Proteomic study on the cellular responses to non-thermal low electric field in *Saccharomyces cerevisiae*

Ali Fanous[1, 2], Angelika Görg[2], Harun Parlar[1,*]

[1] Department for Chemical-Technical Analysis and Chemical Food Technology; Research Center Weihenstephan for Brewing and Food Quality, Technische Universität München, Weihenstephaner Steig 23, D-85354 Freising-Weihenstephan, Germany

[2] Department for Proteomics, Technische Universität München, Am Forum 2, D-85354 Freising-Weihenstephan, Germany

*Corresponding author: parlar@wzw.tum.de, phone: +49-(0)8161-71-3283, fax: +49-(0)8161-71-4418

Running title:

S. cerevisiae response to non-thermal low electric field

Abstract

In this work we examined the effect of non-thermal low electric treatment on the protein expression of *Saccharomyces cerevisiae.* The electric field was produced from therapeutic device Powertube QuickZap, which is intended for the electronic stimulation of nerves. It is used for self-treatment of symptoms and pain. Previous experiments have shown that low frequency electric or magnetic fields cause changes in protein induction. The present study confirms these observations. Two-dimensional gel electrophoresis (2-DE), Progenesis SameSpots software and matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) were used to examine changes in protein expression. In response to 3 hours non-thermal low electric field exposure, the enzyme homoserin dehydrogenase was overexpressed. This result suggests that this enzyme may play an important role in the adaptation of *Saccharomyces cerevisiae* to electric stress. The role of an electric field as a co-stressing factor is discussed.

Keywords:

Non-thermal low electric field; Stress response; Matrix assisted laser desorption ionization-time of flight mass spectrometry; Two-dimensional gel electrophoresis

1 Introduction

Recent proteomic research has provided evidence that environmental stress or stimuli induce the expression of characteristic stress-related proteins in living organisms. This opens the possibility (at least in principle) of using these proteins, which are involved in adaptive and protective mechanisms of living organisms, for the detection of any kind of environmental stimuli with the help of their highly specific „fingerprint". The stress response is an important protective mechanism that enables cells from animals, plants and bacteria to survive environmental stressors with the aid of stress proteins, which are associated with sensing and repairing damage to DNA, helping damaged proteins to refold and to regain their conformations and also acting as chaperones for transporting cellular proteins to their destinations in cells (Kültz et al. 2005). By developing an analytical assay based on these protein expression signatures, it may provide a better understanding of the damage and toxicity mechanisms of these stimuli which are not fully understood yet.

The effect of a non-thermal low electric field on biological systems has been reported by various workers (Harakawa et al. 2005; Türközer el al. 2008). The results however of low electric field research are contradictory, and only little is known about the possible mechanisms of interaction between the electric field and living organisms. Ranalli et al. (2002) studied the effect of low electric treatment on *S. cerevisiae* microflora. They demonstrated that a low voltage treatment using graphite electrodes had a considerable effect on the viable and growth of *S. cerevisiae.* Low electric field could be attributed to many mechanisms, including changes in the electrochemical membrane potential, membrane transport and membrane permeability (Rols et al. 1990). An electric current, even at a low intensity, can cause increases in hydrogen ion concentration inside the cytoplasm (Avery et al. 1996) and disturbance of membrane functionality causing the alteration of cell metabolism. Another important work however showed that extremely low frequency electric field had no

effect on plasma lipid peroxide level and antioxidant activity in rats (Harakawa et al. 2005). Türközer el al. (2008) on the other hand observed effects of 50 Hz electric field exposure on lipid peroxidation levels and antioxidant defence mechanisms in brain tissue of guinea pigs. Goodman and Blank (1998) studied the effect of electromagnetic field (EMF) on *Drosophila* chromosomes and observed that EMF causes changes in chromosome and that heat shock genes were overexpressed. It has been shown in many laboratory studies that levels of hsp70 transcripts were induced when *S. cerevisiae* and human cells are exposed to low frequency electromagnetic fields (Goodman et al. 1994; Lin et al. 1997).

