6th European Symposium on Enzymes in Grain Processing

28th -30th November 2011
at Carlsberg Research Center
Gamle Carlsberg Vej 10, Copenhagen, Valby

Program and Abstract Book

DTU Systems Biology

Carlsberg Laboratorium

We are extremely grateful to the following sponsors for financial support for ESEGP6

Dear Colleagues and Friends

It is a pleasure to welcome you to Copenhagen for the 6th European Symposium on Enzymes in Grain Processing (ESEGP). Started by the 1996 initiative of TNO (The Netherlands), ESEGP has been successfully organized by VTT (Helsinki, Finland 1999), KUL (Leuven, Belgium, 2002), INRA (Nantes, France, 2005), and IFR (Norwich, UK, 2008). We have a program with 34 talks and 42 posters that we hope will promote an atmosphere of stimulating free discussion in continuation of the spirit of previous ESEGP Symposia.

The scope of this meeting maintains a balance of academic and applied research into classical topics such as enzyme structure-function, baking and brewing, as well as the new genomic technologies and emerging areas for grain research covering bioenergy, feed, biorefineries and functional ingredients. ESEGP is truly an international event in the cereal research calendar.

We are indebted to Karen Marie Jakobsen, Käthe Bundgaard, and Merete Yding for their hard work in coping with the administration of the meeting. Anette Henriksen (web-design and graphic design of ESEGP logo) and Pernille Seier (maintenance of the conference website) are cordially acknowledged.

Finally we are grateful to the Carlsberg Laboratory for generously hosting us all.

We wish you a stimulating and enjoyable symposium and stay in Copenhagen.

Monica and Birte
November 2011

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William Willats (KU-Life)
Karen Marie Jakobsen (DTU Systems Biology)

Program

Monday 28th November 2011

08:00 Registration desk opens and poster setup

Opening Session

09:00 Words of welcome by Jens Duus (Carlsberg)

Chair: Christine Finnie (DTU) & Alison Smith (JIC)

09:10 Hans-Peter Mock (IPK-Gatersleben, Germany)
Advanced techniques in mass spectrometry and their potential to provide novel insights into plant seed biology

10:00 Coffee

Session 1: Cereal breeding, GMO and quality
Chair: Henrik Brinch-Pedersen (Flakkebjerg) & Hans-Peter Mock (IPK)

10:40 Preben Bach Holm (Aarhus University, Flakkebjerg, Denmark)
Cereal breeding, GMO and quality

11:10 Vanja Tanackovic (KU-Life, Denmark)
***Starch bioengineering in* Brachypodium distachyon**

11:30 Kim Henrik Hebelstrup (Aarhus University, Flakkebjerg, Denmark)
Cereal bioengineering: amylopectin-free and hyper-phosphorylated barley starch

11:50 Lunch and poster viewing

Session 2: Tools to describe cereal systems and individual components
Chair: Andreas Blennow (KU-Life) & Peter Koehler (DFA)

14:00 Alison Smith (John Innes Centre, Norwich, UK)
Manipulating the synthesis and degradation of barley starch

14.30 Henrik Brinch-Pedersen (Aarhus University, Flakkebjerg, Denmark)
Unraveling the complement of cereal phytases: getting a handle on phosphate and minerals

15:00 Gabriel Paës (INRA, Reims, France)
Fluorescent probes as tools to investigate secondary plant cell wall architecture by FRAP in arabinoxylan gels

15:20 Rob Field (John Innes Centre, Norwich, UK)
The in vitro generation of starch-like surfaces on SPR chips for the analysis of starch-active enzymes

15:40 Coffee and Poster Session

Session 3: Enzyme structure / function relationships. Oxido-reductases, Lipases, Proteases
Chair: Maher Abou Hachem (DTU) & Barry McCleary (Megazyme)

16:30 Peter Koehler (Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany)
Detoxification of gluten by means of enzymatic treatment

17:00 Olof Björnberg (DTU Systems Biology, Lyngby, Denmark)
Dissecting molecular interactions involved in recognition of target disulfides by the barley thioredoxin system

17:20 Claus Krogh Madsen (Aarhus University, Flakkebjerg, Denmark)
The genetics of cereal mature grain phytase activity

17:40 Theresa Schwalb (Deutsche Forschungsanstalt für Lebensmittelchemie, Freising, Germany)
Gluten-specific peptidase activity of different cereal species and cultivars induced by germination

18:00 Close of session

Tuesday 29th November 2011

Session 3 (continued): Enzyme structure/function relationships. Carbohydrate-active enzymes

09:00 Bernhard Henrissat (AFMB, Marseille, France)
Exploring microbiomes for digestive carbohydrate-active enzymes

09:30 Gabrielle Potocki-Véronèse (INSA, Toulouse, France)
Functional metagenomics to get novel insights into plant polysaccharide breakdown by human gut bacteria

10:00 Barry McCleary (Megazyme International, Ireland)
Action of endo-1,4-beta-xylanase and endo-1,4-beta-glucanase on polysaccharides

10:20 Marie Sofie Møller (DTU Systems Biology, Lyngby, Denmark)
Barley limit dextrinase and its proteinaceous inhibitor – The complex structure

10:40 Coffee

Session 4: Binding domains, their nature, and potential functionality in grain processing

Chair: Anne Meyer (DTU) & Bernard Henrissat (AFMB)

11:10 Christophe Courtin (KU Leuven, Belgium)
Secondary binding sites - An efficient way of compensating for the lack of CBMs in single-module xylanases?

11:40 Morten Munch Nielsen and Jose Cuesta-Seijo (Carlsberg, Valby, Denmark)
Exploring the substrate specificity and processivity of three barley starch synthases (HvGBSSI, HvSSI and HvSSIIa)

12:00 Darrell Cockburn (DTU Systems Biology, Lyngby, Denmark)
Detection of surface binding sites in carbohydrate active enzymes

12:20 Jonas Willum Nielsen (University of Copenhagen, Department of Biology, Denmark)
Analysis of degradation of the starch component amylopectin by barley alpha-amylase 1

12:40 Photo session

13:00 Lunch and Poster Session

Session 5: Non-food applications in cereal-based industry. Feed, Bioenergy and Biorefinery

Chair: Jørn Dalgaard Mikkelsen (DTU) & Maija Tenkanen (Univ. Helsinki)

14:45 Dan Pettersson (Novozymes, Bagsværd, Denmark)
A new generation of enzymes for improving the nutritive value of cereal grains in animal feed

15:15 Guiseppe Dionisio (Aarhus University, Flakkebjerg, Denmark)
Cereal hydrolytic enzymes important for liquid feed application: screening and cloning of triticale enzymes for improving phosphorus and nitrogen bioavailability

15:35 Coffee

16:15 Jim Robertson (Institute of Food Research, Norwich, UK)
Integrated protocols for the sustainable recovery of natural constituents from cereal bran

16:35 Outi Santala (VTT, Finland)
Xylanase-aided modification of wheat bran at high and low water content

16:55 Davinia Salvachúa (CSIC, Madrid, Spain)
Exploitation of cereal processing sidestreams for bioethanol production: Improved pretreatment of wheat straw

17:15 Kenzi Clark (Institute of Food Research, Norwich, UK)
Functional characterization of metagenomic enzymes from the chicken cecal microbiome

17:35 End of session

17:45 Conference Dinner at Carlsberg Museum

Wednesday 30th November 2011

Chair: Preben Bach Holm (Flakkebjerg) & Craig Faulds (VTT)

Session 6: Application of enzymes in cereal-based industries. Baking, Brewing and Functional Foods

09:00 Stefan Kreisz (Carlsberg, Valby, Denmark)
Enzymes in brewing technology

09:30 Charlotte Poulsen (Danisco Genencor, Brabrand, Denmark)
The use of enzymes in baking applications

10:00 Karolien Decamps (KU Leuven, Belgium)
Pyranose oxidase improves dough stability in breadmaking

10:20 Maija Tenkanen (Univ. Helsinki, Finland)
Comprehensive characterisation of molar mass and solution properties of native and enzymatically tailored cereal arabino-xylans by HPSEC and AsFIFFF

10:40 Coffee and posters

Session 7: Innovative processes and design of cereals for the future

Chair: Monica Palcic (Carlsberg) & Christophe Courtin (KUL)

11:10 Jean-Guy Berrin (INRA, Marseilles, France)
Post-genomic analyses of fungi for the degradation of plant cell wall

11:40 Peter Falck (Lund University, Biotechnology, Sweden)
Hydrolysis of xylans by a thermostable family 10 xylanase from Rhodothermus marinus and fermentation of hydrolysis products by intestinal bacteria

12:00 Ernst Meinjohanns (Carlsberg, Valby, Denmark)
Profiling and "in-vivo"imaging" of barley proteases in germinating barley by activity based probes: Methods and application

12:20 Closing Speaker Alain Buléon (INRA, Nantes, France)
Enzymatic hydrolysis of concentrated raw starch and breakdown of crystalline structures

12:50 Presentation of Poster Prizes

Close of Symposium

13:00 Lunch

Opening Session

OP1

Advanced techniques in mass spectrometry and their potential to provide novel insights into plant seed biology

H.-P. MOCK

Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany

Within the last decade, application of mass spectrometry has tremendously increased in biological sciences. First mass spectra were obtained by J.J. Thomson already in 1912 and since then the underlying principle has been widely adopted in physics and chemistry. Matrix-assisted laser desorption and electro-spray ionization techniques were introduced in the nineties and acknowledged by the noble prize in 2002. Their development enabled and stimulated the analysis of larger biological molecules such as proteins. The continuous improvement of instrumentation allowed extending the field of comprehensive “omics”-techniques by metabolomics and proteomics. In the presentation, data from our studies on the barley seed proteome will be given. We have used gel-based as well as gel-free approaches to access the seed protein composition. The developmental changes of the seed proteome during seed maturation have been studied by a label-free LC-based approach and evaluation of the peptide data set has been assisted by a clustering algorithm prior to protein identification. Results from the proteome study on the developmental patterns of specific proteins have been confirmed by using enzyme assays and Western blotting. Next, we attempted to obtain spatial resolution in our analysis of the seed proteome. Laser micro-dissection has been applied to excise endosperm transfer cell tissue as well as of the nucellar projection. Samples were analysed by LC-MS of peptides resulting from tryptic digestions and peptide profiles were compared in a quantitative manner to demonstrate the specific proteome composition of each tissue. Most recently, MS-based imaging techniques have been introduced in plant biology to analyse the spatial distribution of metabolites and proteins. We will present our first data sets obtained by this novel approach in the analysis of developing barley seeds.

Session 1:

Cereal breeding, GMO and quality

Cereal breeding, GMO and quality

P.B. HOLM

Aarhus University, Denmark

Cereals are in a global and a European context highly significant for human as well as livestock nutrition and have recently also attracted interest as a source of biomass. Since their domestication in the Near East 10-12.000 years ago extensive breeding has been undertaken to meet the demands for high nutritional quality and brewing and baking quality. In the current presentation I will comment on cereal breeding and in particular focus on the potential of genetic modification for improving quality, its current limitations and possible solutions. Since this meeting is a European symposium I will restrict myself to barley and wheat.

Due to their economic and nutritional importance barley and wheat and in particular the barley and wheat grain are among the most heavily researched plant organs. The genetic basis for a range of baking, brewing and nutritional traits are today well known providing breeding with a large number of well-defined breeding targets. In particular barley has been the subject for extensive mutation breeding. A large number of transcriptomic, proteomic and metabolomic studies have been undertaken and although barley and wheat not yet have been fully sequenced there are very abundant resources in the form of molecular markers of various types, mapping populations, BAC libraries and TILLING populations. Barley and wheat breeding has today also significantly benefitted from tissue culture in the form of anther and microspore cultures from where double haploids can be generated. In consequence, two or more years may be saved in the breeding program for pure lines.

However, transformation provides the breeder with a number of options that cannot be met by conventional breeding. Transformation technologies based on biolistics were reported in the early nineties for wheat and barley. Since then Agrobacterium mediated transformation techniques have been implemented. They work in particular well for barley while biolistics still appears to be the most efficient transformation technology for wheat. The well characterized basis for a range of traits in the cereal grain, often enzymes encoded for by single genes, inspired at an early stage molecular researchers and breeders to generate transgenic plants with modulated expression of genes encoding enzyme or storage compounds or to introduce genes encoding enzymes with novel or modified properties such as heat stability. Although impressive results have been obtained with transformation it is on the other hand apparent that there is substantial room for improvement. It is increasingly necessary to get a better understanding of the regulatory networks of the cereal grain as well as the functional divergence between the individual cells of the major tissues of the grain. The transformation procedures also need to be improved and meet the demands of the skeptical public with respect to safety and usefulness. Novel strategies for meeting these concerns will be described.

