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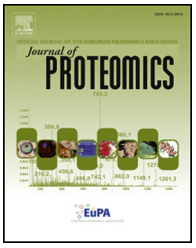

Absolute quantification of *Corynebacterium glutamicum* glycolytic and anaplerotic enzymes by QconCAT

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ABSTRACT

The soil bacterium *Corynebacterium glutamicum* is one of the best-studied production hosts for industrial biotechnology, and it is primarily used for the large-scale production of essential amino acids, such as L-lysine. For rational strain development, detailed knowledge of intracellular protein concentration is crucial to determine metabolic capacities and limitations. We developed a QconCAT approach for the accurate absolute quantification of key enzymes of *C. glutamicum* glycolysis and anaplerosis. Following well-defined batch cultivations, 10 metabolic enzymes were quantified, accounting for approximately 6% of the total cell dry weight. Copy numbers per cell ranged from 36,700 ± 3500 for phosphofructokinase (PFK) to 507,700 ± 40,300 for enolase (ENO), which is considerably lower than the corresponding data obtained from *Saccharomyces cerevisiae*. Moreover, accurate measurement of the biovolume permitted an estimation of molar concentrations of intracellular enzyme catalysts ranging from 7.6 ± 1.9 μM (PFK) to 105.2 ± 28.6 μM (ENO). Finally, model-assisted data evaluation demonstrates that our method provides an important cornerstone toward a more detailed mechanistic understanding of *C. glutamicum* metabolism.

Biological Significance

Determination of absolute proteins amounts using quantitative concatemers (QconCAT's) has already been successfully demonstrated for various species including human, animal and yeast. Interestingly, application of the QconCAT methodology for the determination of cytoplasmic enzyme concentrations in a prokaryote has not been described so far. This study is concerned with a novel targeted approach for the absolute quantification of 10 key enzymes from the central carbon metabolism of the industrially important organism *Corynebacterium glutamicum*. We demonstrate a method that enables complete cell lysis of this robust soil bacterium, thus allowing for accurate quantification of cytoplasmic enzymes. By linking measured enzyme amounts with respective biovolume data, intracellular enzyme concentrations were estimated, which are of special importance for any systems biology approach studying *C. glutamicum*'s metabolism at the mechanistic level. To our knowledge this is the first report of applying the QconCAT methodology for determining intracellular enzyme concentrations in a prokaryote.

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Abbreviations

ANA,	Anaplerotic reactions
AQUA	Absolute quantification
CDW	Cell dry weight
CE	Crude extract
DTT	Dithiothreitol
EMP	Embden–Meyerhof–Parnas-Pathway
MWCO	Molecular weight cutoff
PSAQ	Protein standards for absolute quantification
PTM	Post translational modification
QconCAT	Quantitative concatemers

Enzyme identifiers

AceE	Pyruvate dehydrogenase decarboxylating subunit
FBA	Fructose bisphosphate aldolase
ENO	Enolase
PCx	Pyruvate carboxylase
PDH	Pyruvate dehydrogenase
PEPCk	Phosphoenolpyruvate carboxykinase
PEPCx	Phosphoenolpyruvate carboxylase
PFK	Phosphofructokinase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PK	Pyruvate kinase

1. Introduction

Corynebacterium glutamicum is a gram-positive soil bacterium that is closely related to several pathogenic *Mycobacterium* species. This bacterium has long been used for the fermentative production of amino acids. Currently, more than 2.2 million tons of L-glutamate and 1.5 million tons of L-lysine are produced with optimized production strains based on the original wild type *C. glutamicum* that was discovered more than 50 years ago [1,2]. Due to its importance for industrial biotechnology, *C. glutamicum* has been comprehensively studied, and its genome sequence is available along with its transcriptome, metabolome and fluxome data [3,4]. The first proteomic analysis of *C. glutamicum* was conducted by creating a reference map based on two dimensional gel electrophoresis [5]. In recent years, gel-free approaches have been used to study the impact of factors such as external stress caused by pH shift and osmotic pressure on the *C. glutamicum* proteome [6,7]. Furthermore, the effects of different carbon sources [8,9] and protein turnover have been investigated [10].

These investigations have established a broad knowledge base rendering *C. glutamicum* a model organism for systems biology. The first genome-wide metabolic networks are currently available, thus enabling the elucidation of the metabolic capacities and limitations of this organism for specific applications [11,12]. The next phase of *C. glutamicum* analysis entails gaining a deeper system-level understanding, which requires the integration of metabolic networks with genome regulation networks [13] and the interconnecting proteome. This, however, requires absolute quantitative protein data that are crucial for the construction and validation of such "vertical" networks [14]. Previously, these data were not available for *C. glutamicum*.

In recent years, several methods for the absolute quantification of proteins by mass spectrometry have been presented. The use of synthetic, isotope-labeled peptides or absolute quantification (AQUA) peptides allows the rapid quantification of proteotypic peptides in complex biological samples [15]. Complete proteolysis is mandatory for accurate results because these peptide standards are usually added after tryptic digestion. Moreover, the synthesis of AQUA peptides is still relatively expensive.

Alternatively, quantification standards can be produced by the individual purification and calibration of all targeted proteins in an isotope-labeled form. This method, called PSAQ, promises highly accurate results, even under sub-optimal digestion conditions because proteotypic peptides will be released from their native sequence background [16]. Unfortunately, PSAQ becomes extremely laborious for multiplexed studies that involve numerous proteins.

The QconCAT approach, first introduced for the quantification of proteins in chicken skeletal muscle, offers a reasonable compromise between both of the methods described above [17]. In the QconCAT approach, short DNA sequences, coding for a set of proteotypic peptides, are concatenated into one QconCAT gene that is subsequently expressed and purified in a heavy-labeled form. This approach has been successfully applied for the absolute quantification of proteins from numerous species such as human, yeast, chicken, cattle and the human parasite *Schistosoma mansoni* [18–22]. Recently, Al-Majdoub et al. applied the QconCAT technology for the quantification of ribosomal proteins in the prokaryote *Escherichia coli* [23]. For multiplex studies, the QconCAT approach is cheaper compared to AQUA peptides and less laborious than a PSAQ strategy. However, it requires a fully purified QconCAT protein, and complete tryptic digestion is crucial if the standard is externally calibrated.

In this study, we present the first application of QconCAT for the absolute quantification of cytoplasmic enzymes in a prokaryote. We demonstrate a reliable method for the complete cell lysis and cytoplasmic protein extraction from a robust soil bacterium for the accurate quantification of 10 metabolic key enzymes.

2. Materials and Methods

2.1. Strains, media and cultivation conditions

Wild type *C. glutamicum* ATCC130332 [24] was cultivated in 1 l CGXII defined medium [25] with 10 g l^{-1} glucose as the sole carbon and energy source. Cells were grown to mid-exponential phase in lab-scale bioreactors (DASGIP AG, Germany) at 30 °C, pH 7.0 (adjusted by the addition of 4 M sodium hydroxide) and 30% dissolved oxygen (1 vvm, 400–1200 rpm). Growth was monitored by optical density, and glucose concentration was determined offline by enzymatic conversion to 6-P-gluconate and photometric measurement of the produced NADH. Cells were harvested by centrifugation and they were immediately

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