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Combining Proteomic Strategies and Molecular Display Technology for Development of Vaccines against *Candida albicans*

Seiji Shibasaki[1]*, Miki Karasaki[1] and Mitsuyoshi Ueda[2]

[1]*General Education Center, Hyogo University of Health Sciences, Chuo-ku, Kobe 650-8530, Japan*
[2]*Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan*

Abstract

Recent developments in pharmaceutical technology allow systematic investigation of molecules operating in a living cell, facilitating identification of key players, which show potential as novel drug targets. One of the tools that has emerged for identifying potential antigens for use as vaccines against infectious diseases is time-course proteome analysis. The model virulent microorganism *Candida albicans* has been well studied in terms of both physiological characteristics and clinical aspects for developing pharmaceutics in treatments. In this article, we review a time-course proteome study of *C. albicans* during adaptation to a serum. Furthermore, we introduce a novel biotechnological strategy for developing vaccines using characteristic proteins that have been identified as virulence-related molecules.

Keywords: *Candida albicans*; Quantitative proteome; Serum; Antigen; Molecular display; Vaccine

Introduction

Infectious disease is a major cause of morbidity and mortality. Following the development of penicillin [1], the first antibiotic, public health has been greatly improved through the development of subsequent antibiotics. A wide variety of antibiotics have demonstrated efficacy against infectious diseases, although they cause several adverse effects and their uses have led to the evolution of multidrug-resistant microorganisms. For example, strains of pathogenic microorganisms such as *Mycobacterium tuberculosis* [2], *Staphylococcus aureus* [3], and *Pseudomonas aeruginosa* [4] exhibit increased resistance to almost all antibiotics at present. A comprehensive understanding of the relationships between cellular molecules and the pathogenesis of infectious diseases is thought to be important for development of new strategies to prevent infection. In this review, *Candida albicans*, which causes candidiasis, was selected as a model pathogenic microorganism for systematic analysis of its cellular molecules. *C. albicans* is a pathogenic fungus that is an important cause of superficial mucosal and disseminated infections. Superficial and systemic candidiasis is observed when host immunity is compromised by AIDS, chemotherapy for treatment of cancer, or the administration of immunosuppressants. Indeed, the number of immunosuppressed patients has increased greatly, and these patients are at risk for opportunistic candidiasis infections [5].

Recently, life science and biotechnology researchers have begun to use "omics" technologies to analyze the molecular profile of individual cells [6]. Techniques of genomics [7], transcriptomics, proteomics [8], or lipidomics [9] have been utilized in studies of a variety of organisms, including *C. albicans*, for gaining a systematic understanding of physiological phenomena combined with molecular profiles. Concerning a search for vilurence-related proteins from *C. albicans* by using omics technology, proteomics is superior to other technologies because direct analysis of proteins is going to be performed. Here, we first review studies that have used a proteomic approach to analyze proteins of *C. albicans* with liquid chromatography and tandem mass spectrometry (LC-MS/MS) equipped with long monolithic silica capillary columns [10]. Furthermore, biotechnological applications of the data obtained using these analyses to oral vaccination are described.

Time-Course Proteomic Analysis of *C. albicans*

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) has been widely used for proteomic investigations of *C. albicans* [11,12]. 2D-PAGE has typically been used for protein separation for proteome analysis. The shotgun approach is an alternative strategy for proteomic analysis that combines LC-MS/MS. This method can identify many kinds of proteins, including a small quantity of proteins in a high-throughput manner. In addition, systems with ultra-performance chromatographic separation have been constructed, and have demonstrated excellent performance [13]. For instance, a system employing a long monolithic silica capillary column (350 cm) successfully identified 2,602 proteins produced in *Escherichia coli* cells in a single experiment [14]. Aoki et al. [15] applied the LC-MS/MS system to a time-course proteomic analysis of *C. albicans* during adaptation to fetal bovine serum (FBS). In this study, *C. albicans* was first grown in a YPD medium and transferred to a YNB ± FBS medium. This experiment presents a model for early systemic candidiasis in which *C. albicans* adapts to the bloodstream. *C. albicans* incubated in YNB ± FBS media for 0–60 min could provide a good model to investigate the dynamics of proteome variation in the early stages of infection. In the next step, prepared peptides derived from proteins identified from *C. albicans* grown in YNB ± FBS media were subjected to LC-MS/MS measurement using a long monolithic silica capillary column (200 cm) [16], and a lot of proteins were successfully identified. Aoki et al. [15] identified 1130, 1012, and 701 proteins from the 0, 60 min cultures in YNB −FBS, and 60 min of YNB +FBS samples. In addition, 1034, 933, and 868 proteins were identified from time-course samples of 10, 20, and 40 min of YNB +FBS, respectively (Table 1). A total of 1418 unique proteins were identified (Figure 1A) [15]. Of these