OP3

Starch bioengineering in *Brachypodium distachyon*

V. TANACKOVIC[1], *J.T. SVENSSON*[1], *A. BULEON*[2], *M.A. GLARING*[3], *M. CARCIOFI*[4], *S. LANGGÅRD JENSEN*[1], *A BLENNOW*[1]

[1]*VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen;* [2]*INRA, UR1268 Biopolymeres Interactions Assemblages;* [3]*Department of Agriculture and Ecology, Faculty of Life Sciences, University of Copenhagen;* [4]*Department of Genetics and Biotechnology,Faculty of Agricultural Sciences, Aarhus University*

Brachypodium distachyon was recently introduced as a model plant for temperate cereals (Opanowicz et al. 2008; IBI 2010). In order to explore pre-domesticated and novel features of cereal starch metabolism we aim to establish Brachypodium as a model.

Bioinformatics analysis identified starch biosynthesis genes including seven soluble starch synthases (SS), two granule bound starch syntheses (GBSS), four starch branching enzymes (SBE), two glucan- and one phosphoglucan- water dikinases (GWD, PWD). Transit peptides and putative carbohydrate-binding modules (CBMs) of the families CBM20, CBM45, CBM48 and CBM53 were identified. The gene setup for starch biosynthesis is very similar to barley and a phylogenetic analysis based on the SS genes provided evidence for a close relation to barley and wheat.

To investigate the starch structural features, grain starch from two lines Bd21 and Bd21-3 were characterized. Microscopic, chemical and structural data including amylopectin chain length distribution, phosphate content, and amylose content of the starch granules provided evidence for a close structural relationship to temperate cereals. Close relationship can be observed even though kernel starch content and starch granule size were considerably lower and β-glucan content was much higher than that for barley (*Hordeum vulgare*). Small-angle and wide-angle X-ray scattering (SAXS and WAXS) show low crystallinity of Brachypodium starch granules as compared to barley. These data were confirmed by differential scanning calorimetry (DSC) data. Polarization microscopy indicated ordered chain arrangements only in the outer layer of the granules.

We are currently identifying mutants and biolistic *Agrobacterium*-mediated transformation directed towards silencing and performing overexpression of key starch biosynthesis genes aiming at providing evidence for differential or conserved actions of specific genes in this grass as compared to domesticated cereals.

Our data show that *Brachypodium distachyon* can provide a valuable and efficient model for starch bioengineering in temperate
cereals.

Opanowicz, M., Vain, P., Draper, J., Parker, D. and Doonan, J.H. (2008) Brachypodium distachyon: making hay with a wild grass. Trends Plant Sci. 13, 172-177.

The International Brachypodium Initiative (2010) Genome sequencing and analysis of the model grass Brachypodium distachyon. Nature 463, 763–768

OP4

Cereal bioengineering: amylopectin-free and hyper-phosphorylated barley starch

K.H. HEBELSTRUP[1], M. CARCIOFI[1], S.L. JENSEN[2], S.S. SHAIK[2], A. BLENNOW[2], J.T. SVENSSON[2], E VINCZE[1], A. HENRIKSEN[3], A. BULÉON[4], P.B. HOLM[1]

[1]*Dept. of Molecular Biology and Genetics, Aarhus University;* [2]*VKR Research Centre Pro-Active Plants, Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Copenhagen, Frederiksberg;* [3]*The Protein Chemistry Group, Carlsberg Laboratory, Denmark;* [4]*UR1268 Biopolymeres Interactions Assemblages, INRA, F-44300 Nantes, France*

Barley lines producing grains with either amylopectin-free or hyper-phosphorylated starches were engineered by transgenic methods.

Amylopectin-free barley was generated by simultaneously silencing the three genes encoding the starch branching enzymes (SBEIIa, SBEIIb and SBEI) by a chimeric hairpin. This construct was inherited as a single locus with a 3:1 segregation, which makes the method useful for breeding as compared to combing alleles of the three different SBE genes segregating independently. Transgenic grains were wrinkled. This is a phenocopy of Mendel's wrinkled peas, which were also based on a non-functional allele of an SBE gene. Amylopectin content was below detection level (< 1%) measured by both size exclusion chromatography (SEC) and differential scanning calorimetry (DSC). Scanning electron microscopy (SEM) showed that this amylopectin-free starch is organized into amorphic granules. Plants were propagated for three generations. Yield and grain phenotypes were determined in grains grown under semi-field conditions. Loss of SBE activity was compensated by the cereal grain by up-regulated gene expression of starch synthases. As an effect, yield loss is limited to 20%. This demonstrates for the first time a way for production of pure amylose in plants with limited yield loss.

In a different barley line endosperm specific overexpression of glucan water dikinase from potato (StGWD) was conducted. The content of phosphate esters in starch from consecutive generations (T0 and T1) of transgenic grains was tenfold higher than from vector control and wild type grains. Amylose content was not affected in hyperphosphorylated grains. Hyper-phosphorylated starch granules had several pores on the surfaces, similar to pores seen on enzymatically semi-degraded granules. This provides support for the presence of a general mechanism in starch degradation in the plant kingdom where phosphorylation carried out by ectopic expression of StGWD tags barley starch granules for degradation by endogenous enzymes.

Together this work shows two new strategies for in planta starch bioengineering of cereals. It demonstrates that bioengineering may be used to obtain novel and technologically interesting cereal starches, and to elucidate the complex pathways of starch biosynthesis and the roles of individual starch biosynthetic enzymes.

Session 2:

Tools to describe cereal systems and individual components

OP5

Manipulating the synthesis and degradation of barley starch

A. SMITH

John Innes Centre, Norwich, UK

I will present recent research on the roles of enzymes of starch degradation in germinating barley grains, and the exploitation of variation for starch synthesis in developing grains. We have applied complementary chemical-genetic and reverse-genetic approaches to understand the importance of α-glucosidase in endosperm starch metabolism during the first days of seedling growth. The enzyme converts maltose to glucose, but previous *in vitro* studies indicated that it can also attack starch granules. We identified iminosugar inhibitors of recombinant HvAGL97, an α-glucosidase previously identified from barley endosperm, and applied them to germinating grains. All inhibited conversion of maltose to glucose, but some also reduced starch degradation and seedling growth. Transgenic seedlings carrying an RNAi silencing cassette for *HvAgl97* also showed a strong inhibition of conversion of maltose to glucose, but were unaffected in starch degradation and seedling growth. These results indicate the presence of unknown glucosidase(s) necessary for starch degradation and seedling growth, and also raise the interesting possibility that iminosugars could be used to control starch loss and rootlet production during malting.

It is well known that barley genotypes with altered starch synthesis have unusual and potentially valuable grain properties. However exploitation is hindered by lack of understanding of the importance of genetic background in determining these properties. In collaboration with colleagues at NIAB, Cambridge, UK, we have transferred four different mutations affecting starch synthesis in a UK elite line of barley. Our analyses are shedding new light on the influence of genetic background on starch and grain properties.

OP6

Unraveling the complement of cereal phytases: getting a handle on phosphate and minerals

H. BRINCH-PEDERSEN, G. DIONISIO, C.K. MADSEN, P.B. HOLM

Aarhus University, Science and Technology, Dept. of Molecular Biology and Genetics, Research Centre Flakkebjerg, DK-4200 Slagelse, Denmark.

Phytases (myo-inositol hexakisphosphate phosphohydrolase) [EC 3.1.3.26 and EC 3.1.3.8] are phosphatases which can initiate the sequential hydrolysis of orthophosphate groups from phytate (InsP6, myo-inositol 1,2,3,4,5,6-hexakisphosphate) thus providing phosphate, inositol phosphates and inositol required for a range of cellular activities. Barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) possess a significant phytase activity in the mature grains. Maize (*Zea mays* L.) and rice (*Oryza sativa* L.) possess little or virtually no pre-formed phytase activity in the mature grain and depend fully on *de novo* synthesis during germination. Here it's demonstrated that wheat, barley, maize and rice all possess purple acid phosphatase genes which expressed in *Pichia pastoris* gives fully functional phytases (PAPhys) with very similar enzyme kinetics. Preformed wheat PAPhy was localized to the protein crystalloid of the aleurone vacuole. Phylogenetic analyses indicated that PAPhys possess four conserved domains unique to the PAPhys. In barley and wheat, the *PAPhy* genes can be grouped as *PAPhy_a* or *PAPhy_b* isogenes. In rice and maize only the "b" type (*OsPAPhy_b* and *ZmPAPhy_b*, respectively) were identified. HvPAPhy_a and HvPAPhy_b1/b2 share 86% and TaPAPhya1/a2 and TaPAPhyb1/b2 share up to 90% (TaPAPhy_a2 and TaPAPhy_b2) identical amino acid sequences. In spite of this, *PAPhy_a* and *PAPhy_b* isogenes are differentially expressed during grain development and germination. In wheat it was demonstrated that "a" and "b" isogene expression is driven by different promoters (~31% identity). *TaPAPhy_a/b* promoter reporter gene expression in transgenic grains and peptide mapping of TaPAPhy purified from wheat bran and germinating grains confirmed that the *PAPhy_a* isogene set present in wheat/barley but not in rice /maize are the origin of high phytase activity in mature grains.

OP7

Fluorescent probes as tools to investigate secondary plant cell wall architecture by FRAP in arabinoxylan gels

G. PAES, V. AGUIÉ, B CHABBERT

INRA, Riems, France

Controlled fractionation and upgrading of lignocellulosic biomass is limited by multiple factors related to their structural and functional characteristics, but also to their complexity. In order to investigate the lignocellulosic plant cell architecture, microscopic techniques are well suited, and different types of probes (antibodies, carbohydrate binding modules,...) have been designed to specifically bind to some components. They can be localised through immunolocalisation or fluorescence techniques. By using techniques such as fluorescence recovery after photobleaching (FRAP), confocal laser scanning microscopy can provide additional information on the mobility of these probes (diffusion and mobile/immobile fractions), which is crucial in applications such as grain processing where enzyme diffusion can be a limiting factor.
Therefore, we have devised a series of experiments in order to analyse the mobility of various size probes grafted with FITC as the fluorophore: dextrans (10, 70 and 250 kDa) and BSA as a model protein. These probes have been placed into secondary plant cell wall inspired hydrogels, which allow assaying the impact of various physiochemical and biochemical parameters on the probes diffusion. Gels are made of water extracted arabinoxylans (WEAX) which are cross-linked through ferulic acid moieties with the action of a laccase. WEAX gels have been prepared at different concentrations in order to modulate the gel mesh size, while probes are added directly into the gel before cross-linking was triggered and followed by rheological measurements. The gel systems have also been complexified by adding cellulose nanocrystals (CNCs) at different concentrations.
Diffusions measured for the different probes show great variations, depending both on the probe size and the gel concentration (mesh size). Together with rheological analysis, presence of the CNCs seem to make the gels more organized (with more compartments and less entanglements) and interestingly, CNCs have no impact on the probe diffusion but decrease mobile fractions.
Overall, FRAP technique allows rapid and reliable determination of probes diffusion in simple hydrogels and paves the way for future analysis of closer-related lignocellulosic systems which should bring more information on glycoside hydrolases diffusion in plant cell walls.

OP8

The *in vitro* generation of a starch-like surfaces on SPR chips for the analysis of starch-active enzymes

R. FIELD

John Innes Centre, Norwich, UK

The synthesis and degradation of starch granules involves the complex interaction ofmany enzymes. Starch biochemistry is complicated by the insoluble nature of the granule and the fact that the enzymes involved show both tissue and species variation. In this study we set out to develop starch-like surfaces and spectroscopic approaches that would enable the investigation of prospective starch-active enzymes.
Phosphorylases catalyse the reversible phosphorolysis of carbohydrates and are used extensively in metabolism. PHS2, the cytosolic α-1,4-glucan phosphorylase from Arabidopsis, was identified as a suitable candidate enzyme to synthesise amylose-like chains in vitro. In this study, crystal structures of the enzyme were obtained and the activity of the enzyme was determined in solution by phosphate release methods and through analysis of glycan chain extension by CE-LIF. In addition, with short glucans immobilised on a gold surface, extension and degradation of the surface was monitored in real time using surface plasmon resonance (SPR). These studies provide both substrates and methods with which to analyse the action of enzymes potentially involved in starch granule metabolism or of enzymes with applications in starch modification.

Session 3:

Enzyme structure/function relationships. Oxido-reductases, Lipases, Proteases, Carbohydrate-active enzymes

OP9

Detoxification of gluten by means of enzymatic treatment

P. KOEHLER, H. WIESER

German Research Center for Food Chemistry, Freising, Germany

Celiac disease (CD) is an inflammatory disease of the upper small intestine in genetically predisposed individuals caused by glutamine- and proline-rich peptides from cereal storage proteins (gluten) with a minimal length of nine amino acids. Such peptides are insufficiently degraded by gastrointestinal enzymes; they permeate into the lymphatic tissue, are bound to celiac-specific, antigen-presenting cells and stimulate intestinal T-cells. The typical clinical pattern is a flat small intestinal mucosa and malabsorption. At the moment the only therapy is a strict lifelong gluten free diet. Recent research has shown that gluten and gluten peptides can be degraded by bacterial, fungal and animal prolyl endopeptidases, by a combination of lactobacilli and fungal peptidases as well as by peptidase preparations from germinating cereals. These peptidases can be used to produce gluten-free foods from gluten-containing raw materials. In addition, preparations containing these enzymes have been suggested for an oral therapy of CD, in which dietary gluten is hydrolyzed by co-ingested peptidases already in the stomach thus preventing CD-specific immune reactions in the small intestine. This would be an alternative for CD patients to the gluten-free diet. Beside peptidases microbial transglutaminase could be used for the detoxification of gluten either by selectively modifying glutamine residues of intact gluten by transamidation with lysine ethyl ester or by crosslinking gluten peptides in beverages via isopeptide bonds so that they can be removed by filtration.

