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Comparative Proteomic Analyses of the Yeast *Saccharomyces cerevisiae* KNU5377 Strain Against Menadione-Induced Oxidative Stress

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Abstract The *Saccharomyces cerevisiae* KNU5377 strain, which was isolated from spoilage in nature, has the ability to convert biomass to alcohol at high temperatures and it can resist against various stresses [18, 19]. In order to understand the defense mechanisms of the KNU5377 strain under menadione (MD) as oxidative stress, we used several techniques for study: peptide mass fingerprinting (PMF) by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (MS) followed by two-dimensional (2D) gel electrophoresis, liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), and surface-enhanced laser desorption ionization-time of flight (SELDI-TOF) technology. Among the 35 proteins identified by MALDI-TOF MS, 19 proteins including Sod1p, Sod2p, Tsa1p, and Ahp1p were induced under stress condition, while 16 proteins were augmented under normal condition. In particular, five proteins, Sod1p, Sod2p, Ahp1p, Rib3p, Yaf9p, and Mnt1p, were induced in only stressed cells. By LC-ESI-MS/MS analysis, 37 proteins were identified in normal cells and 49 proteins were confirmed in the stressed cells. Among the identified proteins, 32 proteins were found in both cells. Five proteins including Yel047cp and Met6p were only upregulated in the normal cells, whereas 17 proteins including Abp1p and Sam1p were elevated in the stressed cells. It was interesting that highly hypothetical proteins such as Ynl281wp, Ygr279cp, Ypl273wp, Ykl133cp, and Ykr074wp were only expressed in the stressed cells. SELDI-TOF analysis using the SAX2 and WCX2 chips showed that highly multiple-specific protein patterns were reproducibly detected in ranges from 2.9 to 27.0 kDa both under normal and stress conditions. Therefore, induction of antioxidant proteins, hypothetical proteins, and low molecular weight proteins were revealed by different proteomic techniques. These results suggest that comparative analyses using proteomics might contribute to elucidate the defense mechanisms of KNU5377 under MD stress.

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Saccharomyces cerevisiae is one of the most extensively used microorganisms in industrial applications [20] and clinical research. *S. cerevisiae* has been used for the production of protein- and small-molecules drugs in medicine as well as in the food and wine fermentation industries. *S. cerevisiae* is also important as a model in research laboratories to study fundamental biological processes [20]. Nonpathogenic *S. cerevisiae* has several features that make it useful for research; rapid growth, cultivation under well-controlled conditions, and effective genetic manipulation. *S. cerevisiae* can be an informative predictor of human gene functions. Approximately 50% of human genes involved in heritable diseases have yeast homologs [1].

Changes in the environmental conditions of cells that impose a negative effect demand rapid cellular responses. Molecular mechanisms, which are induced upon the exposure of cells to such adverse conditions as oxidative stress, are commonly referred to as stress responses [14, 29]. Such cellular stress responses cause a variety of changes under transcriptional and translational levels. Different methods can be used to analyze these changes in yeast-presented oxidative stress. An examination of the transcriptome provides an accurate indication of which genes are active in cells, but less precise information of the proteins that are present is given [14]. Therefore, proteomics, the field of proteome analysis, is a very attractive technology because proteins perform cellular functions. Proteomics is the systematic study of the many and diverse properties of proteins in a parallel manner with the aim of providing detailed descriptions of the structure, function, and control of biological systems involved in health and disease [27]. Advances in methods and technology have elevated the

field of biological research from the reducible biochemical analysis of single proteins to proteome-wide measurements [27]. Among the various technologies used in proteome research, 2D gel electrophoresis separates proteins using an isoelectric point and relative molecular weight, on the basis of protein mobility in a polyacrylamide gel matrix.

The separated proteins are identified by MS-based techniques that enable high-throughput protein identification after Coomasie or silver staining. The obtained results, at multiple points such as cellular organs and specific conditions, are databased as a reference map. Therefore, 2D maps in protein levels have been constructed to document the important characteristics of industrial yeast strains, such as an ale-fermenting strain, a wine strain, and a lager-brewing strain [20], and clues regarding biomarkers involved in human disease are provided. In addition, 2D gel electrophoresis has been used to obtain a global pattern of changes in the yeast proteome [20], as a functional and physiological response regarding stimuli in environment challenges such as cadmium [31], lithium [3], hydrogen peroxide [9], sorbic acid [7], and amino acid starvation [37].

Although 2D PAGE separation provides excellent resolution, the new need for protein sample handling and identification is required because of the drawbacks of 2D gel electrophoresis; labor intensiveness, poor gel-to-gel reproducibility, and difficulty in separating low-abundant proteins or membrane proteins. LC-based technology is an important analysis methodology in the proteomics field because it compensates for the drawbacks of 2D gel electrophoresis [20]. LC-MS (nanocapillary chromatography interfaced directly with an ESI mass spectrometer; LC-ESI-MS/MS) has the following advantages: automation, high-speed, high resolution, and the high-sensitivity separation of extremely complex peptide mixtures. In addition, SELDI-TOF MS is a new technique in biomarker discovery that combines two powerful methods: chromatography and mass spectrometry [13]. SELDI-TOF is ideal for high throughput analysis because it is rapid, and it requires only microliter amounts of a crude sample [29].

Here, our focus is to understand how the regulation of protein levels contributes to the execution and coordination of cellular metabolism and physiology under MD-induced oxidative stress, by two major proteomic methodologies now available: 2D gel electrophoresis and liquid chromatography coupled to mass spectrometry (LC-MS/MS). The spectrum of proteins expressed in the KNU5377 cells are compared under MD-induced stress condition.

