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Proteomic Analysis of Fructophilic Properties of Osmotolerant *Candida magnoliae*

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***Candida magnoliae*, an osmotolerant and erythritol producing yeast, prefers D-fructose to D-glucose as carbon sources. For the investigation of the fructophilic characteristics with respect to sugar transportation, a sequential extraction method using various detergents and ultracentrifugation was developed to isolate cellular membrane proteins in *C. magnoliae*. Immunoblot analysis with the Pma1 antibody and two-dimensional electrophoresis analysis coupled with MS showed that the fraction II was enriched with membrane proteins. Eighteen proteins out of 36 spots were identified as membrane or membrane-associated proteins involved in sugar uptake, stress response, carbon metabolism, and so on. Among them, three proteins were significantly upregulated under the fructose supplying conditions. The hexose transporter was highly homologous to Ght6p in *Schizosaccharomyces pombe*, which was known as a predominant transporter for the fructose uptake of *S. pombe* because it exhibited higher affinity to D-fructose than D-glucose. The physicochemical properties of the ATP-binding cassette transporter and inorganic transporter explained their direct or indirect associations with the fructophilic behavior of *C. magnoliae*. The identification and characterization of membrane proteins involved in sugar uptake might contribute to the elucidation of the selective utilization of fructose to glucose by *C. magnoliae* at a molecular level.**

Keywords: *Candida magnoliae*, fructophilic yeast, membrane proteins, transporter, two-dimensional electrophoresis

Candida magnoliae, isolated from honey combs, is an osmotolerant yeast and produces erythritol mainly from sugars for the protection of cells against a high osmotic pressure [5, 13]. Erythritol is a natural sweetener with a low calorie and anticariogenic effect. In Japan, it has been used as a sugar substitute for candies, soft drinks, and yogurt [20]. Most of ingested erythritol is not metabolized by the human body and is excreted in the urine without changing blood glucose and insulin levels. Thus, it could also be used for diabetic patients [11].

C. magnoliae showed unique physiological characteristics compared with other typical yeasts. *C. magnoliae* is able to grow in a wide range of pH values in the presence of high concentrations of sugars [16] and does not produce ethanol, but produces erythritol, glycerol, and mannitol from hexoses [14, 26]. The most interesting characteristic of *C. magnoliae* is a peculiar preference of fructose to glucose as a carbon source. Although a molecular basis for the selective utilization of fructose by *C. magnoliae* remains unclear, several mechanisms of such a fructophilic property have been elucidated in other yeasts. *Candida shellata* and *Zygosaccharomyces bailii* have a preference for fructose, whereas *S. cerevisiae* in general appears to be glucophilic [6, 25]. Recently, a gene coding for a fructose-specific transporter in *Z. bailii* was cloned and characterized by the functional complementation of a *S. cerevisiae* mutant strain deficient in hexose transporters [22]. In *Z. bailii*, fructose promoted the inactivation of glucose transporters, preventing the utilization of glucose when fructose was available. Moreover, fructose crossed the plasma membrane through the fructose-specific transporter at a higher rate than glucose. The mode of sugar metabolism has been investigated for the explanation of fructose-specific uptake by *C. magnoliae* [27]. More investigations on the fructophilic property of *C. magnoliae* are desirable to use this strain for erythritol production in an industrial scale. However, the genome of *C. magnoliae* is not sequenced yet and hence its biological information is limited to explore the fructophilic mechanism at a protein level.

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To investigate the fructophilic characteristics of *C. magnoliae*, a differential centrifugation method using various detergent solutions was developed to obtain its membrane fractions. Proteomic techniques including two-dimensional electrophoresis (2-DE), MS, and homology searching were used to identify membrane-associated proteins including sugar transporters. This comprehensive proteomic overview of the *C. magnoliae* membrane compartments could, to some extent, reveal the molecular basis of its fructophilic property.