OP10

Dissecting molecular interactions involved in recognition of target disulfides by the barley thioredoxin system

O. BJÖRNBERG[1], *P. HÄGGLUND*[1], *K. MAEDA*[1, 2], *B. SVENSSON*[1]

[1]*Department of Systems Biology, Technical University of Denmark;* [2]*EMBL, Heidelberg, Germany*

Structural disulfides in inhibitors can be reduced and thereby inactivated by thioredoxin. In a structure in complex with barley α-amylase/subtilisin inhibitor (BASI), two loops in barley thioredoxin h2, containing a conserved cis-proline and glycine residue, respectively, surround the active site cysteines, and seem to contribute to binding [Maeda, K., Hägglund, P., Finnie, C., Svensson, B., and Henriksen, A. (2006) Structure 14, 1701]. In the present study, nine mutants were investigated to evaluate the significance of backbone hydrogen bonds and van derWaals contacts from the two loops. M88 precedes the completely conserved cis-proline and exchange to smaller residues (M88G and M88A) strongly decreased the rate of BASI disulfide reduction as measured by NADPH oxidation via thioredoxin reductase. Replacement by proline, removing the possibility to donate a hydrogen bond from the main chain amide extinguished activity but the mutant was deficient with respect to reactivity of its active site dithiol/disulfide. Also in the cis-proline loop, the mutant E86R, displayed a three-fold increase in activity. A106 is just C-terminal to the conserved glycine residue. Mutant A106P, which lacks possibility to donate a hydrogen bond from the main chain amide showed only 10% residual activity but was fully active with respect to recycling by thioredoxin reductase. Mutants were also analysed with respect to reduction of the protein disulfides in glutathione peroxidase and insulin. The results point at an important role of main chain interactions in target recognition.

OP11

The genetics of cereal mature grain phytase activity

C.K. MADSEN, G. DIONISIO, I.B. HOLME, P.B. HOLM, H. BRINCH-PEDERSEN

Aarhus University, Department of Molecular Biology and Genetics, Research Centre Flakkebjerg

A high mature grain phytase activity (MGPA) in cereals promotes the sustainability of animal intensive agriculture and reduces the need for phosphate supplements. Furthermore it may help counter some forms of human malnutrition such as zinc deficiency which affects millions

Cereal species show great variation in MGPA from <100 FTU/kg for rice, maize and oat, to ~5000 FTU/kg for rye. However, all cereals with more than 100 FTU/kg belong to the Triticeae. Cultivars of the same species also show variation. In wheat we have found a 5-fold variation, in rye two fold but so far we have found little variation in barley. Understanding the molecular basis for this variation would be valuable for the effort to increase MGPA by breeding or cisgenic approaches.

Two families of phytases are known in cereals, the Multiple Inositol Phosphatases (MINPP's) and the Purple Acid Phosphatase Phytases (PAPhys). Each family is represented by potentially 1-5 loci in diploid cereal genomes. It can be demonstrated biochemically that the PAPhy's are responsible for the high MGPA of the Triticeae. The PAPhys are highly conserved at the mature protein level and variation in specific activity of enzymes characterized thus far is small and do not correlate with the variation of the phytase activity in grains when different species are compared. Hence we propose that the variation in phytase activity between species and cultivars are mainly attributable to gene regulation. To investigate this hypothesis, we have used genomic library screening, PCR and IPCR to isolate phytase genes from wheat, barley, rye and wild / relict relatives. The promoters were analyzed for known regulatory elements and compared. We have demonstrated that the PAPhy genes are regulated by two distinctly different types of promoters that are active primarily during grain filling and germination, respectively. Cross reference with the sequenced genomes of rice, maize and sorghum showed that the PAPhy_a gene which is active during grain filling is unique to the Triticeae. We can therefore ascribe the high MGPA of the Triticeae to this one gene. Furthermore, we have identified variants of the PAPhy_a gene that are either inactive or correlates with higher MGPA.

Gluten-specific peptidase activity of different cereal species and cultivars induced by germination

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In the last years a number of bacterial and fungal peptidases has been discovered, which are capable of detoxifying gluten-containing foods and raw materials. Our previous studies have shown that gluten-specific peptidase activity can also be induced by germinating cereal grains, and enzymatic activity is primarily enriched in the bran. However it is not yet known how gluten-specific peptidase activity is affected by cereal species and cultivars. Therefore, the goal of this research was to study the peptidase activity of different germinated cereals by using gliadin as a protein-based substrate as well as the coeliac-toxic peptide PQPQLPYPQPQLPY. Grains of spelt (cv. Oberkulmer Rotkorn and Franckenkorn), einkorn (cv. FR7037 and UH36582), emmer (cv. Osiris and Ramses), barley (cv. Conchita and Marthe), oats (cv. Ivory and Scorpion), maize (cv. Grosso and Ricardinio), rye (cv. Conduct and Guttino) and common wheat (cv. Winnetou and Hermann) were germinated for seven days at 15 and 25 °C, lyophilized and milled into flour and bran. The bran was extracted with a sodium acetate buffer (0.2 mol/L, pH 4.0). The resulting extract was incubated with gliadin (50 °C, pH 4.0 and 6.5, 2.5 h) or with the celiac-toxic peptide PQPQLPYPQPQLPY (50°C, pH 4.0 and 6.5, 1 h) as substrates. The reactions were stopped by heating to 90°C for ten minutes. Gliadin or peptide degradation was quantified by RP-HPLC. Residual peak areas of the starting materials after incubation were recorded at 210 nm.

The experiments showed that some of the brans were capable of degrading both gliadin and the celiac-toxic peptide extensively. For example, bran of emmer cv. Orisis germinated at 25 °C degraded 66.8 % of the gliadin substrate after incubation at pH6.5. Also very high degradation rates were obtained for the brans of emmer cv. Osiris germinated at 25 °C (66.1 %), einkorn cv. UH36582 germinated at 15 °C (55.3 %) and barley cv. Marthe germinated at 25 °C (51.7 %). The peptide PQPQLPYPQPQLPY was also hydrolyzed quite efficiently. The highest degradation rates were 72.3 % (wheat cv. Hermann), 77.2 % (emmer cv. Ramses) and 78.6 to 88.6 % (emmer cv. Osiris). In general, both assays for measuring gluten-specific peptidase activity of bran from germinated grains could be performed relatively simple and generated reproducible results.

OP13

Exploring microbiomes for digestive carbohydrate-active enzymes

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While microbiology has long focused on isolated and cultivated microbes, it is now well established that certain environments like the human gut are inhabited by a community of thousands of microbial species that collectively enable many functions that are not encoded by the host's genome. For instance the human genome does not encode any enzyme for the breakdown of the multitude of plant cell wall polysaccharides, and this breakdown is entirely catalyzed by our digestive flora. Traditionally bacterial communities were studied by sequencing 16S rRNAs from the community in order to infer the taxonomical distribution of the organisms within the community. With the rapid drop in sequencing costs, it is now possible to sample large amounts of the community DNA, and this provides an unprecedented view of the functional properties of the community. The sheer volume of data that are needed to correctly sample such communities for functional profiling is a difficulty that can be overcome with massively parallel computer clusters or grids. However, in the case of carbohydrate-active enzymes, the problem is made even more difficult due to the poor relationship between sequence and function (or specificity). We will present how we have adapted the Carbohydrate-active enzymes database pipeline to carry out the search of carbohydrate-active enzymes in large metagenomic datasets and we will describe several of our latest results in the investigation of human and animal digestive microbiomes.

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OP14

Functional metagenomics to get novel insights into plant polysaccharide breakdown by human gut bacteria

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The human gut hosts a complex bacterial community that plays a major role in nutrition and in maintaining human health. Intestinal bacteria, of which most have never been cultured, produce a battery of carbohydrate active enzymes (CAZymes) that are very effective in degrading dietary fibers, these plant polysaccharides being resistant to the action of the human digestive enzymes. To explore the diversity of these microbial CAZymes, a multi-step function-based approach was developed to guide the in-depth pyrosequencing of specific regions of the human gut metagenome that encode the enzymes involved in dietary fiber breakdown. From few mega-bases of metagenomic DNA, this strategy allowed identification of dozens of novel CAZymes involved in cellulose, pectin, hemicellulose, galactan and starch breakdown. Very original associations of biocatalysts acting synergistically to deconstruct plant cell walls were also revealed. The results gave new insights on the way that gut microorganisms evolve to adapt their gene machinery to the enzymatic degradation of complex carbohydrate structures. They also highlighted the power of this generic strategy for exploring the functional diversity encoded by metagenomes, and for the discovery of novel biocatalysts of interest for plant polysaccharide processing.

OP15

Action of endo-1,4-beta-xylanase and endo-1,4-beta glucanase on polysaccharides

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Endo-1,4-beta-Xylanase (xylanase) and endo-1,4-beta-glucanase (cellulase) enzymes vary in their ability to hydrolyse branched xylans or 1,4-beta-glucans. The action of 10 different xylanases (range of Families) and 5 different cellulases on substituted and mixed linkage polysaccharides has been studied with an aim of obtaining information on the subsite binding requirements of the enzymes. Wheat and rye endosperm arabinoxylans are highly substituted and thus subject to just limited hydrolysis by xylanases. Controlled acid hydrolysis of wheat arabinoxylan has been performed to reduce the arabinose content from 39% to 22%. This modified arabinoxylan is very susceptible to xylanase hydrolysis with the release of a range of arabinose containing xylosaccharides. Some of these have been characterised.

OP16

Barley limit dextrinase and its proteinaceous inhibitor – The complex structure

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Starch is the most abundant storage carbohydrate in cereal grains. It is composed primarily of amylopectin, a branched polymer of glucose in which α-1,4-linked glucan chains are branched with α-1,6-bonds. Enzymatic mobilisation of starch in germinating barley seeds involves an initial solubilisation, mainly by α-amylase, 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. Among these enzymes only LD is able to cleave off α-1,6-linked branches in α- and β-limit dextrins. In germinating seeds, LD occurs in a free active form and as a bound inactive form, possibly in complex with a proteinaceous inhibitor; limit dextrinase inhibitor (LDI) [1, 2]. Due to the presence of LDI, LD in malt is ineffective resulting in significant amounts of unfermentable branched dextrins in wort and beer, which in turn cause lower yield of alcohol. LDI, therefore, has important implications in the malting and brewing industries [3]. Recently, successful recombinant production of functional barley LD and LDI was established in *Pichia pastoris* [4, 5], which made it possible to characterise the two proteins and their interaction in detail. Before recombinant production of LD and LDI being available only detailed insight into the LD:LDI interaction was that the proteins form a 1:1 complex as determined by electrospray time-of-flight mass spectrometry [6]. The stoichiometry was now confirmed by inhibition assays and moreover for the first time it has been possible to determine the dissociation constant (K_D) of the LD:LDI complex which by using surface plasmon resonance was found to be 40 pM at pH 6.0, 150 mM with a k_{off} of 6•10-5 sec-1. Recently, we solved the crystal structure of the LD:LDI complex to a resolution of 2.7 Å. This achievement will enable detailed analysis of the key structural elements in the protein-protein interactions by aid of site-directed mutagenesis.

This work was supported by The Carlsberg Foundation, The Research Council for Natural Science, DTU PhD stipends (to MVBC and MSM) and an Oticon foundation MSc scholarship (to JMJ).

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Session 4:

Binding domains, their nature, and potential functionality in grain processing

OP17

Secondary binding sites - An efficient way of compensating for the lack of CBMs in single-module xylanases?

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Apart from non-catalytic binding to independent carbohydrate-binding modules, various structural studies have revealed that non-catalytic carbohydrate binding sites can also occur on the surface of the catalytic module of glycoside hydrolases. Such sites are referred to as secondary substrate binding sites (SBSs). In contrast to CBMs, which are often attached to the catalytic module through flexible linkers, SBSs mostly have a fixed position relative to the catalytic site. However, SBSs have been linked to a wide variety of putative functions that have also been attributed to CBMs in modular enzymes. These include (1) enzyme targeting towards its substrate and keeping it in the proximity of its substrate, (2) assisting catalysis by loading substrates into the active site groove, (3) disruption of the natural structure of substrates to facilitate catalysis, (4) keeping a substrate chain in contact with the enzyme for subsequent reactions (processivity), (5) allosteric activation of the enzyme, (6) retention and passing on of reaction products for further processing by other enzymes or for import in the parent microorganism and (7) anchoring of the glycoside hydrolase to the cell wall of the parent microorganism. Also in xylanases, SBSs have been observed in crystal structures of several single module enzymes. To elucidate the functional significance of SBSs for GH11 *Bacillus subtilis*, GH11 *Aspergillus niger* and GH8 *Pseudoalteromonas haloplanktis* xylanases, enzymes with a modified SBS were created. Characterization of these mutant enzymes suggests that the SBS in the GH11 xylanases has a different role than in the GH8 xylanase. In spite of the SBSs in the GH11 xylanases being located in different regions on the enzyme surface and their totally different architecture, a similar functionality was demonstrated for these sites. Activity and binding affinity towards polymeric substrates were significantly decreased upon weakening of their SBS. The SBS was suggested to play a role in targeting the xylanase towards its substrate but also in assisting substrate hydrolysis by guiding substrate into the active site groove. For the GH8 xylanase, binding properties were also significantly affected by modifying the SBS. However, in contrast to GH11 xylanases, the SBS only impacted the xylanase activity towards insoluble substrates, whereas the activity towards soluble substrates remained unaffected. Analogous to many CBMs, this SBS probably has a role in targeting the enzyme towards insoluble substrate surfaces. Although the exact biological function of SBS in xylanases is yet to be unraveled, the obtained results suggest that SBS modification can be a helpful tool to alter their functional properties.

