Protein.DTU

3rd WORKSHOP

16th March 2010

DTU Chemical Engineering

DTU Nanotech

DTU Informatics

DTU Physics

DTU Vet

DTU Food

DTU Electro

DTU Systems Biology

DTU Chemistry

Protein.DTU
3rd WORKSHOP
16th March 2010

DTU

Contents

1. Program
2. List of abstracts
3. Abstracts
4. List of participants

WHAT'S GOING ON IN PROTEIN SCIENCE AT DTU?

Workshop and networking for young scientists in Protein.DTU
Tuesday, 16th March 2010, 08:30 – 17:30 hrs
Building 306, Lecture Hall 31

08:30 - 09:00 Arrival: registration, poster setup and breakfast

09:00 - 09:15 Welcome by **Birte Svensson** (Head of task force, Protein.DTU)

Chair: Birte Svensson (Professor, DTU Systems Biology)
09:15 - 09:30 Introductory address **Niels Axel Nielsen** (Director, Public Sector Consultancy, DTU)

Chair: Günther Peters (Associate Professor, DTU Chemistry)
09:30 - 10:15 **Karen Martinez** (Associate Professor, BioNano Group, Nanoscience Centre, University of Copenhagen)
"Novel functional assays for membrane proteins: Taking advantage of surfaces and nanomaterials"

Chairs: Jenny Emnéus (Professor, DTU Nanotech), Pernille Hammar Andersson (Learning Lab DTU), Peter M.H. Heegaard (Professor, DTU Vet), and Kristoffer Almdal (Professor, DTU Nanotech)
10:15 – 10:30 **Introduction, objectives and procedure of Part I and II of the Protein.DTU net-working activity during the day**

10:30 – 11:20 **Part I - Oral poster appetizers** – 1 min, 1 overhead transparency each
Session 1: Proteomics (posters 1–9)
Session 2: Analysis and Nanotechnology (posters 10–17)

11:20 – 11:35 Coffee break

11:35 – 12:25 **Part I (continued) - Oral poster appetizers** - 1 min, 1 overhead transparency each.
Session 3: Biotechnology and Modelling (posters 18–26)
Session 4: Protein structure and function (posters 27–36)
Session 5: Expression, Pathways and Bioinformatics (posters 37–45)

12:25 – 12:30 Short introduction to the afternoon's activities

12:30 – 13:15 Lunch

Chairs: Jenny Emnéus (Professor, DTU Nanotech), Pernille Hammar Andersson (Learning Lab DTU), Peter M.H. Heegaard (Professor, DTU Vet) and Kristoffer Almdal (Professor, DTU Nanotech)
13:15 – 14:55 **Part II – What happens tomorrow?**

14:55 – 15:00 Rounding up of Part II (Protein.DTU task force)

REG-no. DK 30 06 09 46

15:00 – 15:15 Coffee break

Chair: Flemming Jessen (Senior Scientist, DTU Food)

15:15 – 15:45 **Jens S. Andersen** (Professor, Department of Molecular Biology and Biochemistry, University of Southern Denmark, Odense)

"Dynamic organellar proteomics linking centrosomes, cilia, and diseases"

Chair: Christine Finnie (Associate Professor, DTU Systems Biology)

15:45 – 16:15 **Bente Vestergaard** (Associated Professor, Department of Medicinal Chemistry, University of Copenhagen, Faculty of Pharmaceutical Sciences)

"Proteins that ruin your brain - SAXS analysis pinpoints the toxic species in Parkinsons"

Chair: Birte Svensson (Professor, DTU Systems Biology)

16:15 – 16:20 **Conclusions**

16:20 – 17:30 **Time for "a beer and a snack" and continued discussions**

List of abstracts

Proteomics:

1. Per Hägglund: *Identification of thioredoxin targets using a quantitative proteomics approach based on isotope-coded affinity tag.* (Systems Biology)
2. Anders Laurell Blom Møller: *Plant plasma membrane proteomics: what is possible?* (Systems biology)
3. Avishek Majumder: *A proteomic approach to study prebiotic effect on the probiotic bacterium Lactobacillus acidophilus NCFM.* (Systems Biology)
4. Ofir Gilad: *Comparative membrane proteome analysis of a probiotic bacterium grown in the presence of prebiotics.* (Systems Biology)
5. Alexander Holm Viborg: *Production and characterization of prebiotics-related enzymes from Bifidobacteria* (Systems Biology)
6. Plaipol Dedvisitsakul: *Glycosylation and carbonylation study in* Lactobacillus acidophilus *NCFM and* Lactococcus lactis *MG1363.* (Systems Biology)
7. Morten Ejby: *Proteomic investigation of the probiotic* Bifidobacterium lactis *Bl-04 response to different carbohydrates.* (Systems Biology)
8. Anne Knudsen: *Exploring specific plant carbohydrate prebiotic interactions of* Bifidobacterium*.* (Systems Biology)
9. Bjarne Ersbøl: *Identification of protein groups using dimension reductive methods.* (Informatics)

Analysis and Nanotechnology:

10. Agnieszka Mech-Dorosz: *Development of biomimetic membrane array sensors.* (Nanotech)
11. Fozia Shah: *Real-time monitoring of stem cell differentiation in brain tissue using microfluidic on-line culture systems.* (Nanotech)
12. Claus R. Poulsen: *Detection of proteins using impedance and antibody coating in a microsystem.* (Nanotech)
13. Sania Ibragimova: *Hydrogel for* in situ *encapsulation of multiple black lipid membranes*. (Physics)
14. Christian Vestergaard: *Optimal estimation of the diffusion coefficient from noisy time-series measurements*.(Nanotech)
15. Kim Mortensen: *Optimized localization-analysis for single-molecule tracking and super-resolution microscopy*. (Nanotech)
16. Lisa Lystbæk Andersen: *Purification and characterization of bioactive peptides from fish protein hydrolysates*. (Food)
17. Susanne Jacobsen: *Protein core facility: Amino acid analysis, protein sequencing and mass spectrometry*. (Systems Biology)

Biotechnology and Modeling:

18. Britt Guillaume Jensen: *Study of hydrophobins in* Aspergillus *species*. (Systems Biology)
19. Mona Højgaard Pedersen: *Heterologous expression of hydrophobins RodA and RodB from* Aspergillus fumigatus *in host* Pichia Pastoris. (Systems Biology)
20. Hiroyuki Nakai: *Chemoenzymatic synthesis of novel oligosaccharides as prebiotics*. (Systems Biology)
21. Anders Jørgensen: *Targeted enzyme modification of starch for encapsulation of flavours*. (Systems Biology)
22. Abida Sultan: *Exploiting barley first wave enzymes activities for better feed.* (Systems Biology)
23. Arun Kumar Subramanian: *Molecular docking studies to gain insights into binding interactions of novel DACA analogs - G-quadruplex DNA*. (Chemistry)
24. Jonas Pedersen: *Modeling of DNA in nanochannels using linear elasticity theory*. (Nanotech)
25. Alessandro Corozzi: *ReaxFF modeling of lipase B from* Candida antarctica *and subtilisin savinase*. (Chemistry)
26. Henrik Bohr: *The quantum aspects of proteins and their biological function*. (Physics)

Protein structure and function:

27. Sandro Bottaro: *Local moves for Monte Carlo sampling of proteins around the native state.* (Electro)
28. Maja Martic: *Extending the functional diversity of iron-sulfur proteins by generation of heterometallic iron-sulfur clusters*. (Chemistry)
29. Anikó Czene: *New type of chimeric metallonucleases for gene therapy*. (Chemistry)
30. Marie Møller: *Structure, function and protein engineering in starch debranching enzyme systems*. (Systems Biology)
31. Olof Björnberg: *Reduction of a structural disulfide (C144-C148) in barley alfa-amylase/subtilisin inhibitor (BASI) by thioredoxin*. (Systems Biology)
32. Kristine Groth Kirkensgaard: *Reaction mechanism of NADPH-dependent thioredoxin reductase (HvNTR2) from* Hordeum vulgare *(barley).* (Systems Biology)
33. Johanne Mørch Jensen: *Characterisation of the redox-dependent interaction between recombinant barley limit dextrinase inhibitor and thioredoxin h.* (Systems Biology)
34. Nicolas Navrot: *Exploiting the barley redox proteome: Expression and characterization of barley thioredoxin target proteins*. (Systems Biology)
35. Mikkel Glaring: *Expression and characterisation of starch binding domains from Carbohydrate Binding Module family 45.* (Systems Biology)
36. Folmer Fredslund: *Properties of the glycoside hydrolase family 36 α-galactosidase from the probiotic bacterium* Lactobacillus acidophilus *NCFM*. (Systems Biology)

Expression, Pathways and Bioinformatics:

37. Bent Petersen: *NetSurfP - real value solvent accessibility predictions with amino acid associated reliability.* (Systems Biology)
38. Jonas Nørskov Søndergaard: *Mapping of signalling pathways in dendritic cells to identify novel drug targets against latent pathogenic microbes.* (Systems Biology)
39. Petr Efler: *The* Lactococcus lactis *thioredoxin system*. (Systems Biology)
40. Gabriella Christina van Zanten: *Stimulation of the growth of two probiotic bacteria,* Lactobacillus acidophilus *NCFM and* Bifidobacterium lactis *BL-04, by selected prebiotic candidates*. (Systems Biology)
41. Trine Vammen Vendelboe: *Expression and purification of human Dopamine β-monooxygenase*. (Chemistry)
42. Irina Borodina: *Allergen display on the cell surface of yeast*. (Systems Biology)
43. Kirsten Lindegaard Bovbjerg: *The induction of anti-inflammatory porteins in stimulated equine whole blood.* (Vet)
44. Peter Heegaard: *MBL-A concentrations and MBL 1 genotypes in european wild boars, large White pigs, and wild boar/large White crossbreds*. (Vet)
45. Kasper Olsen: *Overwinding of DNA explained*. (Physics)

Identification of thioredoxin targets using a quantitative proteomics approach based on isotope-coded affinity tags

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Thioredoxin is a protein disulfide reductase implicated in a wide range of cellular processes, e.g. metabolic control, protection against oxidative stress, transcriptional regulation, and cell division. However, the detailed target specificity is still not well understood. To further the understanding of the molecular recognition of thioredoxin, we have developed a novel quantitative proteomics approach for reliable identification of specific targets *in vitro* based on isotope-coded affinity tags (ICAT) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). By exploring the thiol-specific reactivity of the iodoacetamide-based ICAT reagent, specific target disulfides are identified and thioredoxin-mediated reduction is quantified by measuring ratios of peptides labeled with ICAT reagents containing "heavy" (^{13}C) and "light" (^{12}C) carbon isotopes. We applied the method to identify targets of a cytosolic h-type thioredoxin (HvTrxh1) in dissected barley embryo. Previously characterized target proteins such as peroxiredoxin and cyclophilin were identified as well as a range of novel putative target proteins including several ribosomal proteins.

