Biochemical Engineering Journal 58–59 (2011) 25–32

Contents lists available at SciVerse ScienceDirect

Biochemical Engineering Journal

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

High-level production of uricase containing keto functional groups for site-specific PEGylation

Hai Chen, Yingfei Lu, Zhengzhi Fang, Jingxian Liu, Hong Tian, Xiangdong Gao**, Wenbing Yao*

State Key Laboratory of Natural Medicines, China Pharmaceutical University, Nanjing 210009, People's Republic of China

ARTICLE INFO

Article history:
Received 21 June 2011
Received in revised form 4 August 2011
Accepted 11 August 2011
Available online 10 September 2011

Keywords:
Orthogonal tRNA/aminoacyl-tRNA synthetase
Optimization
Fermentation
Enzyme production
Site-specific PEGylation
Recombinant DNA

ABSTRACT

We describe an *E. coli*-based optimized system for the production of uricase with keto functional groups incorporated efficiently and site-specifically. In the process, the orthogonal suppressor tRNA/aminoacyl-tRNA synthetase (aaRS) pair specific for *p*-acetylphenylalanine (*p*AcF) was optimized to be effective at *p*AcF incorporation, showing no toxicity to the host cells. The efficiency of *p*AcF incorporation was further improved by coupling five copies of the T-stem mutant suppressor tRNA gene omitted the 3′ terminal CCA with two constitutive copies of the D286R mutant aaRS gene in a single-plasmid construct. To assay the utility of the optimized system, we incorporated *p*AcF in response to three independent amber nonsense codons (Lys21TAG, Phe170TAG, Lys248TAG) into uricase. Under optimized expression conditions, 24 mg/L mutant uricase was produced, corresponding to 40% of the yield of wild-type uricase (UOXWT). The desired specificity for incorporation of *p*AcF into uricase was confirmed. Kinetic measurements and spectroscopic study performed by CD did not show any relevant differences in the substrate affinity, the catalytic activity and protein secondary structure between native and mutant uricase. Additionally, the mutant uricase was site-specifically modified with methoxy-PEG-oxyamine ($mPEG_{5K}$-ONH_2). This efficient system provides reactive handles for a rational PEGylation to manipulate uricase structure and function and will be beneficial for enhancing the incorporation of other unnatural amino acids into proteins.

1. Introduction

Uricase (EC 1.7.3.3) is an enzyme involved in purine metabolism and is responsible for the oxidation of uric acid to allantoin, which is more soluble and more readily excreted in urine than uric acid [1]. Since this enzyme can effectively lower serum uric acid levels, it is an attractive alternative for the treatment of gout and hyperuricemia [2]. The recombinant *Aspergillus flavus* uricase, Rasburicase (Elitek®, Sanofi-Aventis), is effective to prevent acute tumor lysis syndrome [3]. However, uricase is not naturally present in humans [4]. Being a heterologous protein, it is associated with immunogenicity, which has limited its tolerability and efficacy as a therapeutic agent [5].

PEGylation is a successful strategy for enhancing the biochemical and biopharmaceutical properties of proteins through the covalent attachment of polyethylene glycol chains [6]. Uricase from *Bacillus fastidiosus* [7], *Arthrobacter protoformiae* [8], *Candida utilis* [9] and mammals [10] has been PEGylated to diminish its immunogenicity and improve the half life and patient compliance. However, problems in controlling the conjugation chemistry above frequently arise because more than one residue of the targeted amino acid in native uricase is surface-accessible and the location of reactive groups on protein is random. Thus, the stoichiometry of the uricase–PEG conjugate and the attachment sites of the PEG to the uricase cannot be precisely controlled [11]. The lack of selectivity and positional control in the attachment of PEG chains can lead to significant losses in biological activity and the generation of a heterogeneous mixture that is difficult to separate [12].

A particularly attractive method of circumventing issues related to natural amino acids mediated protein bioconjugation is the use of unnatural amino acids [13]. Schultz and co-workers have developed a technology for truly site-specific incorporation of unnatural amino acids by reengineering protein synthesis [14]. In this method, an orthogonal tRNA/mutant tRNA synthetase pair is exploited to introduce an unnatural amino acid at a desired location. Using this strategy, an impressive array of amino acid derivatives bearing various reactive groups have been site-specifically incorporated into protein targets, which can be subsequently modified via click chemistry [15,16]. The keto group has chemical reactivity orthogonal to the common 20

* Corresponding author. Tel.: +86 25 83271218; fax: +86 25 83302827.
** Corresponding author. Tel.: +86 25 83271543; fax: +86 25 83271249.
E-mail addresses: xiangdong_gao@yahoo.com.cn (X. Gao), wbyao@cpu.edu.cn (W. Yao).

