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EFFECT OF SODIUM NITRATE (V) ON *SACCHAROMYCES CEREVISIAE* STRAINS OF DIFFERENT ANTIOXIDATIVE STATUS AND ENERGETIC METABOLISM

Agata Święciło

Chair of Biochemistry and Environmental Chemistry, Faculty of Agricultural Sciences, University of Agriculture, Lublin

Key words: sodium nitrate (V), yeast, antioxidating system, reactive oxygen species (ROS)

The *Saccharomyces cerevisiae* yeast, differing with respect to the efficiency of antioxidating system and activity of mitochondrial processes, was used in the experiment. Sensitivity of these cells to 40-min incubation with sodium nitrate (V) was determined. Respiratory-competent cells deprived of the main antioxidating enzymes and the cells subjected to oxidative stress generated by antimycin A showed a greater sensitivity to sodium nitrate (V) than the cells deprived of functional mitochondria or the cells taken during the stationary phase of growth. The obtained results show that reactive oxygen species do not play an important part in the mechanisms of toxicity induced by the presence of sodium nitrates (V) in the case of cells with oxygen metabolism.

INTRODUCTION

Nitrates (III) and (V) are chemical compounds which are very common in the natural environment. They are also used in the food industry in the production of cured meats, ready-made products and cheeses. Excessive consumption of nitrates is very harmful to mammalian organisms and the effects are the most destructive in the case of young and old mammals [Faivre *et al*., 1976; Weyer *et al*., 2001]. It has been found that the sodium nitrate (V) induced the shift of prooxidative-antioxidative balance in mammalian blood plasma [Kirpichenok *et al*., 1997].

The main purpose of the study was to examine the effect of these salts on the cells of the *Saccharomyces cerevisiae* yeast, which are characterised by a changed activity of the antioxidating system. The yeast represents lower eukaryotes. Due to the structural and functional similarity of its cell organelle it has been successfully used for years as a model organism in the study of a potential toxicity of food additives. Yeast is equipped with an antioxidating system which in many respects resembles the system found in the cells of higher eukaryotes.

The main antioxidating enzymes are superoxide dismutase and catalase. Superoxide dismutase (SOD) is an enzyme which catalyses the reaction of deprotonation of superoxide anion-radical. In the cytoplasm of *Saccharomyces cerevisiae* yeast cells there appears zinc and copper (CuZn--SOD) dismutase, whereas in the mitochondria – a manganese (Mn-SOD) dismutase is observed to occur. Catalase catalyses the reaction of disproportionation of hydrogen peroxide to water and oxygen. Yeast has two types of catalase: cytoplasmic catalase T and peroxisomal catalase A. These proteins are coded by two different nuclear genes and their composition of amino acids is also different [Aamerer *et al*., 1981].

It is known that there are yeast strains which are deprived of the activity of antioxidating enzymes. The strains deprived of the activity of one or both of catalases in standard conditions of growth are similar to the cells of wild strains as far as their phenotype is concerned. However, the mutations which lead to a lack of activity of one or both of superoxide dismutases result in significant changes in the dynamics of ageing of these cells. According to Müller *et al*. [1980], the life span of yeast cells can be measured by the number of buds that single stem cells are able to produce. The so-called replicating life span of yeast cells determined in this way may, to some extent, be a parameter analogous to the life span of dividing mammalian cells. A lack of activity of one of the superoxide dismutases shortens the replicating life span of these mutants by half. On the other hand, depriving yeast cells of the activity of both enzymes shortens the life span of the cells even more. The effects of these deficiencies are additive [Wawryn *et al*., 1999].

In the case of higher eukaryotes mutations concerning *sod1* structure gene are related to a progressing destruction of motor neurons [Reaume *et al*., 1996], but mutations of *sod2* gene are neonatal lethal [Li *et al*., 1995]. Changes in the activity of SOD were observed in cancerous diseases and syndromes of accelerated ageing.