MATERIALS AND METHODS

Strain and Stress Conditions

The KNU5377 strain [18, 19] was aerobically grown in a nutrient-rich YPED medium (1% yeast extract, 2% peptone, 2% dextrose) overnight at 30°C with shaking at 160 rpm. The precultured cells were transferred to fresh YPD media and then cultured for 5 h at 30°C with continuous shaking until the log phase (A_{600}=1.0) was reached. To generate oxidative stress, the mid-log cultured cells were subjected to 120 μM of superoxide-generating agent MD for 1 h at 30°C.

Sample Preparation for Two-Dimensional Gel Electrophoresis

After the harvesting of cells and washing, cells were disrupted five times for 5 min by a MicroMixer with a lysis buffer containing 80 mM of Tris-HCl, pH 8.0, 2% SDS, 1.5% β-mercaptoethanol, 10% glycerol, 1 mM of PMSF, and a protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany) and an equal amount of glass beads (400–600 μ, Sigma, St. Louis, U.S.A.). The supernatants were immediately boiled for 5 min and cooled on ice for 5 min. The supernatants were collected by centrifugation, precipitated by trichloroacetic acid (final conc. 10%) for 1 h on ice, centrifuged at 13,000 rpm for 20 min at 4°C, and then washed three times with HPLC-grade ethanol. The washed pellets were dried by a Speed Vac. The pellets were resupended in a sample buffer containing 9 M of urea, 4% CHAPS, 0.1 M DTT, and 0.2% Bio-Lyte (3-10, Bio-Rad, Hercules, U.S.A.) at room temperature for 1 h, and then centrifuged at 15,000 rpm for 30 min. The supernatants were transferred to a new Eppendorf tube. The protein concentration level was measured by a modified method with a Protein Assay reagent (Bio-Rad).

Two-Dimensional Gel Electrophoresis

The first-dimensional isoelectric focusing (IEF) experiment was carried out on commercial 17-cm, pH 4–7 immobilized pH gradient (IPG) strips at 20°C, with a maximum current limitation of 50 μA/strip in a Protean IEF Cell (Bio-Rad). After active rehydration at 50 V for 12 h, 90 μl (1.2 mg) of the protein sample was loaded onto the bottom of an IPG strip. IEF was performed according to the following steps: 250 V for 1 h, 1,000–10,000 V for 6 h, and 90,000 Vh at 10,000 V. After focusing, the strips were equilibrated twice for 15 min each. The first equilibration solution contained 30% (w/v) glycerol, 6 M of urea, 2% (w/v) sodium dodecyl sulfate (SDS), 50 mM of Tris-HCl, pH 8.8, 65 mM of dithiothreitol (DTT), and a trace of bromophenol blue. The second equilibration was performed with the same equilibration solution, except that DTT was replaced with 260 mM of iodoacetamide. The gels were subjected to second-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separation for the second dimension was performed on 1.0 mm thick, 12% polyacrylamide gels at 10°C, at a constant current of 30 mA per gel in a Protean II xi Cell (Bio-Rad). After

protein fixation in 40% methanol and 10% acetic acid for 12 h, the gels were stained with Coomassie Brilliant Blue R-250 (Sigma) for 3 h, and then destained. Spots of interest were excised and the following mass spectrometry was performed.

MALDI-TOF Analysis

Protein spots were excised and destained with 30% methanol. Two-hundred mM of ammonium bicarbonate was added to the gel-piece and mixed for 30 min. The liquid was discarded and the washing process was repeated. The gel-pieces were shrunk by dehydration in 100% acetonitrile, removed, and then dried in a Speed Vac. Enzymatic digestion was performed by adding 20 μl of 0.0125 μg/μl sequence grade modified trypsin (Promega, Madison, U.S.A.) in 50 mM ammonium bicarbonate and 5 mM calcium chloride. The mixture was incubated for 16 h at 37°C. Extracted digestion mixtures were dried using a Speed Vac, and then suspended in 0.1% trifluoroacetic acid (TFA). Two μl of the extracted sample were dispensed onto a MALDI sample plate with 2 μl of matrix solution (40 mg/ml α-cyano-4-hydroxy-cinnamic acid in 0.1% TFA and 50% acetonitrile), and then the amount was dried under ambient conditions.

Mass spectra were obtained by using a Voyager-DE STR MALDI-TOF mass spectrometer (Applied Biosystems, CA, U.S.A.) with delayed extraction and reflectron. Spectra were calibrated upon acquisition using a ProteoMass Peptide MALDI-MS Calibration Kit, MS-CAL2 (Sigma, St. Louis, U.S.A.) with angiotensin II (1046.5423), $P_{14}R$ (1533.8582), and ACTH fragment 18-39 (2465.1989) as external calibration. Database searches were carried out with ProFound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe).

LC-ESI -MS/MS

The KNU5377 cells were grown to log phase in YPED medium, and then the cells were exposed to 120 μM of MD. After harvest and washing, the cells were disrupted five times for 1 min by a MicroMixer with a lysis buffer containing 50 mM of HEPES, pH 7.0, 1 mM of PMSF, and a protease inhibitor cocktail and an equal amount of glass beads (400–600 μ, Sigma). The supernatants were collected by centrifugation and vacuum-dried. The pellets were suspended in a 50 mM ammonium bicarbonate (ABC) buffer, pH 8.7, and heated for 20 min at 90°C. Each sample solution was reduced by 10 mM of dithiotreitol (DTT) for 20 min and then alkylated by 100 mM of iodoacetamide (IAA) in the dark. After reduction and alkylation, the sample solutions were digested with trypsin (0.1 μg/μl) containing 1/20–1/100 of activity in proportion to the protein concentration overnight at 37°C, which were vaccum-dried. The pellets containing peptide mixtures were used for LC-ESI-MS/MS. The analysis of LC-ESI-MS/MS was entrusted to ProTan at the College of Dentistry at Kyungpook National University. The analysis was a similar method as reported by Wei *et al.* [33].

