UNIVERSITY OF PÉCS

Ph.D. program for Biology

Molecular analysis of microorganisms' life processes

The analysis of stress processes in yeast species

Ph.D. thesis

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Introduction

Every cell type, including the multi-cellular organisms are able to respond to the changes of the environmental factors. Stress refers to the reactions of the organisms to every stimulus that offsets the organism from its homeostasis, and induces adaptation. Unicellular organisms, like yeasts are more exposed to hostile environmental changes than the cells of the multi-cellular organisms, therefore, it is essential for them to possess quick response-mechanisms enabling them to adapt to abrupt environmental changes.

The first defense line of cells against oxidative stressors is the group of non enzymatic antioxidant molecules. These compounds directly inactivate the stressors or the rising free radicals. In the next stage, cells sense the rising damages or changes through their signal systems, and activate complex transduction pathways. In fission yeast the main stress-response system is the MAPK cascade. The signal transduction system regulates the activity of the response specific genes by transcription factors.

Transcription factors have operative role in the regulation of the homeostatic processes. In active state, they bound to the general stress-response elements in the promoter region of a wide-range of genes, thus regulate the gene transcription activity. By the help of the emerging gene products, the cell re-establishes its homeostatic condition, adapts to the environmental factors and restores its proliferation ability. Abrupt changes in the environmental factors cause stress even when the new conditions are preferential to the cells, for example the rise of the temperature from 24 °C to 37 °C induces stress in yeast cells, and temporally slows down the proliferation until adaptation occurs.

External environmental factors and intracellular mutation can cause stress. In case of external environmental factor induced stress there are stressors. These can be xenobiotics such as heavy metals, micotoxins etc. or irregular conditions.

Micotoxins are seconder metabolits of filamental fungi with toxic effect on animal cells. Their medical hazard became known only at the 1970th. They are important feed pollutants, inducing no or slight taste alteration, thus there presence can easily avoid notice. The information about their mode of action and about the caused diseases are meager, and the detoxification process is made difficult by their thermo stable property.

To defend against micotoxins it is essential to survey there mechanism of action, permitting the development of reliable, quick and low-priced detection mechanisms, effective detoxification methods and selective therapies against micotoxicoses.

Every process taking part in the maintenance of the homeostatic balance is under the control of an interchanged, interconnected and overlapping control-system. Intracellular activated processes are regulated by positive and negative feedback systems, sensing the different steps of the

activation. Positive feedback mechanisms induce cascade processes that amplify the signal intensity until the activity of the process gets outside of the normal range. The negative feedback blocks the activity of the actual process by the signal induced reaction-cascade, holding the activity of the process in a small range.

The mutation generated abnormal gene products and the absence of the normal gene products induces changes in the normal metabolic processes and in the homeostatic balance system of the cells. Cells try to compensate for these changes trough there regulation systems, inducing different physiological changes compared to their normal metabolism. The degree of the compensation depends strongly on the effect of the mutation affected gene product to the physiological processes.

Aims

At the Department of General and Environmental Microbiology, Faculty of Sciences, University of Pécs one of the programs is the study of the oxidative stress processes of unicellular fungi (*Schizosaccharomyces pombe, Phaffia rhodozyma, Candida albicans* etc.).

1. One part of my Ph.D. study was the comparison of the redox system of a carotenoid producing parental and a carotenoid deficient mutant *P. rhodozyma* strain. The purpose of the study was, to define a physiological stress (the absence of carotenoids) induced oxidative balance alterations. It was important to decide whether the detected oxidative stress tolerance was caused by the carotenoid deficient phenotype inducing mutation or another mutation in the redox system.

The aims of our examinations were:

1.1. How does the tolerance of the mutant strain against oxidative stress and heavy metals change?

1.2. How does the concentration of intracellular reactive oxygen species change in the mutant?

1.3. Is there any significant difference in the specific activity of the antioxidant enzymes or in the glutathione concentration in the mutant compared to the parental strain?

