Sustainable use of silver warehou (*Seriolella punctata*): effects of storage, processing conditions and simulated gastrointestinal digestion on selected in-vitro bioactivities

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Abstract Australian underutilised fish species may serve as a potential source of valuable proteins and potent bioactive peptides. This novel research is the first to investigate the effects of storage-processing conditions and an in-vitro simulated gastrointestinal digestion (pepsin–pancreatin) on bioactive peptides’ release during storage of fish fillet, derived from Australian silver warehou (*Seriolella punctata*). In-vitro bioactivities including angiotensin-converting enzyme and trypsin inhibitory and antioxidant activities were analysed. The antioxidant power was evaluated by DPPH free radical scavenging activity, Cu²⁺ chelating and Fe³⁺ reducing abilities. Fillets were stored at chilled (4 and 6 °C) and freezing (−18 °C) temperatures for 7 and 28 days, respectively. Results indicated that during postmortem storage, endogenous enzymes released from fillets an array of polypeptides during storage. The demonstrated physiological activities were further increased during simulated digestion. Bioactivities were greater at 4 °C, increasing over 7 days as compared to at 6 and −18 °C. An increase by 2 °C for chilled temperature was enough to cause significant changes in activities. The crude extracts obtained by pancreatin treatment demonstrated the highest metal chelating activities at 4 °C

(86.3 ± 0.1 % on day 7). Physiological potency, especially metal chelating activity, of fillets obtained from silver warehou may be manipulated by storage conditions that would consequently be further enhanced during simulated digestion.

Keywords Underutilized fish · By-catch · Storage · Digestion · Bioactivity

Introduction

Silver warehou (*Seriolella punctata*) constitutes an important commercial species for the Australian fisheries. It can be captured either intentionally by bottom trawling and retained, or unintentionally and rejected due to lack of profitable interest (Australian Fisheries Management Authority 2015). A large proportion of silver warehou catch is landed during the spawning months, deflating prices, causing financial losses to the seafood industry. Its constant name changing also resulted in poor identification and a lack of acceptance by local and international markets (McLaughlin et al. 2009). Moreover, the poor sensory qualities, principally, its off-white colour after filleting have reduced its consumer demand. Thus, these consequences do not make silver warehou a prime catch, but it is instead considered as by-catch. It is categorized as under-valued fish species (McLaughlin et al. 2009). Considered as waste, by-catch species are disposed of into the ocean, posing substantial threat to the species and jeopardizing the marine ecosystem (Blanco et al. 2007). Alternatively, they are converted into commercial and low-value products such as fertilizers, fish silage, meals, and baits, amongst others (Blanco et al. 2007).

Interestingly, fish waste, an excellent source of proteins, can be converted into more marketable, functional and

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health value-added products by novel enzymatic hydrolytic technology (Nurdiani et al. 2015). Enzymatic hydrolysis has been widely used to improve the functional properties (water-holding, emulsification, gelling and solubility) of mainly myofibrillar proteins (Kristinsson and Rasco 2000). Moreover, utilizing intact marine-derived proteins to produce bioactive peptides by enzymatic hydrolysis is increasingly becoming the focus of today's scientific world. Bioactive peptides, derived from various food sources, display an array of physiological functions, including anti-hypertensive, anti-oxidative, opioid activities, just to name a few (Kim and Wijesekara 2010).

Since Australian consumers (1) have specific fish requirements for consumption, including inexpensive, boneless and skinless white-flesh fillets (Department of Agriculture 2015), (2) prefer domestic seafood and (3) are prepared to pay more for home-branded products (Calogeras et al. 2011), the seafood industry should meet the above requirements to promote sale of silver warehou. Therefore, enzymatic proteolysis could be an effective approach to improve utilization of silver warehou fillets by making it a potential medium for production of bioactive peptides, with prospective physiological properties, which have not been previously documented.

Generating bioactive peptides by silver warehou endogenous and/or exogenous enzymes whilst maintaining freshness and balance of amino acids may be challenging, but of great importance. Chilling and freezing are two major ways to preserve freshness of fish, due to their perishable nature (Garthwaite 1997). However, these preservation techniques may lead to myofibrillar proteins denaturation and aggregation (Tejada 2001). These biochemical changes may essentially alter biofunctional parameters of fish peptides. No study has hitherto investigated the effects of chilled and frozen storage on in-vitro bioactivities of underutilised fish species.

