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Identification of Proteins Affected by Iron in *Saccharomyces cerevisiae* Using Proteome Analysis

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Abstract To study the effect of iron on *Saccharomyces cerevisiae*, whole-cell proteins of *Saccharomyces cerevisiae* were extracted and subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), and differentially expressed proteins were identified. The proteins separated were further identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and were compared with a protein database. Of more than 300 spots separated by molecular weight and isoelectric points, 27 differentially expressed spots were identified. Ten proteins were found to be differentially expressed at high iron concentration. Triosephosphate isomerase (TPI), YDR533C hypothetical protein, superoxide dismutase (SOD), 60 kDa heat-shock protein (HSP60), pyruvate dehydrogenase beta subunit 1 (PDB1), and old yellow enzyme 2 (OYE2) were upregulated, whereas thiol-specific antioxidant (TSA), regulatory particle non-ATPase subunit 8 (RPN8), thiol-specific peroxiredoxin 1 (AHP1), and fructose-1,6-bisphosphate adolase (FBA) were downregulated by iron. Based on the result, we propose that SOD upregulated by iron would protect the yeast from oxidative stress by iron, and that TSA downregulated by iron would render cells hypersensitive to oxidative stress.

Key words: Iron, MALDI-TOF, *Saccharomyces cerevisiae*, two-dimensional electrophoresis

Iron is a critical element for most organisms and is present in the active site of many enzymes, which are involved in metabolic pathways such as the tricarboxylic acid cycle, respiration, oxygen transport, and DNA synthesis [13]. However, excess iron stores in cells can lead to oxidative damage, as free iron readily participates in redox reactions within cells that can produce the reactive oxygen species and associated cellular and molecular damage [3]. To investigate the effect of iron on gene expression in eukaryotes, approaches such as differential display and subtractive hybridization have the advantage of surveying the entire genome [1, 24]. However, they are technically demanding and have given way to microarray techniques [5]. To date, however, numerous microarray experiments with various organisms have not yielded any definitive picture of the role of iron.

Yeast has the same defense mechanisms as higher eukaryotes, and offers the information of genome and proteome studies, since *S. cerevisiae* was the first eukaryote whose genome was completely sequenced and the number of each expressed gene has been determined [6, 23]. This information renders *S. cerevisiae* to be an ideal eukaryotic model to study the effect of iron on cellular control in eukaryotes. The proteomic investigation of yeast by 2D-PAGE has bean performed with solubilized yeast proteins [8], followed by other studies using similar approaches to investigate glucose exhaustion, oxidative, hyperosmolarity, and cadmium stress responses [2, 22]. Proteomic studies can help coupling genome and proteome analyses to cellular functions, and they are complementary to genomic studies, since an abundance of mRNA has not necessarily been correlated with gene expression quantitatively [7]. Many studies have focused on the molecular mechanisms relevant to the utilization of iron in yeast [18, 21]. However, the effect of iron on *S. cerevisiae* has not yet been reported, using the proteomic analysis.

To address the effect of iron in *Saccharomyces cerevisiae*, we measured the level of iron in yeast cultured in iron-containing medium and identified differentially expressed proteins by proteome analysis, employing 2D-PAGE and MALDI-TOF mass spectrometry analysis.

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MATERIALS AND METHODS

Materials

All reagents used in 2D-PAGE were obtained from Amersham Biosciences (Uppsala, Sweden) and Bio-Rad (Hercules, CA, U.S.A.). All these reagents were of analytical grade. Sequencing grade trypsin was obtained from Promega Company (Madison, WI, U.S.A.). Trifluoroacetic acid and acetonitrile were obtained from Sigma.

Yeast Culture

S. cerevisiae S11-39 was cultured in YM medium containing 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 1% (w/v) glucose, and 0.5% (w/v) peptone. Ferric citrate was used as the iron source when the absorbance of cells (optical density at 600 nm) reached 0.4. Exponential phage cells, grown in YM media at 30°C, were adjusted to a concentration of 2×10^8/ml. Cultured cells were harvested by centrifugation (15 min, 5,000 ×*g*) and washed twice with 1 ml of sterile 0.1 M sodium phosphate buffer (pH 7.0).

