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Targeted Proteomic Analysis of Central Metabolic Enzymes Using Nano-LC-Triple Quadrupole Mass Spectrometry

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Life Science

■ Abstract

Targeted proteomics is an analytical technique that measures the expression levels of pre-selected target proteins. Nano-liquid chromatography (nano-LC) is used in targeted proteomics because its low flow rates allow greater analytical sensitivity when dealing with samples that contain very small amounts of peptides. Mass spectrometers capable of high-speed operations, such as those with ultra-fast mass spectrometry (UFMS™) technology, are also needed in targeted proteomics to perform the simultaneous analysis of large numbers of targeted proteins. This article describes applying targeted proteomics with nano-LC and ultra-fast mass spectrometry to the analysis of central metabolic enzymes in yeast and cultured human cells.

1. Introduction

A central metabolism is a basic feature shared by a wide range of organisms. For example, baker's yeast (*Saccharomyces cerevisiae*) uses central metabolic pathways to perform a type of fermentation that converts glucose to ethanol and carbon dioxide. The outstanding fermentation performance of *S. cerevisiae* has been a feature of food production since ancient times, such as in baking and the brewing of Japanese sake and wine, and in more recent times for the production of bioethanol. Metabolic engineering of *S. cerevisiae* is also becoming more prevalent, with gene recombination and gene editing techniques used to modify the central metabolic pathways of *S. cerevisiae* to produce a variety of useful compounds.

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Central metabolic pathways also play a role in the ability of the oleaginous yeast *Lipomyces starkeyi* to accumulate large amounts of lipids. In the field of cancer research, associations between a central metabolism and mechanisms of disease are a focus of interest, with cancer cells known to specifically activate metabolic pathways that create lactate from glucose.

The central metabolism functions thanks to some 100 enzymes, which catalyze the reactions on central metabolic pathways. Measuring the expression levels of enzymes on central metabolic pathways offers a shortcut to understanding how the production of useful substances can be enhanced through central metabolism regulation and the associations between disease and central metabolic functions.

Targeted proteomics offers a method for measuring protein expression levels based on mass analysis, which involves obtaining a crude enzyme extract from a biological sample, digesting the enzyme extract with trypsin, and analyzing the resulting mixture of trypsin-digested peptides using the liquid chromatograph-triple quadrupole mass spectrometer.[1,2] Before performing this analysis, trypsin-digested peptides that allow quantitative analysis of the target protein must be selected in advance for each targeted protein, after which a list of multiple reaction monitoring (MRM) transitions is used to selectively detect and quantify these peptides. Nano-LC is also used in targeted proteomics because its low flow rates allow greater analytical sensitivity when dealing with samples that contain very small amounts of peptides. Mass spectrometers capable of high-speed operations, such as those with ultra-fast mass spectrometry (UFMS) technology, are also needed in targeted proteomics to perform the simultaneous analysis of large numbers of targeted proteins. This article applies targeted proteomics using nano-LC and ultra-fast mass spectrometry to analyze central metabolic enzymes in yeast and cultured human cells.

2. Experiments

2-1. Reagents and Culture Conditions

All solvents used for LC/MS were of LC/MS grade (FUJIFILM Wako Pure Chemical). *S. cerevisiae* was batch cultured in a 200 mL baffled flask filled with 50 mL of SD culture medium (yeast nitrogen base w/o amino acid 6.7 g/L, glucose 20 g/L) at 30 °C and shaken at 120 rpm. Cells were recovered in the logarithmic growth phase. *L. starkeyi* was cultured under the same conditions as *S. cerevisiae* using 50 mL of synthetic culture medium.[3] 1.0×10^6 MCF-7 cells were seeded on a 10 cm plate and cultured in DMEM culture medium.

2-2. Preparing Samples for Analysis

The three major steps undertaken to prepare samples for analysis were extracting proteins from biological tissue, reductive alkylation and trypsin digestion of the extracted proteins, and desalination. Proteins were extracted from yeast cells by the following protocol: a 50 mL specimen of culture broth was centrifuged (5000 × g, 4 °C, 5 min); the supernatant was discarded; and the precipitate was resuspended in an equal amount by weight of cell lysis buffer (50 mM HEPES, 5 % glycerol, 15 mM DTT, 100 mM KCl, 5 mM EDTA, and 1 tablet of cOmplete protease inhibitors cocktail (Roche)). This suspension was then transferred to an Eppendorf tube filled with zirconia beads (enough 0.6 mm beads for 1 PCR tube, 6 mm bead × 1) and homogenized (3000 r/min, 6 min) in a Beads Crusher µT-12 (TAITEC). The resulting mixture was centrifuged (15000 rpm, 4 °C, 5 min), and the supernatant was transferred to an Eppendorf tube designed for use in proteomics (low protein binding tube) for analysis. The reductive alkylation and trypsin digestion of proteins was performed according to a published method.[4] Protein extraction from MCF-7 human breast cancer cells and reductive alkylation and trypsin digestion of proteins were performed using the Proteomics Sample Preparation Kit for Cultured Cells (#FMR-001, Funakoshi). Desalination was performed using the commercially available MonoSpin C18 (GL Sciences) or StageTips that were prepared in-house by layering three Empore Octadecyl C18 47 mm Extraction Disks 2215 in a pipette tip designed for use in proteomics.[5,6]

