1.0 mg/mL, 70 μL), with previously reported cardio system activity,[23,24] were also tested in the HRSA assay.

$$\text{HRSA \%} = (\text{AbsS} - \text{AbsNC})/(\text{AbsBC} - \text{AbsNC}) \times 100\% \quad \text{(Equation 1)}$$

2,2'-azinobis-(3-ethylbenzothiazole-6-sulponate radical cation ($ABTS^{\bullet+}$) decolorization assay. A 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) dilution series (0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 mM) was made by diluting a standard solution of Trolox (2.5 mM) with PBS (5 mM, pH 7.4). Each of the Trolox dilutions was mixed with $ABTS^+$ radical solution. OD values were measured at 734 nm, after 6 min incubation at room temperature in the dark.[25,26]

$ABTS^+$ radical solution (3.7 mM) was prepared by mixing 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) solution (7.4 mM) with potassium persulfate (2.6 mM), 1:1 (v:v). The solution was reacted in the dark at room temperature for approximately 20 hours and then diluted with PBS (5 mM, pH 7.4) until reaching an absorbance of 1.116 (734 nm). Fish samples were diluted in PBS (5 mM, pH 7.4), 1.5 mg protein/mL sample. Fish samples were then mixed with diluted $ABTS^+$ radical solution (3.7 mM) and incubated in the dark for 30 min at room temperature. OD values were measured at 734 nm. Salmon mix samples were further diluted until OD values were within the Trolox standard curve. Activity was expressed as μmol Trolox equivalent (TE)/mg protein.

Total protein concentration. The total protein concentration of the samples was measured by the Bradford method according to the literature procedure.[27]

RESULTS

Total protein concentration. The mean protein concentration of cod liver, cod mix, and cod skin was 6.577, 2.587, and 0.880 mg/mL, respectively. Salmon mix and salmon skin had a mean protein concentration of 4.667 and 2.485 mg/mL.

Bioactive peptide motif identification. Two samples from each sample group were analysed by LC-MS/MS followed by bioinformatics analysis and in-house BioPepDB matching. Peptides/motifs with bioactive properties were identified in all fish samples (Table 2, a list of all identified peptides can be found in Table S1 in supplementary material).

Hydroxyl radical scavenging activity (HRSA)

The results from the antioxidant activity assay are reported in Table 3. In general, the mean antioxidant activity was higher in salmon samples compared to cod samples. All samples exhibited a trend of proportionality between antioxidant activity and protein concentration (Figure 3).

Lower antioxidant activity was detected in the three synthetic di-peptides compared to the activity measured for the fish samples at the same concentration (1.0 mg/mL). The synthetic di-peptide AH displays a higher % of HRSA activity than the other two tested di-peptides, viz. VW, and VK.

$ABTS^+$ radical scavenging activity. The results from the $ABTS^+$ radical scavenging assay are reported in Table 4. These data were in agreement with the one obtained from the HRSA assay. Salmon samples generally had higher antioxidant activity than cod samples.

Table 2. Bioactive peptide motifs found in cod liver, cod mix, cod skin, salmon mix, and salmon skin.

Sample type Activity	Cod liver	Cod mix	Cod skin	Salmon mix	Salmon skin
Antioxidant	AH, EL, FKK, FL, GGE, IKK, LPF, MY	AH, EL, FL, IKK	AH, FL	AH, EL, FL, GGE, MQIFVKTLTG, MY, PEL	AH, EL, FIKK, FL, GGE, IKK, MY, PEL
Cardio system	AP, DP, ER, EY, FE, FY, GPM, GPV, IY, KLP, KP, KY, LAP, LEQ, LF, LGP, LKA, LL, LY, MF, PE, RVY, RY, ST, VK, VM, VR, WA, YL, YP, VY	AFL, AP, APL, AVF, DP, ER, EY, FE, FY, GPV, IY, KLP, KP, KY, LAP, LF, LKA, LKP, LL, LRP, LY, PE, RY, ST, VFK, VK, VM, VR, VY, WA, YL, YN, YP	AP, DP, IPA, IVE, KLP, LGP, LL, PE, ST, VK, VM, YL, KY	AFL, AP, APL, DP, ER, EY, IAP, IPA, IVE, IW, KP, KY, LGP, LKP, LL, PE, ST, VK, VM, VW, VY, YL, YN, YP	AEL, AKK, AP, APL, DP, ER, EY, FY, GTG, IVE, KP, KW, KY, LAP, LF, LL, LW, LY, PE, ST, VK, VM, VR, VY, YN, YP
Immunomodulation	YG	YVL	YVL	-[a]	YQY
PEP inhibitor	VF	VF	VF	-[a]	VF
Type 2 diabetes	-[a]	GPL	-[a]	GPL	GPL