***Corresponding author:** Seiji Shibasaki, General Education Center, Hyogo University of Health Sciences, Chuo-ku, Kobe 650-8530, Japan, Tel: +81-(0)78-304-3159; Fax: +81-(0)78-304-2859; E-mail: seiji@huhs.ac.jp

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Table 1: The number of identified proteins at each periods.

Sample name		Protein concentration (mg mL^{-})	The number of identified proteins
0 min		3.21	1130
YNB -FBS	60 min	3.80	1012
	10 min	3.43	1034
	20 min	3.43	933
YNB + FBS			
	40 min	3.40	868
	60 min	3.49	701

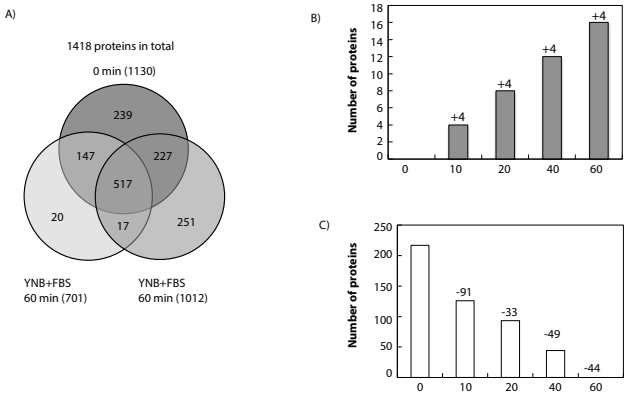


Figure 1: Summary of proteins identified in time-course proteome analyses of *C. albicans* [15]. A) The Venn diagram of identified proteins in the sample at 0 and 60 min in YNB ± FBS. The total number of unique proteins identified was 1418. B) The number of newly produced proteins during adaptation to the serum. Totally, 16 proteins were produced. C) The number of disappearing proteins.

proteins, 517 were found in all three samples and recognized as a core set of the *C. albicans* proteome. Protein identification was performed using MASCOT (Matrix Science, London, UK) against the assembly 21 protein database at Candida genome database (CGD).

A group of proteins (i) that were not found in the 60 min YNB – FBS or 0 min samples and (ii) that persisted in the 60 min YNB +FBS once identified during any period were defined as 'newly produced proteins.' These proteins were recognized as important effectors that positively contributed to cellular integrity during their presence in the serum. Four proteins (RHR2, HGT1, ATP16 and orf19.3767) were first identified at 10 min, and these were continuously detected during the latter periods. In the same manner, four additional proteins were identified as newly produced proteins at each period (20, 40, or 60 min) (Figure 1B). A total of 16 proteins (RHR2, HGT1, ATP16, SPT14, ERG6, PEX12, orf19.3767, orf19.713, orf19.3686, orf19.4825, orf19.4620, orf19.4594, orf19.5342.2, orf19.4123, orf19.2439 and orf19.6211) were classified as newly produced proteins.

In contrast, proteins (i) that were continuously detected from 0 min to a certain time, (ii) that were not detected after they disappeared at any period, and (iii) that were not detected in the 60 min YNB–FBS sample were defined as "disappearing proteins." Although these proteins may provide advantages in a nutrient-rich situation, they may also have disadvantages in a harsh environment and they are thought to be unnecessary. The analysis identified 217 proteins as disappearing proteins (Figure 1C).