2-3. Analytical Conditions for Nano-LC-Triple Quadrupole Mass Spectrometer

This study performed nano-LC with the LC-20ADnano and triple quadrupole mass spectrometry with the LCMS-8040 and LCMS-8060. The analytical conditions are shown in Table 1. Skyline was used for all data analysis.[7] The MRM conditions used to measure the central metabolic enzymes of *S. cerevisiae* were taken from an LC/MS/MS MRM library of metabolic enzymes (yeast).[8] The MRM conditions used to measure the central metabolic enzymes of *L. starkeyi* were prepared in-house (unpublished data). For human MCF-7 cells, text data on 133 target peptides and 398 MRM transitions were taken from published data,[9] input into Skyline, and output as a method for the LC/MS systems. The collision voltage was obtained from the Q1 *m/z* value using the formula CE = 0.03 × [Q1 *m/z*] + 4.0.

Table 1 Nano-LC-Triple Quadrupole Mass Spectrometry System Setup and Analytical Conditions (Example)

LC:	**LC-20ADnano**
Eluent A:	0.1 % formic acid + 5 % acetonitrile aq. solution
Eluent B:	0.1 % formic acid + 95 % acetonitrile aq. solution
Eluent C (for Trap):	0.1 % formic acid aq. Solution
Online Degasser:	One unit for eluent A and one for eluent B (to prevent mixing)
Piping:	nanoViper fingertight fitting (Thermo Scientific)
Flow Rate:	400 nL/min
Gradient:	B. conc. 0 % (0-7 min) – 65 % (45 min) – 100 % (50-65 min) – 75 % (67 min) – 0 % (75-90 min) Injection valve switched to sample introduction side at 5 minutes
Trap Flow Rate:	40 µL/min
Column Temperature:	Not controlled
Column:	L-column Micro L-C18 0.1 × 150 mm, 3 µm (CERI)
Trap Column:	L-column Micro L-C18 0.3 × 5 mm, 5 µm (CERI)
Tip:	Fortis tip 150-20 (AMR)
Interface:	nano-spray interface (AMR)
MS:	**LCMS-8040 or LCMS-8060**
Nebulizer Gas Flow:	None
DL Temperature:	150 °C
Heat Block Temperature:	200 °C
Drying Gas Flow:	None
Q1 Resolution:	Low
Q3 Resolution:	Low
CID Gas:	310 kPa (LCMS-8040) 270 kPa (LCMS-8060)
Interface Voltage:	1.4 to 1.7 kV
Detection Method:	MRM mode