This work was supported by the Danish Research Council for Technology and Production Sciences and the Carlsberg Foundation.

Plant plasma membrane proteomics: what is possible?

Møller, A.L.B.[1], Witzel, K.[2], Kaspar, S.[2], Matros, A.[2], Svensson, B.[1], Mock, H. P.[2], Barkholt, V.[1], Andersen, B.[1] and Finnie, C.[1]

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2: Institute for Plant Genetics and Crop Plant Research, Gatersleben, Germany.

Almost all environmental changes to the surroundings of a plant cell are initially perceived via plasma membrane-embedded proteins, many of which act as sensors and facilitators of transport. Knowledge of the plasma membrane protein profile is needed if we are to use targeted breeding or gene technology to develop crop plants for the future.

Plasma membrane proteomics is still in its infancy due to the hydrophobic nature and low abundance of integral membrane proteins. Existing classical 2-dimensional gel-based methods are not ideal for analysis of membrane proteins which tend to precipitate in the IEF gel at their pI. We have used reversed-phase chromatography combined with 1-dimensional SDS-PAGE (Hynek et al. 2006; 2009) and 2-DE with benzyldimethyl-n-hexadecylammonium chloride (16-BAC) electrophoresis in the first dimension and SDS-PAGE in the second, for separation of proteins. In combination with mass spectrometry these approaches have resulted in identification of proteins from plasma-membrane enriched fractions and have potential for recording the dynamics of the most abundant integral membrane proteins. However, for a more complete and quantitative analysis of plasma membrane proteomes, label-free nanoLC-ESI-Q-TOF MS will be applied.

The project is funded by The Danish Research Council for Technology and Production Sciences, The Danish Natural Science Research Council, The Danish Centre for Advanced Food Studies and COST FA0603

A proteomic approach to study prebiotic effect on the probiotic bacterium *Lactobacillus acidophilus* NCFM

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Prebiotics are non-digestible food components that selectively stimulate the growth or activity of certain bacteria in the intestinal microbiota, thereby conferring a beneficial effect on host health[1]. Probiotics are live microorganisms that, administered in adequate amounts, confer health benefits to the host[1]. Different types of carbohydrates have been found to stimulate the growth of probiotic bacteria and act as potential prebiotics. *Lactobacillus acidophilus* NCFM (NCFM) is a low G+C Gram-positive bacterium adapted to survive in the gastrointestinal tract and is a well documented probiotic. It encodes 1862 predicted open reading frames (ORFs) (NCBI:CP000033) including 37 glycoside hydrolases (GH) (http://www.cazy.org/) involved in utilization of carbohydrates[2]. By the use of negative transcriptional regulation, NCFM is able to express GH gene products for utilization of carbohydrate sources. In the present study, a proteome map (pH 3–7) of NCFM whole cell extract was generated to aid investigations on metabolic adaptation to prebiotic food sources by analyzing 650 protein spots resolved by 2D electrophoresis (pH 3–7) using tryptic *in-gel* digestion and mass spectrometry. A total of 625 proteins were identified representing 302 unique gene products of which 280 had pI within pH 3–7 corresponding to 39.7% coverage of the theoretical acidic proteome. The identified proteins were analyzed *in silico* for functionality, cellular localization, codon-anticodon adaptability index and hydropathy index. The dynamics of the proteome and the abundance of GH's in presence of the prebiotic lactitol were characterized using 2D-DIGE (DIfference Gel Electrophoresis) (pH 4–7) for NCFM grown on glucose and lactitol, revealing 68 spots with altered relative intensity. Thirty-two unique proteins were identified to be up- or down-regulated in 42 of these spots by adaptation of NCFM to growth on lactitol. Regulated proteins involved in metabolism of lactitol included β-galactosidase small subunit (GH2), galactokinase, galactose-1-phosphate uridylyltransferase and UDP-glucose 4-epimerase showed 1.6-12.7 fold change. The β-galactosidase small subunit was found to be significantly up-regulated with a fold change of 12.7. Moreover proteins like the phosphotransferase system enzyme II, fructose-bisphosphate aldolase, and pyruvate kinase showing a fold change of 1.7–2.2 are suggested to play a role in carbon catabolite repression. Similar studies are carried out with raffinose and cellobiose.

This project is supported by the Danish Strategic Research Council's Programme Committee on Health, Food and Welfare (FøSu). AM thanks the Technical University of Denmark for a H.C. Ørsted postdoctoral fellowship and AS thanks Danisco for a M Sc. fellowship grant.

References

[1] S. Macfarlane, et al. Alim. Pharmac. Ther., 24, 701-714 (2006)
[2] E. Altermann, et al. Proc. Natl. Acad. Sci. U. S. A., 102, 3906-3912 (2005)

Comparative Membrane Proteome Analysis of a Probiotic Bacterium Grown in the Presence of Prebiotics

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Prebiotics are commonly indigestible complex carbohydrates that are specifically fermented by a single (or a few) probiotic bacteria, and hence elicit a beneficial effect on the human host. The application of prebiotics, accompanied by probiotics (synbiotics), or as an independent application, attracts growing attention from both a scientific and a commercial point of view. In spite of the experimental data attained regarding the metabolism of prebiotics in bifidobacteria, the knowledge of the transport mechanisms by which these carbohydrates are imported into the cell is not established yet. The current Industrial PhD project aims at investigating these mechanisms. As a part of a comparative, whole-cell proteome analysis of cultures of an extensively applied probiotic strain, *Bifidobacterium animalis* subsp. *lactis* BB-12, grown in the presence of two prebiotic carbohydrates (fructo- or xylo-oligosaccharides, respectively), membrane fractions will be obtained by a complementary experimental approach. The experimental layout consists of a classical gel-based method, a tryptic method and a proteinase K method [as described by Hahne *et al.*, (2008)]. The protein content from these fractions will be then identified by LC-MS/MS. The results obtained by these analyses are expected to broaden the insight into the membrane proteome of the bacterium in general and the transport systems it is utilizing for the intake of fructo- and xylo-oligosaccharides.

Acknowledgments
The projected is supported by grants from the Danish Ministry of Science, Technology and Innovation (for further details see www.erhvervsphd.dk).

References:

H. Hahne, S. Wolff, M. Hecker and D Becher, From complementarity to comprehensiveness--targeting the membrane proteome of growing Bacillus subtilis by divergent approaches; Proteomics. 2008, 8(19):4123-36.

Production and characterization of prebiotics-related enzymes from Bifidobacteria

Alexander Holm Viborg[1,2*], Kim Ib Sørensen[2],Ofir Gilad [1,3,] Susanne Jacobsen[1], Birte Svensson[1] and Birgitte Stuer-Lauridsen [3]

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Probiotic strains such as those belonging to the genus *Bifidobacterium* are widely used as dietary supplements to a variety of foodstuffs. Pre -and probiotics play a significant role in gastrointestinal well-being as a result of the beneficial effects they exert on human hosts. Xylo-oligosaccharides (XOS) are regarded as emerging prebiotics which are used as food ingredients and have been shown to trigger a specific bifidogenic effect on the gut microflora (1). In spite of the generally accepted importance of pre-and probiotics in gastrointestinal well-being and the growing interest in the field, the knowledge of the underlying molecular mechanisms is limited. In order to shed more light on the underlying molecular mechanisms, the current study focuses on the interactions between the probiotic strain *Bifidobacterium animalis* subsp. *Lactis* BB-12 (BB-12) and the prebiotic substrate XOS.

Glycosylation and Carbonylation study in *Lactobacillus acidophilus* NCFM and *Lactococcus lactis* MG1363

Plaipol Dedvisitsakul, Per Hägglund, Christine Finnie, Susanne Jacobsen and Birte Svensson

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To gain deeper understanding on the physiology of *Lactobacillus* and *Lactococcus*, the proteome information is required and this includes a strict requirement for identification also of protein post-translational modifications (PTMs). There is substantial evidence for eukaryotic and bacterial proteins that PTMs contribute to determine the function, structure, localization, turnover and interaction with other proteins (1, 2). The aim of this project is to screen and identify post-translationally modified proteins in *Lactobacillus acidophilus* NCFM and *Lactococcus lactis* MG1363. In preliminary experiments, periodic acid-Schiff staining of proteins after one-dimensional gel electrophoresis suggested that some proteins in *Lactobacillus acidophilus* NCFM were glycosylated. However, no putative glycosylated proteins were detected by this method in *Lactococcus lactis* MG1363. On the other hand, however, *Lactococcus lactis* MG1363 was found to contain carbonylated protiens as shown by Western blot analysis using specific antibodies for the modification. The bacterial proteins will be separated by two-dimensional gel electrophoresis and subsequently post-translationally modified proteins will be identified by mass spectrometry.

