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Salinity Affects Fatty Acid and Extracellular Glycoprotein Composition of *Dipodascus australiensis*

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Summary

Significant changes in the fatty acid composition of the cell lipids and extracellular glycoproteins of the yeast-like species Dipodascus australiensis grown under NaCl stress or in salt-free conditions were observed in the different growth phases. In the cells which were cultivated under hypertonic conditions, oleic acid content increased during the exponential growth phase while mean percentage of linoleic acid decreased. Significant changes in the fatty acid composition of the cell lipids occurred between the late exponential phase and early stationary phase of growth between ca. 3rd to 5th day for salt-free cultivation medium and between 6.5 and 8.5 day for hypertonic conditions.

The resistance of yeast cells to osmotic-stress (caused with higher concentration of NaCl) can be correlated with the production of the extracellular glycoproteins. NaCl-stressed samples contained more mannose, galactose, and less glucose and glucosamine. The glycoproteins containing glutamic acid were produced under the influence of 8% NaCl predominantly during the early stationary phase of the growth.

Keywords: Dipodascus australiensis, fatty acids, extracellular glycoproteins, NaCl-stress

Introduction

The biological stress may be defined as a biological factor that can potentially cause injury to the organism. The metabolism of many microorganisms is adaptable in order to allow the cells to respond to extreme change in the environment *(1)*. In yeast cells, enzyme systems are repressed or derepressed in response to the changes in nutrients, concentration of salt *(2)*, temperature and oxygen. Most of the enzymes are located on the external surface of the plasmalema compartments or in the periplasmic space of the cell wall and can be excreted in the extracellular medium *(3,4)*. Glucans and mannoproteins are interwoven in a cell wall in a certain ratio *(5)*, which is not evenly maintained throughout the wall *(6)*. Mannoproteins overlay the glucan and cover the cell surface *(7)*. The internal glucan layer determines cell wall rigidity *(5)* while the external mannoprotein layer determines cell wall porosity *(8)*.

Salt-tolerant yeasts may be characterized by their ability to change the membrane functions in order to keep solutes within the cells in response to osmotic stress. The membrane lipids play a crucial role in controlling membrane fluidity *(9)*.

In this work we investigate changes in the lipid composition of the whole cells and in crude extracellular glycoproteins of *D. australiensis* grown in the medium with or without 8% NaCl.

Materials and Methods

Yeast strain and growth conditions

Dipodascus australiensis CCY 52-6-1 was obtained from the Culture Collection of Yeasts, Bratislava, Slovakia. The strain was cultivated on a reciprocal shaker at 28 °C in 1 000 mL flasks with 500 mL of a liquid mineral salt medium containing 2% (w/v) glucose *(10)*. The NaCl concentration in cultivation medium was performed by the addition of 80 g NaCl per litre of cultivation medium. The initial pH of medium was adjusted to 6.2.

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Analytical methods

The growth of cultures was monitored by the measuring of the absorbance at 660 nm.

The cells were separated by centrifugation. Crude glycoproteins were isolated by precipitation from the supernatant after addition of two volumes of 96% (volume fraction) ethanol, and subsequent centrifugation. Ethanol precipitate was dissolved in distilled water, dialysed against distilled water and freeze-dried.

Fatty acid composition was determined according to Šajbidor *et al. (11)*. Protein content was measured as described by Lowry *et al. (12)*. Glutamic acid was analyzed (after an 20 h hydrolysis at 105 °C with 6 mol/L HCl) on an amino acid analyzer (Model T-339, Microtechna, Praha). The carbohydrate content was determined by the phenol-sulphuric acid method. Monosaccharide components (after an 8 h hydrolysis at 110 °C with 4 mol/L HCl) were determined as alditol trifluroacetates by GC--mass spectrometry.

Paper chromatography of monosaccharide and aminosaccharide components (after an 8 h hydrolysis with 4 mol/L HCl) was performed by the descending method on Whatman No.1. paper using elution system: ethyl acetate – pyridine – water (8:2:1) as described *(13)*.