Early investigations performed using 2D-PAGE could not identify dynamic differences between reference maps [17,18]. These results could be attributed to a bias toward abundant proteins, samples containing complex protein mixtures, and the low dynamic range of 2D-PAGE. However, the time-course proteome study used monolithic LC-MS/MS-based technique can identify less-abundant proteins with difficult biochemical properties. Thus, identification of the dynamic variation in the proteome which includes disappearance was instructive for understanding of candidiasis.

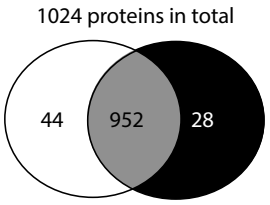
Quantitative Time-Course Proteomics

Aoki et al. [15] has also reported a quantitative time-course proteomics study of *C. albicans* during the early stages of adaptation to serum (0–180 min) [19]. Quantitative time-course proteome analysis is a promising approach. It requires a high-throughput strategy to measure a large number of samples. In this case, an LC-MS/MS system with a monolithic silica capillary column longer (470 cm) than that described in the previous section [15] was used. Using this system, the researchers were able to identify several thousand proteins in a high-throughput manner, without laborious sample fractionation. Comprehensive characterization of the adaptation process using quantitative time-course proteome analysis is expected to profoundly enhance our understanding on the virulence of *C. albicans*.

C. albicans cells were collected and transferred to YPD media (named as YPD series) or YPD+FBS (named as FBS series). The media were immediately shaken for 0, 30, 60, 120, and 180 min at 37°C. Extracted proteins were labeled using tandem mass tagging (TMT) and evaluated, and LC-MS/MS analysis was performed with a long monolithic silica capillary column (470 cm), using the proteome samples taken at 0 min on YPD as a control. The sample (named as 0 min YPD) was aliquoted into 3 tubes by volume at a ratio of 1:0.5:2, and these samples were labeled with TMT-126, TMT-127, or TMT-128, respectively. The proteomes of these YPD and FBS series were analyzed using the LC-MS/MS system. In the analysis, 1,024 proteins were identified and quantified. Of these proteins, 44 were categorized as YPD-specific and 28 were categorized as FBS-specific (Figure 2). The mass spectrometry data of each sample were used for protein identification, and quantification was performed using Proteome Discoverer 1.2 (Thermo Fisher Scientific). Protein identification was performed using MASCOT.

To interpret the proteomic changes in detail, proteins were hierarchically clustered (on a vertical axis) and associated with 12 characteristic categories, labeled A to L. Four types of groups were identified, i.e. groups that showed an increasing trend both in the YPD and FBS series (A–D), a cluster that showed an increasing trend in the YPD series (E), groups that showed an increasing trend in the FBS series (F and G), and other groups (H–L). To categorize these groups at a functional level, the proteins were subjected to gene ontology (GO) enrichment analysis using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (http://david.abcc.ncifcrf.gov/). Groups A–D were shown to be enriched in proteins related to cellular homeostasis, redox regulation, and glycoprotein metabolism.

Proteins in group E (YPD-specific) were shown to be associated with aminoacyl-tRNA biosynthesis. Group G (FBS-specific) was shown to be enriched with proteins involved in intracellular processes such as catabolic acetyl-CoA catabolism and coenzyme catabolic processes related to the citrate cycle. Proteins in the citrate cycle were upregulated in the FBS series compared to the YPD series; many proteins involved in the citrate cycle (for example, Aco1, Idp1, Sdh12, and Mdh1) were enriched in group G. *C. albicans* cells appear to optimize their protein profiles by upregulating proteins in the citrate cycle to efficiently acquire energy in the blood. This observation is in accord with the previous microarray analysis that noted that human blood and a