2. In the second part of my work, our aim was to study the effect of patulin on the plasma-membrane.

The aims of our examinations were:

2.1. Electron Spin resonance spectroscopy was used to investigate the structural alterations of the plasma-membrane by patulin. The possibility of a patulin induced phase transition temperature modification and its dependence on the patulin concentration was examined on *S. pombe* protoplasts.

2.2. To demonstrate the biological effect of structural plasma-membrane alterations the efflux of substances absorbing at 260 nm was detected by spectrophotometer.

3. Than the effect of patulin (an external oxidative stress inducing species), was examined on the redox system of *S. pombe*.

The aims of our examinations were to answer:

3.1. How do cells control the effect of patulin treatment? How does the specific activity of the antioxidant enzymes and the glutathione concentration change in response of patulin?

3.2. What kind of changes occur in the intracellular concentrations of reactive oxygen species ($O_2^{\bullet-}$, H_2O_2, $^{\bullet}OH$) in response of patulin?

3.3. How does the reduction capacity change?

3.4. Does a noticeable adaptation process exist?

Methods

The parental astaxanthin-producing *P. rhodozyma* strain (ATCC 24202, MCP 324) was used to obtain a carotenoid-less mutant MCP 325 (Microbial Collection of Pécs) by induced mutagenesis. The mutant produced white colonies and proved to be auxotrophic (arg-, leu-, lys-). For the maintenance of cultures, YM agar (0.5% malt extract, 0.25% yeast extract, 1% glucose, 0.25% peptone and 1% agar, pH 5.3) or YM broth (without agar) with the addition of the necessary amino acids (150 μg ml^{-1}) was employed at 20 °C.

The fission yeast, *Schizosaccharomyces pombe* uracil auxotrophic (ura4-D18) heterothallic (h-) strain was used in the second part of our experiments. *S. pombe* cells were incubated in SM liquid medium contained: glucose 1%, $(NH_4)_2SO_4$ 0.5%, KH_2PO_4 0.05%, $MgSO_4$ 0.01%, and Wickerham vitamin solution 0.001%, supplemented with 100 mg l^{-1} uracil pH 5.6 at 30 °C.

The cells were precultured overnight and mid-log phase cultures were prepared in 3.33 Hz incubator shaker at with a starting cell number 10^6 cell ml^{-1}, and used after washing two times with 9 g l^{-1} NaCl in each experiment. Multiplication rate and generation time was determined by haemocytometer and by the monitoring of the optical density (OD) increment on λ=595nm.

Stock solution of patulin (405.5 mM) was prepared in 100 % acetonitrile. The final concentration of the solvent was 0.8% in all experimental systems.

Determination of growth inhibition, minimal inhibitory concentrations, survival rates, adaptation, and Minimal Inhibiting Concentration measurements

Growth inhibition of patulin at 0 μM, 5 μM, 50 μM, 500 μM and 1000 μM concentrations was measured at 30 °C with 10^6 cell ml^{-1} starting cell number. The multiplication of cells was followed by spectrophotometer (OD595nm). Survival rates and adaptation of cells were estimated with 10^8 cell ml^{-1} starting cell number in HEPES buffer at pH 7.4.

The Minimal inhibitory concentration (MIC) of patulin was determined by the standard microdilution method of NCCLS M27-A. The Minimal inhibitory concentrations against heavy metals, oxidative stressors and osmotic stressors were carried out on the surface of stressor containing plates.

EPR measurements

Washed cells were transferred into the protoplasting solution [1% (w/v) Trichoderma lysing enzyme in 0.6 M KCl pH 6.0] and incubated on 30 ºC for 30 min with occasional shaking.

For EPR measurements protoplasts were washed twice in 0.6 M KCl solution, cell number was adjusted to 10^8 cell ml^{-1} and cells were treated with different concentrations (0 μM, 50 μM, 500 μM, and 1000 μM) of patulin for 20 min on 30 °C. Protoplast lyses inducing effect of patulin was examined by spectrophotometer.

Spin-labeling: 3 µl aliquot of 5-SASL of stock solution (5 mg ml-1) was added to 500 µl protoplast suspension, and the mixture was vortexed for 30 seconds and rested for another 30 seconds on room temperature. This procedure was repeated for three times to facilitate spin probe incorporation. The suspension was than sedimented and resuspended in 100 µl of 0.6 M KCl. The suspension was placed in a 100 µl capillary tube and centrifuged again at 4 °C, and the supernatant was removed.

