Molecular biology and physiology of sulphur metabolism of *Schizosaccharomyces pombe*

PhD thesis

Tibor Simonics

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Name: Doctoral School of Food Sciences

Field: Food Sciences

Head: Prof. András Fekete
Corvinus University of Budapest

Supervisor: Prof. Anna Maráz
Department of Microbiology and Biotechnology
Faculty of Food Science
Corvinus University of Budapest

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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Signature of Head of School Signature of Consultant

1.Introduction

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Sulphur is an essential element for every living organism, it is necessary for growth. Cells take up sulphur from the environment as different organic and inorganic compounds and they during metabolism build up into their organic molecules. For most living organisms sulphate is the main inorganic sulphur source, it is one of the most frequently occuring anion in the environment.

As other inorganic anions, sulphate is taken up by cells during specific membrane transport. Sulphate transport is dependent on energy and usually taken up by several permeases. The reduction of sulphate is a conservative biochemical process catalysed by similar enzymes in most organisms. The reduction of sulphate to sulphide is carried out by four enzymatic steps. In the first step the sulphate ion is bound to an ATP molecule by the ATP-sulphurylase enzyme, and a biphosphate is released. A phosphate group is connected to the newly generated 5'-adenylil-sulphate (5'-adenosin-phospho-sulphate=APS) molecule by the APS-kinase and then the newly generated 3'-phospho-5'-adenylil-sulphate (phospho-adenozin-phospho-sulphate=PAPS) is

reduced to sulphite by PAPS reductase enzyme. The sulphite-reductase reduces sulphite into sulphide in one enzymatic step. Sulphide can be utilised in different ways, depending on the organism in question.

In *S. cerevisiae* sulphide is incorporated into organic molecules through the O-acetylhomoserine and homocysteine is formed. From homocysteine either methionine or through cystathionine cysteine is produced. Homocysteine can be formed from either cysteine or methionine, therefore any of the two S-containing amino acids can serve as the sole sulphur source (Thomas and Surdin-Kerjan, 1997).

In *Schiz. pombe* the sulphide reacts with O-acetylserine and cysteine is formed. In the reaction of cysteine with O-acetylhomoserine cystathionine is released, and methionine is formed from cystathionine through homocysteine. Homocysteine can be produced by two other reactions as well, from the reaction of sulphide and O-acetylhomoserine or from methionine through S-adenosyl-methionine and S-adenosyl-homocysteine.

Sulphide is incorporated into amino acids in *Aspergillus nidulans* and *Neurospora crassa* in a way similar to that described in *Schiz. pombe*. In filamentous fungi cysteine can be formed from homocysteine through cystathionine, however, the enzymes (cystathionine-β-syntase and cystathionine-γ-lyase)

involved in this reaction are missing in the fission yeast, thus cysteine cannot be formed from methionine through homocysteine and cystathionine (Brzywczy and Paszewski, 1994).

One of the most intensively studied yeast is *Schizosaccharomyces pombe*. Although much is known about its physiology and genetics, its sulphur metabolism is unclear. Until the beginning of my research work not any genes playing a role in sulphate metabolism has been described. During my work a research group from Bern (Schweingruber et al.) published 4 genes (*SAM1, MET25, MET9, MET11*) catalising the incorporation of sulphur into organic molecules. Kohli et al. (1975) isolated 5 methionine auxotrophic strains (*met1-met5*) and mapped by classical way into the chromosomes.

Using selenate-resistant/sulphate non-utilizing strains our group managed to prove that *Schiz. pombe* is able to transform methionine into cysteine by degradation of methionine to sulphate and then it forms cysteine from the sulphate through the sulphate reduction pathway. Therefore the selenate resistant/sulphate non-utilizing mutants having defects in any steps of the sulphate assimilation cannot utilize methionine as sole sulphur source.

If too much sulphide is produced the sulphur is released from the cell in the form of hydrogen-sulphide. This process can cause serious problems in brewing or during wine-making. The high-efficiency microbiological degradation of malic acid occuring in wine is theoretically possible in two ways: 1., lactic acid bacteria during malolactic fermentation can transform malic acid to lactic acid 2., during maloethanolic fermentation done by *Schiz. pombe* ethanol and carbon-dioxide are formed from malic acid. Presently the malolactic fermentation is used in wine-making. The disadvantage of this process is that it is not operating at high sulphite concentration, low pH, low temperature and high ethanol concentration; while the maloethanolic fermentation works also at low pH and at high sulphite concentration. The disadvantage of maloethanolic fermentation is that during total degradation of malic acid the fruitty flavour of wine also disappears and that during fermentation undesirable sulphur-containing flavour and aroma compounds are formed, like hydrogen-sulphide. Application of immobilized cells would be a solution to preserve the fruitty flavour of wine.