Subsequently, evaluating bioactive peptides release during simulating gastrointestinal digestion (SGID) is vital because it demonstrates the types of peptides produced during digestion, and thus most likely to survive the GI tract (Korhonen and Pihlanto 2006). Because of the importance of maximizing freshness and high quality of silver warehou fillet while simultaneously benefiting from the bioactive peptides liberated upon SGID, the main emphasis of our study was to establish combined effects of storage conditions (temperature, storage time) on the quality-related changes as well as in vitro biofunctionalities of undigested and digested fillets during storage. Our exploration could provide the Australian seafood industry with a better understanding on the plausible means of developing silver warehou fillets to maximize their use, increase commercial value and enhance consumers' demand. The study would also provide a foundation for

future research in regards to identification of muscular proteins that contain potential bioactive peptides as well as structural characterization of these peptides.

Materials and methods

Samples collection

Silver Warehou was kindly supplied by Barwon Foods (Seafood and Food Service Specialists; North Geelong, Australia). After having caught off the south-eastern coast including Tasmania and Bass Strain on the continental slope, fresh whole silver warehou was transported on ice in a polystyrene foam box to Werribee campus, Victoria University, within 12–24 h. Upon arrival to our laboratory, fish was immediately stored in the cold room (4 °C) and on ice, prior to handling, within 2 h.

Experimental design

The experimental design, depicted by Fig. 1, was set up to investigate the effects of storage and processing conditions on the quality and in vitro bioactivities of the commercially important silver warehou fillets. The weight and length of each whole fish were approximately 900 g and 35 cm, respectively. From each fish, two fillets were obtained, weighing almost 150–200 g. On day 0 (the day of samples' arrival), each fillet portion was individually glad-wrapped, further sealed with aluminium foil, randomly placed into labelled locked containers, and stored at the selected temperatures in temperature-controlled fridges and freezer.

Chilled temperatures of 4 and 6 °C were selected for the following reasons: (1) home fridges are usually stabilized between 4 and 6 °C, depending on the setting. Therefore, it was important to investigate the effects of these 2 primary cold temperatures on the release of bioactive peptides and their in vitro bioactivities; (2) fish major endogenous enzymes, such as calpain and cathepsins, fundamentally involved in the hydrolysis of myofibrillar proteins are active at cold temperatures (Ahmed et al. 2013a). Hence, a difference by 2 °C would potentially impact on their hydrolytic abilities, thus, deserving attention. The 7 days storage period for chilled samples were appropriate since in real life, fish are not stored in the refrigerator for more than a couple of days, due to rapid microbial growth. In addition, the frozen storage studies for 28 days were appropriate since a decrease in bioactivities was observed over time, and hence the storage period was discontinued.

Two portions of fillets, representing the duplicates, were removed daily for analyses for refrigerated samples and for first week of frozen fillet, with a further weekly analysis, up

Fig. 1 Flowchart depicting the experimental design applied in the current study

until 28 days. On each day of analysis, frozen samples were defrosted in the cold room (4 °C) for a couple of hours, prior to processing. Fish fillets were also subjected to an in vitro simulated digestion, with gastrointestinal enzymes, viz, pepsin and pancreatin to investigate the ability of releasing physiologically important peptides. The quality assessments involved macronutrient compositions, pH determination and microbiological analysis. All the 5 in vitro bioactivities were monitored during undigested and digested states. The laboratory and all equipment used during the daily processing of fish were aseptically maintained at all times. The procedure was repeated on three different occasions reflecting seasonal differences.

Chemicals

Angiotensin-I-converting enzyme, *N* α -benzoyl-L-arginine-4-nitroanilide hydrochloride (BAPNA), copper sulphate, 1,1-diphenyl-2-picrylhydrazyl, hippuryl-histidyl-leucine, iron(III) chloride, pancreatin (P7545; porcine pancreas), pepsin (P7000; porcine stomach mucosa), potassium ferricyanide, pyridine, pyrocatechol violet, sodium phosphate, trichloroacetic acid and trypsin (Type II-S from Porcine pancreas) were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). Dimethyl sulfoxide (DMS), ethyl acetate and glacial acetic acid were from Merck Pty Ltd (Darmstadt, Germany). All other chemicals used for chemical compositional analyses and preparation of buffering solutions were of analytical laboratory grade.

