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Australian Eastern School Whiting *(Sillago flindersi)* – a potential source of metal reducing agents and free radical scavengers

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Abstract

Underutilised Australian Eastern School Whiting *(Sillago flindersi)* fish was investigated for *in-vitro* bioactivities, after exposure to fish endogenous and gastrointestinal (pepsin and pancreatin) enzymes. The study comprised of storing fish at chilled (4 and 6°C) and freezing (-18°C) temperatures for 7 and 28 days, respectively. Hydrolysis by endogenous enzymes only, resulted in increased bioactivities for the 4°C samples, whereas significant decreases ($p < 0.05$) were observed for the 6 and -18°C samples. However, bioactivities of these samples increased significantly ($p < 0.05$) after further hydrolysis under simulated digestion conditions. Proteolysis by digestive enzymes, mainly pancreatin considerably enhanced the antioxidant activities. To benefit from the health properties of eastern school whiting fish, it is suggested to consume the fish fresh. The intent is to enhance full use of fish and not certain parts such as fish oil. For proper utilization and sustainability, whole fish must be used.

Introduction

Endemic to South-Eastern Australia, eastern school whiting *(Sillago flindersi)* is one of the most important fish species in Australian fishery, considering the annual landings by the New South Wales Ocean Trawl Fishery exceeding 800 t annually (Wild Fisheries Research Program 2010). However, owing to their small shaped size, delicacy, slight sweetness, bony and fine texture, such species are often discarded, posing a significant sustainability issue and severe economic loss to the seafood industry (Wild Fisheries Research Program 2010).

Fish, a poikilothermic, aquatic chordate, is nutritionally and economically important; and must be utilized efficiently. From a nutritional perspective, fish constitute a rich source of essential fatty acids, vitamin and minerals and majorly, essential amino acids (high protein quality) (Khora, 2013). Besides the recovery of fish oil, with potential therapeutical benefits and to prevent fish wastage, a novel technique; enzymatic hydrolysis, could be developed to also recover proteins or their derivatives peptides (Nurdiani *et al*., 2015). Fish is a rich source of easily digestible proteins, which are highly sensitive to proteolytic digestion, with a digestibility of more than 90% (Khora, 2013). This high digestibility could correspond to the high proportion of myofibrillar proteins (60-80%) as compared to collagenous fibres (3-10%) in fish muscle (Delbarre-Ladrat *et al.*, 2006).

Myofibrillar proteins from fish muscle undergo hydrolysis or proteolysis during storage at refrigerated or freezing temperatures by the endogenous enzymes present in the fish muscle. These enzymes are of mainly the calpain and/or the cathepsins family. Their enzymatic activity impacts on the breakdown of the proteins (Delbarre-Ladrat *et al*., 2004). For instance, eastern school whiting exhibited high endogenous activity of calpain-like enzyme in comparison to silver warehou and other fish species (Ahmed *et al*., 2013). This high enzymatic activity may cause rapid softening during chilled storage of fish fillets, affecting textural quality, constituting further reasoning for its low commercial value. Interestingly though, autogenous muscle enzymes, active at post mortem storage conditions, often lead to proteolysis of fish muscle, which may either cause quality losses during cold storage (Ahmed *et al*., 2013) or release of peptides, with beneficial in vitro bioactivities, such ACE-inhibitory activity and potential anti-oxidant capacities (Manikkam *et al*., 2016a). It is therefore essential to identify if eastern school whiting fillets could similarly exert significant in vitro bioactivities, which could be an asset in improving its commercial

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value.

Moreover, it is equally crucial to explore the stability of these proteins/peptides in the gastrointestinal tract (GIT) upon digestion. *In vitro* methods, known as the simulated gastrointestinal digestion (SGID) simulate the human GIT and are extensively used, since they are rapid and safe (Medenieks and Vasiljevic, 2008; Samaranayaka and Li-Chan, 2008; You *et al.*, 2010). Therefore, we hypothesized that manipulating the storage conditions of fish fillets could release an array of polypeptides with potential in vitro bioactivities. Hence, the aim of our study was to explore the combined effects of refrigerated and freezing storage on the quality-related changes as well as bioactivities of eastern whiting during human digestion. It is important to maximize the utilization of this species to boost up the seafood industry sustainability.