Determination of the relative molecular masses (M_r) of ethanol precipitates was performed on a FPLC instrument (Pharmacia, Sweden) using gel-permeation chromatography on Superose 12 HR column (10×30 mm) in 0.05 M phosphate buffer, pH = 7.0 containing 0.15 M NaCl. Flow rate was 0.5 mL min^{-1}. The content of proteins in the fractions was measured at 280 nm and phenol-sulphuric acid method was used for content of carbohydrates. The following standards have been used: ribonuclease – 13.7 kDa, chymotrypsinogen A – 25 kDa, ovalbumin – 43 kDa, bovine serum albumin – 67 kDa, aldolase – 158 kDa, catalase – 232 kDa, ferritin – 440 kDa, thyroglobulin – 669 kDa and blue dextran – 2 000 kDa.

Results

Quantitative changes in the fatty acid composition and in extracellular glycoproteins of the yeast species *D. australiensis* grown under NaCl stress or salt-free conditions were found during the different growth phases. Early stationary phase of strain CCY 52-6-1 growing in non-saline medium was used as inoculum into medium without NaCl (reference medium) or to medium supplemented with 8% (w/v) NaCl (stress medium). The 5-day duration of lag-phase was required for adaptation (Fig. 1) of the culture to the hypertonic conditions.

The ratio of unsaturated fatty acids (C16:1, C18:1) to saturated fatty acids (C16:0, C18:0) influences membrane fluidity, *i.e.* an increase in the proportion of unsaturated fatty acid side chains in cellular lipids tends to increase membrane fluidity *(14)*. As shown in Table 1, these ratios were determined from cells in NaCl-stress conditions and were dependent on the age of the culture. The ratio of C16:1/C16:0 did not change in the NaCl-free medium.

The observed variations in the composition of fatty acids and absorbance (A_{660}) of the culture at different

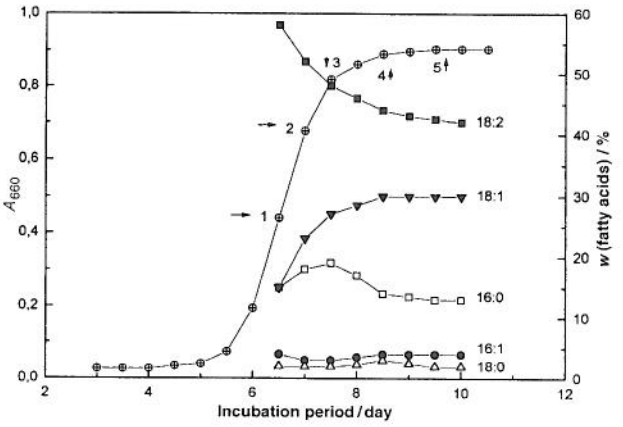

Fig. 1. Dependence of the changes in the cellular fatty acids composition on the age of *D. australiensis* grown in hypertonic medium. ⊕—⊕ growth curve.

Table 1. The ratio of unsaturated fatty acids to saturated fatty acids

Growth phases[a]	Ratio of fatty acid[b]			
	C16:1/C16:0		C18:1/C18:0	
	0	8	0	8
1	0.06	0.16	5.6	6.8
2	0.02	0.15	5.6	10.5
3	0.02	0.16	5.7	11.5
4	0.01	0.26	5.8	11.7
5	0.02	0.26	6.2	15.2

[a]1, 2, 3, 4, 5-phases of growth (see Fig. 1, 2); **0** - NaCl-free medium, **8** – medium with 8% NaCl, [b]mean percentage values (fatty acids in cell lipids) of three independent experiments.