SELDI-TOF Analysis

The strong anion-exchange (SAX2) and weak cation-exchange (CWX2) Protein Chip arrays used in this experiment were provided by Ciphergen Biosystems (Fremont, CA, U.S.A.). The array processes were operated using a PBS II laser desortion/ionization, time-of-flight (TOF) mass spectrometer (Ciphergen Biosystems). Yeast crude extracts containing proteins expressed under normal and oxidative stress conditions were directly deposited onto the Protein Chip array surfaces. Two types of arrays, WCX2 and SAX2, were used respectively. Subsequent experiments followed protocols reported by Weinberger *et al.* [34].

Microarray Analysis

Microarray analysis was performed as a previously reported method [17]. We calculated the ratios (Cys5 of the stressed cells/Cys3 of normal cells) of hybridization intensity. Ratios of hybridization intensity (Cys5/Cys3) over 2.0 were determined to be upregulated, and ratios below 0.5 were regarded as being downregulated.

Transcriptional Analysis by RT-PCR

After fermentation following each temperature, cells were harvested by centrifugation. Total RNA was isolated by a Qiagen RNA/DNA Mini Kit (Qiagen, Hilden, Germany), which was used to synthesize cDNA. The cDNA was synthesized by an AccuPower RT PreMix (Bioneer, Daejun, Korea). The PCR reaction was performed by an AccuPower PCR PreMix (Bioneer) with each primer. After initial denaturation at 94°C for 4 min, the reaction mixture was cycled 35 times. The PCR conditions were the following: 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min. After 35 cycles, there was a final extension at 72°C for 10 min. After PCR, the PCR products were electrophoresed, stained with ethidium bromide, visualized, and photographed. PCR amplicon of *PDA1* was used as a reference control [35]. The used oligonucleotides are as follows: PDA-F: 5'-CTTCATTCAAACGCCAACCA-3' and PDA-R: 5'-GAGGCAAAACCTTGCTTTTTG-3'; HSP60-F: TTGAGATCATCCGTTGTTCGT and HSP60-R: TATTATCACCGAAACCAGGCG; HSP31-F: TGGCCC-CAAAAAA AGTTTT AC and HSP31-R: AAAGCGTCG-ATGGATCTTACG. Each primer was designed by a program of the *Saccharomyces* Genome Database (SGD) (http://seq.yeastgenome.org/cgi-bin/web-primer).

Immunoblotting Analysis

SDS-PAGE was performed by the Laemmli method [21]. Fifty μg of denatured proteins were analyzed in 10–12% polyacrylamide gel and electrophoretically transferred to

a PVDF membrane (Bio-Rad), using a transfer buffer (25 mM of Tris-base, 192 mM of glycine, and 20% methanol). The PVDF membranes were blocked for 60 min at room temperature in a TTBS buffer (0.05% Tween-20, 10 mM of Tris-HCl, pH 7.6, 150 mM of NaCl), containing 5% non-fat skim milk and 0.02% sodium azide. The blotted membranes were incubated overnight at 4°C with each primary antibody, rabbit anti-Sod1p, rabbit anti-Sod2p, mouse anti-Hsp60 (Stressgen, Victoria, Canada), rabbit anti-Ssb antibody (kindly provided by Dr. Elizabeth A. Craig), and rabbit anti-Tsa1p (kindly provided by Prof. Park Jeen Woo of Kyungpook National University, Korea). After washing four times with TTBS, the membranes were incubated for 90 min with a secondary antibody such as anti-rabbit IgG (H+L) HRP Conjugate (Promega, Madison, U.S.A.) or anti-mouse IgG (Amersham, Uppsala, Sweden). The membranes were washed 4 times with TTBS, developed by enhanced chemiluminescence (ECL kit, Amersham, Uppsala, Sweden), and then processed.

RESULTS

Peptide-Mass Fingerprinting Analysis by MALDI-TOF Following 2-D PAGE

A comparison of quantitative changes was performed by mass spectrometry. As shown in Fig. 1 and Table 1, 35 proteins of the approximately 200 spots analyzed were identified by PMF using MALDI-TOF following 2D PAGE. Among these proteins, 19 proteins were induced under the stressful condition, whereas 16 proteins were depressed. For the stressed cells, the elevated proteins were mainly antioxidant proteins containing Sod1p, Sod2p, Tsa1p, and Ahp1. Several heat-shock proteins (Hsps) or HSP-related proteins were also detected. Ssc1p, Ssa2p, and Ssb2p of the HSP70 family, the protein coding complex of HSP90 and p50, Hsp31p and Hsp60p were repressed (Fig. 1 and Fig. 2B). Heat-shock protein 42 (Hsp42p), however, was induced in the stressed cells. It is interesting to note that several hypothetical proteins were identified. Proteins encoded by Ycr099c, Ydr071c, and Yer165w were augmented in the drug-treated cells, but proteins encoding Ylr456w, Yil001w, and Yfr044c were overexpressed in normal cells. In particular, 6 proteins containing Sod1p, Sod2p, Ahp1p, Rib3p, Yaf9p, and Mnt1p were observed on the gel of the only-stressed cells. In addition, spot number 35 was highly induced in the affected cells. Unfortunately, the protein could not be identified by PMF using MALDI-TOF MS. The transcriptional expression levels of Sod1, Sod2, and Tsa1 were upregulated, which corresponded with the results of microarray analysis. Transcriptional and translational expression patterns of Hsp60 gene were not corresponded (Fig. 2).