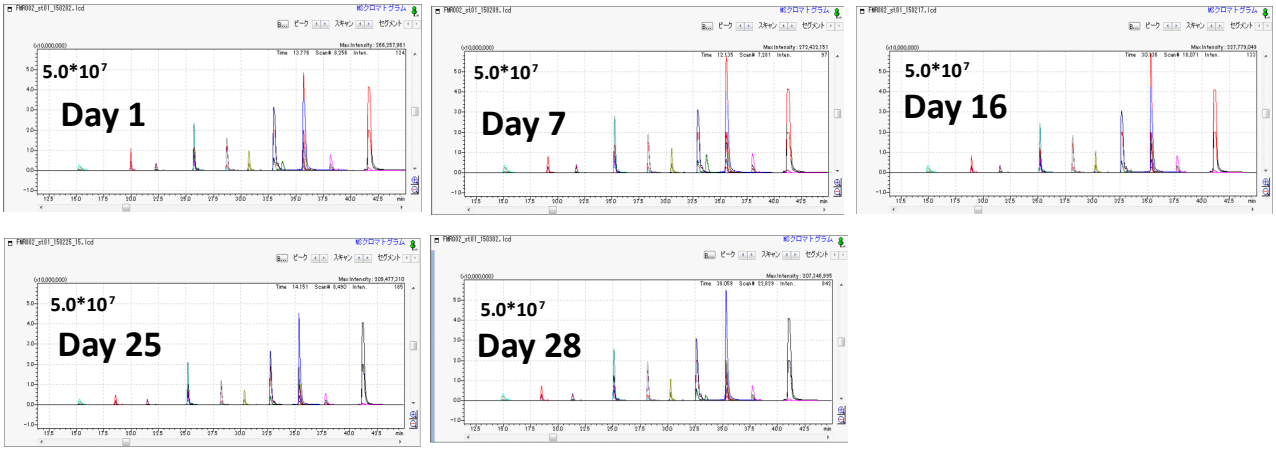

Fig. 1 Confirming the Stability of Nano-LC Retention Times
When analysis was performed continuously over a period of 1 month, ion source cleaning and MRMplus Retention Time Marker (FMR-002, Funakoshi) analysis were performed once per week.

3. Results

3-1. Confirming the Stability of Nano-LC Retention Times

Retention time stability on the nano-liquid chromatograph was tested using the MRMplus Retention Time Marker (FMR-002, Funakoshi) as a test proteomic peptide sample. The sample contained a mixture of 12 peptides. (There are published SRM transitions that allow for the detection of each peptide.) When analysis was performed continuously over a period of 1 month, ion source cleaning and FMR-002 analysis were performed once per week (Fig. 1). This weekly analysis of FMR-002 showed that the shift in retention times was within 0.2 minutes and with very little variation in detection sensitivity, confirming that the nano-LC retention times and ion source were stable enough for quantitative analysis (Fig. 1).

3-2. Confirming High-Speed Performance of Triple Quadrupole Mass Spectrometer

Because triple quadrupole mass spectrometers are now capable of operating at increasingly high speeds, the LCMS-8060 needs as little as 0.8 seconds to acquire a single MRM channel of data (dwell time). Paired with a required pause time of 1.0 msec, the LCMS-8060 is capable of acquiring data on 500-555 channels/sec in parallel. One concern with this improvement is that shorter dwell times will lead to mass chromatograms with a lower S/N ratio, which has a negative effect on quantitative accuracy. This concern was tested by acquiring data on a single sample using an MRM method with different dwell times and then comparing the quantitative results. Trypsin-digested proteins were prepared from *S. cerevisiae* strain S288C cultured in a fully labeled (^{13}C) glucose medium and a *ZWF1* gene knockout strain (zwf1Δ) cultured with non-labeled glucose. Samples for analysis were then prepared by mixing the two tryptic digests at a ratio of 1:1. An MRM method for analyzing central metabolic enzymes was used to measure the relative amounts of non-labeled and ^{13}C-labeled peptides ([zwf1Δ]/[S288C]). A data acquisition cycle time of 1200 msec/cycle was used for all dwell times. Examining the results obtained from the LCMS-8040 showed no major variation in relative amounts when the dwell time was reduced from 10 to 3 msec (Fig. 2a). Even when the dwell time was reduced from 10 to 1 msec, the variation in relative amounts remained low and within a factor of 2 (Fig. 2b).[10]

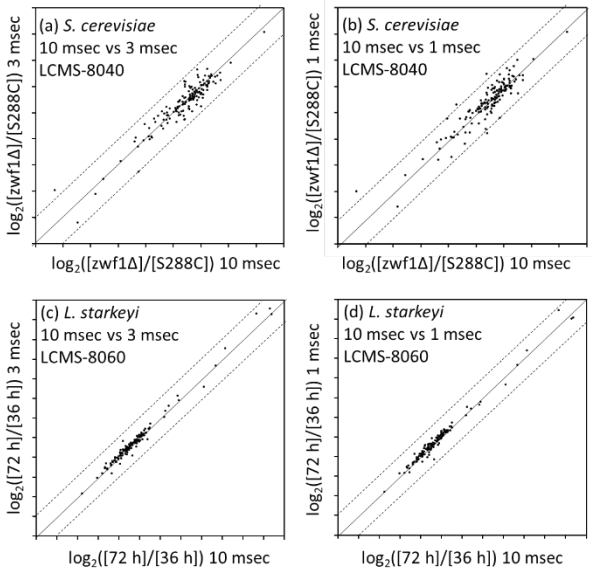

Fig. 2 Effect of Dwell Time on Relative Amounts of Peptides
This shows the amounts measured on the system relative to the reference sample.[10]
The dotted lines show variations by factors of 0.5 and 2.