Atomic Absorption Spectrometry

For iron-uptake assay, cells were grown, harvested, washed three times with nano-pure water, and dried at 50°C for 2 days. The dried cells (0.04 g) were digested with 6 ml of concentrated nitric/perchloric acid (2:1, v/v) in volumetric flasks in a flame for 10–15 min. Digested samples were put into a flask, nano-pure water was added to a final volume of 100 ml, and then the samples were filtered. The quantities of iron were determined by atomic absorption spectrometry AA-6401 (Shimazu, Kyoto, Japan). The instrument settings were made according to the standard procedure recommended by the manufacturer. The wavelength was 248.3 nm with a deuterium background correction, and the burner gas mixture was air-acetylene. The iron atomic absorption standard solution was a commercial product (Accustandard, New Haven, U.S.A.). Working standards were prepared from the stock standard solution by diluting with dilute nitric acid solution.

Protein Preparation

Yeast cells in the late mid-exponential phase were harvested by centrifugation, when they reached an optical density of 1.0, washed in ice-cold deionized water, and spun at 15,000 ×*g* for 5 min. Yeast cell pellets were transferred into individual 1.5-ml eppendorf tubes and stored at −70°C if not processed immediately. Cells (10–20 mg of dry weight) were disrupted with sonication (Ultrasonic vcx 400; Sonics & Materials Inc): 400 ml of sample buffer (100 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM PMSF) was added to the yeast cell pellet, and they were sonicated six times for 10 sec each with intermediate cooling on ice. After cell breakage, 1 ml of thiourea/urea lysis buffer [7 M urea, 2 M thiourea, 1 mM EDTA, 1 mM PMSF, 4% (w/v) CHAPS, 0.5% IPG (v/v) buffer, and 1% (w/v) DTT] was added, and the mixture was incubated for 20 min at 4°C with gentle shaking and centrifuged for 30 min at 20,000 ×*g* [8]. After the supernatant was withdrawn, at least 4 volumes of ice-cold acetone was added into the protein extract, and the mixture was allowed for at least 3 h to precipitate at −20°C. The precipitated protein was centrifuged for 30 min at 20,000 ×*g* and residual acetone was removed, and the pellet was dried by an air dryer. Dried protein pellet was resuspended in an equal volume of lysis buffer, and then transferred to a new 1.5-ml tube after centrifugation for 20 min at 20,000 ×*g*. The protein concentration of the extract was determined using a protein assay kit (Bio-Rad). Extracts were stored at −70°C before isoelectric focusing.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

2D-PAGE was carried out by IPG (immobilized pH gradient) phor (Amersham Biosciences) for the first-dimensional isoelectric focusing using an immobiline DryStrip (Bio-rad), and by a protean II 2-DE system (Bio-Rad) for the second-dimensional SDS-PAGE [10]. Protein (100 μg) was loaded onto the IPG (Immobilized pH 4–7 linear gradient) strips by rehydration loading IPGphor. The strip gel was rehydrated for 6 h at 30 V, and the proteins were separated using the following stepwise increases in voltage and running times: 150 V for 1 h, 300 V for 1 h, 600 V for 1 h, and 5,000 V for 13.35 h (to a total of ~ 67 kVh). After the above isoelectric focusing, the strips were incubated with an equilibration buffer I [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 1% (w/v) DTT] and buffer II [50 mM Tris-HCl, pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue, 2.5% (w/v) iodoacetamide] for 15 min each. The equilibrated strip was placed on a 13% polyacrylamide gel. Separation was continued at 20 mA/gel in the tank buffer [25 mM Tris-HCl, pH 8.8, 198 mM glycine, and 0.1% (w/v) SDS] until bromophenol blue reached the bottom of the gel. After electrophoresis, the gel was treated with a fixing solution [40% (v/v) methanol, 10% (v/v) acetic acid] for 1 h. After fixation, the gel was rinsed twice with 30% (v/v) ethanol for 20 min each and washed with distilled water for 20 min. Subsequently, the gel was treated with a sensitizing solution [0.02% (w/v) sodium thiosulfate] for 1 min and washed with distilled water. The gel was treated with an $AgNO_3$ solution [0.2% (w/v) $AgNO_3$, 0.02% (v/v) formaldehyde] for 25 min and washed with distilled water. The gel was incubated with a developing solution [3% (v/v) sodium carbonate, 0.05% (w/v) formaldehyde] until protein spots were clearly visible. The development reaction was stopped by replacement of the developing solution by a stop solution [1.4% (w/v) EDTA] for 45 min. The silver stained gel was scanned using a densitometer 800 (Bio-Rad), and the digitalized

image was analyzed using PDQUEST software (V. 6.1, Bio-Rad).