The enzymes involved in sulphate assimilation are also used for the reduction of toxic analogues of sulphate, e.g. selenate, chromate and molibdenate which would become toxic for the cell above certain concentrations. The mechanism of toxic effect of

selenate is well-known. In *S. cerevisiae* selenate is reduced via the sulphate assimilation route to selenite. The toxic effect of selenite is attributed to the high amount of H_2O_2 and O_2^- ions formed during reduction route (Pinson et al., 2000). Tolerance of cells to selenite depends on the detoxification mechanisms. During this process selenite reacts non-enzymatically with the reduced form of glutathion during which selenium (Se^0) is formed (Gharieb et al., 1995). The general toxicity of selenium is attributed to its incorporation into sulphur-containing amino acids instead of sulphur. Selenium-containing amino acids change the tertiary structure of proteins, thus inactivating the enzyme (Lauchi, 1993).

The fact that in eukaryotic microganism the sulphate utilization is closely connected with the resistance to selenate was firstly described for *Aspergillus nidulans* by Arst in 1968. Strains which were able to utilize sulphate were selenate-sensitive, but strains not utilizing sulphate become selenate-resistant. Surdin-Kerjans research group characterized *S. cerevisiae* mutants having defect in one of the sulphate assimilation genes by different selenate resistance levels. Our research group was the first who isolated and characterized selenate-resistant *Schizosaccharomyces pombe* strains. The main goal of my PhD thesis was to characterize selenate-resistant/sulphate non-

utilizating strains and then to investigate the hydrogen-sulphide production of the wild type and selenate-resistant mutant strains in an immobilized cells system.

1.2.Objectives

1. Indentification of the inactivated gene by cloning in selenate-resistant/sulphate non-utilizing strain isolated by our group earlier. Firstly I had to develop a high-efficiency transformation method which was then used for transformation of *Schiz. pombe* mutant by genomic libraries.
2. Isolation, subcloning and characterization of the gene encoding an enzymes playing a role in the sulphate reduction pathway.
3. Investigation of the expression of the cloned gene in *S. cerevisiae*.
4. Investigation of the applicability of the cloned gene as a selection marker in a cloning vector.
5. Measuring of the enzyme activity of ATP-sulphurylase and the hydrogen-sulphide production of wild type and selenate-resistant strains.
6. Localization of the selenate-resistant gene on the *Schiz. pombe* gene map, and investigate whether it is allelic with any of the *Schiz. pombe met1-met5* mutant genes isolated by Kohli et al. (1975).

7. Investigation of the possibility of using the selenate-resistant mutant in wine-making for maloethanolic fermentaion.

2. Materials and methods

2.1. Strains used

The strains listed below were used in my work:

-*Schiz. pombe* strains

a., selenate-resistant strains: 0-82 h^- *ade5*$^-$ Se^R-2; B-579 h^- Se^R-2; 0-121 h^- *trp1arg1* Se^R-2; L-972 h^- Se^R-2

b., methionine auxotrophic strains: 3-112 h^- *met1-1;* 16-635 h^+ *met2-2;* 3-117 h^+ *met3-1;* 20-763 h^+ *met4-6;* 0-162 h^- *met5-1*

c., cloning strain: D-18 *ura4*$^-$

-*S. cerevisiae* methionine auxotrophic strain: CC371-4B Matα *his3 leu2 ura3 met3*

2.2. Plasmids, genomic banks

For the cloning experiments the *Schiz. pombe* pUR18N shuttle vector, for cloning of the desired gene into *S. cerevisiae* the Yeplac181 vector with autonomous replication, was used.

2.3. Brief review of experimental methods

For screening the *Schiz. pombe* gene libraries, prepared and published by Zsigmond Benkő were used.

Because not any of our selenate-resistant strains carried the *ura4*$^-$ auxotrophic mutation, our first goal was to construct selenate-resistant strain with *ura4*$^-$ mutation. This strain was isolated after protoplast fusion of 0-82 *ade5*$^-$ Se^R-2 and D-18 *ura4*$^-$ strains and a haploid mitotic segregant was isolated after benomyl treatment.

The transformation method published for *Schiz. pombe* has not proved to be effective, therefore we developed a new method. The highest transformation efficiency was achieved by the following treatment. 1.,10 mM ditiothreitol, 0.1 M litium-acetate, 100 mM Tris, 5 mM EDTA (pH8.5) and 2., 100 μg/mL lysing enzyme (Sigma).