OP18

Exploring the substrate specificity and processivity of three barley starch synthases (HvGBSSI, HvSSI and HvSSIIa)

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Starch is the main polysaccharide in which carbon and energy are stored in plants. It is a principal source of energy in human nutrition and used in several industrial processes including papermaking and first generation bioethanol. Starch is an insoluble glucan composed of two polymers of glucose, the highly-branched amylopectin (α-1,4-glycosidic linked with α-1,6 branch points) and the linear amylase (α-1,4-glycosidic linked). In higher plants starch is synthesised in plastids of both photosynthetic (leafs) and nonphotosynthetic cells (e.g. stems, roots, seeds and tubers). Starch synthesis in cereal grains is complex and involves several carbohydrate active enzymes including starch synthases (ADP-glucose: [1–>4]-α-D-glucan 4-α-D-glucosyltransferase; EC no. 2.4.1.21, GT5), branching and debranching enzymes. Five gene classes of starch synthases are encoded in barley (granule-bound starch synthase (HvGBSSI), starch synthase I (HvSSI), II (HvSSIIa), III, and IV) and all catalyse the transfer of a glucosyl moiety to an existing α-glucan chain. The overall aim of the project is to clone, express, purify and characterise each gene class of starch synthases to understand their contribution to the synthesis of the starch granule, which still is unclear. HvGBSSI being the exception is exclusively linked to amylose synthesis. Several natural cultivars of barley containing mutations in their waxy loci (which encodes HvGBSSI) produce starch granules with very low amylose content[1]. The genes of HvGBSSI, HvSSI, and HvSSIIa were expressed in *Escherichia coli* and purified to homogeneity by IMAC and gel filtration. Activity was determined using a continuous enzyme-linked glycosyltransferase assay[2]. The acceptor and donor substrate specificity were screened using a selection of different maltooligosaccharides, UDPGlc, and ADP-Glc. Fluorescently labelled maltooligosaccharides were also tested as acceptor substrates for starch synthases. The fluorescent dye facilitates analysis of the maltooligosaccharide product distribution by MALDI-TOF MS and capillary electrophoresis with a fluorescence detector. Furthermore the maltooligosaccharide products profiles will assist determination of the level of processivity by barley starch synthases.

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OP19

Detection of surface binding sites in carbohydrate active enzymes

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Carbohydrate active enzymes face a number of challenges when acting upon polymeric substrates. These can include localization to the correct substrate in a complex environment (e.g. a plant cell wall), isolating a single strand of the polymer and in the case of processive enzymes, translating along that strand. Some of these challenges can be met by accessory domains such as carbohydrate binding modules (CBMs). While common, these modules are not universal, with many enzymes that are active on polymers lacking such aids to catalysis.

There are several examples of surface binding sites that have been found on catalytic domain, distant from the active site. This includes several α-amylases (1-2), xylanases (3-4) and an agarase (5). It is currently unknown how widespread these surface binding sites are, but they may play an important and underappreciated role in the degradation of polymeric carbohydrates.

In the current study a screen of a wide variety of carbohydrate active enzymes is being undertaken to determine the ubiquity of these surface sites and their importance for substrate binding and catalytic activity. The study encompasses enzymes from a number of families among the glycoside hydrolases, carbohydrate esterases and polysaccharide lyases. Most of the enzymes chosen for the study are active on polysaccharides and lack a CBM.

Several techniques are being used to detect these surface binding sites. This includes affinity electrophoresis, carbohydrate microarrays and surface Plasmon resonance. The utility of these techniques in detecting surface binding sites is being validated using an enzyme known to contain a surface site, the α-amylase Amy1 from barley. Binding has been detected in several other enzymes, suggesting that surface binding sites are relatively common. Screening continues and the next step will be to determine the location of the binding sites and study them through mutagenesis, which should provide insight into their role in carbohydrate catalysis.

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OP20

Analysis of degradation of the starch component amylopectin by barley alpha-amylase 1

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Barley alpha-amylase isozyme 1 (AMY1) is an alpha–1,4 endo-glycoside hydrolase involved in starch depolymerization. Recent studies point at two distinct binding sites (SBS1 and SBS2) with high affinity for starch granules (1,2). Although fundamental to the understanding of the mechanism of AMY1, the role of SBS1 and SBS2 in degradation of the starch components, amylose and amylopectin, is not yet fully understood. In order to address this matter, we have focused on the depolymerization of amylopectin, amylose and beta-limit dextrin, and since beta-cyclodextrin has been shown to bind to both SBS1 and SBS2, the inhibition by beta-cyclodextrin. Progress curves revealed a bi-exponential depolymerization of amylopectin and beta-limit dextrin in contrast to the mono-exponential depolymerization of amylose. The biexponential progress curve divided the substrate into two fractions of approximately 60 and 40% that were degraded with a relatively slow and a fast reaction rate, respectively. The presence of beta-cyclodextrin inhibited the fast rate of the biexponential progress curve of amylopectin and beta-limit dextrin depolymerization, whereas the depolymerization of amylose was not affected. These results point to a role for either SBS1, SBS2, or both as an important binding site in depolymerization of branched starch components. The results have led us to suggest a putative mechanism of allosteric control of AMY1 activity by small oligo-saccharides during the depolymerization of branched substrates.

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Session 5:

Non-food applications in cereal-based industry. Feed, Bioenergy and Biorefinery

OP21

A new generation of enzymes for improving the nutritive value of cereal grains in animal feed

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Enzymes have been used in the animal feed industry on a regular basis since the beginning of the nineties. Enzymes for improving the nutritive value of barley were the first to be studied with data going back to the late fifties[1,2]. Enzymes for improving the filtration of barley wort were used in broiler diets based on barley since it was hypothesised that the same polysaccharides that were causing filtration problems in brewing also were responsible for the poor performance of barley fed broilers. Today we know that it was the mixed linked (1-3),(1-4)-ß-D-glucans (or simply ß-glucans) present in the barley cell walls that were responsible for filtration problems in brewing as well as the detrimental effects on broiler performance. As feeding standards changed and antibiotics slowly were phased out wheat became important as a feed cereal, replacing the fibre rich barley and allowing the formulation of more energy dense diets suitable for the new breeds of fast growing broiler chickens. As a response to this development fibre degrading enzymes targeting the arabinoxylans of wheat became of interest and xylanases gained focus and finally became more important than ß-glucanases in broiler production. Environmental concerns in The Netherlands regarding water eutrophication from phosphorus pollution triggered the marketing of phytases around 1995 with a tremendous world wide sales increase after the year 2000 due to the meat and bone meal ban and, in recent years, a trend for increasing prices on feed phosphate (a limited resource). However, the effects of phytase had been studied in a broiler trial already in 1971[3]. The latest commercial feed enzyme on the market is protease. Proteases were originally developed for the detergent industry and their pH optima were therefore highly alkaline. As a consequence their resistance to low pH values were quite poor, but today there is at least one protease on the market that we know does not denature when encountering the pH conditions of the stomach. These are the major feed enzyme preparations on the market while there also exists some minor products assumed to target the oligosaccharides and dietary fibre fraction of vegetable protein sources (oil seeds and pulses) although their efficacy regularly is up for discussions in the industry. All enzymes used in the feed industry so far are hydrolases and in addition there are discussions in academia and industry regarding the possible use of microbial amylases and lipases, also belonging to the hydrolase class of enzymes. We can anticipate that this also will be the case in the near future. However, current research in the bio-fuel industry, first and second generation ethanol production, has launched a huge screening effort for finding significantly improved hydrolases: amylases, xylanases, cellulases and auxiliary enzymes for improving degradation. These developments have generated a spin-off of new enzymes that also may be suitable for use in feed and food applications. In the animal feed industry there is also an ongoing quest for finding new phytases capable of an improved release of phytate bound phosphorus. Phytase application is also finding its way to the food and alcoholic beverage industries.

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OP22

Cereal hydrolytic enzymes important for liquid feed application: screening and cloning of triticale enzymes for improving phosphorus and nitrogen bioavailability

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The grains from a series of spring and winter triticale cultivars were biochemically screened for phytase, protease and xylanase activities. Triticale cultivars possessing the highest protease, xylanase or phytase activity were selected. Since the triticale genome has not been sequenced and the rye genome is only partly known, candidate gene sequences for the enzymes were selected mainly based on barley sequences. Primers for cloning of candidate genes and for semi-quantitative RT-PCR were designed. The main proteases expressed during grain development were found to belong to the cysteine proteases (gliadains) and the aspartic proteases (phytepsins). The protease are currently being expressed in *Pichia pastoris* for biochemical characterization and evaluation in liquid feed. For xylanases, only class one (with CBM present) was found to be expressed during grain development, and only to a very limited level. For the triticale phytases, their expression resembled what has already been seen in wheat and barley, with one isoform expressed mainly during grain development and one isoform expressed mainly during grain germination (Dionisio etal., 2011).

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OP23

Integrated protocols for the sustainable recovery of natural constituents from cereal bran

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The Joint EU - India Collaborative project, NAMASTE, has the objective to identify and introduce innovative and industry-relevant approaches for the valorization of fruit and cereal wastes and by-products. This will be achieved through an holistic conversion into functional and health-benefiting beverages, foods and feeds by means of environmentally and economically sustainable protocols and technologies attractive to the food industry. Recent approaches with cereal bran have involved the use of processing technologies to fractionate and recovery potentially high value components such as residual starch and protein, along with bioactive components such as dietary fibre (DF) natural antioxidants and functional compounds, like oligosaccharides with possible prebiotic activity.

Integrated protocols have been developed for the enzymatic extraction of wheat bran components and the production of a DF concentrate. The protocols use food-grade enzyme preparations. A water soluble, maltose / peptide rich fraction, (~55% of the original bran) has potential for use as a fish feed. The water-insoluble residue(45% of the original bran) represents a dietary fibre concentrate and a substrate suitable for the enzymatic release of water soluble fibre-based ingredients with potential prebiotic activity. Enzymatic treatments used so far can release approximately 25% of the fibre concentrate mass as water soluble polysaccharides and oligomers. The residual enzyme-recalcitrant residue remains available for further processing and this is the subject of further physicochemical treatments to enhance its enzymatic digestibility.

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OP24

Xylanase-aided modification of wheat bran at high and low water content

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Enzymatic reactions are typically conducted at high water content, but enzymatic treatment at reduced water content could be economically beneficial, especially when targeting dry end products. We have previously demonstrated that enzyme-aided solubilisation of bran arabinoxylan (AX) can be efficiently performed even at low water content (40%). The aim of the current study was to elucidate the impacts of high (90%) and low (40%) water content xylanase-aided treatment on the chemical, structural and physicochemical characteristics of wheat bran and its main biopolymers.

Analysis of monosaccharide composition showed that during both treatments, the A/X ratio of water extractable AX (WEAX) of bran decreased with increasing WEAX content. Sequential extraction and SDS-PAGE analysis of salt-extractable and SDS+DTT extractable proteins showed that the solubility of bran proteins decreased especially during the treatment at 40% water content, and protein aggregation was detected in the SDS+DTT-extractable fraction. The bran treated at 40% was characterised by smaller average particle size and more degraded microstructure than the bran treated at 90% water content. This indicates the impact of the high shear forces at the 40% treatment, enhanced by the compact texture of the material. Also the physicochemical properties of bran were affected, as the water retention capacity (determined for the freeze dried sample) was lower after treatment at 40% than at 90% water content. The study showed that water content of bran processing can have a significant impact on bran proteins and physicochemical characteristics of bran.