Quality assessment

Macronutritional compositional analysis

The protein, ash, and fat contents were determined using the methods of AOAC (2000).

pH determination

Ten grams of fish fillet was homogenised with MilliQ water in the ratio of 1:10 (w/v), and pH of the homogenate measured using a calibrated pH meter (Merck Pty Limited, Germany) at room temperature.

Microbiological analysis

The number of viable cells from the total plate count was determined as colony forming units/g (Log CFU/g). Plate containing 25–250 colonies was considered (AOAC 2000).

Processing conditions

Undigested condition

On each sampling day, 25 g of fish fillets from each selected temperature was minced using a mortar and pestle, followed by addition of 250 ml MilliQ water (MilliQ plus, Millipore Australia) and homogenization. The homogenate was centrifuged at 11,000×g (JA20 rotor, Beckman

Instruments Inc., Palo Alto, CA, USA) for 20 min at 4 °C. On each removal day, frozen fillets were defrosted in the cold room (4 °C) for 2 h prior to homogenization. The supernatant obtained from each homogenate after centrifugation was filtered into clean tubes as crude protein/peptide extracts, and stored at −20 °C until further assayed.

Digestion condition

Fresh (day 0), frozen and chilled samples were subjected to an in vitro pepsin–pancreatin artificial digestion, to investigate the effects of digestive enzymes on silver warehou fillets during storage, according to the method described by Medeniek and Vasiljevic (2008), with modifications. Fillets (25 g) were ground and mixed with 100 ml MilliQ water; this step presented time 0 of the digestion process. All samples were then acidified with 1 M HCl to pH 2 and 5 ml of pepsin (Porcine gastric mucosa) solution was added. The samples were then incubated for 2 h at 37 °C at 100 rpm. After pepsin digestion, pH was adjusted to 6.3 with 1 M NaHCO₃ solution and further to 7.5 with 1 M NaOH, prior to addition of addition of pancreatin solution. The mixture was carefully mixed and incubated at the same above conditions. After 2 h of incubation, the tubes were immersed in boiling water bath for 15 min to halt the enzymatic reactions. They were then cooled on ice for 5–10 min. The cooled samples were then centrifuged at 1500×g (Sorvall® RT7 centrifuge, DuPont, Newtown, CT, USA) for 15 min at 4 °C. To investigate the changes in bioactivities of digests during digestion, aliquots of digests were removed at 30 min intervals for 4 h. All GI digests were filtered using 0.45 µm membrane filter (Schleicher & Schuell GmbH, Germany) before storing at −20 °C for additional analyses.

In vitro bioactivities

ACE inhibitory (ACE-I) activity

The ACE-I activity was established spectrophotometrically (NovaSpec®—II Spectrophotometer; Pharmacia, Cambridge, UK) assayed by measuring the absorbance at 228 nm. The extent of inhibition was calculated using Eq. 1 (Donkor et al. 2007).

$$\text{ACE inhibitory activity}(\%) = \left[1 - \left(\frac{C - D}{A - B} \right) \right] \times 100 \quad (1)$$

where, A = absorbance in the presence of ACE and without the ACE-I component; B = absorbance without the ACE-I component; C = absorbance with ACE and the ACE-I component; D = absorbance without ACE and with the ACE-I component.

Trypsin inhibitory activity

The TIA was determined according to Medeniek and Vasiljevic (2008). A volume of 250 µl of crude extracts was pre-incubated at 37 °C for 10 min with 625 µl of BAPNA–DMS–Tris buffer solution. This was followed by the addition of 250 µl trypsin enzyme solution (40 mg trypsin enzyme in 200 ml of 1 mM HCl) before incubating for 10 min at 37 °C. The reaction was terminated by adding 250 µl of 30 % glacial acetic acid and vortex-mixed. The absorbance of each sample was read at 410 nm. The inhibitory activity was evaluated using Eq. 1.

