Report from workshop on

Bioactive peptides from aquatic raw materials

Copenhagen, 2 March 2010
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Bioactive peptides from aquatic raw materials

2 March 2010
Copenhagen

Edited by
Lisa Lystbæk Andersen
Henrik Hauch Nielsen
Flemming Jessen

National Food Institute
Technical University of Denmark
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Preface

The open workshop “Bioactive Peptides from Aquatic Raw Materials” took place in Copenhagen on 2 March 2010 at Ingeniørforeningens (IDA) Mødecenter. The workshop was arranged by Flemming Jessen (project leader), National Food Institute, Technical University of Denmark (DTU), in cooperation with Gestur Hovgaard, director of the marine biotech company Atlantic Biotechnology S/P, Faroe Islands. Other project participants: Hordur G. Kristinsson (Matís, Reykjavik, Island), Turid Rustad (Norwegian University of Science and Technology (NTNU), Trondheim, Norway), Ingmar Høgøy (Seagarden ASA, Haugesund, Norway), Jan Stagsted (University of Aarhus, Denmark), Hóraldur Joensen (University of Faroe Islands, Tórshavn, Faroe Islands), Eddy G. Torp (DueMiljø AS, Oslo, Norway), Frank Hansen (Hansen-Øye Consult, Sortland, Norway).

This report consists of a synopsis of the presentations at the workshop with the programme and the slides accompanying each presentation presented in Appendix 1-12.

The workshop was supported by the Working Group for Fisheries Cooperation (AG-Fisk), Nordic Council of Ministers for Fisheries and Aquaculture, Agriculture, Food and Forestry.

March 2011
Summary

A workshop was held in Copenhagen in March 2010 addressing issues of documentation of bioactivity and the commercial utilisation of bioactive peptides and other compounds derived from aquatic raw materials. Ongoing research activities and commercial initiatives were presented by representatives from both research institutions and Nordic companies specialising in developing products containing bioactive peptides.

In conclusion of the workshop, three areas where further research is needed to fully document the bioactive effect of marine peptides were identified. These were: 1) Studies on the uptake and stability of bioactive peptides in the intestinal system, 2) the implementation of more extensive human trials to confirm positive results from \textit{in vitro} and animal studies, and finally, 3) more research is needed to determine whether the observed effect of marine peptides on the metabolic syndrome is a general peptide effect or directly attributable to specific peptides found only in fish or other aquatic materials. In general, more studies are needed to explore the sources, bioavailabilities, and the physiological/functional properties and the mechanisms of action of bioactive peptides.
Workshop synopsis

Introduction

The aim of the workshop was to generate an overview of the current state of the art of research and development within the field of utilising bioactive peptides and other compounds of marine origin for health beneficial purposes. The ambition was furthermore to provide a knowledge platform to facilitate Nordic cooperation and research coordination between industry and research institutions through the establishment of new collaborations and joint applications for funding under national as well as EU auspices.

The aquatic ecosystem represents an as of yet not fully explored resource of biodiversity with a huge variety of organisms adapted for living conditions very different from terrestrial animals. For example, fish generally have an eminent bioactive defence system for protection against the high amounts of potentially harmful bacteria in the water. In consequence, fish and other inhabitants of the aquatic ecosystem expectedly possess a great number of biocomponents of both nutritional and pharmaceutical value, in line with what has been found in the rain forest.

During industrial processing of fish for human consumption only about half of the total fish weight is converted to edible products. The remaining part, the secondary products, is mainly used for animal feed products. Internationally, there is increasing documentation of the fact that fish contain large amounts of bioactive peptides. These bioactive peptides often have beneficial effects in respect to both human and animal health, as for example strengthened immune response, better blood pressure regulation, inhibition of cancer cell proliferation, reduction of obesity, diabetes, and antibacterial activity. Products with bioactive peptides from fish have a potential usage in healthy foods, as nutritional supplements, as ingredients, as alternatives to antibiotic growth promoters in animal feeds, and as pharmaceutical products.

Despite many years of focus on better exploitation of the secondary marine products, there is still a great need for product and market development. The access to ever more advanced technologies and research-based knowledge increasingly enables the potentials of the marine raw materials to be developed into specific innovations. New applications within the area of “bioactive peptides” are developed in a complex interplay between industries, technologies, research, and markets. This workshop has its main focus on the “niche” that bioactive peptides represent within the broad spectrum of marine ingredients.

11 presentations were given by invited speakers from both academic research institutions and Nordic companies developing health promoting products.

Health aspects

Biologically active peptides released from fish proteins, either during digestion or during food processing, may have crucial influences on the regulation and modulation of the human metabolism. Thus such peptides are potential nutraceuticals providing health benefits and prevention or treatment of diseases. Many studies have reported that fish peptides have effects on important
health aspects including blood pressure, obesity, diabetes, cancer, coronary vascular disease, inflammation, and immunological diseases (Hordur G. Kristinsson, Appendix 8, page 85).

Central to many studies on the health effects of peptides are in vitro investigations of the mechanisms known to be involved. Antihypertensive peptides are mainly found and studied by their inhibitory effect on angiotensin-converting enzyme (ACE). Apart from inhibiting ACE, the ACE inhibitors have the ability to influence the metabolic syndrome as they reduce fat mass in adipose tissue (Stéphanie Bordenave-Juchereau, Appendix 2, page 23). In relation to obesity the bioactive effects are registered as reduced fat depositing, probably due to a regulation of the adipocyte life cycle (Stéphanie Bordenave-Juchereau, Appendix 2, page 23), but also as reduced liver lipid and reduced adipose tissue mass (Bjørn Liaset, Appendix 3, page 31). The mechanisms of action of bioactive peptides influencing diabetes seem to be both by a reduced post-prandial blood glucose response and by effects on the insulin response (Einar Lied, Appendix 4, page 43). Antimicrobial peptides from fish show direct effects on microbes and they are also potentially able to enhance the immune function (Jan Stagsted, Appendix 5, page 57). To document anticancerous effects the peptides must possess dual effects and induce programmed cell death (apoptosis) as well as inhibition of cell proliferation (Flemming Jessen, Appendix 12, page 143).

Industrial perspectives

Marine peptide hydrolysates are expected to have a great potential as supplements in a number of future products. Functional foods such as sports drinks, pet food, dietary supplements, but also cosmetics are seen as some of the most promising areas where, today already, there is a large and growing market. This is promoted by the increasing consumer awareness that a number of diseases (both concerning humans and animals) may be avoided by a healthier lifestyle, among this an intake of foods containing health promoting ingredients such as bioactive peptides (Hordur G. Kristinsson, Appendix 8, page 85).

Today already, there are a number of commercial marine peptide products with claimed bio-active properties such as activity against hypertension, intestinal diseases, stress, and insulin resistance on the market (Hordur G. Kristinsson (Appendix 8, page 85), Einar Lied (Appendix 4, page 43)).

A number of Nordic companies producing marine based hydrolysates have been established within the recent years, e.g. NutriMarine Life Science AS from Norway (Einar Lied, Appendix 4, page 43) and Atlantic Biotechnology S/P, Faroe Islands. Already established companies such as Seagarden ASA, Norway, have extended their production to also include marine hydrolysates/peptides (Bjarte Langhelle, Appendix 10, page 115). One of the major challenges for existing and future commercial peptide products is valid documentation of the functional and health promoting properties. Presentations at the workshop showed that a considerable number of research studies are taking place in collaboration with private companies to document the bioactivity of the products. The company NutriMarine Life Science AS has tested a protein hydrolysate, NutriPeptin, in a human trial investigating blood glucose concentration and insulin sensitivity (Einar Lied, Appendix 4, page 43). The Norwegian company Seagarden ASA has collaborated with International Research Institute of Stavanger (IRIS), Norway, on identifying peptide sequences with known stimulatory effects on the gastro-
Bioactive peptides from aquatic raw materials

intestinal system in peptide products (Anne Hjelle, Appendix 11, page 121). Similarly, protein hydrolysates from the Danish company Marinova ApS, which produces ingredients and foods from fish protein, are tested in collaboration with the National Food Institute, DTU, and University of Copenhagen, Denmark, for anticarcinogen and antioxidative properties (Flemming Jessen, Appendix 12, page 143).

Research activities

Bioactive peptides can be found as naturally present in e.g. the mucus of fish, where they may function as an outer defence against bacteria. However, bioactive peptides are potentially present in any protein as part of the protein sequence and are only released in their functional forms upon protein degradation, e.g. during digestion. These hidden peptides or “cryptides” are usually small containing 2-20 amino acids and have functions distinct from the parent protein (“Cryptein”, Stéphanie Bordeneave-Juchereau, Appendix 2, page 23). It has been found that some of the bioactive peptides are multifunctional; i.e. they may possess both ACE inhibitory activity and influence the proliferation and differentiation of fat cells (Stéphanie Bordeneave-Juchereau, Appendix 2, page 23). In this way, intake of marine peptides may be able to help prevent the development of the human disease known as “Metabolic syndrome”, which involves a number of cardiovascular risk factors such as obesity and hypertension.

The general focus in research, as well as on industrial scale, is on producing protein hydrolysates from fish or algae using either fermentation or for the greater part enzymatic hydrolysis using food grade proteases. Elucidating the functional mechanisms behind the observed health effects of e.g. fish protein hydrolysates is not always the immediate objective as the raw hydrolysates may display health beneficial effects in themselves. The Norwegian company Nutri-Marine Life Science AS (Einar Lied, Appendix 4, page 43) has developed a very promising fish hydrolysate product from saithe fillet (NutriPeptin). The positive health effects of NutriPeptin are supported by two clinical trials. NutriPeptin is suitable for application in various foods.