MATERIALS AND METHODS

Strain and Culture Conditions

An osmophilic yeast, *C. magnoliae* KFCC 10900, was inoculated into a 500-ml baffled flask containing 50 ml of growth medium (yeast extract 1%, bacto-peptone 2%, and glucose or fructose 2%) and cultured at 30°C and 250 rpm for 24 h. The culture broth (50 ml) was transferred to a 2.5-l jar fermentor (KoBioTech, Incheon, Korea) containing 1 l of fermentation medium with 300 g/l glucose or fructose, 10 g/l yeast extract, and 20 g/l bacto-peptone. Batch fermentation was performed at 30°C, 700 rpm, and an air flow rate of 1 vvm. Optical density was monitored by measuring the absorbance of the culture broth at 600 nm with a spectrophotometer (UltroSpec 2000; GE Healthcare Life Sciences, Uppsala, Sweden) [12]. Dry cell weight was estimated by the multiplication of a conversion factor (0.26) with the optical density. Cells were harvested by centrifugation at 20,000 ×*g* for 5 min at 4°C and washed two times in five volumes of ice-cold water. Samples of cell pellets were immediately stored at −80°C for further analysis.

Isolation of Membrane Proteins

All isolation steps were performed at 4°C. The frozen cell pellet was washed two times in five volumes of ice-cold PBS buffer (pH 7.4). Cells were resuspended in the ice-cold phosphate-buffered saline (PBS) buffer containing a protease inhibitor cocktail (Sigma, St. Louis, MO, U.S.A.), of which the wet concentration was about 5 g/l. Disruption of resuspended cells was done five times at 20,000 psi using a French Press (Thermo Spectronic, Rochester, NY, U.S.A.), followed by boiling for 5 min and cooling in an ice bath. To remove debris containing cell walls and unbroken cells, the disrupted suspension was centrifuged at 7,000 ×*g* for 10 min. The cleared supernatant was then incubated with lysis buffer {2 M thiourea, 7 M urea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 1% dithiothreitol (DTT), and 2% carrier ampholytes; pH 3–10} and SDS buffer [1% sodium dodecyl sulfate (SDS), 0.1 M Tris-HCl, pH 7.0] for 1 h under gentle agitation. The solid pellet of membrane vesicles was separated from the supernatant by ultracentrifugation at 300,000 ×*g* for 1 h using a Beckman Ti90 rotor (Fullerton, CA, U.S.A.). This supernatant was transferred completely to a new microcentrifuge tube and named as PM I. The following solutions in a row were added to a tube with the pellet: 200 µl of SDS buffer, 500 µl of lysis buffer, and 1% sarcosine. Until uniform suspension was obtained, this mixture was completely resuspended by sonication and then incubated again for 1 h for protein solubilization, followed by ultracentrifugation at 200,000 ×*g* for 1 h. The supernatant was taken and named as PM II. The final pellet was resuspended in 200 µl of SDS-buffer and 500 µl of lysis buffer and clarified by ultracentrifugation at 100,000 ×*g* for 60 min. This final supernatant was named as PM III. Protein concentration was determined by the Bradford method [2] using bovine serum albumin (BSA) as the standard. All recovered PM fractions were stored in 20% (w/v) glycerol resolved in 50 mM Tris-HCl (pH 8.0) at −80°C until further analysis.

SDS-PAGE and Immunoblot Detection

Isolated samples were fractionated by 12% (w/v) SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and then immediately transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Eschborn, Germany) according to the standard method [4]. Immunodetection of plasma membrane ATPase 1 (Pma1) on the PVDF membranes was carried out using a Pma1 monoclonal antibody and an anti-rabbit IgG-HRP purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) as primary and secondary antibodies, respectively. Both immunoreactions were performed at room temperature for 1 h. Antibody binding was visualized using a chemiluminescence reaction kit (Opti4-CN Substrate Kit, Bio-Rad) according to the manufacturer's instruction. Coomassie blue staining followed the standard method [19].