Identification of Proteins

Proteins were identified by using MALDI-TOF mass analysis [17]. The selected protein spots were cut from the gel with a spot cutter (Bio-Rad). Excised gel spots were destained with 100 μl of destaining solution (30 mM potassium ferricyanide, 100 mM sodium thiosulfate) with shaking for 5 min. After removal of the solution, gel spots were incubated with 200 mM ammonium bicarbonate for 20 min. The gel pieces were dried in a speed vacuum concentrator for 5 min, and the dried gel pieces were rehydrated with 20 μl of 50 mM ammonium bicarbonate, containing 0.2 μg of modified trypsin (Promega, WI, U.S.A.), for 30 min at 4°C. The digestion was performed overnight at 37°C. The peptides were eluted with 0.8 μl of matrix solution [70% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid, 10 mg/ml alpha-cyano-4-hydroxycinamic acid]. The eluted peptides were spotted onto a stainless steel target plate, and masses of peptides were determined using a MALDI-TOF mass spectrometer (Applied Biosystems Inc.). Calibration was carried out by using the internal mass of trypsin. Peptide masses were matched with the theoretical peptides of all yeast proteins in the NCBI database using Mascot software and profound software.

Results and Discussion

Iron Storage in *S. cerevisiae*

To investigate the effect of iron in yeast, *S. cerevisiae* was grown with or without 20 mM ferric citrate for 48 h at 30°C. The grown yeast culture was collected and analyzed by atomic absorption spectrometry. The iron content of *S. cerevisiae* in the medium with 0, 10, and 20 mM ferric citrate was 310±36 μg, 5,310±520 μg, and 15,925±2,023 μg per 1 g dry cell, respectively. This iron content in *S. cerevisiae* was about 9-fold higher than that in *Pichia pastoris* transformant [12]. The above results showed that *S. cerevisiae* grown in high iron medium has a functional property of iron storage *in vivo*. These yeast cells were further investigated by proteome analysis.

Comparison of Protein Profiling of *S. cerevisiae*

For better understanding of iron storage in yeast, the differential protein expressions in yeast at 20 mM ferric citrate were examined by 2D-PAGE. Twenty-seven spots from a 2D gel were analyzed by MALDI-TOF mass spectrometer. Among these, ten spots were identified by mass mapping. Identification of these proteins would be valuable for understanding the mechanism of iron metabolism. For this, we carried out 2D-PAGE three times to identify protein spots that showed consistently differential expression levels at 20 mM iron concentration. Within the experimental window of 2D-PAGE, an average of 27 spots was detected. Figure 1 shows a comparative proteome analysis of yeast. The differences in spot intensity between *S. cerevisiae* S11-39 grown in culture with or without 20 mM ferric citrate were quantified by PDQUEST software. We identified ten proteins that showed differential expression levels between *S. cerevisiae* S11-39 grown in culture with or without 20 mM ferric citrate from database analysis. Table 1 lists the theoretical pI and molecular mass of proteins whose expression levels showed differences between *S. cerevisiae* S11-39 grown in culture with or without 20 mM ferric citrate: The experimental and theoretical pIs and molecular masses agreed in most of the identified spots. However, we