[a]Not detected; PEP = prolyl endopeptidase; R = arginine; F = phenylalanine; H = histidine; I = isoleucine; L = leucine; K = lysine; T = threonine; W = tryptophan; V = valine; A = alanine; N = asparagine; D = aspartic acid; C = cysteine; Q = glutamine; E = glutamate; G = glycine; P = proline; S = serine; Y = tyrosine.

Table 3. Antioxidant activity, reported as HRSA % ± standard deviation. The antioxidant activity from cod skin was based on one sample per concentration, due to the low protein concentration. na = not applicable; nd = not detected, AH = Alanine-Histidine, VW = Valine-Tryptophan, VK = Valine-Lysine, GSH = Reduced Glutathion.

Concentration (mg/mL)		1.0	0.5	0.25	0.125
Sample type (number of samples)					
Cod	Liver (3)	65 ± 19.8	51 ± 17.4	44 ± 15	27 ± 16.7
	Mix (7)	82 ± 35.2	56 ± 26.6	42 ± 18.6	25 ± 15.8
	Skin (1)	60	34	29	22
Salmon	Mix (7)	98 ± 16.8	73 ± 11.7	59 ± 16.9	49 ± 17.8
	Skin (5)	125 ± 21.3	88 ± 49.9	53 ± 16.3	25 ± 21.1
Dipeptides	AH (na)	64	nd	nd	nd
	VW (na)	19	nd	nd	nd
	VK (na)	39	nd	nd	nd
Positive control	GSH (na)	94	45	19	11

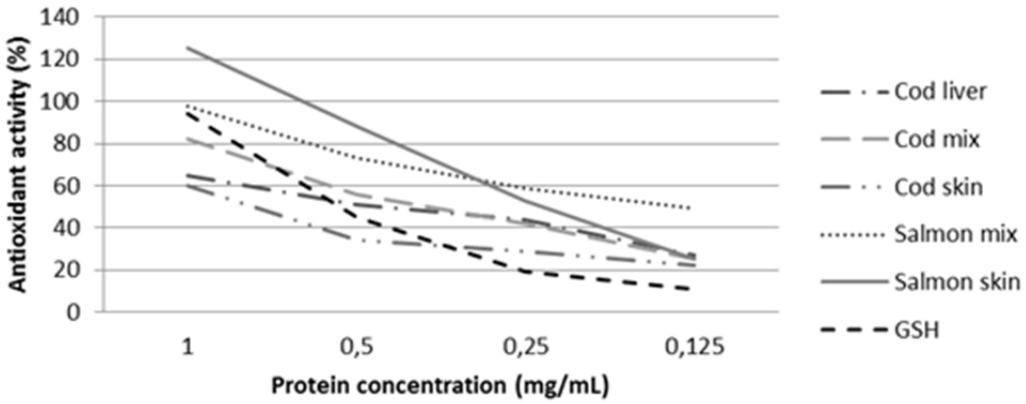

Figure 3. Antioxidant activity results of the hydroxyl radical scavenging activity (HRSA) assay (GSH = positive control).

Table 4. $ABTS^{+}$ radical scavenging activity in cod and salmon samples expressed as μmol Trolox Equivalents (TE)/mg protein. The results for skin, of both cod and salmon, were based on one replicate due to low protein concentration.

