Session 6: Plant Biochemistry

Oral presentations

O6.1

Plant bioreactors — *in* and *ex situ* production of recombinant proteins

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In the past decade, accomplishments in biochemistry and molecular biology and genetics enabled scientists not only to gather a large amount of experimental data on structural and functional organization of genomes and metabolomes but also to develop tools for manipulation and highly specific modification of the gene expression in *Eukaryota*, including higher plant species. Development of genetic engineering resulted in creating technologies which enable the construction of plants characterized by novel, desirable traits. With the advent of biotechnology and gene transfer technology, a broad and extensive research brought about different expression systems for recombinant/heterologous proteins of industrial and pharmaceutical value. In the past decade, plants have been actively considered as important expression systems of numerous recombinant proteins as they undergo acetylation, phosphorylation, and glycosylation as well as other post-translational modifications required for the biological activity of many eukaryotic proteins. Today, the plant expression system is recognized as a new platform useful for the production of a large amount of proteins that can be either accumulated in plant tissues (*in situ*) or, after rhizosecretion, recovered from root exudates of hydroponic cultures (*ex situ*). Plants, as an alternative system for recombinant protein production, require specific methods of protein product isolation and purification. Currently, most of recombinant proteins are extracted from biochemically complex plant tissues using solvents, which makes downstream processing and purification of individual protein a laborious, difficult and costly process and a major obstacle to large-scale protein manufacturing. An alternative for this protein extraction and purification system is rhizosecretion of target protein–**E**lastin-**L**ike **P**olypeptide fusion protein in the hydroponic medium and application of thermo-sensitive characteristics of ELP domain for its purification. ELPs are biopolymers consisting of the elastin-based repeat pentapeptide motif Val-Pro-Gly-Xaa-Gly (where the "guest residue", Xaa, is any amino acid except Pro) which undergo a sharp inverse temperature phase transition (T_t 2–3ºC). The T_t depends on ELP molecular weight, amino acid substitution of Xaa, applied solvent, and ELP concentration as well as ionic strength of the solution. Reduced concentration of ELP results in a decrease of T_t which can be balanced by an increase of salt concentration, a process which enables the recovery of the protein fused with ELP from the solutions of high dilution degree such as medium of hydroponically grown plants or hairy root cultures. Recent developments and perspectives of the future application of *in* and *ex situ* production of recombinant proteins using plant bioreactors will be discussed.

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O6.2

The evolution of PR-10 proteins: the changing function and the structural conservation

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Plants respond to pathogen infection or environmental stress by increasing the expression of a number of genes that encode pathogenesis-related proteins. Up to date, PR proteins have been classified into 14 families based on their biological role and/or physicochemical properties. The biological functions of most classes of the defense proteins have been recognized. The role of some PR proteins, including PR-10, in defense response still remains to be elucidated. Amino acid sequence alignment as well as phylogenetic analysis allows distinguishing three major groups of that protein class: (i) Intracellular pathogenesis-related (IPR). Many IPR proteins have allergenic properties and can be found in pollen grains, fruits and vegetables. Almost all of these proteins cause IgE-mediated type I allergy. On the basis of weak sequence homology, ranging from 17 to 20%, the presence of characteristic sequence motifs and secondary structure predictions, two additional distinct groups of proteins have been tentatively included in the PR-10 class: (ii) Major latex proteins (MLP) present in the latex of some plants (opium poppy, bell pepper) and (iii) Cytokinin-specific binding proteins (CSBP), for which the ability of plant hormones binding have been shown. The phylogenetic tree clearly distinguishes the three groups. The presence of sequences from the same species in different branches can be explained by numerous gene duplications which occurred in the past and led to creation of numerous copies of the same gene in a common ancestor. The oldest of these duplications took place before the separation of monocots and dicots and resulted in arising of the three major groups described above. Subsequent duplications occurred before the emerging of present species. It is clearly visible in legume subtree where, for example, the sequences of yellow lupine IPR proteins group in different branches resulting in the presence of different PR-10 protein subclasses within one species. Within subclasses, most likely due to concerted evolution, a large number of very similar genes (97–99% identity) coexist. The consequence of the numerous gene duplications is coding of PR-10 proteins by multigene families. All studied PR-10 proteins, including CSBP proteins represent the same canonical model of the crystal structure. It is possible that the CSBP proteins have evolved from the PR-10 group to specifically bind only one group of ligands, cytokinins. Since they are cytosolic proteins, a protective or transport function within the plant cell could be proposed. The low level of sequence conservation between the CSBP and PR-10 groups suggests an early divergence or fast mutation rate. In addition to mutations affecting the volume and chemical character of the ligand binding cavity, there are numerous other changes. Nevertheless, the evolutionary pressure has left the general protein fold intact, suggesting its importance and confirming the ligand-binding role for PR-10 proteins.

