Short communication

Detection of small bioactive peptides from Atlantic herring (Clupea harengus L.)

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Recent research has shown that fish residual materials contain a range of components with interesting biological activity. Therefore, there is a great potential in the marine bioprocess industry to utilize these by-products as starting material for generating more valuable products. The aim of the present study was to search for bioactive peptides (in particular small natural bioactive peptides with molecular weight lower than 10 kDa) in Atlantic herring (Clupea harengus L.) by-products such as skin and more general residual materials. By such means a range of peptides with claimed interesting biological activities was found. Herein the activity of the detected bioactive peptides and strategies for isolating peptide fragments containing the bioactive motif is discussed. Identification of bioactive peptides in crude peptide/protein sources (skin and residual materials) was performed directly using a combination of mass spectrometry (Orbitrap), bioinformatics and database search. This method was a good angle of approach in order to map the potential in new species and species that have been very little studied.

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1. Introduction

Historically pelagic fish, and in particular herring, have been of great importance to the population alongside the west coast of Norway with herring fisheries being an important contributor to this part of the country’s economy. The majority of the pelagic fish caught is used for human consumption. Large quantities of the catch go to export, and in 2009 herring contributed to 30% of the total export of Norwegian seafood. That year the herring catch totaled about 900,000 tons. Approximately 25% of the herring becomes residual material after food processing\([2]\). Residual material in this context includes trimming, fins, frames, heads, skin and viscera. Totally in the European Union for all types of fish processing waste and by-products from fisheries totals about 5.2 million tons per year\([15]\). With these quantities of residual material available there is an increasing interest in the fishery sector to find new possible utilization for these resources\([7]\). Up to recently, large quantities of these raw materials were sent to fish meal plants for use as animal feed or discarded. However most of these products possess low economic value. Recent research has shown that fish residual materials contain a range of components with interesting biological activity\([11]\). Therefore, there is a great potential in the marine bioprocess industry to utilize more of these by-products as starting material for generating more valuable products.

Fish derived bioactive peptides represent a source of health enhancing components. These peptides can potentially be released during gastrointestinal digestion or food processing. The peptides are considered to promote various activities including, immunomodulatory, antimicrobial, opiate-like, antioxidant, antithrombotic, hypocholesterolemic and anti-hypertensive actions\([6, 11, 16, 18]\). Many of the reported bioactive peptides are multifunctional and can exert more than one of the mentioned effects. These peptides usually contain 2–20 amino acid residues per molecule. Many research groups and companies have therefore focused their attention on converting the remaining proteins into bioactive peptides by enzymatic treatment, thus generating various hydrolysates containing short peptides\([5, 11, 12, 18]\).

Bioactive peptides are of commercial interest as components of functional foods and nutraceuticals with certain health claims. The latter fact and the fact that Atlantic herring fisheries are of great importance on the South-West Norwegian coast in addition to the fact that this species had not previously been studied with similar intent, it was natural to focus the present research on this fish. The aim of the present study was to search for bioactive peptides (in particular small natural bioactive peptides with molecular weight lower than 10 kDa) in Atlantic herring by-products such as skin and more general residual materials. By such means we found a range

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2. Materials and methods

2.1. Sample collection

Herring skin and herring residual material (the remains from the fish when the filet is taken out) were provided by NOFIMA, Stavanger, Norway. In this study Atlantic herring (Clupea harengus L.) caught by purse-seine in the North Sea (about 1 h west of Kristiansund) (February, 8, 2011) was used. The fresh herring was transported to Stavanger at −1.2 °C, frozen in seawater two days after being caught and stored for 4 months at −30 °C until the samples were prepared for analysis.

2.2. Sample preparation

Residual materials from three fish were pooled (total of three samples) and skins from 3 different herring were pooled (total of three samples). The resulting samples with weight between 0.5 and 2 g were homogenized in PBS buffer (50 mM, pH 6), 1:1 (w/v) while cooled on ice. The samples were then centrifuged and the supernatant was divided equally into two replicates. This procedure gave a total of 6 samples containing herring residual material extracts and 6 samples containing skin extracts. Samples were then filtered through a 10 kDa cut-off filter (Microcon Centrifugal Filter Devices, Millipore, 10000 x g, 30 min, +4 °C) before being diluted with mobile phase A prior to LC–MS analysis.

