Purification of Polygalacturonase Enzyme from Strain F58 of *Fusarium oxysporum*

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Polygalacturonases catalyze the random hydrolysis of 1, 4-α-D-galactosiduronic linkages of plant cell wall pectin. In nature they are agents of fungal pathogenicity but these enzymes are also economically important in industry for fruit juice clarification. The purpose of this study was to extract and purify polygalacturonase enzyme by ion exchange chromatography techniques. Polygalacturonase was assayed from sample of 10 days old inoculated Pectin zymogram medium in pH 4.5. Purification of enzymes was achieved by ammonium sulfate precipitation, followed by cation exchange chromatography on CM Sepharose fast flow column equilibrated to pH 5.5 with 50 mM sodium acetate buffer. Elution of the column carried out with NaCl 1 mol gradient. 3 ml fractions were collected and assayed for enzyme activity. Fractions with highest enzyme activity were pooled and the precipitated proteins were added onto SDS PAGE. The results of chromatography showed one enzyme activity peak between 0.2–0.4 of gradient salt in purification profile. Fractions with highest enzyme activity were pooled and the precipitated proteins were used for Electrophoresis by SDS-PAGE methods. SDS-PAGE showed two distinct bands about 34 and 32 KDa. These two bands showed PG activity in gel containing pectin as substrate (Activity staining). Regarding the importance and value of this enzyme, scientific improvement and cost effectiveness of production processing, extraction, purification and production of this enzyme could be an important due to its application in industry.

Monitoring the Dynamics of Transcription and Translation within the Time Course of Recombinant *E. coli* Cultivations

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Among the key objectives for the optimization of recombinant protein production on industrial scale high yield of the product is one of the most important issues. To achieve high quantities of recombinant protein strong host/vector systems are utilized, generally leading to an overburden of the host cell metabolism. To cope with different types of stress cells have evolved complex regulatory entities, such as the stringent response network, enabling up- and down regulation of promoters by highly specific signal molecules (like ppGpp and sfactors).

In this work we describe a broad screening approach for the detection of marker proteins inferred from variations of genome and proteome patterns to quantify the actual metabolic load of host cell. A combination of microarray analysis, difference gel electrophoresis (DIGE) and MALDI-MS technology together with the application of time series experiments during defined recombinant cultivation processes of *E. coli* HMS 174(DE3)pET11a provide an insight into interactions between metabolic pathways and regulatory networks [Ong et al., Bioinformatics 18, Suppl. 1, S241–S248]. Microarray and DIGE data showed a significant increase in the number of altered genes or proteins, whereof a dramatic change in the cell during this stress response could be derived.

After MS-identification regulatory networks will be reconstructed by clustering proteome and genome data and correlated to process relevant data like growth of BDM. Bioinformatic based tools provide means to identify bottlenecks of metabolism enabling reverse engineering of host cell for the optimal exploitation for recombinant protein production.
30.3 Proteomic Approach for the Establishment of Substantial Equivalence of a Transgenic CRY1AB Expressing Rice with Its Traditional Counterpart, Cultivar Tarom Molaii

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Substantial equivalence may be considered as a part of food safety assessment to survey risk of genetically modified food crops. The concept implements the establishment of sufficient (substantial) levels of similarity (equivalence) between a transgenic crop/food and its traditional counterpart which is in most cases the original variety used for transformation.

During the international year of rice (2004), Iranian scientists released the first insect resistance genetically modified (GM) rice for human consumption. The transgenic rice contained a cry1Ab gene under the control of a PEP-carboxylase promoter. Before commercial release intensive food safety assessment was conducted that included the establishment of substantial equivalence of this GM rice with its traditional counterpart the Iranian high quality aromatic rice variety Tarom Molaii. For the realization of this objective, in addition to measurement of more than 40 agronomic and biochemical traits of the plants randomly selected from the experimental plots in the field, two-dimensional gel electrophoresis was used to compare proteome of dehulled seeds of those plants. Of about 700 protein spots reproducibly detected on 2-D gels analyzed, no significant qualitative or quantitative differences, was detected between GM and non-GM seeds. Our results showed that the expression of major proteins and nutritional components of the seeds and the measured agronomic traits were not affected by the genetic alteration of this independent transgenic event.