OP25

Exploitation of cereal processing sidestreams for bioethanol production: Improved pretreatment of wheat straw

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Wheat straw, the most abundant agricultural sidestream in Europe and the second in the worldwide after rice straw (Kim and Dale, 2004), is a very cheap animal feed but is also becoming an attractive renewable resource within a biorefinery concept, especially for the production of bioethanol. Utilisation of this cereal processing sidestream is hindered due to the high costs associated with the need of pretreating the material to disrupt the lignocellulose matrix to improve the accessibility of hydrolytic enzymes to cellulose and hemicellulose. Currently, acidic steam explosion is the most effective pretreatment for this purpose. Nevertheless, this approach is expensive, requiring high pressures and temperatures. However, it also generates byproducts that adversely affect both enzyme performance and downstream processing (Jurado et al., 2009). In this work, the potential of combining a fungal pretreatment with a mild alkali treatment as a possible alternative to steam explosion, has been investigated. Twenty one strains of basidiomycetes from the CIB culture collection were tested for their ability to grow on wheat straw under solid state fermentation (SSF) conditions. Changes in substrate composition, enzyme secretion, hydrolysis efficiency and ethanol production yield after 7, 14 and 21 days of each biopretreatment were evaluated. Most fungi degraded the lignin component within the wheat straw with variable degrees of selectivity, although only eight of them improved sugar recovery compared to untreated samples. Glucose yields after 21 days of pretreatment with *Poria subvermispora* and *Irpex lacteus* reached 69% and 66% of cellulose available in the initial straw, respectively, with an ethanol yield of 62% in both cases. Conversions from glucose to ethanol reached around 90%, showing that no inhibitors were generated during this pretreatment. Our results are still far from the high fermentation yields to ethanol obtained used combined steam explosion and alkaline peroxide pretreatments (Chen et al., 2008) but the results show that fungal pretreatments used in this study can replace certain current chemical processes1 (Chen et al., 2007), resulting in similar or even better ethanol yields. Moreso, our results demonstrate the highest fermentable sugars yield from wheat straw after enzymatic hydrolysis reported so far (Salvachua et al.,2011). The use of the enzyme and oxidative systems generated by fungi such as *I. lacteus* and *P. subvermispora* growing on biomass coupled with mild alkali pretreatments has the potential to be a powerful tool in the processing of many cereal sidestreams leading to the development of new value chains for the cereal processing industry.

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OP26

Functional characterization of metagenomic enzymes from the chicken cecal microbiome

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A variety of commercially available enzymes (β-glucanases, xylanases, and phytases) and enzyme mixtures are added to grain-based poultry feed in order to improve nutrient absorption, feed conversion, bird performance, litter and manure quality, and poultry house hygiene. The efficacy of these enzymes relies on their activity within the gastrointestinal tract of the birds, where they release phosphorous from phytate (phytases) or break down polysaccharides with anti-nutritive effects (xylanases and glucanases). In order to identify enzymes with improved performance in the gastrointestinal environment, we have used a functional metagenomic approach targeted towards symbiotic bacteria that naturally inhabit the gastrointestinal tract of chickens. It is expected that enzymes produced by these bacteria would be better candidates for poultry feed supplementation, as they are perfectly adapted to the intestinal milieu, and are likely to be well tolerated by the birds.

Two metagenomic fosmid libraries were prepared (a total of 20,000 clones) containing DNA from the chicken cecal microbiome. A fosmid vector system was used for construction of the libraries, allowing for putative expression of genes and operons needed to break down the substrates and for a reasonable inference to the phylogenetic origins of the inserts. Next, the libraries were screened for clones producing enzymes with relevance to poultry nutrition. β-glucanase, lipase, and xylanase activities were detected using agar plates containing the substrates lichenin, tributyrin, and arabinoxylan, respectively. The screen for phytases included an initial agar plate screen for phosphatase activity with X-phosphate (5-bromo-4-chloro-3-indolyl phosphate) followed by a colorimetric phytase assay measuring released inorganic phosphate from phytic acid. Using this approach, we were able to identify 36 genetically stable metagenomic clones producing lipase, β-glucanase, phytase, and xylanase activity. The fosmid inserts were sequenced, assembled, and annotated to identify the gene responsible for enzyme activity, as well as other genes that contribute to the utilization of the substrate and the metabolic potential of the chicken cecal microbiome. A selected set of genes, 3 phytases, 2 β-glucanases (GH16), and a xylanase (GH10), have been PCR amplified from 6 metagenomic fosmid inserts and cloned into the pOPINF expression vector for heterologous expression in *Escherichia coli*. Purification and enzymatic characterization is underway. We aim to demonstrate that enzymes of gut bacteria, due to their unique adaptation to the intestinal environment, have advantageous functional characteristics when compared to enzymes that are currently used in poultry feed supplementation.

Session 6:

Application of enzymes in cereal-based industries. Baking, Brewing and Functional Foods

OP27

Enzymes in brewing technology

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Brewing is one of the oldest food processing technologies existing. Beer has a long tradition and has been part of the human diet for more than 9000 years. Invented mainly to safeguard water and store cereals in a “different” way it soon became a very special product, not only because of the alcohol content but also because the business itself is an integrated part of our social life, first as craftsmanship, and later as an industry. From the food chemistry point of view, brewing can be described as processing and fermenting cereals (and hops) in a cascade of enzymatic processes. Single steps like malting or mashing were empirically designed to support enzymatic reactions long before enzymes had been described. All main production steps are dedicated to the main three enzymatic processes in brewing called proteolysis, cytolysis and amylolysis referring to the substrate used which are proteins, cell wall polysaccharides and finally starch. The paper will give an overview how the endogenous enzymes from barley malt, as well as modern exogenous enzymes, are used in brewing, and how enzyme research can lead to innovations in the brewing and beverage industry.

OP28

The use of enzymes in baking applications

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The technical benefits of using exogenous enzymes in baking applications will be presented. With a starting point in the wheat substrate the different classes of enzymes (amylases, xylanases, lipases, oxidative enzymes, proteases) relevant for baking will then be reviewed. Protein engineering work on the development of a new amylase for antistaling will be dealt with in more detail. Finally some health and nutrition aspects of using xylanases in whole grain baking will be shown.

OP29

Pyranose oxidase improves dough stability in breadmaking

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The use of glucose oxidase (GO) can improve processing or end product quality of a variety of food stuffs including bread. Knowledge on the functionality of pyranose oxidase (P2O), an enzyme with a similar reaction mechanism as GO in breadmaking, is, however, not available. While GO oxidizes C1 of s-D-glucose in the presence of oxygen with the production of H_2O_2, P2O acts on C2 of α- as well as s-D-glucose and on other sugars. Kinetic analysis of representatives of both enzyme classes showed that P2O (*Trametes multicolor*) has a higher affinity towards glucose and O2 than GO (*Aspergillus niger*). Furthermore, for the same activity of P2O and GO on glucose, the in vitro oxidation of ferulic acid and thiol groups was more efficiently catalyzed by P2O than by GO. Supplementation of P2O or GO to dough clearly had an impact on dough handling and rheology, probably mediated through the production of H_2O_2. However, neither P2O nor GO significantly affected the volume of straight dough bread produced with fermentation and proofing times of 90 and 36 min, respectively at dosages below 1.00 nkat/g flour. Supplementation with higher enzyme levels (1.00 nkat/g flour) significantly decreased bread loaf volume due to too high dough strength. When a breadmaking procedure with reduced fermentation time (20 min), extended proofing time (56 min) and an additional proofed dough handling step, imitating the mechanical stress to which dough is subjected in industrial practice, was used, inclusion of different enzyme levels in the dough recipe substantially increased the stability of the proofed doughs. This resulted in less collapse and higher bread loaf volumes. These results show that, in industrial large-scale breadmaking, P2O, similar to GO, can be a very valuable enzyme.

OP30

Comprehensive characterisation of molar mass and solution properties of native and enzymatically tailored cereal arabinoxylans by HPSEC and AsFlFFF

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Arabinoxylans are the main hemicellulose component in cell walls of monocots such as cereals and grasses consisting of the (1–>4)-beta-D-linked xylopyranosyl backbone with alpha-L-arabinofuranosyl substituents attached at positions O-2, O-3 or both. The arabinose-to-xylose ratio as well as distribution of arabinose along the xylan chain varies between plant species and plant tissues. The role of arabinoxylans as a health-promoting dietary fibre component is well-known and has been widely studied. However, the physico-chemical characteristics of structurally variable arabinoxylans differ, and need to be studied thoroughly in order to better understand their structure-function properties.

The molecular characteristics and solution properties of several structurally different arabinoxylans were thoroughly analysed applying two multidetector techniques, high-performance size-exclusion chromatography (HPSEC) in aqueous and DMSO solutions, and asymmetric flow field-flow fractionation (AsFlFFF) in aqueous solution [1,2]. Arabinoxylans studied were from wheat (high, medium, and low viscosity) and rye (high viscosity) endosperm. Two alpha-arabinofuranosidases (AXH-m and AXH-d3) with distinct specificities were used to modify further the arabinose-toxylose ratio of arabinoxylans [3]. Not only the arabinose-to-xylose ratio but also the position of the arabinofuranosyl substituents was found to affect the watersolubility of arabinoxylans. DMSO was a better solvent than water in HPSEC for all arabinoxylans studied as chain entanglement occurred in aqueous eluent. On the other hand, water-based AsFlFFF possessed more separation power for the differentiation of arabinoxylan aggregates from individual chains than HPSEC. The results obtained reveal details on solution behaviour of cereal arabinoxylans, and can be further exploited when applications involving arabinoxylans are developed.

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Session 7:

Innovative processes and design of cereals for the future

OP31

Post-genomic analyses of fungi for the degradation of plant cell wall

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Filamentous fungi are potent degraders of plant cell wall due to their ability to thrive in ligno(hemi)cellulose-rich environments. During the last decade, fungal genome sequencing initiatives have yielded abundant information on the genes putatively involved in the breakdown of polysaccharides. At present, additional experimental studies are essential to provide insights into the fungal secreted enzymatic pools involved. We have performed a wide analysis of 20 filamentous fungi for which genomic data are available to investigate their biomass hydrolysis potential. A comparison of fungal genomes and secretomes using enzyme activity profiling revealed discrepancies in CAZyme sets dedicated to plant cell wall. Investigation of the contribution made by each secretome to the degradation of wheat straw demonstrated that most of them individually supplemented the industrial *T. reesei* CL847 enzymatic cocktail. Unexpectedly, the most striking effect was obtained with the phytopathogen *Ustilago maydis* that improved the release of total sugars by 57% and of glucose by 22%. Proteomic analyses of the best-performing secretomes indicated a specific enzymatic mechanism of *U. maydis* that is likely to involve oxido-reductases and hemicellulases. This study provides insight into the lignocellulose-degradation mechanisms by filamentous fungi and allows for the identification of a number of enzymes that are potentially useful to further improve industrial lignocellulose bioconversion process.

OP32

Hydrolysis of xylans by a thermostable family 10 xylanase from Rhodothermus marinus and fermentation of hydrolysis products by intestinal bacteria

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Xylan is the most abundant hemicellulose polymer in many cereal grains and can be hydrolyzed into oligosaccharides using xylanolytic enzymes [1]. These enzymes selectively cleave the s 1-4 linked xylose backbone and release xylooligosaccharides (XOSs) proven to selectively stimulate a group of intestinal bacteria important for gut health [2]. Different XOSs can be obtained after enzymatic hydrolysis depending on the origin of the xylan polymer and on the properties of the enzyme. In order to evaluate the thermophilic bacterial xylanase RmXyn10A [3] from *Rhodothermus marinus* different commercial xylans were hydrolyzed and the XOSs were tested in batch cultivation of intestinal bacteria important for a balanced and healthy intestinal tract. Anion exchange chromatography (HPAE-PAD) was applied to analyze the XOSs produced from xylan hydrolysis and to confirm bacterial utilization in the spent fermentation broth. RmXyn10A proves suitable to hydrolyze different xylans into XOSs with prebiotic potential [4] intended for use in functional food products.