Materials and Methods

Samples collection

Barwon Foods (Seafood and Food Service Specialists; North Geelong, Australia) generously provided the fresh eastern school whiting (ESW), which were conveyed on ice in polystyrene box to our Food Science laboratory (Werribee campus, Victoria University), within 24 h of catch. The fish were processed immediately once arrived to our laboratory.

Chemicals

Angiotensin-I-converting enzyme, Nα-Benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPNA), copper sulphate, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Hippuryl-Histidyl-Leucine, iron (III) chloride, pancreatin (P7545; porcine pancreas), pepsin (P7000; from porcine stomach mucosa), potassium ferricyanide, pyridine, pyrocatechol violet, sodium phosphate, trichloroacetic acid (TCA), and trypsin (Type II-S from Porcine pancreas) were all purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Acetonitrile, dimethyl sulfoxide (DMS), ethyl acetate, glacial acetic acid and trifluoroacetic acid (TFA) were from Merck Pty Ltd (Darmstadt, Germany). All other chemicals used for preparation of buffering solutions were of analytical lab grade.

Experimental design

The experimental design (Figure 1) was established to investigate the impact of fish endogenous enzymes and in combination with digestive enzymes on the release of crude peptides from fish fillets during storage at chilled and frozen

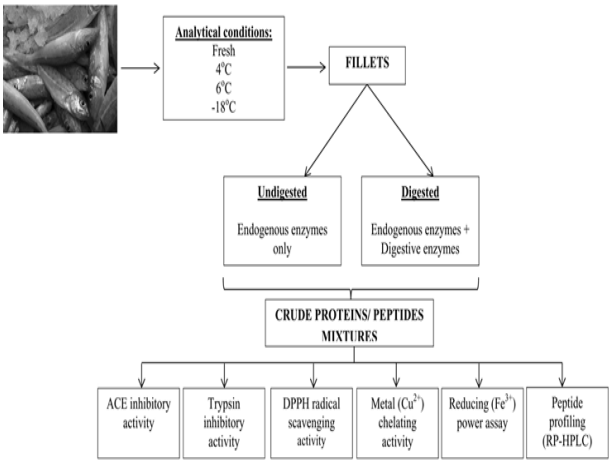

Figure 1. Experimental design

temperatures. Whole fish, with approximate length of 20-30 cm, were separately packaged, randomly divided, placed in labelled locked containers and stored at 4, 6 (chilled) and -18°C (frozen), for 7 and 28 days respectively. The reasoning for the selection of storage temperatures and times has already been underlined by Manikkam *et al.* (2016a), as a similar experimental design was developed for silver warehou fish. On day 0, the fish were stored whole at the selected conditions since ESW are small sized and delicate to handle. Sampling small number of fish each day minimized contamination and biased results. On each sampling day, fish were removed from storage and fillets acquired aseptically to avoid unnecessary contamination. Same procedure was repeated on 3 different occasions, reflecting seasonal differences; and results were presented as means of three replicates and two subsampling.

Processing of fillets (undigested and digested)

Ten grams of fish fillets were minced using a mortar and pestle, followed by addition of 100 ml MilliQ water and homogenization. The homogenate was centrifuged at 5600 x g (JA20 rotor, Beckman Instruments Inc., Palo Altro, CA, USA) for 20 min at 4oC. Frozen fillets were defrosted in the cold room for 2-3 hours prior to filleting. The supernatant obtained from each crude homogenate after centrifugation was filtered into clean tubes, which were stored at -20°C until further assayed. Moreover, a simulated GI digestion, described by Manikkam *et al.* (2016a) was carried out to study the combined effects of endogenous and GI enzymes on generation of bioactive peptides from ESW fillets.