Yeast cells also exhibit a significant similarity to mammalian cells as far as the organisation and number of mitochondria are concerned. Many yeast mitochondrial proteins are homologous to human proteins [Chatterjee & Singh, 2001; Singh *et al*., 2001]. The main function of mitochondria is to produce energy as a result of the activity of electron transport

Author's address for correspondence: Agata Święciło, Chair of Biochemistry and Environmental Chemistry, Faculty of Agricultural Sciences, University of Agriculture of Lublin, ul. Szczebrzeska 102, 22-400 Zamość, Poland; e-mail: amyszka@wnr.edu.pl

chain associated with oxidative phosphorylation. Reduced activity of mitochondria caused by the accumulation of oxidative damage in mitochondrial DNA may lead to the appearance of diseases and ailments typical of old age [Simic, 1992]. Total blocking of mitochondrial functions in mammalian cells usually leads to their death [Scheffler, 1999]. Yeast cells are able to function in such conditions only if the process of obtaining energy by fermentation is not disturbed [Shadel, 1999]. Mitochondrial mutants of yeast exhibiting big deletions constituting about 50-99% of mitochondrial DNA genome (mt DNA) or its total loss (the *rho*$^-$ and *rho*^{0} mutants) can be easily obtained in laboratory conditions. They constitute a good object to be used in the study of the role of oxygen metabolism in toxicity induced by environmental substances.

MATERIALS AND METHODS

Characteristics of *Saccharomyces cerevisiae* strains used in the experiment

The following strains were used in the study: SP-4 wild-type strain (*wt*) of genotype: Mat*α leu1 arg4* [Biliński *et al*., 1978]; A50DSCD1-9C5b, deprived of the activity of catalase type A and T of genotype: Mat*a leu1 arg4 ctt1 cta1* [Wawryn *et al*., 1999]; DSCD6-6B, deprived of the activity of cytosolic and mitochondrial superoxide dismutase of genotype Matα *ura3 sod1 sod2*; and SP-4*rho*$^-$, A50DSCD1-9C5b*rho*$^-$, DSCD6--6B*rho*$^-$ respiratory mutants obtained by threefold passaging isogenic cells of respiratory-competent strains (*rho*$^+$) in the presence of ethidium bromide three times. Change into a stable *rho*$^-$ phenotype was verified on the basis of a lack of growth in YP medium containing 20 g/L of ethanol.

Media and growth conditions

The yeast was grown in liquid and solid YPG medium containing: 10 g/L of yeast extract, 10 g/L of peptone, and 20 g/L of glucose. Agar (20 g/L) was added to the solid medium. The yeast culture in liquid medium was grown in aerobic conditions at 28°C for 24 h in order to obtain cells at the logarithmic phase of growth (LOG cells) or 48 h in order to obtain cells at the stationary phase of growth (STAT cells). The density of logarithmic yeast cultures used in the experiment ranged from $1\text{-}5\times10^7$ cell/mL, whereas the density of stationary cultures ranged from $4\text{-}8\times10^8$ cell/mL.

Sodium nitrate (V) and antimycin A treatments

The suspension of yeast cells (LOG or STAT cells) was incubated for 40 min in the presence of selected concentrations of sodium nitrate (V), in the presence or absence of antimycin A at a concentration of 10 μg/mL. The following concentrations of sodium nitrate (V) were used: 0, 0.5, 1, 1.5, 2, 2.5, and 3 mol/L.

Determining the viability of yeast cells in the presence of substances under study

Portions of 0.1 mL of a yeast cell suspension were inoculated onto the surface of solidified medium in Petri dishes. The dishes were incubated at 28°C for 48 h. The number of colonies grown on the medium indicated the viability of the cells in incubated suspension. The number of colonies which grew on the medium without sodium nitrate (V) obtained for the experiment and not containing sodium nitrate was taken as 100%.

A preparation of antimycin A in a lyophilised form was bought from Sigma Chemical C.O., whereas sodium nitrate (V) was bought from POCH.

The presented results constitute a mean of at least four independently conducted experiments.