O6.3

Wide contribution of protein phosphatase ABI1 (PP2C) to the *Arabidopsis thaliana* abiotic stress response

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To better understand the role of ABI1 in abiotic stress response and ABA signaling, we characterized the phenotype of a null allele for the *ABI1* gene selected from SALK T-DNA insertion collection. The absence of the ABI1 protein was manifested by a hypersensitivity to ABA and glucose in germination assays and also in root growth. The ABA-induced stomatal closure was also more pronounced in *abi1* compared with WT, which resulted in substantial drought avoidance in the mutant line. We demonstrated that ABI1 interferes with guard cell signaling upstream of ROS production and cytosolic calcium elevation. Furthermore, to identify candidate genes associated with abiotic stress responses, we performed a microarray screen of *abi1* and WT Col-0 plants. Microarray analysis has shown a modified transcriptional profile in *abi1* upon ozone and drought stress. Using 2 ANOVA testing we obtain 154 and 193 genes that showed significantly different responses between WT and *abi1* mutant to drought and ozone stress, respectively. In both treatments, some genes showed a stronger transcriptional response in *abi1* than in WT ('gain of regulation'), whereas other genes displayed a weaker response in *abi1* than in WT ('loss of regulation') where regulation refers to either up- or down-regulation in response to the stress in the *abi1* mutant. Furthermore to verify that the microarray approach to functional discovery is effective we demonstrated an ABI1-dependent regulatory mechanism controlling both ABA and ethylene biosynthesis. In summary, performed analysis revealed a complex ABI1 signaling network in ozone and drought stress conditions, suggesting a relationship between ABI1, stress signaling, and metabolic management. Thus, the results provide relevant information to integrate ABI1 with processes related to plant stress tolerance.

O6.4

Analysis of expression and function of glucosyltransferase

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Glucosylation is a prominent modification reaction in plant secondary metabolism. In many cases glucosylation is the last step in the biosynthesis of a number of secondary plant products including flavonoids, cyanohydrins, steroidal alkaloids, and saponins.
Glucosyltransferases are found in many organisms, and their particular importance for plants has been suggested In plants where a variety of enzymes has been detected, they exhibited diverse functions and anthocyanin metabolism regulation, auxin metabolism modulation, and an unknown function induced by methyl jasmonate and salicylic acid are among them.
Recently the gene encoding *Solanum sogarandinum* glucosyltransferase has been cloned during a screening of cold induced library. The functional characterization of both coding end regulatory sequences was performed.
First for *in vitro* analysis, the glucosyltransferase (GT) was expressed in pET wector and the protein was used for antibody generation and establishing enzyme specificity. Activity against both antocyanins and kampferol was confirmed.
In parallel the promoter specificity was analyzed by GUS method. It was established that glucosyltranferase promoter is strongly activated by UV light, cold and ABA. The obtained results were confirmed with Northern and Western blot analysis.
To study glucosyltransferase function *in vivo* two sets of transgenic plants were created- overexpressing glucosyltransferase alone and in tandem with dihydroflavonol reductase (DRF), a key enzyme of flavonoid synthesis in potato plants. Overexpressing GT in potato plants resulted in increased flavonoids content. The transgenic plants displayed increased resistance to pathogen infection accompanied by a significant increase in tuber yield. In contrast in plants over-expressing both the transgene for DFR and the transgene for GT, the synthesis of phenolic acids was diverted away from the anthocyanin branch- large amounts of kaempferol, chlorogenic acid, isochlorogenic acid, sinapic acid and proanthocyanins were detected. The study of various metabolite levels in transgenic plants confirmed the importance of glucosyltransferase in regulation of metabolite flux in phenylpropanoid pathway.