The nucleotide sequence of the cloned DNA fragment was determined by Perkin-Elmer automatic DNA sequencer (model ABI373) in the Biological Research Center Szeged by dideoxi

chain termination method. Primers used in sequencing were always synthetized according to the newly determined nucleotide sequences. The sequences were evaluated using the programs BLASTN, BLASTX (http://www.ncbi.nlm.nih.gov), FramePlot 2.3.2. (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl; Ishikawa and Hotta, 1999) and ClustralW (http://decypher.stanford.edu/index_by_algo.htm, Thompson et al., 1994).

Plasmids were isolated both from *Schiz. pombe* and from *S. cerevisiae* by using the plasmid rescue in *Escherichia coli*. In the case of *Schiz. pombe* the efficiency of plasmid rescue was much lower than in *S. cerevisiae.*

In the case of measuring the hydrogen-sulphide production, the liberated hydrogen-sulphide was trapped by cadmium-hydroxide solution. The quantity of cadmium-sulphide produced from the reaction of hydrogen-sulphide and cadmium-hydroxide was assessed according to colorimetrically.

For measuring of enzyme activity of ATP-sulphurylase, I used the method published by Segel et al. (1987). The principle of this method is that ATP-sulphurylase liberates pyrophosphate from ATP. Pyrophosphatase hydrolyses pyrophosphate and the liberated phosphate was assessed by colouric reaction and

measured by spectrophotometer. The intensity of the blue color is proportional to enzyme activity.

To prepare immobilized cells I mixed the cell suspension with Na-alginate solution in the ratio 1:3 and then solidified by dropping into 0.2 M $CaCl_2$ solution. After 2 hours the immobilized cells were rinsed by sterile destilled water.

The malic acid contents of the samples were determined by malic-acid test (Boehringer-Mannheim) according to instructions of the manufaturer.

3. Summary of results

In the beginning of my work I fused a selenate-resistant/sulphate non-utilizing mutant and a transformable *ura4*$^-$ mutation-containing strain. Selenate-resistant, uracil auxotroph recombinant strain was isolated on benomyl-containing medium, which was suitable for transformation by a vector containing the *ura4* prototrophic gene as a selection marker. In *Schiz. pombe* the most frequently used high-efficiency transformation method is the lithium-acetate method. Because this method did not resulted sufficient transformation efficiency in the case of the newly isolated strain, I tried the electroporation transformation technique. I developed a high-efficiency electro-transformation

method for S 18-82 *ura4*$^-$ Se^R-2 strain. Before electroporation I disintegrated the cellural and membrane structure by using different detergents, enzymes or lithium-acetate solution. The best results, which proved to be enough for screening the gene libraries, was 6.10^4 transformants/μg DNA.

The two electro-transformation methods of highest-efficiency were used for screening the three genomic libraries containing DNA inserts of different sizes. Positive transformants were isolated on minimal medium containing sulphate as the sole sulphur source. The complemented gene responsible for sulphate utilization provided suffiecient growth of the transformans. Selenate sensitivities of the transformants were equal to that of the wild type strain. I isolated plasmid from the transformants and then sequenced one of the cloned fragment. By searching for homologous sequences I compared sequence of the cloned DNA with sequences found in BLASTN data base. In the mentioned data base one sequence was found (cosmid 1228) which contained the whole cloned genomic DNA fragment. This cosmid had been sequenced as the part of *Schiz. pombe* "GENOM PROJECT". It is close to the centromere on the left arm of chromosome II.

On the 4.5 kbp long DNA fragment two ORFs were found. It was proved by subcloning that the gene encoding ATP-

sulphurylase enzyme is responsible for the selenate-resistant phenotype. This gene was named as *sua1*.

The 1473 bp long *sua1* gene codes for a 440 amino acid containing protein. The start codon is the ATG, encoding for methionine and TAA is the STOP codon. The gene does not contain any introns. In the downstream region gene there is an adenine and thimine rich fragment, the A+T content of the 200 bp long downstream region of the gene is 73 %.

As in other living organisms conservative regions responsible for substrate binding (^{190}VxAFQxRNP, "GRD-loop") can be found in the Sua1p. Before the GRD region one amino acid change can be found, in the position 286 isoleucine is found instead of valin typical to other organisms. The molecular weight of the Sua1p is 54.7 kDa.

The selenate-resistant strain practically had no ATP-sulphurylase activity, but the enzyme activity of positive transformants and the wild type strain were the same.