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OP33

Profiling and "*in-vivo*" imaging" of barley proteases in germinating barley by activity based probes: Methods and application

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Plant proteases play a pivotal role in all aspects of regulation in plants from disease control, fungal resistance to germination, regulation of phenotype and yield. In the distillery and brewing industry barley proteases are essential and involved in malting, mashing and fermentation. Plant proteases exert finely balanced and potent post-translational control over a diverse range of functions in the cell by recognizing an even larger number of different and selective substrates. Despite annotation of several proteases in the plant genome, their role, control, regulation and inhibition of many proteases is still remaining unexplored. This is largely due to the fact that proteases exert their effect in amplification / signalling cascades and regulatory pathways, in the presence of an array of interacting proteins and substrates in often distinct sub-cellular compartments. Thus, *in-vitro* experiments alone provide only a limited picture of their role at cellular level under physiological "*in-vivo*" conditions. Recent progress in the development of activity based near-infrared fluorescence (NIRF) probes (ABPs) in our research group has opened up the possibility of detection and visualizing protease activity by molecular NIRF imaging techniques. Light in the NIR region between 650-900 nm can penetrate deep into living tissue, thereby offering a unique opportunity to visualize fluorescent probes "*in-vivo*". The presentation will detail some of the recent progress in the expression of recombinant barley proteases, their activation and substrate specificity by on-bead barley protease substrate screening. The preferred barley protease substrates are then synthesised as fluorescence and NIRF activity *in vivo* probes by solid phase synthesis. The application of the NIRF probes in *in-vivo* imaging of barley protease activity during the different phases in germinating barley malting is presented. Specifically the following aspects will be covered:

- Expression of barley proteases
- On-bead substrate screening for barley proteases
- Design approach: From protease profiling to NIRF activity probes
- Synthesis of NIRF-activity probes for plant proteases
- "*in-vivo*" imaging of NIRF-activity probes for barley proteases during germination

Enzymatic hydrolysis of concentrated raw starch and breakdown of crystalline structures

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Two new alpha-amylases from *Rhizomucor species* (RA) and *Anoxybacillus flavothermus* (AFA), found to be effective in biofuel and low temperature glucose syrup production, were studied in detail due to their very high efficiency on concentrated suspensions of native raw starch granules below gelatinization temperature.
Hydrolysis rate reaches 85% for 31% maize starch suspensions. In the first stages of hydrolysis crystallinity and amylopectin fraction decrease rapidly, evidencing a specific preferential degradation of the crystalline structures. At the same time, the amylose-lipid complexes present in maize starch are more resistant and hydrolyzed in further stages releasing amylose fragments. Then, these fragments rearrange into a very resistant B-type structure which prevents a complete hydrolysis [1.2].
The way the granular architecture and the crystalline structures are degraded was assessed in detail. A very new synchrotron UV fluorescence technique was used for the first time for tracking *in situ* the enzymatic breakdown of single starch granules. Diffusion of enzyme into the starch granules at 283 nm resolution, using amylase tryptophan fluorescence without added fluorescent probe, and resulting morphological changes, in visible light, were monitored [3]. It allowed to record 3D maps of the location of amylase within single starch granules and the related modification of their morphology at different times of hydrolysis. Transmission electron microscopy (TEM) experiments on crystalline lamellae, extracted from native starch by mild hydrolysis, demonstrated that both RA and AFA attack the crystalline regions by the side of the double helices and not by their ends.
Amylase binding onto starch granules, disentanglement of the double helices present in the starch crystalline lamellae and degradation of crystalline structures at high starch concentration are discussed. Potential conformational changes of RA are also considered, on the basis of preliminary results originating from synchrotron SAXS (Small Angle X-ray Scattering) and SRCD (Synchrotron Radiation Circular Dichroism) measurements.

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Poster Presentations

PP1

Enzymatic production of added-value compounds from lignocellulosic lipophilic extractives

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Plant sterols, specially their fatty acid esters, are considered as nutraceuticals with plasma cholesterol lowering action [1]. Natural sources of these compounds are seeds and cereals, being the last also rich in glycosylated sterols and sterols esterified with phenolic acids (which combine cholesterol lowering and antioxidative properties). Furthermore, taking into account the biorefinery concept, lignocellulosic residues, such as wheat straw, would be a good source to obtain them [2].

Sterol esterases and lipases can catalyze in low water media esterification reactions between free phytosterols and fatty acids to obtain the corresponding esters, which show higher solubility, to be used for the manufacture of functional foods. In this sense, both the sterol esterase produced by the ascomycete *Ophiostoma piceae* and its recombinant variant expressed in *Pichia pastoris* have been employed with this aim, and the results have been depicted in a patent application [3]. Briefly, when both enzymes were used to acylate a mixture of soy phytosterols with a small excess of lauric acid in a biphasic system isooctane:water the overall yield after 48h was 80%, what meant an increment of 13% regarding to that obtained with an enzymatic commercial preparation from *Candida rugosa*. In addition, production of phytosterol esters was higher with enzymes from *O. piceae* than with the commercial enzyme at low doses of biocatalyst (1.5 U/mL).

Since both native and recombinant proteins from *O. piceae* show better catalytic properties than the esterases/lipases currently available, we also propose their use to catalyze the synthesis of steryl ferulate mixtures, exploiting the health benefits provided by these compounds through their incorporation into different kinds of foods [4].

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PP2

Foaming properties of tryptic wheat gliadin peptide fractions

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Wheat gliadins typically show a central hydrophilic domain (CD), particularly rich in Gln and Pro and two terminal Hydrophobic domains (TD) containing almost all chargeable amino acids. The N-TD is very short, while the C-TD represents about half of theprotein sequence. Aqueous solutions of gliadins, the 70% ethanol extractable proteins of wheat gluten, show excellent foaming ability. However, gliadin is poorly soluble near neutral pH. Enzymatic proteolysis of gliadin leads to peptides with improved solubility, but does not automatically lead to similar or improved foaming properties compared to native gliadin. More insight is required about the structure of gliadin peptides in relation to their foaming properties.

Tryptic hydrolysis of gliadin, followed by graded ethanol precipitation, resulted in four peptide fractions, which were, according to differences in amino acid levels, related to the CD or TD of gliadins. Foams made from aqueous solutions of these tryptic gliadin peptide fractions were studied at different pH conditions. Foam volume (FV) of both CD related peptide fractions were similar over the total pH range, while foam stability (FS) was decreased at extreme pH conditions and optimal at pH 6.7. FS was lower at pH 12.0 compared to pH 2.0. In contrast, FV of the TD related peptide fractions were low at acidic pH values but increased towards more alkaline conditions. One TD related peptide fraction showed a clearly lower FS at acidic pH compared to more alkaline conditions, while the FS of the other fraction was less correlated with the pH. The hydrophobic distribution of peptides within foam and solution revealed that, for the CD related peptide fractions, the peptides within foam were enriched in hydrophobic peptides, while, for TD related peptide fractions, smaller differences in hydrophobic distribution was observed.

Influence of organic co-solvents on the activity and substrate specificity of feruloyl esterases

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Organic co-solvents can expand the use of enzymes in cereal processing through making substrates more soluble and thus more accessible. In choosing the most adequate co-solvent for feruloyl esterases, hydrolysis of methyl p-hydroxycinnamates by three pure enzymes and the multi-enzyme preparation Ultraflo was evaluated. Low concentrations of dimethylsulfoxide (DMSO) enhanced hydrolysis while at levels >20% (v/v) activity was reduced. DMSO also enhanced acetyl esterase-type activity in these enzymes. The co-solvent effect was different for each enzyme-substrate couple, indicating that other factors are also involved. Kinetic studies with a *Talaromyces stipitatus* feruloyl esterase (TsFaeC) showed low concentrations of DMSO enhanced the hydrolytic rate while Km also increased. Moreover, long-term incubation (96 h) of an *Aspergillus niger* feruloyl esterase (AnFaeA) in DMSO:water induced the enzyme's ability to hydrolyze methyl p-coumarate, suggesting an active-site rearrangement. Dimethylsulfoxide (10-30%) is proposed as an adequate co-solvent for feruloyl esterase treatment of water-insoluble substrates.

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Disassembly of brewer's spent grain by multi-enzymatic mixtures

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Cereal grain residues arising from established industries such as brewing and baking amounts to millions of tonnes each year. Traditionally these have either gone mainly to animal feed or been composted. As industrial processes have expanded due to consumer demands, more of this material now ends up as landfill. This causes environmental impact and the loss economically important material, particularly rich high value components, like arabinoxylans. However, further knowledge is required on how to fractionate and isolate them.

As part of a "green" approach to fractionation, commercial food grade enzyme preparations have been examined for their ability to liquefy cereal-derived material, such as brewer's spent grain (BSG). The importance of carbohydrases, feruloyl esterases and proteases, as well as physical factors, in opening up the structure of BSG and its subsequent hydrolysis has been examined. We have screened enzyme mixtures for key activities and how these activities behave under different hydrolytic conditions. Minimal enzyme preparations based on these studies have been prepared. These can show similar solubilisation to the whole enzyme mixture. Selective inhibition studies have also been performed. Results show, for example, protease activity is required to enhance polysaccharide-cleaving activities, but other non-catalytic factors can also be important to facilitate hydrolysis. This approach has identified key points required to understand enzymatic disassembly of BSG and what enzyme mixtures may be most suitable under different treatment conditions.

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PP5

Characterization of a novel cyclomaltodextrin glucanotransferase from *Thermoanaerobacterium thermosulfurigenes*

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Cyclodextrin glucanotransferase (CGTase, also known as cyclomaltodextrin glucanotransferase or cyclodextringlycosyltransferase, EC 2.4.1.19) belongs to the same family of glycoside hydrolases (glycoside hydrolase family 13) as the α-amylases.

GH13 is a very large family of enzymes displaying a great variety in specificities and product formation profiles. Enzymes classified as CGTases are capable of catalyzing more than one reaction; cyclization, coupling, disproportionation and hydrolysisusing substrates such as starch, amylose, amylopectin, and cyclodextrins. The starch and carbohydrate industries are in need of biocatalysts in order to modify polysaccharides and their derivative compounds for the production of industrial and consumer products, and there is a demand for robust enzymes among the industries in order to run the catalytic process at moderate to high temperature for lowering the viscosity of polysaccharides, along with an interest to find novel enzymes with altered properties.

In line with the need to search for novel enzymes, we have within the framework of the EU-project “AMYLOMICS” expressed and purified a novel enzyme from *Thermoanaerobacterium thermosulfurigenes*, which based on sequence similarities is most closely related to cyclodextrin glucanotransferases (75% id to a known CGTase from the same species). The domain structure of the enzyme contain the typical GH13 catalytic domain (A, B and C), an IPT-domain (D), and a C-terminal CBM20 (carbohydrate-binding module, family 20) domain. The CBM20 domain is common in a large number of starch degrading enzymes including alpha-amylase, beta-amylase, glucoamylase, and CGTase (cyclodextrin glucanotransferase). At the time of writing the characterization of this novel enzyme is ongoing and result from this work will be presented in the symposium.

PP6

Branching-enzyme treatment of high-concentration starch slurries

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In this study enzymatic modification of starch was investigated as an alternative to traditional chemical and physical modification techniques used in the food industry. Such processes can be considered as more environmentally friendly and also the products can be used in clean-label foods since they, as opposed to traditionally modified starches, do not require an E-number. It is further expected that new and interesting properties of enzymatically treated starches can be discovered. Low water content during enzyme treatment saves energy during treatment and subsequent drying and is necessary for production on industrial scale. However, the low water content can affect the enzyme action due to suppression of starch gelatinization during modification. A thermostable branching enzyme (E.C. 2.4.1.18) from *Rhodothermus obamensis* (Novozymes, Bagsværd) has been used in various concentrations to modify starch from potato and maize.

The enzymatically treated samples were evaluated based on apparent amylose content assessed by iodine staining. Enzymatic modification resulted in a highly branched product. Interestingly, the botanical origin of the starch affected the molecular composition of the final product. The observed differences were further characterized at molecular level using Dionex amylopectin chain-length profiling, size exclusion chromatography and NMR. Our data indicate the production of a molecularly more homogeneous and compact product being potentially more stable in solution.

PP7

Bread and breakfast cereal enrichment with γ-amino butyric acid, a blood pressure lowering component

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Breakfast cereals and bread make up an important part of the human diet and energy intake in the Western world and, hence, are extremely suited to associate health benefits with. Intake of γ-amino butyric acid (GABA), a major inhibitory neurotransmitter ofthe nervous system of animals, has been claimed to have different beneficial effects, including a lowering of blood pressure of hypertensive individuals. GABA is released by glutamate decarboxylase (GAD) from glutamic acid. GAD is an enzyme of both prokaryotic and eukaryotic species. We here enriched bread and breakfast cereals with GABA by optimizing the food recipes and production process. The dynamics of glutamate and GABA levels in bread and breakfast cereals were monitored during their manufacture. Addition of exogenous GAD boosts the production of GABA and a well-thought ingredient choice and strict control of the production process eventually results in foods significantly enriched with GABA. The consumption of one portion of the produced breakfast cereals can e.g. deliver 60% of the daily intake recommended to lower blood pressure*. Furthermore, it was not necessary to combine GAD with other enzymes increasing the free glutamic acid concentration in the mixture (e.g. transglutaminase, peptidases, …), which offers the unique opportunity to produce GABA enriched foods without drastically impacting food structure.

*Inoue, K., Shirai, T., Ochiai, H., Kasao, M., Hayakawa, K., Kimura, M. and Sansawa, H. (2003). Blood-pressure-lowering effect of a novel fermented milk containing gamma-aminobutyric acid (GABA) in mild hypertensives. European Journal of Clinical Nutrition, 57(3): 490-495.

PP8

Concerted action of acetyl esterase and α-glucuronosidase enzymes in xylan degradation

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Acetyl esters (Ac) and glucuronic acid or its 4-O-methyl ether (GlcA/meGlcA) are common substituents of xylan in the lignified parts of cereals and dicotyledonous plants. meGlcA is almost exclusively α(1->2) linked to the xylan backbone, whereas Ac can be present either as mono- or disubstitution on 2-O and/or 3-O. Acetyl groups are removed by acetyl esterase enzymes, which are known to act either on actyls on xylan oligomers (acetyl esterase, AE) or alternatively on polymeric xylans (acetyl xylan esterase, AXE). The same is true for α-glucuronosidases (GLUR) as well. We have studied the effect of different esterase enzymes on the deacetylation of acetylated xylo-oligosaccharides (XOS), either alone or in combination of α-glucuronosidase enzymes. Neutral linear XOS were nearly completely deacetylated with the best polymer-acting AXEs alone. Deacetylation of meGlcA-substitued XOS was a challenge to all enzymes, since even a concerted action of AXE and AE resulted in only up to 80% deacetylation, indicating that meGlcA substituents hinder the action of all esterases. Deacetylation was enhanced after addition of GLUR to the reactions, but strong differences in the action of different GLUR enzymes was observed. Site specificities of esterase enzymes will also be discussed.