RESULTS AND DISCUSSION

Low sensitivity of yeast cells to sodium nitrate (V) made it necessary to use a rather high concentration of this salt. Figure 1 presents the results concerning viability of respiratory-competent logarithmic cells and respiratory mutants in the presence of selected concentrations of sodium nitrate (V).

Respiratory-competent yeast cells of wild-type strain *wt*, the *sod1sod2* mutant and the *cta1ctt1* mutant reacted to the presence of sodium nitrate (V) in the environment in a similar way (Figure 1). A 50% death rate of the yeast population was observed in the presence of sodium nitrate (V) in the concentration of about 1 mol/L.

The *rho*$^-$ respiratory mutants of the strains under study exhibited a higher resistance to sodium nitrate (V) than the *rho*$^+$ isogenic strains. A 50% death rate of these cells was observed in concentrations ranging from 2 to 2.5 mol/L. Only the cells of the *sod1sod2rho*$^-$, triple mutant exhibited a lower level of resistance to sodium nitrate (V). However, also in this case an increase in the resistance to this factor was observed as compared to isogenic respiratory-competent strain.

Relatively high resistance of yeast cells to sodium nitrate (V), comparable to the resistance to NaCL [Święciło & Krzepiłko 2005], may result from the fact that nitrates (V) are removed from cells efficiently. Mammalian cells also have such a mechanism. The time of half-expulsion of nitrates from human organism is *ca*. 5 h [Green *et al*., 1981].

The activity of the main antioxidating enzymes is not so significant for the toxicity induced by sodium nitrate (V) in the case of respiratory-competent cells. However, the cells that are wholly dependent on cytoplasmic processes for obtaining energy (the *rho*$^-$ mutants) exhibit differences in their sensitivity to sodium nitrate (V). In this case, a lack of activity of both superoxide dismutases leads to a significant increase in the level of sensitivity of these cells.

According to other studies, the cells of the *sod1sod2* double mutant exhibit a higher sensitivity to prooxidating substances than the cells of wild-type strains and the *sod* single mutants [Święciło & Krzepiłko, 2004; Viau *et al*., 2006].

As compared to dismutases activity, catalase activity plays a less significant role in protecting cells from harmful environmental factors [Biliński *et al*., 1985; Demasi *et al*., 2006].

Dysfunction of mitochondria manifesting itself as a lack of activity of respiratory chain (the *rho*$^-$ mutation) seems to play a key role in the appearance of resistance to sodium nitrate (V).

In other studies there have been many examples confirming positive effects of removing mitochondrial function in yeast in stress conditions. The *rho*$^-$ mutation protects cells

from the toxic effects caused by the presence of 100% oxygen, salinity or Fe^{+2} salts [Wawryn *et al*., 1999; Wiśnicka *et al*., 1998; Święciło & Krzepiłko, 2005]. It has been suggested that in these conditions reactive oxygen species (ROS) act as agents in the mechanisms of toxicity. Consequently, eliminating mitochondria, which are the main source of ROS [Boveris, 1984; Rasmussen *et al*., 2003], both in physiological and in stress conditions may bring yeast cells notable benefits.

The results obtained (Figure 1) suggest that in the case of respiratory-competent cells the mechanism of toxicity of sodium nitrate (V) does not depend on ROS. Thus, it can be supposed that the effects of the *rho*$^-$ mutation in this case will not depend on the concentration of reactive oxygen species.

In order to verify this hypothesis a response of yeast cells to sodium nitrate (V) in the conditions of oxidative stress was examined. Oxidative stress was generated by the presence of 10 μg/mL of antimycin A. This antibiotic reduces the electron transfer activity of the bc l cytochrome complex and induces the synthesis of superoxide anion-radical [Müller *et al*., 2002].

