

FISH PHYSIOLOGY AND BIOCHEMISTRY (FIBP)

- A NETWORK AND A PH.D. STUDY PROGRAM

SAGSNR: 5007-00-045

OPDATERET D. 1. JANUAR 2004

Faglig præsentation og formål

- At overføre og udnytte viden om en række fysiologiske og biokemiske processer i celler fra pattedyr til fiskeceller, samt at studere fysiologiske processer i fiskemusklens, specielt i forbindelse med iltmangel. Dette har relevans for *post mortem* udviklingen af fisken og dermed for dens kvalitet som fødevarer. Formålet skal opnås gennem etablering og studier af fiskecellekulturer fra væv af særlig betydning for fiskens kvalitet som fødevarer som f.eks. muskel, bindevæv og underhudsfedt.
- At styrke den grundlagsskabende forskning i enzymet *trimethylamin-N-oxid aldolase* og bringe den i position på højest niveau. Derved skabes basis for efterfølgende ph.d. studier indenfor området. Enzymet er ansvarligt for den meget uønskede dannelse af formaldehyd under opbevaring af fisk.
- At etablere et ph.d.-studieprogram i "Fish muscle physiology and biochemistry" med henblik på at kunne tilbyde relevante kurser i et studieforløb for biokemikere og fysiologer med interesse for fisk samt for levnedsmiddelstuderende.

Baggrund og statistik

- Projektleder, Docent Else Kay Hoffmann, ph.d.
- Delprojekter:

1: Calcium, pH and cellular water balance in determination of fish meat quality

Antal personer: 2 seniorforskere, 1 postdoc (6 md) og 2 ph.d.-studerende (startet henholdsvis august 2001 og juli 2002). Den sidst startede ph.d. holdt op efter at have brugt 9 måneder af stipendiet, fordi ægtefællen fik sit drømmejob i udlandet. En ny ph.d. studerende er startet 1. februar 2004, de 9 manglende måneder er betalt med 8 fra Københavns Universitet og 1 måned fra "Fishnet".

Antal publikationer:

4 publikationer i internationalt anerkendte tidsskrifter (inklusive indsendte og under udarbejdelse):

Ossum, C.G., Hoffmann, E.K., Vijayan, M.M., Holt, S.E. and Bols, N.C. (2004.) Characterisation of a novel fibroblast-like cell line from the rainbow trout and responses to sublethal anoxia. *Journal of Fish Biology*, 64, 1-14.

Ossum C.G. and Hoffmann, E.K. Effects of chemical anoxia and reoxygenation on the regulation of the MAPKinase, ERK in RTHD fibroblasts (to be submitted).

Marshall W.S., Ossum C.G., and Hoffmann, E.K. Osmosensing chloride cells rapidly regulate ion transport in an estuarine teleost fish (in prep).

Marshall W.S. and Hoffmann, E.K. Rapid regulation of ion transport by mitochondria rich cells in opercular epithelium of an estuarine teleost (in prep)

C. Hougaard, C.G. Ossum, E.K. Hoffmann: Activation of Ca²⁺-activated and inwardly rectifying K⁺ channels by chemical anoxia in rainbow trout myosatellite cells in primary culture (needs a few additional experiments).

2: Biochemical and physiological properties of the enzyme responsible for formaldehyde formation in fish during storage

Antal personer: 2 seniorforskere, 1 post-doc. (15 md)

Antal publikationer: 1 artikel, 1 projektrapport

Nielsen, M. K. and Jørgensen, B. M. (2004): Quantitative relationship between *trimethylamine-oxide aldolase* activity and formaldehyde accumulation in white muscle from gadiform fish during frozen storage. *J. Agric. Food Chem., submitted.*

Nielsen, M. K. (2003): Biochemical and physical properties of the enzyme responsible for formaldehyde formation in fish during storage. *Progress report, DIFRES/FF.*

- Antal kurser: 2 (bilag 5)
Symposiumdeltagere: 42(2002) og 48 (2004).
Deltagere i praktisk kursus: 12 (2002) og 12 (2003).
- Internationale foredragsholdere ved symposium:
2002
Prof. Ian A. Johnston, University of St. Andrews, Scotland
Dr. Richard Taylor, INRA de Theix, France
Dr. David J. McKenzie, University of Birmingham, England
Prof. Gert Flik, University of Nijmegen, The Netherlands
Prof. Geoffrey Goldspink, University Collage London, England
Dr. John Fleng Steffensen, University of Copenhagen, Denmark