In this study we compared the protein profile of a sample treated with Powertube QuickZap to this of an untreated sample (control), and for each sample, three analytical replicates were run on a pH-range of 4,9–6,9. The eukaryotic microorganism S. *cerevisiae* was used as a model organism to investigate the induction of stress proteins to exposure to non-thermal low electric treatment. *S. cerevisiae* was chosen based on the fact that it is safe to handle and that its genome has been completely sequenced (Goffeau et al. 1996). Furthermore, *S. cerevisiae* was used as a model organism in many medical studies (Lagali et al. 2003; Simon et al. 2000). We analysed the protein expression in *S. cerevisiae* upon non-thermal low electric stress with 2-DE and identified overexpressed proteins using MALDI-TOF-MS.

2 Material and methods

2.1 Yeast strain growth and exposure conditions

The strain BMA64-1A of *S. cerevisiae* (Institute for Molecular Bioscience, Frankfurt, Germany) was grown at 30 °C in defined synthetic YNB medium (6.7 g/L) supplemented with 20 g/L w/v glucose and buffered with sodium hydroxide (6 g/L w/v) succinic acid (10 g/L w/v) to pH 5.8. When the cells reached to the late mid-exponential phase, the cell culture was divided into two cultures; the first one was treated with the therapeutic device Powertube

QuickZap (Fritonex AG, Switzerland), which was operated with a 9-volt battery. The electric impulses within the frequency range of 124–217 kHz were produced from its electrodes, which were inserted into a flask containing 100 ml of the cell culture for time period of 3 hours.

The second culture was not treated with Powertube QuickZap and was considered as a control. The cells were harvested by centrifugation in the late mid-exponential phase once the culture reached an optical density (OD) of 1.0, washed afterwards in ice-cold deionized water, and then spun for 5 min (5000 × g). Yeast cell pellets were each transferred into 1.5 mL Eppendorf tubes and stored at –78 °C if not processed immediately (Harder et al. 1999; Blomberg et al. 1995).

2.2.1 Cell lysis and protein solubilization

The amount of 200 µl of hot (95 °C) sodiumdodecylsulphate (SDS) sample buffer (1% w/v SDS, 100 mM Tris-HCl, pH 8.5) were added to the yeast cell pellet and sonicated (15 × 1 s). After cell lysis, the sample was boiled for 5 min, cooled to room temperature in an ice-bath, diluted with 500 µL of Thiourea/urea lysis buffer (2 M Thiourea, 7 M urea, 4% w/v CHAPS, 1% w/v dithiothreitoland 2% w/v carrier ampholytes, pH 3–10), and spun (16,000 × g, 5 min). The clear supernatants were removed and stored at –78 °C until analysis (Harder et al. 1999).

2.2.2 2-DE with Immobilized pH-Gradients (IPGs)

For the detection of stress-induced proteins in *S. cerevisiae*, 2-DE with IPGs was applied according to Görg et al. (2000). The rehydration buffer of the IPG strips contained 6 M urea, 2 M thiourea, 1% CHAPS w/v, 0.4% DTT w/v, and 0.5% pharmalyte 3–10 v/v. All Chemicals were of analytical or biochemical grade (Amersham Biosciences, Freiburg, Germany). The sample was applied by cup-loading (2 cm from the anode). The protein load was 150 mg for analytical gels, and 450 mg for micropreparative gels, respectively. For the