Sample type (number of samples)		μmol TE/ mg protein
Cod	Liver (3)	93.80 ± 29.12
	Mix (7)	112.52 ± 5.6
	Skin (1)	109.8
Salmon	Mix (7)	637.52 ± 190.12
	Skin (1)	141.59

DISCUSSION

Oxidative stress caused by reactive oxygen species (ROS) is one of the major factors in ageing,[28] and age-related diseases such as cancer,[29] Alzheimer's disease,[30] and Parkinson's disease.[31] The major intracellular source of ROS is mitochondrial generated superoxide anions ($O_2^{\bullet -}$).

In order to balance the prooxidant/antioxidant conditions, aerobic organisms contain antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase as well as endogenous antioxidant molecules such as ubiquinol,[32] and melatonin (Figure 4).[33] Dietary supplements of antioxidants, such as α-tocopherol (Vitamin E), ascorbic acid (Vitamin C), and peptides, also play an important role.[34-36]

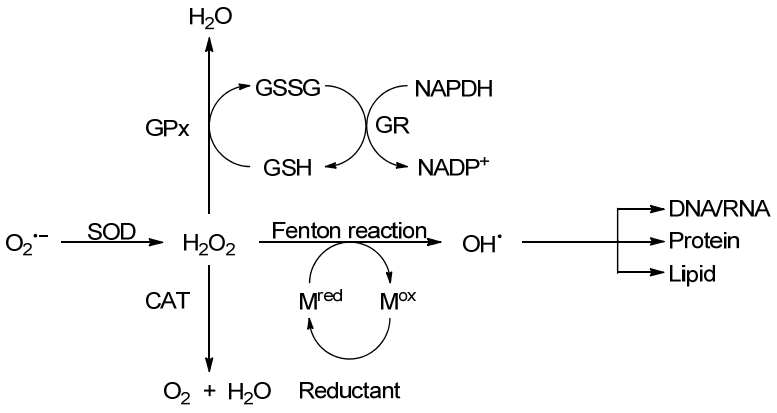

Figure 4. Outline of the defence systems against reactive oxygen species. M^{red} = reduced form of the metal; M^{ox} = oxidized form of the metal; SOD = superoxide dismutase; GSH = glutathione reduced; GSSG = glutathione oxidized; GR = glutathione reductase; GPx = glutathione peroxidase; CAT = catalase; NADPH = reduced nicotinamide adenine dinucleotide phosphate; $NADP^{+}$ = oxidized nicotinamide adenine dinucleotide phosphate.[37]

Peptides with antioxidant activity from natural raw material could help to decrease oxidative stress. Research has therefore been focused on documenting the presence of bioactive peptides in various matrixes. Bioactive peptides have been identified in residual material from a range of fish species,[10] such as Atlantic salmon,[4] Atlantic herring,[6] and Alaska pollack.[38] These peptides can be inactive as part of a parent protein and released naturally during gastrointestinal fermentation,[39] or they can be released by enzymatic hydrolysis prior to consumption.

In the present study, natural bioactive peptides were identified by using a combination of mass spectrometry (LC-MS/MS, Orbitrap), bioinformatics and database search up against an in-house database of peptides with known bioactivity. The results from the analysis of cod liver, cod mix, and salmon mix samples contained a number of peptides with amino acid combinations (motifs) known to possess cardio system activity. These samples also had many identified motifs with well documented antioxidant properties. In addition, a few motifs with immunomodulation, prolyl endopeptidase (PEP) inhibitor, and activity towards type 2 diabetes were found (Table 2). Few bioactive peptides were identified in skin samples of both cod and salmon. This is probably due to the low peptide/protein concentration in these samples, most likely due to low amounts of water soluble peptides/proteins in fish.

