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Extracellular lipase enzymes from zygomycetes fungi: production, isolation and examination of biotechnologically relevant properties

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Lipase enzymes (E.C. 3.1.1.3) hydrolyze the triacylglycerols, which are the major constituents of fats and oils, to produce free fatty acids, glycerol and partial acylglycerols. Moreover, many lipases can catalyze the synthesis and translocation of ester linkages resulting biotechnologically important ester compounds. Recently, there is a growing interest for microbial lipases due to their low production costs and wide range of industrial applicability. Accordingly, identification and biochemical characterization of novel microbial lipases have special importance for industrial process development purposes. Zygomycetes are good producers of lipase enzymes; however, only a few enzymes have been isolated and characterized from this fungal group to date. Our knowledge regarding to their synthetic activity in organic media is also limited.

In our studies, 204 zygomycetes fungi were tested on culturing media contained tributyrin for preliminary detection of their extracellular lipase activities. Many *Rhizomucor, Rhizopus, Mucor, Umbelopsis* and *Mortierella* strains showed high enzyme activity and selected for further submerged and solid-state fermentation assays. In those studies, effect of different inductor oils on the enzyme activity is also tested. Enzyme yield of some isolates was outstanding when wheat bran was used as supplement in both submerged and solid state fermentations. Addition of mineral salt solution and olive oil to the solid fermentation medium resulted in at least 1.5-fold increment in the enzyme activities. Lipase production was also tested using oat bran, pressed hempen-, line-, poppy-, pumpkin seed as substrate with high lipid-content. The pumpkin-, and poppy seed residues proved to be promising substrates for lipase induction.

Transesterification assays were performed in non-aqueous conditions using lyophilized crude lipases of selected 11 strains. Enzymes from *Rhizomucor miehei, Rhizopus oryzae, Rhizopus stolonifer, Mucor corticolus* and *Mortierella echinosphaera* exhibited the highest transesterification activity in *n*-heptane using *p*-nitrophenyl palmitate as fatty acid donor and ethanol as acceptor. To characterize the reaction, effect of incubation time and temperature, various reaction media and acceptor alcohol on synthetic activity were also tested. Results showed that prolonged incubation time and high temperature (40 and 50 °C) generally enhanced the product yield. Considering the results of fermentation and transesterification tests, purification and biochemical characterization of *R. miehei*, *Rh. oryzae*, *M. corticolus* and *Mo. echinosphaera* lipases were carried out. SDS-polyacrylamide gel electrophoresis indicated a molecular mass of about 52, 56, 20 and 30 kDa for the purified enzymes, respectively. Biochemical characterization assays including temperature and pH tolerance studies, substrate specificity determination, and examination of the effect of some ions, alcohols and organic solvents on the activity were also performed. Purification of lipase produced by *Rh. stolonifer* is in progress, and synthetic esters formed by transesterification and esterification reactions are also being researched using gas chromatography technique.

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Molecular characterization of ROP GTPase activated kinases in *Arabidopsis*

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The Rho-type GTPases have central roles in cellular processes associated with cytoskeletal dynamics (e.g. cell movement, cell division, cell shape, and cell polarity). These proteins operate as molecular switches: they activate signal transduction pathways when they are in GTP-bound conformation, but their signalling activity cease when they are GDP-bound. If the Rho GTPase is in the GTP-bound form, it can further activate a diverse set of downstream signalling effector proteins.

Plants has a specific group of Rho-type GTPases, the „Rho of plants" (ROP) family. Our knowledge about the signalling pathways associated with ROPs is yet incomplete. ROPs differ from other Rho-type GTPases in the regions which are responsible for effector binding, suggesting that ROP GTPases have specific effectors. Indeed, plants lack the Rho GTPase-activated PAK kinases, which are very important mediators of Rho GTPase signalling in yeast as well as in animals. Therefore our question was: are there any ROP GTPase-activated kinases, which may have PAK-like functions in plants? Due to a yeast two-hybrid screening approach two ROP-interacting kinases could be identified. These kinases interacted with the GTP- but not with the GDP-bound ROP form what is typical for ROP effectors. Furthermore, the in vitro activity of these kinases was dependent on the presence of GTP-bound ROP. These ROP-activated kinases belong to the subfamily VI of receptor-like cytoplasmic kinases (RLCKs) of *Arabidopsis*. They have a receptor kinase-like catalytic domain, but they don't have extracellular or transmembrane regions and that's why they can found in the cytoplasm. Based on their primary structure, the 14 *Arabidopsis* RLCK VI kinases can be classified into two groups (A and B). Only the members of group A have ROP GTPase-binding ability. Based on in silico comparison, several positions were identified where the amino acids are characteristically different in the sequences