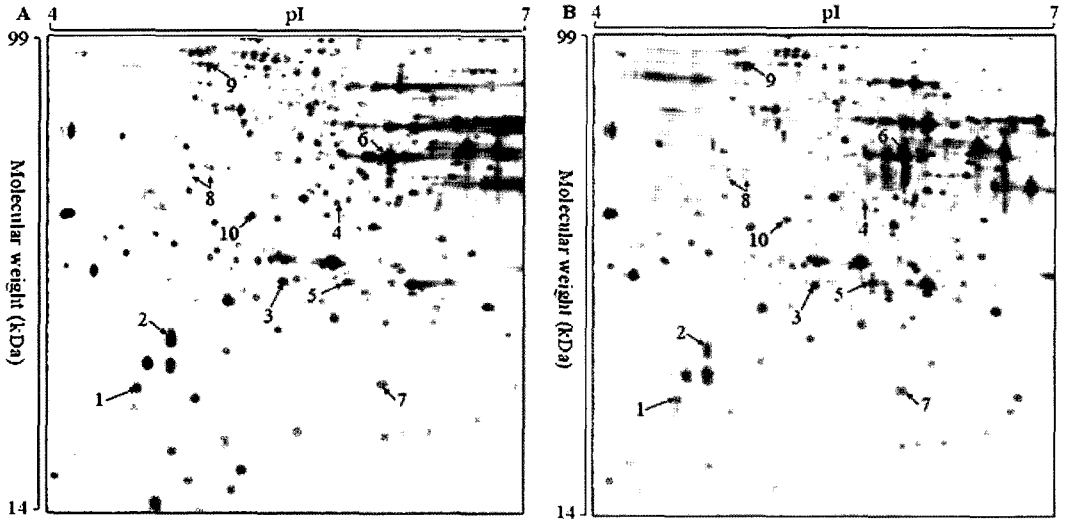

Fig. 1. Comparative proteome analysis of *S. cerevisiae* S11-39 grown in culture without (**A**) or with (**B**) 20 mM ferric citrate.
Gel images were analyzed using PDQUEST software. Differentially expressed proteins are numbered in both gels.

Table 1. Identification of differentially expressed proteins by iron in *Saccharomyces cerevisiae* S11-39.

Spot number	Regulation	pI	Molecular mass (kDa)	Protein name	Accession No.
1	Down	5.03	21.69	TSA (Thiol-specific antioxidant)	6323613
2	Down	5.01	19.27	AHP1 (Thiol-specific peroxiredoxin 1)	6323138
3	Up	5.3	25.55	YDR533C hypothetical protein	47168642
4	Down	5.4	38.47	RPN8 (Regulatory particle non-ATPase subunit 8)	6324835
5	Up	5.7	26.76	TPI (Triosephosphate isomerase)	230406
6	Down	5.5	39.89	FBA (Fructose-1,6-bisphosphate adolase)	6322790
7	Up	5.48	15.84	SOD (Superoxide dismutase)	16730103
8	Up	5.2	40	PDB1 (Pyruvate dehydrogenase beta subunit 1)	6319698
9	Up	5.2	61.02	HSP60 (60 kDa heat-shock protein)	6323288
10	Up	6.1	45	OYE2 (old yellow enzyme 2: NADPH dehydrogenase)	6321973

Spot numbers of proteins correspond to those shown in Fig. 1.

could also find some differences between experimental and theoretical values for some spots in Fig. 1. This difference could be explained by the following explanations. One reason could be that proteins were modified as a result of cellular processes, specifically post-translational modifications such as phosphorylation. Secondly, incomplete binding and denaturation of proteins by SDS during the equilibration step could be responsible. It is also possible that the homologs identified from the database represent different protein isoforms with different charges and size properties [4, 14].

The expression levels of the following six proteins were increased in *S. cerevisiae* S11-39 grown on YM broth with 20 mM ferric citrate (Figs. 1, 2A). Copper-zinc superoxide

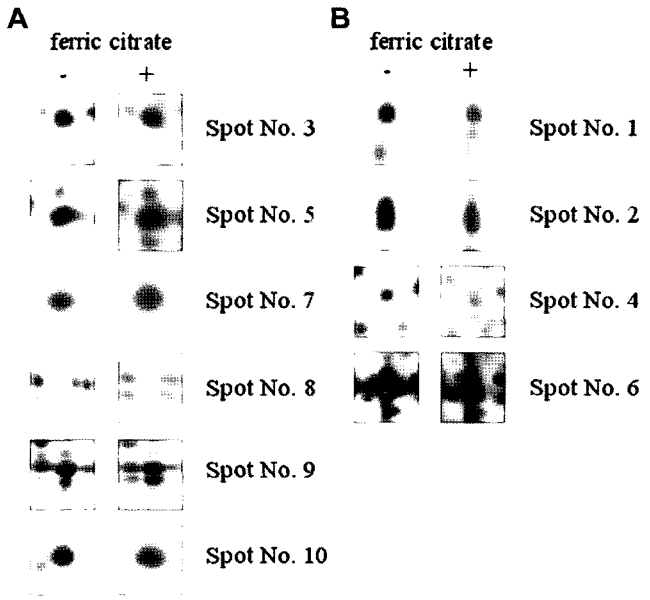

Fig. 2. Relative intensities of upregulated (**A**) and downregulated (**B**) protein spots obtained through 2D-PAGE.
Left panels: protein preparation from *S. cerevisiae* S11-39 grown under normal condition. Right panels: protein preparation from *S. cerevisiae* S11-39 grown in culture with 20 mM ferric citrate.

