Session VIII: The role and/or use of particular enzymes in mitosis regulation, sensor construction and yeast cell engineering

Lectures

L8.1

Subcellular localization of mTOR and rictor: New insights in the mTORC2 functioning

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Keywords: mTOR; rictor; subcellular localization; immunofluorescence; chromatin; mitosis

Aim: The mammalian target of rapamycin (mTOR) is well conserved kinase from yeast to mammals. The mTOR kinase forms two multicomponent protein complexes (mTORC1 and mTORC2) which differs both structurally and functionally. In contrast to mTORC1, comparatively little is known about functioning and regulation of mTORC2. Previous studies showed that phospho-mTOR (Ser2481) located at the midzone and cleavage furrow of the dividing cells. It was proposed that phospho-mTOR could be a mitotic kinase, however, its role and targets during mitosis are unknown. To provide novel insights regarding functioning of mTORC2 complex we analyzed the particularity of mTOR and Rictor (unique component of the mTORC2) and its phosphorylated forms subcellular localization during different stages of mitosis in MCF7 cell line.
Methods: Immunofluorescence assay using confocal microscopy was applied.
Results: We examined the peculiarity of mTORC2 subcellular distribution in MCF7 interphase and mitotic cells with anti-mTOR mAb and unique anti N-terminal Rictor mAb. Immunostaining of Rictor revealed the primarily a uniform speckled cytoplasmic distribution as well as its localization in nuclei and on the nuclear envelope. Similar pattern of immunostaining was observed for mTOR. Further analysis of Rictor and mTOR localization revealed a different pattern of immunostaining in cells that was depended on cell cycle phase. For the first time it was shown that Rictor and phospho-mTOR (Ser2481) associated with condensed chromatin. Moreover following analysis of cells revealed that character of this association depends on mitosis phases.
Conclusions: Our data for the first time indicate possible involvement of mTORC2 in mitosis regulation.

L8.2

Metabolic engineering of the yeast *Saccharomyces cerevisiae* for redirection of carbon flux towards glycerol instead of ethanol

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Keywords: yeast engineering; glycerol production

Glycerol is used in cosmetic, paint, automotive, food, pharmaceutical industries. Despite increase of glycerol accumulation as by-product of biodiesel production, purification of this polyol for subsequent application in food and cosmetic industry is too expensive. There are known *Candida* yeast strains effectively converting glucose to glycerol, however, they need aeration which elevates process costs. Therefore there is an interest in development of microbial strains effectively converting cheap feedstocks to glycerol under anaerobic conditions. Facultative anaerobic yeast *Saccharomyces cerevisiae* could be a good platform for this goal.
In *S. cerevisiae*, glycerol synthesis occurs from dihydroxyacetone phosphate by subsequent action of glycerol-3-phosphate dehydrogenase (Gpd1) and glycerol-3-phosphate phosphatase (Gpp2). Synthesized glycerol is exported from the cells via channel formed by aquaglyceroporin Fps1. However, in physiological conditions dihydroxyacetone phosphate is predominantly isomerized to glyceraldehyde-3-phosphate by triose phosphate isomerase (Tpi1) and subsequently converted to ethanol through several sequential reactions. We aimed to redirect consumed carbon toward glycerol production instead of ethanol.
To decrease Tpi1 activity, recombinant *S. cerevisiae* strain with shortened to 25 bp version of *TPI1* gene promoter was constructed. This strain revealed 2-2.5 folds increase in glycerol production. In order to enhance the activities of enzymes involved in glycerol synthesis, we transformed *S. cerevisiae* with vector containing hybrid *GPD1-GPP2* ORF (encoding artificial fusion of Gpd1 and Gpp2 enzymes) under the control of strong constitutive promoter of the alcohol dehydrogenase gene (*ADH1*). Glycerol production reached 4 folds increase in the best of obtained recombinant strains. Combination of *GPD1-GPP2* fusion overexpression and decrease in Tpi1 activity resulted to 5-fold increase of glycerol production as compared to the WT strain.
The best results were obtained for the strain with decrease in Tpi1 activity, overexpression of hybrid *GPD1-GPP2* ORF (encoding artificial fusion of Gpd1 and Gpp2 enzymes) and overexpression of modified form of *FPS1* gene which provides constitutive glycerol export. This strain produced approximately 8 fold more glycerol during alcoholic fermentation than that of parental strain reaching 18 g/l.