of group A and B kinases. It was supposed that these residues might be responsible for the difference in ROP GTPase-binding. Although these amino acid motifs are dispersed along the kinase sequences, on 3D kinase models most of them form a common surface. We replaced these amino acids one by one in the ROP-activated RLCK VI_A2 kinase with typical amino acids of B-type kinases, using site-specific mutagenesis. Changing the motifs prevented ROP GTPase binding in yeast two hybrid system. The mutant kinases were produced in and purified from a bacterial protein expression system. The in vitro auto-phosphorylation activity of the kinase was completely eliminated in the case of certain mutations, while in other instances the activity become independent of the presence of the ROP GTPase. These results were confirmed with the RLCK VI_A3 kinase. As this kinase (in contrast to VI_A2) well phosphorylates the myelin basic protein substrate, we could demonstrate that the mutations affect substrate phosphorylation and auto-phosphorylation in the same way.

We were interested in how ROP-activated kinases are widespread in the plant kingdom. Therefore the evolutionary conservation of the position of ROP-binding amino acids was investigated in kinase sequences of different plant taxa with available whole genome sequence. We could conclude that RLCK VI A-type kinases exist in multicellular plants but not in unicellular algae. Consequently, their function is probably not essential for basic cell functions, but is rather required for the development of a multicellular organisms. This is supported by our experiments using transgenic and mutant plants with altered kinase expression. These studies show that the kinases regulate plant growth and morphology. That the in planta functions of these kinases are dependent on ROP GTPases, still need to be justified. The ROP-binding mutant kinase forms we produced may serve as a tool to answer this question.

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Cell response to UV irradiation: Bomapin is a new member in the damage response

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Ultraviolet light-induced DNA damage response is an extensively studied process because improper or delayed repair of the errors could lead to cancerous malformations. Although several proteins involved in DNA damage repair have been identified, the entire repair cascade and the protein-protein interactions remain to be explored. Based on the wavelengths UV light is classified as UV A, B and C. UV C irradiation is absorbed mostly by the ozone layer, therefore under normal circumstances, UV A and UV B are the major causes of DNA damage in epidermal cells. In order to study the process of DNA damage response upon UV B irradiation, we used keratinocyte cells to test their DNA repair abilities and analyze the possible mechanism which could lead to skin cancer progression.

In a primary screen we identified Bomapin as one of the most dramatically up-regulated gene in keratinocytes following UV B irradiation. Bomapin (SPB10) is a serine protease inhibitor which promotes cell proliferation under normal circumstances in hematopoietic and myeloid leukemia cells. In the absence of growth factors Bomapin can induce apoptosis. However, only a few pieces of data have been reported about the function of Bomapin. Based on these findings, it seemed to be interesting to determine whether this protein has a gain-of-function effect in cancer evolution. To learn about the possible biological function of *Bomapin* induction upon UV B treatment we used immortalized keratinocyte (Hker E6SFM, HaCat) and melanoma (A375) cell lines. As a first step we analyzed the kinetics of UVB induction by determining the mRNA level in different time intervals following irradiation (2h, 8h, and 24h). We found that *Bomapin* mRNA level was increased 2 and especially 8 hours after UVB irradiation (80 J/m^2), while 24h after the treatment the mRNA level was decreased in each the examined cell lines. The mRNA level of *Bomapin* was also increased upon a low intensity (20 J/m^2) UV B irradiation in primer keratinocyte cells. Accordingly, the transient change of mRNA level suggests that Bomapin might be involved in the UV B induced stress response. In accord with the elevated Bomapin mRNA level, higher amount of Bomapin protein could be detected after DNA damage induced by ultraviolet irradiation (2 h, 8 h, and 24 h) in Hker cells, suggesting Bomapin involvement in UV B damage response. Since the protein seems to be a possible player in UV B repair in the following step we investigated the subcellular localization of Bomapin protein. We found that under normal circumstances, Bomapin protein was mostly present in the cytoplasm and co-localized with tubulin. Few hours after DNA damage Bomapin is transported to the nucleus from the cytoplasm and it is detectable in chromatin bound foci suggesting its possible importance in the repair process of damaged DNA.

In summary, our results suggest that Bomapin protein could be an essential player in the UV B induced stress response. Further experiments are needed to clarify its regulatory function in UV B response.

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