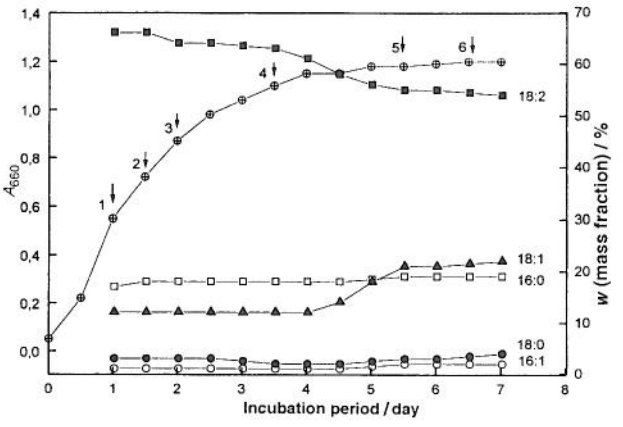

Fig. 2. Dependence of the changes in the cellular fatty acids composition on the age of *D. australiensis* grown in salt-free medium. ⊕—⊕ growth curve.

stages of growth are illustrated in Figs. 1 and 2. Culture age and hypertonic conditions did not affect the relative amounts of myristic acid (C14:0, in trace amounts not shown), palmitic acid (C16:0, about 17% mass fraction) and stearic acid (C18:0, about 2% mass fraction).

Significant changes in the fatty acid composition of the cell lipids occurred between the late exponential

phase and early stationary phase of growth, ca. 3rd to 5th day for salt-free cultivation medium and between 6.5 and 8.5 days for hypertonic conditions. In lipids of the cells cultivated in hypertonic conditions was the content of oleic acid (C18:1) increased from 15% to 30% while mean percentage of linoleic acid (C18:2) decreased from 58% to 43%.

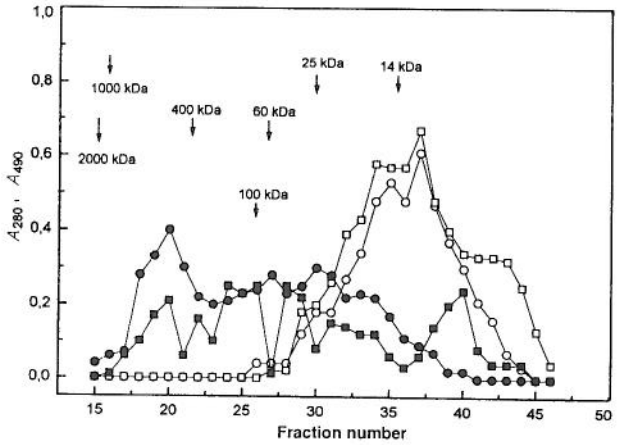

Fig. 3. Changes in the molecular mass distribution of extracellular glycoproteins produced in different growth phases under salt-free conditions.
Proteins (as A_{280} □—□), carbohydrate (as A_{490} ■—■) early exponential growth phases,
proteins (as A_{280} ○—○), carbohydrate (as A_{490} ●—●) early stationary growth phases.

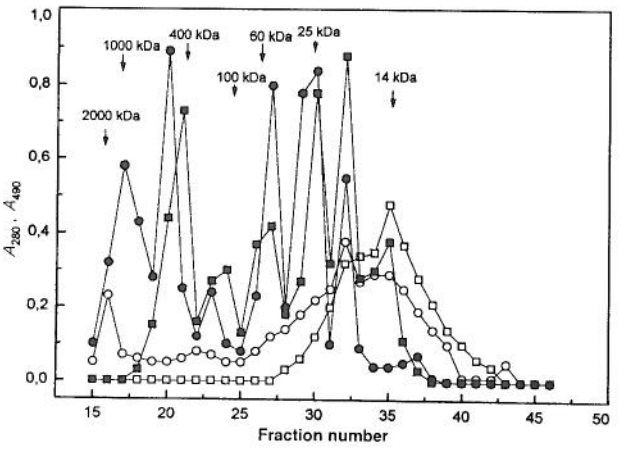

Fig. 4. Changes in the molecular mass distribution of extracellular glycoproteins produced in different growth phases under hypertonic conditions (8% NaCl).
Proteins (as A_{280} □—□), carbohydrate (as A_{490} ■—■) from early exponential growth phases,
proteins (as A_{280} ○—○), carbohydrate (as A_{490} ●—●) from early stationary growth phases.