O6.5

Plants defense mechanisms in response to wounding

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Plants respond to mechanical wounding or herbivore attack not only in damaged tissue (local response) but also at distal site of wounding (systemic response). The plant hormone jasmonic acid (JA) and its methyl ester (MeJA), collectively referred to as jasmonates, represent the well characterized class of endogenous signals involved in long-distance defense response. Other components are implicated in these signaling pathways including: systemin, oligosaccharides, reactive oxygen species, abscisic acid and ethylene. Jasmonic acid-dependent and -independent wound signal transduction pathways have been identified and well characterized in *Solanaceae* and *Arabidopsis* thaliana. Reversible protein phosphorylation regulates both JA-dependent and -independent wound signaling pathways. Some of the specific for plants calcium dependent protein kinases (CDPKs) are involved in wound signal transduction. ZmCPK11 is a wound-responsive maize CDPK. In leaves, kinase activity and *ZmCPK11* mRNA accumulation were stimulated by wounding. The level of *ZmCPK11* is also increased in noninjured neighboring leaves. The results suggest that the maize protein kinase is involved in a systemic response to wounding. (Szczegielniak *et al.*, 2005). The recent results demonstrate that ZmCPK11 is essential component of wound induced JA-dependent signaling pathway.

Reference:

Szczegielniak J, Klimecka M, Liwosz A, Ciesielski A, Kaczanowski S, Dobrowolska G, Harmon AC, Muszyńska G (2005) A wound-responsive and phospholipid-regulated maize calcium-dependent protein kinase. *Plant Physiol* **139**: 1970–1983.

Posters

P6.1

Mutagenesis and expression of an active-site mutant of yellow lupine L-asparaginase

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In plants, L-asparagine is the most abundant metabolite for the storage and transport of nitrogen that is utilized in protein biosynthesis. Asparagine hydrolysis is catalyzed by the enzyme asparaginase. Kinetic studies of asparaginase from *Lupinus luteus* (LlA) show that the enzyme is predominantly active as isoaspartyl aminopeptidase. In this light, LlA gain in relevance as enzyme responsible for the degradation of misformed proteins, in which a peptide bond occurs at the side chain of asparagine or aspartic acid. The crystal structure of LlA demonstrates that it is composed of two subunits, a and b, generated during autoproteolytic cleavage of the precursor molecule at Gly192-Thr193 peptide bond. The threonine residue that becomes an N-terminus of subunit b plays crucial role in both, enzymatic reaction and autoactivation. We have designed, cloned and expressed an active-site mutant of LlA with the catalytic threonine residue replaced by an alanine in order to prevent autocleavage and preserve precursor molecule. We intend to make biochemical tests on LlA mutant to confirm decline enzymatic activity as well as determine its crystal structure to analyze structural basis of maturation mechanism.

P6.2

Analysis of three groups of pollen hydrolytic enzymes of tristylous *Lythrum salicaria* using two-dimensional electrophoresis

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Lythrum salicaria, now a widespread invasive species, exhibits tristyly, a form of heteromorphic selfincompatibility. In tristyly, each plant exhibits one (and only one) of three morphologically different floral forms. A flower of *L. salicaria* produces two types of stamens, with a characteristic sort of pollen, and these two exhibit different incompatibility reactions. Each flower has also one of three types of styles. These generative elements in one particular flower always have three different lengths. Differences between stamens of a single flower could be the result of epigenetic phenomena. We performed two-dimensional gel electrophoresis (2-DE) to analyze some protein extractomes including three groups of hydrolytic enzymes (esterases, proteases and acid phosphatases) derived from the six different stamen types (two from each of three floral forms). Herein, we present the results concerning enzyme analyses. We revealed that there were significant quantitative differences between esterases detected in two pollen types from the same flower. Furthermore, analysis of proteases and acid phosphatases showed also qualitative differences between these enzymes in two pollen types derived from the same flower.