L8.3

Mathematical model of enzymatic kinetics of acetylcholinesterase inhibition by aflatoxin B1: basis for the general approach to the potentiometric biosensor

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Keywords: mathematical model; biosensor; acetylcholinesterase;, aflatoxin B1; inhibitory analysis; enzyme kinetics

Mathematical modeling is used for optimization of the analytical characteristics of biosensors. The recent trend using biosensors based on enzyme inhibition for environmental monitoring makes their study highly relevant. Aflatoxin B1 (AFB1) is a highly toxic and carcinogenic secondary metabolite of the fungus *Aspergillus*. It contaminates a wide range of foodstuff and poses a threat to human and animal health. The aim of this study is to develop a mathematical model for the operation of a potentiometric acetylcholinesterase-based biosensor for aflatoxin B1 determination. In this work, a mathematical model was developed and validated by using a potentiometric biosensor based on immobilized acetylcholinesterase. The biosensor operation is described by a system of kinetic rate equations, determining the dynamics of biochemical reactions in the biosensor. Each equation describes the concentration of enzyme, substrate, inhibitor, and product, or the concentration of enzyme-substrate, enzyme-inhibitor, and enzyme-substrate-inhibitor complexes, as a function of time. This experiment determined the boundary conditions used in the model, which were the initial enzyme, substrate, and inhibitor concentrations in the biomembrane.

The simulation results were correlated with the experimental data. AFB1 reversibly inhibits acetylcholinesterase according to mixed inhibition-type kinetics. To conclude, the classical scheme of mixed-type inhibition enzyme kinetics can be successfully applied to potentiometric biosensors based on acetylcholinesterase inhibition.

Thus, this work has demonstrated that mathematical modeling can be successfully applied in the optimization of analytical characteristics of a potentiometric acetylcholinesterase-based biosensor for aflatoxin B1 detection.

Posters

P8.1

Co-localization of phospho-mTOR (Ser2481) and condensed chromosomes in MCF-7 cells

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Keywords: mTOR; phospho-mTOR; mitosis; metaphase

The mammalian target of rapamycin (mTOR) is a protein kinase involved in the regulation of numerous subcellular processes resulting in protein biosynthesis, autophagy inhibition, cell growth, metabolism regulation and aging. mTOR overactivation accompanies different types of malignancies, type II diabetes, and neurodegenerative disorders. Previous studies showed that phospho-mTOR (Ser2481) located at the midzone and cleavage furrow of the dividing cells. It was proposed that phospho-mTOR (Ser2481) could be a mitotic kinase. However, the role of Ser2481 phosphorylation and targets of phospho-mTOR (Ser2481) during mitosis are unknown. Double immunofluorescent analysis was performed on cell lines MCF-7 to study the peculiarities of the phospho-mTOR (Ser2481) subcellular localization. Mitotic cells demonstrated bright staining in the form of dots, which subcellular localization changed dramatically during mitosis progression. We have shown for the first time that phospho-mTOR (Ser2481) localized as discrete foci at metaphase plate during mitosis in human cell line MCF-7. Our data support the hypothesis proposed earlier that phospho-mTOR (Ser 2481) could act as a CPP-like kinase during mitosis. Our finding also raises a question about phospho-mTOR (Ser2481) co-localization with centromere proteins and its possible participating in chromosomal passenger complex functioning.

P8.2

cat8 gene involvement in regulation of xylose alcoholic fermentation in the thermotolerant yeast *Ogataea* (*Hansenula*) *polymorpha*

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Keywords: xylose; alcoholic fermentation; *CAT8* gene knock out; yeast engineering

Xylose is considered as semi-fermentative carbon source showing features of both fermentative and respiratory substrates. Being important carbon source for alcoholic fermentation, search for approaches which activate fermentative abilities of this pentose and simultaneously block its respiration is of great interest.

We pay attention to *CAT8*, the global transcriptional regulator involved in regulation of gluconeogenesis and utilization of alternative to glucose carbon sources in *Saccharomyces cerevisiae*. However, the functions of *CAT8* homologue in the thermotolerant methylotrophic yeast *Ogataea* (*Hansenula*) *polymorpha* were not studied.

Homologue of *S. cerevisiae CAT8* gene was isolated from the sequenced strain *O. polymorpha* NCYC495 and used for construction of the deletion cassette. The strains with knock out in *CAT8* gene were constructed on the background of the wild-type strain and proved to be the best ethanol producers from xylose. Both types of deletion strains have defect in growth on gluconeogenic substrates (glycerol, ethanol) whereas growth on glucose and xylose was not affected. The mutants Δcat8 isolated from the wild-type strain did not show changes in ethanol production in glucose medium whereas accumulated 2-3 times more ethanol in the medium with xylose. The Δcat8 mutants isolated from the most advanced ethanol producer from xylose also did not show any differences in ethanol production in glucose medium whereas accumulated 12.5 g of ethanol per Liter at 45 °C during xylose alcoholic fermentation.