30.4 Differential Proteomic and Phosphoproteomic Analysis of Meiotic Maturation of Oocytes

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Mammalian oocytes play a key role in many areas of reproductive biology. Technologies such as stem cell therapy, assisted reproduction and preservation of genetic diversity place an increased demand for mature oocytes with full developmental competence. Meiotic division of oocytes (maturation) is a complex process regulated by precise timing of protein synthesis and degradation as well as by protein phosphorylation. This work is focused on characterization of global protein patterns of pig oocytes during in vitro maturation (IVM) and on identification of proteins/phosphoproteins that are typically associated with different stages of meiotic division. We employed proteomic approach for analysis of pig oocytes during IVM, in particular in germinal vesical (GV) stage and metaphase of the first and second meiosis (MI and MII). The oocyte lysates were separated by mini 2-DE, protein profiles were compared and proteins typically expressed in various stages of IVM were identified by mass spectrometry. Because pig genome is far from completeness, MALDI peptide mass mapping itself revealed only a few porcine proteins. Majority of proteins was identified based on cross-species homology to other mammals and their identity was confirmed by PSD-MALDI MS or LC-MS/MS sequencing. The phosphoproteome analysis was performed by 2-DE immunoblotting using specific antibodies followed by MS identification. The comparative analysis of GV, MI, and MII protein profiles revealed proteins/phosphoproteins characteristic for the individual stages of maturation. Proteins differentially regulated during IVM may present potential biomarkers reflecting the oocyte quality and help to understand mechanisms of oocyte maturation at the protein level.

30.5 Meta-proteomics, a New Way to Explore Microbial Function in Natural Environments

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Natural environments provide diverse habitats for microorganisms. However, we know little about the in situ functions of the microbes. Metagenomic sequencing has significantly expanded the current gene and protein database and makes post-genomic research feasible. In this study, we applied proteomics to explore protein expression profiles of various marine microbial communities. Soluble proteins extracted from three sources, Chesapeake Bay picoplankton (<3 μm), bacterial epibionts of the hydrothermal vent polychaete worm Alvinella pompejana, and sponge-microbe complexes, were analyzed by two-dimensional gel electrophoresis. The proteome fingerprints from these samples are distinct. Abundant protein spots are excised and characterized using either the mass fingerprinting assay (MALDI-TOF mass spectrometry) or the de novo sequencing (tandem mass spectrometry). With the limited database for environmental proteomes, MALDI-TOF MS did not provide significant hits. However, three proteins from Chesapeake Bay were analyzed by tandem mass spectrometry and found closely related with environmental clones within Craig Venter’s metagenomic database. Sample CB1 corresponding to hypothetical protein is not significantly similar to any known proteins in sequence databases. Sample CB3 may correspond to subunit 7 of the NADH:ubiquinone oxidoreductase (complex I) while Sample CB6 is similar to a family of predicted amino peptidases. Metaproteomics will become a useful method compliment to current metagenomics to explore the microbial functions in natural environments.
30.6 Identification of Growth Factors by Functional Selection on Combinatorial Libraries of Affinity Ligands

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Objective: To identify proteins that support cell growth by functional selection using affinity ligands.

Methods: Conditioned cell medium from stimulated PMBC cells was incubated with a combinatorial library of thousands of affinity ligands synthesized on chromatography resin beads. Each bead has a millions of copies of a single, unique ligand. Proteins in the medium were fractionated on the beads; trace proteins were concentrated and abundant proteins were diluted. After washing to remove unbound and weakly bound proteins, the bead-bound proteins were co-cultured with NK-92 cells in 384-well plates for 48 hours.

Results: Clumps of live cells grew on or near about 30 beads. The ligand on one of the beads was sequenced, scaled up and used to identify the active factor by affinity purification. Purification was enhanced by narrowing the protein concentration range of the medium on a combinatorial peptide ligand library before incubation with the affinity ligand. SDS-PAGE and silver stain analysis of protein bound to the ligand detected a ~15 kDa band that correlated with cell growth when protein-loaded beads were co-cultured with cells. ELISA and Western blot demonstrated that IL-2 was captured by the affinity ligand.

Conclusion: We have developed a discovery method in which the identity of neither the active factor nor its affinity ligand need be known before selection. In this study we have identified IL-2 from conditioned cell medium in an assay for cell-growth; we have also used intact plasma, and serum in this discovery method. This method enables the discovery of novel proteins or activities, which cannot currently be detected or purified from unfractionated biological materials due to interferences from abundant species.