EPR spectra were recorded on an ESR 300E spectrometer (BRUKER, BIOSPIN, Germany) equipped with diTC2007 temperature variator. Spectra were taken in temperature range from 0 to 30 ºC. Measurement parameters: microwave power of 5 mW, field modulation of 100 kHz, amplitude of 2 Gauss.

Detection of the leakage of substances absorbing at 260 nm

Since patulin has absorption at 260 nm (maximum at 276 nm) the loss of these substances of cells was measured after lysing of cells in boiling water (100 °C). Suspension (10^8 cell ml^{-1}) was treated with patulin with different concentrations for 0, 30 and 60 min at 30 °C, than samples were centrifuged. The pellet was suspended in boiling (100 °C) water for 30 min. After repeated centrifugation the optical density (OD260nm) of the supernatant was determined.

Detection of ROS

Dihydroethidium and dihydrorhodamine 123 (DHR 123) was used to detect the intracellular concentrations of peroxide and superoxide levels, respectively. A BD FACSCalibur flow cytometer and a Perkin-Elmer LS50B spectrofluorimeter were used to the measurements. The working concentrations of the dyes were 10 µM. The signal of 20000 cells was detected. The excitation wave-length was 488 nm for both dyes and the emission wave-length was 585 nm in case of dihydroethidium and 530 nm in case of DHR 123.

EPR Spectroscopy was utilized to measure the hydroxyl-radicals and chromium reduction.

Detection of antioxidant enzyme and glutathione

The specific intracellular activities of GR, GST, GPx, G6PD, catalase, Cu/Zn-SOD and Mn-SOD, and the specific intracellular concentrations of GSH and glutathione disulphide (GSSG) and the total protein concentration measurements were determined by using well-established colorimetric assays.

Results and discussion

Study of the carotenoid deficient *Phaffia rhodozyma* strain

The mutant strain exhibited significantly altered sensitivity or resistance to oxidative stressors such as Menadione, lipid peroxide and $K_2Cr_2O_7$. The white mutant contrary to the parental strain did not contain any benzene-soluble carotenoid intermediates. The absence of carotenoids in this white mutant did not explain the high level of stress resistance/sensitivity observed, because no such phenomenon was detected in any other carotenoid deficient *P. rhodozyma* mutant. These data suggested that the carotenoids play a limited part in stress tolerance. Accordingly, the oxidative stress resistance detected may be a consequence of a second mutation in this mutant MCP 325.

Te generation time of the parental strain and its mutant proved to be the same. In comparison with the parental strain, under uninduced conditions the mutant MCP 325 contained a significantly lower specific cellular $O_2^{\cdot -}$ concentration. This result could be interpreted in terms of the significantly higher specific SOD activity of the mutant strain. Te peroxide levels were about 6 times higher in the mutant strain, which can be explained by it's 16 times lower specific catalase activity.

The concentration of $^{\cdot}OH$ was 61±12% higher in the parental strain than in the mutant strain. These results were supported by a 4.8 times higher Cr(VI)-reducing capacity and significantly lower intracellular H_2O_2 and GSH concentrations.

The specific activity of GR proved to be the same in both strains. The unbalanced, oxidized state of the mutant cells was indicated by the significant increases in the Cr(V) and $^{\cdot}OH$ concentrations after the addition of NADPH. The very low catalase activity in the mutant in response to H_2O_2 induction resulted in an 11.6-fold increase in the specific activity of catalase. It seems reasonable that the elevated H_2O_2 concentration in the mutant cells under uninduced conditions was not sufficient to induce up regulation of the catalase gene so as to restore the unbalanced oxidized state of the cells.

General characterization of the *S. pombe* strain

5 μM patulin had no detectable effect on the cell proliferation, 50 μM patulin slightly delayed cells from entering the logarithmic phase and 500 μM and 1000 μM patulin caused 32% and 83% decreases in the cell proliferation, respectively

This observation suggested the possibility of adaptation processes, which had not been investigated in the case of patulin. Treatment with 10, 30, 50 or 500 μM patulin resulted in a 22.4%, 27.4%, 61.9% or 95.7% decrease, respectively, in the colony-forming ability of the cell population after 60 min.