2003
Dr. C. Louise Milligan, University of Western Ontario, Canada
Dr. Ian McCarthy, University of Wales, Bangor, United Kingdom
Dr. Richard Taylor, INRA, France
Dr Frank Bo Jensen, University of Southern Denmark, Denmark

Status

- Resultaterne og deres betydning
Forskningsprojekt 1.
1.a. Det er lykkedes at etablere primærkulturer af myoblaster fra regnbueørred og disse er anvendt til en elektrofysiologisk karakterisering af hvilke ionkanaler der aktiveres når disse celler udsættes for iltmangel. Det viser sig at iltmangel aktiverer både en calciumaktiveret kaliumkanal og en ”inwardly-rectifying” kaliumkanal, hvilket fører til kalium- og vandtab fra musklen. Vi er påbegyndt calcium- og pH-målinger i disse celler under iltmangel for at vurdere mekanismen bag kaliumkanal aktivering. Projektet mangler nogle data og er netop blevet genoptaget.
Se bilag 1: **Primary culture of myosatellite cells from rainbow trout and activation of whole-cell currents by chemical anoxia.**

1.b. Der er etableret en permanent cellelinie (RTHDF: Rainbow trout hypodermal fibroblasts) fra underhudsfedtvevet. Cellelinien er karakteriseret med hensyn til vækstrate, næringsbehov og telomeraseaktivitet, der er et mål for celleliniens evne til at blive udødelig. Der er gennemført studier af anoxi og reoxydering af disse celler med henblik på studiet af stressproteiner og de involverede signaltransduktionsveje. Se bilag 2: **Cell line development and signal transduction during chemically anoxic stress**

1.c. På RTHDF har vi dernæst ved 2D-gel elektroforese undersøgt proteinexpressionen i kontrolceller og celler udsat for anoxi i perioder af forskellig længde. Allerede 30 minutters iltmangel giver signifikante ændringer i proteinexpressionsmønstret og ved langtidsiltmangel ses dramatiske ændringer. Projektet har ligget stille efter at den phd studerende rejste til udlandet, det er netop blevet genoptaget af en ny ph.d studerende. Se bilag 3: **Proteome changes in RTHD- cells after Na-azide incubation for 30' or over night, analysed by 2D-gel electrophoresis.**

Fiskecellelinier fra væv, der benyttes til menneskelig konsum er en mangelvare, hvorfor etableringen af permanente cellelinier har stor betydning for denne forskning. Desuden åbnes der muligheder for økotoxikologiske studier på celleniveau samt vækststudier i forbindelse med akvakultur.

Forskningsprojekt 2.

Der er blevet udviklet en oprensningsprocedure for *trimethylamin-N-Oxid aldolase*, og der er oprenset store mængder enzym fra galleaft fra sej med henblik på videre analyse.

Enzymaktiviteten fra både milt og muskel er blevet undersøgt hos en lang række arter af torskfamilien. Det er blevet påvist, at der er store forskelle i enzymaktiviteten mellem arter og individer. Det er fundet at enzymaktiviteten ikke korrelerer med indholdet af *trimethylamin-N-oxid* (substrat).

Der er påbegyndt en undersøgelse over hvorvidt enzymets funktion *in vivo* er knyttet til cholinestofskiftet. Se bilag 4: **Biochemical and physiological properties of the enzyme responsible for formaldehyde formation in fish during storage.**

- Internationalt samarbejde og samarbejde i netværket (FIBP og Fishnet)
I projektet samarbejdes omkring etableringen og karakteriseringen af cellelinierne med professor Niels C. Bols, University of Waterloo, Canada og professor Shawn E. Holt, Virginia Commonwealth University, Richmond, USA. Ph.d. studerende Carlo G. Ossum har haft 2 succesrige ophold (henholdsvis 3 og 2 måneder) hos Niels Bols.
Desuden har vi indledt et samarbejde med professor William S. Marshall, Biology Dept. St. Francis Xavier University, Antigonish NS Canada om osmoregulatoriske problemer i fisken. Else Hoffmann har arbejdet på projektet i professor Marshalls gruppe som St. Francis Xavier professor i 7 uger og Carlo Ossum har tilbragt 3 uger i gruppen i Antigonish.