30.7 A Shotgun Approach to Identify Proteins Acetylation by LC-MS

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Acetylation is one of important post-translational modifications which play important roles in the regulation of gene expression and signal transduction pathways. Although histones are the most well-studied acetylated proteins, other nonhistone acetylated proteins which are regulated by histone acetyltransferase (HATs) and histone deacetylases (HDACs) remain unknown. Instead of targeting specific acetylated protein and identifying acetylation sites by GST-pull down, we are developing a shot gun approach to study the global acetylation with HDAC inhibitors treatment. Immunoprecipitation with non-specific anti-acetylated lysine antibodies has been applied to enrich and purify the chemical acetylated standard proteins. The captured acetylated proteins are identified by analysis of GluC digested peptides on LC-MS/MS and the modification sites are located by LC-MS/MS. The results were compared with immunoprecipitation of GluC digested peptides and protein identification with LC-MS. The development of this approach will be applied to study the global acetylation of cell lysate from Jurkat cells treated with HDAC inhibitors and it will facilitate the study of the mechanism of post-translational modification in gene expression and signal transduction pathways.

30.8 Proximity Ligation; a Method That Converts Proteins into DNA Tags

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Proximity Ligation is a protein detection method for highly sensitive and specific protein analysis. This method applies a ligation based mechanism to convert protein into amplifiable DNA tags using affinity reagents. These affinity reagents are attached to DNA strands which can be ligated upon binding and thus form a new sequence representing the protein. The sequence can be amplified and detected using conventional DNA amplification methods such as real time PCR. Several independent recognition events are required to create a signal in proximity ligation which makes this method highly specific. The DNA based signal amplification gives the method great sensitivity and the highly parallel character of DNA provides fundamental advantages in multiplexed applications. Different classes of binders can be used in proximity ligation such as antibodies or DNA-Aptamers. We have developed protocols to convert antibodies into proximity ligation probes enabling highly sensitive detection of a number of proteins. Proximity ligation reactions can be adapted to homogenous and solid phase assay formats. We are working on a triple recognition format of the proximity ligation assay where three binding events give rise to a DNA encoded signal. This method enables very sensitive assays by reducing background ligations and highly specific detections as three independent recognition events are required. We are developing multiplexed proximity ligation formats utilizing the highly parallel character of the assay.

30.9 Fully Integrated Chip for 2D Protein Separation (IEF+MW)

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We report here the theory and experimental results of new 2D device for fast IEF and MW separation. The IEF part is a dielectric membrane with separate conducting channels filled by immobile gels of varying pH (we use novel “parallel IEF”). The MW part is separate open channels with gradient gels for MW separation. This device work in native and denaturative conditions. The 2D device is a consumable chip designed for protein expression analysis and biomarker screening. Measuring approximately 40 mm x 20 mm, it is a single-use product which separates samples in 20 minutes. This product is intended for researchers who want to track protein regulation through protein expression profiling. The 2D device high resolution can resolve an individual protein’s isoelectric point to 0.05 pH units. This makes computer analysis determining the isoelectric point of a protein precise and prevents typical 2D gel errors arising from variable gels and software vagaries.
A Completely Automated Cell Pretreatment Unit for Proteomics

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We present the development of a completely automated cell-pretreatment unit for proteomics, which integrates all procedures from cell culture to lysis without any manual operation. Protein expression profiling is necessary for studying cellular mechanisms, where cell handling is essential. Conventional cell handling method needs the indispensable centrifugation and pipetting for washing cells and changing media, where cell loss is unavoidable. To solve such a labor intensive cell handling procedures and low reproducibility, we challenged to integrate all pretreatment steps in a unit, where procedures and temperature are controlled automatically by sequential programming. The unit is composed of a fabricated microdevice that has a micropore membrane, through which medium exchange, cell wash and protein extraction can be performed without centrifugation. To reduce protein loss in this device, we also developed a novel coating reagent, extraction from seaweed (0.01%, H11021), which coats well even for micro- and nano-scales without any clogging, and completely suppressed the adsorption of proteins. The performance of this coating reagent is superior to that of the conventional coating such as polyethylene glycol (PEG). We will show the results of protein adsorption by scanning electron microscopy (SEM) and fluorescent microscopy. Finally, we analyzed 0.6 pg of a marker protein, stathmin (18 kDa), using these systems resulting in a ten-fold increase in intensity and reducing the total analysis time by one tenth compared with the conventional method using centrifugation. The new cell-pretreatment unit treated with novel coating reagent will be useful for proteomic research.