P6.3

Comparative analysis of 2a polymerase protein sequence of chosen Polish strains of Peanut stunt virus, PSV

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Peanut stunt virus (PSV) is a member of the genus *Cucumovirus* in the family *Bromoviridae*. It is one of the most important pathogens of the legumes all over the world. Its genome consists of positive single strand RNAs: RNA1 and RNA2 encode 1a and 2a protein, respectively, RNA3 encodes 3a movement protein and a coat protein translated from the subgenomic RNA4. In some strains along with their genomic and subgenomic RNAs may be packed fifth component designated as satellite RNA (satRNA).
The proteins 1a and 2a are the compounds of the viral replicase complex. The N-terminal region of the PSV 1a protein contains methyltransferase domain involved in capping of genomic strands, whereas the C-terminal encodes helicase domain. The PSV 2a protein has the characteristic conserved motif of RNA-dependent RNA polymerase (RdRp).
In our study we have analysed the 2a polymerase protein sequence of chosen Polish strains of PSV from celery (PSV-Ag) and pea (PSV-G) and we have compared them to other known sequences from the genus *Cucumovirus*.
RNA from purified virus particles was isolated by phenol/SDS extraction followed by ethanol precipitation. Then we have carried out the RT-PCR reactions to amplify the 2a protein of examined PSV strains. The specific primers were designed on the basis of known sequences of PSV RNA2. The products of amplification were subsequently cloned, sequenced and analysed in BLAST. The phylogenetic analysis was performed in PAUP 4.0 software. The comparison of 2a protein nucleotide sequences has shown the highest similarities (84%) to PSV-ER and PSV-J belonging to subgroup I of PSV. Furthermore, the amino acid identities with mentioned above PSV strains range from 86–87%, whereas the similarities 90–92%. The analysis of RdRp domain shows also high amino acid identities to other *Cucumovirus* ranging from 79–96%. Obtained data confirms our previous research on coat protein sequences of Polish isolates which indicated the high homology with subgroup I of PSV and their close genetic relation to each other.

P6.4

Quantitative analysis of yellow lupine csbp genes expression

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The presense of CBP proteins (*Cytokinin Binding Protein*) was demonstrated in many of plant species, algae and bacterias. The investigations resulted in isolation of a cytoplasmal mung bean protein with high affinity to these phytohormones (Fujimoto *et al.*,1998). The protein mass was determined at 17 kDa (*Vigna radiata* cytokinin specific binding protein).
Searching of a protein data bases and comparison of amino acid sequences of PR-10 (PR, *pathogenesis related proteins*) and CSBP proteins showed less than 20% identity. However, on the ground of their secondary structures' similarities it was proved that both protein groups have analogous construction of β sheets and α helics (Pasternak *et al.* (2006) *Plant Cell* **18**: 2622–2634). However, phylogenetic analysis clearly distinguish CSBP proteins as a separate subclass.
Two homologue nucleotide sequences were found in yellow lupine expression library: *Llcsbp*1 and *Llcsbp*2. These homologues differ in terms of sequences and number of coding amino acids: LlCSBP1-158 aa, LlCSBP2-155 aa.
The goal of this study was quantitation of *Llcsbp*1 and *Llcsbp*2 gene expression level in variuos organs of yellow lupine, using reverse transcriptase real-time PCR method with SYBR Green I as a detection format. The obtained quantitative results were normalized towards ubiquitin expression level as a control gene. The specificity of the reaction was confirmed by melting curve analysis and agarose gel electrophoresis for all fragments of the investigated genes. Finally, statistical tests were performed.
The results showed significantly higher *Llcsbp*1 gene expression level than *Llcsbp*2 in such organs as young and old leaves, pods and roots. The situation appeared to be different only in seeds, where the expression level of *Llcsbp*1 gene was by half lower than the expression level of *Llcsbp*2.
This experiment confirms diverse and low levels of expressed both homologue genes in organs under investigation. That suggests, they might be involved in signal transduction induced by cytokinins, during various physiological processes.