Yeast cells of wild-type strain (*wt*) taken at the logarithmic and stationary stage of growth incubated with antimycin A exhibited a similar level of sensitivity to sodium nitrate (V) to the cells which have not been subjected to the activity of this antibiotic. Lack of effects of activity of antimycin A in both cases suggests that the level of induction of ROS in mitochondria does not play a significant role in sodium nitrate (V) toxicity. It should be stressed that yeast cells taken at the stationary phase of growth exhibit a higher resistance to sodium nitrate (V) than logarithmic cells; however their resistance is lower than that of the cells of the *rho*$^-$ respiratory mutant. Higher resistance of yeast cells at the stationary phase of growth was also observed after application of $SnCl_2$ [Viau *et al*., 2006]. It should be added that they tolerated over thousand-fold higher doses of $SnCl_2$ than the cells of the same strain taken at the logarithmic phase of growth. The high resistance at the stationary phase of growth results from initiating two independent defensive mechanisms. The first one is initiated during the diauxic-shift, when the cells are adjusting to an oxygen way of obtaining energy and when mechanisms of a general response to environmental stress appear [Boy-Marcotte *et al*., 1998]. The second mechanism may be initiated by ROS inducible transcription activators [Maris *et al*., 2001]. A considerably lower level of resistance to sodium nitrate (V) as compared to the one induced by $SnCl_2$ observed in stationary cells most probably results from initiating only one of the defensive systems discussed above. Taking into account the results presented in this study (Figures 1 and 2), the participation of ROS in inducing defensive mechanisms generated by the presence of sodium nitrate (V) in the case of cells with oxygen metabolism should be excluded.

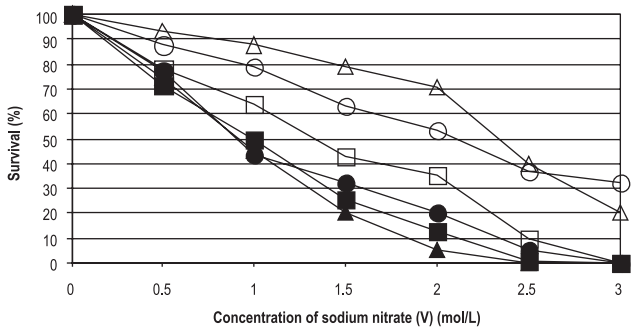

FIGURE 1. Viability of respiratory-competent logarithmic cells and respiratory mutants: Sp4*wt*, DSCD6-6B*sod1sod2*, A50DSCD1-9C5b*ctt1cta1*, SP4*rho*$^-$, DSCD6-6B*sod1sod2rho*$^-$, A50DSCD1-9C5b*ctt1cta1rho*$^-$ in the presence of sodium nitrate (V).
Legend: closed shapes – respiratory-competent strains (*rho*$^+$), open shapes – respiratory mutants (*rho*$^-$), triangle – *wt*, square – the *sod1sod2*, circle – *cta1ctt1* mutant.
Error bars showing standard errors of the mean were eliminated for clarity.

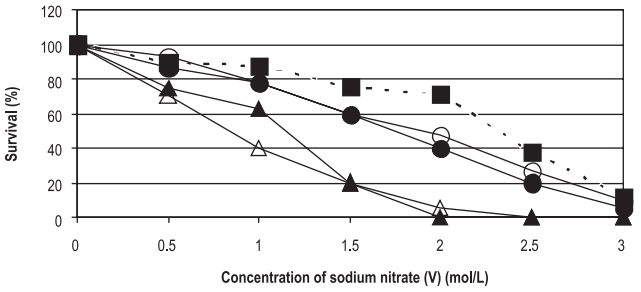

FIGURE 2. Viability of respiratory-competent logarithmic cells of wild-type strain taken from logarithmic and stationary stage of growth in the presence of sodium nitrate (V) and antimycin A.
Legend: closed shapes – lack of antimycin A, open shapes – addition of 10 μg/mL of antimycin A, square – *rho*$^-$ respiratory mutant, triangle – logarithmic cells of wild-type strain (SP4*wt*), circle – stationary cells of wild-type strain (SP4*wt*).
The survival curve of the *rho*$^-$ respiratory mutant was presented for comparison only and that is why it is marked with a dotted line.

CONCLUSIONS

1. Yeast cells deprived of catalase and dismutase activity and the cells of wild-type strain react to the presence of sodium nitrate (V) in a similar way, which suggests that these enzymes do not play any important role in the mechanisms of toxicity induced by sodium nitrate (V).

