Synthetic and Systems Biotechnology xxx (2018) 1–8

Contents lists available at ScienceDirect

Synthetic and Systems Biotechnology

journal homepage: http://www.keaipublishing.com/en/journals/synthetic-and-systems-biotechnology/

Cell-free synthesis of stable isotope-labeled internal standards for targeted quantitative proteomics

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ARTICLE INFO

Article history:
Received 26 December 2017
Received in revised form
16 February 2018
Accepted 18 February 2018

Keywords:
Absolute quantification
Mass spectrometry
Cell-free protein synthesis system
In vitro translation
Targeted quantitative proteomics
PURE system

ABSTRACT

High-sensitivity mass spectrometry approaches using selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) methods are powerful tools for targeted quantitative proteomics-based investigation of dynamics in specific biological systems. Both high-sensitivity detection of low-abundance proteins and their quantification using this technique employ stable isotope-labeled peptide internal standards. Currently, there are various ways for preparing standards, including chemical peptide synthesis, cellular protein expression, and cell-free protein or peptide synthesis. Cell-free protein synthesis (CFPS) or in vitro translation (IVT) systems in particular provide high-throughput and low-cost preparation methods, and various cell types and reconstituted forms are now commercially available. Herein, we review the use of such systems for precise and reliable protein quantification.

Contents

1. Introduction

Cells survive by constituting very complicated molecular networks involving various small molecules and macromolecules including nucleic acids, proteins, carbohydrates and lipids. A wide variety of complex biological functions such as migration, predation and metabolism are orchestrated by functioning networks that contribute to prevent entropy from increasing in non-equilibrium systems, as discussed by Schrödinger [1], which essentially forms the basis of all life activities. While individual reactions in a network operate via simple mechanisms governed by thermodynamics, complexity is generated by the enormous number of reactions in the network. Therefore, the most intuitive way to

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Peer review under responsibility of KeAi Communications Co., Ltd.

https://doi.org/10.1016/j.synbio.2018.02.004

understand this complexity is to accurately measure the information present among the individual molecules over time, and to clarify the characteristics of the entire reaction network by data assimilation with the model of an entire cellular network [2] or a partial molecular network [3]. Among the molecules involved, proteins, the expression products of genetic information, are the major cellular components responsible for almost all biochemical processes. Thus, a systems-level biological approach based on quantitative proteomics [4] is crucial for understanding life.

Traditionally, quantification of proteins has been conducted using radioactive isotopes or antigen-antibody interactions. Recently, methods using intracellular detection of protein dynamics by fluorescence or luminescence imaging coupled with genetic engineering have also been adopted [5–7]. However, for detecting and quantifying proteins as they occur in biological samples without using special procedures employing antibodies and/or genetic engineering, mass spectrometry (MS) is most suitable method, in terms of both throughput and sensitivity. MS-based quantitative global proteomics researches have revealed whole proteome maps of yeast, mouse and human cells [8–13], detecting more than 10,000 proteins in the process, demonstrating its high-throughput capabilities.

In standard liquid chromatography (LC)-MS approaches, biological samples are processed with proteases such as trypsin, which cleaves peptide bonds between the carboxyl group of arginine or lysine and the amino group of the adjacent amino acid. The resultant proteotypic peptides, specific to their parent proteins, are separated by LC and individually subjected to MS analysis, which separates each peptide peak into two dimensions based on their individual chemical properties and masses. The addition of a pre-fractionation step such as strong cation exchange (SCX) column chromatography can further increase the sensitivity by allowing three- or more-dimensional separation [14,15]. The introduction of tandem mass spectrometry, also termed MS/MS, can improve the detection resolution by determining the peptide sequences through the generation of fragment ions from precursor ions. MS/MS is an indispensable technique for both global and targeted proteomics. Sequence determination by MS/MS is necessary for shotgun analysis of various peptides in global proteomics approaches. For selected reaction monitoring (SRM) or multiple reaction monitoring (MRM) methods that are frequently employed in targeted proteomics experiments, selection of both precursor and fragment ions by a triple quadrupole mass spectrometer is necessary to limit the number of peptides observed for each target, which enables highly sensitive analysis of target peptides in samples.