DPPH free radical scavenging activity (RSA)

The antioxidant capacity of samples was evaluated by measuring the free RSA following the method of Donkor et al. (2012), with slight modifications. Briefly, 4.0 ml DPPH solution (0.075 mM DPPH in methanol) was added to 0.1 ml diluted (in 1 ml methanol) sample followed by 30 min incubation in the dark, after which, the absorbance was read at 517 nm with a Pharmacia UV spectrophotometer (Cambridge, UK). The scavenging activity was calculated as percent inhibition, using Eq. 2.

$$\text{Inhibition}(\%) = \left[1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of blank}} \right) \right] \times 100 \quad (2)$$

Metal chelating activity

The chelating activity of crude in vitro fish peptide extracts on pro-oxidative copper ions (Cu²⁺) was investigated according to (Zhu et al. 2008), with modifications. Briefly, 500 µl of 2 mM CuSO₄ was mixed with 500 µl of pyridine (pH 7.0) and 10 µl of 0.1 % pyrocatechol violet. An aliquot of 500 µl peptide extract was then added and allowed to quiescently stand for 5 min. The disappearance of the blue colour was then recorded by the measuring the absorbance at 632 nm. An equivalent volume of MilliQ water instead of sample was used as the blank. The Cu²⁺ chelating activity of the crude protein/peptide extracts was calculated as shown below.

$$\text{Cu}^{2+} \text{ chelating activity} = \left[\frac{A_o - A_s}{A_o} \right] \times 100 \quad (3)$$

where, A_s = absorbance of the sample; A_o = absorbance of the blank solution using distilled water instead of sample.

Reducing power assay

The potential to act as a reducing agent by the ability of donating an electron to Fe³⁺ ions reducing it to Fe²⁺ was

determined by investigating the reducing power of fish crude protein/peptide extracts according to (Zhu et al. 2008), with adjustments. Briefly, 1.0 ml of samples was mixed with 1.0 ml of sodium phosphate buffer (0.2 M, pH 6.6) and 1.0 ml of 1 % potassium ferricyanide. The mixture was incubated at 50 °C for 30 min. Trichloroacetic acid (10 %) was added to the mixture, and centrifuged at 1500×g (Sorvall® RT7) for 10 min. Finally, the supernatant was mixed with MilliQ water and ferric chloride solution (0.1 %). After quiescent standing at room temperature for 10 min, the absorbance was measured at 700 nm. An equivalent volume of MilliQ water instead of the sample was used as the blank. Increase absorbance represented increased reducing power of samples.

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 (Fig. 1) was triplicated and sub-sampled twice resulting in at least 6 independent observations ($n \geq 6$). Results were analyzed 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

Quality assessment

The proximate composition of fresh fillets (day 0) was 18.9 % protein, 1.8 % fat, 77.5 % moisture and 1.5 % ash. Proximate composition of fillets was also determined on respective sampling days to identify changes during storage (data not shown). Protein, moisture, fat, and ash contents did not show any significant change during storage ($p > 0.05$). Similar observation were reported earlier (Gandotra et al. 2012). Similarly, pH of fillet on day 0 was 6.8 and did not change much ($p > 0.05$) irrespective of storage temperatures and time (data not shown). On the other hand, an increase by 4 and 5 log cycles in bacterial load in raw fish muscle was observed (Table 1). A total plate count value surpassing 6 logs CFU/g was considered as bacteriological spoilage of fish muscle and inedible for human consumption (International Commission on Microbiological Specifications for Foods 1986). Accordingly, our fillets were still consumable until day 3 when refrigerated at 4 °C and day 14 when frozen. After day 3 at 4 °C, fish fillets quickly deteriorated, with increased

bacterial load, noticeable pungent smell and colour change. The present findings showed that it was not advisable to stow fish muscle at 6 °C due to rapid microbial spoilage. Psychro-tolerant Gram-negative bacteria (*Pseudomonas* spp. and *Shewanella* spp.) could grow on chilled fish, above 4 °C (Gram and Huss 2000).