Much of the health effects associated with intake of fish can be attributed to the presence of n-3 fatty acids. However, fish peptides, minerals/trace elements, vitamins, and amino acids, and not least the in seafood abundant free sulfonated organic acid taurine, which has importance for energy metabolism, may also be important contributors (Edel O. Elvevoll, Appendix 5, page 53). Various seafood items display antioxidative capacity during simulated digestion experiments. Human clinical trials with combined n-3 fatty acids and taurine supplementation resulted in reduced total cholesterol and LDL-cholesterol in the blood. Similarly, saithe fish protein hydrolysate (FPH), which contains high amounts of taurine, has been shown to reduce visceral adipose tissue in rats (Bjørn Liaset, Appendix 3, page 31). However, taurine does not seem to be solely responsible for the observed effects and, furthermore, it remains to be tested if the positive effect of FPH is specific due to its marine origin or whether protein sources from other species display the same properties.

Three fish hydrolysates produced by Seagarden ASA (Bjarte Langhelle, Appendix 10, page 115) were subjected to fractionation, liquid chromatography purification, and mass spectrometry with the aim of identifying and categorising as many bioactive peptides as possible based on known bioactive peptide sequences (Anne Hjelle, Appendix 11, page 121). Focus was put on identifying bioactive peptides with respect to gastrointestinal diseases specifically and all three
Bioactive peptides from aquatic raw materials

Hydrolysates did to varying degrees contain bioactive peptides known to stimulate the gastrointestinal system. The PEPFISH project (Flemming Jessen, Appendix 12, page 143) has focus on identifying and characterising bioactive peptides from enzymatic hydrolysates from underutilised or low-value materials from fish processing. The hydrolysates will be tested in vitro for effects in relation to cancer cell proliferation and apoptosis, cancer cell migration, blood pressure regulation (ACE inhibition), immunological responses, and antibacterial activity.

Fish peptides both from laboratory scale and commercial hydrolysates display antioxidative properties in different oxidative systems (Turid Rustad, Appendix 7, page 77). This property is potentially useful for the food industry as addition of fish peptides can help avoid problems with oxidation and hence rancidity in products containing unsaturated oils. Furthermore, the fish protein hydrolysates have also been found to contain calcitonin gene-related peptide (CGRP)-like and Gastrin/Cholecystokinin (G/CCK)-like molecules. CGRP is a potent arterial and venous vasodilator involved in the control of hypertension. Gastrin and cholecystokinin are hormonal regulators of various digestive processes and feeding behaviours. Interestingly, the amount of CGRP-like molecules was highest when fresh fish material was used for hydrolysis.

A potentially enormous value addition is to be expected if peptides with documented health promoting or other effects can be derived from low-value fish material (Hordur G. Kristinsson, Appendix 8, page 85). However, most claimed bioactive effects still need to be verified. Also, a problem exists with consumer acceptance of products with added fish hydrolysates due to problems with lipid oxidation and subsequent rancid tastes and flavours. Some of these problems can be reduced by using hydrolysates prepared from protein isolates of homogenized fish materials, which contain lower amounts of lipids and pro-oxidants.

A large number of naturally occurring bioactive peptides, such as e.g. piscidines, have also been identified. The Nanofish project (Jan Stagsted, Appendix 6, page 67) has focus on isolating naturally occurring antimicrobial and immunostimulating peptides from fish. The Nanofish project has developed so-called nanoparticles containing antimicrobial fish peptides coated with a protective layer of chitosan or alginate. The nanoparticles facilitate protection of the peptides from degradation in the upper digestive tract and safe release in the lower digestive tract.

As a whole other aspect of bioactive compounds derived from fish, a variety of enzymes has been described by the Norwegian company Nofima AS (Inge W. Nilsen, Appendix 9, page 103). These include the well-known shrimp alkaline phosphatase and a heat labile shrimp double-strand specific DNase. Inhibitors of HIV-protease have been identified in marine invertebrates with very interesting pharmaceutical prospects. Currently, salmon lysozymes with antibacterial effects are being characterised.

Future challenges/research areas

The presentations and discussions at the workshop identified three main areas where further research is necessary in order to increase the knowledge on bioactivity of marine peptides and how they can be commercialized:

1) Research on uptake of bioactive peptides from the gut and how bioactivity is influenced by variations in pH, digestive enzymes, and transport through the intestinal membrane
2) More extensive controlled human trials are needed to confirm results obtained in in vitro and animal experiments
3) There is a need to determine whether the positive effect of marine peptides on the metabolic syndrome is a general peptide effect or due to specific peptides, and whether the observed effect is dependent on a synergistic interplay of several peptides. In this connection, it should also be determined what role other marine components present in marine hydrolysates such as the organic acid taurine has on bioactivity.

In conclusion, more research is necessary to obtain the valid and reliable documentation of bioactivity that is needed in order to introduce marine peptide products into the market and to obtain possible health claims. In general, more studies are needed exploring the sources, bioavailabilities, and possible physiological/functional properties and the mechanisms of action of bioactive peptides.
Sammenfatning

En workshop blev afholdt i København i marts 2010 omhandlende dokumentation af bioaktivitet og kommerciel udnyttelse af bioaktive peptider og andre forbindelser fra akvatiske råvarer. Igangværende forskningsaktiviteter og kommercielle initiativer blev præsenteret af repræsentanter fra både forskningsinstitutioner og nordiske virksomheder med speciale i at udvikle produkter, der indeholder bioaktive peptider.

Tre områder hvor yderligere forskning er nødvendig for fuldt ud at dokumentere den bioaktive effekt af marine peptider blev afslutningsvis identificeret på workshoppen. Disse var: 1) Undersøgelser af optagelsen og stabiliteten af bioaktive peptider i mave-tarmsystemet, 2) genemførelse af mere omfattende humane studier der kan bekræfte de positiveresultater fra in vitro- og dyreforsøg, og endelig 3) er der behov for mere forskning for at afgøre, hvorvidt den observerede effekt af marine peptider på det metaboliske syndrom er en generel peptid effekt, eller direkte kan henføres til specifikke peptider der kun findes i fisk og andre akvatiske råmaterialer. Generelt er flere undersøgelser nødvendige for at udforske ressourcerne, biotilgængeligheden, og de fysiologiske/funktionelle egenskaber og virkningsmekanismer af bioaktive peptider.
Appendices

Appendix 1. Programme

**BIOACTIVE PEPTIDES FROM AQUATIC RAW MATERIALS**

**Workshop, 2 March 2010, 10:00 a.m. - 17:00 p.m.**

Venue: Ingeniørføringens Medecenter, Kalvebod Brygge 31-33, DK-1780 København V

<table>
<thead>
<tr>
<th>Time</th>
<th>Presenter</th>
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<tbody>
<tr>
<td>9:00 - 10:00</td>
<td>Arrival</td>
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<tr>
<td>10:00 - 10:10</td>
<td>Welcome by Henrik Hauch Nielsen (Senior scientist, National Food Institute, Technical University of Denmark)</td>
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<tr>
<td>10:10 - 10:30</td>
<td>Rolf K. Berge (Professor, Institute of Medicine, University of Bergen, Norway)</td>
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<td>&quot;Improved health through novel peptides of marine origin&quot;</td>
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<td>10:30 - 10:50</td>
<td>Stéphanie Bordenave-Juchereau (Senior scientist, University of La Rochelle, France)</td>
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<td>&quot;Marine cryptides as a tool to fight the metabolic syndrome&quot;</td>
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<td>10:50 - 11:10</td>
<td>Bjørn Liaset (Scientist, National Institute of Nutrition and Seafood Research (NIFES), Norway)</td>
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<td>&quot;Fish protein hydrolysate reduces visceral adipose tissue mass in rats&quot;</td>
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<td>11:10 - 11:40</td>
<td>Coffee break</td>
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<td>11:40 - 12:00</td>
<td>Einar Lied (Managing director/Professor, NutriMarine Life Science AS, Norway)</td>
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<td>&quot;Marine peptides: a tool of blood glucose lowering and stabilisation&quot;</td>
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<td>12:00 - 12:20</td>
<td>Edel Elvevoll (Dean, Faculty of Biosciences, Fisheries and Economics, University of Tromsø, Norway)</td>
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<td>&quot;Seafood and health - more than n-3 fatty acids&quot;</td>
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<td>12:20 - 12:40</td>
<td>Jan Stagsted (Senior scientist, Department of Food Science, Aarhus Universitet, Denmark)</td>
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<td>&quot;Nanofish – utilization of natural fish antimicrobial peptides as nanoparticles?&quot;</td>
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<td>12:40 - 13:40</td>
<td>Lunch</td>
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<td>13:40 - 14:00</td>
<td>Turid Rustad (Professor, Department of Biotechnology, Norwegian University of Science and Technology (NTNU), Norway)</td>
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<td>&quot;Antioxidative and bioactive activities of fish protein hydrolysates&quot;</td>
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<td>14:00 - 14:20</td>
<td>Hordur G. Kristinsson (Head of division, Matís, Iceland)</td>
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<td>&quot;Production, quality and bioactivity of fish derived peptides&quot;</td>
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<tr>
<td>14:20 - 14:40</td>
<td>Inge W. Nilsen (Senior scientist, Nofima AS, Norway)</td>
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<td></td>
<td>&quot;Marine enzymes and enzyme inhibitors&quot;</td>
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Note:
Rolf K. Berge and Gestur Hovgaard were unable to give their presentations at the workshop.
Appendix 2. “Marine cryptides as a tool to fight the metabolic syndrome” by Stéphanie Bordenave-Juchereau
**CRYPTIDES?** (Autelitano et al. 2006)

- Hidden peptides with bioactivities
- Often unpredicted
- Role often totally distinct to the parent protein

**BIOLOGICAL ACTIVITIES:**

- Hypcholesterolemic, antioxidative, antithrombotic
- Mineral binding, opioid agonist and antagonist
- Antimicrobial, immunomodulatory...