first dimension, the isoelectric focusing (IEF), IPG gels were cast with pH gradients between 4.9 and 6.9. IEF was performed using the IPG phor IEF unit from Amersham Biosciences. After IEF, the IPG strips were equilibrated in a IPG strip equilibration solution containing 30% w/v glycerol, 6 mol/L urea, and 0.25% w/v bromophenol blue in 0.05 mol/L Tris-HCl pH 8.8 (Görg et al. 1988). Dithiothreitol (DTT) (65 mM/L) was added to the first equilibration step and 260 mM/L iodoacetamide to the second, respectively (Görg et al. 1987). Both equilibration steps lasted for 15 min at room temperature. The second dimension (SDS-PAGE) was performed in vertical gels (13% T) using Ettan Dalt II from Amersham Biosciences. For determining the molecular weight, precision protein standard markers (BioRad, Hercules, CA, USA) were used. The resolved proteins were stained with silver nitrate (Blum et al. 1987), or with a Coomassie Brilliant Blue (CBB G-250) solution (Roti Blue; Roth, Karlsruhe, Germany) (Candiano et al. 2004). The images from stained gels were digitized with a flatbed scanner (Epson expression 1680 Pro). The computer based analysis of the 2-D gels for protein detection, spot matching between gels, and change of protein expression levels was performed with SameSpots software (Molecular Dynamics, Newcastle, UK) according to the manufacturer's instructions. The analysis of the images was performed fully automatically. The normalization factors were determined based on the total volume, and matched spot volumes were normalized across the whole match. After automatic spot detection, matching, and quantification, the data were ranked according to p-value, and the maximum fold change was observed across all the groups compared. For the control and treated sample, only protein spots showing a statistically significant up- or down-regulation ($p = 0.005$) were considered as differentially expressed. The normalized spot volumes of the image in the match set were compared (if the data were identical, all ratios equal 1; for different data, the ratios were < 1 or > 1).

2.3 Protein identification by peptide mass fingerprinting (PMF) using MALDI-TOF-MS

The protein spots of interest were excised from CBB stained gels and destained for 40 min using 100 mL ACN (50%) and 5 mM of $(NH_4)HCO_3$ (50%), and dried at 40 °C for 30 min using a Speedvac. For the tryptic digestion, 20 mg of trypsin was dissolved in 100 mL 1 mM HCl, and immediately before use, 150 mL 5 mM $(NH_4)HCO_3$ was added to the trypsin solution (final concentration: 12.5 ng/mL). Afterwards 10 µl of this solution was pipetted on each dried protein spot and incubated for at least 8 h at 37 °C. The elution solution (30 mL) was pipetted each into a sample vial, which were then incubated in an ultrasonic bath for 20 min. The supernatant was taken and dried using a Speedvac. The digested sample (0.5 mL) was added to the slide and mixed with 0.5 mL of the matrix solution (15 mg/mL alpha-cyano-4-hydroxy-cinnamic acid (CHCA) in 50% acetonitrile (ACN) and 0.5% trifluoroacetic acid (TFA)) and air-dried for 10 min. Mass spectrometry was performed using an Ettan z^2 MALDI-TOF (Amersham Biosciences) equipped with a UV nitrogen laser (337 nm) in harmonic reflectron mode: positive-ion reflectron mode at 20 kV with delayed extraction mode and low mass rejection; calibration: peptide samples (Angiotensin II, ACTH 1–39) and internal standard (trypsin autodigestion fragments) (Wildgruber et al. 2002). For each spectrum, 200 single shots were accumulated. The proteins were considered as identified when at least five peptides were matched, and/or the expectation value was 0.005.

3 Results and discussion

3.1 Temperature monitoring

To ensure that the possible biological effects observed in our study were not related to thermal confounding, the temperature in electric field-exposed samples was monitored over the course of the entire exposure period. The temperature data in the media of each samples were

collected every 60 s. No major changes in temperature were detected during the entire exposure period.

3.2 Differential protein expression

The exposure of *S. cerevisiae* cells to low electric field dose did not cause a dramatic change in protein expression. We analyzed the proteomic response to the electric field exposure in order to identify differentially expressed proteins, which could be important for the resistance to this stress. The *S. cerevisiae* cells were treated with the therapeutic device Powertube QuickZap for 3 h, and the extracts from untreated as well as treated cells were then subjected to comparative 2-DE (Fig. 1). Afterwards, the changes in spot intensity between untreated and treated cells were quantified by image analysis software and the differences by a 1.5-fold change between treated and untreated cultures and a p-value (expectation) of 0.005 were considered as significant. After treating the *S. cerevisiae* cells with the therapeutic device Powertube QuickZap, only one considerable up- regulated protein in the pH range between 4.9–6.9 was found (Fig. 1).