Since the intensity of peptide peaks depends on their physical properties, absolute quantification of peptides of interest by MS is achieved by utilizing a stable isotope-labeled peptide internal standard with the same sequence at a known concentration [16]. Apart from the difference in mass, all other chemical and physical properties of the two peptides are identical; hence retention times in LC, ionization efficiencies and fragmentation patterns in MS/MS are all identical. Thus, quantitative determination of peptides of interest can be carried out only by comparing the intensities of the two peaks (Fig. 1). It is over three decades since this concept was first proposed [17], and it is now possible to prepare peptide standards in various ways. In recent years, targeted quantitative proteomics using internal standards synthesized by cell-free protein synthesis (CFPS) or in vitro translation (IVT) systems have gradually attracted attention. This review focuses on the cell-free synthesis of stable isotope-labeled internal standards for targeted quantitative proteomics studies.

2. Chemical peptide synthesis and cellular protein synthesis

Various approaches currently employed for preparing stable isotope-labeled peptides are shown in Fig. 2. The methods can be roughly divided into two types, chemical synthesis from the C-terminus, and biological synthesis from the N-terminus using ribosomes, among which chemically synthesized peptides represented by Absolute Quantification (AQUA) is the most widely used method [18]. AQUA peptides are generated by solid-phase synthesis [19], in which peptides bound to a solid matrix with a free N-terminal amino group are reacted with an N-terminally protected amino acid, followed by deprotection of the amino group and washing of the solid phase. Peptides can be elongated at their N-terminus by consecutive reactions. Stable isotope labels can be introduced by replacing one residue with a stable isotope-labeled amino acid. Moreover, by using modified amino acid such as phosphorylated serine as a substrate, modified peptides that mimic post-translational modification can be produced, demonstrating the versatility of this approach.

However, there are some limitations associated with this method. It requires large amounts of isotopically labeled amino acids as substrates. Synthesis can be difficult depending on the peptide sequence due to sequence-dependent side reactions. These limitations make it difficult to reduce the cost per peptide, which is a barrier to multiplexed synthesis. To overcome these drawbacks, multiplexed chemical peptide synthesis, termed SPOT synthesis [20], is applied to establish SRM assays in a high-throughput manner using crude (impure) peptides without laborious purification [21]. A stable isotope dimethyl labeling method [22,23] where the chemically synthesized peptides are labeled with stable isotope-labeled formaldehyde is another cost-effective alternative for MS-based quantification [24,25].

Biological synthesis can be further divided into cellular and cell-free synthesis approaches (Fig. 2). Stable Isotope Labeling by Amino acids in Cell culture (SILAC) is a representative cellular synthesis method [26]. In this method, the composition of the medium is adjusted so that a stable isotope-labeled essential amino acid is necessarily introduced into the protein as it is, so that a specific amino acid of the intracellular protein is completely replaced with a labeled residue. Unlike chemical synthesis methods, stable isotope labeling can be introduced nonspecifically to intact cell proteins, enabling various peptides to be synthesized simultaneously, which is more effective for global proteomics analysis. In recent years, SILAC-based global quantitative proteomics has been applied at the tissue, organism and individual level by growing on a diet containing isotope-labeled amino acids [27–31].

Development towards targeted quantitative proteomics has also progressed. In the Absolute SILAC method, targeted analysis can be carried out by constructing expression systems for specific proteins in cells, and tryptic digests of the produced proteins are used as internal standards [32]. Cellular protein expression systems are also used in the QconCAT method, which uses tryptic digests of artificial proteins comprising a concatenation of proteotypic peptides [33,34].

3. Cell-free protein synthesis systems

In general, to prepare a specific protein sample, a recombinant protein expression system is employed in which the gene encoding the protein of interest is inserted into an expression vector and protein is produced in *Escherichia coli*, yeast or other cells. Once the method is established, it has an advantage that the target protein samples can be obtained reproducibly, and therefore, it is a method indispensable for preparation of protein samples. However, to perform protein production using this system, it is necessary to go

Please cite this article in press as: Narumi R, et al., Cell-free synthesis of stable isotope-labeled internal standards for targeted quantitative proteomics, Synthetic and Systems Biotechnology (2018), https://doi.org/10.1016/j.synbio.2018.02.004