CKS1	HRT2	orf19.1545	orf19.5833
PLB4.5	ARG5,6	orf19.1564	orf19.6250
URE2	MLT1	orf19.1865	orf19.6438
TOS1	MRT4	orf19.1956	orf19.6503
PGA52	PTC7	orf19.2265	orf19.6705
HGT7	SIN3	orf19.3173	orf19.7034
BUB3	RFC1	orf19.3242	orf19.7368
OBPA	NOG1	orf19.3290	orf19.7485
HSE1	KIN2	orf19.3367	orf19.7580
VTC4	DJP1	orf19.3613	orf19.808
LYS22	orf19.132	orf19.5666	orf19.997

STF2	CAF16	orf19.5194.1
HGT1	orf19.1336.2	orf19.6534.2
PUT1	orf19.688	orf19.6602
MRP20	orf19.2544	orf19.6625
RPP2B	orf19.319	orf19.4406
HAP5	orf19.4947	orf19.925
DTD2	orf19.1495	orf19.5565
RPF2	orf19.2920	orf19.4504
HYR1	orf19.6923.1	
APA2	orf19.5161	

Figure 2: Summary of proteins identified in "quantitative" time-course proteomics of *C. albicans* in YPD series and FBS series [19]. The names of categorized proteins in these series are listed bottom.

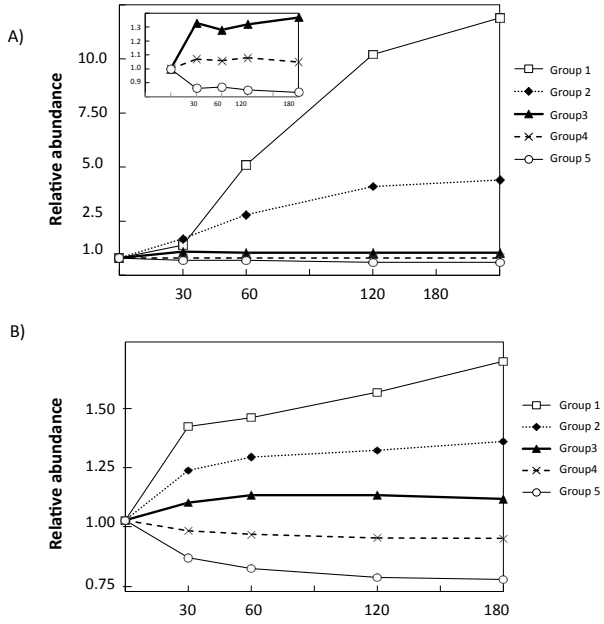


Figure 3: The individual protein profiles were grouped 1 by K-means clustering for A) FBS or B) YPD series. For the FBS series, the detailed profiles of group 3–5 are dipicted in the magnified panel [19].

polymorphonuclear cell fraction could transcriptionally activate the citrate cycle [20]. Further, another investigation has demonstrated that Gcn4 [21], a transcriptional activator, plays an important role in upregulation of the citrate cycle [22]. In light of these results, the validity of this proteomic method was successfully demonstrated.

To analyze treatment-specific proteome patterns, time-course profiles of the FBS series and the YPD series were categorized using non-hierarchical K-means clustering (Figure 3). Two protein clusters with significant upregulation in the FBS series (Group 1: 10-fold, Group 2: 5-fold) were identified (Figure 3A), whereas the proteins in the YPD series showed only modest changes, with a maximum of about 2-fold increase (Figure 3B). In the FBS series, only 2 (Sod5 and Blp1) of the 1,024 identified proteins were clustered in Group 1, and 4 proteins (Ece1, Ucf1, Stf2 and Hgt1) were clustered in Group 2. Other proteins in the FBS series showed moderate abundance changes [19]. Based on these results, we suggest that *C. albicans* employed the following adaptation strategy: first, *C. albicans* tuned its proteome to adapt to a new environment, in which most proteins were upregulated 2-fold higher than has been suggested by previous studies [23]. Second, a few proteins were upregulated >5-fold, which might suggest that these proteins were critical for adaptation to the novel environment. These findings are not in accord with all the data presented in previous transcriptome analysis reports, which have shown that dozens of proteins were upregulated >5-fold after blood treatment [20,24], indicating a low correlation between transcriptome and proteome analyses of *C. albicans*, owing to differences in the stability of transcripts and proteins.