In vitro bioactivities during storage (undigested condition)

The impact of storage conditions in the presence of endogenous enzymes only, on the biochemical functionalities of crude extracts from fillets were investigated. Results shown in Table 1 indicated that endogenous enzymes from silver warehou fillets liberated polypeptides during *postmortem* storage due to limited breakdown of the protein matrix. As a result, all samples contained peptides that exerted assessed bioactivities to a varying degree ($p < 0.05$). Interestingly, storage temperature exhibited a varying ($p < 0.05$) effect on fillets stored at 4 °C, showing increasing bioactivities over a period of 7 days, likely due to relative stability of cathepsins L (Yang et al. 2015). Furthermore, the high levels of cathepsins B + L activities in silver warehou (Ahmed et al. 2013b) likely enhanced the hydrolysis of myofibrillar proteins during storage and consequently improved the physiological properties in vitro at 4 °C.

Over time, a slight change in reducing power (data not shown) of crude extracts of undigested fillets was observed at 4 °C. Specific amino acids Trp, Met, Cys and Lys constituted an important asset for a peptide or functional component to act as a reducing agent, especially in reducing iron(III) to iron(II) (Carrasco-Castilla et al. 2012). Our fillets could be lacking these specific amino acids. However, an amino-acid profile analysis of the crude-extracts would have enabled identification of the types of amino acids present in our samples. Moreover, endogenous cathepsins L is often implicated in the release of peptides during storage at 4 °C, as demonstrated in pacific hake muscle (Samaranayaka and Li-Chan 2008), explaining the high DPPH radical scavenging and metal chelating activities of our undigested samples at 4 °C.

On the other hand, chilled samples at 6 °C showed a decreasing trend in biofunctional activities (Table 1). There is a distinct difference in all 4 assessed bioactivities between storage at 4 and 6 °C. The values increased during the 7 days of storage at 4 °C, but decreased significantly ($p < 0.05$) at 6 °C. The difference in temperature by only 2 °C would have been enough to create favourable conditions within the fillets matrix, increasing microbiological activity. More specifically, psychotropic bacteria are able to grow at temperature below 7 °C, correlating with our

Table 1 Microbial count and in vitro bioactivities of undigested fillets as influenced by storage conditions

Storage temperatures	Microbial count (log CFU/g)			ACE inhibition (%)			Trypsin inhibition (%)			DPPH RSA (%)			MCA (%)		
	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
<i>Sampling days</i>															
1	3.3	4.2	3.4	45.6	38.6	36.8	44.0	40.0	37.3	40.9	40.6	37.7	60.4	60.1	58.6
3	5.5	6.3	3.4	49.1	35.7	31.6	49.5	37.3	34.5	42.2	39.6	36.0	62.0	57.4	57.0
5	7.1	7.8	3.7	52.6	28.7	26.3	56.6	31.3	30.9	46.0	37.8	33.8	63.7	48.2	55.6
7	7.8	8.8	4.7	55.3	28.7	22.8	58.3	30.9	28.7	48.5	36.5	28.4	64.8	44.3	53.5
14	nd	nd	5.3	nd	nd	17.5	nd	nd	24.0	nd	nd	22.5	nd	nd	48.7
21	nd	nd	6.1	nd	nd	12.3	nd	nd	16.2	nd	nd	15.6	nd	nd	41.6
28	nd	nd	7.2	nd	nd	5.3	nd	nd	10.3	nd	nd	10.2	nd	nd	33.6

The means present the average of 6 independent observations ($n \geq 6$)

SEM—pooled standard error of the mean, nd: not determined—microbial count and in vitro activities were not determined on these specific days due to practicality reasons

On day 0, the total plate count of fresh fillet was 3.3 Log CFU/g

On day 0, the in vitro bioactivities of fresh fillet were as follows: ACE inhibition: 42.1 %; trypsin inhibition; 42.3 %; DPPH RSA: 37.2 %; MCA: 62.1 % and RPA: 18.6 %

bacterial count (Table 1). Generally, high activity of endogenous muscle proteases during initial days of refrigerated storage may be an indicator of texture-associated degradation (Delbarre-Ladrat et al. 2004). Postmortem conditions such as pH and temperature may influence the level of activity of muscle proteases and consequently impacting on the properties of resulting properties (Ahmed et al. 2013a). Silver warehou exhibited the lowest endogenous calpain-like enzymatic activity (Ahmed et al. 2013a), supporting the observed decline in in vitro bioactivities at 6 °C during storage.