**MARINE CRYPTEINES:**

- Vertebrate, invertebrate
- Protein from frame, muscle, skin...
- From by-products...

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**Some marine cryptides**

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<thead>
<tr>
<th>activity</th>
<th>origin</th>
<th>sequence</th>
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<tbody>
<tr>
<td>Antioxidant</td>
<td>Sardine muscle</td>
<td>MY</td>
</tr>
<tr>
<td>ACE inhibitory/hypotensive</td>
<td>Bonito</td>
<td>LKP, NM, LKP</td>
</tr>
<tr>
<td></td>
<td>Limanda frame protein</td>
<td>MIFPGAGGPEL</td>
</tr>
<tr>
<td></td>
<td>Oyster</td>
<td>AW, VW, FY</td>
</tr>
</tbody>
</table>

Obtained by fermentation and/or enzymatic hydrolysis

Alcalase, thermolysin, pepsin, trypsin

Some sequences (short) appear in various species.
Some cryptides are multifunctional
Unknown peptides are still hidden in biologically active hydrolysate
Hypertension and ACE
Molecules able to inhibit Angiotensin Converting Enzyme...

- Reduce hypertension: no degradation of KININE and no generation of ANGIOTENSIN II
- Prevent anomalies like type 2 diabetes (Bradykinin: vasodilator which potentiate adipocytes insulin sensitivity. McCarty 2003)
- Decrease fat storage (RAS in adipocytes, Goossens 2003)

Metabolic syndrome
Cluster of common cardiovascular risk factors:
- central obesity,
- hyperglycaemia,
- low HDL-cholesterol concentrations
- Hypertension
- Hypertriglyceridemia.

The association of MetS with the risk of developing diabetes and cardiovascular disease implies a greater risk of mortality.
**Obesity**

Imbalance between energy intake and expenditure leading to excessive body fat deposition.

**OBESITY**: Fat tissue pathology

With physiological, psychological and social consequences

**ADIPOCYTE**

- Hypertrophy of adipocytes
- Hyperplasia of adipocytes
- Both: enlargement and multiplication of adipocytes

**RAS** components are over-expressed in case of obesity.

**HYPERTENSION**

**CRYPTIDES ACE-I**
Bioactive peptides from aquatic raw materials

Dedicated to the regulation of adipocyte activity and the prevention of obesity and related diseases.

Adipocyte Life Cycle
- Preadipocyte
- Adipocyte
- Hyperplasia
- Maturing adipocyte
- Lipid filling
- Hypertrophy
- Hyperplasia

Treatments that regulate both size and number of adipocytes provide better therapeutic approach for treating obesity and associated pathologies.

Decrease of adipose tissue mass needs:

- Lipolysis or apoptosis of mature adipocytes
- Regulating proliferation and differentiation of fat cells
Could marine cryptides/ hydrolysates have incidences on adipocyte life cycle?

Our cell model: Immortalised human adipocytes

Lipid estimation by oil red O staining, glycerol and NEFA release measurement.

Adipocyte life cycle:
- Preadipocytes
- Hypertrophy
- Mature adipocytes

Clonal expansion

Determinant

Adipocyte

Mature adipocyte

Lipolysis

Apoptosis

Hyperplasia

Preadipocyte

Mature adipocyte
Some results obtained with immortalised human adipocytes:

Example of the lipolytic activity of an Algae Hydrolysate (AH)

Common lipolytic agents were tested on immortalised adipocytes which react as other cells lines used for lipolytic agents screening.
Algae hydrolysate (60µg/ml protein content) allowed a glycerol release as caffeine 0.25mM.

At this concentration, a reduction of 33% of fat storage was observed

Incubation: 22h

Marine cryptides....

- ACE inhibitory activity
- Incidence on adipocyte development

...and on the development of the metabolic syndrome...
Financial support and research projects:

BIOTECMAR
BIOTECHNOLOGICAL EXPLOITATION OF MARINE PRODUCTS AND BY-PRODUCTS

Pr. Fabienne Guérard

Projet cofinancé dans le cadre du programme PSDR Grand Ouest par:

GESTION DURABLE (PSDR Grand Ouest)
Pr. Patrick Bourseau

Thanks!
Appendix 3. "Fish protein hydrolysate reduces visceral adipose tissue in rats" by Bjørn Liaset
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**Incidences of obesity increase—so what?**

[Diagram showing various health issues related to obesity]

www.biocytis.com

**Obesity and the metabolic syndrome**

**Definitions for the Metabolic Syndrome**

**WHO 1999**

- Dysglycemia (DM, IFO, IGT, IR) + 2 of:
  - BMI \(>30\) or TAHK \(>0.25\) males/\(0.85\) females
  - Dystipidemia (Trig \(\geq 1.7\) mmol/l low HDL \(<0.9\) males/\(<1.0\) females)
  - BP \(>140/90\) monthly
  - Micro alb (abs excl \(>20\) μg/min)

**ATP III**

- 3 or more of:
  - Waist \(>102\) cm (males)/\(<88\) females
  - Dystipidemia (Trig \(\geq 1.7\) mmol/l low HDL \(<1.0\) mmol/l low HDL \(<1.3\) mmol/l)
  - BP \(>135/85\) mmHg
  - FPG \(>6.1\) mmol/l

http://img.medscape.com
Bioactive peptides from aquatic raw materials

5

Why obesity?

- Proteins !!!

Lowell & Spiegelman 2000
Nature 404, 652

6

Dietary protein intake and heat production

Data fig TS Hamilton 1939 J Nutr

- Recommended intake: 15 (10-35) energi%
Dietary protein and energy expenditure

- Very-low dietary protein induces energy expenditure
- High dietary protein induces energy expenditure
- However, protein intake quite constant over last decades (15-20 en%)
- Can different types of dietary proteins induce energy expenditure differently at average dietary level

Fish protein hydrolysate

Enzymatic hydrolysis

Dried fish protein hydrolysate (FPH)
Bioactive peptides from aquatic raw materials

Principle of FPH production

Hydrophobic

Hydrophilic

Taurine

FPH

Insoluble

Principle of sodium-caseinate production

NaCl

Na-caseinate
Rat study with saithe FPH

- Dietary saithe FPH compared to dietary soy protein and casein

<table>
<thead>
<tr>
<th>Component</th>
<th>Saithe FPH [g/kg]</th>
<th>Soy protein</th>
<th>Casein</th>
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</thead>
<tbody>
<tr>
<td>Saithe FPH</td>
<td>239</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soy protein isolate</td>
<td>-</td>
<td>230</td>
<td>-</td>
</tr>
<tr>
<td>Casein</td>
<td>-</td>
<td>-</td>
<td>227</td>
</tr>
<tr>
<td>KCl</td>
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Saithe FPH reduces energy-efficiency

A. Growth chart

B. Energy efficiency
Bioactive peptides from aquatic raw materials

Saithe FPH contains taurine – and taurine important for bile acid (BA) conjugation

Dietary Taurine

Liver BA concentrations

Liver

Conjugated BA

Taurine

Bile acids (BA)

Cholesterol

Saithe FPH reduces TAG in liver and blood

Liver fatty acids

Liver TAG

Plasma TAG

VLDL

Liver β-oxidation

Plasma CH3-β-hydroxybutyrate

Series B

Series C
Saithe FPH elevates plasma bile acid concentration

Bile acids (BA) → Cholesterol

Liver

Blood BAs

Conjugated BA

Intestinal BAs

Fecal BA excretion

Saithe FPH Soy protein Cassin

Plasma BA concentrations


Bile acids induces heat production in brown adipose tissue

Bioactive peptides from aquatic raw materials

17

FPH alters gene-expression and decreases visceral adipose mass

18

Suggested mechanism of action
Conclusions:

- At equal energy-intake, the rats that receive dietary sain FPH have:
  - Reduced visceral adipose mass
  - Reduced fasting plasma TAG
  - Reduced hepatic TAG concentrations

- FPH is promising for preventing development of the metabolic syndrome in rats, relative to soy protein and casein

FPH and the metabolic syndrome

**Definitions for the Metabolic Syndrome**

**WHO, 1989**
- Dysglycemia (DM, IFO, IGT, (IR)) + 2 of:
  - BMI ≥ 30 or TMIH (>0.90 males/0.85 females)
  - Dyslipidemia (Trig ≥ 1.7 mmol/l, low HDL (<0.9 males/1.0 females)
  - BP > 140/90 monthly
  - Micron alb (albumin > 20 μg/min)

**ATP III**
- 3 or more of:
  - Waist (>102 cm males/88 females)
  - Dyslipidemia (Trig > 1.7 mmol/l, low HDL (<1.0 mmol/l, 1.3 mmol/l)
  - BP > 135/85 mmHg
  - FPG ≥ 6.1 mmol/L

http://img.medscape.com
Collaborators:

- University of Copenhagen
  - Qin Hao
  - Karsten Kristiansen

- University of Southern Denmark
  - Philip Hallenborg

- University of Bergen
  - Gunnar Møllgren

- Karolinska University
  - Hans-Ulrich Marschall

- NIFES
  - Lise Madsen
  - Marit Espe
  - Gabriel Criales
  - Åse Heltveit
  - Jacob Wessells
  - Livar Frøyland

Financial support:

- Rubin/ FHF
- Innovation Norway
- Danish Natural Science Research Council (Danish Obesity Research Centre)
- Program Committee on Nutrition, University of Bergen

Large scale enzymatic hydrolysis:

- Novozymes, Bagsvaerd, Denmark
Thank you for your attention!

https://dak.com/people/bigger-beer-belly-in-the-world/
Appendix 4. ”Marine peptides; a tool of blood glucose lowering and stabilisation” by Einar Lied
Bioactive peptides from aquatic raw materials

3. Raw material: Mixed fresh or fresh frozen fish and shellfish. Fish and shellfish used exclusively to high quality raw material is used in the process.

