Quantification of *Saccharomyces cerevisiae* pentose-phosphate pathway intermediates by LC-MS/MS

Mirjam Wamelink
Metabolic Unit, VU University Medical Center Amsterdam, de Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
Erwin Jansen
Metabolic Unit, VU University Medical Center Amsterdam, de Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
Eduard Struys
Metabolic Unit, VU University Medical Center Amsterdam, de Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
Hans Lehrach
Max Planck Institute for Molecular Genetics, Ihnestrasse 73, 14195 Berlin, Germany
Cornelis Jakobs
Metabolic Unit, VU University Medical Center Amsterdam, de Boelelaan 1117, 1081 HV Amsterdam, The Netherlands
Markus Ralser
Max Planck Institute for Molecular Genetics, Ihnestrasse 73, 14195 Berlin, Germany

Method Article

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Abstract

Introduction

The pentose phosphate pathway \(PPP) is a central pathway of the cellular carbohydrate metabolism. The PPP is directly interconnected with glycolysis and composed of a irreversible, oxidative- and a reversible, non-oxidative part. The PPP provides the cell with intermediates for several bioorganic syntheses, and acts as balancer for the redox state in response to oxidant exposures [1,2]. Here, we provide a liquid chromatography tandem mass spectrometry \(LC-MS/MS) based protocol for accurate quantification of PPP intermediates in _S. cerevisiae_.

Reagents

Yeast growth media \(dependent on the desired strain and experiment) Hanks Balanced Salt Solution \(HBSS) without Phenol red \(e.g. Sigma H1387 + 4.2 mM sodium bicarbonate) Perchloric acid Acid washed glass beads \(425-600 µm, Sigma) Phosphate buffer, 1 M, pH= 11.5 $^{13}C_6$-glucose-6-phosphate prepared by glucokinase using $^{13}C_6$-glucose [3] Acetonitrile \(Merck) Octylammonium acetate prepared from octylamine \(Sigma) and acetic acid Dihydroxacetone phosphate \(dhap), ribose 5-phosphate \(r5p), erythrose 4-phosphate \(e4p), xylulose 5-phosphate or ribulose 5-phosphate \(x5p), glucose 6-phosphate or fructose-6-phosphate \(g6p), sedoheptulose 7-phosphate \(s7p) and 6-phosphogluconate \(6pg) to generate calibration curves for an accurate quantification.

Equipment

Microcentrifuge Spectrophotometer Vortex with TurboMix equipment \(e.g. Scientific Industries Vortex Genie 2 with Turbo Mix holder SI-0563), or FastPrep-24 machine \(MP Biomedicals) Triple quadrupole tandem mass spectrometer with classic HPLC-unit \(we used a PE-Sciex API-3000 tandem mass spectrometer equipped with an electrospray source \(Turbo Ion Spray) and a Perkin-Elmer series 200 HPLC unit) Dedicated 3.9 x 150 mm Symmetry C_{18} HPLC column, bead size 5 µm \(Waters)

Procedure

1. inoculate 2 x 3ml media with the yeast strain to be analyzed 2. shake or rotate overnight at 30 °C 3. use this overnight cultures to adjust 2 x 30ml yeast media to an OD_{600} of 0.1 - 0.2 4. grow the cells to mid-log phase, record OD_{600} value 5. There are two possibilities how to proceed a) lyse the complete 30 ml culture. This has the advantage of yielding in higher concentrated metabolite extracts or b) analyze 1.5 ml aliquots, which is the method of choice if a fast sample handling is required. The lysate procedure requires breaking the cells in a buffer containing 2% perchloric acid; this guarantees, that metabolic enzymes are immediately inactivated upon lysis **Lysate Procedure A \(30 ml culture)** i) centrifuge the 30 ml culture in a 50 ml tube, 3 min, 3000 g, RT ii) re-suspend the pellet in 1 ml HBSS buffer iii) transfer