Omkring proteomanalyserne samarbejder seniorforsker Flemming Jessen og docent Else K. Hoffmann direkte og ph.d.-studerende Søren Wilhjelm arbejder både på August Krogh Institutet og på Danmarks Fiskeriundersøgelse, Afd. for Fiskeindustriell Forskning i Lyngby (FF).
Desuden er der afholdt 3 orienterende møder i FIBP, og projektgruppen har deltaget i Fishnet-symposiet på Fuglsøcentret med et foredrag samt i Fishnet-symposiet på Landbohøjskolen med to foredrag.

Projektet har synergi til et nystartet projekt ved Danmarks Fiskeriundersøgelser, omhandlende forudsigelse af produktkvalitet udfra proteomstudier hos ”familier” af regnbueørred.
Der knyttes en specialestuderende på August Krogh Institutet til projektet fra 1. september 2003.

Vurdering

Det har været positivt, at det så hurtigt er lykkedes at etablere en permanent fiskecellelinie og at de primære cellekulturer fra fiskemuskel viste sig meget velegnede til elektrofysiologiske undersøgelser. Arbejdet med effekten af iltmangel på signalvejene i disse celler er forløbet særdeles tilfredsstillende. Det har derimod givet en del problemer, at fiskecellerne har vist sig tilsyneladende uegnede til mærkning med en række fluorescerende stoffer, som normalt benyttes til måling af calcium og pH i celler. Det tog en del tid at finde årsagen, som sandsynligvis er, at de anvendte celler fra fedtvævet under huden indeholder meget ”multidrugresistent-protein” i cellemembranen og derfor ekskluderer fremmede stoffer meget effektivt.

Proteomarbejdet er startet senere og er yderligere blevet forsinket på grund af at den ph.d. studerende der var knyttet til projektet rejste til udlandet. Projektet er netop genoptaget med en ny meget dygtig ph.d. studerende.

Det har været vanskeligere end forventet at opnå den nødvendige renhed af *trimethylamin-N-oxid aldolase* med henblik på sekventering og fremstilling af antistoffer, og under arbejdet med optimering af oprensningsproceduren blev udgangsmaterialet en begrænsende faktor. Der er imidlertid nu under togter med DFU's forskningsskib indsamlet tilstrækkeligt materiale til at de nødvendige procedurer kan gennemføres, og en præparation af høj kvalitet forventes fremstillet i løbet af 1. halvdel af 2004.

Fremtid

- Resterende periode.
Kaliumkanalerne i de primære myoblastceller vil blive yderligere karakteriseret og korreleret med ændringer i cellulært calcium og pH, med henblik på publicering. De problemer, som vi havde med at mærke fedtcellerne med de fluorescerende stoffer, som vi bruger til måling af Ca og pH er heldigvis langt mindre i myoblasterne.

I RTHDF cellerne vil vi forsøge at beskrive så mange trin som muligt i den signaltransduktion, der er involveret i dødsprocessen, som initieres efter langvarig anoxi.

En ny cellelinie fra bindevæv (RTM#2) er under etablering og vil specielt blive udnyttet til studier af matrix metalloproteinase ekskretion under iltmangel.

De anoxi inducerede ændringer i proteinexpressionen i RTHDF cellerne vil blive verificeret og de involverede proteiner søgt identificeret ved massespektroskopi.

En molekylær karakterisering af *trimethylamin-N-oxid aldolase* vil blive gennemført, ligesom der vil blive fremstillet monoklonale og polyklonale antistoffer mod enzymet.

Med henblik på at kunne bestemme enzymets fysiologiske funktion vil fundne aminosyresekvenser blive sammenlignet med kendte sekvenser i internationale databaser.

Ph.d.-kurset vil blive gentaget i 2004 i den udvidede form, inkluderende proteomanalyse som blev indført i 2003

Opfølgning og anbefalinger

De etablerede permanente fiskecellelinier vil udgøre et vigtigt grundlag for fremtidige sammenlignende studier mellem pattedyrceller og fiskeceller, ikke kun til studiet af virkningen af anoxia men også til studier af en lang række andre fysiologisk vigtige processer. Primærkulturer af fiskeceller og studiet af isolerede fiskevæv vil herudover være et vigtigt redskab i sammenlignende studier mellem forskellige fiskearter og fisk adapteret til forskellige omgivelser. Det sidste kan have speciel værdi, da der eksisterer fisk tilpasset en mangfoldighed af biotoper med ekstreme betingelser. Dette sidste vil vi fortsætte med at studere i samarbejde med prof. Marshalls gruppe i Canada.