Performing the same comparison on the LCMS-8060 using an MRM method for analyzing central metabolic enzymes in the oleaginous yeast *L. starkeyi* also showed that the variation in relative amounts remained smaller than a factor of 2 both when the dwell time was reduced from 10 to 3 msec and from 10 to 1 msec (Fig. 2c, and d) (unpublished data). These results confirmed that data can be acquired at high speed on both the LCMS-8040 and LCMS-8060 while maintaining the accuracy of quantitative data.

3-3. Checking MRM Assay Method Transfer

MRM assay development is becoming more expansive and trending towards the ability to measure all proteins across the entire genome of key species, such as humans and mice. Once an MRM assay method is created, it can be transferred between the mass spectrometry systems of different manufacturers. For example, Drabovich *et al.*[9] built an MRM assay that targets 78 human central metabolic enzymes and in the same report published text data on 133 target peptides and 398 transitions for a Thermo Scientific triple quadrupole mass spectrometer. We input this text data into Skyline and then output the data as an MRM assay method for Shimadzu's LC/MS systems. The collision voltage was calculated from the Q1 *m/z*. (See section **2-3**.) Multiple peptides were detected when the MRM assay reprocessed for the LCMS-8040 was used for quantitative proteomic analysis of cultured human MCF-7 cells (breast cancer cells). A linear relationship was also observed between the measured retention time of each peptide and the retention times noted in the literature, showing that the retention times could also be transferred between systems (Fig. 3, unpublished data).

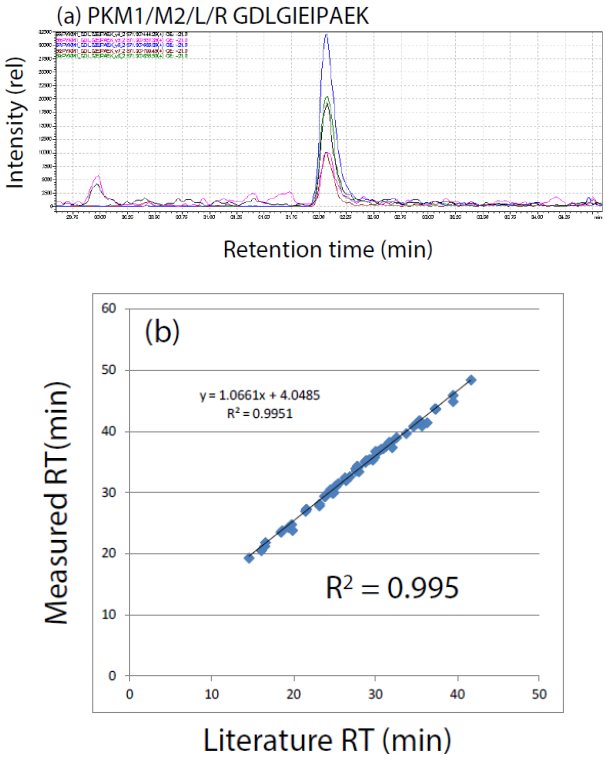

Fig. 3 Checking MRM Assay Method Transfer
The transferred version of the published MRM assay method was used to perform targeted proteomic analysis of central metabolic enzymes in MCF-7 breast cancer cells: (a) a chromatogram showing detection of GDLGIEIPAEK assay peptide derived from PKM, and (b) a comparison of published retention times for assay peptides and measured retention times after method transfer.

3-4. Targeted Proteomic Analysis of Central Metabolic Enzymes in Single-Gene Knockout Mutant Strain of *S. cerevisiae*

S. cerevisiae has an innately powerful ability to produce ethanol. In *S. cerevisiae*, pyruvate decarboxylase (Pdc1) is a key enzyme on the pathway that generates ethanol (ETOH) from pyruvate (PYR), a product of the glycolytic system. When the gene that codes for Pdc1 is knocked out in an attempt to switch to the biosynthesis of another compound from PYR, the switch does not occur as expected, and levels of ethanol conversion only fall slightly. In a previous study, quantitative proteomic analysis of the *PDC1* gene knockout strain pdc1Δ and the wild type strain BY4741 was performed and the expression profile of central metabolic enzymes compared.[11] The results showed a Pdc1 protein expression level of zero in the pdc1Δ strain (Fig. 4a). However, Pdc5, which exhibits the same function as Pdc1 and is barely expressed under normal circumstances, was expressed at substantially higher levels, and it acted as a backup for Pdc1. We also observed a simultaneous increase in the expression of Hxk1 and Tdh1, enzymes that function at the entrance and at an intermediate point in the glycolytic system, respectively. This shows that other parts of the glycolytic system are also affected by the *PDC1* gene.