In vitro *bioactivities of fish crude extracts*

In our study, five important in vitro bioactivities of the obtained fish crude extracts were analyzed according to Manikkam *et al.* (2016a). The assayed bioactivities included ACE- and trypsin- inhibitory

Table 1. *In vitro* bioactivities of undigested fillets upon storage at chilled and frozen conditions

	ACE Inhibition %			Trypsin Inhibition %			DPPH RSA %			MCA %			RPA %		
Storage temperatures	4°C	6°C	-18°C	4°C	6°C	-18°C	4°C	6°C	-18°C	4°C	6°C	-18°C	4°C	6°C	-18°C
Sampling days															
1	34.35	32.93	34.15	36.41	36.09	36.41	46.93	45.86	45.61	60.02	54.99	54.07	17.50	17.10	17.40
3	36.59	31.30	33.85	36.87	35.02	36.41	48.30	44.29	41.09	63.21	48.13	48.13	17.80	15.80	16.30
5	38.61	28.05	32.32	37.33	31.80	33.79	49.56	37.89	37.33	65.73	40.14	43.79	18.50	15.10	14.00
7	40.24	26.22	29.27	38.63	28.72	30.72	52.76	34.13	35.26	67.33	34.88	38.31	19.50	14.00	12.80
14	nd	nd	26.83	nd	nd	27.42	nd	nd	32.43	nd	nd	34.42	nd	nd	10.30
21	nd	nd	24.39	nd	nd	24.88	nd	nd	29.42	nd	nd	31.91	nd	nd	9.50
28	nd	nd	21.95	nd	nd	18.89	nd	nd	25.28	nd	nd	26.88	nd	nd	7.70
SEM		0.22			0.12			0.01			0.00			0.00	

The means present average of 6 independent observations (≥ 6); SEM: pooled Standard Error of Mean
DPPH RSA: DPPH radical scavenging activity; MCA: Metal chelating activity; RPA: Reducing power assay; nd: not determined
On day 0, the *in vitro* activities of fresh raw fillets were as follows: ACE inhibition: 35.37%; Trypsin inhibition: 37.48%; DPPH RSA: 46.36%; MCA: 56.82%; and RPA: 17.50 %

activities in addition to determination of antioxidant power by i) measuring the free radical DPPH scavenging activity, ii) metal (copper) chelating ability and iii) acting as a reducing agent by donating an electron to Fe (III) ions.

Reverse-phase HPLC analysis of crude peptide extracts

Crude peptide extracts before and after simulated digestion were profiled using a Varian HPLC (Varian Analytical Instruments, Walnut Creek CA, USA) equipped with a reverse-phase C-18 monomeric column - 5µm, 300 Å, 250 mm x 4.6 mm and a guard column (Grace Vydac, Hesperia, CA, USA) (Donkor *et al.*, 2007). Samples were applied using a 10 µl injection loop. The peptides were eluted by a linear gradient from 100% to 0% solvent (0.1% TFA in deionised water) in solvent B (0.1% TFA in 90% v/v, acetonitrile in deionised water) over 90 min. All samples and mobile phase solvents were filtered through a 0.45 µm membrane filter (Schleicher and Schuell GmbH, Germany). Peptides separation was conducted at room temperature with a flow rate of 0.75 ml/min. The eluted peptides were monitored at 214 nm using a Varian 9050 variable wavelength UV/Vis detector.

Statistical analysis

All experimental analyses were conducted using a randomized, split plot in time blocked design. The digestion time was included as an additional factor in a subplot when required. The replications served as the block. The experimental design was triplicated and subsampled twice resulting in at least 6 independent observations ($n \geq 6$). Results were analysed using a General Linear Model (GLM) procedure of the Statistical Analysis System (SAS). The level of significance was preset at $p < 0.05$.

Results and Discussion

Impact of fish endogenous enzymes and storage conditions on in vitro bioactivities

Table 1 indicates that hydrolysis of fish muscle protein in the presence of endogenous enzymes is possible. However, it is crucial to determine the effects of storage conditions (temperature and time) on the hydrolytic ability of these enzymes in releasing potential bioactive peptides. Storage temperature and time had significant impact ($p < 0.05$) on releasing peptides from fish samples, with varying degree of bioactivities (Table 1). Chilled samples, at 4°C showed increasing trend of ACE inhibition, DPPH radical scavenging and metal chelating activities from 0-7 days. A similar pattern was observed with crude extracts from Australian silver warehou fish (Manikkam *et al.*, 2016a). Endogenous enzymes may be more active at 4°C than at 6 or -18°C. Moreover, the stability and/or autolytic activity of fish enzymes post mortem are also fish species-specific (Delbarre-Ladrat *et al*., 2004; Ahmed *et al*., 2013).