P6.5

Application of AFLP and GISH techniques for identification of introgressions conferring abiotic stress resistance in a Lolium-Festuca complex

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Good-quality forage cultivars from *Lolium* are not sufficiently resistant to environmental conditions in less favoured areas, contrary to *Festuca* winter hardy, drought tolerant and persistent species. Hybridization between *Lolium* and *Festuca* takes place with relatively ease, their chromosomes pair, and recombine freely in their hybrids and derivatives. However hybrids found in nature are usually sterile therefore breeding procedures are applied to restore partially or completely their fertility. They can be next utilized in introgression backcross breeding programmes to improve *Lolium* species abiotic stress resistance, which allow to transfer of limited number of *Festuca* genes to the single *Lolium* genotype. To identify *Festuca* chromosome segments in recipient *Lolium* species and determine their chromosome location genomic *in situ* hybridization (GISH) is used. Furthermore AFLP method can be utilized, what additionally leads to generation of genomic markers linked with selected agronomic traits.
This studies demonstrate application of two different introgression breeding programmes where hybrids between *Lolium* and *Festuca* were used to obtain *Lolium* forms with enhanced winter stress resistance. The pentaploid F_1 hybrids *F. arundinacea* (2n = 6x = 42) × *L. multiflorum* (2n = 2x = 14) was backcrossed into diploid *L. multiflorum* cv. Tur. *L. multiflorum* genotypes with single *Festuca* chromosome segments were used to obtain two diploid populations, representing BC_3 and BC_4 generations, containing more freezing resistant plants. In the second programme partially fertile triploid hybrids were obtained by crossing *F. pratensis* (2x) and *L. perenne* (4x). F_1 plants used as male parent were backcrossed to diploid *L. perenne.* Genomic structure of BC_1 genotypes was defined by GISH and their winter hardy were scored. Genotypes obtained from both breeding procedures having improved stress tolerance comparing with their parental forms were analysed through AFLP technique. Using different primer combinations it was possible to detect introgressions linked with freezing resistance and winter hardy, which can be used as selective markers.

P6.6

Biological interaction between chlorogenic acid derivatives and calcium ions in lettuce (*Lactuca sativa*)

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Chlorogenic acid (3-*O*-ceffeoyl-D-quinic acid), an ester of caffeic acid with quinic acid, is one of the phenolic compounds from the group of hydroxycinnamic acids found in lettuce. It has many properties beneficial to health, among other things antioxidant, antimutagenic and anticarcinogenic. In this work the effect of calcium ions on the content of chlorogenic acid in fresh and storaged leaves of lettuce were studied. The changes of total phenolic compounds expressed as chlorogenic acid equivalents and antioxidant activity were also evaluated in this research.
The plants from pot experiment were treated with 0.1 M $CaCl_2$ solution as spray on leaves and then divided into three parts one of that was processed directly after harvest and others were being storaged for 7 and 14 days at 5°C. In this studies lyophilized samples of lettuce "Omega" cultivar were used from whitch chlorogenic acid was extracted with 70% (v/v) aqueous methanol solution. After evaporation to dryness the residues were dissolved in water and cleaned by means of SPE extraction using Sep-Pak C_{18} column. Sampling fraction containing phenolic compounds was eluted with 60% (v/v) aqueous methanol solution and next was separated on individual components by HPLC, Eurosil Bioselect 300-5 C_{18} Column (300 × 4 mm) with gradient elution: A-5% formic acid in water, B-methanol. The content of chlorogenic acid was calculated with the use of calibration curve. Total phenolic compounds of isolated fraction was determinated according to the Folin-Ciocalteu procedure. Antioxidant activity was determined by DPPH assay.
Results from our experiment indicated that calcium ions used in our research have beneficial effect on formation of chlorogenic acid and dicaffeoyl tartaric acid in the leaves of lettuce. There was found approximately threefold increase of the level of chlorogenic acid and 30% increase of the level of diceffeoyl tartaric acid in compare to control. High correlation coefficient between antioxidant activity of isolated fraction, the level of chlorogenic acid and calcium ions indicates on high contribution of this ions in biosynthesis of phenolic acids.