Derudover er studiet af cellulære dødsprocesser (nekrose og apoptose) i fiskeceller dels interessante i sammenligning med den forskning, der foregår på området i pattedyrceller, dels interessante for forståelsen af de processer, der foregår efter fiskens død, og som har betydning for hvordan den senere kvalitet af fisken bliver. Vi mener således, at opfølgende projekter er væsentlige og bør inddrages i de deltagende institutioners fremtidsplaner.

De opnåede resultater på *trimethylamin-N-oxid aldolase* kan danne basis for studier i enzymets reaktionsmekanismer med henblik på en mulig hæmning af enzymet og dermed en formindskelse af problemerne med dannelse af formaldehyd i fisken som fødevare. Alt efter enzymets virkelige identitet kan resultaterne støtte forskningen i tilsvarende enzymer fra andre organismer.

Sammenfattende har hele projektet haft succesrige grundforskningsmæssige og anvendelsesorienterede aspekter. Forskningsfeltet trives godt i sin nuværende form, og det vil være oplagt at fortsætte samarbejdet mellem DFU, DTU og KU samt eventuelt at inddrage andre institutioner som f.eks. Syddansk Universitet (Biologisk Institut, økofysiologigruppen) i samarbejdet.

Bilag 1.

Primary culture of myosatellite cells from rainbow trout and activation of whole-cell currents by chemical anoxia.

Post-doc. C. Hougaard and Reader E.K. Hoffmann Ph.D, August Krogh Institute, University of Copenhagen.

Background

To study the cellular signalling cascades activated during ischemia/anoxia in fish muscle cells, we aimed at establishing a permanent culture of myoblasts from rainbow trout as such a culture is not commercial available. The idea was that a culture rich in myoblasts could be established from a mixed culture of fibroblasts and myoblasts isolated from rainbow trout muscle by growing the culture under conditions where the growth of myoblasts is favoured.

Methods/Results

In the first attempts to establish such a culture, we used muscle tissue isolated from one-year old rainbow trout. The yield of myoblasts in these cultures was however very low (the myoblast/fibroblast ratio was approximately 1:25). It is known from other cell systems (e.g. rat) that the yield of myoblasts decreases as a function of age. Hence, to optimise the myoblast yield we tried to make primary cultures of myoblasts from newly hatched larvae and rainbow trout less than 4 months old. Several different methods were used in our attempts to establish a primary culture of myoblasts from larvae and young rainbow trout, including methods applied with success at the August Krogh Institute when establishing muscle-cell cultures from rats and the procedure used by Ma and Collodi (1999) to establish myosatellite cell cultures from lamprey. The best results were, however, obtained when using a slightly modified version of the method described by Matschak & Stickland (1995) to isolate the cells, and the method described by Koumans et al (1990) to enrich the percentage of myoblasts in the culture by adhesion to laminin-coated surfaces. In this culture the ratio between myoblasts and fibroblasts is, originally approximately 1:1. As fibroblasts and not myoblasts grow well in culture, the ratio will decrease rapidly and within weeks the culture appears to contain only fibroblasts. Although we have not been successful in establishing a pure myoblast culture it has been possible to perform electrophysiological single-cell experiments as the two types of cells are easily distinguished when looked at in a microscope.

Electrophysiological experiments were performed using the whole-cell patch-clamp technique. This method is extremely useful when studying ion transport via channels and has the great advantage that the cell interior can be controlled with regards to ion concentrations and thus the activated channels are easily distinguished. Anoxia was induced by exposing the cells to 10 mM Na-azid in the presence of 10 mM glucose. This procedure inhibits the oxidative metabolism but ensures a certain energy level by glycolysis.

Exposing myoblasts to Na-azid (10 mM) induces a large outward whole-cell current and, in addition, the reversal potential of the current approximates the reversal potential for K^+ , indicating that the current activated by Na-azid is primarily carried by K^+ leaving the cell via K^+ channels. At the present time it is unclear what types of K^+ channels contribute to the Na-azid induced current in myoblasts isolated from rainbow trout, but preliminary investigations indicate that Ca^{2+} -activated and inwardly-rectifying K^+ channels may contribute to the current.

Future plans

We want to further characterize the K^+ channels activated by anoxia in myoblasts isolated from rainbow trout and combine these with single-cell measurements of $[Ca^{2+}]_i$ and pH_i and p38 MAP kinase activity using fluorescence microscopy.

Planned paper

C. Hougaard, C. Ossum, E.K. Hoffmann: Activation of Ca^{2+} -activated and inwardly rectifying K^+ channels by chemical anoxia in rainbow trout myosatellite cells in primary culture.