Protein Separation by 2-DE

Separation of the fractionated protein samples by isoelectric point was carried out with 18 cm immobiline dry strips providing a nonlinear pH gradient (pH 3–10; GE Healthcare Life Sciences) [15, 17]. After equilibration for 30 min, a horizontal SDS-PAGE system (18 cm× 20 cm, Protein II XI Cell; Bio-Rad) was used to separate the proteins by molecular weight. Analysis using 2-DE was performed in triplicate. An SDS-PAGE gel of 12% polyacrylamide with 1.5 mm thickness was prepared.

Visualization of Protein Spots and Image Analysis

Protein spots on the 2-DE gels were visualized using an automatic gel staining unit (Process Plus; GE Healthcare Life Sciences) with a silver staining kit (GE Healthcare Life Sciences) based on the acidic/silver nitrate method. Silver-staining without glutaraldehyde treatment followed the procedure recommended by the supplier. The silver-stained gel was scanned by a calibrated densitometer (GS-710; Bio-Rad) and its raw image was processed using the 2-dimensional software PDQuest (Bio-Rad). The image file was stored in 16 bit TIFF format and analyzed using the Phoretix 2-dimensional program (V. 2004; NonLinear Dynamics, Newcastle, U.K.). After automatic spot detection with default parameters, detected artifacts were removed manually. The average values and standard deviations of spot area and density were used as parameters to determine differences in protein expression level.

In-Gel Tryptic Digestion

Manual preparation of MS analysis samples followed the previous protocol with some modifications [24]. Briefly, protein spots of interest were excised from 2-DE gels and destained as described previously [9]. Gel pieces were washed with water several times until the yellow color disappeared and were incubated in 200 mM ammonium bicarbonate (pH 8.0) for 20 min. After incubation, the gel pieces were washed with water and acetonitrile, dried by vacuum centrifugation, and then rehydrated in a minimal volume of 25 mM ammonium bicarbonate (pH 8.0) containing 25 µg/ml of a sequencing grade modified trypsin for overnight at 37°C. The excess

liquid was removed and the peptides in the gel pieces were extracted twice with 20 μl of the 50% acetonitrile and 5% trifluoroacetic acid solution. The corrected elute was dried, desalted, and concentrated with a Zip-Tip C_{18} (Millipore, Bedford, MA, U.S.A.) according to the manufacturer's instructions prior to MS analysis.

MALDI MS and Protein Identification

The digest was analyzed by a Voyager-DE short tandem repeat (STR) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrophotometer (Applied Biosystems, Framingham, MA, U.S.A.) using the thin-layer method. Briefly, the matrix solution was composed of 10 mg α-cyano-4-hydroxycinnamic acid (CHCA) dissolved in 1 ml of 10 mg/ml nitrocellulose (NC) in the acetone/isopropanol [1/1 (v/v)] solvent. The matrix solution of 0.5 μl was first loaded on the target plate prior to drying. Next, 0.5 μl of the digest was placed on the plate, dried, and washed several times with 0.1% TFA. MS spectra were obtained in the delayed extraction modes using an accelerating voltage of 20 kV and 150 ns delay. A nitrogen laser at 337 nm desorbed and ionized the sample. At least two peptide ions generated by the autolysis of trypsin were used as internal standards for calibrating the mass spectra. Protein identification using monoisotopic peptide masses obtained from MALDI-TOF MS was carried out by searching of the NCBInr database using the Mascot (http://www.matrixscience.com) algorithm under the standard parameters. Protein identification with a confidence interval (CI%) score greater than 95% was accepted.