2. Homogenizer: The fish mixture is mixed with pure water and made into a homogenate in a homogenizer.

3. Inoculation & Hydrolysis: The temperature in the homogenate is raised to 65 °C. The mixture of enzymes is added and the homogenate is stirred for 30 minutes.

4. Enzyme Inactivation: The temperature in the homogenate is raised to 90 °C, and kept for 30 minutes. Other inactivating the enzyme and ensuring product stability.

5. Separation: The hydrolysate is followed by separation of the water soluble peptide fraction and the undigested protein rich fraction in order to obtain a pure peptide product.

6. Concentrating: The aqueous soluble and peptide-rich fraction is concentrated by a simultaneous dry material content by precipitation.

7. Spray drying: The concentrate is spray dried into an easy flowing white powder.

8. Packaging: The spray dried powder is packed in vacuum plastic bags, which are sealed in baled form.

---

**Chemical Characterization**

**Proximate Composition (%)**
- Protein: 44.2
- Fat: 0.1
- Water: 6.8
- Ash: 0.8

**Minerals (mg/kg)**
- Calcium: 220
- Magnesium: 2.1

**Amino Acid Profile (g/100g)**
- Nonessential amino acids: 95.2
- Essential amino acids: 157.5
- Total amino acids: 255.7
- Protein: 64.3
- Tyrosine: 12.4
- Arginine: 56.0

Nutraceuticals:
- *Abalone* bioactive peptides: 25.4
- *Turmeric* bioactive peptides: 32.0
- *Moringa* bioactive peptides: 27.2
- *Fenugreek* bioactive peptides: 37.1
- *Valine* bioactive peptides: 40.2
- *Isoleucine* bioactive peptides: 38.4
- *Leucine* bioactive peptides: 39.5
- *Proline* bioactive peptides: 30.9
- *Tryptophan* bioactive peptides: 6.8

*Glu* and *Asp* (g/100g):
- Glutamic acid: 98.7
- Aspartic acid: 270.0
- Glycine: 620.0
- Proline: 129.9

Estimated content of *Glutamine*: 70.0

---

The Making of Nutraperp...
Bioactive peptides from aquatic raw materials

The Health Problem

About 3% of the population in Western communities suffers from Diabetes 2, another 9% suffers from persistent elevated blood glucose levels. It is estimated that about 7% of the population will suffer from Diabetes type 2 by the end of 2018, and twice that number from highly elevated and unhealthy blood glucose levels.

Obesity and diabetes have turned out to be a great and fast growing health problem.

Diabetes may lead to:

- **Cardiac disease**: the risk of developing different cardiac diseases is increased by 4-5 fold in people suffering from diabetes.
- **Elevated blood pressure**: 70% of diabetic patients have blood pressures higher than 140/90 (normal blood pressure)
- **Kidney disease**: Diabetes increases the risk of severe kidney failure and dialysis treatments.
- **Neuropathy**: Diabetic is the most frequent cause of blindness in people less than 60 years.
- **Impairments**: Diabetic affects blood circulation, the risk of amputations below the knee is increased 36 fold.

Growing health problems related to overweight and elevated blood glucose levels spare for innovative nutrition preparations and food supplements based on natural and functional ingredients to reduce health hazards.

Clinical study with healthy individuals

**Objective**: Investigate the effects of NutraPeptide in the postprandial blood glucose and serum insulin in comparison with soy protein, casein and fish protein from tilapia (fresh fish processed).

**Test individuals**: 17 healthy individuals, all women ranging from 35 to 55 years, participated in the study.

**Implementation**: The studies were always performed in the morning. The test meal was the first meal of the day-and was given as an ordinary breakfast meal containing white bread with butter, marmalade, tomato, cucumber, a cup of tea and a soup consisting of the test protein. In the peptide meal the source of protein was composed of peptides and full protein from fish filet in the ratio 20:80. All meals (containing the different proteins) were standardized in energy and macronutrients. Each combination of meals were tested in all participants of the study, totalling 17 observations per protein tested. The meals were taken within a 15 min period. Blood was sampled from the arm before the meal and then at 20 min intervals for 240 minutes. The test persons was resting in bed during the whole measuring period.

**Analysis**: Blood samples were analysed for whole blood glucose and serum insulin concentrations.

Bioactive peptides from aquatic raw materials

The study
The study was carried out at Uppsala University and comprised 17 healthy women 31±5 years old. They were given a test meal of a standardized 75 g oral load of white bread with butter, jam and tomato, a soup containing the test protein and a cup of tea. All meals were isonitrogenic and isonitrogenous. The meals were spaced within 15 minutes of which the test persons were sampled for blood at 10 minutes interval for 3 hrs.

Conclusion
Marine peptides reduced the mean the blood sugar postprandial response significantly compared to fish protein (p<0.05) and casein (p<0.05).

The postprandial insulin response from fish protein was significantly lower (p<0.005) than the casein response from fish protein hydrolysate (fish peptides).

Maeve van der Wel & Bengt H Morgan 2005, manuscript in preparation.

MUSCLE CELL (→ MUSCLE FIBER)

Increased insulin sensitivity leads to increased facilitated diffusion of glucose through the cell wall and into the muscle cell.

Nutri-Peppein™ increases receptor sensitivity for insulin, and consequently reduce insulin resistance.
Bioactive peptides from aquatic raw materials
Bioactive peptides from aquatic raw materials

The bioactive marine peptides were evaluated in a study on type 2 diabetes patients. The study was conducted in 2010.

**Diabetes 2 and NutriPeptan™**

**The studies continue in 2010**

**Conclusion:** 1% NutriPeptan™ reduced the blood glucose response in Diabetes type 2 patients by 47%.

**NutriPeptan™ as a tablet**

**The studies continue in 2010**

**Conclusion:** 1% NutriPeptan™ reduced the blood glucose response by 47%.

**The studies:** Each weighing 80 mg, containing 500 mg of NutriPeptan™ and 35 mg of marine alginates, were consumed 30 minutes before eating and compared with placebo which resulted in a decrease in blood glucose in the stomach.

**The test persons:** 35-65 years, healthy by test and women.

**The experiment:** The test persons took 100 mg of NutriPeptan™ in an empty stomach in the morning. 3 tablets equivalent to 10 mg of NutriPeptan™ per day were taken 10 minutes before the meal. Blood glucose measurements were performed at 15 minutes intervals until the glucose levels returned to the level or below before taking the test meals.
Bioactive peptides from aquatic raw materials

**SPORT**

NutriPeptin™ is used as a functional ingredient in the sport nutrition preparation Restitution™. Professional Sport Nutrition: the first in a series of 3 different sport nutrition preparations with NutriPeptin™ inside: "PreCompl. - On the Road" and "Restitution" aimed to increase performance and satisfy the specific nutrition demands before, during and after work-outs and competitions. Restitution™ Sport was tested and used by Team CSC / Tour de France 2005 and 2006, and in Giro d’Italia 2006.
NutriPeptin™ is manufactured and categorised as a food ingredient; neither is it classified as "Novel Food" since marine hydrolysates has been produced and used in foods before January 1st, 1998.

Consequently, NutriPeptin™ and its applications in foods is only subject to food manufacture control and regulations in Norway as well the EU without any further documentation.

---

Bottom Line:

NutriPeptin™ lowers the postprandial blood glucose response and may be used as a tool to:

- stabilise the blood glucose level in individuals suffering from persistent elevated blood glucose and diabetes 2 without any kind of side effects.
- the claim is supported by 2 clinical studies in Sweden and the UK.
- 2 clinical studies are being started at the University of Bergen - Institute of Medicine in collaboration with Haukeland University Clinic.
Appendix 5. “Seafood and health – more than n-3 fatty acids” by Edel O. Elvevoll

Seafood and health - more than n-3 fatty acids

Edel O. Elvevoll
Faculty of Biosciences, Fisheries and Economics (BFE)
University of Tromsø


Antall døde per 1 000 innbyggere

Kilde: Mømelund og Borgen (1996) oppdatert med tall fra Statistisk sentralbyrå
Fish Consumption: Effects on Disease Outcomes

- **Heart**
  - Coronary Death / Sudden Cardiac Death
  - Nonfatal Coronary Events
  - Atrial Fibrillation
  - Congestive Heart Failure

- **Brain**
  - Neurodevelopment (in utero, infancy)
  - Ischemic Stroke
  - Mood and Depression
  - Cognitive Decline and Dementia
  - Postpartum Depression

- **Other**
  - Inflammatory Diseases
  - Cancer
  - Bone Health

Advice on fish consumption: benefits and risks

“Seafood choices – balancing benefits and risks” 2006. Institute of Medicine, USA

“Advice on fish consumption: benefits and risks” 2004. SACN/COT, Storbritannia

“Helhedssyn på fisk og fiskeværer” 2003. Fødevarédirektoratet, Danmark

EPSA (2005), WHO/FAO(2003, 2009?), etc

Undeland et al., 2009 Seafood and health - what is the full story? MARIFUNC

“Et helhetssyn på fisk og annet sjømat i norsk kosthold” 2006: VNM, Norway
Getting information on health effects

**Human studies, clinical trials**
- Highest level of evidence
  - Firm control of diet, study length, type of subjects, etc.
  - Expensive
  - Impossible to conduct on some diseases

**Observational (Epidemiological) studies**
- Segment of population observed
  - Fish intake associated with diseases
  - Indication of correlation
  - Many confounding factors

**Animal Studies**
- Extrapolation to humans is limited
  - Can exert tight control over experimental conditions
  - Insights into designing human studies

**In vitro (test tube) studies**
- Hardest to extrapolate to humans
  - Important in early phases
  - Can give directions

---

**Fish consumption and CHD mortality**

- Meta-analysis, observational, 222,364 individuals
- Inverse association between fish consumption and CHD mortality rates
- Fish meal once a week, reduced risk of CHD death by 15% compared to
current consumption
- May be further reduced with additional consumption
  - 1% decrease with per additional serving per week

He et al., Circulation, 2004
Meta-analysis of 16 prospective cohort studies (n=326,572) and 4 randomized controlled trials (n=29,456) from the U.S., Europe, and Asia.

