A search for bioactive peptides in fish mucus

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Introduction

Bioprospecting, i.e., search for bioactive compounds, in fish by-products has a potential to convert low-value products into more valuable products. Bioactive compounds from various natural sources have been found to possess a range of biological activities e.g. anti-cancer, anti-inflammatory, anti-microbial or immunomodulatory activities, and they have been very valuable in the therapeutic armory (aspirin, penicillin, cyclosporine, and rapamycin are but a few examples). There are several recent examples of anti-microbial compounds, often of peptidic nature, from fish (Bergsson et al., 2005; Luders et al., 2005; Patrzykat and Douglas, 2005; Narvaez et al., 2010; Bragadeeswaran et al., 2011; Rajanbabu and Chen, 2011). Still, this source of biodiversity has been seriously underexploited in bioprospecting terms (Patrzykat et al., 2003), but often overexploited in commercial fisheries. Nations heavily dependent on fishing could create more value from the same amount of catch by bioprospecting the secondary products.

The aim outlined herein is to find bioactive compounds from fish secondary products. The main emphasis in this project will be on compounds in the fish skin mucus that have anti-bacterial activity, using cod, salmon and monkfish as starting materials (see Figure). We will here establish a bioprospecting pathway, where the analytical methods and obtained results also can be valuable for other areas of applied research, in particular in the fight against salmon (sea) louse (*Lepeophtheirus salmonis*).

Working plan and methods

Initial study (first year)

We will initially focus on the mucus of the fish skin. The mucus is a first-line of defense against the surrounding environment, and interesting bioactive compounds have previously been detected in the mucus from several fish species (Rajanbabu and Chen, 2011). We will make a comparative study of the three species using mass spectrometry (MS) and other proteomic tools to identify as many potentially bioactive compounds as possible.

Identification

As outlined in the figure the bioprospecting pathway includes sampling the mucus and filtration of samples resulting in two initial fractions. Low molecular weight fractions will be directly analyzed by LC-MS/MS. High molecular weight fractions will be subfractionated by isoelectric focusing in a gel free system (Agilent OffGel) and further by polyacrylamide electrophoresis. Detected proteins will be digested with trypsin and analyzed by LC-MS/MS.

Identification of peptides will first be done by searches against available genome and EST databases. Non-identified peptides with good spectra will be analyzed manually. Some low-molecular weight compounds that are not standard peptides (i.e., do not contain the standard unmodified amino acids) can have biological activities (Falkenberg et al., 2011). These compounds might contain modified amino acids, or they might be other classes of molecules. By MS analysis of exact mass together with the isotopic peak distribution of the molecules we will be able to approach the chemical formulas.

Bioactivities

As for investigation of the antibacterial activities, we will first concentrate on the low molecular weight fractions (Rajanbabu and Chen, 2011). The fractions will be tested with a standard bacterial growth assay using OD measurements. We also aim at developing two alternative bacterial assays. The simplest is an assay with poor bacterial growth conditions. Bacteria are much less sensitive to antibiotics under poor growth conditions (Eng et al., 1991; Nguyen et al., 2011). Thus, fractions that can kill off bacteria under poor growth conditions are even more interesting. The other assay takes into account that the environment usually contains a mixture of bacteria. We will combine a metagenomic approach for testing of anti-bacterial effects, and investigate if certain bacteria are susceptible to the antibacterial effects while other bacteria are resistant.

Later studies (second and third year)

In the first year studies, we will hopefully have detected bioactivities. The main aim is now to identify the compounds that possess these activities. We will then concentrate on the species that have shown the highest measurable bioactivities in its mucus, and we must develop methods to obtain larger amounts of the mucus. More detailed fractionations will be needed. The initial fractionation methods will be by molecular weight (using filtration, dialysis, size exclusion chromatography, etc.) and by physicochemical properties (preparative HPLC using reverse phase chromatography, ion exchange chromatography, etc.). The results will be analyzed statistically by multivariate analysis and principal component analysis in support of the search for the bioactive compounds.

In cooperation with the Department of Food Science, Aarhus University, it is possible to test for antibacterial and/or immunomodulatory activity using *in situ* pig intestinal microbial survival model and *in vivo* infection model. The former model uses the natural intestinal flora of pigs. Immediately after killing a pig the proximal small intestine is exposed, sutured at 10 cm intervals, and cut to preserve intestinal content and saline. Peptides are added and the intestinal "bags" are incubated a couple of hours. The content is added to bacterial growth media and plated for counting of colonies the next day. The *in vivo* infection model uses weaned piglets of a certain genotype that is susceptible to infection with the E. coli strain HB104. The piglets will receive the test material and will subsequently be inoculated with the E. coli strain. Growth of E. coli HB104 and diarrhea will be scored.

Future studies (four to ten years +)

When the structures of the bioactive compounds are at hand, it can be determined whether the best approach for making industrial scale amounts of the compounds are by processing the secondary products from the fish industry, or by synthetic chemistry. Commercialization will be attempted if the compounds are promising. Furthermore, having established the necessary methods and competence, more complex starting materials can be entered into the bioprospecting pathway.

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Figures

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- 5 http://www.nature.com/nprot/journal/v7/n4/full/nprot.2012.007.html?WT.ec_id=NPROT-201204
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Expected results and effects

We expect that there should be some differences between bottom-dwelling and pelagic species, as they probably have to be protected against partially different microbial and parasitic environments.

Through the work described here, we expect to isolate bioactive compounds, most likely with anti-bacterial activities, from the mucus of fish. The most potent compounds will be structurally determined, and commercialization will be attempted. Furthermore, a thorough characterization of mucus from salmon will also be a cornerstone in the fight against salmon (sea) louse (*Lepeophtheirus salmonis*).