2. Deprivation of cells of the possibility of oxygen respiration (the *rho*$^-$ mutation) leads to an increase in the resistance to sodium nitrate (V), most probably through a mechanism independent of ROS.

3. Stationary yeast cells exhibit a higher resistance to sodium nitrate (V) as compared to logarithmic cells, most probably due to the activity of defensive mechanisms induced by stressogenic factors.

REFERENCES

1. Ammerer G., Richter K., Hartter E., Ruis H., Synthesis of *Saccharomyces cerevisiae* catalase A *in vitro*. Eur. J. Biochem., 1981, 113, 327-331.
2. Bilinski T., Krawiec Z., Liczmanski A., Litwinska J., Is hydroxyl radical generated by the Fenton reaction *in vivo*? Biochem. Biophys. Res. Commun., 1985, 130, 533-539.
3. Bilinski T., Lukaszkiewicz J., Sledziewski A., Demonstration of anaerobic catalase synthesis in the cz1 mutant of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun., 1978, 83, 1225-1233.
4. Boveris A., Determination of the production of superoxide radicals and hydrogen peroxide in mitochondria. Methods Enzymol., 1984, 105, 429-435.

5. Boy-Marcotte E., Perrot M., Bussereau F., Boucherie H., Jacquet M., Msn2p and Msn4p control a large number of genes induced at the diauxic transition which are repressed by cyclic AMP in *Saccharomyces cerevisiae*. J. Bacteriol., 1998, 180,, 1044-1052.
6. Chatterjee A., Singh K.K., Uracil-DNA glycosylase-deficient yeast exhibits a mitochondrial mutator phenotype. Nucleic Acids Res., 2001, 29, 4935–4940.
7. Demasi A.P., Pereira G.A., Netto L.E., Yeast oxidative stress response. Influences of cytosolic thioredoxin peroxidase I and of the mitochondrial functional state. FEBS J., 2006, 273, 805-816.
9. Faivre J., Faivre M., Klepping C., Roche L., Methemoglobinemias caused by ingestion of nitrites and nitrates. Ann. Nutr. Aliment., 1976, 30, 831-838 (in French; English abstract).
10. Green L.C., Ruiz de Luzuriaga K., Wagner D.A., Rand W., Istfan N., Young V.R., Tannenbaum S.R., Nitrate biosynthesis in man. Proc. Natl. Acad. Sci. U S A, 1981, 78, 7764-7768.
11. Kirpichenok L.N., Gidranovich L.G., Kheidorov V.P., The joint action of nitrates and gamma radiation on the blood plasma proteinase-inhibiting and antioxidative systems in rats. Radiats. Biol. Radioecol., 1997, 37, 297-302 (in Russian; English abstract).
12. Li Y., Huang T.T., Carlson E.J., Melov S., Ursell P.C., Olson J.L., Noble L.J., Yoshimura M.P., Berger C., Chan P.H., Wallace D.C., Epstein C.J., Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. Nat. Genet., 1995, 11, 376-381.
13. Maris A.F., Assumpcao A.L., Bonatto D., Brendel M., Henriques J.A., Diauxic shift-induced stress resistance against hydroperoxides in *Saccharomyces cerevisiae* is not an adaptive stress response and does not depend on functional mitochondria. Curr. Genet., 2001, 39, 137-149.
14. Müller F., Crofts A.R., Kramer D.M., Multiple Q-cycle bypass reactions at the Qo site of the cytochrome bc1 complex. Biochemistry, 2002, 41, 7866-7874.
15. Müller I., Zimmermann M., Becker D., Flömer M., Calendar life span *versus* budding life span of *Saccharomyces cerevisiae*. Mech. Ageing Dev., 1980, 12, 47-52.
16. Rasmussen A.K., Chatterjee A., Rasmussen L.J., Singh K.K., Mitochondria-mediated nuclear mutator phenotype in *Saccharomyces cerevisiae*. Nucleic Acids Res., 2003, 31, 3909-3917.
17. Reaume A.G., Elliott J.L., Hoffman E.K., Kowall N.W., Ferrante R.J., Siwek D.F., Wilcox H.M., Flood D.G., Beal M.F., Brown R.H.Jr., Scott R.W., Snider W.D., Motor neurons in Cu/Zn superoxide dismutase-deficient mice develop normally but exhibit enhanced cell death after axonal injury. Nat. Genet., 1996, 13, 43-47.
18. Scheffler I.E., Mitochondria. 1999, Wiley-Liss, NewYork, N.Y, pp. 123-125.
19. Shadel G.S., Yeast as a model for human mtDNA replication. Am. J. Hum. Genet., 1999, 65, 1230-1237.
20. Simic M.G., The rate of DNA damage and aging. EXS., 1992, 62, 20-30.
21. Singh K.K., Sigala B., Sikder H.A., Schwimmer C., Inactivation of *Saccharomyces cerevisiae* OGG1 DNA repair gene leads to an increased frequency of mitochondrial mutants. Nucleic Acids Res., 2001, 29, 1381–1388.
22. Święciło A., Krzepiłko A., The role of antioxidant system in response of yeast *Saccharomyces cerevisiae* cells to strong salt stress. Zesz. Prob. Post. Nauk Rol., 2005, 505, 451-458 (in Polish; English abstract).
23. Święciło A., Krzepiłko A., The role of superoxide dismutases and catalases in cells of *Saccharomyces cerevisiae* yeast response to oxidative stress induced by menadione. Zesz. Prob. Post. Nauk Rol., 2004, 496, 519-525 (in Polish; English abstract).
24. Viau C., Pungartnik C., Schmitt M.C., Basso T.S., Henriques J.A., Brendel M., Sensitivity to Sn^{2+} of the yeast *Saccharomyces cerevisiae* depends on general energy metabolism, metal transport, anti-oxidative defences, and DNA repair. Biometals, 2006, 19, 705-714.
25. Wawryn J., Krzepilko A., Myszka A., Bilinski T., Deficiency in superoxide dismutases shortens life span of yeast cells. Acta Biochim. Pol., 1999, 46, 249-253.
26. Weyer P.J., Cerhan J.R., Kross B.C., Hallberg G.R., Kantamneni J., Breuer G., Jones M.P., Zheng W., Lynch C.F., Municipal drinking water nitrate level and cancer risk in older women: the Iowa Women's Health Study. Epidemiology, 2001, 12, 327-338.
27. Wiśnicka R., Krzepilko A., Wawryn J., Krawiec Z., Bilinski T., Protective role of superoxide dismutase in iron toxicity in yeast. Biochem. Mol. Biol. Int., 1998, 44, 635-641.