Total risk reduction = 36% (95% CI: 20 to 50%; p<0.001)

Hibbeln et al., Feb 17, 2007  
- Lancet 369:578-585

Relative to health and consumption advisories:
"mothers who ate small amounts (< 340 g/wk) of seafood were most likely to have suboptimum neurodevelopment outcomes than children of mothers who ate more seafood than the recommended amounts"

340g/wk = 12 oz./wk = Four 3 oz. Servings /wk
To a large extent attributed to n-3 fatty acids...

Other possible contributors

- Peptides
- Amino acids
- Minerals and trace elements
- Vitamins
Antioxidative Capacity (ORAC) of Seafood Items and Blueberry during digestion


Antioxidative Capacity (FRAP) of Seafood Items and Blueberry

Antioxidative Capacity of Seafood Items and Blueberry during digestion

- Oxygen Radical Absorbance Capacity (ORAC)
  - Preformed peroxyl radicals attack a fluorescent probe and decrease the fluorescence. Antioxidants have a protective effect: donate H-atom and delays the decrease in fluorescent.
  - pH 7.4 and 37°C. Trolox as standard.

- Ferric Reducing Ability of Plasma (FRAP)
  - Measures the reducing capacity of an antioxidant to reduce a Fe$^{3+}$-complex to a Fe$^{2+}$-complex with an intense blue colour.
  - pH 3.6 and 37°C. Trolox as standard.

- AOC
  - Increase up until the phase simulating the small intestine.
  - ORAC: muscle > blueberry
  - FRAP: muscle < blueberry

- FRAP assay pH 3.6:
  - stomach
  - appropriate to use when assessing the antioxidative protection in this environment.

- ORAC assay pH 7.4
  - better method to assess the antioxidative protection in the intestine and elsewhere in the human body.

- Cellular antioxidative assay
Bioactive peptides from aquatic raw materials

**Taurine**

\[
\begin{align*}
\text{O} & \quad \text{H} \\
\text{HO-S-C-CH}_2 & \\
\text{O} & \quad \text{H} & \quad \text{NH}_2
\end{align*}
\]

- Free sulfonated amino acid.
- Shown to be a marker of seafood consumption.

**Dietary markers on IHD mortality**

- Associations between various dietary markers and mortality from IHD
- Males, n=2462
- "In our present study, taurine excretion, in fact, appears as the most significant factor (inversely) in IHD mortality"
Bioactive peptides from aquatic raw materials

Taurine supplementation

- Without TAU: 1 g n-3 PUFA
- With TAU: 1 g n-3 PUFA and 420 mg taurine
- Control

Changes in serum lipids (mmol/L)

- Total Cholesterol
- HDL-C
- LDL-C

* Significantly different from the control group

- Effects of taurine supplementation on blood lipid levels comparable to a diet high in seafood
- Randomized, double-blind, parallel intervention study
- 320 healthy individuals
- 2 weeks, fish oil
- Reduced total cholesterol (4%) and LDL (
- HDL increased (6%)
- More clinical trials needed

Taurine is abundant in seafood

- Intervention studies in animals and humans have suggested that protective effect may act through several mechanisms
  - Antioxidative properties
  - Reduce pro-inflammatory products
  - Suppress atherosclerosis
  - Platelet aggregation, reduce blood cholesterol, improved cardiac performance

- Study
  - The mechanism(s) needs to be studied
  - The relevance and the effect of taurine in amounts normally acquired through the diet should be studied
  - Possible interactions with other components abundant in seafood
**Setup, mouse dietary experiment**

- Apolipoprotein-E<sup>-/-</sup>-mice (background C57Bl/6, black 6)
- Atherrogenous diet to speed up the pathological processes
  - Western diet (TestDiet)
    - 21.0% fat (gm) \(\Rightarrow 41.7\%\) kcal
    - 0.2% cholesterol, no cholate
    - isocaloric and isonitrogenous
- 4 dietary groups (♀), n=9
  - Western diet alone
  - Western diet + Seal oil (1% wt:wt)
  - Western diet + Seal oil (1% wt:wt) + taurine (0.5% wt:wt)
  - Western diet + taurine (0.5% wt:wt)
- 14 weeks, Blood sampling 0, 7 and 14 weeks

**Analyses**

- Heart + aortic arch \(\rightarrow\) Embedded in paraffin for subsequent histological analyses
- Whole mount *en face* analysis of lesion formation in the aorta
- Measurement of cholesterol, triglycerides, cytokines, chemokines, and soluble adhesion molecules in plasma
- Determination of fatty acids in whole blood/plasma
- Gene expression:
  - Isolation of RNA from various tissues (heart, liver, kidney, blood) and subsequent real time PCR analysis
Other analyses

I. Atherosclerosis
   a. Heart-aortic arch
   b. Embedded in paraffin for Histological analyses
   c. Whole mount *en face* analysis of the aorta
      - Opened longitudinally
      - Oil red staining

II. Gene expression

*en face lesion analysis, aortic arch*

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<th>Males</th>
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<tr>
<td>SO</td>
<td></td>
</tr>
<tr>
<td>CO</td>
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</table>
Bioactive peptides from aquatic raw materials

25

![Graph showing taurine in fish products (mg/g ww)]

26

![Graph showing taurine in some raw and prepared muscle foods]
The end – thank you

Karl-Erik Eikertsen,
Hege Devold,
Hanne K. Mæhre,
Ida J. Jensen,
Bjørn Tore Dragne,
Rune Larsen,
Svein Kristian Stormo,
Jan Ole Olsen,
Jan Brox,
Bjarne Østerud

Foto: Ole Torrisen, III
Appendix 6. "Nanofish – utilization of natural fish antimicrobial peptides as nanoparticles?" by Jan Stagsted

Nanofish

Utilization of natural, fish antimicrobial peptides as nanoparticles?

<table>
<thead>
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<th>Peptide</th>
<th>Sequence</th>
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<th>Mann/De</th>
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<td>2667</td>
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</tbody>
</table>


University of Aarhus
Department of Food Science
Jan Stagsted
Nanofish 2010
Bioactive peptides from aquatic raw materials

Nanofish

YouTube - Antimicrobial Peptides
Welcome to the Antimicrobial Peptide Database and Analysis System

Number of species: ~6,300  ~30,000

Number of AMP: 548  50
Bioactive peptides from aquatic raw materials

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<th>Peptide</th>
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<td>Peptide 4</td>
<td>VTYSYTYCNIV</td>
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</table>
Monkfish peptides in an aqueous extract inhibit growth of E. coli

Nanofish
Monkfish peptides in an acidic extract modulate innate immune response

![Graph showing luciferase activity vs. 1/dilution for solvent control and monkfish peptides.]

Nanofish

![Diagram showing various foods and their effects on NF-κB and Curcumin.]

University of Aarhus
Department of Food Science

Jan Stagsted
Nanofish 2010
Protection against digestive proteases in the upper GI tract

Release of AMP in the lower GI tract

Protecting AMP’s from degradation

Chitosan for anionic AMP
Alginate for cationic AMP
Protecting AMP’s from degradation

Chitosan for anionic AMP
Alginate for cationic AMP
Nanofish

Thank you for your attention!
Appendix 7. “Antioxidative and bioactive activities of fish protein hydrolysates” by Turid Rustad

Antioxidative and bioactive activities of fish protein hydrolysates

Turid Rustad
Department of Biotechnology
Norwegian University of Science and Technology (NTNU)

Outline

- Process overview
  - What influences composition and properties
- Antioxidant activity of FPH
  - Model systems
  - Different prooxidants
  - Mechanisms
- Bioactive activity
  - CGRP-like molecules
  - Gastrin/Cholecystokinin-like molecules
- Concluding remarks
Bioactive peptides from aquatic raw materials

Raw material

- Cod backbones after removal of flesh
  - Frozen or fresh backbones
    • Different time of hydrolysis
- Commercial fish powders
  - Norland HFC
  - Aroma (New Zealand)
  - Powders from Danish fish protein
    • MariPep C
    • MariPep P
    • MariPep CK
Antioxidative properties

- Peptides from fish proteins have antioxidative properties in different oxidative systems
- Antioxidative mechanisms related to the ability of peptides and hydrolysates to:
  - scavenge radicals
  - chelate transition metals
  - alter the development of rancidity in unsaturated oils by adducting volatile aldehydes
- Antioxidative properties depend on
  - amino acid sequence
  - molecular weight
  - pH

DPPH radical scavenging activity

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<tr>
<th>Sample</th>
<th>Radical scavenging %</th>
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<td>P</td>
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</tr>
<tr>
<td>CK</td>
<td>30</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
</tr>
<tr>
<td>Norland</td>
<td>35</td>
</tr>
<tr>
<td>FPH20</td>
<td>40</td>
</tr>
<tr>
<td>FPH50</td>
<td>35</td>
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<tr>
<td>Aroma</td>
<td>20</td>
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Iron chelating ability

<table>
<thead>
<tr>
<th></th>
<th>g pr/uM Fe</th>
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<td>MariPep P</td>
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<tr>
<td>Norland HFC</td>
<td>5.0</td>
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<tr>
<td>Aroma powder</td>
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</table>

Fe²⁺ concentration - 4mMol/mL
Interaction time between protein and iron - 60 min

Kinetics of iron chelation

Protein concentration: C, Norland, CK, FPH20, FPH50 - 8 mg/mL;
P sample - 1.0mg/mL; Aroma - 0.08mg/mL
The concentration of added Fe²⁺ in the final reaction mixture - 4mMol/mL
**Fe and haemoglobin mediated oxidation**