PP9

Heterologous expression, purification and characterization of barley (*Hordeum vulgare* L.) endoprotease B2

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During germination of barley seeds, cysteine proteases accounts for more than 90 % of the total proteolytic activity degrading the grain storage proteins. Cysteine proteases are synthesized as pro-enzyme and are activated through reduction of the active site cysteines and by removal of the pro-domain. The complement of cysteine proteases is comprehensive and for detailed studies of the individual components of this complement, a fast and efficient eukaryotic expression platform is highly desirable.

In the current study, the barley key cysteine endoprotease B2 (HvEPB2ΔC) was expressed in *Pichia pastoris*. In order to achieve this, a HvEPB2ΔC cDNA clone was inserted into the *P. pastoris* expression vector pPICZ Aα, which was electrotransformed into *P. pastoris* strain KM71H. Heterologous protein production was induced with 2% MeOH and maximum yield were obtained when harvesting the supernatant after 4 days. Purification of the recombinant HvEPB2ΔC (rHvEPB2ΔC) from the supernatant were performed with IMAC by FPLC. The purified fractions were analyzed via SDS-PAGE, western blotting and via activity assaying. Kinetic parameters, effect of protease inhibitors, thermal stability, temperature and pH optimum was obtained by activity assays.

From the IMAC purification a yield of 4.26 mg purified rHvEPB2ΔC per l supernatant was obtained. rHvEPB2ΔC follows first order kinetics (Km = 8,6 µM) for the chromogenic substrate Z-Phe-Arg-pNA and shows significant inhibition in the activity by the cysteine protease specific inhibitors E64 and leupeptin. The temperature optimum for rHvEPB2ΔC was determined to be 55°C and the thermal stability T50 value to 44°C. Activity of rHvEPB2ΔC at different pH values revealed a pH optimum at 4.5. Incubation of purified rHvEPB2ΔC with Osborne fractionated barley seed storage proteins for 12 hrs revealed after SDS-PAGE a significant degradation of the storage proteins. The degradation did not occur in the presence of E64.

PP10

Redox regulation of transferases involved in starch biosynthesis in *Arabidopsis thaliana*

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Starch is an abundant storage carbohydrate in plants, and essential for human nutrition. The properties of starch are also exploited in industrial applications such as paper and bioethanol production. Starch consists of two glucose polymers; amylose, a linear chain of residues connected by α-1.4-glucosidic bonds, and amylopectin a highly branched polymer, residues are linked with both α-1.4-glucosidic bonds and α-1.6-glucosidic bonds (branch points). Starch is present in leaves, stem, tubers and grains of the plant. Multiple enzymes are involved in the synthesis of cereal starch, starch synthases, branching and debranching enzymes among others. Due to the complex synthesis of starch grains, many important structural and catalytic functions of the enzymes involved in the grain synthesis, including the redox state of the cell, remain unknown. Redox regulated enzymes implicated in starch synthesis and degradation have been identified in vitro in *Arabidopsis thaliana*. These are ADPglucose pyrophosphorylase, beta-amylase (BAM1) and starch phosphorylator GWD1 among others. With the use of an activity screen based on manipulation of redox potentials in *A. thaliana* extracts, several enzymes including, starch synthase I (SSI), branching enzyme II (BEII), and starch synthase III (SSIII), where shown to be dependent on redox potential. The aim of the current study is to clone and express *A. thaliana* SSI, SSIII and BEII in *Escherichia coli*. The dependence on redox-potential of each individual enzyme will be determined in addition to kinetic characterization.

PP11

The role of the thioredoxin-regulated β-amylase BAM1 in diurnal starch degradation

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With the aim to compensate for their sessile nature, plants have evolved sophisticated mechanisms both essential for their normal growth and in response to environmental changes. Post-translational modifications take part in plant metabolic plasticity, regulating the activity of metabolic enzymes. Common modifications of plant enzymes include protein phosphorylation and thiol-disulfide redox modulation, typically involved in the control of plant metabolism.
The most convenient way for plants to store large amounts of chemical energy and organic matter without altering the osmotic balance of the cell is represented by starch. Leaf starch is transiently accumulated in a daily cycle and soluble carbohydrates resulting from starch degradation are mainly exported to sink tissues. Alternatively, starch-derived soluble sugars can also participate to osmotic adjustment under water stress or take part in the osmotic regulation of specialized cells, notably guard cells.
The *Arabidopsis thaliana* BAM1 (At3g23920) is demonstrated to be a plastid-targeted β-amylase, specifically activated by reducing conditions, while it is catalytically inactive under oxidizing conditions. Among all major plastid thioredoxin isoforms, BAM1 is most efficiently activated by thioredoxin f1, followed by thioredoxins m1, m2, y1, y2, and m4. Alternative activation of BAM1 was also achieved by plastid-localized NADPH-thioredoxin reductase (NTRC), which allowed the recovery of about half of the BAM1 maximal activity.
In contrast with the timing of starch metabolism in mesophyll cells, redox regulation of BAM1 activity suggests that this enzyme would be mainly active in the light rather than in darkness. To elucidate this inconsistent behaviour, knockout (KO) mutants and promoter activity of BAM1 were analyzed. Differently from KO mutants for BAM3 (At4g17090; the major chloroplastic β-amylase, insensitive to redox regulation) which show a common sex phenotype (starch excess), characterized by stunted growth and starch accumulation at night, a T-DNA insertion line in which BAM1 expression was nil displays normal growth but reduced stomata opening and increased starch content in illuminated guard cells.
The promoter activity of BAM1 was studied by placing the reporter genes GUS and YFP under the control of BAM1 promoter, and Arabidopsis transgenic plants were analyzed. In non flowering plants, both YFP- and GUS-expression of BAM1 are shown in leaves and roots, but expression in leaves was mainly restricted to guard cells, in agreement with the regulatory properties of the enzyme.

Interestingly osmotic stress triggers BAM1 expression in mesophyll cells of young plants. Total β-amylase activity also increased, together with its redox-sensitive fraction, in osmotically stressed wild type plants but not in KO mutants. Taken together these data suggest that thioredoxin-regulated BAM1 acts in the diurnal pathway of starch degradation in guard and can similarly activate a starch degradation pathway in illuminated mesophyll cells under osmotic stress.

PP12

Effect of a milling pre-treatment on the enzymatic hydrolysis of carbohydrates in brewer's spent grain

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Brewer's spent grain (BSG), an abundant side stream from the brewing industry, has the potential to be a valuable source of food, chemicals and energy if cost-efficient fractionation methods can be developed. The hydrolysis yield of BSG carbohydrates was improved 2-fold through the introduction of a milling step prior to the enzymatic hydrolysis. Course and fine milled fractions were characterized by particle size distribution and light microscopy. The fine milling decreased particle size down to the micron level and this in turn improved the carbohydrate solubility yield by a multi-enzyme mixture from 23 to 45%. Carbohydrate solubilisation could be further increased through the further addition of cellulase. The physical degradation caused by the milling also liberated a small amount of soluble carbohydrates without the requirement of any enzymatic treatment.

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Phytase activity in yeast

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The low absorption of minerals from cereal based food, such as bread, was attributed to the high content of phytic acid salts (phytates) in cereals. Phytic acid (IP6; myo-inositol hexaphosphate) is the principal storage of phosphorus in plants, particularly in cereal grains and legumes. It is highly charged with six phosphate groups extending from the central myo-inositol ring and binds minerals, such as Zn^{2+}, Fe^{2+}, Ca^{2+}, Mg^{2+}. Formed phytate are insoluble at psychological pH, and, therefore, minerals and phosphate are unavailable for absorption in the human intestine. The bioavailability of minerals and phosphate will increase if phytate is degraded. Characterized phytases, are enzymes, that catalyses the stepwise dephosphorylation of phytate to myo-inositol and phosphoric acid via penta- to mono- phosphates. This enzymatic activity produces available phosphate and non-chelated minerals for human absorption.

Mineral bioavailability in bread can be increased, using high phytase active yeasts, in addition to native cereals phytase. There are no yeast strains with high phytase activity available for bread industry today, so the potential of identification of yeast strains to be used for bread making with high content of bioavailable minerals is of outstanding importance.

The objective of this study was to screen phytase activity in yeasts, isolated from food and drinks. Screening of phytase positive yeast strains was carried out at conditions, optimal for bread making: pH 5.5 and 30 ºC, in order to identify strains which could be used for baking industry.

A total of 41 yeast strains, belonging to *Saccharomyces cerevisiae*, *S. pastorianus*, *S. bayanus*, *S. exiguus*, *Candida krusei*, and *Arxula adeninivorans* species, were screened for their ability to grow in minimal liquid and on solid media, supplemented with phytic acid dipotassium salt, as the only phosphorus source. Eleven yeast strains were selected for further determination of phytase activity due to their rapid growth in liquid and on solid minimal media. Two yeast strains were selected for further determination of phytase activity due to their very slow growth in liquid minimal medium, in order to check the trustiness of primary screening - growth test in liquid medium.

PP14

Highlighting the action of carbohydrate oxidases in wheat flour aqueous extract

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Measurements of oxygen consumption in wheat flour aqueous extract added with carbohydrate oxidases (GOX or COX) confirmed the oxidase activity of these enzymes. For amounts of GOX and COX showing equal activity (oxygen consumption rate) towards glucose 0.22 M, COX is more active than GOX towards a wheat flour extract. Hydrogen peroxide is produced during the oxidation of the flour carbohydrates by oxygen catalyzed by GOX or COX. Then, the endogenous peroxidase present in the wheat flour extract can catalyze the oxidation of glutathione (GSH) and phenols, such as ferulic acid (FA) and tyrosine (Tyr). The addition of increasing concentrations of GSH, FA and Tyr in a wheat flour extract shows that oxygen consumption by COX is inhibited by low concentrations of FA and unaffected by GSH and Tyr while oxygen consumption by GOX is only inhibited by high concentrations of Tyr and unaffected by GSH and FA. HPLC-UV analyses confirm that the oxidation of glutathione and phenols is effective with the formation of oxidized glutathione (GSSG) and oxidation products of FA and Tyr but with significant differences between COX and GOX.

In a wheat flour aqueous extract, the presence of COX or GOX is compulsory to observe the oxidation of FA and Tyr whereas, a low oxidation of GSH is already observed in the absence of COX and GOX. The amount of produced GSSG is more important with GOX than with COX.

The consumption of FA and the formation of its two dimers (8 5'benzofuran diFA and 8-8'-□-lactone diFA) increase with the amount of added FA with the 8 5'benzofuran diFA being always predominant. The amount of dimers reaches a plateau with COX while it increases with GOX.

The oxidation of Tyr is less effective with COX than with GOX, dityrosine being always produced in greater quantities than trityrosine.

With a wheat flour extract, COX seems more efficient than GOX to produce the hydrogen peroxide to activate the peroxidase activity of the flour. Assuming that the reactivity of Tyr, -SH (cysteine) and ferulic acid are the same when these molecules are in the free state or bound to macromolecules, in the presence of COX (or GOX) the peroxidase can then participate to the oxidative reactions leading to the cross linking of gluten proteins and arabinoxylans. The system POD/ carbohydrate oxidase can then contribute to the development of networks during the formation of the dough but with differences in the oxidation products obtained. The question remains to know if COX is at least as effective as GOX as processing aid in breadmaking.

2-Oxidation of tyrosine in the presence of glutathione or ferulic acid in three different systems : wheat flour aqueous extract / carbohydrate oxidases, purified wheat peroxidases / H_2O_2 and wheat flour extract / H_2O_2

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Added to a wheat flour aqueous extract (WFAE), carbohydrate oxidases (GOX or COX) catalyze the oxidation of the flour carbohydrates by oxygen and produce hydrogen peroxide (H_2O_2). This latter compound can then be used by wheat peroxidases (POD) to catalyze the oxidation of glutathione (GSH), ferulic acid (FA) or tyrosine (Tyr) when they are separately added in WFAE. This leads to the formation of oxidized glutathione (GSSG), dimers of ferulic acid, dityrosine and trityrosine. This study compares the oxidation of Tyr in the presence of GSH or FA by different enzymatic systems: WFAE / carbohydrate oxidases (COX or GOX), purified wheat germ cationic PODs / H_2O_2 and WFEA / H_2O_2. As long as the flour extract does not lack of oxygen or carbohydrates, H_2O_2 is continuously produced in the first system WFAE / carbohydrate oxidases. For the two other systems, successive additions of small amounts of H_2O_2 are carried out for a controlled oxidation of Tyr. When Tyr and GSH are both added:

- in the WFAE / carbohydrate oxidases system, less Tyr is consumed in the absence of GSH than in its presence. Neither dityrosine nor trityrosine are detected. - in the purified wheat germ PODs / H_2O_2 system, the formation of dityrosine is detected but not in the WFEA / H_2O_2 system.
- regardless the enzymatic system used, GSSG is formed in higher amounts in the presence of Tyr than in its absence but with differences between COX and GOX in WFEA / carbohydrates system and between the purified wheat germ PODs / H_2O_2 and WFEA / H_2O_2 systems. It is likely that the WFAE contains a catalyst that oxidizes GSH and the addition of tyrosine increases the formation of GSSG by coupled oxidation.