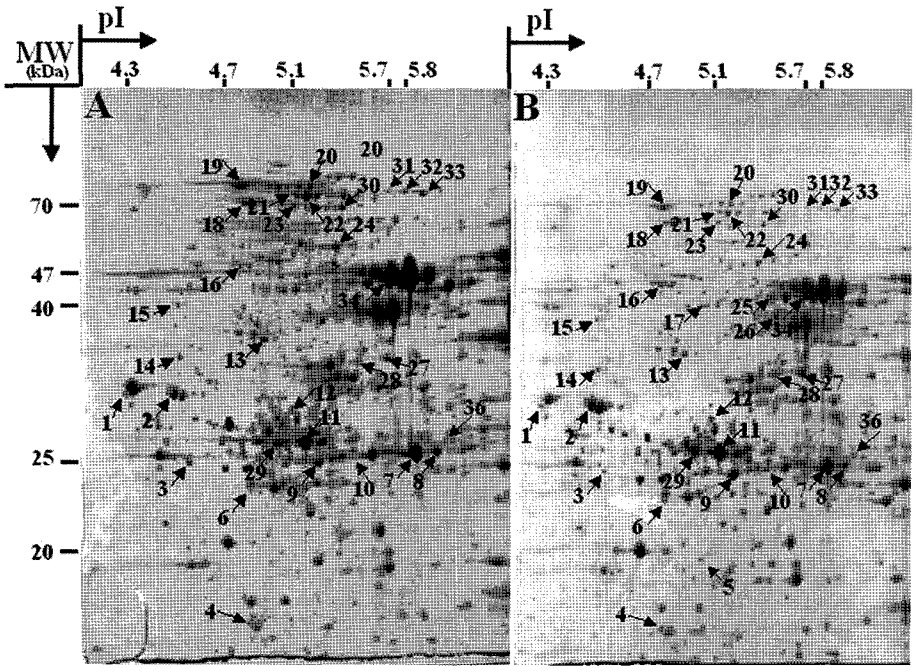

Fig. 1. Comparative analysis of 2D gel electrophoresis against menadione in *S. cerevisiae* KNU5377.
Total soluble protein samples were obtained from exponentially growing KNU5377 cells, which were treated with 120 μM of MD. The first dimension was taken after in-gel rehydration was conducted with IPG 4-7 (ready-made); separation distance, 170 mm; sample application (1.2 mg protein). The running conditions were as follows: Step 1: for 1 h at 250 V; Step 2: linear ramping between 250 V and 10,000 V for 6 h; and Step 3: for 90,000 Vh at 10,000 V. Second dimension, vertical SDS-PAGE (12%); CBB staining; (left) not treated, (right) after treatment of 120 μM of MD for 1 h.

Table 1. Proteins identified by peptide mass fingerprinting using MALDI-TOF.

Spot no.	Score	Coverage (%)	MW (kDa)	pI	Protein name	Change	Microarray (ratio)
1	2.43	22	28	4.6	RpsOa	down	0.45
2	1.61	34	26.1	4.7	Shu1p	up	2.15
3	2.01	18	24.16	4.8	Complex of HSP90 and p50	down	-
4	1.82	27	17.9	4.9	Ycr099cp: hypothetical protein	up	3.06
5	1.96	25	19.1	5	Ahp1p: thiol-specific peroxiredoxin	up	3.47
6	2.3	44	21.5	5	Tsa1p: thioredoxin peroxidase	up	6.25
7	1.83	26	23.2	5.9	Ylr456wp: hypothetical protein	down	0.33
8	2.43	52	24.2	6	Adk1p: adenylate kinase	down	0.49
9	1.69	60	21.9	5.6	Ydr071cp: hypothetical protein	up	2.67
10	1.83	42	22.5	5.5	Rib3p	up	7.58
11	2.21	32	24.2	5.3	Cdc33p: mRNA cap-binding protein eIF-4E	up	2.36
12	1.77	51	25.9	5.3	Yaf9p	up	2.37
13	1.88	36	29.5	5	Hcr1p	down	0.28
14	1.63	18	32.6	4.7	Bcp1p	up	2.02
15	2.06	42	37.2	4.7	Sgt2p	up	4.04
16	2.15	15	42.8	5	Hsp42p: heat-shock protein	up	10.72
17	2.43	21	45.8	5.1	Tlg2p: tSNARE	up	2.21
18	2.21	26	59.7	5	Yil001wp: hypothetical protein	down	0.49
19	2.43	29	69.4	4.9	Ssa2p: HSP70 family	down	0.22
20	2.43	30	70.6	5.5	Ssc1p: mitochondrial matrix ATPase	down	1.03
21	1.66	28	68.01	5.3	Rck2p: protein kinase	up	2.69
22	2.43	23	68	5.4	Ssb2p: heat-shock protein of HSP70 family	down	7.74
23	1.56	20	60.7	5.2	Hsp60p: chaperonin	down	8.22
24	1.74	34	52.8	5.4	Yfr044cp: hypothetical protein	down	0.38
25	2.43	48	15.71	5.6	Sod1p: Cu, Zn superoxide dismutase	up	12.44
26	2.19	23	40.6	5.7	Mnt1p: alpha-1,2-mannosyltransferase	up	2.05
27	2.43	25	33.1	5.8	Atc1p: nuclear protein	up	2.19
28	2.43	33	26.7	5.7	Tpi1p: triosephosphate isomerase	up	2.11
29	2.43	61	26.65	5.3	Hsp31p: heat-shock protein	down	2.74
30	1.92	22	64.5	5.5	Asi2p; predicted membrane protein	down	0.6
31	2.33	26	64.3	5.7	Yer165wp: hypothetical protein	up	2.18
32	2.31	26	64.3	5.7	Pab1p: poly(A)-binding protein	down	0.18
33	1.84	21	64.4	5.8	Polyadenylate-binding protein	down	-
34	2.16	31	46.9	5.9	Eno2p: enolase	up	2.72
36	1.96	25	22.7	6	Sod2p: Mn-containing superoxide dismutase	up	4.74