dismutase (CuZnSOD), a 32-kDa homodimeric protein in the cytoplasm of yeast, catalyzes the disproportionation of superoxide into dioxygen and hydrogen peroxide [9]. This enzyme is a metalloenzyme that scavenges potentially harmful superoxide anion and represents a critical component of the oxidant stress protection system. The enzyme has recently been reported to be responsible for maintenance of cellular redox state [11, 19]. Therefore, upregulated superoxide dismutase in high iron concentration would protect cells from iron stress. Triosephosphate isomerase (TPI) catalyzes the interconversion of the three-carbon sugars, dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate, in the glycolytic pathway. YDR533C, which encodes a hypothetical protein of unknown function, is transcriptionally upregulated by azetidine-2-carboxylic acid [20]. Pyruvate dehydrogenase beta subunit 1 (PDB1) is an evolutionarily-conserved multiprotein complex found in mitochondria. The 60-kDa heat-shock protein (Hsp60), a tetradecameric mitochondrial chaperonin required for ATP-dependent folding of precursor polypeptides and complex assembly, prevents aggregation and mediates protein refolding after heat shock. Old Yellow Enzyme 2 (OYE2) is a highly conserved NADPH oxidoreductase containing flavin mononucleotide and may be involved in sterol metabolism [15].

The following four proteins in *S. cerevisiae* were downregulated by iron (Figs. 1, 2B). A thiol-specific antioxidant enzyme (TSA) reduces reactive oxygen, nitrogen, and sulfur species, using thioredoxin as hydrogen donor, and provides protection against the inactivation of other enzymes by the thiol/Fe(III)/oxygen system [25]. Alkylhydroperoxide reductase 1 (AHP1), with a distinct thiol peroxidase activity supported by thioredoxin, reduces hydroperoxides to protect against oxidative damage [16]. The regulatory particle non-ATPase subunit 8 (RPN8) of the yeast 26S proteasome with multisubunit protease is responsible for degrading ubiquitinated proteins. Fructose-1,6-bisphosphate aldolase (FBA), a cytosolic enzyme required for glycolysis and gluconeogenesis, catalyzes the conversion of fructose

A

Observed	M_r (expt.)	M_r (calc.)	Start amino acid	End amino acid	Peptide sequences consistent with mass
3,202.49	3,201.48	3,201.72	32	60	GKYVVLAFIPLAFTFVCPTEIIA
1,563.72	1,562.71	1,561.8	111	124	DYGVLIEEEGVALR
1,235.42	1,234.41	1,233.68	137	147	HITINDLPVGR
316.9	815.89	815.41	148	154	NVDEALR
1,237.36	1,236.36	1,235.62	155	164	LVEAFQWTDK
972.02	971.01	970.44	189	196	EYFEAANK

B

1 MVAQVQKQAP TFKKTAVVDG VFDEVSLDKY K**GKYVVLAFI PLAFTFVCPT**

51 **EIIAFSEAAK** KFEEQGAQVL FASTDSEYSL LAWTNIPRKE GGLGPINIPL

101 LADTNHSLSR **DYGVLIEEEG VALR**GLFIID PKGVIR**HITI NDLPVGRNVD**

151 **EALRLVEAFQ WTDK**NGTVLP CNWTPGAATI KPTVEDSK**EY FEAANK**

Fig. 3. Identification of thiol-specific antioxidant.
A. The masses of the tryptic peptides were matched with thiol-specific antioxidant. **B.** The sequence of protein is represented by capital letters for the amino acids. The sequence coverage by the tryptic peptides is indicated by bold capital letters.

1,6-bisphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.

Figure 3 shows the identification of thiol-specific antioxidant enzymes. An indication of the quality of these data is evidenced by the example of thiol-specific antioxidant enzyme, in which six measured peptide masses were within 0.11 of the values calculated for it.

In conclusion, the identification of proteins differentially expressed between *S. cerevisiae* grown in culture with or without iron is critical for understanding the mechanism associated with iron storage in yeast.

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