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ODDZIAŁYWANIE AZOTANU (V) SODU NA SZCZEPY DROŻDŻY *SACCHAROMYCES CEREVISIAE* O RÓŻNYM STATUSIE ANTYOKSYDACYJNYM I METABOLIZMIE ENERGETYCZNYM

Agata Święciło

Katedra Biochemii i Chemii Środowiskowej, Wydział Nauk Rolniczych, Akademia Rolnicza, Lublin

Oddziaływanie azotanu (V) sodu badano na komórkach drożdży *Saccharomyces cerevisiae* różniących się sprawnością systemu antyoksydacyjnego i aktywnością procesów mitochondrialnych. Oznaczono wrażliwość tych komórek na 40 minutową inkubację z azotanem V sodu. Kompetentne oddechowo komórki pozbawione głównych enzymów antyokydacyjnych oraz komórki poddane stresowi oksydacyjnemu, generowanemu za pomocą antymycyny A charakteryzowały się większą wrażliwością na azotan V sodu niż komórki pozbawione funkcjonalnych mitochondriów lub pobrane ze stacjonarnej fazy wzrostu (rys. 1 i 2). Uzyskane wyniki sugerują, że reaktywne form tlenu nie odgrywają istotnej roli w mechanizmach toksyczności indukowanych obecnością azotanu V sodu w przypadku komórek o metabolizmie tlenowym.