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<tr>
<th>Prooxidants</th>
<th>Fe</th>
<th>Haemoglobin</th>
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<tbody>
<tr>
<td>Effect of prooxidant</td>
<td>Linear</td>
<td>Michaelis-Menten</td>
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<tr>
<td>Dissolved oxygen</td>
<td>Not dependent</td>
<td>Dependent (1st order kinetics)</td>
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<tr>
<td>pH</td>
<td>Optimum at pH 4 - 5</td>
<td>Increasing with pH</td>
</tr>
<tr>
<td>Temperature</td>
<td>$\text{Ea} = 60-86 \text{kJ/mole K}$</td>
<td>$\text{Ea} = 48 \text{kJ/mole K}$</td>
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<tr>
<td>Phosphate</td>
<td>Antioxidant</td>
<td>No effect</td>
</tr>
<tr>
<td>EDTA</td>
<td>Antioxidant</td>
<td>No effect</td>
</tr>
</tbody>
</table>

**A system to measure the oxidation kinetics**

**Liposomes**

- Cod roe phospholipids
  - PUFAs: 41 ± 9%
  - EPA: 11 ± 2%
  - DHA: 26 ± 9%

**Oxidation rate measurement**

- Prooxidant
- Antioxidant
- Solution of oxidation
Bioactive peptides from aquatic raw materials

Reduction of oxygen uptake rate (OUR)
Fe³⁺ catalysed oxidation – effect of pH

Protein concentration - 1.25mg/mL
Fe³⁺ concentration - 15μM
Liposomes (lipid concentration 6mg/mL)

Hb induced oxidation: pH ~ 6.6

- Fish proteins reduced Hb induced oxidation
- C and CK showed identical effect
- FPH20 was the most effective of the tested proteins
Bioactive peptides from aquatic raw materials

Reduction of oxygen uptake rate (OUR)
Haemoglobin as prooxidant - effect of pH

Inhibition of oxidation, %

Protein concentration - 4 mg/mL
Fe^{3+} concentration - 15 μM
Liposomes (lipid concentration 6 mg/mL)

Antioxidative properties - Summing up

<table>
<thead>
<tr>
<th></th>
<th>FPH 20</th>
<th>FPH 50</th>
<th>MariPep P</th>
<th>MariPep C</th>
<th>MariPep CK</th>
<th>Norland</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH radical scavenging activity</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Iron chelating ability</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fe^{2+} (pH 4.5)</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fe^{3+} (pH 5.5)</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fe^{3+} (pH 6.5)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hb (pH 4.5)</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hb (pH 5.5)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hb (pH 6.5)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

2 4 3 5 0
Model food system for evaluation of effect of added fish proteins

The recipe for salmon pate

- Salmon (cooked) 41.6 %
- Salmon (smoked) 10.8 %
- Rainbow trout oil 17.5 %
- Whey powder 3 %
- Water 24.7 %
- Salt 0.5 %
- Vinegar 0.8 %
- Fish proteins 1.1 %

*20 min of baking at 190°C (water bath)*
Appendix 8. “Production, quality and bioactivity of fish derived peptides” by Hordur G. Kristinsson
Many different applications

Food ingredients
Aquaculture
Farm animals
Process aids
Cosmetics
Food supplements
Nature medicine
Medical products

USA – functional foods

$30-80 billion in 2009

2011
25% omega-3 products
Animal food market – opportunities

USA - petfood
$50 billion in 2009

Health effects

Increased consumer awareness about natural bioactive compounds

- Might work against:
  - Oxidative stress
  - Increased blood pressure
  - High cholesterol
  - Inflammation
  - Cancer
  - Diabetes
  - Obesity

Could play a role in a more healthy living
Important to document and verify bioactivity

In-vitro
Test tubes

In-vitro
Cell cultures

In-vivo
Animals

In-vivo
Clinical trials

Cost

Substantial value addition

Fishmeal  SeaCure  PeptACE

0.13 ISK/g protein  81 ISK/g protein  132 ISK/g protein

623-1015 fold difference!
Bioactive peptides from aquatic raw materials

Peptide products

- Katsuobushi oligopeptide (Vasotensin®) Nippon Supplements
- Sardine peptide SP100N Senmilesau
- Seacure® Proper Nutrition
- PeptACE Natural Factors
- Peptides de Poisson Grand Ocean
- PeptiStress D)Fusion
- AntiStress Forté Pharma

Foods with fish peptides

- Apertizers with low GI: Nutripeptin
- Cakes and orange juice with Collagen HM
- Bread with Phescallim
- Chocolate with Protizen: relaxing properties

Copalis, France
Cosmeceuticals with fish peptides

The hydrolysis process

- Fish Muscle + Water
- Homogenization
- Enzyme
- Hydrolysis
- Reaction Termination
- Cooling
- Centrifugation or Filtration
- Drying or Concentrating
- FISH PROTEIN HYDROLYSATE

Vselena, Poland
A new approach - isolate hydrolysate

Patented pH-shift process by MPF IIC.

Some benefits of using isolate

Better color and cleaner product
- ↓ lipids
- ↓ pro-oxidants
- ↑ protein
Some benefits of using isolate

Less lipid oxidation

Major problem of fish peptide products

Lipid oxidation

Consumer acceptance threshold
Antioxidants can be effective

The isolate process can improve quality
Many different species/materials studied

- Cod
- Haddock
- Saithe (pollock)
- Blue whiting
- Capelin
- Herring
- Salmon
- Clams
- Shrimp
- Lobster
- Sea cucumber
- Seaweed
- Tilapia
- Channel catfish

FOCUS: Byproducts

Hydrolysis Ultrafiltration

Investigations

Antioxidative properties
- ORAC
- DPPH
- Metal chelation
- Radical scavenging
- Monocytes
- Protein carbonyls
- Washed fish model (food model)
- Fish protein isolates (food model)
- Clinical trials

Anti-hypertensive properties
- ACE
- Clinical trials

Anti-carcinogenic properties
- Alamar blue
- Protein expression

Anti-inflammatory properties
- Dentic cell model
Fish peptides
Antioxidative activity

ORAC

Blue whiting peptides

9.5% DH
10.5% DH
12.5% DH

ORAC value (μmol TE/g protein)
Bioactive peptides from aquatic raw materials

**ORAC**

- < 5 KDa
- 5 - 10 KDa
- 10 - 30 KDa
- > 30 KDa

**Capelin peptides**

- Cryotin (North ltd)
- Protamex (Novo Enzymes)

**DPPH radical scavenging**

- Blue whiting peptides

- Degree of hydrolysis (%)
  - 9.5% DH
  - 10.5% DH
  - 12.5% DH
Metal chelation

Mononuclear cell assay
27

**Antioxidant effect in food model**

![Graph showing antioxidant effect in food model](image)

**Tilapia peptides**

Enzyme: Flavourzyme

- Control
- Flavor 20% >30 kDa
- Flavor 7.5% >30 kDa
- Flavor 20% 10-30 kDa
- Flavor 7.5% 10-39 kDa
- Flavor 20% <10 kDa
- Flavor 7.5% <10 kDa

- >30 kDa
- 10-30 kDa
- <10 kDa

28

**Aquatic food peptides**

ACE inhibition

![Diagram showing aquatic food peptides and ACE inhibition](image)
ACE inhibition of various marine peptides

<table>
<thead>
<tr>
<th></th>
<th>Protein [mg/mL]</th>
<th>ACE inhibition [%]</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; [mg/mL]</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;30 kDa</td>
<td>20,6</td>
<td>94,9</td>
<td>1,4</td>
</tr>
<tr>
<td>&lt; 30 kDa</td>
<td>19,1</td>
<td>85,9</td>
<td>0,8</td>
</tr>
<tr>
<td>&lt; 10 kDa</td>
<td>16,3</td>
<td>70,5</td>
<td>0,2</td>
</tr>
<tr>
<td>&lt; 5 kDa</td>
<td>9,8</td>
<td>78,2</td>
<td>0,1</td>
</tr>
</tbody>
</table>

Lower IC<sub>50</sub> value => higher activity
HPLC separation of < 5kDa fraction

ACE inhibition of fractions (IC_{50})
Appendix 9. “Marine enzymes and enzyme inhibitors” by Inge W. Nilsen

Bioactive compounds from marine organisms: studies on cold-adapted enzymes

Copenhagen, March 2010
Inge W. Nilsen

Marine bioprospecting group – interests / main areas

Extremophilic enzymes: cold-adaptation
- unique activities
- in vivo functions
- in vitro applications

Antibacterial enzymes: lysozymes
- innate immunity
- in vivo functions
- in vitro applications

Antiviral low-MW molecules - marine enzyme inhibitors
In focus: enzymes with unique features

- Cold-adapted
- ± Low-temperature activities
- ± Heat lability
- High catalytic rates
- Fish & marine invertebrates
- Basic / molecular and applied research

Past – recent - present studies →

---

Stories from "the past" - 1

1. Shrimp alkaline phosphatase (SAP): successful commercial product
   - Sold as native & recombinant product
   - Heat labile (efficient & irreversible heat inactivation), not cold-active
   - Dephosphorylates DNA and nucleotides
   - Used prior to DNA sequencing and in cloning

---


Stories from "the past" - 2

2. Shrimp double-strand specific DNase (dsDNase); commercial product
   - Sold as recombinant product
   - Heat labile (efficient & irreversible heat inactivation), not cold-active
   - Degrades dsDNA
   - Used to prevent false positives of carry-over products in PCR amplification reactions

References:

A method of removing nucleic acid contamination in amplification reactions. US patent 6,541,236.

The enzymes and the DNA sequences of a thermolabile and double-strand specific DNase from
hemolymph shrimps (Penaeus borealis). Publ. ONE (in press).