The utilised MS approach was valuable in order to document and identify the presence of peptides/motifs with well-known biological activity, as previously reported for herring.[6] However, the activity needs to be documented since the bioactive motifs detected by MS could be present in conformations that are not bioactive due to steric hindrance. In this study we were particularly interested in documenting and confirming the antioxidant activity of the samples. Two independent *in vitro* antioxidant assays were therefor performed in order to evaluate the activity of the samples. The HRSA assay measure the ability that antioxidant molecules have to neutralize hydroxyl radicals ($OH^{\bullet}$)[40,41] generated by a Fenton-like reaction.[42,43] The $ABTS^{+}$ radical scavenging assay measure the ability to reduce $ABTS^{\bullet+}$, which is generated upon treatment of ABTS with potassium persulfate. Three synthetic di-peptides (AH, VW, and VK) were included in the HRSA assay as positive controls. Where the former di-peptide, viz. AH, is a well proven antioxidant,[20] and VW has antioxidant activity as its secondary activity.

All the analysed fish samples (cod liver, cod mix, salmon mix, and salmon skin) had higher hydroxyl radical scavenging activity than AH at the concentration of 1.0 mg/mL, thus confirming that the peptides/motifs identified by MS were in a confirmation enabling them to function as antioxidants. The synthetic di-peptide AH displayed higher % of HRSA activity, as expected, compared to the other synthetic dipeptides VW and VK, known to have cardio system activity as the major function.[23,24]

The antioxidant activity detected in salmon mix and skin by the HRSA assay exceeded 100% for some tested concentrations (i.e. the highest ones). This assay results are reported as % of hydroxyl radical ($OH^{\bullet}$) scavenging capacity of antioxidants in the sample. In theory, the amount of hydroxyl radicals is proportional to the amount of H_2O_2 initially added to each sample due to the reaction with $Fe(phen)_3^{2+}$. The HRSA % is based on the scavenging of a fixed concentration of hydroxyl radicals ($OH^{\bullet}$).[40] A percentage exceeding 100 could possibly be related to the presence of oxidized species already present in the sample.

The high lipid content in salmon resulted in poor phase separation during centrifugation of the salmon fillet tissue appeared to be caused by the high fat content, a challenge that has previously been reported by others.[44] During the extraction of water soluble peptides, some fish oil may have been included in the samples. Problems related to the high lipid content in salmon have previously been experienced by Picot et al.[45] Fish oil is readily susceptible to autoxidation generating peroxides.[46] If these peroxides, in addition to the added H_2O_2, generated hydroxyl radicals in reaction with $Fe(phen)_3^{2+}$, the antioxidant peptides could reduce more free radical species than those generated from H_2O_2 in the assays and by such means resulting in results exceeding 100%.

Although the salmon samples had a lower motif count then the cod liver and cod mix samples as identified by LC-MS/MS analysis the activity for these samples were higher. This difference in activity could be related to the presence of some specific peptides in the salmon samples. In fact, the salmon mix samples had two peptides that were not identified in the cod samples (i.e. MQIFVKTLTG and PEL) and the salmon skin samples had the peptides FIKK and PEL that were not present in the cod samples (Table 3).

The fact that the antioxidant activity for three out of the five sample types (i.e. cod mix, salmon mix and salmon skin) was significantly higher than the one recorded for the di-peptide AH show that residual material from the fish processing industry has a potential as an antioxidant supplement source. Taking into consideration the large quantities of residual material from fish available worldwide, there is a good potential in converting this material into products for human consumption with health beneficial properties. The sustainability of the future fisheries requires that more of the residual material is utilized in a more valuable way.

CONCLUSIONS

Water soluble bioactive peptides with antioxidant, cardio system, immunomodulation, and PEP inhibitor activity were identified by LC-MS/MS analysis of residual material from cod and salmon. The antioxidant activity of the samples was confirmed by two independent *in vitro* assays. It was found that the salmon samples had a higher antioxidant activity then the cod samples, which likely is due to the presence of a few peptides in the salmon samples that were not found in the cod samples. However, all fish samples had higher antioxidant activity than the di-peptide AH (positive control), a peptide with well proven antioxidant properties. The sample preparation strategy utilized in this study is simple and can easily be scaled up.

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