LC-Tandem MS and Protein Identification

According to the procedure of Gevaert *et al.* [8], the digest solution was dried by a centrifugal vacuum concentrator and redissolved in 1 μl of formic acid, followed by the addition of 9 μl of water. An Ultimate HPLC (LC Packings, a division of Dionex, San Francisco, CA, U.S.A.) was coupled with a LCQ-DECA ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.A.) containing an electrospary source. The sample was separated by a C_{18} column (300 μm i.d.×15 cm, 100 Å with 3 μm particles, LC Packings) connected with a 300 μm i.d. C_{18} guard column (LC Packings) in a capillary flow rate mode (4 μl/min). The analytical column was connected to the electrospray source with 100 μm i.d. fused silica. No switching valves were used in this step. Injection was made manually with a 10 μl low-dispersion loop. For LC-MS data acquisition, LCQ (Thermo Finnigan, U.S.A.) was operated in the data-dependent mode, where the top four peaks in every full MS scan were subjected to tandem MS (MS/MS) analysis. MS/MS conditions were set as follows: default charge state, 3; default isolation width, 3; normalized collision energy, 35%; activation q, 0.250; activation time, 30.00 ms. The LCQ sequence data were analyzed with the Sequest algorithm using the following parameters: threshold, 100,000; enzyme, trypsin; number of internal missed cleavage points, 4; peptide mass tolerance, ±3.0 (average mass); fragment ion mass tolerance, ±0.4 (monoisotopic mass); parent mass range, 300–5,000 Da; filters, 4 of 5; Xcorr, 1.0; DelCN, 0.1; sp, 500; Rsp, 5.0; 30% fragment ions. The subcellular location of identified proteins was obtained by searching of the Swiss-Prot/TrEMBL database at the ExPASy proteomics server (http://expasy.org).

Sequence Alignment

The sequence alignment with yeast hexose transporters was carried out by the CLUSTALW program at the Biology Workbench (version 3.2; the San Diego Supercomputer Center, the University of California, San Diego [http://www.workbench.sdsc.edu]) using the standard parameters.

Results and Discussion

Separation of Membrane Proteins in *C. magnoliae*

Numerous approaches have been described to improve the isolation of desired proteins in yeast plasma membrane and to reduce sample complexity by using various detergents and organic solvents [21, 29]. Membrane proteins from cell extracts are notably underrepresented because of poor solubility by their intrinsically hydrophobic natures [3]. Hydrophobicity likely causes self-aggregation during isoelectric focusing (IEF), leading to a 2-DE gel with poor resolution, and horizontal or vertical strips [7]. To isolate membrane proteins from *C. magnoliae* cells, an array of membrane proteins according to their hydrophobicity was achieved by a strategy of the disruption of intact cells with a French Press and combination of ultracentrifugation and various detergent treatments. About 1,400 μg/ml of cell membrane proteins were recovered in each fraction. Three fractions of PM I, PM II, and PM III were collected, analyzed by SDS-PAGE, and visualized with the Coomassie blue staining and western blotting using an antibody against Pma1 (Fig. 1). Pma1 with an apparent molecular mass of 99 kDa has been used as a typical marker of the yeast plasma membrane proteins [1]. Pma1 was not observed by the blue protein staining on SDS-PAGE gels, caused by a low expression level of Pma1 (Fig. 1A). Pma1 was detected by the immunoblotting in total proteins of *S. cerevisiae* and *C. magnoliae,* which confirmed its ubiquity (Fig. 1B). The immunoblotting membrane for PM II showed a protein band with the same size of Pma1 expressed in *S. cerevisiae* (Lane S) and *C. magnoliae* (Lane C). Pma1 was not observed in the immunoblotting of PM I and PM III. This result indicated that the second

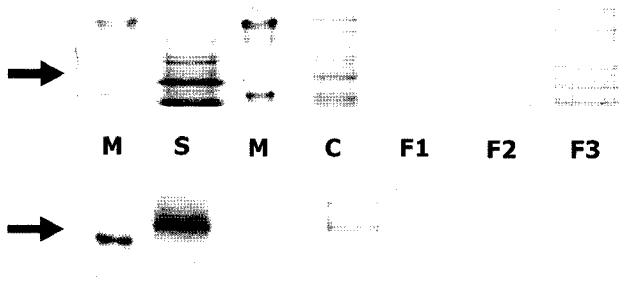

Fig. 1. SDS-PAGE analysis of three membrane protein fractions of *C. magnoliae*. Proteins were visualized by the Coomassie blue staining (**A**) and immunoblotting with an anti-Pma1 antibody (**B**).
Arrows indicate the size of Pma1 and symbols denote the following: M, protein size marker; S, total protein of *S. cerevisiae*; C, total protein of *C. magnoliae*; F1–F3, PM I–PM III, respectively.

