Journal of Industrial and Engineering Chemistry 37 (2016) 90–94

Contents lists available at ScienceDirect

Journal of Industrial and Engineering Chemistry

journal homepage: www.elsevier.com/locate/jiec

Enhanced production of unnatural amino acid-containing proteins in a cell-free protein synthesis system

Kyung-Ho Lee, Christy Catherine, Dong-Myung Kim *

Department of Chemical Engineering and Applied Chemistry, Chungnam National University, Daejeon 305-764, Republic of Korea

ARTICLE INFO

Article history:
Received 23 July 2015
Received in revised form 6 September 2015
Accepted 3 March 2016
Available online 10 March 2016

Keywords:
Cell-free protein synthesis
Unnatural amino acids
Protein engineering
Residue-specific incorporation

ABSTRACT

Replacement of canonical amino acids with unnatural amino acids (UAAs) can provide proteins with novel physicochemical properties and biological functions. In this study, as an alternative option to conventional cell-based methods, we used a cell-free protein synthesis system as a flexible platform for facile and efficient production of UAA-containing proteins. We designed a cell-free protein synthesis system derived from the extract of *Escherichia coli* cells to maximize the selective incorporation of UAAs into the protein structure. First, for the purpose of avoiding competitive incorporation of canonical amino acids and UAAs, the cell extract was extensively washed using a diafiltration process to remove residual amino acids, thereby making the protein synthesis reaction completely dependent upon the exogenous addition of amino acids. In addition, the relatively low affinity of UAAs for cognate aminoacyl-tRNA synthetase was kinetically overcome by increasing the concentration of UAAs to nonphysiological levels. As a result of these modifications of the cell-free synthesis systems, we were able to produce UAA-containing proteins at comparable yields to those of proteins made of canonical amino acids.

Introduction

Genetic incorporation of unnatural amino acids (UAAs) can be used to engineer proteins to acquire new structures and functions that cannot be attained with the natural set of 20 amino acids [1–5]. Several methods have been described for the incorporation of UAAs into proteins during cell-based expression of recombinant proteins [6,7]. Many codons can be decoded with UAAs when they are added to the media in place of structurally analogous canonical amino acids. This approach relies on the relaxed substrate specificity of aminoacyl-tRNA synthetases (AARSs) and is particularly suitable for global modification of proteins.

However, the efficiency of cell-based incorporation of UAAs is often limited by intrinsic cellular regulation mechanisms. Because they inefficiently transverse the cell membrane, most UAAs do not accumulate in the cytoplasm at concentrations high enough to achieve adequate aminoacylation of tRNA [8]. Although intracellular accumulation of certain UAAs can be improved by manipulating growth conditions [9], this approach only works for some amino acid groups and cannot be applied generally. The structural similarities of UAAs and their natural counterparts can also lead to inhibition of metabolic reactions that are essential for cell growth. For example, while norleucine is a good substrate for methionyl-tRNA synthetase and thus can replace methionine in recombinant proteins [10], it inhibits DNA methylation and replication, leading to host restriction and degradation of chromosomal DNA [11]. These deterrents generally lower the cumulative efficiency of UAA incorporation during cell-based protein expression.

In contrast, cell-free protein synthesis can avoid many of the limitations caused by cellular regulatory processes [12–14]. In particular, the absence of a cell membrane allows for direct delivery of amino acids at the desired concentrations without toxicity concerns. In this study, we describe the efficient incorporation of different UAAs into a protein using a modified cell-free protein synthesis system derived from *Escherichia coli*. We first extensively washed cell extracts to ensure that the protein synthesis is strictly dependent upon the addition of exogenous amino acids (either natural amino acids or UAAs). In addition, because aminoacylation is a major rate-limiting step in translation, UAA concentrations in the reaction mixture were increased far beyond physiological levels to kinetically overcome the high K_M values of AARSs for UAAs. Taken together, we were able to introduce different tyrosine analogues into superfolder green fluorescence protein (sfGFP) [15] successfully. Using washed cell

* Corresponding author. Tel.: +82 42 821 5899; fax: +82 42 822 6637.
E-mail address: dmkim@cnu.ac.kr (D.-M. Kim).

http://dx.doi.org/10.1016/j.jiec.2016.03.008
1226-086X/

extract, exclusive incorporation of UAAs was achieved with minimal contamination from cognate amino acids. Our results demonstrate that the open nature and flexibility of cell-free protein synthesis can be applied to the engineering of proteins from an expanded catalogue of amino acids.

Experimental

Reagents

Nucleotide triphosphates, creatine phosphate, and creatine kinase were purchased from Roche Applied Science (Indianapolis, IN). UAAs [3,4-dihydroxy-L-phenylalanine (L-DOPA), 3-fluorotyrosine, and 3-chlorotyrosine] were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nickel-nitrilotriacetic acid (Ni-NTA) resin was purchased from Qiagen (Valencia, CA). Polyvinylidene difluoride (PVDF) membrane was obtained from Millipore (Billerica, MA). L-[U-^{14}C]leucine (11.8 GBq/mmol) was obtained from Amersham Biosciences (Uppsala, Sweden). All other chemical reagents were purchased from Sigma (St. Louis, MO) and were used without further purification. The standard S12 extract was prepared as described previously [16].