PMF analysis revealed that the protein homoserin dehydrogenase was significantly upregulated (1.8-fold) as a response to the electric field exposure (Fig. 2). This enzyme catalyzes the synthesis of homoserine which participates in energy metabolism and helps the cell to gain $NADH/H^+$. In many studies the induction of a large number of genes which are involved in energy metabolism was reported after the treatment of *S. cerevisiae* with NaCl or ethanol as stress factors (Alexandre 2003; Yale and Bohnert 2001). Fanous et al. (2007) found that the protein long-chain acyl-CoA synthetase, which is involved in energy metabolism, was upregulated after the treatment of the soil bacteria *Corynobacterium glutamicum* with the herbicide 2,4-dichlorophenoxy acetic acid (2,4-D). The expression of this enzyme may reflect the increased need of the cell to generate energy that is necessary to counteract electric stress.

Homoserin dehydrogenase was induced and considered as stress protein when *Escherichia coli* was grown in the presence of salt (NaCl); osmotic stress (Weber et al. 2006). The induction of heat-shock proteins has been reported as defense mechanism against a wide range of stress conditions, including exposure to electric and magnetic fields (Goodman et al. 1994; Lin et al. 1997). Heat-shock proteins are molecular chaperones that assist abnormal proteins to accumulate under stress conditions, to regain their proper folding, or to assist their proteolytic degradation (Godon et al. 1998). Surprisingly, in this study we did not find any alteration in expression of heat shock proteins (HSP). As in good agreement with our results, Morehouse et al. (2000) reported that exposure to low-frequency electromagnetic fields does not alter the HSP70-expression in HL60 cells.

4 Conclusion

The results of this study suggest that one identified protein upon with non-thermal low electric field is involved in the cell's adaptation phase. The cell may need homoserin dehydrogenase to cope with increased energy demands after the exposure to the electric field and the enzyme may enable the cell to protect itself from this stress. To the best of our knowledge, this protein has not been previously reported as a stress protein in *S. cerevisiae* cells after the exposure to the electric field or any kind of stress. We suggest that non-thermal low electric field does not have a big effect on *S. cerevisiae* because the cell could induce only one defense mechanism, although *S. cerevisiae* has the ability to induce many defense mechanisms. It indicates that this defense mechanism suffices to protect the cell from external stress.

Declaration of interest

The authors declare no conflicts of interest. The valuable scientific input to this work from Dipl.-Ing. Mr. Martin Frischknecht (Fritonex AG, Switzerland) is gratefully acknowledged.

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Figure Legends

Fig.1. 2D gel images of *S. cerevisiae* cells untreated (= control) or treatment with Powertube QuickZap. The protein that was induced > 1.5-fold was selected as stress protein and identified by MALDI-TOF-MS.

Fig.2. Overexpression of homoserin dehydrogenase after treatment with Powertube QuickZap; "Zoom in" from IPG 4.9–6.9 with left control and right treatment sample.