Classification of Microorganisms Contained in EM (Effective Microorganisms) Based on Automated MALDI-TOF MS Fingerprinting and Pattern Recognition Software

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MALDI was shown to be a fast and reliable method for classification and identification of microorganisms. Applications are conceivable in clinical diagnostics, environmental research, food production control, and homeland security. In combination with dedicated analysis software the identification of bacteria, yeasts and fungi is possible. Here we present the classification and identification of unknown microorganisms in EM (Effective Microorganisms). Due to the fast and easy measurement with MALDI it is possible to monitor the composition of the microorganism community during several days. Species identification was done by comparison with culture type strains.

Bacteria, yeasts and filamentous fungi were grown overnight on agar plates. Small amounts of cell material or cell extracts were placed directly as a thin film onto a MALDI target. HCCA matrix (saturated in 50% acetonitrile, 0.1% TFA) was overlaid and dried by air. 500 MALDI spectra were summarized for each sample. The peak lists were used in the analysis software for identification of the microorganisms by comparison with the stored reference mass lists.

In this study we investigated the species identity and composition of microbial community during the fermentation process into “EMa.” The simple sample preparation resulted in very characteristic profile spectra for each microorganism. Spectra were containing about 5 to more than a hundred peaks depending on the respective species. Complete inactivation in case of potential pathogenic cells could be reached by prior treatment with high concentrations of TFA. The detection limit for living bacterial cells is about 10^4 cells. These results were equal or even superior compared to traditional methods like biochemical activities or 16S RNA sequences.
30.12

**OmicsLink Open Reading Frame Expression Clones of Human Genes (a Platform for Systems Biology and Reverse Proteomics)**

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The advent of systems and integrated biology necessitates the cloning of the entire set of protein-encoding open reading frames (ORFs) of human genes in order to study on proteome level the biophysical and biochemical properties of individual proteins and their molecular interactions, which plays important roles in elucidating the mechanisms of physiological and pathological processes. By using high fidelity PCR system and proprietary RecJoin cloning technology, we have cloned more than 16,000 unique full length ORFs of human genes from 72 high quality human cDNA libraries into 15 types of expression vectors with eight different peptide-tags. These expression-ready clones, named as OmicsLink ORF expression clones, are designed and constructed to be suitable for expression and functional analysis of proteins in different host cells and cell-free systems. We have expressed, in *E. coli* cells, 16,000 individual full length ORF clones with His6-tag under control of T7 promoter. The difficult challenges and problems in data mining, database building, pre-cloning and post-cloning sequence analyses to ensure the authenticity, quality and utilities of OmicsLink ORF expression clones in reverse proteomics research will be discussed. The protein expression database for over 16,000 full length ORFs of human genes in *E. coli* will also be presented.

30.13

**Subcloning and Expression of Human Alpha-Fetoprotein Gene in *Pichia pastoris* Yeast**

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Alpha-fetoprotein protein (AFP) is a fetal and tumor secreted protein which is used as a diagnostic tool. The objective of this study was to produce and purify AFP protein using DNA recombinant technology for applying in diagnostic kits. In this study *Pichia pastoris* as metylotrophic yeast were used for producing AFP protein. For amplifying of interest gene PUC18 vector and for protein expression pHIL-S1 vector were used. After creating recombinant plasmid of pS1-AFP electroporation and lithium chloride methods were used for transferring to susceptible strains of GS115-HIS-. Exotrophic media lacks of histidine were used for screening. In order to identify the cloned gene to yeast genome and produced phenotypes PCR method was applied. The two used culture media were YPG and YPM. The quantity and quality of produce protein were checked by SDS-PAGE and ELISA methods. Restriction analysis of pS1-AFP recombinant and transformed plasmid revealed that contain a piece of 1.78 kbp AFP gene and was shown on 1% agarose gel electrophoresis. That was also confirmed by PCR method. Selection of transformed mutant strains of muts in comparison to mut+/H11001 and culturing of those in glycerol media (YPG) until OD600 × 6 and then transferring to methanol media (YPM) with addition methanol of 1% final concentration resulted in inducing protein production in exotrophic media with lack of histidine. The rate of induced protein was 10 mg/mL. In summary, including acid phosphatase gene as a signal, and promoter AOX1 at the beginning of AFP gene is suitable for expression and secretion. It is postulated that it could be useful for monoclonal antibody and designing diagnostic kit.