The *GCR2* gene is a global regulator of the glycolytic system, and performing the same analysis with a *GCR2* gene knockout strain (gcr2Δ) resulted in the decreased expression of many enzymes in the glycolytic system (Fig. 4b). Various other effects were also observed outside of the central metabolism, including decreased expression of enzymes in the amino-acid biosynthesis pathway and increased expression of enzymes in the trehalose pathway. Metabolic engineering aims to improve the efficiency of target compound production by expressing enzymes that catalyze additional reactions and eliminating enzymes that catalyze unwanted reactions. This study suggests that manipulation of a single site can affect all central metabolic pathways.[11]

4. Conclusion

This study applied targeted proteomics with nano-LC and ultra-fast mass spectrometry to analyze central metabolic enzymes in yeast and cultured human cells. The results confirmed that nano-LC retention times and the mass spectrometer ion source were stable enough for quantitative analysis (Fig. 1) and that ultra-fast mass spectrometry can be used to acquire data at high speeds while maintaining accurate quantitative data (Fig. 2). Furthermore, this study confirmed that MRM assay methods taken from published data can be transferred for use on different equipment (Fig. 3). Analysis of a single-gene knockout strain of *S. cerevisiae* also showed that manipulation of a single site can affect all central metabolic pathways (Fig. 4). In conclusion, using targeted proteomics with nano-LC and ultra-fast mass spectrometry for the simultaneous quantitative analysis of central metabolic enzymes can provide insights into how the central metabolism can be regulated for improved production of useful substances as well as the associations between disease and central metabolic functions.

Acknowledgments

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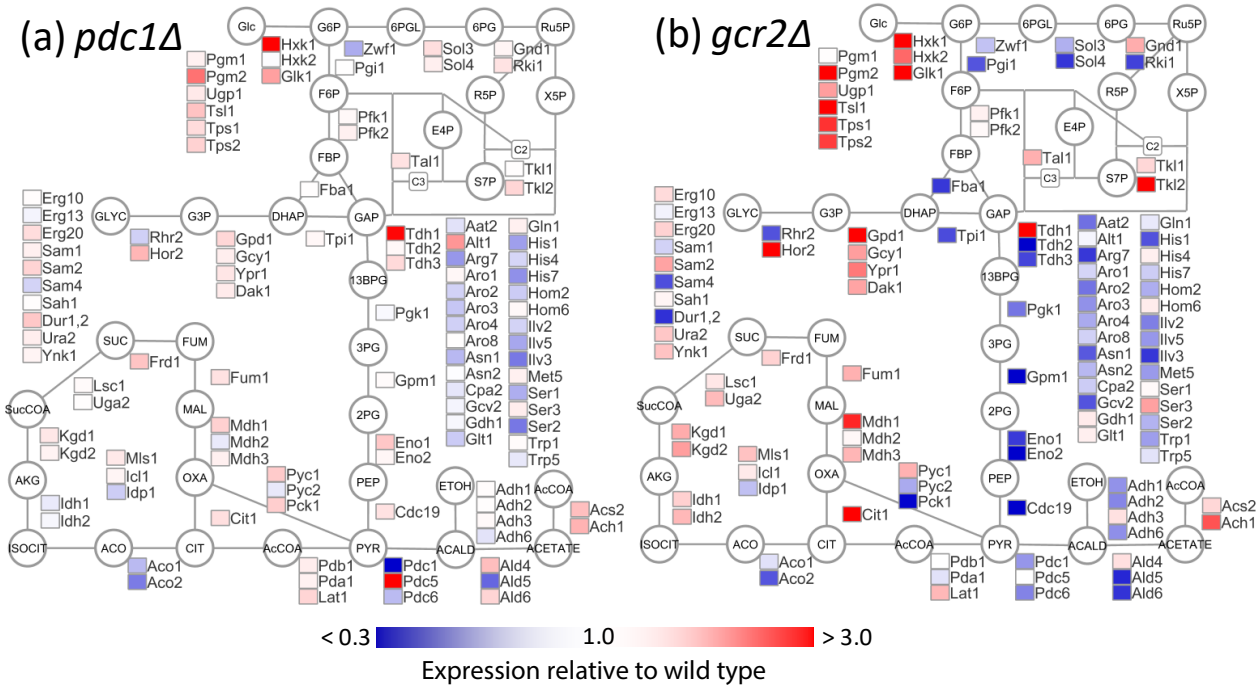

Fig. 4 Targeted Proteomic Analysis of Central Metabolic Enzymes in Single-Gene Knockout Mutant Strain of *S. cerevisiae*[11]
The heat map shows the expression of each enzyme in (a) the pdc1Δ strain and (b) the gcr2Δ strain relative to the wild type strain.

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