LC-ESI-MS/MS Analysis

As shown in Table 2, LC-ESI-MS/MS analysis of the protein mixtures identified 37 proteins in normal cells, and 49 proteins in stressed cells. Among the identified proteins, 32 proteins, such as pyruvate decarboxylase and the hypothetical protein Ylr179cp, were identified both in the normal and the stressed cells. A quantitative analysis of the matched proteins, however, could not be achieved. In spite of this limitation, several proteins were specifically expressed in either cell. Five proteins, such as Yel047cp and Met6p, were only identified in the normal cells, whereas 17 proteins, such as Abp1p, Tal1p, and Sam1p, were elevated in the stressed cells. It is interesting to note that the stressed cells expressed highly hypothetical proteins such as Ynl281wp, Ygr279cp, Ypl273wp, Ykl133cp, and Ykr074wp.

SELDI-TOF Analysis

A SELDI-TOF analysis, by using SAX2 and WCX2 chips, showed that highly multiple-specific protein patterns were reproducibly detected in the range between 2.9 and 27.0 kDa of the normal and stressed cells. As shown in Fig. 3 and Table 3, for an analysis using the WCX2 chip, 99 mass values were detected, whereby 84 mass values (84.8%) obtained from normal cells showed a higher intensity than the stressed cells. In particular, mass values in the range between 2.9 and 5.0 kDa and between 9.8 and 26.6 kDa showed increasing magnification of the spectra of the normal cells. The peak in the range between 5.4 and 9.1 kDa in the affected cells was somewhat higher than in the normal cells. The peak at *m/z* 2988, 3093, 3512, 3525, 4071, 4784, 16888, 17090, and 17168 in the normal cells

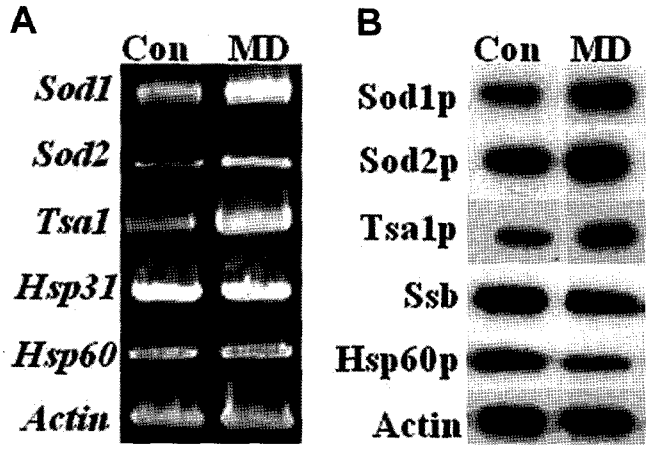

Fig. 2. RT-PCR and immunoblotting of cell-rescue proteins.
Total RNA was prepared from KNU5377 cells that were grown to OD_{600} of 1.0 in a YPED medium at 30°C and then exposed to 120 μM of MD for 1 h at 30°C with shaking. After conducting cDNA synthesis with reverse transcriptase, a PCR was carried out with each PCR primer. Each band was normalized by *PDA1* (**A**). The total cellular proteins from the log-phase cells exposed to MD were separated by SDS-PAGE, transferred to a PVDF membrane, and allowed to react with each first and second antibody. The intensity level of Cu/Zn Sod (Sod1p), Mn Sod (Sod2p), thioredoxin peroxidase I (Tsa1p), heat-shock protein family (Ssbp), and heat-shock protein 60 (Hsp60p) was processed by ECL Plus (**B**). Con: Non-treated cells; MD: Stressed cells.

was over 4 times higher than those in the stressed cells. Analysis using the SAX2 chip showed a total of 69 expression patterns. The level of magnification for the normal and stress-induced cells was similar. Mass values in the range between 4.0 and 10.4 kDa and between 11.6 and 16.4 kDa, however, indicted high intensity in the stressed cells, whereas those in the broad range between 10.6 and 11.3 kDa turned out high values in the unaffected cells. The peaks at *m/z* 5111, 5163, 14285, 15197, and 16494 in the stressed cells were two times higher than those in the normal cells.

DISCUSSION

Until recently, three types of MS analyses have been used to perform proteome analysis. First, analyses in the linear mode are commonly used to measure the molecular masses of protein. Second, the reflector mode of operation can be used to analyze peptide mixtures, which are data commonly termed PMF (peptide mass fingerprinting). In the third type, post-source decay (PSD) is used to gain peptide sequencing. Although many instruments can be used to analyze samples of different organs, MALDI-TOF has been frequently used for the analysis of gel-separated proteins. The same type of arrangement has been used to perform SELDI, which is attracting increasing interest as a tool for screening biological samples to identify biomarkers associated with various pathologies [12].