References

- Koumans et al (1990) Cell Tissue Research 261:173-181
- Ma & Collodi (1999) Methods in Cell Sciences 21:39-46
- Matschak & Stickland (1995) Experientia 51:260-266

Bilag 1a. (new project started in August 2003)

Rapid regulation of ion transport by mitochondria rich cells in opercular epithelium of an estuarine teleost

Professor William S. Marshall, Biology Dept. St. Francis Xavier University, Antigonish NS Canada, Else K Hoffmann and Carlo G Ossum, August Krogh Institute.

Background

Small estuarine fish such as the Killifish, *Fundulus heteroclitus*, forage in shallow water following advancing tides and are exposed regularly to very dilute fresh water (FW) microenvironments. Typically the salinity in these shallows is low, *ca.* 3‰ seawater (1.0 g/l) or lower. Because the animals return to high salinity between tides, only temporary "coping" mechanisms are required, not permanent adaptation to FW. In the first few hours, blood osmolality and ion content are reduced, producing a hyposmotic cue to ion transporting cells.

Killifish opercular epithelium and related teleost membranes are model systems containing mitochondria rich cells used to study the regulation of salt transport. Reduction in ion transport after transfer to fresh water includes an inhibition of active Cl⁻ secretion and passive diffusive ion loss in a three stage process spanning approx. 30 min. There is a combination of sympathetic neural reflex mediated by α_2 -adrenoceptors operating via intracellular inositol tris phosphate through intracellular Ca²⁺, a rapid cellular hypotonic shock response (Marshall et al. 2000) and finally a covering over of ion secreting cells by pavement cells (Daborn et al. 2001). These three steps effectively minimize salt loss in dilute media. The upregulation of salt secretion on return to full strength sea water (SW) may be via hormones (arginine vasotocin and urotensin I) and neurotransmitter (vasoactive intestinal polypeptide) in combination with hypertonic shock (Hoffmann et al. 2002). Because in nature the rapid inhibition of Cl⁻ secretion is short lived (a few hours), voluntary (involving shoaling behavior) and mediated by autonomic reflex and neurohormones in combination with direct effects of blood tonicity on ion transporting cells, the concept of a *nonstressful* salinity change is put forward.

Methods/Results

We exposed isolated opercular epithelia mounted in Ussing chambers to hypotonic shock in the presence of various protein kinase (PK) inhibitors as well as serine/threonine protein phosphatase (PP) inhibitors and blockers of volume activated anion channels (VSOAC). By Western analysis we tested activation (phosphorylation) of various kinases. Finally we have used confocal laser scanning microscopy to look at localization of various kinases in the operculum epithelium.

On the basis of these results we are presenting a hypothetical model for rapid control of salt secretion across fish gills. The project is also supported by NSERC.

Future plans

We want to further characterize the regulation of salt secretion across fish gills using the operculum epithelium as a model. The operculum epithelium will in addition be an excellent model in comparative studies between various fish species adapted to various biotopes.

Papers in preparation

Marshall W.S, Ossum C.G. and Hoffmann, E.K. Osmosensing chloride cells rapidly regulate ion transport in an estuarine teleost fish (in prep).

Marshall W.S. and Hoffmann, E.K. Rapid regulation of ion transport by mitochondria rich cells in opercular epithelium of an estuarine teleost (in prep).

References

- Daborn K, Cozzi R.F.F, Marshall W.S. 2001. Dynamics of pavement cell-chloride cell interactions during abrupt salinity change in *Fundulus heteroclitus* J Exp Biol 204: 1889-1899.
- Hoffmann EK, Hoffmann E, Lang F, Zadunaisky JA. 2002. Control of Cl⁻ transport in the opercular epithelium of *Fundulus heteroclitus*: long- and short-term salinity adaptation. Biochim Biophys Acta 1566:129-139.
- Marshall WS, Bryson SE, Luby T. 2000. Control of epithelial Cl⁻ secretion by basolateral osmolality in euryhaline teleost *Fundulus heteroclitus*. J Exp Biol 203:1897-1905.

Bilag 2.

Cell line development and signal transduction during chemically anoxic stress

Ph.D. student Carlo G. Ossum and Reader Else K. Hoffmann, Ph.D.

Aims of study

The focus of this project is the development of cell lines originating from edible tissue of the rainbow trout, *Oncorhynchus mykiss* W. and studies of anoxia and reoxygenation of these cells.