Proteins uniquely identified or specifically upregulated in the FBS series may be important factors for adaptation to serum. Aoki et al. [15] selected proteins upregulated only in the FBS series, identifying 22 proteins that were specifically upregulated in the FBS series. In addition, 28 proteins were uniquely identified in the FBS series. These 50 proteins were designated "FBS-induced proteins." Several previously identified virulence factors, for example, Alo1, Nag6, Phr1, Rpf2, and Sod5 [25-29], were included in this group, indicating that these proteins are potential virulence factors.

Examination of a Novel Antigen Candidate

C. albicans malate dehydrogenase (Mdh1p) was identified by the time-course proteome studies discussed above as the group 4 (Figure 3), and was thought to be a candidate for a vaccine against candidiasis because it was identified through every periods without large variation in relative abundance. Indeed, Mdh1p was also identified in a proteome study for screening *C. albicans* immunogenic proteins by a two-dimensional electrophoresis (2-DE/MS system [30]. As an antigen candidate of *C. albicans*, a commonly existing protein in every situations and associated with an important metabolic pathway is thought to be suitable for a vaccine. Mdh1p is associated in the citrate cycle. Therefore, recombinant Mdh1 protein (Mdh1p) with a His-tag was produced in *E. coli* and evaluated as an immunogenic protein and a candidate vaccine against candidiasis [31]. Recombinant Mdh1p was purified using an endotoxin column and administered to mice via subcutaneous injection or intranasal administration before they were given a lethal dose of *C. albicans*. After vaccination, IgG antibody responses were evaluated by enzyme-linked immunosorbent assay (ELISA). Furthermore, survival tests were performed to evaluate the efficacy of Mdh1p as a vaccine. All control mice died within 25 d. In contrast, 100% and 80% of mice treated with subcutaneous and intranasal administration of Mdh1p, respectively, survived. These results indicated that, among the *C. albicans* antigens examined thus far, such as hyphal wall protein (Hwp1p), phosphoglycerate kinase (Pgk1p), and glyceraldehyde-3-phosphate dehydrogenase (Gap1p) [32], Mdh1p is currently the most effective antigen for use as a vaccine for *C. albicans*. Furthermore, an investigation of time-course variation in *C. albicans* under serum-containing conditions to identify virulence-related molecules could also provide novel and effective antigenic proteins.

A Convenient Tool for Preparation of Antigenic Proteins

To produce larger quantities of antigenic proteins identified by proteomic investigation, a convenient biotechnological tool is required for pharmaceutical development. One technique that shows potential,

molecular display technology, has been developed in recent years. The technology uses genetically engineered microorganisms to produce foreign proteins via a very simple strategy [33]. The approach consists of fusion of a heterologous protein such as an antigen to a cell-wall protein, thereby anchoring the foreign protein to the cell surface. The molecular display method to prepare target proteins is especially well established for use with the yeast *Saccharomyces cerevisiae*, and it is also known as "cell surface engineering" [34-39]. For example, an antigen from red sea bream iridovirus (RSIV) was displayed on the surface of yeast cells to create an oral vaccine for use in fisheries [40]. β-glucan, an abundant component of the yeast cell wall, is thought to function as an adjuvant [41]. Further, yeast is a generally recognized as safe (GRAS) organism, suitable for the preparation of oral vaccines without antigen purification, while that process is required for recombinant proteins produced by *E. coli*. Therefore, molecular display could provide a method for production of antigenic proteins selected in proteome studies that is more rapid and convenient than conventional vaccine production methods.