Yeasts, like other organisms living in aerobic conditions, are continuously exposed to oxidative stress-derived reactive oxygen species (ROSs) such as singlet oxygen, superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radical ($OH^·$) via the metal-catalyzed Haber-Weiss and Fenton reaction [6, 8, 10, 30]. These ROSs are also the result of normal metabolism, including the energy generation process of aerobic respiration and the β-oxidation of fatty acids, and also exposure to extracellular physical stress levels containing heat, osmotic pressure, and irradiation, or chemical stress levels including ethanol, hydrogen peroxide, MD, and xenobiotics [28]. The produced ROSs can directly damage a wide range of cellular biological components including carbohydrates, nucleic acids, lipids, peptides, and proteins [4]. Owing to the harmful effects of these ROSs, yeast cells induce and/or repress a great number of proteins such as antioxidants and cell-rescue proteins, in order to minimize or avoid the extent of either the production or damage of free radicals.

On the basis of these facts, we performed protein expression mapping to study the quantitative analysis of global changes in the protein expression of cells using 2D gel electrophoresis coupled with mass spectrometry, LC-ESI-MS/MS, and SELDI-TOF, under MD-induced oxidative stress in KNU5377, which is resistant to various stresses [16, 17, 24, 25, 38]. Protein identification was simultaneously carried out by MALDI-TOF MS based on peptide mass fingerprinting following in-gel digestion with trypsin and by liquid chromatography tandem mass spectrometry (LC-ESI-MS/MS). There are some proteins that cannot be identified by MALDI-TOF MS; for example, the low-molecular mass proteins that yield only a few peptides that can be analyzed by tandem MS, using the Q-TOF approach. By these analyses, approximately 84 proteins were identified. Of the identified proteins, several interesting proteins were expressed under MD stress. Sod1p encoding cytosolic superoxide dismutase, Sod2p encoding mitochondrial superoxide dismutase, Tsa1p and Ahp1 encoding cytosolic thioredoxin peroxidase, which were involved in antioxidant proteins and highly induced in the MD-only treated cells. The identified proteins function in that the superoxide anion produced after the MD treatment has been converted to hydrogen peroxide by superoxide dismutase, which is removed by peroxidases such as thioredoxin peroxidase. *Δsod1* is hypersensitive to redox cycling agents such as paraquat and MD, and to high O_2, but not to hydroperoxides [2, 11]. Tsa1 and Ahp1 are dominant regarding their transcript levels and their protective roles against stress in the exponential growth phase. Tsa1, however, is more specific toward H_2O_2 [5], whereas Ahp1 is more specific toward t-butylhydroperoxide (t-BOOH) [25, 26, 30]. In the

Table 2. Proteins identified by LC-ESI-MS/MS.

Proteins induced both under stressful and not stressful conditions		
No.	Protein name	MW (kDa)
1	Circularly permuted phosphoglycerate kinase from yeast:Pgk P72	44.7
2	Asymmetric yeast enolase dimer complexed with resolved 2'-phosphoglycerate and phosphoenolpyruvate	46.6
3	Pyruvate decarboyxlase	61.5
4	Yeast triosephosphate isomerase (Mutant)	26.6
5	Yeast Cu/Zn superoxide dismutase room temperature (298K) structure	15.7
6	Refined structure of yeast inorganic pyrophosphatase and its K61r mutant	31.7
7	Crystal structure of threonine synthase from yeast	57.3
8	Crystal structure of the *S. cerevisiae* Ydr533c protein	25.7
9	Structure of the ribosomal 80s-Eef2-sordarin complex	38.9
10	Glyceraldehyde-3-phosphate dehydrogenase	35.5
11	Fk-506-binding protein (12 kd, yeast) complex with Fk-506	12.0
12	Crystal structure of yeast Fsh1YHR049W, a member of the serine hydrolase family	27.3
13	Pmi40p: mannose-6-phosphate isomerase	48.2
14	Unnamed protein product: YGR086C	38.3
15	Ylr179cp	22.1
16	Crystal structure of yeast cytosine deaminase apoenzyme: inorganic zinc bound	17.6
17	Crystal structure of domain 2 of the yeast copper chaperone for superoxide dismutase (Lys7)	15.4
18	Yel030wp	70.0
19	NMR structure of apo calmodulin from yeast *Saccharomyces cerevisiae*	16.0
20	Structure of the ribosomal 80s-Eef2-sordarin complex	38.9
21	Yeast Eef1a:eef1ba in complex with gdpnp	10.5
22	RPL31A	12.9
23	RPS3	26.5
24	Structure of the ribosomal 80s-Eef2-sordarin complex	11.3
25	RP30	21.6
26	Yeast cytochrome Bc1 complex with bound substrate cytochrome C	12.0
27	The yeast actin Val 159 Asn mutant complex with human gelsolin segment 1	41.7
28	Yeast initiation factor 4a N-terminal domain	24.9
29	Yeast iso-2 cytochrome C	12.4
30	Structure of yeast triosephosphate isomerase	26.6
31	Yeast profilin	13.5
32	Structure of the ribosomal 80s-Eef2-sordarin complex	16.5

KNU5377 cells, 2D analysis and microarray analysis showed that Tsa1 and Ahp1 were remarkable under MD stress (Fig. 1, Fig. 2, and Table 1) [16]. In addition, we identified the overexpression of various types of antioxidant proteins containing glutathione and thioredoxin systems and antioxidant molecules such as glutaredoxin and trehalose against MD stress [16]. It is interesting that the overexpression of these genes increased the yeast's thermotolerance [32].

