Bioorganic & Medicinal Chemistry Letters 20 (2010) 5613–5616

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

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

Condensed *E. coli* cultures for highly efficient production of proteins containing unnatural amino acids

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ARTICLE INFO

Article history:
Received 3 July 2010
Revised 7 August 2010
Accepted 10 August 2010
Available online 13 August 2010

Keywords:
Unnatural amino acid
tRNA
Ubiquitin
Aminoacyl-tRNA synthetase

ABSTRACT

Current biosynthetic methods for producing proteins containing site-specifically incorporated unnatural amino acids are inefficient because the majority of the amino acid goes unused. Here we present a universal approach to improve the efficiency of such processes using condensed *Escherichia coli* cultures.

In vivo unnatural amino acid mutagenesis is now an increasingly common technique used to study protein structure and function. Among the many uses are controlling protein function with light,[1–5] trapping transient protein–protein interactions,[6,7] or providing chemically reactive 'handles' for the attachment of fluorescent dyes or polymers.[8,9] While some of the amino acids that have been described in the literature are commercially available, many must be prepared by the user through multi-step chemical syntheses. This can complicate or prevent the use of UAAs in laboratories not equipped for synthetic chemistry or at the very least make synthetic UAAs precious materials. Indeed current protocols for in vivo UAA mutagenesis are inefficient in that an extremely small molar equivalent of amino acid is used in the synthesis of protein. Typically millimolar concentrations of UAA are added to protein expression media to produce micromolar or nanomolar yields of protein. In other words, >99% of the synthetic UAA goes unused and is discarded with spent media. Moreover, it is common for protein translation yields using amber codon suppression technology to be significantly lower than that of a wild-type protein.[10] Thus any methods that can improve the efficiency of UAA use will be valuable to protein scientists.

In the course of working with synthetic amino acids we were interested if the waste of material used during protein expression could be minimized. Unfortunately the UAA concentration in the media must be at least 1 mM to ensure optimum cell uptake and substrate selection by engineered aminoacyl-tRNA synthetases. The only alternative was to examine if *Escherichia coli* cultures could be condensed to dramatically smaller volumes than would be typical for protein expression. Such strategies have been used previously to minimize the cost associated with isotopic labeling of protein samples for nuclear magnetic resonance (NMR) studies.[11,12] Provided a robust purification protocol is available, one can generate large quantities of cell mass from small volumes of labeled media.

We first chose to conduct protein expression studies using N^{ε}-benzyloxycarbonyl-L-lysine (Z-lysine). This protected lysine analog has recently been added to the genetic codes of *E. coli* and mammalian cells using an evolved pyrrolysyl-tRNA synthetase/$tRNA_{CUA}$ pair from *Methanosarcina* species.[13,14] As a protein target we expressed human ubiquitin (Ub) containing a TAG stop codon mutation in place of the codon for Lys 48. Importantly Ub is a small (8 kDa), stable protein that can be easily distinguished from endogenous *E. coli* proteins in a crude lysate. We performed expressions in a typical rich media format but just prior to induction the cells from an appropriate portion of a larger culture were harvested by centrifugation and then resuspended in a smaller volume (2 mL) of induction media containing 2 mM Z-lysine. For example, to obtain a 50-fold condensation ratio, cells from 100 mL of log-phase culture ($OD_{600} = 0.8$) are resuspended in 2 mL resulting in a final $OD_{600} = 40$. After expression these cultures were diluted back to the original cell density to allow for equal comparison of all conditions. These samples were then analyzed for protein production using SDS–PAGE of the total cellular protein content. As

Abbreviations: UAA, unnatural amino acid; Ub, ubiquitin; sfGFP, superfolder green fluorescent protein.

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doi:10.1016/j.bmcl.2010.08.049

an internal standard and for quantification purposes we added 10 µg of bovine serum albumin (BSA) to each protein sample.

As can be seen in Figure 1, we surveyed a range of condensation ratios from 1×, or normal cell density (OD_{600} = 0.8), to ratios of 100×. In the case of Ub, clear protein expression can be seen in all samples except 100× at which point the condensation experiment reaches a point of diminishing returns. This indicates that UAA can be added to cells at the time of induction and be utilized for protein expression at such high cell densities. The lack of protein production in the absence of UAA indicates that the fidelity of amber codon suppression and incorporation is not affected by changes to culture density. We performed these analyses in triplicate and used the presence of a BSA internal standard to quantify protein yields and the relative efficiency of UAA usage under each scenario (Table 1 and Supplementary data). Using Ub_K48_Z-lysine as an example, we observed that the optimum protein production occurs when cultures are condensed by a factor of 25. These conditions generated 1.8 mg of target protein from a 2 mL expression and represent a significant improvement in yield. Importantly, ~93% less UAA is needed. When expression cultures are not condensed (1×), it is evident from the cell density and the observed protein quantities that there is some continued growth after IPTG induction but little overall increase in protein produced per amino acid.