Enolase1 (Eno1p) from *C. albicans* has been selected as a model antigenic protein to be displayed on the surfaces of *S. cerevisiae* cells [42,43]. Eno1p has been identified as the group 5 in quantitative time-course proteome analyses (Figure 3). For a reason as same as Mdh1p in the previous section, Eno1p was though to be suitable for an antigen candidate of vaccine. Eno1p is also associated in an important metabolic pathway, glycolysis. To construct an oral vaccine, Eno1p was displayed on the yeast surface via introduction to the yeast strain BY4741 using the pULD1-eno1 plasmid; successful transformation was confirmed. Display of Eno1p on the cell surface of BY4741/eno1 cells harboring the plasmid was observed by immunofluorescence microscopy. For quantitative analysis of the relative amounts of Eno1p displayed on the surface of yeast cells during cultivation in a liquid medium, the fluorescence of yeast cells bound with AlexaFluor488-labeled IgG was measured. Eno1p-displaying yeast cells were grown for oral administration to mice; cells displayed a large number of Eno1p-fusion proteins on their surfaces.

To evaluate the efficiency of Eno1p-displaying yeast cells as a vaccine, mice were vaccinated by oral administration of the cells 4 times over the course of a 7-week period before challenge with a lethal dose of *C. albicans*. The average titer of antibody against Enop1 generated after oral administration of yeast cells displaying Eno1p was evaluated by ELISA. An average titer of antibody against Eno1p generated after the administration was 5.2×10^3, although the value varied substantially between animals, from 1.0×10^2 to 5.2×10^4. Thus, the yeast could positively present sufficient immunological stimuli to immunize almost all mice. In the evaluation of the protective effect of the oral vaccine, examination of survival rate after challenge with a lethal dose of *C. albicans* for 35 days indicated that 60% of mice that received an oral dose of Eno1p-displaying cells survived longer than mice that received an oral dose of yeast cells displaying no Eno1p (Figure 4). This survival rate is better than that found in conventional antigen administration trials using subcutaneous injection (12.5%) or intranasal administration (25%). These results suggest that cell-surface display of an antigen with adjuvant potential selected in proteome studies and administration of cells constructed by molecular display may provide a convenient and effective type of oral vaccine against not only candidiasis but also various infectious diseases. In addition, this yeast oral vaccine can be prepared rapidly because, unlike proteins produced in *E. coli*, it does not require a complicated purification step, and mutations can be easily generated because the plasmid DNA is easy to be manipulated. Although only a yeast system for molecular display is described here, bacterial systems can also be used [44,45].

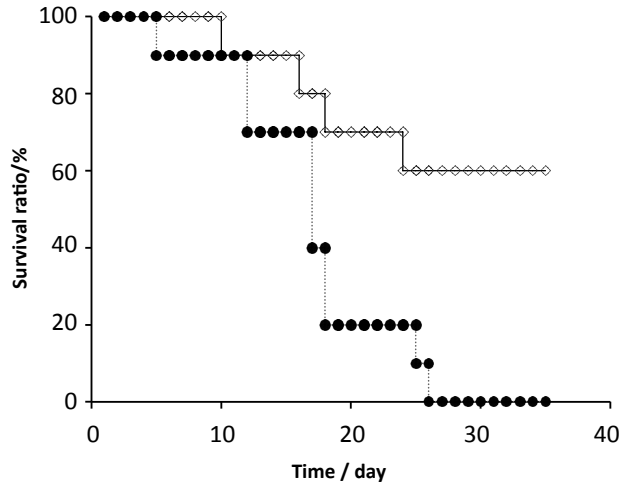


Figure 4: Survival investigation after oral administration of yeast cells. Open diamonds, administration of yeast displaying Eno1p [41]. Closed circles, administration of yeast displaying no Eno1p (host cell).

Conclusions

Here we reviewed studies that identified dynamic variations in the *C. albicans* proteome by using quantitative time-course proteome analyses using a LC-MS/MS system with a long monolithic silica capillary column. This research strategy enabled identification of virulence-related molecules that could be used in creation of novel pharmaceuticals such as vaccines. We also reviewed the oral delivery of yeast cells displaying surface antigens, which were shown to protect more than 50% of the mice studied against candidiasis. This demonstrated the potential of molecular display of immunogens selected by proteomic analysis for convenient generation of oral vaccines against various infectious diseases. Although further demonstrations using other candidate proteins are indispensable to establish reliable technology for vaccine development, the combination of a proteomic approach with molecular display technology could be developed into a convenient and powerful tool for creation of vaccines against various infectious diseases.

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