However, trypsin inhibition of 4°C samples declined from 37.47% (day 0) to 36.87% (day 3) and increased slowly from 4-7 days during the cold storage. The rate of hydrolysis/degradation of bioactive peptides by fish endogenous enzymes during the one week of storage likely depended on the type of peptide (amino acid sequence) formed. The end results may indicate that some peptides of interest may disappear and new ones may appear during prolonged storage (Donkor *et al*., 2007). On the other hand, extended storage of fish fillets at 4°C could have potentially increased the trypsin inhibition of the crude extracts; nonetheless from a consumption viewpoint, fish stored at 4°C for more than 1 week would be drastically loaded with microbial organisms and could be detrimental to human health. But, should the peptides indicate high

trypsin inhibition after 1 week of storage at 4°C, fish protein hydrolysates could potentially be developed (Kristinsson and Rasco, 2000; Cudennec *et al*., 2008).

In contrast, a reduction in bioactivities at 6°C was observed (Table 1). Fish flesh is subjected to spoilage during higher temperatures, with the growth of psychrophilic bacteria (Mol *et al.*, 2007). These bacteria were possibly using fish proteins and peptides as energy source and released amino acids for growth (Kristinsson and Rasco, 2000) hence, reducing the release of potential bioactive peptides from ESW fillets at 6°C. Moreover, as explained by Manikkam *et al.* (2016a), the difference in chilled temperatures by only 2°C would have been enough to create favourable conditions within the fillets matrix to promote microbial activity.

Freezing has long been recognised as an effective means of preservation to extend shelf life of fish. Nonetheless, freezing and frozen storage of fish muscle may however lead to negative effects, such as denaturation and aggregation of especially myofibrillar proteins. These conformational changes often result in altered functional properties (solubility, water holding capacity), changed textural attributes (hard, dry texture) and juiciness (Barros *et al.*, 1998). Moreover, freezing-thawing cycle induces biochemical changes owing to mechanical damage, denaturation of muscle proteins and loss of water-holding capacity (Hallier *et al*., 2008). Furthermore, myosin, the major contractile proteins of skeletal muscle become unstable and easily denatured due to formation of disulphide bonds, as water is separated in the form of disruptive ice crystals (Ramírez *et al*., 2000). Therefore, hydrophobic amino acid groups normally associated with the interior of a protein molecule become exposed to biochemical damages.

In addition, freezing and thawing may result in i) fragmentation of cell membranes and lysis of intra-cellular organelles, and ii) decline in myosin and actomyosin Ca^{2+}-ATP-ase activities, resulting in a change of the myosin head. These changes could result in unfolding tertiary conformation of myosin owing to weakening in intra-molecular hydrophobic bonds (Tejada, 2001). The above explanation supports the significant decline in examined *in vitro* bioactivities ($p < 0.05$) observed for the frozen samples, in accordance with Manikkam *et al.*, (2016a), whereby a similar tendency was perceived with silver warehou fish.

During muscle storage, cathepsins B and D may be released from the lysosomal matrix into the cytoplasm and intracellular spaces as a consequence of lysosomes breakdown (Bechet *et al.*, 2005). In this regards, ESW demonstrated lower cathepsin D activity compared to silver warehou (Ahmed *et al.,* 2013), which likely explained the lower bioactivities at 6°C and -18°C in the present study. Additionally, cathepsins B and L activities increased significantly during post mortem storage in both super-chilled and ice-stored Atlantic salmon muscle (Gaarder *et al*., 2012), suggesting that i) the principal cause of post mortem degradation of fish muscle may be attributed mainly to cathepsins B and L and ii) more proteolytic peptides may be released as a result of such hydrolysis. Consequently, the high antioxidant activities observed with ESW at 4°C, 6°C and -18°C in the current study.