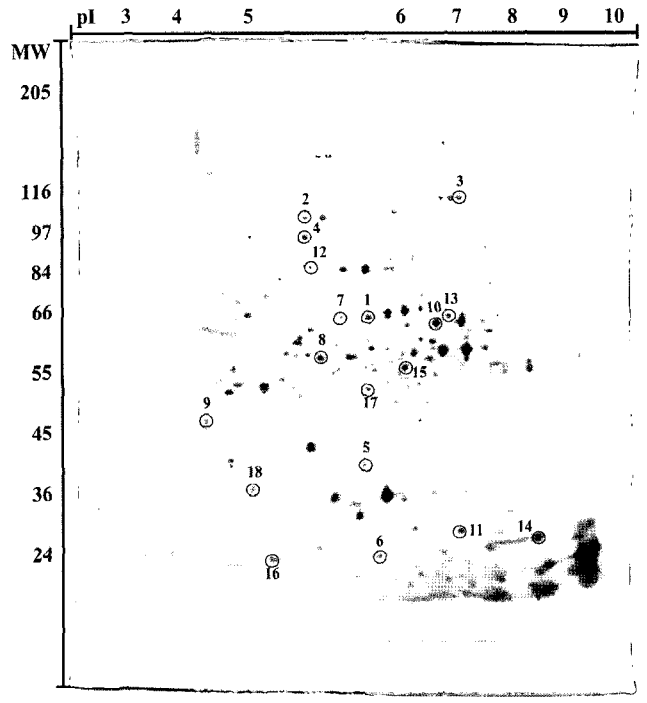

Fig. 2. A representative 2-DE gel image of the membrane protein fraction PM II.

Protein spots were visualized by the silver staining. Horizontal and vertical axes indicate isoelectric point and molecular weight, respectively. Analysis using 2-DE was carried out in triplicate. Numbers indicate protein spots under MS analysis of which results are shown in Table 1.

fraction of PM II was enriched with membrane-associated proteins.

Identification of Membrane Proteins from *C. magnoliae*

The developed membrane preparation method allowed the analysis of the constituent proteins of each fraction by 2-DE and MS. A silver-stained 2-DE gel of PM II is shown in Fig. 2. Seventy-four spots were cut out of the three gels, enzymatically cleaved with trypsin, and analyzed by MALDI-MS peptide mass fingerprinting (PMF). Some proteins that were not conclusively identified by PMF were analyzed by electrospary ionization tandem MS (ESI MS/MS). Preliminary identification of 38 protein spots present in PM I and PM III revealed large contamination (35/38) by cytosolic proteins. However, eighteen different proteins from 36 spots in PM II were identified as membrane-associated. As confirmed by the immunoblot analysis, these results demonstrated the validity of the adapted detergent and ultracentrifugation method for membrane protein preparation in *C. magnoliae*. The number of identified proteins is relatively low when compared with that from 2-DE gels of soluble proteins. All the identified proteins are listed in Table 1 and classified according to their biological functions; sugar uptake, stress response, carbon metabolism, and others.