The *sua1*$^{-}$ mutant were transformed with vector containing *sua1* gene as a selection marker. The advantage of using *sua1* gene as a selection marker is that both the host strain and the complemented transformant can be isolated using positive selection, according to selenate-resistance and sulphate-

utilization, respectively. By culturing the transformants on non-selective medium plasmid-loss can be quantitatively determined.

The cloned *sua1* gene was transferred to a bifunctional vector and used to transform a *S. cerevisiae met3* mutant. The ATP-sulphurylase gene of *Schiz. pombe* could complement the *S. cerevisiae met3* mutant gene and its termination signal could ensure gene expression.

The first methionine auxotrophic mutants were isolated in 1975 by Kohli et al. I investigated which sulphur sources can be utilized by these methionine mutants. Using these results, map positions of the mutant allels on the chromosomes as determined by Kohli and the published *Schiz. pombe* genom sequences I concluded that not any is allelic with *sua1* gene. The results showed that in *met1* and *met2* two different homocysteine-methyltransferases, in *met3* the cystathionine-γ-synthase, in *met4* mutant the homoserine-O-acetyltransferase and in *met5* the metyl-tetrahydrofolate-reductase gene was inactivated. It is likely that the last one is allelic with the *MET9* gene described by Naula et al (2002).

Luca Bánszky in her PhD work investigated the malic acid degradation and H_2S production ability of selenate-sensitive (wild type) and selenate-resistant strains in free cells system. The disadvantage of the free cells system is that after decreasing the

malic acid concentration to the desired level the cells cannot easily be removed from the wine and can cause undesirable quality changes in wine. To avoid this problem I investigated the malic acid degradation and H_2S production of selenate-sensitive and selenate-resistant strains in immobilized cells system. In organic and inorganic sulphur-containing medium it was proved that mutants did not produce measurable H_2S in contrast to the wild type strains. The malic acid degradation and the changes in wine aroma spectrum were the same in both cases.

4. New scientific results

- I developed a high-efficiency transformation method for *Schiz. pombe* which can be used for screening of gene library.
- I cloned and characterized the gene encoding the ATP-sulphurylase enzyme in *Schiz. pombe* which was named *sua1*.
- Application of the *sua1* gene as a selection marker allows positive selection. Both the selenate-resistant cloning host strain or the transformants can be selected positively and the plasmid-loss at transformants can be determined easily and quantitatively.
- I proved by cloning of *sua1* gene into *S. cerevisiae* that *Schiz. pombe* ATP-sulphurylase gene is able to complement *S.*

cerevisiae met3 mutant. It shows that ATP-sulphurylase is a conservative gene.

- I showed that any of the methionine auxotrophic genes mapped by Kohli et al. (1975) is identical with the ATP-sulphurylase gene.
- By measuring H_2S production I proved that the selenate-resistant strains in contrast to wild type strains did not produce H_2S either in organic or in inorganic sulphur source containing medium or in wine.
- I proved that immobilized selenate-resistant mutant cells both in wine and in organic or in inorganic sulphur containing media degradated malic acid at the same level as the wild type strain.

5. Futher development and utilization of the results

- Construction of *Schiz. pombe* cloning vectors containing *sua1* gene as the only selection vector. It would be used in preparation of *Schiz. pombe* gene libraries.
- Characterization of the promoter region of the *sua1* gene and the substrate binding region of the encoded protein .

- The *sua1*$^-$ mutant could degradate malic acid and did not produce detectable H_2S, therefore it is recommended to use them in wine-making for decreasing the malic acid content of wines.
- In wine-making using of the immobilized cell system is recommended more than free cell system because immobilized cells degraded malic acid better and their removing from wine is easier. As the preparation of immobilized cells is expensive, this step can be simplified by using flocculent *sua1*$^-$ mutants. It is necessary to investigate the applicability in wine deacidification in laboratory and semi-pilote, then pilote scale.

6. List of publications

Articles published in journals with impact factors

2000: **Simonics T.**, Kozovska Z., Michalkova-Papajova D., Delahodde A., Jacq C., Subik J.:
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Hsp90 inhibition accelerates cell lysis. Anti-Hsp90 ribozyme reveals a complex mechanism of Hsp90 inhibitors involving both superoxide- and Hsp90-dependent events.
J Biol Chem. 2003;278(37) 35231-35240

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Articles published in peer-reviewed journals without impact factors

2001: **Tibor Simonics**, Luca Bánszky, Anna Maráz
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A *Schizosaccharomyces pombe* szulfát metabolizmusában szerepet játszó gén molekuláris vizsgálata.
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