Synergistic effects of endogenous enzymes, pepsin and pancreatin on the bio-functionalities of fillets

Hitherto, our study has elucidated that a mixture of crude polypeptides, oligopeptides and/or peptides can be generated during storage over a period of time, by the hydrolytic nature of endogenous enzymes on fish muscle proteins, correlated with our previous study on silver warehou (Manikkam *et al.*, 2016a). However, released peptides exhibited lower bioactivities; raising concerns of whether further proteolytic processes may promote breaking down of these concentrated mixtures of proteins into simpler/shorter peptides with greater in vitro activities. In this regard, it is valuable to assess the effects of GI enzymes, physiologically active at certain pH, on the release of potent bioactive peptides as well as on their increasing or decreasing functional activities.

Endogenous calpains and cathepsins may act synergistically with post mortem physiochemical conditions including low temperature, reduced pH and relatively increased muscle ionic strength to modify the interaction and structural conformation of proteins. (Delbarre-Ladrat *et al.*, 2006). Biochemically, proteins' digestion begins in the stomach by action of pepsin at acidic pH 2. In the luminal phase of the small intestine, the polypeptides are further cleaved by the pancreatin proteases, at more alkaline pH (6.3), resulting in a mixture of oligopeptides and free amino acids (Vermeirssen *et al.*, 2004). In Figure 2, overall significant increase ($p < 0.05$) in bioactivities of fresh fillets (day 0) was observed at the end of digestion in comparison to undigested crude peptide mixtures. This indicated that SGID with pepsin and pancreatin had influentially impacted on the hydrolytic pathways of muscle proteins into peptides, exhibiting higher activities. The peptide profiling clearly indicated increased peptide content at the end of digestion (240 min) of fresh fillets on day 0 (Figure 4B).

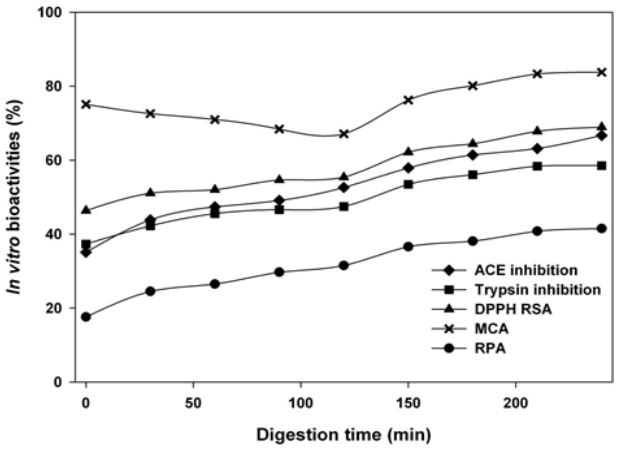

Figure 2. *In vitro* bioactivities of fresh fillets on day 0 during the stages of simulated gastrointestinal digestion. ACE, Angiotensin-converting enzyme; DPPH RSA, DPPH radical scavenging activity; MCA, metal chelating activity; RPA, reducing power assay.
Standard error of mean (SEM): ACE inhibition = 0.07; Trypsin inhibition = 0.05; DPPH RSA = 0.00; MCA = 0.04; RPA = 0.00

ACE-I activity of crude extracts

An overall significant increase ($p < 0.05$) in ACE inhibition was observed at 4oC from day 0 to 7. No change in ACE-I activity for day 5 samples stored at 4°C, (210 and 240 min). A similar observation was made for day1 samples held at 4°C for 90 and 120 min with pepsin digestion, day7 (6°C) at 180, 210 and 240 min and day14 (-18°C) at 210 and 240 min of pancreatin digestion (data not shown). The unchanged activities observed with the above mentioned samples could be due to i) hydrolysis reaching its maximum and no further break down of peptides or ii) disappearance of peptides during hydrolysis, as observed by Donkor *et al.* (2007). Furthermore, at the end of frozen storage (day 28), a significant decrease ($p>0.05$) in ACE-I activity was observed (data not shown). This decline could be explained by the susceptibility of proline amino acids residue at the C-terminus, generally known as the most favoured amino acids for ACE-I peptides, to freezing damage (Vermeirssen *et al.*, 2004, Manikkam *et al*., 2016b).