To determine if this methodology is general we next decided to use a different system for UAA mutagenesis coupled with an alternative protein target. We used the *Methanococcus jannaschii* system developed for genetic encoding of *p*-benzoylphenylalanine (pBpa),[7] to perform mutagenesis on superfolder green fluorescent protein (sfGFP).[15] We repeated the condensation experiments using a V150TAG mutant of sfGFP and analyzed triplicate expression experiments by SDS–PAGE (Supplementary data). We observed a similar trend in the protein yields albeit with lower overall production (Table 1). There was very little protein observed by SDS–PAGE in condensation experiments above 10×. Nevertheless these conditions resulted in a reduced consumption of UAA

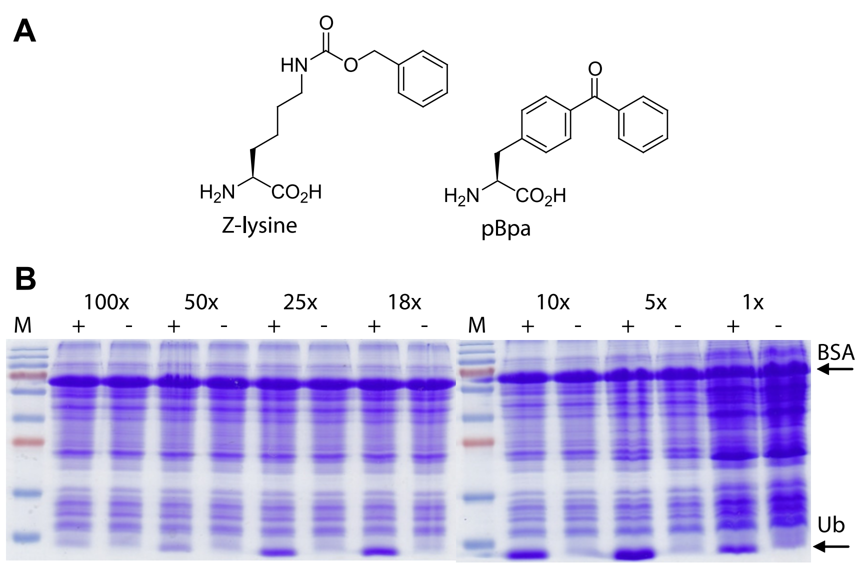

Figure 1. (A) Structures of Z-lysine and pBpa. (B) Protein production of Ub (K48_TAG) in the presence (+) or absence (−) of 2 mM Z-lysine. Total cellular protein observed by Coomassie-stained SDS–PAGE analysis.

Table 1

Condensation ratio	Culture loaded (µL)	Protein on gel[b] (µg)	Total protein produced[c] (µg)	Total UAA used (µmol)	Protein produced/UAA used (nmol/µmol)
Ub_K48_Z-Lys					
1×	50	3.6 ± 0.7	140	4	4.1
5×	20	7.4 ± 1.8	740	4	22
10×	10	6.3 ± 1.7	1300	4	38
18×	5.6	4.0 ± 0.7	1400	4	41
25×	4.0	3.6 ± 0.5	1800	4	53
50×	2.0	1.3 ± 0.3	1300	4	38
100×	1.0	ND	ND	4	ND
sfGFP_V150_pBpa					
1×	25[a]	2.1 ± 0.2	170	4	1.6
5×	20	3.8 ± 0.5	380	4	3.5
10×	10	2.5 ± 0.5	500	4	4.6
18×	5.6	0.7 ± 0.1	250	4	2.3
25×	4.0	ND	ND	4	ND
50×	2.0	ND	ND	4	ND
100×	1.0	ND	ND	4	ND

[a] Because this culture grew substantially a smaller sample was loaded. This is considered in the calculations.
[b] Amount of protein on gel was calculated using ImageJ software and normalized to BSA standard (10 µg). Average of three experiments ± standard deviation.
[c] Calculated total protein normalized to the full 2 mL expression.