Among the cell-rescue proteins, several heat-shock proteins (Hsps) or Hsp-related proteins that are involved directly in the degradation or reactivation of damaged proteins, by various stressful conditions [32], were also identified. Ssc1p, Ssa2p, and Ssb2p of the Hsp70 family, protein encoding complexes of Hsp90 and p50, Hsp31p and Hsp60p, except Hsp42p decreased in the stressed cells. The mRNA transcripts of the *HSP60* and *HSP31* genes were induced or unchanged when exposed to MD, but the translational level of these genes decreased (Fig. 1 and Fig. 2). In particular, the heat-shock protein 70 (Hsp70) family and the Hsp60 protein are the main targets of protein damage against oxidative stress such as hydrogen peroxide [4]. These proteins are induced by various types of stresses such as heat shock and high concentration of ethanol [23].

We previously reported that PMF analysis of MALDI-TOF showed that Ssa2p sustained damage following immunoblotting with anti-DNP [16] and it was confirmed that Hsp60 was degraded (in press). Therefore, the down-expression of these proteins seems to be a target of protein damage. Hsp104, Hsp90, and small heat-shock proteins (sHSPs), however, were induced two-fold by immunoblotting (Hsp104, Hsp90, Ssap, and Hsp30p), and microarray and RT-PCR (*HSP12* and *HSP26*) (data not shown). It is interesting that heat-shock protein 31, which is a chaperone with similarities to *E. coli* Hsp31 and *S. cerevisiae*

Table 2. Continued.

Proteins induced under menadione stress condition		
No.	Protein name	Peptide sequence
1	Structure of the N-terminal domain of the yeast Hsp90 chaperone	R.ELISNASDALDK.I
2	Cofilin homology domain of a yeast actin-binding protein: Abp1p	R.ASFAANFAAVANNLFK.G
3	Unnamed protein product: Ynl281wp	K.VTNLDTNKDDEDDDGILADGK.L
4	Unnamed protein product: Ygr279cp	R.LYGTDCNQVENVFK.A
5	Structure of the ribosomal 80s-Eef2-sordarin complex (gi 49258849)	R.AVGGEVGASAALAPK.I
6	Yeast cytochrome c peroxidase (E.C.1.11.1.5)	K.EFNDPSNAGLQNGFK.F
7	Sam1p: S-adenosylmethionine synthetase	R.FVIGGPQGDAGLTGR.K
8	Unnamed protein product: Ypl273wp	R.TLSEYNNLLNR.I
9	Structure of the ribosomal 80s-Eef2-sordarin complex (gi 49258858)	R.LTADYDALDIANR.I
10	Tal1p: transaldolase	K.TTEEQVENAVDR.L
11	Solution structure of the yeast translation initiation factor Eif4e	K.FEENVSVDDTTATPK.T
12	X-Ray structure of the C-terminal Ulp1 protease domain	K.VSDGSSEIFFK.I
13	Structure of the ribosomal 80s-Eef2-sordarin complex (gi 49258836)	K.LAAPENEKPAPVR.T
14	Unnamed protein product: Ykl133cp	K.EPESEQCVFMKELLTAR.D
15	Hypothetical Ydr430cp	R.EVVDKLTELQK.Y
16	Unnamed protein product: Ykr074wp	K.NIVLHDYDLADK.T
17	Crystal structure of cystathionine gamma-lyase from yeast	K.QSSPANPIGTYEYSR.S
Proteins induced under normal condition		
No.	Protein name	Peptide sequence
1	Yel047cp	K.LPPGFEIVSALSNNLK.K
2	Chain B, yeast phosphoglycerate mutase-3pg complex structure	K.YVDPNVLPETESLALVIDR.L
3	Chain N, structure of the ribosomal 80s-Eef2-sordarin complex	K.FVQGLLQNAAANAEAK.G
4	Met6p: 5-methyltetrahydropteroyl triglutamate	R.APEQFDEVVAAIGNK.Q
5	RL28_YEAST 60S ribosomal protein L28 (L27A) (L29) (YL24) (RP62)	K.ETAPVIDTLAAGYGK.I

Hsp32p, Hsp33p, and Sno4p [36], was depressed under these stress conditions.

The KNU5377 strain also showed significant changes in hypothetical proteins, which are called unnamed proteins, whose function remain to be elucidated. The stressed cells expressed highly hypothetical proteins encoded by Ycr099c, Ydr071c, and Yer165w, Ynl281w, Ygr279c, Ypl273w, Ykl133c, and Ykr074w, whereas the normal cells expressed unnamed proteins encoded by Yel047c, Ylr456w, Yil001w, and Yfr044c. Although some functions of these proteins are unknown, expression of the hypothetical proteins might contribute to regulate transcriptional factors or protein-protein interactions in yeast cells. Taken together, antioxidant proteins, hypothetical proteins, and proteins of low molecular weight were highly expressed in the KNU5377 under MD stress.