P6.7

The application of nanotechnology for detection of physiologically active peptides

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Cell-based biosensors are novel bioelectronic portable devices containing living cells [1]. Plant cells have a highly sensitive and selective perception system for detection of bioactive agents [2]. The extracellular alikalinisation rate of plant suspension culture is important for detection phisologycal changes in the micro-enviromental space of cells. Screan printed electrode containing ruthenium dioxide is able to detect of such changes and have been used as the electrochemical transducer. The electrode response dependet upon H^+ concentration in extra cellular solution.

We show that cells of tobacco and protoplasts of maize attached to the electrode sulface can be used to the detection of flagellin the virulence factor of *Agrobacterium tumefaciens* and systemin-like peptides from pollen of maize. Such obtained disposable sensor made possible to measure the pH changes in a distance of 10–50 nm from the cell plasma membrane, with good sensitivity and reproducibility. The elaborated cell-based biosensor with tobacco cells can be useful for detection of 10 nM purified flagellin or of crude extract of bacteria [3]. The results demonstrate that this kind of device has potential to monitor changes of living cell for a long term, and to evaluate bioactive components of pathogens. Therefore, the objective of the presented research was to develop a rapid procedure for detection of other bacteria or viruses by developing of this system.

Next work was the direct electrochemical detection bioactive systemin-like peptides from maize pollen. Such peptides interact with specific receptors on protoplast membrane and inactivated H^+-ATPase. It is possible that short peptides play important function in pollen stigma interaction and particulary in self-incompatible plants. Biosensor containing plants living cells can be very useful tool for detection of self-incompatibility.

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P6.8

Comparison of the content of triterpene acids in edible berries of three wild *Vaccinium* species occurring in Poland

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Compounds present in the fruits of *Vaccinium* species, regarded as one of the richest sources of antioxidant phytonutrients, are reported to play several roles in human health maintenance. Meanwhile, another important group of dietary nutraceutics, i.e. triterpenoids, was not well characterized in these plants. Thus, the purpose of this work was the investigation of triterpenoidal constituents in three wild species bearing edible fruits, i.e. bilberry *Vaccinium myrtillus* L., cowberry *V. vitis-idaea* L. and cranberry *V. oxycoccus* L., naturally occurring and readily harvested in Poland.

Collected berries were used either dried to study the contents of triterpenoids in the entire fruits, or fresh to isolate the constituents of epicuticular wax layer. Dried berries were powdered and extracted in Soxhlet apparatus with diethyl ether. The surface of fresh undamaged berries was gently extracted by chloroform and ethyl ether. Obtained extracts were separated by TLC and isolated triterpene compounds were assayed by GLC.

Two isomeric acids, oleanolic and ursolic, were identified as the main triterpenoid constituents in all berries studied. The total content of both acids were rather similar, amounted to 0.7% of dried weight in bilberry, 1% in cowberry and 0.9% in cranberry. However, the ratio of oleanolic to ursolic acid was different, equaling in bilberry 1:0.58, in cowberry 1:2.8 and in cranberry 1:4.7. Thus, oleanolic acid is almost twice more abundant than ursolic acid in bilberry, whereas in two other berries the amount of ursolic acid is higher, in cowberry more than twice than oleanolic, in cranberry almost five times. In fruit epicuticular wax layers the same acids were detected, with the ratio of oleanolic to ursolic acid respectively 1:0.46, 1:3.8, 1:5.1 in bilberry, cowberry and cranberry. As we discovered previously, the oleanolic acid is more abundant also in other parts (i.e. leaves and rhizomes) of the bilberry plant, whereas ursolic acid is dominant in cowberry and cranberry plants.