Comparison of Membrane Proteins from Glucose- and Fructose-grown Cells

Using the Phoretix 2-D software, approximately 140 and 160 spots were detected on the silver-stained gels resolving the membrane proteins from glucose- and fructose-grown cells, respectively. The differential quantitative analysis of each matched protein spot between glucose- and fructose-grown samples using the Phoretix 2-D software revealed that three proteins among the isolated membrane proteins from fructose-grown cells were enhanced at least 2-fold, including the ATP-binding cassette (ABC) transporter, hexose transporter, and inorganic phosphate transporter. Other protein spots showed no considerable change in their densities. Protein spots and their histograms with significant changes under the fructose stress condition are shown in Fig. 3 and their molecular characteristics, such as amino acid fragment, isoelectric point (pI), and molecular weight (MW), are presented in Table 1. Three proteins displayed in Fig. 3 were also found in Fig. 2, such as spot 1 for the ABC transporter, spot 2 for the hexose transporter, and spot 3 for the inorganic phosphate transporter. Interestingly, the hexose transporter and inorganic phosphate transporter showed multiple spots on the 2-D gels. Under the fructose-induced osmotic stress condition, two spots of the three inorganic phosphate transporter isozymes, located at both sides, were expressed by a 2-fold enhancement (Fig. 3C). Expression levels of the two hexose transporter isoforms increased significantly and were also analyzed by ESI MS/MS, which verified their enhanced amounts in the fructose-grown cells. The physiological implications of the fructose-affected proteins are discussed below.

ATP Binding Cassette Transporter

Analysis of the digestion mixture of spot 1 by nanoelectrospray MS/MS yielded the amino acid sequence of YSSWSFSVKG with a doubly charged precursor ion at *m/z* 453.27. Subsequent searching of the *Saccharomyces* Genome Database (SGD) showed a high homology to the amino acid sequences of *S. pombe* ABC1. The second peptide denoting the sequence of LAIKKLLQYLQN was also found in this ABC1 protein sequence. The ABC transporter was known to be able to solely deliver fructose into cells. A proteomic analysis of *Bifidobacterium longum* identified a fructose-transporting protein and an ABC transporter, which were upregulated in a fructose-enriched medium [28].

Hexose Transporter

Nanoelectrospray MS/MS analysis of the digestion mixture from spot 2 resulted in a doubly charged precursor ion at *m/z* 612.82 yielding a molecular mass of 1,223.59 Da. The complete sequence of the peptide was determined to be AIMKAQWRVSW by the Mascot. Two other peptides

Table 1. A list of *C. magnoliae* protein spots identified in this study.

Spot No.	Protein name	Accession number	Subcellular location	Amino acid sequence	Type of analysis	MW/pI	Protein score	Mass coverage[a]	Sequence coverage (%)[b]
	Sugar uptake								
1	ABC transporter	Q92337	Membrane	YSSWSFSVKG LAIKKLLQYLQN	ESI/MS/MS	66,999/5.5	41	7/12	11
2	Hexose transporter	P78831	Membrane	AIMKAQWRVSW KDNYFFYYGTLVFT RDEYFSCLFIFSFAQTWG	ESI/MS/MS	51,801/8.7	51	15/64	30
3	Inorganic phosphate transporter	P25297	Membrane	FKDFCRHFGQW LPHVMEIFALFMLL	ESI/MS/MS	57,634/6.8	57	11/71	34
	Stress response								
4	Heat-shock protein 10	NP_014663	Mitochondria /cytoplasm	SGIFLPEKSVEK IVPLLDRILV	ESI/MS/MS	84,572/5.4	40	10/94	46
	Carbon metabolism								
5	Aldehyde dehydrogenase, mitochondrial precursor	P46367	Mitochondrial matrix	EDDVEEAVQA TVVLKTAESTPL	ESI/MS/MS	90,313/5.6	39	10/69	29
6	Xylulose reductase	Q07993	Membrane /cytoplasm	ITLTNVSIPKISDPNEVII YCQGDYSDSIELVSSR	ESI/MS/MS	24,479/ 5.7	37	10/145	48
7	Vacuolar ATPase assembly integral membrane protein	P32341	Membrane	LEFLKYQEQELEY AIWYWTG IKELLKPLE	MALDI	63,879/5.3	51	16/44	14
8	Amino-acid permease 1	Q92367	Membrane	SWLYFWIWLT ARSYGECEF	MALDI	53,999/5.3	52	13/91	36
9	NADH reductase	P36060	Mitochondria outer membrane	ESTHLFVCGPPPFMNA DQGELIGILNNLGYS	ESI/MS/MS	27,009/4.3	35	7/76	41
10	Malate dehydrogenase-mitochondrial precursor	P17783	Mitochondria matrix	LGLLPSASLSSLPD KGASSTTEVG	MALDI	43,910/7.0	32	7/88	33
11	Peroxisomal hydratase-dehydrogenase-epimerase	Q02207	Peroxisome	PTLAKAVKFPTP	ESI/MS/MS	28,281/7.8	40	11/43	14
12	Hypothetical zinc finger membrane protein	AHTY5	Transmembrane	TAEADRAAQRQQF GAIALVGTFM	ESI/MS/MS	65,183/5.8	44	11/71	31
13	Palmitoyltransferase AKR1	Q09701	Golgi apparatus	AALNQQIPIC LATDEDKMTPLHW	MALDI	66,634/6.8	48	18/82	36
	Other functions								
14	Pheromone a factor receptor	P06783	Membrane	LTMNLTCIVDA IARYNGCQNLLSP PIYNIW	MALDI	28,021/9.1	46	11/40	35
15	Deoxyribonuclease I precursor	P49183	Nuclear envelope	EPLGRKSYKEQYLFV TLVNLLQLA GTLRIA	ESI/MS/MS	34,677/7.9	24	8/51	26
16	Hypothetical protein	Q8STZ1	Unknown	REVEMLQR RELDEGYEK	ESI/MS/MS	30,009/5.2	33	9/47	30
17	Dolichyldiphosphatase	P53223	Endoplasmic reticulum	IKQPRPVSFG	ESI/MS/MS	57,634/6.8	35	15/85	26
18	Cell division control protein	Q06697	Peripheral membrane	ILVDHNSALRGAKPINFGYLIK	MALDI	35,039/4.6	57	34/269	53