Many proteins were identified by LC-ESI-MS/MS. Among the proteins identified, we tried to conduct a comparative analysis with data values from common proteins induced simultaneously under normal and stressful conditions. For example, cytosolic superoxide dismutase (Sod1p), from transcript analyses (microarray and RT-PCR) (Table 1) and translational analysis (Western blot) (Fig. 2), was strongly induced against MD stress. LC-ESI-MS/MS data, however, cannot conduct quantitative analyses. As shown in Table 2, the values of score, coverage, and peptide in normal cells were higher than those in the MD-treated cells. The transcripts of genes (*FRR1*, *FRR2*, *FRR3*, and *FRR4*) involved in the FK-506 binding protein were downregulated under MD stress (data not shown). The analyzed values of the stressed cells were higher than those of the control cells. Interestingly, protein expression was in inverse proportion to data values. This approach, however, cannot clearly support quantitative analysis. Therefore, the result suggests that a subsequent analysis is necessary when comparative and quantitative analyses of the coinduced proteins are carried out.

SELDI-TOF analysis, using the SAX2 and WCX2 chips, showed that highly multiple-specific protein patterns were also reproducibly detected in the range between 2.9 and 27.0 kDa for normal and stressed cells. The results of each chip, however, showed fine and different mass values with mutual advantage. These results suggested that there is an intensive direction for future study involved in low molecular weight proteins, particularly from 2.0 to 20.0 kDa. SELDI-TOF, which is proteomic technology involved in the quantitative analysis of protein mixtures, could be applied to biological samples of normal or stressed cells

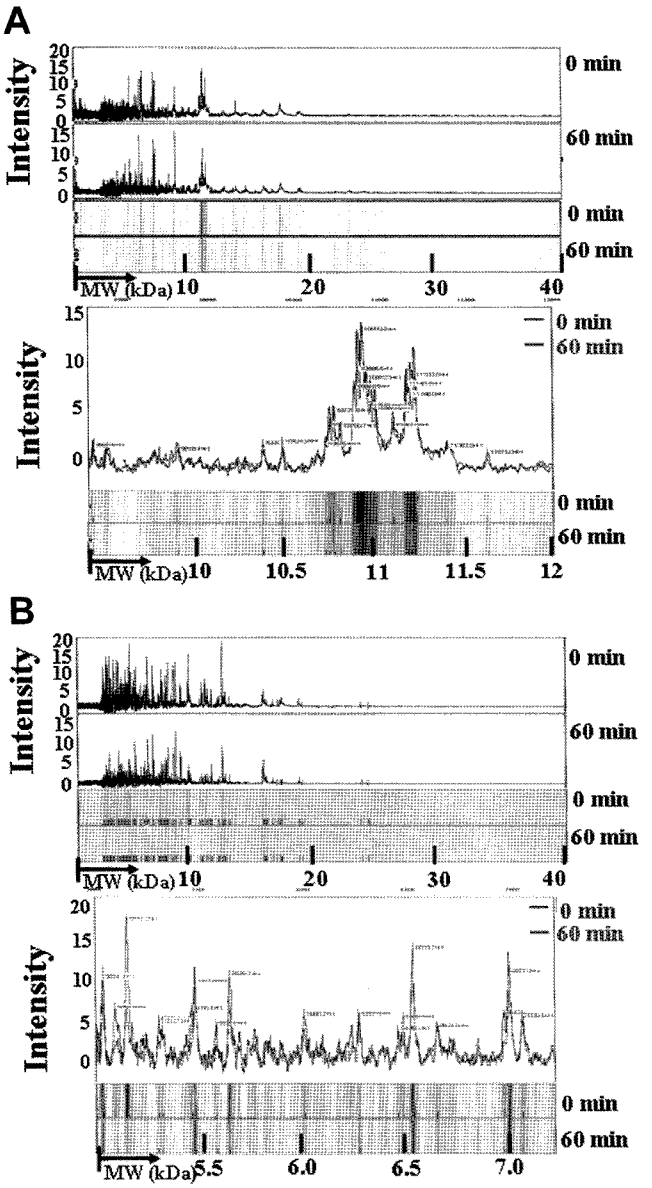

Fig. 3. Identification of biomarkers for the menadione-treated cells of the KNU5377 strain using SELDI-TOF.

Samples from non-stressed and stressed cells were analyzed by using a Ciphergen system with a C16 reversed, phase chip surface. Analysis of the total data set, using an iterative searching algorithm, revealed a pattern of *m/z* values that was characteristic of the stressed cells. **A**. Upper panel: SAX2 full spectrum (~40 kDa); Bottom panel: SAX2 spectrum between 9.3 kDa and 12 kDa. **B**. Upper panel: WCX2 full spectrum (~40 kDa); Bottom panel: WCX2 spectrum between 5.0 kDa and 7.2 kDa.

in order to detect high-throughput biomarkers after MD exposure.

From the above-mentioned proteomic techniques, we identified many proteins (about 84 proteins). A comparison of cell lysates by these techniques, from normal versus stressed cells, can potentially provide new strategies or guides in understanding cellular stress responses against oxidative stress involved in human diseases. Furthermore, we will focus on low molecular weight protein identification on the basis of SELDI-TOF results.

Table 3. Intensities of mass values analyzed by SELDI-TOF using the SAX2 chip.

Peaks (*m/z*)	Intensities	
	Without treatment	With treatment
4035	3.476	6.404
4635	4.422	8.460
5111	2.036	5.928
5127	2.055	3.709
5163	1.657	5.654
5583	3.408	5.468
5750	10.440	16.845
5966	10.902	5.828
5990	13.051	6.130
6012	13.309	8.712
6796	5.301	2.872
8674	8.622	17.840
10938	8.528	5.955
13578	4.538	2.051
13784	1.203	0.557
14285	0.465	0.705
15197	0.466	0.825
16494	0.536	1.177
16898	0.866	0.465
22778	0.497	0.774

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