Model system

In order to study the effects of anoxia in piscine cell lines, anoxia is induced chemically by challenging the cells with sodium azide, which block the electron transfer to O₂ in mitochondria. The cell line used is RTHDF (see below).

Results

To this end, one fibroblast-like cell line from connective tissue of the hypodermis and surface of the muscle has been established, characterised and named RTHDF for Rainbow Trout Hypodermal Fibroblasts. In addition to an analysis of the basic properties of cell growth, serum requirements and detection of telomerase activity, the time-course of MAP kinase (p38^{MAPK}) activation and expression profile of Hsp70 during anoxia and recovery were studied (Ossum et al., Journal of Fish Biology in press). We find that sublethal anoxia rapidly activates p38^{MAPK} by phosphorylation and later induces an increase in the amount of the inducible heat shock protein Hsp70.

At present, focus is on the regulation of the MAPK family member extra cellular signal-regulated kinase, ERK1/2. ERK is typically activated by mitogens and play a crucial role for cell survival. We find that ERK is downregulated during anoxia and upregulated by subsequent reoxygenation. Upregulation of the ERKs by reoxygenation seems to be dependent on generation of reactive oxygen species. However, if the ERK-activity is completely abolished, the cells cannot be rescued by recovery. In addition, experiments indicate involvement of PKC isoforms, although the role of PKC is still not clear. Another interesting observation is differences in isoform usage between the RTHDF cells and cells of mammalian origin, i.e. NIH 3T3 fibroblasts and Ehrlich ascites tumour cells; In the RTHDF cells, ERK1 is the preferred isoform, while in the mouse, ERK2 is predominantly activated.

In addition, a new cell line – RTM C#2 – is under development. These cells are derived from rainbow trout muscle and appear to be fibroblastic. However, it is too early in the process of cell line establishment to give any further characteristics of the cells.

Publications

Ossum, C.G., Hoffmann, E.K., Vijayan, M.M., Holt, S.E. and Bols, N.C. (2004.) Characterisation of a novel fibroblast-like cell line from the rainbow trout and responses to sublethal anoxia. *Journal of Fish Biology*, 64, 1-14.

Ossum C.G. and Hoffmann, E.K. "Effects of chemical anoxia and reoxygenation on the regulation of the MAPKinase, ERK in RTHD fibroblasts" (paper to be submitted)

Future perspectives

Working with fish cells in culture is extremely time-consuming. Therefore, the listing below is prioritized with respect to the time available.

1. Complete an article describing the above mentioned ERK-data in further detail.
2. Analysis of mechanisms inducing Hsp70 by anoxic stress.
3. Studies of death pathways induced by anoxia.
4. Analysis of possible secretion of matrix metalloproteinases during anoxia and anoxic cell death. Here, establishment of RTM C#2 is of particular interest.
5. Establishment and characterisation of RTM C#2.

Bilag 3.

Proteome changes in RTHD-cells after Na-azide incubation for 30' or over night, analysed by 2D-gel electrophoresis

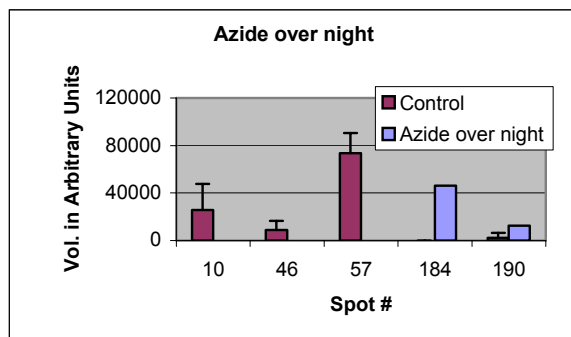
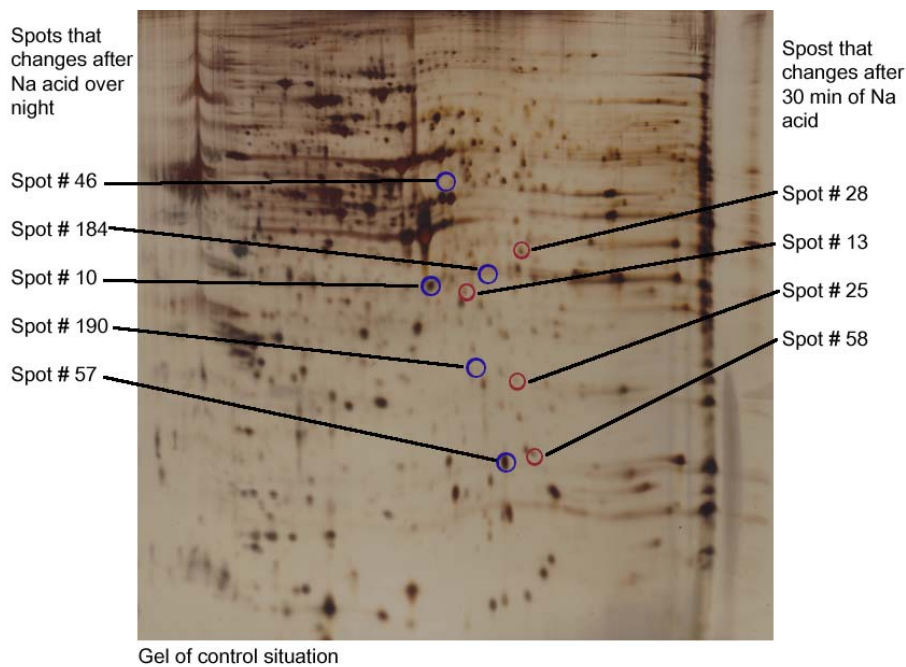
Ph.D. student Søren Wilhjem, senior scientist Flemming Jessen, Ph.D. and Reader Else K Hoffmann, Ph.D.