Similar to 6 °C samples, frozen samples (−18 °C) showed a decreasing trend in selected in vitro bioactivities (Table 1). Freezing and thawing may result in (1) fragmentation of cell membranes and lysis of intra-cellular organelles, (2) decreased water-holding capacity of fish muscle and (3) drop in myosin and actomyosin Ca^{2+} -ATP-ase activities, resulting in a change in the myosin head (Makri 2010). These changes could result in unfolding tertiary conformation of myosin owing to weakening in intra-molecular bonds (Makri 2010). Reduction in bioactivities during frozen storage could also be explained by instability and sensitivity of fish myosin to denaturation and degradation as well as the cross-linking and aggregation of myofibrillar proteins (Tejada 2001). When water is separated as ice, proteins, mainly myofibrillar proteins, became unstable and protein denaturation began. Therefore, the hydrophobic and hydrophilic amino acid groups normally associated with the interior of a protein molecule became more exposed (Tejada 2001), supporting our low biochemical activities of frozen samples.

In-vitro bioactivities during storage and digestion

Human digestive enzymes, such as pepsin, chymotrypsin and pancreatin, may further hydrolyse polypeptides fragments producing a range of smaller peptides exhibiting important physiological properties (Manikkam et al. 2015). After oral administration, gastrointestinal enzymes may break down the active peptides, thereby increasing or decreasing their functional activities (Vermeirssen et al. 2004). The following section will thus discuss the impact of gut enzymes on the release of bioactive peptides and their bioactivities.

ACE-inhibition of crude extracts

On day 0, fresh fillets exhibited an ACE-inhibition of 70.2 % at the end of pancreatin digestion (Fig. 2a). This effectiveness of pancreatin at increasing ACE-I activity was observed throughout the storage stability experimentation, irrespective of storage temperature and time. Moreover, an overall increase in ACE-I activity of 4 °C samples was observed throughout week 1 (Fig. 2b), whereas a significant decline ($p < 0.05$) in activity was observed at 6 and −18 °C. Di- or tri-peptides, especially proline amino acid residue at the C-terminus are generally resistant to degradation by digestive enzymes, and the most favoured for ACE-I peptides (Vermeirssen et al. 2004). It was also predicted that a C-terminal Gly is not very favourable residue for exhibiting ACE inhibition (Wu et al. 2006), which could possibly have been the case for our 6 °C and frozen samples. However, further investigation of the amino acid profiles of the crude extracts is required to fully understand the discrepancy in ACE-I activity of the various samples.

Fig. 2 Physiological activities of fresh fillets at day 0. Legend: filled triangle angiotensin converting enzyme inhibition (ACE-I); filled circle trypsin inhibition (TI); filled square antioxidative (DPPH) activity; filled diamond metal chelating (MC) activity; open square reducing power assay (RPA)

Trypsin inhibition of crude extracts

Digestion also improved the trypsin inhibition by the fish digests. Unlike ACE-I activity, cold storage at 4 °C and time did not significantly ($p > 0.05$) impact on trypsin inhibition (data not shown). In general, fresh samples appeared to be a source of more potent peptides with greater trypsin inhibitory activity than the frozen fish, in line with the study of Medeniek and Vasiljevic (2008). During freezing, the formation of ice crystals caused cryo-concentration of solutes, partial dehydration and dislocation of water molecules in the muscle, accelerating protein denaturation and aggregation (Makri 2010), which could have impacted on the release of bioactive peptides and consequently on the trypsin inhibition ability. At the beginning of chilled storage (4 °C), trypsin inhibition was not high ($p > 0.05$), reaching to a nearly constant activity at the end of 7 days. It was likely that peptides of interest were not released, in line with Donkor et al. (2007). Similar to ACE-I activity, trypsin inhibition declined during storage at 6 and −18 °C (data not shown). It has been very difficult to compare our results with previous studies since no research has been done in this particular area, requiring further investigations.