Nevertheless, pretreatment of cells for 1 h with a sub inhibitory concentration (10 μM) of patulin caused significantly increased survival rate when these cells were subsequently treated with 50 μM or 500 μM patulin.

The Minimal inhibition concentration of patulin against fission yeast cells proved to be 162.2 μM.

The effect of patulin on the plasma membrane of *S. pombe* protoplasts

After treatment for 120 min, the patulin concentration had decreased by 20%, that means that yeast cells are able to uptake a large amount of patulin in a short time period.

The experiments revealed that patulin in concentrations as high as 1000 μM did not cause either shrinking or lyses of the protoplasts.

The probe mobility increased in response to patulin. The phase-transition breakpoint for the *S. pombe* control cells was found to be 14.1 ^{o}C. Treatment of the cells with 50 μM, 500 μM or 1000 μM patulin resulted in a decrease of the breakpoint to 13.9^{o}C, 10.1 ^{o}C or 8.7 ^{o}C, respectively. These results clearly demonstrate the significant dose-dependent interaction of patulin with the lipid components of the yeast plasma membrane. No hysteresis was observed in the hyperfine spitting constants. This means that in the presence of patulin the heat-induced mobility change does not give rise to irreversible alterations in the plasma membrane.

It has been already verified that the main targets of patulin are nucleophiles, and in particular glutathione and the thiol and amino groups of proteins, the interaction leading to both crosslinked and non-crosslinked products. These crosslinks exert considerable effects on the biological function and activity of proteins. Accordingly, it appears reasonable that patulin has direct effects on plasma membranes with the consequence of its ability to modify membrane protein structures and functions.

Measurement of the efflux of compounds absorbing light at 260 nm (several nucleotides, nucleosides and free bases) which appear to originate in the free intracellular pool, has proved to be the a suitable and simple assay for comparative studies of membrane barrier function .

In the presence of 50 μM, 500 μM or 1000 μM patulin, the cells lost 25%, 30.5% or 34% of these materials, respectively, within 20 min. This efflux process slowed down significantly between 20 min and 60 min. The quantity of substances loss is proportional to the alteration in the of patulin-induced phase-temperature decrement

Patulin-induced loss of plasma membrane barrier function might be one of determinative part of its citotoxicity . Moreover, it should contribute to delaying cells from entering the logarithmic phase. And, during this elongated resting phase of the cultures, the cells certainly modify their membrane composition and their patulin detoxification processes to adapt to the patulin-induced membrane alterations.

The oxidative stress inducing effect of patulin

500 μM patulin treatment for 60 min induced significant increment in the specific enzyme activity of Cu/Zn SOD, CAT, GST and decrement in the intracellular concentration of GSH and GSSG. These processes caused a decrease in the rate of the intracellular $O_2^{\bullet-}$ and H_2O_2 formation sixteen minutes after the treatment, because catalase is a very reactive enzyme and patulin caused a 2,5 fold increment in it's specific enzyme activity. 1000 μM patulin caused no such phenomena: the concentration of H_2O_2 and $O_2^{\bullet-}$ increases measurably, probably because the concentration of patulin is too high, and cells are not able to adapt, and there is no change in the transcription.

To study the control of the oxidative stress inducing ability of patulin, the MICs of the MAPK deletion mutants of *S. pombe* against patulin were measured. The pap1- mutant exhibited a very significant sensitivity against patulin, and other mild sensitivities were found in case of the wis1- and sty1- strains.

The drastic decrement of the intracellular GSH concentration induced significant Cr(VI) reduction capacity decrease, because GSH is able to reduce Cr(VI) directly. Still there was only a slight $^{\bullet}OH$ concentration increment because the intracellular concentration of H_2O_2 (required for the $^{\bullet}OH$ generation) was close to the normal rate 60 minutes after the patulin treatment. The addition of NADPH caused considerable increment in the Cr(VI) reduction capacity both in the control and the patulin treated samples.