Another important observation was that irrespective of storage time and temperature, maximum ACE inhibition was noted with pancreatin digestion. Pepsin and/or pancreatin digestion has mainly been applied to release peptides from plant products, such as soy protein (Lo and Li-Chan 2005), peas (Vermeirssen *et al.*, 2003), dairy proteins (Pihlanto-Leppala *et al*., 2000), and some marine sources (Hai-Lun *et al.*, 2006, Samaranayaka and Li-Chan 2008, Darewicz *et al.*, 2014), with limited research findings. It is therefore difficult to draw a conclusion on how effective the pepsin-pancreatin digestion released peptides with ACE inhibition activity. However, Based on our findings, peptides with ACE inhibition potential can be derived from ESW fillets when stored at 4oC for a week.

Trypsin inhibition of crude extracts

Similar to ACE-I activity, storage conditions (time and temperature) had significant ($p < 0.05$) effect on trypsin inhibition (data not shown). The inhibitory effect of trypsin enzyme in stomach is important to boost CCK production in the presence of food. The satiety hormone CCK controls the amount of food one consumes. High protein content foods, such as soy (Nishi *et al*., 2003), whey (Zhou *et al*., 2011) and fish have gained considerable attention in ability to reduce hunger and maintain satiety (Cudennec *et al.*, 2008, Cudennec *et al.*, 2012) by inducing CCK production. In comparison to undigested crude extracts, digestion improved TIA of fish digest. Maximal trypsin inhibition of 62.67% was achieved on day 7 (4°C), at 240 min of digestion, demonstrating that ESW fillet can be a potent trypsin inhibitor; however further investigation need to be carried out to identify peptide sequence associated with the activity.

Antioxidant activities (DPPH free radical scavenging activity, metal chelating activity and reducing power assay)

The scavenging abilities of DPPH radicals by crude extracts changed depending on temperature and time (data not shown). An increase in activity from day 1-7 (4°C) and a decline for 6°C and frozen samples was observed. Frozen samples exhibited weak antioxidant activity, possibly due to physical aggregation of peptides (Ren *et al*., 2010). In further studies, to better understand the aggregation of peptide and/or protein aggregation during frozen conditions, monitoring the reactive changes in sulphide groups is crucial, since the latter impacts on myosin proteins (Ramírez *et al*., 2000). Various studies have investigated antioxidant properties of hydrolysates or bioactive peptides from animal sources, mackerel (Wu *et al*., 2003) and grass carp myofibrillar proteins (Ren *et al*., 2010) amongst others. Nevertheless, the current study investigated the release of peptides from underutilised Australian fish species, with potent antioxidant activity. Antioxidant properties are more related to the composition, structure and hydrophobicity of proteins or peptides present in original protein sources. Some examples of amino acids that cause antioxidant activity include Tyr, Trp, Met, Lys, Cys and His (Sarmadi and Ismail 2010). The sulphide group in cysteine has an independently

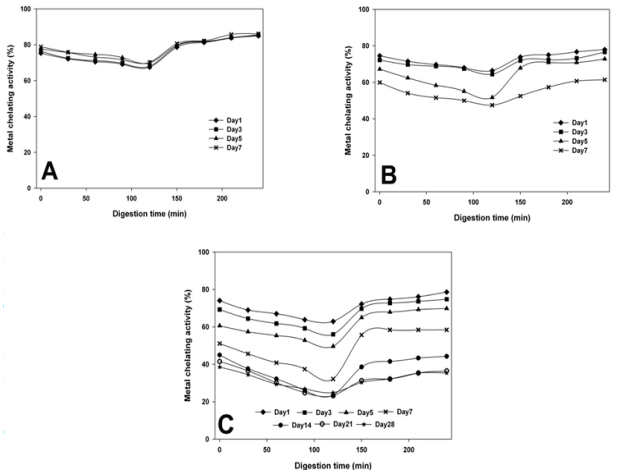

Figure 3. Effects of storage temperatures and times on the metal (Cu^{2+}) chelating activity of the digests during various stages of the simulated digestion of ESW fillets stored at 4°C (A), 6°C (B) and -18°C (C); Standard error of mean (SEM) = 0.04

crucial antioxidant action due to its direct interaction with radicals (Sarmadi and Ismail 2010). Therefore, further studies should focus on the fractionation and purification of peptides to identify the amino acid sequence contributing to the high antioxidant activity of ESW fillets.