When FA and Tyr are added together:

- in the WFAE / carbohydrate oxidases system, less Tyr is consumed in the presence of FA than in its absence. Similar results are observed with the two other systems. In addition, the Tyr oxidation is more effective with COX than with GOX.
- FA disappears progressively in the presence of WFEA / GOX and quickly in the presence of WFEA / COX.
- in the purified wheat germ PODs / H_2O_2 system, the FA consumption is complete whereas there are still traces of FA in the WFEA / H_2O_2 system.

The differences observed among the three systems could be due to the fact that in the WFAE / carbohydrate oxidases system, the POD activity is dependent on the H_2O_2 produced by COX or GOX which depends on the oxygen and carbohydrate availability in the WFAE whereas in the two other systems, the POD activity is only dependent on the amount of added H_2O_2. In addition, the differences observed between COX and GOX could be explained by an higher H_2O_2 production by COX which involves an higher POD activity towards oxidation of phenolic compounds.

PP16

Investigation of surface binding sites in barley alpha amylase 1

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Cereal alpha-amylases (EC. 3.2.1.1) are involved in the degradation of starch by hydrolysis of 1,4-alpha-D-glucosidic bonds and represent important industrial enzymes in baking and brewing processes. Thus, the preferred binding of these enzymes to starch and related oligosaccharides is of central importance and has been investigated in the two alpha-amylases identified in barley, referred to as isoform 1 (AMY1) and 2 (AMY2), sharing 80% sequence identity [1]. AMY1 possesses two secondary carbohydrate binding sites on the surface at a certain distance from the active site, called starch binding site 1 and 2 (SBS1 and 2) [2-3]. SBS1 contains two consecutive tryptophans (W278 and W279), whereas SBS2 has a tyrosine residue (Y380) as the key side chain in binding of oligosaccharides. Both sites are conserved in AMY2 but only SBS1 is possibly involved in the binding of oligosaccharides [4]. Additionally, mutational studies of SBS1 and SBS2 in AMY1 revealed that both sites are important for the activity and substrate binding [5-6]. However, the influence of SBS1 and SBS2 on the function of barley a-amylase is not known in detail and in order to examine interactions with substrates without hydrolysis, the inactive catalytic nucleophile mutant (D180A) was combined with surface site mutations in a series of AMY1 mutants involving W278, W279 and Y380 either alone or in combination. Binding affinities of these inactive mutants for different malto-oligosaccharides (cyclic and linear) are measured by Surface Plasmon Resonance (SPR) to describe the surface binding sites recognition contributed for different structural elements of substrates.

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PP17

Enzymatic and chemical constraints on the solubilisation of brewers' spent grain

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The enzymatic hydrolysis of Brewer's spent grain (BSG) has been investigated through treatment with commercial carbohydrases and proteases. Treatments to improve the release of polysaccharides have considered the effects of pre- and post-digestion with commercial enzyme cocktails on the profile and composition of polysaccharides and bound phenolics. Residues arising from enzymatic treatments were chemically fractionated using alkali and also chemically delignified to determine the loss of non-polysaccharide material present and to estimate the relative importance of lignin in controlling the deconstruction of BSG. Enzymatic treatments released 25-30% of the BSG mass. A controlled fractionation using alkali solubilised ~40% of untreated BSG and ~30% of the enzyme treated residue. In each case there was a selective solubilisation of arabinoxylan but this represented only ~15% of the mass in the untreated BSG and ~11% from the enzyme treated residue. No differences in fractionation behavior were apparent due to prior enzyme treatment but in each case there was a significant loss of non-polysaccharide components during alkali treatment. This could not be accounted for by protein and suggests the presence of a high proportion of alkali-soluble lignin in BSG. Chemical delignification of the alkali-insoluble residues and further alkali fractionation released the remaining hemicellulose, to yield a residue which was >90% cellulose. Further knowledge of the properties and interaction between BSG polymers will facilitate an improved enzyme-assisted total deconstruction of BSG and hence the exploitation of its biomass.

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PP18

Phenotypic comparison of single mutants lacking of (phospho)glucan-water dikinase

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Starch is the most prominent storage carbohydrate in plants. Starch metabolism involves numerous enzymes, all together attending to the major carbon flow in plants. Common feature of starch metabolic enzymes, is their clustering in protein (or gene) families. For example, the genome of the model plant *Arabidopsis thaliana* is supposed to code three α-amylases, nine β-amylase-like proteins, three isomaylases, three (phospho)glucan-water dikinases and so on (1). In most cases, the redundancy of the members of one protein family is only apparent. For example, recent results clearly indicate that two of the nine β-amylase-like proteins work as transcriptional factors (2). The aim of the present work is to investigate the role of (phospho)glucan-water dikinases and their specific contribution to starch metabolism. The (phospho)glucan-water dikinases (PWD and GWD, respectively) are a recently discovered class of enzymes, required for starch metabolism (3; 4). GWD and PWD catalyze the same reaction, with the only difference that GWD does not require pre-phosphorylation of the substrate starch, while PWD only phosphorylates pre-phosphorylated starch. Two T-DNA insertion mutants for each of the following genes, GWD1 (At1g10760), GWD2 (At4g24450) and PWD (At5g26570), were obtained from the European Arabidopsis Stock Center (NASC, Nottingham, UK). Insertion site of the T-DNA was confirmed by PCR on genomic DNA and homozygous lines (GWD1: SAIL_165_B11; GWD2a: SALK 152327C; GWD2b: SALK 080260C; PWD: SALK_110814) were used in experiments. As previously reported, mutants lacking GWD1 showed the most severe phenotype, characterized by small size and inability to degrade starch during the night (average starch content was 7-fold higher than in WT). In comparison, pwd mutants presented a less drastic phenotype (starch content was about double in respect to the WT), while gwd2 plants displayed identical starch content to WT plants. Despite the differences in starch content, X-ray powder diffraction patterns of starch granules extracted from Arabidopsis leaves of mutants and WT plants were identical, suggesting that none of these enzymes are involved in modifying the fine architecture of starch granules. All mutant lines displayed a lower growth rate of both rosetta leaves and primary root. In comparison to WT plants, the differences in rosette growth varied from 0,185 mm/d in gwd1 to 0,460 mm/d in gwd2a (WT 0,867 mm/d). For primary roots, growth varied from 0,035 mm/h in gwd1 to 0,068 mm/h in gwd2b plants (WT 0,124 mm/h). Other phenotypic parameters, as flowering time (assayed as number of rosette and cauline leaves when the primary influorescence was 10 cm high), flower number, silique number, seed number per silique, seed weight and seed size were determined. The obtained data suggest that none of the analyzed gene had a strong influence on the flowering time, although inflorescence of gwd1 mutants required more time to reach the height of 10 cm. Vice versa the absence of GWD2 or PWD decreased the number of flowers and siliques, without affecting the number of seeds per silique and the average weight of individual seeds. Unexpectedly, the behaviour of gwd1 mutants was similar to that of the WT in this respect. Analyses of seed contents (protein, oil, sugars) in mutant lines, as well as promoter activity of the three genes, are in progress.

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PP19

Xylanolytic activities and endogenous proteinaceous xylanase inhibitors in barley

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Cereals like barley naturally produce endo-β-1,4-xylanases for modification of cell walls during seed development and germination (1,2). In addition, cereals also contain microbial xylanases produced by microbes populating the surface of the kernels. Xylanases (EC 3.2.1.8) hydrolyze internal β-1,4-glycosidic linkages between xylose units in the arabinoxylan (AX) backbone of cell walls. According to the CAZy database, xylanases have been classified into glycoside hydrolase (GH) families 5, 7, 8, 10, 11 and 43. Accumulating evidence suggest that plant xylanases are not inhibited by the known xylanase inhibitors (TAXI, XIP and TLXI), whilst the microbial xylanases are sensitive to inhibition (3). In a similar manner, the efficiency of the regularly applied microbial xylanases in the cereal-processing industry to improve the product quality will be significantly compromised due to the presence of xylanase inhibitors.

The present study sets out to investigate xylanase activities (plant vs. microbial) and xylanase inhibition levels in different barley cultivars. Activity measurements suggest that there is considerable inter-cultivar variation in the level of both microbial and endogenous xylanase activities, as well as at xylanase inhibitor levels. Application of proteomics analyses (2D-gel electrophoresis, MALDI-TOF-TOF mass spectrometry) coupled with 2D-immunoblots enabled detection of the different types and multiple isoforms of xylanase inhibitors. However, additional experiments are needed to estimate the impact of genotype, environment and their interaction to the variation of xylanase activities and inhibition levels. Moreover, the commensal microbial community found on the surface of barley kernels will be analyzed. This will provide additional information if there is a correlation between specific microbes and the surface associated xylanase activity.

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PP20

Synthesis of potential prebiotic α-linked galactooligosaccharides by the transglycosylation ability of a GH36 α-galactosidase from human gut symbiont *Ruminococcus gnavus* E1

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Wholegrain grains and cereals contain a wide range of potentially protective factors that are relevant to gastrointestinal health. The best studied prebiotics are fructans [fructooligosaccharides (FOS), inulin] and galactooligosaccharides (GOS). These oligosaccharides satisfy the strict criteria for being prebiotics, being a nondigestible food ingredient that selectively stimulates the growth and or activity of desirable bacteria in the gut. Since the levels of GOS in foods are relatively low, and purification problematic, efforts are ongoing to synthesize GOS by chemical or enzymatic means. To this aim, α-galactosidases can be used to synthesise GOS using transglycosylation reactions. Here we report the enzymatic characteristics and regulation of expression for two α-galactosidases, AgaE1 and AgaE2, from *Ruminoccocus gnavus* E1, a human gut symbiont. The growth patterns of this strain indicated a preference towards complex (i.e. lactose and GOS) rather than simple carbohydrates (i.e. galactose) and a collaborative action of more than one glycoside hydrolase (GH) for both lactose or GOS hydrolysis and subsequent assimilation. AgaE1 and AgaE2 were heterologously expressed in *Escherichia coli*, purified to homogeneity and their biochemical properties and substrate preferences comparatively analysed. The enzymes belong to GH family 36 with high amino acid sequence similarities (approximately 70 %) to other biochemically characterised α-galactosidases from Firmicutes bacteria. Both enzymes have high sequence similarity to *Lactobacillus acidophilus* NCFM□□□-galactosidase, for which a 3D structure is available. Under optimum pH conditions (pH 6.0) and high melibiose concentration (69 % w/v), AgaE1 was able to synthesise GOS with α-(1-->6) regioselectivity and degree of polymerisation of 4 to 5. Furthermore, linear α-(1-->6) linked GOS up to [Hex]12 were obtained using glucose as acceptor and PNPGal as donor. In order to improve the transglycosylation yields, a nucleophilic mutant AgaE1 (D479A) has been generated and the donor β-D-galactosyl fluoride (β-gal-F) synthesized. The results of the glycosynthase reaction with β-gal-F and different sugar acceptors will be discussed.

PP21

Biochemical characterization of a glycosyl hydrolase protein from *Penicillium funiculosum*

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Enzymes are powerful biological catalysts, environmentally friendly, widely used in industry, and specifically adapted for food and feed industry. Biocatalysts can be used in cocktails, or pure, to improve taste, shape or digestibility. Among the enzyme mixtures on the feed market, there is Rovabio Excel™ used in animal feed in order to improve cereals digestibility. This cocktail is produced by the filamentous fungus *Penicillium funiculosum* strain IMI 378536. Guais et al. (2008) showed that the Rovabio contained various activities including CBH I with two potential proteins belonging to the family of glycoside hydrolase family 7 (GH7).

To date, several enzymes of this consortium have been characterized. From 2-gel electrophoresis we found that the major protein of the present in the cocktail corresponds to a GH7. This class of enzyme is described as cellobiohydrolase, which hydrolyze cellulose chains from their reducing end to release cellobiose. Here, the major protein was further characterized regarding activity and specificity. Firstly, the protein was purified to homogeneity, optimum conditions were determined, as well as thermo- and pH stability, on wheat β-glucan. Then, kinetic parameters were determined (K_m, and k_{cat}) on various model substrates in order to reveal the main activity of the protein. According to these results, the protein from *P. funiculosum* was proven to be a β-glucanase.

PP22

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

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Barley seed limit dextrinase (LD) hydrolyses α-1,6 glucosidic bonds derived from starch. It has been suggested that the disulfide reductase barley thioredoxin h (HvTrxh) regulates the interaction between the LD and its endogenous inhibitor (LDI) in germinating barley seeds. LDI is a 12 kDa CM-protein containing four disulfide bonds and a glutathionylated cysteine. Here full reduction of LDI by thioredoxin is demonstrated to correlate with complete inactivation LDI. HvTrxh not only reduced the disulfide bonds in LDI, but also the mixed disulfide thereby releasing glutathione. 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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