Preparation of washed cell extract

To remove residual amino acids from the S12 cell extract, 2 mL of S12 extract was diafiltered in a Vivaspin centrifugal concentrator (Sartorius Stedim Biotech GmbH, Göttingen, Germany) installed with a 10,000 Da molecular weight cutoff (MWCO) membrane. After the addition of 18 mL of S12 buffer (10 mM Tris–acetate buffer [pH 8.2], 14 mM magnesium acetate, 60 mM potassium glutamate, and 1 mM dithiothreitol) to 2 mL of the S12 extract, the concentrator was centrifuged at 2000 relative centrifugal force (RCF) to reduce the volume of the diluted extract to the original volume (2 mL). This step was repeated three times.

Cell-free protein synthesis

The sfGFP gene was PCR-amplified from pY21sfGFP [17] with primers containing NdeI and SalI sites. After restriction digestion with corresponding restriction enzymes, amplicons were cloned into pK7 plasmids between the T7 promoter and the T7 terminator, as described previously [18]. Purified plasmid (1.53 nM) was added to 15 μL of the standard reaction mixture containing 57 mM HEPES–KOH (pH 7.5); 1.2 mM ATP; 0.85 mM each of guanosine triphosphate (GTP), uridine triphosphate (UTP), and cytidine triphosphate (CTP); 80 mM ammonium acetate; 34 μg/mL 1–5-formyl-5,6,7,8-tetrahydrofolic acid (folinic acid); 1.0 mM each of the 20 naturally occurring amino acids; 0.3 U/mL creatine kinase; 67 mM creatine phosphate; and 4 μL normal or washed S12 extract. The reaction mixtures were incubated at 30 °C for 3 h. To incorporate unnatural tyrosine analogues, tyrosine was replaced by UAAs in varying concentrations in the standard reaction mixture.

Analysis of cell-free synthesized proteins

The fluorescence intensities of the cell-free synthesized sfGFP molecules were measured using a VICTOR X2 multilabel plate reader (PerkinElmer, Waltham, MA) after 10-fold dilution of the reaction samples in phosphate-buffered saline (PBS). The cell-free synthesized proteins were also analyzed by electrophoresis on a 16% Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel, following previously described protocols [15]. Total proteins were stained with Coomassie Brilliant Blue, and the bands of L-DOPA-containing proteins were visualized using nitrobluetetrazolium (NBT) staining after being transferred to a PVDF membrane [19]. To examine the excitation and emission wavelengths of sfGFP, 3 mL of protein samples (200 μg/mL in 10 mM PBS) were loaded and scanned in RF-6000 Spectrofluorophotometer (Shimadzu Corporation, Kyoto, Japan).

To quantify cell-free synthesized proteins, trichloroacetic acid (TCA)-insoluble [^{14}C]leucine radioactivities of the samples were determined as follows. 10 μL of samples were withdrawn after incubation of reaction mixtures, diluted in 0.1 mL of 0.1 M NaOH and left at room temperature for 1 h. Solutions were then spotted on Whatman 3MM filter discs and dried under a heat lamp. Dried filter discs were immersed in 5% TCA resting on ice for the precipitation of proteins. After 15 min, the TCA solution in the beaker was exchanged with a fresh solution. This step was repeated three times. Following the final precipitation step, the discs were washed twice with an excess volume of ethanol. After being dried under a heat lamp, radioactivities on the filter discs were measured with a liquid scintillation analyzer (Tri Carb 2810TR, Perkin Elmer, Waltham, MA, USA) [20].

Mass spectrometry (MS) analysis of UAA-incorporated proteins

The cell-free synthesized proteins were purified in a C4 high-performance liquid chromatography (HPLC) column (GE Healthcare, Pittsburgh, PA). The reaction samples were injected directly into the column and eluted with a mobile phase gradient of 0–70% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) at 1 mL/min. Ultraviolet (UV) absorbance was monitored at 214 nm. Purified proteins were dried in a vacuum concentrator and then dissolved in an aqueous solution of 30% acetonitrile and 0.1% formic acid. The electrospray ionization (ESI) source was coupled to a hybrid quadrupole orthogonal time-of-flight mass spectrometer (SYNAPT G2, Waters, Manchester, UK) and was used to acquire mass spectra in both positive- and negative-ion modes. A capillary voltage of 3.0 kV, a cone voltage of 30 V, and a capillary temperature of 120 °C were applied for both polarities. Desolvation parameters were as follows: desolvation temperature, 600 °C; and desolvation gas flow, 800 L/h. Data were acquired over the mass range from m/z 50–1200 for MS modes. The sample was introduced into the ESI source at a constant flow rate of 20 μL/min using an external syringe pump (Harvard 11 Plus, Instech, Holliston, MA).

Results and discussion

Incorporation of L-DOPA during cell-free protein synthesis

As a model for UAA incorporation, we first attempted to decode tyrosine codons of sfGFP with L-DOPA. L-DOPA can confer unique properties to proteins depending on the oxidation state of its catechol moiety. For example, incorporation of L-DOPA has been investigated in the contexts of protein–protein interactions [21] and the conjugation of biomolecules [19]. In addition, the metal ion-chelating property of L-DOPA has been utilized to develop metal-detecting biosensors [22]. In the initial experiments to substitute tyrosine residues with L-DOPA, sfGFP was expressed in a standard reaction mixture for cell-free protein synthesis where tyrosine had been replaced with the same concentration (1 mM) of L-DOPA. When the fluorescence of the reaction mixture was measured after a 3-h incubation (488 nm excitation, 532 nm emission), substantially reduced sfGFP fluorescence was observed compared with the tyrosine-containing control reaction (Fig. 1a). However, the results of SDS-PAGE analysis showed that the yields of the total and soluble fraction of L-DOPA-containing sfGFP were comparable to those of the control reaction containing tyrosine (Fig. 1b). Decreased fluorescence of sfGFP was attributed to the