The obtained results have shown that in evergreen *Vaccinium* species, i.e. cowberry and cranberry, the ursolic acid is predominant, whereas in bilberry, shedding leaves for the winter, oleanolic acid is the most abundant. It can indicate the presumable difference in physiological activities of these two isomers, as it is known for other isomeric compounds produced by plants (for example antibacterial (+)-catechin and phytotoxic (–)-catechin). Durability and resistance of cowberry and cranberry fruits are ascribed mainly to their high content of benzoic acid, however, the role of the thickness of epicuticular waxes layer rich in ursolic acid also regarded as the potent preservative, cannot be ruled out.

P6.9

The 35 kDa acid phosphatase isolated from lupin (*Lupinus luteus*) cotyledons displaying characteristics of LMW tartrate resistant acid phosphatases (TRAP)

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Phosphorus metabolism in plants is controlled by numerous acid phosphatases. This diversity makes it difficult to isolate and characterize a single native enzyme, especially that of not dominating form in the testing tissue, from among the others displaying similar activity. Introducing a relatively simple procedure comprising a preparative native PAGE as the main purification step, together with DEAE-Sephacel anion exchange chromatography and gel filtration on Superdex 75, let us to separate two low molecular weight (35 kDa and 42 kDa) acid phosphatases from lupin cotyledons. The first of them was the subject of more detailed study.

The 35 kDa enzyme is a thermostable tartrate-resistant metallophosphatase. It exhibits an affinity for phosphate monoesters of various aryl substrates and for inorganic pyrophosphate in a broad pH range with optimum at pH 5.0. It displays neither phosphodiesterase nor acidic or alkaline phytase activity. The high level of hydrolysis of phosphotyrosine but not phosphoserine, may suggest its possible function *in vivo*. This form of acid phosphatase was detected in all vegetative organs of the lupin seedlings but not in the dry seed extract. The level of its activity increases in phosphorus-deficient conditions, especially in roots.

Despite the 35 kDa lupin enzyme appears to be an acidic, ConA non-reactive protein, which differentiates it from the animal LMW TRAP (basic glycoproteins), its overall biochemical characteristics is close to this group of enzymes. The potential mammalian-like phosphatase sequences were detected in a wide range of plants [1-4]. Only one protein representing this group, possessing an acid phosphatase activity, has been isolated from phosphate deprived *A. thaliana* seedlings [4], but its substrate specificity is not known. The conservation of amino acid residues involved in coordination of metal ions in the active centre, as well as similarity of the predicted three-dimensional structure of the plant enzymes to the known structures of the mammalian ones [1] let to presume similar enzymatic activity of both groups. The elucidation of the possible relationship of the isolated lupin enzyme to the plant mammalian-like LMW acid phosphatases is the subject of our ungoing studies.

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P6.10

Proteo-, chitino- and lipolytic enzymes production by entomopathogenic fungus *Conidiobolus coronatus*

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Entomopathogenic fungi posses the ability to invade insects actively, by penetration of their cuticle. Key-role in this process plays the production and secretion of cuticle degrading enzymes such as proteases, chitinases and lipases.

C. coronatus colony 59 (causing 100% mortality of *Galleria mellonella* host) was grown for 30 days in liquid medium Luria Broth (LB). After that time the medium was filtered through filter paper, and mycelium was collected and homogenized by sonication. The activities of elastase, chymotrypsin, N-acetyloglucosoaminidase (NAGase), chitinase and lipase were compared in fungal homogenate and in post incubation medium. The enzymatic activities were measured by spectrophotometry and fluorimetry with the use of suitable synthetic substrates.

In the homogenate the activities of three enzymes were detected: elastase, NAGase and lipase, whereas in the post incubation medium the activities of all five examined enzymes were present.