The program Strategic Scholarships for Frontier Research Network (SFR) of Thailand's Commission on Higher Education is acknowledged for a Ph.D. fellowship.

References

1. Hitchen P.G., Dell A. (2006) Bacterial glycoproteomics. Microbiology 152: 1575-1580.
2. Mann M, Jensen O.N. (2003) Proteomic analysis of post-translational modifications. Nat. Biotechnol. 21: 255-261.

Proteomic investigation of the probiotic Bifidobacterium lactis Bl-04 response to different carbohydrates

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Bifidobacterium animalis subspecies lactis, BL-04 is a probiotic organism that displays the ability to use prebiotic carbohydrates (resistant to host degradation). This project sets out to identify and characterize the important pathways and proteins that enable bifidobacteria to utilize different prebiotic carbohydrates. This is done by a 2D-gel based mass spectrometry combined with differential proteomics approach, where multiple fractions of the proteome are compared in different conditions. The most interesting findings will be selected for cloning, expression, purification and more detailed characterization.

Exploring specific plant carbohydrate prebiotic interactions of *Bifidobacterium*

Anne Knudsen[1,2], Gabriella van Zanten[3], Lene Jespersen[3], Sampo Lahtinen[4], Ole Bandsholm[5], Mark Lawther[6], Maher Abou Hachem[2], Susanne Jacobsen, Birte Svensson[2] and Andreas Blennow[1]

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2Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark, Denmark

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4Danisco A/S, Kantvik, Finland

5KMC AMBA, Denmark

6Biovelop A/S, Denmark

Insight into the molecular mechanisms underlying prebiotic interaction with specific well-known probiotic bacteria lags very much behind. To explore the synbiotic interaction of two widely used probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis animalis* subsp. with selected putative prebiotic carbohydrate candidates, effects were screened using a library of 70 structurally different carbohydrates presented to these two bacterial strains. The carbohydrates consisted of different di-, tri- tetra and pentasaccharides, mixtures of oligosaccharides, polysaccharides and crude potato fibres. Potential prebiotic oligosaccharides were also generated by enzyme assisted hydrolysis of purified oat β-glucan using a endo-1,4-ß-D-Glucanase (*H. insolens*), endo-1,3-ß-D-Glucanase (*Trichoderma* sp.) and lichenase. Likewise, galactans and mannans were used as sources to get putative prebiotic oligosaccharides by aid of endo-1,4-β-Galactanase (*Aspergillus niger*) and endo-1,4-β-mannanase (*Pichia Pastoris*). The resulting oligosaccharide products were analyzed using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD). The screening of the effect of the oligosaccharides on growth of *Lactobacillus* and *Bifidobacterium* strains was performed using a Bioscreen instrument operated under anaerobic conditions. In this instrument the bacterial growth was monitored by measuring optical density (OD) at 600 nm on isolated cultures of the two probiotic strains in combination with the different carbohydrates. Specific prebiotic candidates showed differential effects on the two probiotic strains. Directed by these results, the candidates for the further work was determined. For the work on the *Bifidobacterium* strain, The identification will be done using matrix-assisted laser desorption-ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).
Acknowledgements: This project is funded by the Committee for Food and Health (FøSu) of the Danish Council for Strategic Research, and the Danish Research Council for Natural Science.

Identification of protein groups using dimension reductive methods

Bjarne Kjær Ersbøll, Line Harder Clemmensen, Line Conradsen, Mia Skettrup

DTU Informatics, Richard Petersens Plads B321, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark.

Abstract

Identification of interesting proteins in 2D-electrophoretic gel data is complicated by the fact that there are far more variables (proteins) than observations (gels). Typically, on the order of tens of gels in an experiment and on the order of thousands of individual proteins on a gel. Often interesting proteins are identified by simple and robust methods like t-tests. However, this has many pitfalls: the risk of falsely detecting interesting spots, and the risk of not being able to detect pairs or triplets of interesting spots. Cross-validated selection methods and modern sparse methods of analysis can help solve this problem.

Development of Biomimetic Membrane Array Sensors

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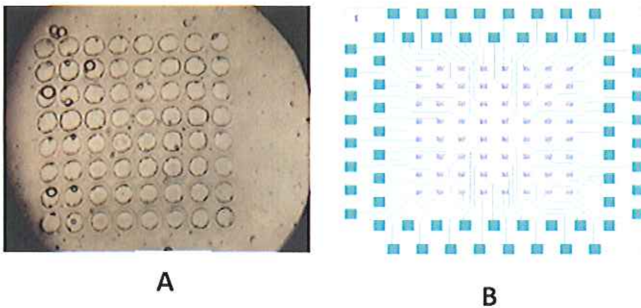
[1]Department of Micro- and Nanotechnology, Technical University of Denmark, Kongens Lyngy, Denmark

[2]Aquaporin A/S, Section DTU, Kongens Lyngby, Denmark

Abstract:

Applying analytical techniques on microchips in a flow format has become an important strategy for rapid screening of substances interacting with their biological targets [1]. Hereby, we focus on the implementation of the biomimetic membrane platform, supported in a polymer based scaffold structure [Fig. 1A], as a powerful tool for drug discovery in miniaturized assays. The central part of this project is the development of a microfluidic system for investigating the interactions between active compounds (ions and ligands) with aquaporins (water channels) and G-protein coupled receptors (GPCRs) reconstituted in biomimetic membranes. The detection of the developed method is based on impedance spectroscopy in a multichannel system using microelectrode arrays [Fig. 1B]. Optical detection strategies will also be applied. The overall aim of this project is to show that the presented method is reproducible and accurate for high through-put screening of potential drug candidates.

Figures:



A B

Figure1: A) Schematic representation of polymer based scaffold structure. B) *Proposed electrode microchip structure (pink squares: individually addressable electrodes; green squares: patches for electrical connections).*

Reference:

1. *R. Davidsson, Microfluidic biosensing system based on enzymes, antibodies and cells. PhD Thesis, Lund University, 2004.*

Real-time monitoring of stem cell differentiation in brain tissue using microfluidic on-line culture systems

Fozia Shah, Arto Heiskanan, Martin Dufva, Jenny Emnéus.

DTU Nanotech -Department of Micro and Nanotechnology, Technical University of Denmark, Kongens Lyngby, Denmark.

Abstract:

The aim of this project is to implant neuronal stem cells (NSC) in the brain region called Striatum, where they differentiate to dopaminergic neurons (Fig. 1A). Earlier studies with implantation of stem cells in organotypic brain tissue slices have established basic knowledge regarding the necessary procedures [1]. However, the studies have not been able to conclusively give information whether the differentiated stem cells function in the same manner as native dopaminergic neurons.

The microfluidic system, schematically illustrated in Fig 1B, will be utilized for culturing brain slices and differentiating stem cells as well as time lapse microscopic and impedance spectroscopic monitoring of stem cell differentiation and integration (axonal growth and innervation of striatal target neurons). Amperometric monitoring of dopamine exocytosis (release of dopamine) on nanometer sized interdigitated electrodes (n-IDEs) (Fig. 2) serves as the conclusive evidence that the differentiated stem cells have acquired the desired properties.

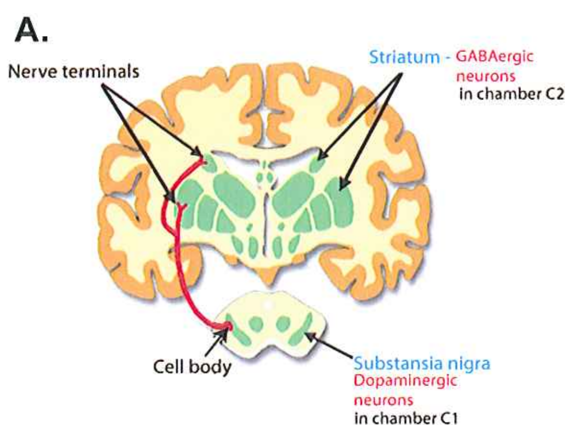


Figure 1:
A. A sketch of the nigro-stratial dopaminergic system in the brain (communication system of neurons in Substansia nigra and Striatum), indicating the brain areas, from which primary cells or tissues are collected for culturing and monitoring in the different chambers C1 and C2 in Fig. 1B.
B. Side view of a fully transparent microfluidic platform for cultivation of cells and tissues to study innervation of two cell or tissue types.

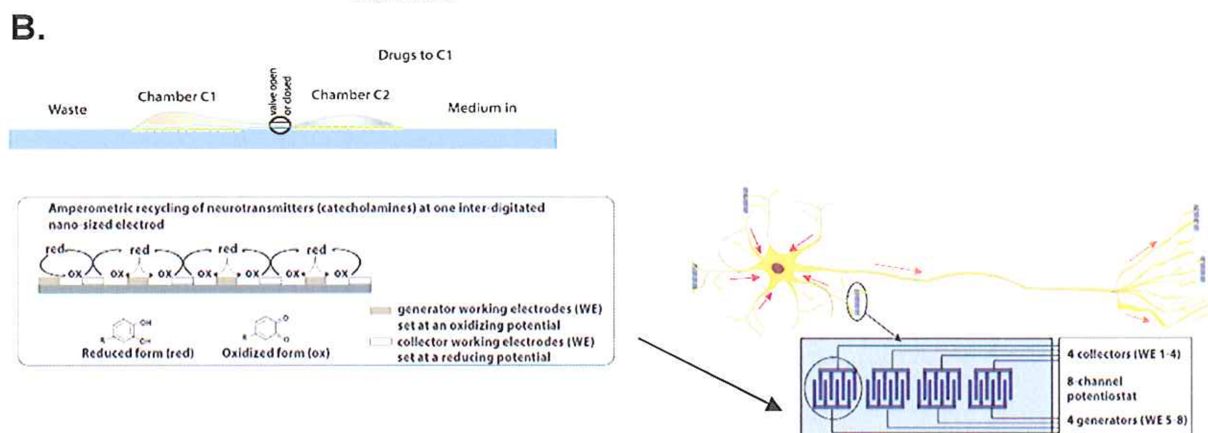


Figure 2. Amperometric recycling of neurotransmittors at one n-IDE nanosized (***left***). An n-IDE array for monitoring neurotransmittor-release from a neuron (***right***).