DPPH free radical scavenging activity

The relatively stable organic radical, DPPH, has been extensively utilized as a free radical to evaluate reducing substances. In regards to 4 °C samples, pepsin digestion for 30 min appeared to release peptides with significant ($p < 0.05$) scavenging activity followed by minimal increase thereafter. During pepsin digestion, more

hydrophobic amino residue side chain groups were expected to be exposed, which would make the peptides more accessible to the DPPH radicals, allowing them to trap the radical more easily (You et al. 2010). However, an increasing trend in activity during pancreatin digestion was observed, not in line with previous studies (You et al. 2010), whereby a sharp decrease in activity was observed with pancreatin digestion. Antioxidative properties of peptides are more related to their amino acids composition, structure and hydrophobicity. Small neutral amino acids include alanine; serine and cysteine (Daniel 2004) often play an important role in antioxidant activities. With respect to 6 °C and frozen samples, a sharp decrease was again observed (data not shown). This might potentially be due to the initial hydrolysis resulting in the release of oligo- or poly-peptides with lower antioxidant activities. Upon further treatment, either proteolytic activity of the antioxidant peptides or their physical aggregation had weakened the scavenging ability of the DPPH free radical. The obtained results suggest that crude peptide extract at the end of digestion of 4 °C samples probably contained peptides, which are electron donors; and could possible react with free radicals, converting into more stable products and terminate the chain reaction (Bougatef et al. 2009).

Cu^{2+} ions chelating activity

The fish digests during in vitro digestion were also evaluated for copper ions chelation, as a measure of antioxidant activity (Fig. 3). Irrespective of storage time and temperature, chelating activity decreased with pepsin treatment, but significantly increased ($p < 0.05$) with pancreatin, in line with You et al. (2010). This may suggest that fewer peptide bonds are broken down with pepsin than with pancreatin. Pancreatin contains many enzymes, including trypsin and additional proteases, which could aid in hydrolysing the fish peptides into even more smaller peptides. The decrease in activity during pepsin digestion could be due to specific peptide structure and amino acid side chain groups (Decker et al. 1992). Pepsin treatment might have disrupted the structure of silver warehou peptides and reduced their ability to bind and trap copper ions (Zhu et al. 2008). Among all the bioactivities assessed, metal chelating activity was the highest, irrespective of storage conditions (Fig. 4).

Ferric reducing power of crude extracts

The reducing power, another indicator of antioxidant activity of bioactive compounds, including peptides, is used to evaluate the ability of an antioxidant to donate an electron or hydrogen. The presence of antioxidants in the crude extracts or hydrolysates causes the reduction of the

Fig. 3 ACE-inhibitory activities of the digests during various stages of the simulated digestion of fillets at 4 °C on filled circle day 1, open circle day 2, filled triangle day 3, open triangle day 4, filled square day 5, open square day 6, filled diamond and day 7

Fe^{3+} /ferric cyanide (FeSCN) complex to ferrous ion (Fe^{2+}) and the colour of the solution changes from yellow to green or blue shade, depending on the reducing potential of the compound (Bougatef et al. 2009). No obvious colour change was observed in our samples, corresponding to the low activity (data not shown). Our fish crude extracts were possibly not effective in producing amino acids that are associated with reducing power, in contrast with previous studies (Bougatef et al. 2009; You et al. 2010).

Conclusion

Endogenous enzymes in silver warehou fillets released an array of polypeptides during postmortem storage due to apparent limited breakdown of the protein matrix. The *in vitro* biofunctionalities of native proteins were definitely enhanced when fillets were subjected to simulated gastrointestinal digestion. Samples at 4 °C and pancreatin digests were more effective in producing high bioactivities. The analysed crude extracts demonstrated higher antioxidant activities with respect to DPPH radical scavenging activity and chelating ability of copper ions. However, lower ACE- and trypsin-inhibitory activities were observed. The comparison of our data with other studies was difficult since no investigations had been previously conducted on the effects of storage temperatures and times on liberated peptides from fish. However, our findings indicated that the underutilised fish analyzed could be valuable food items, containing bioactive peptides. Further studies are needed to identify muscular proteins as the origin of these bioactive peptides as well as primary sequences of these peptides.

Fig. 4 Effects of storage temperatures and times on the metal (Cu^{2+}) chelating activity of the digests during various stages of the simulated digestion of silver warehou fillets at chilled temperatures—4 °C (a) and 6 °C (b) and during frozen storage at −18 °C (c) on filled circle day 1, open circle day 2, filled triangle day 3, open triangle day 4, filled square day 5, open square day 6, filled diamond day 7, open diamond day 14, dashed filled circle day 21, and dashed open circle day 28

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