The copper ions (Cu^{2+}) chelation by ESW crude extracts as affected by storage temperatures, time and digestive enzymes are illustrated by Figure 3. Irrespective of storage conditions (temperature and time), MCA decreased with pepsin digestion, but an upward trend was observed with pancreatin treatment. A similar observation was made with loach protein hydrolysates (You *et al*., 2010). Enzymatic hydrolysis is indeed an effective technology for utilizing low value fish. However, peptide size can affect chelating capacity of peptides, which is further affected by structure, molecular weight and amino acid composition. The first stage of simulated digestion (pepsin) may have disrupted the structure of ESW peptides and reduce its abilities to bind and trap Cu^{2+} ions; a similar observation was made with loach (You *et al*., 2010) and silver warehou (Manikkam *et al*., 2016a) peptides. The second pancreatin-involved digestive stage may have increased the degree of hydrolysis and released more free amino acids. Moreover, any high-affinity metal binding groups, such as carboxylic groups (Zhu *et al.*, 2008) become fully exposed or newly formed (You *et al.*, 2010). Further fractionation, purification and identification of peptides associated with high MCA from the ESW crude extracts deemed necessary.

Direct reaction of a substance is not the only mechanism by which antioxidants may display their activity. Secondary antioxidants do not convert free radicals to more stable products but slow rate of oxidation by one important mechanism; chelation of pro-oxidant metals. Transition metals (copper) promote oxidation by acting as a catalyst of free radical reactions (Končić *et al.*, 2011). Chelation of metals by certain antioxidant compounds, such as ESW-derived peptides, decreases their pro-oxidant effect by reducing their redox potentials and stabilising the oxidised form of the metal (Končić *et al*., 2011). Excess of transition metal ions in human body can result in various anomalies, including neurodegenerative diseases. Based on our results obtained from in vitro study, ESW peptides may serve as efficient and natural metal chelating agent, involved in chelation therapy in the prevention and/or treatment of inflammatory diseases.

The reducing power of crude extracts also changed with respect to storage temperatures, times and digestive treatments (data not shown). A similar trend as the above physiological activities was observed, that is, an increase in activity of 4°C samples, from day 1-7. Pancreatin digests showed higher reducing power, in line with You et al. (2010). The reducing power of a sample or crude extracts of peptides, an indicator of antioxidant activity, is used to evaluate the ability of an antioxidant to donate an electron or hydrogen or acting as a reducing agent. In the presence of an antioxidant derived from proteinaceous food sources, such as from ESW peptides, ferrous chloride is formed, based on the chemical equation below:

Potassium ferricyanide +Ferric chloride → Potassium ferrocyanide +ferrous chloride

The colour of the solution changed from yellow to green or blue shade depends on the reducing potential of the sample (Jin and Wu 2015). Several reports have revealed that there is a direct correlation between antioxidant activities and reducing power of bioactive peptides. Sardinella hydrolysates produced with commercial alcalase enzyme showed potent reducing power (Jeevitha *et al.*, 2014). The lowest reducing power of ESW crude peptides could be due to the peptides associated with reducing power have not been produced with pepsin and/or pancreatin enzymatic reactions. Alcalase enzyme cleavage of peptides demonstrated highest reducing power (Jin and Wu 2015).