Summarizing, it could be concluded that the transcription regulator *CAT8* is apparently involved in repression of xylose alcoholic fermentation and, consequently, its damage strongly activates fermentation.

P8.3

The role of peroxisomal enzymes in xylose metabolism and alcoholic fermentation in methylotrophic yeast *Hansenula polymorpha*

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Keywords: xylose fermentation; peroxisomal enzymes; *DAS1*; *TAL2*; yeast engineering

Hansenula (Ogataea) polymorpha belongs to the natural thermotolerant xylose-fermenting yeasts with ability to ferment at elevated temperatures (up to 50 °C). As xylose is the second most abundant sugar in lignocellulose, *H. polymorpha* is regarded as a potential ethanol producer from lignocellulosic hydrolysates. Moreover, this yeast species is methylotroph capable to grow on methanol as a sole carbon source. Methanol induces growth and division of peroxisomes, as also synthesis of peroxisomal enzymes. Therefore, *H. polymorpha* provides a convenient model to study the mechanisms of peroxisome biogenesis and degradation. Peroxisomes are dynamic organelles, whose dimensions, number in the cell and protein content vary in response to environmental changes. In xylose-containing medium, *H. polymorpha* mutants defective in peroxisome biogenesis were characterized by affected xylose alcoholic fermentation performance. Screening for peroxisomal enzymes putatively involved in xylose utilization in *H. polymorpha* revealed peroxisomal transaldolase and transketolase.

This study focused on characterization of the role of peroxisome localized transaldolase (*TAL2* gene) and specific peroxisomal transketolase, known as dihydroxyacetone synthase (*DAS1* gene), as well as alcohol oxidase (*AOX1* gene) in xylose utilization and fermentation in *H. polymorpha*.

To evaluate the role of peroxisomal enzymes in xylose metabolism, *H. polymorpha* recombinant strains with overexpressed *DAS1, TAL2* or *AOX1* genes were constructed and analyzed. In the wild type strain of *H. polymorpha* overexpression of either of these genes revealed increase in ethanol production during xylose alcoholic fermentation. Moreover, *das1*Δ and *tal2*Δ mutants were impaired in xylose fermentation as compared to the wild-type strain, but not in growth on xylose as a sole carbon source. In contrast, *H. polymorpha tal1*Δ **mutants, lacking cytosolic transaldolase, failed to grow on xylose, however** the growth was totally restored by overexpression of the gene of peroxisomal transaldolase *TAL2*.

Overexpression of both *DAS1* and *TAL2* genes in the obtained earlier advanced ethanol producers from xylose led to further increase up to 30% in ethanol production during xylose alcoholic fermentation at elevated temperatures.

P8.4

Recombinant human arginase I and its apoenzyme as bioanalytical tools for enzymatic assay of *L*-arginine and manganese ions by fluorometric methods

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Keywords: arginase I; Arg assay; manganese ions assay

Arginase I (further − arginase) is a key manganese-dependent enzyme of the urea cycle which catalyses the final cytosolic reaction of urea formation in the mammalian liver – the conversion of *L*-arginine (further − Arg) to ornithine and urea. Arginase may be an effective drug in cancer therapy, causing the Arg starvation of cancer cells as well as an analytical instrument for Arg assay.

In our study, we propose two arginase-based methods for Arg assay using highly purified human liver arginase preparations: from the recombinant yeast strains of *Hansenula polymorpha* and $(His)_6$-tagged arginase from *Saccharomyces cerevisiae*. 2,3-butanedione monooxime was applied to detect urea in the first method (linearity: 0.2 – 250 μM, detection limit – 0.16 μM Arg). The second method for ammonia detection is based on using arginase, urease and *o*-phthalaldehyde (linearity: 0.09 – 6.0 μM, detection limit – 0.080 Arg). The methods were successfully used for Arg assay in blood serum.

A highly purified preparation of apoenzyme of His-tagged arginase (a protein form lacking Mn^{2+} cofactor) has been isolated. This apoenzyme was used as Mn^{2+}-recognizing bioelement for assay of Mn^{2+} ions (linearity: 0.01 – 1.5 nM, detection limit – 0.001 nM). The method was tested on the samples of waste water from the copper and gold mines. The estimated Mn^{2+} contents in tested samples were in a good correlation with the results of reference atomic absorption method (R=0.998).

All developed arginase-based methods are very selective, extremely sensitive and rather simple.

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