the suspension to a 1.5 ml screw tube iv) centrifuge 30 sec, 5000g-7000g in a table-top centrifuge v) remove the supernatant quickly vi) freeze the sample immediately in liquid nitrogen vii) \(at this point, the pellets can be transferred to -80 °C for storage) viii) place the tubes on dry-ice, open the tubes ix) add to the cell pellets an equal volume of glass beads \(Hint: Use a 1.5ml-tube-lid as bucket) x) transfer the tubes to normal ice xi) add 800 µl of 98% HBSS / 2% perchloric acid xii) close the tubes, place them into the Fast-Prep-24 machine \(alternatively, a Vortex with Turbo-Mix equipment can be used, see Procedure B) xiii) start the machine, 6.5 m/s, 40 sec xiv) cool the tubes on ice for 5 min xv) repeat the fast-prep procedure xvi) incubate the samples for 30min on ice xvii) centrifuge the samples for 2 min, at greater than or equal to 14000g, 4 °C xviii) transfer the supernatant to a fresh tube xix) freeze and store the samples at -80 °C until the LC-MS/MS analysis, continue at step 6 **Procedure B \(1.5 ml culture aliquots)** i) aliquot the mid-log culture to a desired number of 1.5ml screw tubes ii) incubate the cultures in a thermo block for at least 5 min at 30 °C iii) centrifuge 5000g-7000g, 30sec iv) remove the supernatant quickly v) freeze the pellet immediately in liquid nitrogen vi) \(at this point, the pellets can be transferred to -80 °C for storage) vii) place the tubes on dry-ice and open them viii) add to the cell pellet an equal volume of glass beads \(Hint: Use a 1.5 ml-tube-lid as bucket) ix) transfer the tubes to normal ice x) add 400 µl of 98% HBSS /2 % perchloric acid xi) in the coldroom, transfer the tubes to the Vortex with Turbo-Mix equipment xii) vortex 4 min, max speed xiii) incubate on ice for 5 min xiv) vortex again, 4 min, max speed xv) incubate the samples for 30 min on ice xvi) centrifuge the samples for 2 min at greater than of equal to 14000g, 4 °C xvii) transfer the supernatant to a fresh tube xviii) freeze and store the tubes at -80 °C until LC-MS/MS analysis, continue at step 6 **LC-MS/MS analysis** 6. thaw the samples 7. Prepare calibrator curves for dihydroxacetone phosphate \(dhap), ribose 5-phosphate \(r5p), erythrose 4-phosphate \(e4p), xylulose 5-phosphate or ribulose 5-phosphate \(x5p), glucose 6-phosphate or fructose 6-phosphate \(g6p), sedoheptulose 7-phosphate \(s7p) and 6-phosphogluconate in the range of the expected concentrations 8. as internal standard, add 50 µl 10 µM $^{13}C_6$-glucose-6-phosphate to 50 µl of the lysates \(and to the calibrators) 9. neutralize the samples with 1 M phosphate buffer \(pH 11.5) to pH 7-8 10. centrifuge for 5 min, at greater than or equal to 14000g, 4 °C. 11. transfer the supernatant to appropriate HPLC vials 12. cap the vials 13. prepare the solvents for the gradient-chromatography. We recommend to use 12.5% acetonitrile in water supplemented with 500 mg/l octylammonium acetate \(pH 7.5) as binary solvent A and 50% acetonitrile in water supplemented with 500 mg/l octylammonium acetate \(pH 7.5) as binary solvent B 14. equilibrate your C_{18} HPLC column with solvent A for several minutes with a flow rate of 1 ml/min. 15. Set a linear gradient from 100% solvent A to 40% solvent A and 60% solvent B in 8 min \(at a flow rate of 1 ml/min) 16. If necessary, split the flow post-column dependent on your mass spectrometer 17. Set the MS to multiple reaction monitoring mode \(MRM) with the electrospray source operating in the negative-ion mode. 18. Configure your MS/MS to record the following MRM transitions: dhap: m/z -169/-97; e4p -199/-97; r5p and x5p: m/z -229/-97; g6p: m/z -259/-97, $^{13}C_6$-glucose 6-P \(IS): m/z -265/-97; 6pg: m/z -275/-97 and s7p: m/z -289/-97. Optimized MS settings for an ABI PE-Sciex API-3000 tandem mass spectrometer equipped with an electrospray source \(Turbo Ion Spray) as well as the detection limits with this setting have been reported earlier [4] 19. For a relative quantification of the PPP intermediates we suggest to normalize the metabolites to the OD_{600}

value of the starter culture rather than to a cell number count. Note: xylulose 5-phosphate/ ribulose 5-phosphate and glucose 6-phosphate/fructose 6-phosphate can not be separately quantified with this method due to chromatographic co-elution.

Timing

2 days + yeast overnight culture

References

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