The activity of elastase was significantly higher in the post incubation medium (5.63 ΔA/min/mg) than in the homogenate (8.6×10^{-2} ΔA/min/mg). The activity of chymotrypsin was minute and detected only in the medium (0.18×10^{-2} ΔF/min/mg). The activity of NAGase was similar in both, the homogenate and the incubation medium (0.68 ΔA/min/mg and 0.52 ΔA/min/mg, respectively). On the contrary, the activity of chitinase was present only in the medium (1.14×10^{-2} ΔF/min/mg). The lipolytic activity in the homogenate was ten times higher than in the medium (2.30×10^{-2} ΔF/min/mg and 0.26×10^{-2} ΔF/min/mg, respectively).

To sum up, the activities of proteolytic enzymes were higher in the post incubation medium than in the homogenate. Release of proteases to the environment reflects adaptation to effective insects' cuticle digestion, an essential part of mechanism allowing fungal invasive hyphae to penetrate insect cuticle. Extracellular presence of NAGase and chitinase reflects this adaptation as well. The insects' cuticle contains 70% of proteins, while chitin together with lipids and fatty acids composes app. 30% of it. The mode of action of lipases in infection process was not intensively studied yet. In this experiment lipase activity remained mainly in intracellular compartment.

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N-acetylglucosaminidase activity of entomopathogenic fungus *Conidiobolus coronatus*: optimization of an assay

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The major problem in pest control is increasing insect resistance to traditional pesticides. Naturally occurring entomopathogens are important regulatory factors of insect populations, therefore several species of entomopathogenic fungi are employed as biological control agents. Entomopathogenic soil fungus *Conidiobolus coronatus* (Entomophtorales) also demonstrates high efficiency in insect paralysis.

Penetration of insect cuticle by fungal pathogens is achieved through a combination of enzymatic and mechanical mechanisms. Entomopathogenic fungi produce a range of cuticle degrading enzymes including proteases, chitinases (exo- and endochitinases) as well as lipases that degrade major components of the cuticle and provide nutrients for the fungus.

N-acetylglucosaminidase (NAGase) belongs to exochitinases and cleaves olygomers of insect chitin to N-acetylglucosamine monomers. Assuming that chitinases play essential role in fungal virulence we decided to optimize reaction conditions for NAGase in order to determine the role of this enzyme in *C. coronatus* virulence.

NAGase activity in mycelial homogenate of *C. coronatus* was assayed spectrophotometrically at 405 nm with 4-Nitrophenyl *N*-acetyl-β-D-glucosaminide (*p*NAG, Sigma) as a substrate. Effect of pH on enzyme activity and stability was measured under 10 mM Tris/HCl buffer at pH range 4–8. The stability of the enzyme at various pH levels was examined after pre-incubation for 30 minutes in a solution of tested pH at 20°C (temperature optimal for growing *C. coronatus*) and 30°C (temperature optimal for insect host, *Galleria mellonella*). Effect of temperature was assayed at 20–80°C range. The thermal stability of the NAGase was tested after 30 minutes incubation at the temperature ranging from 20 to 60°C. Effect of substrate concentration was examined with *p*NAG at various concentrations (6×10^{-1}–0.375×10^{-1} mM).

The optimal conditions for assaying the *C. coronatus* NAGase are as follows: amount of fungal homogenate 4.25×10^{-2} mg of fungal protein/ml, substrate concentration 3×10^{-1} mM, reaction time 15 minutes. The pH optimal for NAGase activity was 7.0. Enzyme was more stable at pH 7.0 in the temperature optimal to cultivate the insect host *G. mellonella* (30°C, 2.9×10^{-2} dA/min) than in the temperature optimal to propagate *C. coronatus* (20°C, 2.6×10^{-2} dA/min). The optimal temperature for enzymatic activity was 60°C. Activity of enzyme incubated for 30 minutes at 60°C remained stable (100% activity), while incubation at 30 and 20°C resulted in the 11 and 29% loss of NAGase activity, respectively.

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