1: Bjorklund LM, Sánchez-Pernaute R, Chung S, Andersson T, Chen IY, McNaught KS, Brownell AL, Jenkins BG, Wahlestedt C, Kim KS, Isacson O. Proc Natl Acad Sci USA. 2002 Feb 19;99 (4):2344-9.

Detection of Proteins using Impedance and Antibody Coating in a Microsystem

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The influenza pandemic at the end of 2009 have brought the attention to the need of having fast, effective, and reliable diagnosis and treatment of virus transmitted diseases. New techniques for detection of virus infections are essential for controlling and containing the spread of virus particles in the future [1].

Our microfluidic system is sensitive and can rapidly detect the binding of a certain specific target protein to the antibody modified electrode surface. The conventional methods for determining type and strain of a virus needs several days to complete, but we estimate that our system can have a diagnosis within 15 minutes. Since the detection is mediated through the use of antibody, the target protein can relatively easily be changed just by binding a different antibody to the electrode. The electrode is connected to a potentiostat, which is used for measuring the impedance of the electrode. This measure is directly proportional to the amount of target protein bound to the antibody on the electrode surface.

In addition to the fast detection, the detection is conducted in a low volume flow chamber (5 x 2 x 0.030 mm), which gives a total volume of only 0.30 μl. Furthermore, the flow chamber opens up the possibility that the sample volume very easily can be varied just by increasing or decreasing the sample flow rate.

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HYDROGEL FOR *IN SITU* ENCAPSULATION OF MULTIPLE BLACK LIPID MEMBRANES

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Abstract:

Hydrogels are hydrophilic, porous polymer networks that can absorb up to thousands of times their own weight in water. They have many applications, one of which is the encapsulation of free-standing black lipid membranes (BLMs) for novel separation technologies or biosensor applications. We investigated gels for *in situ* encapsulation of multiple black lipid membranes across apertures in a hydrophobic ethylene tetrafluoroethylene (ETFE) support. These gels consisted of networks of poly(ethylene glycol)-dimethacrylate or poly(ethylene glycol)-diacrylate polymerized using either a chemical initiator or a photoinitiator. The hydrogels were studied with regards to their material properties such as chemical resistance, swelling behaviour, water permeability and porosity. We found that lifetimes of membranes in gel precursor solutions were short compared to lifetimes in buffer. However, crosslinking the gel stabilized the membranes and improved BLM lifetimes substantially over lifetimes in buffer. Optical images of the membranes and incorporation of the transmembrane peptide gramicidin A showed that the lipid membranes retained their integrity after encapsulation with hydrogel.

Optimal Estimation of the Diffusion Coefficient from Noisy Time-series Measurements

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Recent developments in fluorescent labels have made it possible to track diffusion of single molecules with time-lapse photography, e.g., proteins on DNA, microtubules or actin fibres. Such measurements often contain considerable localization error. The standard method [1] for estimating the diffusion coefficient is based on the mean square displacements. This method is highly inefficient, since it ignores the high correlations inherent in the mean squared displacements. A maximum likelihood method, which takes into account these correlations, is presented and it is shown that it attains the maximum precision possible according to information theory.

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Optimized localization-analysis for single-molecule tracking and super-resolution microscopy

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We present optimal localization procedures for isolated fluorescent molecules and beads imaged as diffraction-limited spots as well as new, reliable formulae for the precisions of various localization methods. Comparing for beads, unweighted least-squares fitting of a Gaussian squanders one third of the available information, a popular formula for its precision exaggerates beyond Fisher's information limit, and weighted least-squares may do worse, while maximum likelihood fitting is practically optimal.

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Purification and Characterization of Bioactive Peptides from Fish Protein Hydrolysates

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A large number of bioactive peptides have been identified in and isolated from various food sources. Milk seems to be a particularly rich source but also different fish species have been found to yield bioactive peptides. Bioactive peptides, usually consisting of 3 to 20 amino acids, can be released from proteins upon degradation by proteolytic enzymes, e.g. in the intestinal tract. The numerous described bioactivities include antihypertensive, anticancerous, antimicrobial, and immumodulating effects.
We intend to isolate and characterize bioactive peptides from fish protein hydrolysates obtained by enzymatic hydrolysis. As the starting point 3 parts of the fish (Trout), which are not normally used for consumption, have been selected for analysis: Skin, belly flap muscle, and jaw muscle. The skin or fish muscle was minced and hydrolysed with the unspecific proteases Alcalase, Neutrase, or UE1 (all from Novozymes, Bagsværd, Denmark) to a hydrolysis degree of 10-15%. The obtained hydrolysates were subjected to ultrafiltration through 3, 5, or 10 kD molecular weight cut-off filters. The resulting permeates were analysed for size distribution using gel filtration. Initial fractionation of hydrolysates was obtained through ion exchange chromatography.
Perspectives: The obtained fractions will be tested *in vitro* for effects in relation to several biological activities such as cancer cell proliferation and migration and antibacterial activity. Preliminary results indicate that the raw hydrolysates as well as fractions from ultrafiltration inhibit angiotensin I-converting enzyme, which is involved in blood pressure regulation. Peptide fractions containing bioactivity will be further fractionated and the individual peptides characterized by liquid chromatography-mass spectrometry (LC-MS). Furthermore, other fish species as well as commercial fish hydrolysates will be analysed for bioactivity.

Protein core facility:

Amino acid analysis, protein sequencing and mass spectrometry

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Enzyme and Protein Chemistry, Department of Systems Biology, building 224, DTU, hosts the protein core facility – for more detailed information and guidelines: see link http://www.epc.bio.dtu.dk/English/Protein%20core%20facility.aspx

The protein core facility offers:

- Amino acid analysis
  - Amount
  - Composition and identification
- Protein identification
  - Identification of proteins
  - Homology search
  - Information of post-translational cleavage
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The services of the core facility are available for researchers and students at DTU, other academia and commercial customers.

For more practical information: You are welcome to contact us at the Protein.DTU workshop.

Study of hydrophobins in *Aspergillus* species

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Hydrophobins are a class of small amphipatic proteins unique to filamentous fungi. They are found in the outermost rodlet layer of fungal spores and are responsible for a high surface hydrophobicity. All hydrophobins contain eight cystein residues, including two pairs of adjacent cysteins, in a conserved pattern. The eight cysteins form four disulphide bonds; Cys1-Cys6, Cys2-Cys5, Cys3-Cys4 and Cys7-Cys8. Furthermore, hydrophobins contain a large proportion of hydrophobic residues, but apart from these two features conservation between hydrophobins is low. During the last few years several *Aspergillus* species have been full genome sequenced, and within some species hydrophobins have been identified. Our aim was to look for new hydrophobins in the genomes of *Aspergillus* by using a perl program. We used the full genome sequence from the following species; *A. fumigatus* (AF293), *Emericella nidulans* (FGSC A4), *A. oryzae* (RIB40), *A. niger* (ATCC1015 and CBS513.88), *A. clavatus* (NRRL 1), *A. terreus* (NIH 2624) and *A. flavus* (NRRL 3357). We have identified between two and eight putative hydrophobins in each of the different species; two in *A. oryzae* and eight in *A. niger*. The remaining species had 5-7 putative hydrophobins. The identified hydrophobins were examined for similarities. In general, conservation between the different hydrophobins was low, but similar hydropathy plots were observed. All identified hydrophobins contained the characteristic eight cystein pattern and furthermore the majority contained a proline situated almost directly after the signal sequence. We are currently looking into the hydrophobin *rodA* and are producing different *E. nidulans* mutants. These mutants will be used to examine the role of *rodA* on spore hydrophobicity.

Heterologous expression of hydrophobins RodA and RodB from *Aspergillus fumigatus* in host *Pichia Pastoris*

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Introduction: Hydrophobins are small amphipatic proteins present on the spore surface of filamentous fungi. They most likely play an important role in the attachment of spores to a solid phase. The pathogenic fungus *Aspergillus fumigatus* expresses the hydrophobins RodA and RodB on the surface of its conidia and these may be of importance to the pathogenesis of the fungus. Although heterologous expression of hydrophobins has proven to be a challenge by past investigators, we made it the aim of this project to produce pure hydrophobins in sufficient quantities for further characterication and investigation using the expression host *Pichia pastoris*.

Methods and materials: The genes encoding hydrophobins RodA and RodB were amplified by RT-PCR with gene-specific primers from the total RNA isolated from the spores of *A. fumigatus* (AF296 strain). The resulting cDNA was cloned into TOPO vectors using TOPO TA Cloning (Invitrogen), and the inserts were sequenced. The genes were further amplified by PCR to generate overhangs with specific restriction sites and cloned into expression vectors pPICZαA and pPICZB while adding a 6xHis-tag to the C-terminal of both hydrophobins. The pPICZαA vector expresses proteins with the signal sequence of alfa-mating factor from *Saccharomyces cerevisia* known to work well for protein secretion from *P. pastoris* and the pPICZB plasmids had proteins cloned with their native signal sequences. The plasmids were linearized, transformed into *P. pastoris* strain X33 and transformants were selected by zeocin resistance. The presence of the RodA and RodB genes in the transformants was confirmed by colony PCR. The expression of RodA and RodB genes was induced by growing cells in culture flasks for three days in buffered complex methanol medium as protein production was dependent on the methanol-induced AOX1 promoter. The protein production was analyzed by SDS-PAGE, coomassie and silver-stained, as well as western blotting using a detection antibody (Penta-His HRP conjugate, Qiagen). Recombinant RodA and RodB were purified using His-select Nickel Affinity gel (Sigma-Aldrich, Saint Louis, MO, USA).