Stories from "the past" - 3

A lysozyme from Icelandic scallops

Not commercialized due to problems in recombinant production
Present stories – background lysozymes

Function: hydrolyze peptidoglycans surrounding bacteria \( \rightarrow \) "antibacterial"

Occurrence in animals:

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Chicken-type</th>
<th>Goose-type</th>
<th>Taurine-type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vorticellids</td>
<td>+</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>Lucinoides</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saccharinae</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Saccharinae (arthropodae)</td>
<td>+</td>
<td>(araneae)</td>
<td>-</td>
</tr>
</tbody>
</table>

Counter-defense in bacteria: "specific" lysozyme inhibitor proteins

Lysozyme studies – Atlantic salmon

- Recombinant goose-type
- Native chicken- and goose-types
- Tissue expressions
Bioactive peptides from aquatic raw materials

Recombinant SaLG – cold-active, heat-labile but reactivates

Recombinant SaLG and bacterial lysozyme inhibitors

SalG – not inhibited by bacterial hy.

Unlike terrestrial g-type lysozymes

And all c-type lysozymes

Used to isolate novel specific

g-type inhibitor from bacteria

PNG – to be published

Collaboration with Christ Michiels,

Catholic University of Leuven, Belgium


salmon g-type lysozyme with high heat tolerance. Curr. Mol.


Kozhuharov P., Mykles B., Brandstad B., Smalls AO, Nilsen H.

and Helgason R. (2015) Thermodynamics and structure of a salmon

cold active g-type lysozyme. (submitted).

Kozhuharov P., Nilsen H, Brandstad B, and Smalls AO

(2006) Structural evidence for loss of inhibition of fish

g-type lysozymes by a bacterial inhibitor of lysozyme.

Native salmon lysozymes: isolation and characterization
Mynaes B, Ørsted K and Nissen IV (unpublished)

• SaLC and SaLG isolated from adult individuals
• Confirmed by bacterial inhibitors PIIG/ivy and MS analyses

• Both native enzymes are cold-active
• Both native enzymes have temperature optimum at 60 °C (!?)
  → Temp-optimum discrepancy for
  recombinant versus native SaLG

• SaLC is bifunctional !!!
  - minimum 50-200 fold higher (endo- and exo-) chitinase activity than SaLG and HEWL

Salmon lysozymes – antibacterial studies

• Expressed interest for activities against enterobacteria E. coli / Salmonella

• Lysozyme enzyme activity of SaLG tested on cells after removal of outer cell wall

• Antibacterial effects tested in different liquid media under various growth conditions

• Lysozymes tested alone or in combination with low-MW material from salmon
Salmon lysozymes – antibacterial studies

- SaLG displays very high enzymatic activity on E. coli (and other) cell walls
  - but:
- No antibacterial effect of SaLG (40 nM - 40 μM → ~1 μg - 1 mg/ml)
- No antibacterial effect of SaLC (max 5 μM)
- No antibacterial effect of SaLG+SaLC
- No synergy in antibacterial effect of SaLG+SaLC+low-MW fraction
  - however:
- Some antibacterial effect of salmon low-MW fractions
- Potential synergistic effect of two different low-MW fractions

Lysozyme gene-expression and enzyme activity studies reveal that goose-type dominates over chicken-type in tissues of juvenile Atlantic salmon (Salmo salar).
Mytines B, Seppola M, Callewaert M, Vanderkleken L, Michels C and Nielsen MW (unpublished)

- Discrimination of lysozyme enzyme activities by specific inhibitors (Ivy and PIG)
- Gene expression studies in agreement with enzyme activities
- Enzyme activities in tissues of adult salmon show the same distribution pattern
**Inhibitors of HIV-1 protease**

Frequent HIV "therapy" - a triple cocktail of inhibitors:
1 inhibitor of reverse transcriptase + 2 inhibitors of protease
Life-time treatment and few available drugs → resistance

**Examples of HIV protease inhibitors:**

- **INDINAVIR (IDV)**
- **AMPRENAVIR (APV)**
- **SAQUINAVIR (SQV)**

---

**Marine inhibitors of HIV-1 protease**

Øverberg K, Arnesen JA, Christoel T, Danielson H, Nilsen IV (unpublished)

- Extracts of marine invertebrates
- Sequential steps of low-MW components purification
- Fractions assayed for inhibition of HIV-protease enzymatic activity
- Compared to enzyme activity inhibition from two anti-HIV drugs (APV, SQV)
- No inhibitor of pepsin activity (another aspartic protease)

- when criteria are met:

  - Analysed for its binding kinetics to HIV-protease (Biacore biosensor)
  - Compared to its binding to human serum albumin (HSA)
  - Compared to binding of clinical drug (IDV) to HIV protease
Marine inhibitors of HIV-1 protease – results 1
- enzyme activity and effect of marine inhibitor

Dose – response profile - similar to two current medical drugs (APV / SQV)
- and additive in combinations

Marine inhibitors of HIV-1 protease – results 2
3 examples – Surface plasmon resonance (SPR, Biacore)

- Binds HIV-protease
- No saturation
- Rapid association
- Rapid dissociation
- Interacts with HSA

- Binds HIV-protease
- Saturation like IDV
- Rapid association
- Medium dissociation
- No HSA interaction

- Binds HIV-protease
- Potential saturation
- Slow association
- Slow dissociation
- No HSA interaction

Sensorgrams and steady states values (RSS) for the interaction between the samples and HIV-protease
Marine inhibitors of HIV-1 protease

- "Reservoir" of marine natural products that inhibit HIV-protease
- These inhibitors vary in specificity and affinity of physical binding
- Some marine inhibitors mimic clinical drugs in interaction with the protease
- Extensive work to screen and characterize such natural products

Lack of funding and no present activity, unfortunately

Start-up story
large project collaboration Nofima marine – SINTEF F&A

Herring ("Norwegian spring spawning herring")
- Enormous amounts (almost 1 million tons landed in Norway)
- Poorly characterized bioactivities
- "Simple" low cost by-products (mainly from ensilage)

To be prospected for
- Novel enzymes, improved lipid products, "drugs" (i.e. antibacterials, inhibitors)
- Is sustainable
- "Advanced" high cost products

Duration: 5 years
Financing: public
Budget: 40 mill NOK (25 % secured)
Bioactive peptides from aquatic raw materials

Thank you for listening!
Appendix 10. “Seagarden ASA, a commercial producer of bioactive peptides from marine protein sources” by Bjarte Langhelle
Bioactive peptides from aquatic raw materials

MARINE SAVOURY INGREDIENTS

WHITE FISH PRODUCTS
- Fish Powders
- Crab Powders
- Squid Powders
- Octopus Powders
- Tilapia Fish Powders
- Pelagic Fish Extract Powder

SHELLFISH PRODUCTS
- Shrimp Powders
- Squid Powders
- Octopus Powders
- Crab Powders
- Skate Powders
- Eel Powders
- Scallop Powders
- Cuttlefish Powder
- Kelp Powder
- Edible Shell Powder
- Seaweed Powder

SAVOURY PASTES
- Fish Paste Bajiki
- Isolated Sardine Paste
- Salmon Paste
MARINE BIO-ACTIVES

COMMERCIAL PRODUCTS

- Minyrex™ (Eua mazzoti 6771UL4) - MIW (microalgae extract) - Functional ingredient for skin care and hair, skin, nail.

- API Chitosan - Approved API (pharma) pharmaceutical ingredient.
- Intermediate for ultra-grade glucosamin and chitosan.

PRODUCT PIPELINE

- Proplig™ (formulated for) - Fish and poultry growth-promoters. 
- Peptides derived from fish used as an alternative to porcine plasma for warming pigs and poultry.
- Collagens - Fish Collagen Hydrolysates.

CURRENT DEVELOPMENT PROJECTS

MARINE SAVOURY INGREDIENTS

- Development of new marine savoury ingredients.
- Chondroitin-specific formulations.
- DME products from processed marine, peptides, and health.

MARINE BIO-ACTIVES

- Proplig™
- MarPlag™
- Collagen hydrolysates.
- Bioactive peptides – IGS.
- Fermentation or growth medium.
- Chitosan formulations.
PeptiGard® - digestion and absorption

Growth Hormone Releasing Peptides

- Enhanced appetite
- Increased HCl production
- Reduced pH in the stomach
- Increased pepsin production
- Increased protein digestion
- Reduced risk of diarrhea
- Enhanced growth

Absorption

Bioactive Peptides

Small intestine

Effect of PeptiGard® on growth performance of weaning piglets - SPAIN

- 4 treatments
- 7 replicates
- 10 pigs (pen)
- 280 pigs
- Initial weight: 5.6 kg
- Age at weaning: 21
- Days in trial: 21

Treatments:
1. 0.7% porcine plasma (APC 920)
2. 5.7% plasma X
3. 3% PeptiGard
4. 0.7% plasma substitute Y

ADG (g)

Days post weaning
Appendix 11. ”Identification and categorisation of bioactive peptides in marine extracts produced by Seagarden ASA” by Anne Hjelle
International trend: (e.g. EU) Nutrition & Health, 7th Framework

- Understanding beneficial and harmful dietary factors
- Interaction between nutrition and physiological/psychological functions
- Reformulation of processed food, development of novel foods and ingredients, dietetic foods and foods with nutritional- and health claims ("Functional food").
- Dietary strategy: development and application of nutrigenomics and systems biology

Example systems biology: Biomolecules (de)activated by onset of atherosclerosis:

Marine ingredients as "functional food"

Seagarden (SG) is a supplier of high quality marine ingredients to customers worldwide, including a wide range of powders and extracts as regards both marine savoury ingredients and marine bioactives. However, this project is limited to evaluation of bioactive PEPTIDES in SG products (fish hydrolysates).