After the addition of H_2O_2 the signal of the Cr(V) decreased significantly in the control samples, but the decrease in the patulin treated samples is mild. The reason of this phenomenon is the CAT activity increasing effect of patulin and the increased enzyme activity degrades the added H_2O_2. This effect is even more obvious in case of the simultaneous addition of H_2O_2 and NADPH.

Summary

1. First an astaxanthin-producing, red colony-forming *P. rhodozyma* parental strain and its stable colorless white mutant were selected to investigate the importance of carotenoids in stress processes.

1.1. The mutant strain exhibited significantly altered sensitivity or resistance to oxidative stressors such as MD, H_2O_2, t-BOOH, $ZnSO_4$ and $K_2Cr_2O_7$. No such differences were found in other carotenoid deficient mutants.

1.2. The intracellular concentrations of ROS and the oxido-reduction status of the mutant and parental cells characterized by the Cr(VI) reduction capacity of cells were measured. Under uninduced conditions the mutant contained a significantly lower specific cellular $O_2^{\bullet-}$ concentration, it's peroxide level was about 6 times higher and the production of $^{\bullet}OH$ 15 min after the addition of $K_2Cr_2O_7$ was significantly lower. These results were supported by a 4.8 times higher Cr(VI)-reducing capacity. The unbalanced, oxidized state of the mutant cells was indicated by the significant increases in the Cr(V) and OH concentrations after the addition of NADPH. The GSH concentration decreased significantly and the concentration of GSSG increased in the mutant.

1.3. The specific activity of SOD increased and CAT activity decreased significantly. The GR activity did not change in the mutant strain.

According to our results, the complete block in carotenoid synthesis plays only limited part in the antioxidant defense mechanisms of *P. rhodozyma*. The other mutation in the genome of the strain caused significant changes in the CAT regulation, inducing endurable oxidative stress in the mutant.

2. The diverse toxic effects of patulin on *S. pombe* were investigated in the second part of my study. It was demonstrated, that in a very short time a considerable amount of patulin can be absorbed by cells.

2.1. It has been verified, that in the same time, patulin in vivo reacts on the plasma membrane of fission yeast, causing functional changes, inducing plasma-membrane fluidity decrease, possibly interacting with the free thiol and amino groups of membrane-proteins.

2.2. Patulin induced membrane-barrier function and the efflux of vital intracellular substrates were demonstrated.

3. Patulin's effect on the oxido-reduction system of *S. pombe* cells was also investigated.

3.1. GSH depletation effect of patulin was affirmed. By the help of this mechanism cells are able to reduce the cytotoxic effect of patulin. The magnitude of this process is affirmed by its acceleration by the specific activity increment of GST.

3.2. The oxidative stress induction, caused by GSH depletation generated ROS increment was demonstrated. Genotoxic inducing effect of these processes was described by other authors. MAPK cascade process activation and specific antioxidant enzyme activities increment was detected. CAT activity increment is especially outstanding, preventing H_2O_2 and $^{\bullet}OH$ concentration increase.

3.3. Patulin induced GSH depletation decreases the reduction capacity of the cells severely.

3.4. Yeast cells are capable to counterbalance even 500 μM patulin concentration in a short time-period, but the survival rate of the cells decreases severely. A sublethal dose of patulin indicates the deceleration of proliferation, and a successful adaptation process against patulin was described.

According to our results, this is the first time that an adaptation mechanism was described against patulin.

Our work presented the first biophysical evidence of patulin induced plasma-membrane fluidizing effect and barrier function decrease

Although patulin induced ROS increment and GSH depletation was described earlier, this is the first study surveying patulin induced oxido-reduction processes in one eukaryote system.

Publications related to the thesis

Horváth, E., Papp, G., Belágyi, J., Gazdag, Z., Vágvölgyi, Cs., Pesti, M. In vivo direct patulin-induced fluidization of the plasma membrane of fission yeast Schizosaccharomyces pombe. Food and Chemical Toxicology. 48: 1898-1904. (2010) IF.: 2,321

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Sum of the impact factors of publications: 2,99

Sum of the impact factors of submited publications: 6,607