Peptide profiling

The peptides released upon simulated gut digestion were analysed by RP-HPLC and qualitative profiles are presented in Figure 4. The peptide profile was clearly affected by storage temperature, time and simulated digestion. Season could be another factor impacted on peptide profile (Medenieks and

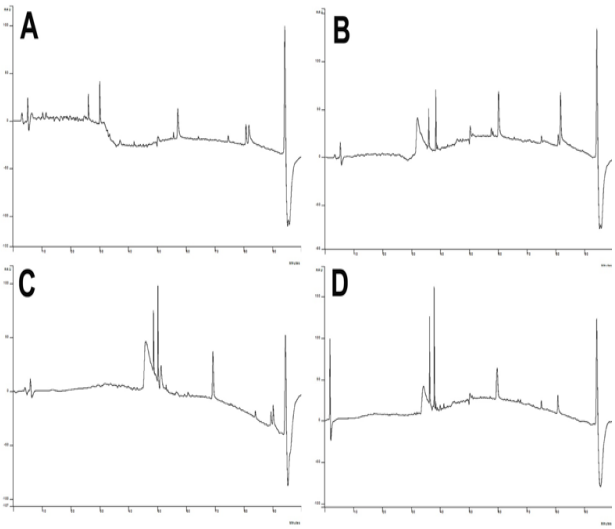

Figure 4A. Peptide profiling undigested crude extracts as affected by storage temperatures (- 4°C) and times by RP-HPLC: day 0-fresh (A), day 3 (B), day 5 (C) and day 7 (D)

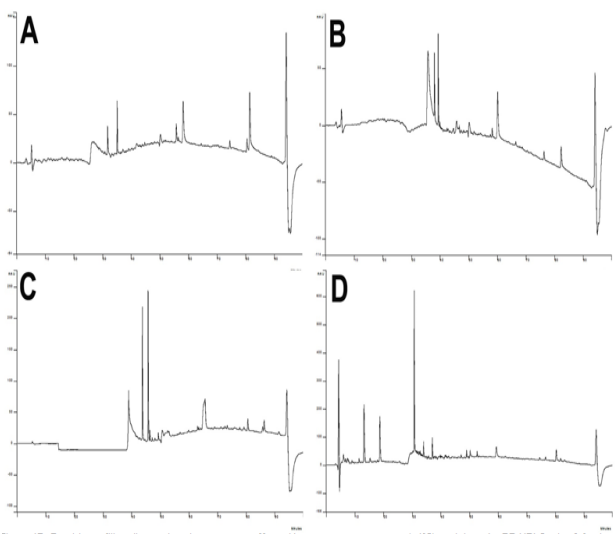

Figure 4B. Peptide profiling digested crude extracts as affected by storage temperatures (- 4°C) and times by RP-HPLC: day 0-fresh (A), day 3 (B), day 5 (C) and day 7 (D)

Vasiljevic 2008), whereby winter fish released fewer peptides compared with summer fish. In this study, peptide content increased at the end of pancreatin digestion (240 min), for 4°C samples (Figure 4B), but decreased for 6°C and -18°C samples (data not shown). This observation correlated with the increased bioactivities of 4°C samples, as explained above. The retention times of observed peaks were similar suggesting that the types of peptides released were similar but their concentrations differ. This implies that some amino acids might have been affected during freezing and cold storage. The lower concentration of peptides released prior to simulated digestion could proportionally relate to the lower proteolytic activity of endogenous enzymes. Moreover, the combination of digestive and fish intracellular enzymes might have contributed to better hydrolytic process, releasing more active peptides.

Conclusion

Results demonstrated that fish endogenous enzymes can hydrolyse proteins into amino acids, but to a lesser extent, whilst simulated gastrointestinal digestion enhanced the degree of hydrolysis. Interestingly, pancreatin treatment was more effective with greater bioactivities compared to digestion with pepsin enzyme. Cold storage at 4°C produced greater physiological activities, mainly Cu^{2+} chelating activity and DPPH radical scavenging activity. Overall, our findings suggested that the undervalued eastern school whiting fish can constitute of a potential valuable functional food entity. The potency to act as a metal chelating agent and antioxidant by scavenging radicals should be further investigated by fractionation of specific peptides. Moreover, the amino acid sequence responsible for the multi-functionalities of ESW-derived peptides should be determined.

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