Addition of 8% NaCl to the medium affected the composition of the extracellular glycoproteins of the studied strain. Data of their monosaccharide composition are shown in Table 2. As long as during growth in the control medium the percentage of mannose remained constant, salt in the medium caused increase of mannose in the glycoproteins. Glycoproteins from the salt-free medium contained a higher amount of glucose and glucosamine (determined by paper chromatography) than the glycoproteins from the medium with 8% NaCl. During growth of the culture in salt-containing medium, the content of glucose decreased, predominantly in early stationary phase of the growth (Table 2). The extracellular glycoproteins obtained from the salt-free medium contained a higher amount of glucose than the glycoproteins obtained from 8% NaCl medium. The glycoproteins from stressed sample were more soluble and content of galactose in saccharide moiety was higher than was the case with the glycoproteins from the reference sample. The glycoproteins containing glutamic acid were produced under the influence of 8% NaCl. During the growth, content of glutamic acid was changed (Fig. 5). Predominatly during the early stationary phase of the growth content of glutamic acid was higher (6.2%, mass fraction), in comparison with late stationary phase in which the content was lower (4.5%, mass fraction) *(15)*. The reference samples contained lower amount of glutamic acid and were relatively constant in the different

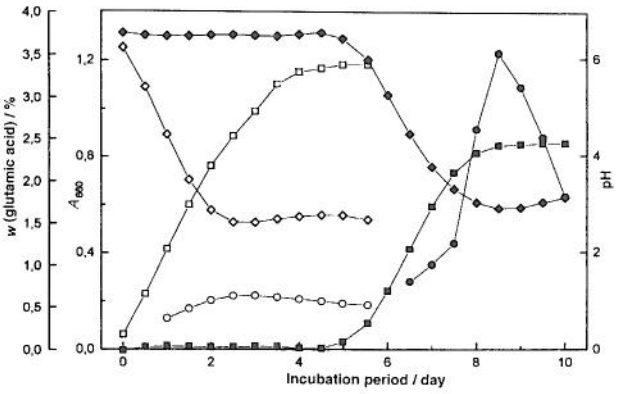

Fig. 5. Changes in the pH of the cultivation medium and content of glutamic acid in the glycoproteins from the different growth phases.
◊—◊ pH salt-free medium, ◆—◆ pH hypertonic medium, ○—○ glutamic acid (salt-free medium), ●—● glutamic acid (hypertonic medium), □—□ growth curve (salt-free medium), ■—■ growth curve (hypertonic medium).

Table 2. The content of the monosaccharides in different growth phases

Sacch[a] (%)	Growth phase and concentration of NaCl									
	1_0	1_8	2_0	2_8	3_0	3_8	4_0	4_8	5_0	5_8
Man	19.7	24.3	18.6	25.6	19.4	29.2	18.4	29.4	19.3	30.5
Glc	40.2	33.3	40.7	35.7	65.3	27.0	63.1	28.7	68.2	29.3
Gal	0	7.1	0	6.2	1.0	6.6	1.2	6.4	3.6	6.7

Man-mannose, Glc-glucose, Gal-galactose; 0 – NaCl-free medium, 8 – medium with 8% NaCl; 1, 2, 3, 4, 5-phases of growth (see Fig. 1, 2); [a]mean percentage values (monosaccharides in glycoproteins) of three independent experiments

growth phases. pH of the cultivation medium decreased during growth and did not depend on the concentration of NaCl in the medium (Fig. 5).

Separation of the glycoprotein fraction on Superose 12 column yielded fractions with M_r ranging from 13 to 2000 kDa. From the comparison of elution volumes of carbohydrates (expressed as A_{490}) and proteins (as A_{280}) it is evident that the changes in relative molecular masses (M_r) have occurred (Fig. 3, 4). In the NaCl-containing medium, the investigated strain produced glycoproteins with higher M_r in comparison with the glycoproteins produced in the reference cultivation conditions.

The M_r values of studied glycoproteins were constant during the growth in the salt-free medium and have changed in NaCl-stress conditions (Fig. 4). In the early stationary phase in the stress conditions the cells produced glycoproteins with higher M_r, than glycoproteins produced in late stationary phase of growth *(16,17)*.