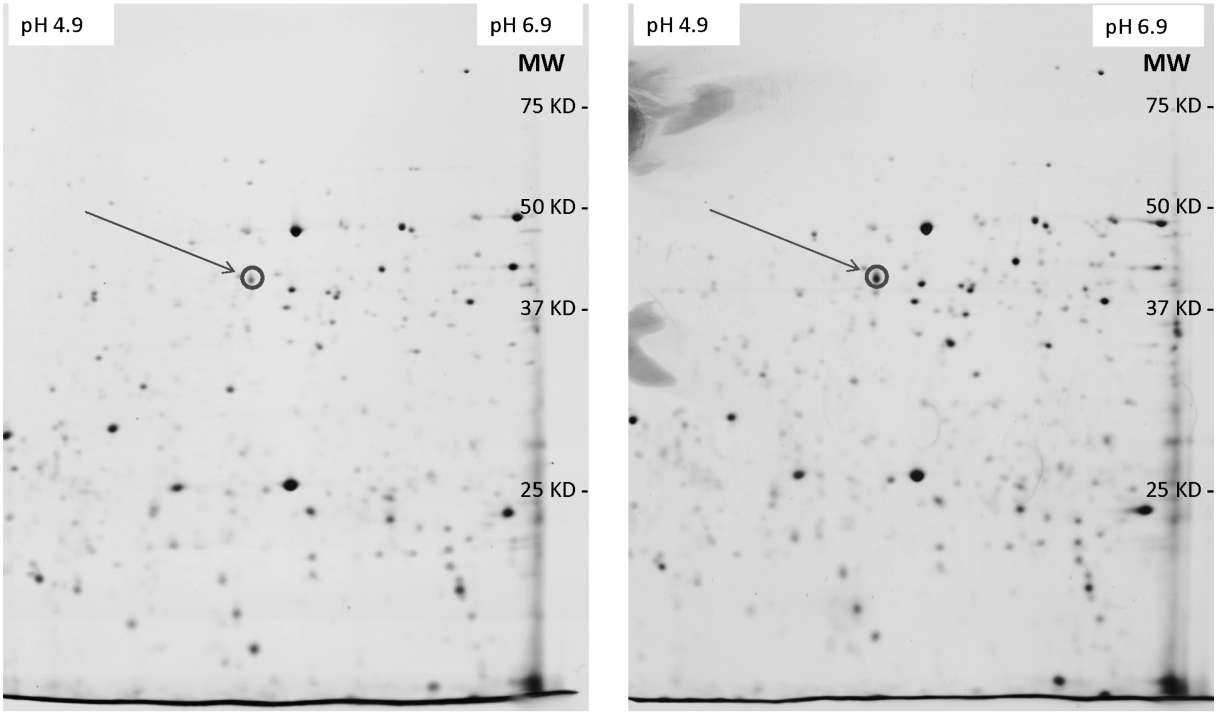

Figure 1

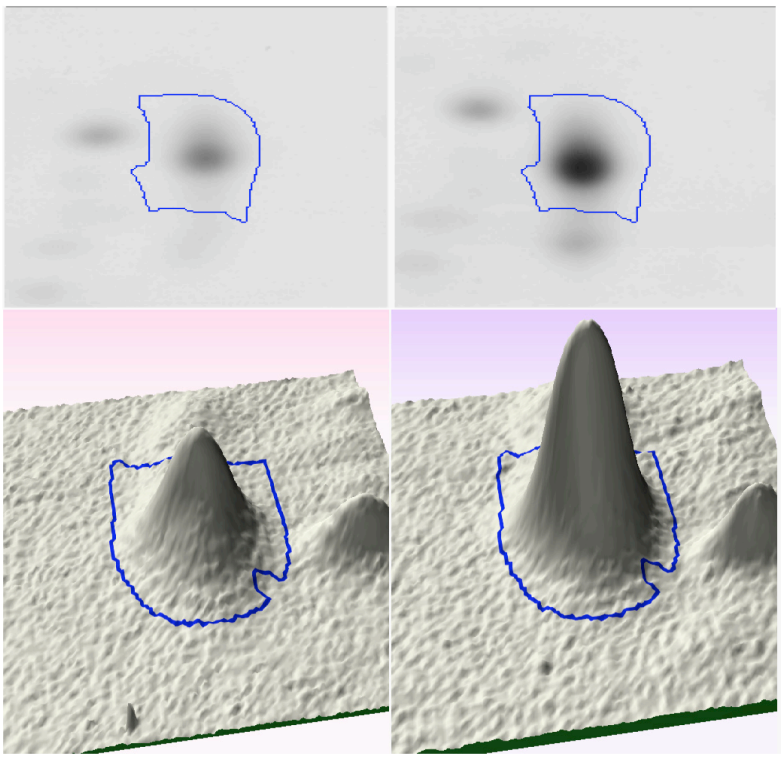

Figure 2