Results: *P. pastoris* cultures expressing hydrophobins resulted in increased foaming, which was attributed to the presence of secreted hydrophobins. Protein bands of expected size were detected in both the fermentation broth and the foam by SDS-PAGE and western blotting. Optimization of the purification of hydrophobins and functional investigations are being carried out at the moment.

Conclusion: Hydrophobins RodA and RodB from *Aspergillus fumigatus* were successfully expressed and secreted by yeast host *Pichia pastoris*.

Keywords: *Aspergillus fumigatus*; *Pichia pastoris*; recombinant protein; hydrophobin; RodA; RodB, fungal spore.

Chemoenzymatic synthesis of novel oligosaccharides as prebiotics

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Various candidates as "prebiotics" inducing selective stimulation of growth of beneficial commensals in gastrointerstinal tract of human were synthesised by inverting phosphorylases [1] and glycoside hydrolases having high capacity to catalyse oligosaccharide-synthetic reaction, 81 out of the identified 111 oligosaccharides are novel. The prebiotic effect by these candidates is being recently evaluated.

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Targeted enzyme modification of starch for encapsulation of flavours

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The taste of foods is limited to five sensations: sweet, bitter, salty, sour, and umami (savoury), whereas thousands of olfactory receptors enable the sensation of tens of thousands flavours in concentrations as low as parts per billion.

Manufactured food substances are usually based on chemically modified starches (i.e. acetylation), which are subject to legislation. The starches are used as thickeners, stabilisers, or flavour carriers. Natural starches are not subject to the same legislation, and are also more appealing to the consumer.

The aim of this study is to use natural starches in the industrial processes to create or enhance flavour delivery systems in manufactured foods. We will study the interactions between selected aromas and selected starches, including enzymatically treated starches, by both by methods mentioned below and also by X-ray studies to reveal the nature of the aroma/starch interaction.

Starch is a mixture of linear amylose and branched amylopectin, 20% and 80%, respectively. Amylose forms a helix-structure with an internal diameter of approximately 10 Å, where guest molecules such as aroma compounds can be accommodated.

By using enzymes, either branching (i.e. amylomaltase, EC 2.4.1.25) or debranching (i.e. isoamylase, EC 3.2.1.68) it is possible to change the structure of the starches to improve the aroma retention.

The aroma content in a given starch can be measured simply by comparing the weight of the aroma/starch mixture and the starch alone. Even though the aroma must be retained sufficiently in the starch to prevent loss of aroma during storage, at least some of the aromas must be released during mastication to provide the consumer with the proper flavour experience. By aid of GC-MS (gas chromatography – mass spectrometry) it is possible to identify the aroma and estimate of the amount.

Analysis of the aroma content in a dry environment is a measure for the total amount of aroma that can be released from the starches, whereas the presence of water creates a situation that mimics the mastication processes. The latter will give an aroma profile that is comparable to the flavour experience.

Exploiting Barley First Wave Enzymes Activities for Better Feed

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Barley *(Hordeum vulgare)* is one of the most important crops worldwide and is predominantly used for livestock feed. The mature barley grain contains a battery of hydrolytic enzymes that are synthesized during seed development and deposited in an inactive form. These enzyme activities (first wave enzymes) are of biological importance as they are required for initiating seed germination and are considered important quality determinants for food and feed. Some of the already known enzymes are among others β-amylases, phytases, proteases, lipases, and cell wall degrading enzymes [1]. Although barley proteins have been substantially studied, the current knowledge about these first wave enzyme activities at molecular level and their potential in feed are scarce. Thus, the aim of this project is to unravel the full complement of first wave enzymes, analyse and characterize their biochemical properties with application of a combination of transcriptomics, proteomics and biochemical and enzymatic techniques. The obtained knowledge will shed some light in the metabolic pathways and regulatory mechanisms governing seed maturation. Exploiting the endogenous first wave enzymes is of great value in future efforts of improving feed quality by maximizing micronutrient uptake and minimizing or even eliminating the necessity of additives to feed.

This PhD project is embedded in the Food Research Program 2008.

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Molecular Docking Studies to Gain Insights into Binding Interactions of Novel DACA Analogs - G-Quadruplex DNA.

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Drugs targeting DNA molecules can have different advantages like: *i*) repairing the genome, or *ii*) suppressing the expression of malfunctioning genes. G-Quadruplex DNA (fig.1A) structures are telomeric DNAs, reported to be in a unique quadruple conformation during non-replication period of the cell, and they are broken down to form single stretches of guanine rich DNA during replication. Stabilizing G-Quadruplex DNA has been attempted to control the fast proliferating cells in cancerous states. Small molecules having good physico-chemical profiles for binding DNA G-Quadruplex structures are potential anti-cancer drugs. Although DACA (dimethyl aminoethyl carboxylic acids – fig.1B) is one of the previously known classes of drugs that can very well recognize DNA structures, they failed in clinical trials due to toxicity. As a part of our ongoing attempts to design novel DACA analogues and to optimize them for selectivity and lower toxicity, several ligands were synthesized. Any attempt to solve the crystal structure of DNA G-Quadruplex – ligand complexes failed. Hence, to gain insights into the binding modes of these novel ligands, we selected a sub-set of molecules from this library and studied them computationally.

Extensive docking, followed by explicitly solvated molecular dynamics simulations were carried out for understanding the interactions of novel DACA drug derivatives using a crystal structure of a parallel DNA G-Quadruplex – daunomycin complex as reference structure. Since conformational sampling is an important issue in docking, we devised an efficient docking protocol, in which, depending on the ligand structure, around 250-1000 conformations were generated for each drug molecule. These conformations were subsequently used for virtual screening (VS). The top scoring 10 hits from VS phase were selected for more detailed Glide XP docking in which 10 best ranking orientations were retained for each docked conformation. Thus obtained pool of conformations was combined and rescored using Glide XP scoring function to get the best binding modes from the whole collection. The top 10 hits from this re-ranked pool were re-studied through QM/MM methods for increased accuracy. The Quantum Polarized Ligand Docking protocol (QPLD) scheme in Schrodinger suite was used for QM/MM docking, in which the whole ligand was treated quantum mechanically (HF method with 6-31G* basis sets) while the receptor was treated by molecular mechanics (OPLS 2005 force field). The ligand partial charges were re-fitted from the QM/MM energies calculated in the presence of receptor which is expected to capture receptor induced polarization. These ligand charges were used with standard OPLS 2005 parameters for re-docking the ligands. The quality of this methodology will be discussed in detail.

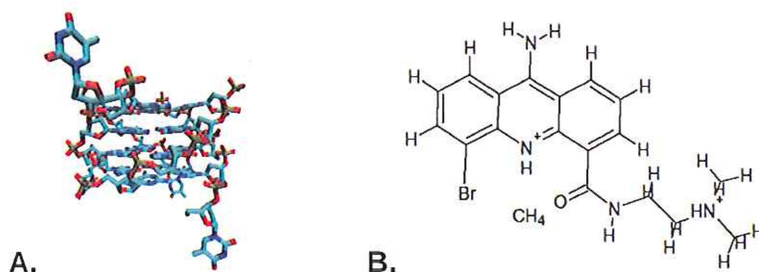

Fig.1.A. The molecular structure of a parallel G-Quadruplex DNA, B. Structure of a DACA drug derivative.

Modeling of DNA in nanochannels using linear elasticity theory

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We model the Brownian dynamics of a single dsDNA molecule in a nanochannel. When such a molecule is placed in a nanochannel, *its extension along the channel is up to 50% of its contour length*, depending on the dimensions of the channel. This linear extension offers the possibility to study, e.g., the binding of site-specific proteins or the sequence-dependent melting of DNA, with the latter giving a coarse-grained representation of the sequence [W. Reisner et al., "Single Molecule Denaturation Mapping of DNA in Nanofluidic Channels." Submitted to *PNAS*].

The resolution achieved with this approach has two limiting factors: *The diffraction limit*, and *thermal density fluctuations* of the DNA in the channel. Density fluctuations make distances measured along the channel correspond to distances measured along the molecule in an unknown, non-linear and random, time-dependent manner. This last problem can be circumvented if *the non-linear map between lab-coordinates and molecule-coordinates can be extracted from images at any point in time*. To this end, we model the thermal motion of the molecule using linear elasticity theory combined with Boltzmann statistics, but also including the photonic shot-noise and the EMCCD camera's *excess noise* from its electron multiplication process. The thermal dynamics of the resulting model is consistent with movies of DNA displaying thermal density fluctuations. These fluctuations set a position-dependent lower bound on the precision with which we can determine positions.