Discussion

We investigated the changes in the composition of the cell lipids and extracellular glycoproteins prepared from cells or cultivation medium of the yeast-like culture *D. australiensis* growing in two media, one containing 8% NaCl and another without NaCl.

Significant differences were observed in the composition of the lipids and extracellular glycoproteins, predominantly in the samples isolated from the late exponential growth phase and early stationary phase (Fig. 1, 2).

Changes in the glucose, mannose, and galactose (Table 2) contents in the carbohydrate moiety of extracellular glycoproteins from the cells grown in the salt-free and 8% NaCl medium were observed. These changes in the composition of extracellular glycoproteins together with the changes of the lipids play an important role in the control of cell water in hypertonic environment (stress conditions). It is known that the external mannoprotein layer determines cell wall porosity *(18)*. In our previous report we suggested that the increased content of mannose and decreased content of glucose in the extracellular glycoproteins were characteristic for effective cryoprotective polymers *(16)*. These compounds protected the cells from dehydratation caused by freeze-thawing process.

The higher content of oleic acid (C18:1) in cell lipids (Fig. 1) represented a part of a general stress response of the cells and can be regarded as an evolutionary advantage for survival and competition under different enviromental conditions *(11,19)*.

It is known that the total cellular activity may be one of the potential factors of interest when trying to elucidate different osmotolerance factors (change of NaCl concentration in the extracellular space). The stationary phase of yeast cells growing in stress conditions is attendant with the production of unsaturated fatty acids of the cell lipids and composition of the extracellular glycoproteins. It was evident from the study that possible major changes in the fatty acid composition and/or in the extracellular glycoproteins composition occurred only during the late exponential and early stationary phases. It has been reported *(14)* that the stationary growth phase was more suitable with respect to tolerance toward the environmental stress *(20)*.

The cell wall porosity is dependent upon the growth phase of the cells and degree of mannosylation of the wall mannoproteins *(8)*. The stress cells adapted to the extreme conditions with the change of the cell wall composition (increased wall porosity and altered composition of the membrane) increased fluidity of the phospholipid bilayer *(9)*.

The production of glycoproteins probably constitutes a part of an adaptation mechanism and extracellular protection against unbalanced growth. In a hypertonic environment the interaction between the bound water molecules and glycoproteins is a stabilizing factor and serves as »osmotic buffer« protecting the cells in a high osmotic-pressure medium *(16)*.

Conclusions

The content of the glucose, mannose, galactose and glucosamine in the carbohydrate moiety of extracellular glycoproteins differed and was dependent on the osmotic stress conditions. The yeast cells under salt-stress produced more oleic acid in cell lipids.

The extracellular glycoproteins produced in later stationary phase of growth are different in comparison with glycoproteins from early stationary phase.

The combination of factors influencing the osmotolerance including the composition of extracellular glycoproteins, composition of lipids and age of the culture is involved.

Acknowledgements

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Slanost utječe na sastav masnih kiselina i ekstracelularnih glikoproteina u *Dipodascus australiensis*

Sažetak

Značajne promjene u sastavu masnih kiselina staničnih lipida i ekstracelularnih glikoproteina opažene su u raznim fazama rasta kvascu slične vrste Dipodascus australiensis, u podlogama bez soli ili pod stresom NaCl. U stanicama uzgajanim u hipertoničnim uvjetima, tijekom eksponencijalne faze rasta, povećava se količina uljne kiseline, a snizuje prosječni postotak linolne kiseline. Značajne promjene u sastavu masnih kiselina staničnih lipida odvijaju se između kasne eksponencijalne i rane stacionarne faze rasta, od približno 3. do 5. dana u podlozi bez soli, te između šest i pol te osam i pol dana pod hipertoničnim uvjetima. Otpornost kvaščevih stanica na osmotski stres (uzrokovan većim udjelom NaCl) usklađena je s proizvodnjom ekstracelularnih glikoproteina. Uzorci pod NaCl stresom sadržavali su više manoze, galaktoze, a manje glukoze i glukozamina. Glikoproteini koji sadržavaju glutaminsku kiselinu nastaju pod utjecajem 8%-tnog NaCl, pretežno tijekom rane stacionarne faze rasta.