[a]Denotes the number of matched *m/z* values per the total number of *m/z* values.
[b]Indicates the number of amino acids spanned by the assigned peptides divided by the number of amino acids of the identified protein.

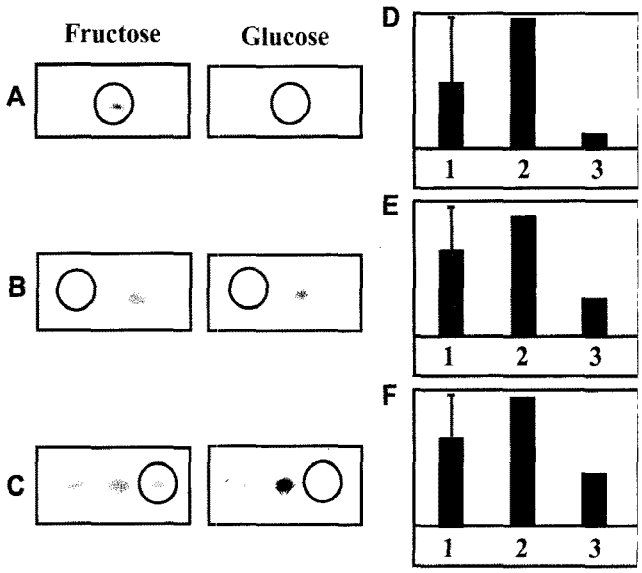

Fig. 3. Protein spot images and histograms of the ABC transporter (**A**, **D**), two hexose transporter isoforms (**B**, **E**), and three inorganic phosphate transporter isozymes (**C**, **F**).
Protein samples were collected from the culture of *C. magnoliae* grown in a complex medium with fructose (2) or glucose (3) as a sole carbon source. The density of each circled spot is displayed in the histogram. Average density of a spot without the change of expression level irrespective of the carbon sources was chosen as the reference (1). This experiment was performed in triplicate.