ReaxFF Modeling of Lipase B from Candida Antarctica and Subtilisin Savinase

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Lipases are a diverse group of enzymes characterized by their drastically increased esterase activity when absorbed to a lipid surface. This process is known as interfacial activation [1].
Candida Antarctica lipase B (CALB), a serine hydrolase, displays enantioselectivity toward secondary alcohols by means of hydrolysis, esterification, or transesterification reactions [2], and aminolysis of their corresponding acetates [3] tolerating non-natural reactants and reaction conditions [4, 5]. CALB is also a very effective catalyst for the production of amines and amides [6] and for the resolution of chiral amines [7]. CALB is a potent catalysts of stereoselective reactions and it is therefore of interest for use in chemical synthesis.
Subtilisins are serine proteases capable of catalyzing hydrolysis of peptide and ester bonds, by binding the substrate in the so-called catalytic triad consisting of a serine, a histidine and an aspartate. The catalytic triad works in a well-understood manner, initiated with a nucleophilic attack by the serine oxygen on the carbonyl carbon of the amide or ester substrate. The serine oxygen is nucleophilic because the histidine acts as a base, thereby removing the proton from the serine during the process [8]. Subtilisin Savinase is an important enzyme used in detergents, because of its high activity in the pH range 8-12 [9] thus additional understanding and development of improved variants is an important research area.
Investigative computational analysis upon the catalytic pathway will be carried out using the so-called "reactive force field", ReaxFF, developed by the Goddard group at the Materials Process and Simulation Center at the California Institute of Technology (MSC, Caltech) [10]. This is a force field capable of describing chemical reactions since the bond order is obtained from the interatomic distance between two pairs of atoms, i.e. the closer the atoms are to each other, the more strongly bonded they are, a concept which dates back to ideas of Linus Pauling. As direct consequence of this approach the ReaxFF method allows bonds to be formed and broken, thus effectively enabling reactions to occur during simulations. We want to compare the method to already known, but time consuming QM/MM methods, and hope to be able to make *in silico* prediction of new enzymes with improved characteristics.

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The quantum aspects of proteins and their biological function. Case of SOD

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Proteins, and especially enzymes, are studied with respect to their quantum behavior which includes electronic states, vibrational states involving the nuclei, excited state dynamics, tunneling phenomena, bio-Auger processes, charge-density fluctuations and general charge transfer. All these aspects are exemplified in the protein SOD (Super Oxide Dismutase) for removing of oxidative radicals.

Local moves for Monte Carlo sampling of proteins around the native state

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ABSTRACT

Recent studies demonstrated that many proteins are highly dynamic and adopt more than one state under physiological conditions. These conformational isomers hold the key to understand many important biological and biochemical processes (i.e. enzymatic catalysis, signal transduction, regulation and misfolding). It is difficult to address these transitions with standard molecular dynamics, due to inaccuracies of the force fields employed and sampling inefficiencies.
We present a new Monte Carlo based method for efficient sampling of compact states. At the heart of this method is the discovery of an analytical solution to the loop closure problem. Formal correctness of the method is verified, and improved computational efficiency relative to the current state-of-art methodologies is shown, suggesting that detailed simulations at the time scales characterizing the conformational switches of proteins can be made feasible.

Extending the functional diversity of iron-sulfur proteins by generation of heterometallic iron-sulfur clusters

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Iron-sulfur proteins form one of the largest classes of metalloproteins, with more than one hundred different iron-sulfur proteins known. These proteins are typically considered as electron transfer mediators but can also catalyze biological reactions, regulate metabolic pathways and gene expression, and serve as biological sensors [1].

Due to unusual ligation mode, some iron-sulfur proteins can be isolated in two different forms either containing an incomplete cuboidal [Fe_3S_4] cluster or a cubane-type [Fe_4S_4] cluster. Under appropriate conditions these two cluster types have ability for interconversion by the loss or uptake of an iron [2]. This interconversion has envisioned the way of incorporating metals, other than iron, into the vacant site of the [Fe_3S_4] cluster, thus generating a series of novel heterometallic iron-sulfur clusters [3, 4, 5].

An incorporation of a heterometal will introduce new properties and possible catalytic functions to the proteins.

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New type of chimeric metallonucleases for gene therapy

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Duchenne Muscular Dystrophy (DMD) is an X-linked lethal disease, which affects muscle wasting in males. Therapeutic possibilities are limited, the current strategies will end up with life-long treatment in the best case. [1] The goal of our work is (i) to develope a simple multiplex PCR method followed by sequencing for the breakpoint determination in DMD gene; (ii) the design and preparation of a new type of DNA sequence specific, functional and non-toxic zinc-finger - HNH (HNH: His-Asn-His rich motif) metallonuclease, targeted towards the mutation point in genomic DNA of a DMD patient. In this chimeric nuclease a domain responsible for non-specific nuclease activity (HNH motif) will be linked to a DNA-binding protein with known or designed DNA sequence-specificity (ZF).
The HNH nuclease domain displays a ββα structure with a central metal ion binding site. It is abundant within the active centre of different nuclease enzymes. An example is the bacterial Colicin E7 (ColE7), which functions in the presence of zinc(II). The ColE7 protein can be overexpressed in *E. coli* only in the presence of an inhibitory protein. Recently, we elaborated a method to express the 42 amino acid containing C-terminal HNH motif of ColE7, and investigated its metal binding ability and nuclease activity. Surprisingly, this protein did not exert cytotoxicity, and *in vitro* nuclease assay also proved to be negative. In the knowledge of the above results we were searching for the minimal functional part of the ColE7. We have designed several mutants, based on the crystal structure of ColE7 [2], revealing the essential amino acid residues. The mutation of the very N-terminal arginine to glycine allowed for only a minor residual nuclease activity, pointing to the essential role of this amino acid in the control of ColE7 activity.
The methods for expression and purification of these mutants as GST fusion proteins have been optimized. The stuctural and functional investigation of the mutants incubated with metal ions and/or DNA will be performed by means of fluorescence, CD and NMR spectroscopy, ESI-MS, crystallography and gel electrophoresis methods (e.g. gel shift assay).

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Structure, function and protein engineering in starch debranching enzyme systems

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Starch is the major carbohydrate reserve in cereal grains and constitutes up to 60% of the total dry weight. Starch consists of the essentially linear 1,4-α-glucan amylose, together with the branched α-glucan amylopectin. The enzyme-catalysed mobilization of storage starch granules in the endosperm of germinating cereal seeds involves an initial solubilization, mainly by α-amylase, which is followed by hydrolysis of the resulting dextrins to oligosaccharides and glucose by the concerted action of α-amylase, β-amylase, limit dextrinase (LD), and α-glucosidase. Of these enzymes only LD has the capacity to hydrolyse α-1,6-glucosidic linkages in branched maltooligosaccharides [1, 2].
Recently an expression system has been successfully established in *Pichia pastoris* for barley LD, and the production and purification procedures have been optimised [3]. Producing LD including various mutants of LD recombinantly gives the possibility of getting high enough amounts of pure protein for crystallisation. The determination of these structures will illuminate substrate binding residues of importance for the specificity and the size of the substrate both with respect to main chain and branch length, and this will allow engineering the enzyme by rational mutational analysis targeting these residues.

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Reduction of a structural disulfide (C144-C148) in barley α-amylase(2)/subtilisin inhibitor (BASI) by thioredoxin

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α-Amylase and subtilisin can be inhibited simultaneously by barley α-amylase/subtilisin inhibitor (BASI) because the interaction sites are localized on opposite sides. BASI contains two structural disulfide bonds, C43-C90, directed towards the protease, and C144-C148 directed towards α-amylase. The latter bond is a target substrate of the protein disulfide reductase thioredoxin.

The crystal structure of BASI in complex with thioredoxin suggested the importance of a few main chain hydrogen bonds (Maeda et al Structure 2006 14, 1701). Two residues from loops in thioredoxin, M88 and A106, participate in a total of three hydrogen bonds only involving the main chain amide and carbonyl groups and we have used site directed mutagenesis to kinetically evaluate them. Exchange to proline residues, that display tertiary amides in the main chain, gave undetectable reduction of the target disulfide in the case of M88 and 10 % residual activity in the case of A106. Exchange to other residues diminished activity to between 5 and 80 % of that of the wild type. However, in these assays thioredoxin was reduced by the flavoprotein thioredoxin reductase and, in contrast to the other mutants, M88P was poorly reduced. The mutant displays a weak general disulfide reductase activity when assayed with other reductants but other methods must be developed to analyze this particular mutant in relation to BASI.

Apart from the loop residues, exchange of E86 in thioredoxin to arginine increased the activity by 300 % presumably by creating a good electrostatic match to a glutamic acid in BASI (E168) in the periphery of the interaction area.

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Reaction mechanism of NADPH-dependent thioredoxin reductase (*Hv*NTR2) from *Hordeum vulgare* (barley)

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Thioredoxins (Trx's) are protein disulfide reductases, which are widely distributed in all types of organisms. They control the intracellular redox environment and are involved in the regulation of a large number of cellular processes including DNA synthesis, oxidative stress response and apoptosis.

Trx is reactivated by specific NADPH-dependant thioredoxin reductases (NTRs). NTRs catalyze the reduction of a redox-active bond in Trx using NADPH as reductant and FAD as cofactor. We here present the structure of *Hv*NTR2 from *Hordeum vulgare* (barley) [1]. *Hv*NTR2 consists of two domains, the FAD and the NADPH binding domains, and so far all previously solved low-molecular-weight NTRs were found to be in one of the two conformations; the flavin oxidizing (FO) or the flavin reducing (FR) conformation. To obtain the latter, one domain has to rotate 66° relative to the other. Only by covalently binding NTR to Trx, the FR can be stabilized for characterization [2]. The structure of *Hv*NTR2 represents a yet unseen conformation, which may correspond to an intermediate between the FO and the FR conformation; the NADPH domain is rotated by 25° and bent by a 38% closure relative to the FAD domain when comparing with *Arabidopsis thaliana* NTR-B [3]. Based on studies of interdomain contacts as well as phylogenetic studies we propose a new reaction scheme for NTR, in which NTR-Trx interactions mediates the FO to FR transformation followed by NADPH binding.