Aim of the studies

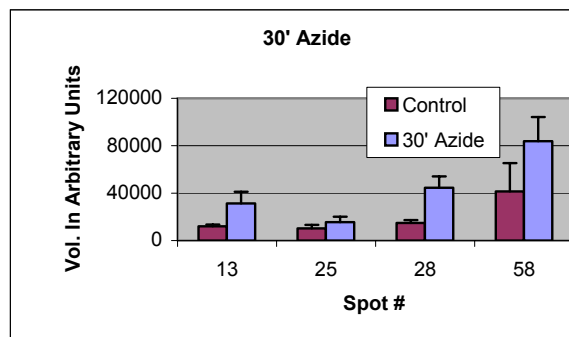
To investigate the proteome changes in various fish cell lines during anoxia.

Results

RTHD-cells were incubated with Na-azide to mimic anoxia for 30 min followed by a recovery phase of 20 hours (n=3) or incubated with Na-azide for 20 hours (killing the cells) without recovery (n=1). Homogenised cell lysate of these samples as well as control cells (n=3) were run on 2D SDS PAGE, silver stained and protein pattern analysed with "Image Master 2D Elite" software from Amersham Pharmacia Biotech. The results are not conclusive, but several proteins of potential interest have been found:



Spots 10,46 and 57 disappear; spot 184 appears



Spot 13, 25, 28 and 58 increases

Future perspectives

Next step will be to repeat these experiments with this cell line as well as other cell lines and identify the proteins involved by Mass spectroscopy in order to get a clearer picture of the effect of anoxia on these fish cell lines.

Bilag.4.

Biochemical and physiological properties of the enzyme responsible for formaldehyde formation in fish during storage

Associate professor Vibeke Barkholt; senior scientist Bo Jørgensen; assistant professor Michael Nielsen

Aim:

To perform basic research on the enzyme *trimethylamine-N-oxide aldolase*.
To create a solid basis for subsequent Ph.D. studies within this field.

Background

Trimethylamine-N-oxide aldolase is an important enzyme from the point of view of applied research and should also be of great interest to basic research due to the vast lack of knowledge regarding

- § Biochemistry: Size and amino acid composition. Reaction mechanism. Is it in fact a well-known metabolic enzyme with activity towards *trimethylamine-N-oxide*? Do different iso-enzymes exist?
- § Physiology: What is its function in organs, muscle and gall juice? Why is it so unevenly distributed? Why is the difference among individuals so large?
- § Technology: How can the activity be controlled (decreased) or at least estimated quickly using prior knowledge of e.g. fishing grounds, water temperature, season, age/size of the fish.

Status

- § A near-optimal enzyme preparation procedure has been developed and used for isolating larger amounts of enzyme from saithe gall juice for further analysis.
- § The enzyme activity in spleen and white muscle from a large number of species within the cod family has been determined. The substantial intra-species, inter-fish variability and the big difference in activity between species have been confirmed. The enzyme activity is not correlated to the muscle content of *trimethylamine-N-oxide*.
- § An idea that the *in vivo* biochemical function of the enzyme was connected to the choline metabolism was explored but without immediate success.