2.3. LC–MS Orbitrap analysis

The nanoflow liquid chromatography-mass spectrometer (LC–MS/MS) analysis were conducted using a Dionex Ultimate 3000 HPLC set up with a 300 μm i.d. x 0.5 cm Acclaim PepMap300 C18 trap column (Dionex) and a 75 μm i.d. x 15 cm Acclaim PepMap100 C18 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. The total run-time of 256 min was used, including the washing step and 30 min re-equilibration of the columns. 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 ≥ 2 and intensity ≥ 10000 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 MS². The mass spectrometer was tuned daily and calibrated weekly using the calibration solution recommended by Thermo Scientific.

2.4. LC–MS Orbitrap data analysis

The raw data files data from the Orbitrap were analyzed using the Proteome Discoverer 1.3 (Thermo Scientific) with the Sequest algorithm to search against the Teleostei (Tax. ID 32443, downloaded from NCBI on the 24/08/2011) database at NCBI (184572 sequences). Precursor mass and fragment ion tolerances were set to 10 ppm and 0.8 Da, respectively. No digestion enzyme was selected according to sample preparation. Max peptide mass was set as 10 kDa due to the cut-off filtration step.

High and medium significance peptide confidence filter was set in Proteome Discoverer, which means that peptide identifications are filtered based on the following combination of charge and Xcorr factor: 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).

2.5. Bioactive peptide database search

An in-house database (BioPepDB, Bioactive Peptide DataBase) was generated from literature studies. It contains 231 motifs, which have been found to have bioactive properties related to various activities, i.e. the cardioxysystem, antioxidant activity, opioid activity, and immunomodulation. The database was made using references up to June 2011 and contained the following information: letter code, amino acid sequence, main activity, bibliographic reference, bioactive peptide source and related/studied species.

The peptides identified by bioinformatics were searched against an 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 compares the motifs (i.e. peptide sequences) to a fasta database, it counts the number of hits and reports the position of the motif for the relevant sequence. The algorithm is currently run from the command line and the output is a text file summarizing the findings.

3. Results

From the Teleost database search, a total amount of 16361 (242 high significant, 16119 medium significant) and of 35437 (472 high significant, 34965 medium significant) unique peptides/proteins were detected in the 6 skin samples and in the 6 residual material samples, respectively. These peptides/proteins identified in the Teleost database were then screened for bioactive sequences using our in-house generated database (BioPepDB).

As outlined in Table 1 and Fig. 1 the majority of motif hits were found for peptides with antioxidant, cardiovascular system, and opioid agonist/antagonist activities.

In total 66 peptides from the herring samples were identified in BioPepDB. Fifty-five of the peptides were present in samples from both skin and residual material while the remaining 11 peptides were found either in only skin samples (4 peptides) or only in residual material samples (7 peptides). The majority of matches (about 50% in both sample types) were related to motifs with potential cardiovascular system activity. Furthermore, the identified peptides also contain motifs that have antioxidant (about 40%) and immunomodulatory (8%) activities. Other activities presented in the database were almost none represented (i.e. ileum contraction, opioid, cytotoxic and antimicrobial). Moreover, both sample types showed a similar distribution of peptides with a given activity (Fig. 1).

According to the obtained results, particular attention was given to the following peptides, which represent the most present motifs in both skin and residual material samples: AH, EL, YG and VK. These four bioactive di-peptides represented between 57% and 59% of the total amount of motif counts in skin and residual material samples.

respectively. Specifically, AH was recorded 376 and 808 times, EL was found 1731 and 3997 times, VK was detected 1006 and 2083 times, and YG was recorded 391 and 826 times in skin and residual material, respectively.