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Characterisation of the redox-dependent interaction between recombinant barley limit dextrinase inhibitor and thioredoxin h

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Abstract:
It has been suggested that the disulfide reductase barley thioredoxin h (HvTrxh) regulates the interaction between the starch debranching enzyme limit dextrinase (LD) and its endogenous inhibitor (LDI) in germinating barley seeds. LDI contains four disulfide bonds and one free cysteine forming mixed disulfides with either glutathione or cysteine. LDI therefore contains five potential targets for HvTrxh reduction. Here we have analysed the reduction of disulfide bonds in LDI upon incubation in the presence of HvTrxh. HvTrxh was capable of reducing the same number of disulfide bonds in LDI as was observed after incubation with the reducing agent dithiotreitol (DTT). Preliminary results from mass spectrometry analysis suggested that HvTrxh not only reduced the disulfide bonds in LDI, but also the mixed disulfide thereby releasing glutathione or cysteine. The effect of HvTrxh treatment on the interaction between LD and LDI was assessed using LD activity measurements. HvTrxh had no apparent effect on LD activity in the absence of LDI or in the LD/LDI complex. However, if LDI was pre-incubated with HvTrxh prior to addition of LD, the inhibitory activity of LDI was greatly impaired. Furthermore, treatment of LDI and the LD/LDI complex with DTT completely eliminated the inhibitory activity of LDI towards LD.

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Systems Biology

Redox control systems in barley: cloning and characterization of dehydroascorbate reductase, glutathione peroxidase and 1-cys peroxiredoxin

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Aerobic organisms in general and photosynthetic organisms in particular are exposed to oxidative stress at the cellular level. Plants have thus evolved advanced redox control systems including the thioredoxin and glutathione pathways as well as the ascorbate-glutathione cycle (1). These systems are involved in many cellular functions, like photosynthesis and sugar metabolism regulation, and are important in critical developmental steps as seed maturation and germination, during which cells are subjected to harsh conditions. Accumulating evidence suggests that these systems are interlinked in a complex manner. We have selected three barley redox proteins for recombinant production based on their potential interactions with the barley thioredoxin system (2). Dehydroascorbate reductase and 1-cys peroxiredoxin were identified as targets in barley embryos using a quantitative proteomics method based on isotope-coded affinity tags (3). Glutathione peroxidase has been identified as a target of thioredoxin in poplar in a previous work, and is highly expressed in the seeds of different plants (4). The genes of barley dehydroascorbate reductase, glutathione peroxidase and 1-cys peroxiredoxin have been successfully expressed in *E. coli* and the corresponding proteins purified to homogeneity by chromatography. Biochemical and structural studies are in progress and will give detailed information about the interaction between thioredoxin and its partners in germinating seeds. Applications of thioredoxin chemistry to different industrial processes involving barley and cereals are also under investigation.

This study was supported by the Danish Research Council for Technology and Production Sciences, The Carlsberg Foundation, and the Centre for Advanced Food Studies (LMC).

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Expression and characterisation of starch binding domains from Carbohydrate Binding Module family 45

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Starch binding domains (SBDs) have been found in CBM families 20, 21, 25, 26, 34, 41, 45, 48 and 53 [1, http://www.cazy.org/fam/acc_CBM.html]. Recently a new SBD family, CBM45 was discovered [2]. This family only contains sequences from the plant kingdom. CBM45 occurs as an N-terminal tandem domain in only two classes of intracellular enzymes, plastidial amylases (EC 3.2.1.1) and glucan water dikinases (GWDs, EC 2.7.9.4). GWD1 is expressed in photosynthetic organisms and phosphorylates transient and storage starch in the plastids, a process which is necessary for subsequent starch mobilisation. Plastidial α-amylases are presumably involved in starch turnover, though the exact role is currently unclear.

In order to probe the domain borders, several single CBM45 constructs of GWD1 from *Solanum tuberosum* (potato) were made and expressed as cleavable His-fusion proteins in *E. coli.* The conformational integrity of a selected protein product was evaluated after removal of the His-tag by differential scanning calorimety (DSC). Even though CBM45s do not contain cysteines, the internally situated CBM, CBM45-2, shows unexpected high thermostability (T_m = 84°C). Binding properties of CBM45 were tested by isothermal calorimetry (ITC) and surface plasmon resonance (Biacore) using soluble low molecular weight starch mimetic motifs. The rather low affinity of CBM45, as compared to most other starch binding domains, suggests reversible or regulated binding of CBM45-containing enzymes. Similar constructs were fused with YFP to probe the ability of isolated CBMs to bind to starch *in planta.* Analyses of tobacco leaves transiently expressing CBM45-YFP-fusions by confocal microscopy suggest *in vivo* binding of CBM45s to transient starch granules. Current characterization focuses on the tandem CBM45 domains from the *Arabidopsis thaliana* plastidial α-amylase AMY3.

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Properties of the glycoside hydrolase family 36 α-galactosidase from the Probiotic bacterium *Lactobacillus acidophilus* NCFM

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Probiotics are defined as live microorganisms that administered in adequate amounts will confer a beneficial health effect on the host and bacteria belonging to the *Lactobacillus* and *Bifidobacterium* genera are often used as probiotics. *Lactobacillus acidophilus* NCFM has been commercially available since the 1970's as a probiotic and many beneficial effects of this bacterium have been reported[1]. The annotated genome of *L. acidophilus* NCFM was published in 2005[2], and revealed the presence of 37 glycoside hydrolases (GHs) assigned into 13 GH families[3] (http://www.cazy.org), thus reflecting the significant carbohydrate catabolism potential of this bacterium. A later study mapped the transport and catabolic machinery involved in carbohydrate utilization using whole-genome cDNA microarrays[4], and this study revealed the pathway for the transport and degradation of α-linked galacto-oligosaccharides such as raffinose and melibiose. The GH36 α-galactosidase encoded in the genome of *L. acidophilus* and implicated in the degradation of these putative prebiotics has been cloned and produced in *Escherichia coli* and the properties of the enzyme have been studied. The enzymatic properties and the substrate preference of this enzyme will be presented illuminating its role in utilization of potential carbohydrate prebiotics.

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NetSurfP - Real value Solvent Accessibility predictions with amino acid associated reliability

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Motivation: Prediction of protein surface accessibility is a competitive field where an ever-increasing performance is both the goal and an opportunity to improve other types of predicted local or global protein features. It is important that the accuracy of any predicted feature for an amino acid is as high as possible, however for most prediction systems, the accuracy is only known as an average performance over a large benchmark data set used for evaluation. Here, we have developed and trained an ensemble of artificial neural networks to predict the real value relative surface accessibility for a chain of amino acids and each prediction is associated with an inherited reliability score.

Results: Artificial neural networks (ANN) have been trained to predict either the relative exposure of an amino acid or a classification as being either buried or exposed using a cutoff of 25% exposure. Both methods have been evaluated against an independent set of 513 protein sequences (CB513) and comparisons have been made to other state of the art predictors. An accuracy of 79.0% correct and a Matthews correlation coefficient of 0.58 was obtained, which is better than otherwise reported. Also, a method was developed and trained to predict the relative surface accessibility (RSA) with an inherited reliability score, in form of a Z-score. A Pearson correlation coefficient of 0.70 was obtained when evaluated against the CB513 dataset. This is also the highest performance reported for a method evaluated against this dataset as we show in a comparison to work reported by other groups. Using the Z-score and only evaluating the performance for the 90% of the most reliable predictions, an accuracy of 79.6% and a Pearson correlation coefficient of 0.72 were obtained. The work presented has been implemented as a webserver named NetSurfP allowing submission of several sequences at the time.

Availability: http://www.cbs.dtu.dk/services/NetSurfP/

Mapping of Signalling Pathways in Dendritic Cells to Identify Novel Drug Targets Against Latent Pathogenic Microbes

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One of the most notorious disease-causing pathogens of our time: human immunodeficiency virus (HIV), harbour the capacity to down-modulate immunity in key immunoregulatory cells, and thereby preventing effective immune clearance. Little is presently known about the intracellular pathways in immune cells that mediate this process, but accumulating evidence suggest that C-type lectin receptor (CLR) crosstalk with Toll-like receptors (TLR) may play an important role. Our project employs a combined experimental and bioinformatics approach to delineate the complex signalling networks that are induced in human plasmacytoid and myeloid dendritic cells (pDCs and mDCs), upon challenge with HIV. The objective is to identify the protein kinases that mediate the specific immune regulation in DCs to discover new drug targets. We are ex vivo isolating the mDCs and pDCs to a >98% purity and stimulating them with very pure HIV TLR and CLR ligands. Because of very limited cell numbers we are analyzing intracellular effector cytokines by phosphoflow and quantitating their relative production. The mRNA level is corresponded to these results to ensure that the immunomodulation is taking place at a pre-transcriptional level. The DC-TLR-CLR system will subsequently be analyzed using a pan-specific phospho-tyrosine antibody to determine important timeframes for phosho-changes, which will be used to perform kinome-wide analysis using mass-spectrometry. Finally, bioinformatics will be used to combine the novel signalling molecules with already existing information to build a more complete picture of the signal transduction cascades involved in immune modulation induced by HIV.