Milestones 2003

- § A further improvement in the enzyme preparation.
- § A molecular characterisation of the enzyme: Size (molecular mass), sub-unit composition, amino acid sequence.
- § Raising poly and monoclonal antibodies against the enzyme protein.
- § Comparison of the amino acid sequence with known sequences found in databases accessible via the Internet.

Publications

A paper concerning the inter-species variability in enzyme activity and the correlation between activity and formaldehyde formation is in preparation.

A paper dealing with identification of the enzyme and proposal of its physiological role awaits further data (the milestones).

Bilag 5.

Ph.D. course in fish muscle physiology and biochemistry

Date: 30th September – 4th October 2002
Place: Danish Institute for Fisheries Research
DTU, Lyngby, Denmark

Senior scientist Flemming Jessen, senior scientist Bo M. Jørgensen and assistant professor Michael K. Nielsen

This course was the first in a series of courses arranged by the network on “Fish Food, Biochemistry and Physiology” ([FIBP](#)). The objective is to promote research in physiological processes in fish muscle that cause or influence post mortem changes, such as *rigor mortis*, changes in water holding ability, production of free fatty acids and break-down of proteins, in order to reveal its implications for fish processing and product quality.

The course comprised

- a one-day seminar (Monday, 30th September) with lectures on selected topics in fish (muscle) physiology and biochemistry (42 participants) and
- 3½ days of exercises (1st – 4th October) on post mortem processes (12 participants).

Lecturers:

- Prof. Ian A. Johnston, University of St. Andrews, Scotland
- Dr. Richard Taylor, INRA de Theix, France
- Dr. David J. McKenzie, University of Birmingham, England
- Prof. Gert Flik, University of Nijmegen, The Netherlands
- Prof. Geoffrey Goldspink, University Collage London, England
- Dr. John Fleng Steffensen, University of Copenhagen, Denmark

Exercises:

- Water pools and their mobility in ice-stored fillets. Correlation to muscle proteins. Changes in the water distribution during chilled storage of cod and salmon are studied by low-field NMR relaxation and (3-way) chemometrics. The amount of bound water is correlated to the amount of muscle protein that can be denatured during heating.
- Formation of formaldehyde and dimethylamine during frozen storage. Activity of the TMAO aldolase enzyme. The dependence of formation of formaldehyde during frozen storage on the presence of white muscle TMAO aldolase (TMAOase) is demonstrated.
- Fatty acid composition. Free fatty acid formation and lipid oxidation during storage. The fatty acid profile of salmon is measured. The formation of free fatty acids and of selected volatile oxidation products during chilled storage is determined, and the effect of a freezing/thawing step on these processes demonstrated.

Future:

The course will be held again in 2003 in an expanded form including an exercise in proteome analysis.

Ph.D. course in fish muscle physiology and biochemistry

Date: 17th – 21st November 2003
Place: Danish Institute for Fisheries Research
DTU, Lyngby, Denmark

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The course comprised

- a one-day seminar (Monday, 17th November) with lectures on selected topics in fish (muscle) physiology and biochemistry (48 participants) and
- 4 days of exercises (18th – 21st November) on post mortem processes (12 participants).

Lecturers:

- Dr. C. Louise Milligan, University of Western Ontario, Canada
- Dr. Ian McCarthy, University of Wales, Bangor, United Kingdom
- Dr. Richard Taylor, INRA, France
- Dr Frank Bo Jensen, University of Southern Denmark, Denmark

Exercises:

- Water pools and their mobility in ice-stored fillets. Correlation to muscle proteins. Changes in the water distribution during chilled storage of cod and salmon are studied by low-field NMR relaxation and (3-way) chemometrics. The amount of bound water is correlated to the amount of muscle protein that can be denatured during heating.
- Accumulation of formaldehyde and dimethylamine during frozen storage. Activity of the TMAO aldolase enzyme. The dependence of formation of formaldehyde during frozen storage on the presence of white muscle TMAO aldolase (TMAOase) is demonstrated.
- Fatty acid composition. Lipid oxidation during chill storage; effect of packaging. The fatty acid profile of rainbow trout is measured. The formation of selected volatile oxidation products during chilled storage is determined, and the effects of vacuum packaging or packaging in nitrogen on oxidation and sensory attributes are determined.
- Proteome analysis of fish muscle. Two-dimensional gel electrophoresis and image analysis of gels will be carried out. As an example the effect of stress on protein expression will be studied by a comparison between samples from unstressed and stressed fish.

Future:

The course will be held again in 2004 in the expanded form including the exercise in proteome analysis.