were identified, as shown in Table 1. Data searching with these sequences resulted in a considerable homology to hexose transporters. The amino acid sequence alignment of hexose transporters from *S. cerevisiae*, *C. glabrata*, and *Schizosaccharomyces pombe* is shown in Fig 4. Two homologous regions contained much similar amino acid sequences to those from the peptide digests of the protein spot 2 such as DNYFFYYGT and CLFIFSFAQTWGP. Hexose transporters comprise a family of proteins involved

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S. cerevisiae    SVEEMRAAGTASWGELFTGKPAMFQRTMMGIMIQSLQQLTGDNYFFYYGT 348
C. glabrata      GVEAEKSAGNASWGELFSTKGKILQRVVMGIMIQSLQQLTGCNYFFYYGT 345
S. pombe         DCEAEMAGGPATWGDILG--ADIRYRTFLGLGVMSLQQLTGDNYYFYYGF 289
                 .*  : * *:**:::    : *..:*: : ******* **:****:
S. cerevisiae    IVFQAVGLSDSFETSIVFGVVNFFSTCCSLYTVDRFGRRNCLMWGAVGMV 398
C. glabrata      TIFKAVGLEDSFQTSIILGIVNFASTFVALYVVDRFGRRKCLLWGSSTMA 395
S. pombe         EVFEGTGMNSPYLSALILDAVNFGCTFGGLFVLEFFGRRMPLIIGGVWQS 339
                  :*:..*:...: :::::. *** .*  .*:.:: **** *: *.
S. cerevisiae    CCYVVYASVGVTRLWPNGQDQPSSKGAGNCMIVFACFYIFCFATTWAPIA 448
C. glabrata      VCMVIFASVGVKSLYPNGKDQPSSTTAGNVMIVFTCLYIFCFATTWAPTA 445
S. pombe         ITFFIYAAVGNRALTR--KNGTSNHRAGAVMIVFSCLFIFSFAQTWGPAA 387
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Fig. 4. CLUSTAL W alignment of the amino acid sequences of hexose transporters from *S. cerevisiae* (NP_011962), *C. glabrata* (XP_445530), and *S. pombe* (NP_587740).
Shade boxes present homologous regions containing the peptide sequences of the *C. magnoliae* hexose transporter. Symbols indicate consensus keys as follows: *, single, fully conserved residue; :, conservation of strong groups; ., conservation of weak groups; blank, no consensus.

in the cellular sugar uptake of various yeasts [18]. A hexose transporter of *S. pombe*, Ght6p, exhibited a slightly higher affinity to D-fructose than D-glucose [10]. This result could explain the fast growth and stable maintenance of *C. magnoliae* in a fructose medium compared with a glucose medium.

Inorganic Phosphate Transporter

MS/MS sequencing for the doubly charged ion at *m/z* 498.81 and 840.97 rendered the amino acid sequences of FKDFCRHFGQW and LPHVMEIFALFMLL, respectively. The same sequences were present in the amino acid sequences of the *S. cerevisiae* inorganic phosphate transporter, which acted as a nutrient signal for the activation of the protein kinase A (PKA) pathway [23]. The PKA pathway occurs in the presence of rapidly fermented sugars like glucose and fructose.

The sequential combination of ultracentrifugation and various detergent treatments allowed the efficient separation of membrane proteins in *C. magnoliae*. Detection of the ubiquitous Pma1 protein and an image analysis of 2-DE confirmed the validation of the developed method for the separation of membrane proteins. Among the membrane or membrane-related proteins of *C. magnoliae* in response to different carbon sources, 18 proteins were identified by MS/MS analysis and the levels of three transporting proteins were enhanced under the fructose stress condition. The identification, biological significance, and fructose-related roles of the ABC transporter, hexose transporter, and inorganic transporter were thoroughly investigated. The sugar uptake mechanism as well as carbon metabolism should be considered for the enhancement of erythritol production by *C. magnoliae*. This proteomic work exploring sugar delivery systems of *C. magnoliae* will be helpful to the efficient preparation of membrane proteins, the elucidation of the fructophilic behavior, and, moreover, the construction of recombinant microbial organisms with efficient sugar transport systems.

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