4. Discussion

Fish derived peptides have been shown to exhibit various effects, such as antihypertensive and antioxidant capabilities, both in in vivo and in vitro studies. In spite of this, very few food products containing fish derived bioactive peptides are commercially available. A defined path for isolation and identification of bioactive peptides has been commonly used, as reported in a recent review [18]. Hydrolysates from protein source are first tested for bioactivities and then fractionated by molecular weight in order to display which molecular size has the highest activity. The peptides/proteins in the fraction resulting in the highest activity are then identified by mass spectrometry followed by bioinformatics (protein sequencing). In the present study, a different approach is utilized. Identification of bioactive peptides in crude peptide/protein sources (skin and residual materials) is performed directly using a combination of mass spectrometry ( Orbitrap), bioinformatics and database search. This method is a good angle of approach in order to map the potential in new species and species that have been very little studied.

Since peptides present in enzymatically digested protein hydrolysates have exhibited different physicochemical properties and biological activities depending on their molecular weights and amino acid sequences [5,6], an appropriate molecular weight cut-off (cut-off filter at 10 kDa) was used in order to be effective in peptide identification, as previously suggested [10].

Here, a relatively large database has been created, containing more than 200 motifs. Studied protein sources, skin and residual material, contained a large amount of bioactive peptides, as expected. In fact, skin from other species of fish has been the source for isolation of a range of bioactive peptides. Like other organisms, fish secrete a variety of immunomodulation peptides involved in host defense mechanisms through their skin [16]. In the present study, one of the most represented di-peptide was YG, recognized as involved in immunomodulation activity [8].

In general, bioactive peptides have been identified in fish by-products from various species [18]. Here the attention is on the more represented motifs: AH, EL, VK and YG. The first two were di-peptides with antioxidant activity [1,20]. Antioxidants are known to be beneficial to human health as they may protect the body against ROS (reactive oxygen species), which can negatively affect membrane lipids, proteins and DNA. Recently, studies have focused on fish and hydrolysates from various species (e.g. mackerel (Scomber australasicus), tuna (Thunnus tongol) in order to find sources for peptides exhibiting antioxidant activity [18]). In particular seven antioxidant peptides were identified in the hydrolysates of sardinele (Sardinella aurita) residual materials (specifically, proteins recovered from waste stream of processing) [3]. It is clear that the possible generation of these two di-peptides from herring by-product have the potential to be incorporated into functional foods and/or feeds as natural antioxidant.

Antihypertensive peptides from fish sources were first identified in sardine meat over twenty years ago. Since then, there have been several reports of crude fish protein hydrolysates containing bioactive peptides with antihypertensive actions, especially in by-products of fish such as salmon (Oncorhynchus keta) [18]. As in this study, the majority have been found in the soluble protein fraction [21], and the di-peptide VK was the most present [13].

To date most of the studies related to bioactive compounds from fish residual material have been focused on red fish (vide supra). The
large quantities of herring caught (900 000 tons in 2009) generating ca. 25% residual material show that there is large amount of material available for bioactive peptide mining. However, as reported herein there is often only a small part of the peptide that is associated with the activity. Often di- and tri-peptides are the most potent compounds. In the future it is therefore desirable to be able to generate specific small peptides with the desired activity. Targeted isolation of the desired peptides can in the future be facilitated by means of, for example, utilizing selective molecular tweezers, an isolation strategy that has been used with great success for other compounds [9,14,17,19,22], attached to solid support such as magnetic beads. The beads can then be isolated by a magnet and the selectively bound peptides removed upon washing. By such means it is possible to isolate pure samples of the interesting peptides and the remaining peptides can still be used in feed. Alternatively, it has been demonstrated that it is possible to obtain serial enzymatic digestions in a system using multi-step recycling membrane reactor combined with ultrafiltration membrane system to separate fish protein hydrolysates based on their molecular weight [4].

5. Conclusions

The preliminary work outlined herein shows the bioactive potential harbored within skin and residual material from Atlantic herring. We have found bioactive peptides mainly with antioxidant, cardio-system, and immunomodulatory activities. It is interesting to note that most of the identified bioactive peptides were in common in skin and residual materials. Given the large quantities of this fish caught annually, and therefore also the large amounts of residual material available, it is obvious that this material can be the source for isolation of peptides with desired activities. The four different di-peptides present in large amounts in both skin and residual materials will be the focus for future studies concerning among other things peptide isolation.

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