The *Lactococcus lactis* thioredoxin system

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L. lactis is a gram-positive micro-aerophilic bacterium producing lactic acid as its primary catabolic end product. The resulting acidification is explored in industrial fermentations where *L. lactis* is used in starter cultures for production of cheese and buttermilk. In the presence of oxygen, *L lactis* is exposed to oxidative stress caused by reactive oxygen species (hydrogen peroxide, superoxide anion radical and hydroxyl radical). Responses to oxidative stress are regulated by various mechanisms among which the thioredoxin (Trx) system plays important roles. Trx contains a redox-active CXXC motif, reduces disulfide bonds in target proteins, and receives reducing equivalents from the NADPH-dependent flavoprotein thioredoxin reductase. Many organisms also rely on the glutathione/glutaredoxin system for disulfide reduction but *L. lactis* lack glutathione biosynthetic apparatus and it can thus be suggested that the thioredoxin system is of particular importance in this organism. *L. lactis* contains an NADPH-dependent thioredoxin reductase (TrxB), two thioredoxins (TrxA, TrxH) and a glutaredoxin-like protein NrdH. The latter one works as source of reducing power for NrdEF ribonucleotide reductase (Jordan *et al.*, 1996). It has similar fold a glutaredoxin, but it is reduced by TrxB. TrxA is similar to the "classical" thioredoxin found in organisms ranging from bacteria to higher eukaryotes with a CGPC active site motif. TrxH, however, display a divergent sequence (14% identity and 30% similarity to TrxA) and contains an atypical CGDC motif that appears to be restricted to gram-positive bacteria. To study the *in vivo* functions of TrxA and TrxH in *L. lactis*, knockout mutants have been constructed and the phenotypes analyzed. The results so far indicate that TrxA is involved in oxidative stress response while the role of TrxH remains unclear.

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Stimulation of the growth of two probiotic bacteria, *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* BL-04, by selected prebiotic candidates

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Introduction: Prebiotics have been shown to support the growth of probiotic bacteria thereby having a beneficial effect on human health. The aim of this work was to evaluate selected and structurally different carbohydrate prebiotic candidates by measuring their capability to stimulate the growth of the two probiotic bacteria *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* BL-04.

Methods: A Bioscreen instrument was used to screen the pre- and probiotics under anaerobic conditions and the optical density at 600 nm was measured to monitor the growth of isolated cultures of the probiotic strains, *L. acidophilus* NCFM and *B. lactis* BL-04, in combination with different prebiotic candidates (1%). By using the area under the growth curves obtained by measuring OD600 during 24 h fermentations, the growth of the two probiotic bacteria on the different prebiotic candidates was compared to the growth supported by a known prebiotic, galactooligosaccharide (GOS).

Results: For *L. acidophilus* NCFM raffinose, isomaltulose, panose, isomaltose, FOS (fructooligosaccharide), galactose, stachyose, trehalose and verbascose supported growth to the same extent as for GOS whilst for gentiobiose and cellobiose the growth was 30 and 35 % higher than GOS, respectively. For *B. lactis* BL-04 isomaltose, melibiose, stachyose and verbascose supported growth to the same extent as GOS whilst for panose, raffinose and maltotriose prepared from hydrolyzed pullullan the growth was 27, 28 and 41 % higher than for GOS, respectively.

Discussion: The fermentation patterns of both *Lactobacilli* and *Bifidobacteria* are strain and species specific. *L. acidophilus* is previously shown to ferment a wide variety of different carbohydrates, but though a gene for a putative transporter of gentiobiose and cellobiose has been identified for *L. acidophilus* NCFM it has not previously been shown that this strain is able to utilize gentiobiose. Most *Bifidobacteria* are able to utilize raffinose but to our knowledge it has not previously been shown that *B. lactis* BL-04 is able to utilize panose and maltotriose. The most promising combinations of pre- and probiotics will be further investigated by differential proteomics to give more understanding of the interactions between the pre- and probiotics. Moreover some of the pre-/probiotic combinations will be used in a colon simulator to simulate their effects on human health.

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Expression and purification of human Dopamine β-monooxygenase

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Human Dopamine β-monooxygenase (hDBM) is a copper-containing monooxygenase that catalyses the conversion of dopamine into norepinephrine [1]. The level of these two neurotransmitters and therefore hDBM are involved in several psychiatric diseases such as depression and schizophrenia [2, 3] and characterization of hDBM is therefore of high importance.
No expression system for hDBM is available [4] and needs to be developed. Expression of soluble hDBM in *E. coli* has been examined by various strategies and has been successful in origami cells with co-expression of DsbC, a protein responsible for the isomerization of disulfide bonds [5]. The expression level is however low. Very little is known about the hDBM structure, but it is a glycoprotein and studies suggest the present of up till 6 intramolecular and 2 intermolecular disulfide bonds [6] which could be the reason for the difficulties with expression in *E. coli*. An eukaryotic expression system in *Drosophila melanogaster* has therefore also been developed. The expression level however needs to be further optimized.
Results from both prokaryotic and eukaryotic expression and preliminary purification of hDBM are presented.

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ALLERGEN DISPLAY ON THE CELL SURFACE OF YEAST

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ABSTRACT

Yeast surface display is a technique, where the proteins of interest are expressed as fusions with yeast surface proteins and thus remain attached to the yeast cell wall after expression. Our purpose was to study whether allergens expressed on the cell surface of baker's yeast *Saccharomyces cerevisiae* preserve their native allergenic properties. We chose to use the major allergens from the common wasp *Vespula vulgaris* venom (phospholipase A1 PLA1, hyaluronidase HYA and antigen 5) as the model.

The proteins were expressed on the surface as fusions with a-agglutinin complex protein AGA2. In order to express PLA1 and HYA, we modified the surface display expression vector to include antibiotic resistance cassette so that constant selective pressure could be applied. PLA1 expression severely inhibited the growth of the yeast cells. PLA1 and HYA expressed on the surface retained their enzymatic activities. HYA and antigen 5 expression was confirmed by fluorescent-activated cell sorting (FACS) analysis of cells stained with antibody against a small c-myc tag attached to the allergens C-terminus. The expression of PLA1 was too low to be detected by FACS. The allergenic properties of the expressed allergens were evaluated by IgE binding and tested by FACS and histamine release (HR). FACS results showed no IgE binding to PLA1 and very limited to HYA but with both wasp venom specific and control IgE. Antigen 5 however bound wasp venom specific IgE antibodies but not control IgE. The HR results supported the FASC data since antigen 5 was the only allergen capable of mediating an allergen-specific histamine release.

In conclusion, the allergens expressed on the surface of yeast retained their IgE binding capabilities, could cause histamine release and remained enzymatically active (where applicable). In perspective the yeast surface display can be used for allergen discovery from cDNA libraries and possibly for sublingual specific immunotherapy (SLIT) as the cells can serve as good adjuvant and can be produced in large amounts at a low price.

The induction of anti-inflammatory proteins in stimulated equine whole blood

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Degenerative joint diseases, including osteoarthritis are an important problem in the human population and also in animals use for sports like horses and an efficient and reproducible method for treatment of such diseases is much demand. A method that has met with some success for treatment of horses is the Irap™ method in which an unstabilized blood sample from the patient is agitated gently and then incubated and allowed to clot overnight at 37°C in the presence of 2.5 mm diameter medical grade glass beads. This results in the formation of serum with anti-inflammatory properties and when injected into the patients affected joint relieves symptoms of osteoarthritis (stiffness, pain) or even cures the disease possibly after a number of treatments. The beneficial effect of the conditioned serum has been ascribed to the presence of anti-inflammatory proteins (cytokines), in particular the so-called interleukin-1 receptor antagonist (IL-1ra) in the serum, counteracting the inflammation present in the joint. However this has not been investigated systematically and the correlation with clinical effects has not been proven. Complemented by studies on the mRNA level we investigated the presence of IL-1ra in normal horse serum and in conditioned horse serum. In order to study expression at the mRNA level we devised a method for extracting mRNA from leucocytes of blood clots, while a cross-reactive anti-mouse IL-1ra immunoassay was used to quantify horse IL-1ra protein. We found that the coagulation process by itself induced IL-1ra (mRNA and protein), IL-1beta and IL-8 and that these factors, including the anti-inflammatory IL-1ra were less prominently induced by the Irap™ method. These preliminary data clearly indicates that IL-1ra can not be the only factor responsible for the anti-inflammatory effect of conditioned serum. Proteome directed methods may be useful for identifying proteins that are differentially induced in conditioned serum and correlated with the anti-inflammatory effect.

MBL-A concentrations and *MBL1* genotypes in European wild boars, Large White pigs, and wild boar/Large White crossbreds

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There is considerable controversy concerning the biological significance of mannan-binding lectin (MBL) and its importance in the human innate immune defense. In pigs, the usage of MBL-A as a breeding parameter has been suggested. Here, we report distinct differences in MBL-A concentration in serum between wild boar x Large White crossbred pigs, and a novel single nucleotide polymorphism (SNP), likely to be functionally important, in the MBL1 gene. MBL-A concentrations in serum were measured in 45 pigs, constituting the second backcross (BC_2) generation in a family material originating from 2 wild boar x Large White intercrosses, before and after vaccination with an *E. coli* vaccine. Moreover, part of the MBL1 gene was sequenced in 16 animals, representing all 5 generations in the family material, and in 6 wild boars. MBL-A concentration in serum showed a bimodal distribution, with one third of the animals at levels between 0.7 and 1.6 µg/ml, close to the detection limit of the assay, and the remaining pigs at levels around 13 µg/ml. Sequencing revealed a total of seven SNPs in exons 1 and 2. One of these – G131A, previously unpublished – gives rise to the substitution of a glycine for an aspartic acid in G-X-Y motif 1 in the collagen-like domain and is likely to interfere with triple helix formation. Furthermore, the presence of a previously described SNP, assumed to affect MBL-A concentrations, was confirmed in the European wild boars, the Large White pigs, and the wild boar x Large White crossbreds. This study provides new pieces of information regarding polymorphism in the porcine MBL1 gene, and provides a link between this polymorphism and the genetically determined subsequent variation in MBL-A serum concentrations in different pig populations.

Overwinding of DNA explained

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Overwinding of DNA was first observed in 2006 [1,2]: DNA does not always unwind as one would intuitively expect, rather it rotates counter to unwinding. Building on earlier work [3], we show that overwinding is geometrical in nature, hence that overwinding is an example of a much more general phenomenon. Overwinding of a double helix can happen when its pitch angle is below 39.4 degrees.

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