Proteins & peptides: "functional building blocks in all organisms"

= unique sequences of Aminoacids:
Bioactive peptides (BAP):

- Normally 2-9 Aa, but can be up till 20 Aa
- Hydrophobic sequences + proline, lysine and arginine
- BAP can be latent as/when part of a "mother" protein
- Proteolysis could be necessary to "release" and activate the peptide for a specific physiological response

My "functional food" spiced with bioactive peptides! Potential effects on...

- Nervous system
- Cardiovascular system
- Gastrointestinal system
- Immune system
**Aim of study:**

- To evaluate effects of bioactive peptides (from SG-fish hydrolysates) on gastrointestinal diseases.
  - by optimising methodology to identify as many bioactive peptides as possible from 3 marine SG-products,
  - by ranking the SG-products’ bioactivity,
  - and by subsequent validation of results by clinical tests (not included in this presentation).

**Project team:**

- **seagarden** → Supplier fish hydrolysates
- **IRIS** → Protein chemistry and bioinformatics
  - Clinical testing (mice)
  - Clinical expertise (humans)
- **prekubator** → Organisation & funding
  - Main funding
Methods

Strategy
1. Literature search for bioactive sequences
2. Chromatography & Mass spectrometry
3. Bioinformatics:
   Sequence identification & product comparison
4. Validation:
   Clinical testing (to be started)
1. Which peptides are bioactive?

- Product evaluation in the present study is based on information from published literature:
- Literature search at 'ISI Web of knowledge' (http://apps.isiknowledge.com)

2A. Sample preparation by chromatography
Challenge:

Sample-COMPLEXITY

Original sample is divided into subfractions

Collection of sample-fractions
2B. (Tandem) mass spectrometry by LTQ Orbitrap

(Tandem) mass spectrometry principle

Orbitrap analysis

Peptide mix in hydrolysate from SG

Peptide Mass Fingerprint

Aminoacid sequence

Method
**Fragmentation spectra:** provide Aa sequence (by bioinformatics)

How to optimise number of identified peptides and Aa sequences:

**Fish hydrolysates from SG**

Optimisation:
1. Separation
2. Fragmentation
Sample separation

Sample composition (watersoluble or not?)

optimise

Time and/or % organic solvent

Optimised separation by gradient D = more identified peptides in the hydrolysates

Gradient A

Gradient D
MS settings (fragmentation)

e.g. Peptide exclusion list to be able to fragment/identify a higher number of peptides.

3. Bioinformatics

A: Database search for sequence ID

B: PERL algorithms for peptide “fishing” and categorisation
A: Sequence identification

Using Sequest algorithm and Proteome Discoverer (+ relevant taxonomic databases available on internet to know the protein-origin of peptides)

B: Categorisation of BAP based on effects

Cardiovascular system
- Hypocholesterolemic
- Antioxidative
- Antithrombotic

Nervous system
- Mineral binding

Gastrointestinal system
- Opioid agonist
- Opioid antagonist

Immune system
- Immunomodulatory
- Antimicrobial

Bioactive peptides from aquatic raw materials

For example:

Opioid peptides:
- Tyr-Pro-Asp-Pro-Gly
- Tyr-Pro-Met-Val
- Tyr-Pro-Met-Val-Ala
- Tyr-Pro-Met-Val-Ala-Tyr
- Tyr-Pro-Met-Val-Ala-Tyr-OH
- Tyr-Pro-Met-Val-Ala-Tyr-OH

Antioxidative properties:
- Triglycerides with Tyrosine Tyr mediate a central role.
- Pro-His-Pro is an active center for antioxidative properties.
- Leu-Leu-Pro-Pro-His
- Val-Leu-Pro-Pro-His
- Gly-Leu-Pro-Pro-His
- Val-Leu-Pro-Pro-His

Cardiovascular – ACE inhibitory:
- Leu-Leu-Pro-Pro-His, Ala-Leu-Pro-Pro-His
- Met-Pro-Pro-His, Tyr-Leu-Val-Val
- Met-Pro-Pro-His, Ala-Leu-Pro-Pro-His
- Met-Pro-Pro-His, Gly-Leu-Pro-Pro-His

Gastrointestinal related:
- His-Ala-Arg-Leu, Arg-Leu-Ala-Arg-Leu
- Val-Ala-Arg-Leu-Ala-Arg-Leu
- Gly-Leu-Pro-Pro-Glu-Leu-Pro-Pro-Glu-Leu-Pro-Pro-Glu

Peptide “fishing” – by PERL algorithms

Part of Gastro List:

<table>
<thead>
<tr>
<th>1191234567890</th>
<th>12345678901234</th>
</tr>
</thead>
<tbody>
<tr>
<td>12345678901234</td>
<td>12345678901234</td>
</tr>
</tbody>
</table>

Output file:

This algorithm might be altered to meet specific needs – it’s currently running in DOS mode, but can be programmed to a windows based interface.

Summary

Analyzed 1513 Fasta entries from file “RR_HuH7_161676_Fasta” with respect to 57 motifs from file: “Bioactive Peptides HgGastro.list”

Total number of matches: 1176
25.01.12, Jun 18, 2009
Results

1A. Effect of method optimalisation (MS gradient)
1B: Effect on fragmentation

1C: “2D” or not “2D”

Number of identified proteins with 1D vs. 2D = 707++ more with 2D
2A. Ranking the bioactivity in SG products (fraction A8)

2B. Same trend in fraction A9
Main conclusions

- The results revealed that all products from Seagarden contain bioactive peptides that is known, from literature, to stimulate the gastrointestinal system.
- The results did also indicated that the level of bioactivity varied among products.
Conclusions II

- Improving chromatography and MS parameters significantly increased the number of identified peptides and proteins (for both 1D and 2D).
- 2-dimensional chromatography further increases the number of identified peptides and proteins compared to 1D.
- A PERL algorithm was successfully used to determine the number of "category-specific" peptides in each product.

The way forward:

Clinical testing by NIFES...

and further peptide analysis
Acknowledgement

- Innovasjon Norway and Prekubator for funding.
- The rest of the project team, and particularly Gunnar Kleppe for keeping the team “on track”.
- The organisers for the invitation
...and thank you for your attention!!!

Contact:
EMail: Anne.hjelle@iris.no
Phone: 0047 97590954
Dr. Anne Hjelle
Acting Vice President
IRIS Biomiljø
Mekjarvik 12
4070 Randaberg, Norway
Appendix 12. ”PEPFISH: Utilisation of bioactive peptides from fish processing – upgrading the value of secondary products” by Flemming Jessen

PEPFISH

Utilisation of bioactive peptides from fish processing – upgrading the value of secondary products

Flemming Jessen

- A 3½ year project initiated April 2008
- Financed by The Danish Council for Strategic Research

- Why
  - Around 50% of the fish is not used for human consumption
  - Increased scientific documentation that fish or hydrolysed fish protein contain large amount of bioactive peptides

- Aim
  - Purification and characterisation of bioactive peptides present in fish or produced by hydrolysis of fish proteins
  - Characterise mechanisms of activity
  - Creating scientific documentation of bioactive fish peptides for use in health food or pharmaceutical products
Project partners

- **Technical University of Denmark, National Food Institute**
  - Lisa Lyttbæk Andersen, Henrik Hauch Nielsen, Michael Engelbrecht, Flemming Jessen
- **University of Copenhagen, Biological Institute**
  - Else K. Hoffmann, Carlo Ossum
- **Rigshospitalet, Dept. of Clinical Microbiology**
  - Leif Percival Andersen, Anna Boschian, Lone Rasmussen
- **Lund University, Division of Bacteriology**
  - Torkel Wadström
- **University of Tromsø, Institute of Marine Biotechnology**
  - Edel Oddny Elvevoll
- **Marinova**
  - Greta Jakobsen, Inez Johansson
- **Biofac A/S**
  - Peter Raviv, Charlotte B. Pipper
- **Novozymes A/S**
  - Gitte Budolfson Lynglev, Steffen Ernst

Project concept

- (Extraction) / hydrolysis of proteins
  - Different commercial and experimental enzymes (Novozymes)
  - Different parts of fish (belly flap, skin)
- Commercial hydrolysates (Marinova, Biofac A/S)
- Fractionation of peptides
  - Filtration (ultra, nano)
  - Gel filtration
  - Ion exchange
- Test for biological activity
  - Different *in vitro* assays (enzymatic, cell culture)
  - *In vivo* (mouse)
- Feedback and further fractionation
- Gastrointestinal digestion effects on bioactivity
- Characterisation of peptides
- Characterise mechanism of activity
**In vitro testing**

- Angiotensin I-Converting Enzyme (ACE) inhibition
- Matrix Metalloproteinase (MMP-9, MMP-13) inhibition
- Antibacterial activity (*Helicobacter pylori*)
- Anticancer activity (pancreas, lymph node)
  - Proliferation (BrdU incorporation)
  - Apoptosis (Caspase-3/7 activity)
  - Migration (Microscopy-based method)
- Antioxidative activity
  - Ion chelating
  - Reducing power
  - Radical scavenging
  - Inhibition of peroxidation (liposome model)

**In vivo testing**

**Mouse: BALB/c, C57**

- Antibacterial activity (*Helicobacter pylori*)
- Immunological effects
Preliminary results

- ACE inhibition activity
  - Found in the main part of hydrolysates or peptide fractions that have been tested.
  - Relevance?

- Anticancer activity
  - Inhibition of cell proliferation at low concentration (0.1 mg/ml) of a couple of hydrolysates and fractions from these.
  - Possible induction of apoptosis by some hydrolysates in the primary tumour cell line, but no effect on the lymph node metastasis cell line.

- Antioxidative effects
  - Activity found in commercial and experimental hydrolysates
  - Low molecular fractions have higher activity than high molecular fractions

National Food Institute, Technical University of Denmark