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doi:10.1016/j.bej.2011.08.006

amino acids and can be chemoselectively ligated with hydrazide, hydroxylamino, and thiosemicarbazide groups under physiological conditions [17]. Previously, Wang et al. had successfully introduced the keto amino acid into proteins and had used it to modify a protein selectively with a small molecule fluorophore and biotin hydrazide [18]. The keto group therefore can provide a reactive handle for the site-specific attachment of a single PEG molecule to a protein. The combination of unnatural amino acid mutagenesis and PEGylation chemistry provides an exciting tool for site-specific derivatization of proteins for improving their biochemical and biopharmaceutical properties.

Recently, we evolved a *Methanocaldococcus jannaschii* (*Mj*) suppressor tRNA/aaRS pair that selectively incorporates *p*AcF into uricase at prescribed locations in *E. coli* [19]. In that system, the suppressor tRNA and aaRS genes were located on two plasmids and did not function optimally with the *E. coli* translational machinery, leading to the low expression level of mutant uricase that could only be analyzed by Western blot analysis. Here, an optimized single-plasmid system with five copies of the T-stem mutant suppressor tRNA gene omitted the 3′ terminal CCA and two copies of the D286R mutant aaRS gene for incorporation of *p*AcF into uricase was reported, which produced acceptable efficiency of *p*AcF incorporation at the amber codon while providing high yield of mutant uricase with full enzymatic activity. What is more, the keto groups present on the mutant uricase were selectively conjugated with $mPEG_{5K}$-ONH_2.

2. Materials and methods

2.1. Strains, plasmids and biochemical reagents

E. coli DH10B (Invitrogen, China) was used for cloning and amplifying plasmids. *E. coli* BL21(DE3) (Invitrogen) was employed for expression. Plasmids pACYC184, pAC-tRNA, pBR322, pBR-EORS, pBR-RS-UOX, and pET28a-UOX were stored in our laboratory. PrimerStar DNA polymerase and *LA Taq* DNA polymerase (Takara, China) were used for PCR. Restriction enzymes were purchased from Takara and antibiotics were purchased from Sangon Biotech (Shanghai) Co., Ltd (China). The *p*AcF was synthesized in our laboratory according to the method reported previously [18]. $mPEG_{5K}$-ONH_2 and methoxy-PEG-succinimidyl carboxymethyl ester ($mPEG_{5K}$-SCM) were obtained from Jenkem (Beijing) Co., Ltd (China). Primers were synthesized by Invitrogen Corporation Shanghai Representative Office (China) and their sequences are listed in Table S1.

2.2. Construction of the pACM series of vectors

The pACM vector was derived from the plasmid pACYC184 with a multiple cloning site replacing its original sequence between EcoRI and AvaI. The multiple cloning site sequence included the mutant *glnS* promotor (*glnS′*) and terminator [20]. Suppressor tRNAs under the control of the *lpp* promoter and *rrnC* terminator and the *proK* promoter and terminator were amplified from pAC-tRNA [21] using PCR with the primers lppF-lppR and proKF-proKR, respectively. Mutations on the T-stem of the suppressor tRNA with or without the CCA trinucleotide at its 3′ terminus (mutRNA-CCA and mutRNA) based on the Schultz research team reports were done using overlapping PCR with the primers proKF-tRNAR, tRNAFI-proKR and proKF-tRNAR, tRNAFII-proKR [22]. All the PCR fragments were then digested with enzymes BglII and AvaI and ligated into the pACM vector, generating pAC-*lpp*-tRNA, pAC-*proK*-tRNA, pAC-*proK*-mutRNA-CCA and pAC-*proK*-mutRNA (pAC-tRNA series of vectors). An *E. coli* codon usage optimized *p*AcFRS was amplified from plasmid pBR-EORS using PCR with the primers aaRSFI and aaRSRI. The PCR fragment was then digested with enzymes KpnI and SacI and ligated into the pAC-tRNA series of vectors under the control of the *glnS′* promotor and terminator, creating plasmids pAC-*lpp*-tRNA-*glnS′*-*p*AcFRS (pAC-a), pAC-*proK*-tRNA-*glnS′*-*p*AcFRS (pAC-b), pAC-*proK*-mutRNA-CCA-*glnS′*-*p*AcFRS (pAC-c) and pAC-*proK*-mutRNA-*glnS′*-*p*AcFRS (pAC-d). The PCR fragment amplified from pBR-EORS was also ligated into the pBAD vector to create pBAD-*p*AcFRS. The resulting plasmid was then used as template for a standard PCR amplification using the primers araBADF and araBADR. The PCR product, containing the entire arabinose operon, was then prepared by restriction digest and inserted by ligation between NdeI and SpeI sites in the pAC-*proK*-mutRNA vector. The result is pAC-*proK*-mutRNA-*araBAD*-*p*AcFRS (pAC-e) containing an arabinose inducible copy of the synthetase. To add the D286R mutation to the *p*AcFRS (*p*AcFRSD286R) [23], an overlapping PCR with the primers aaRSFI-aaRSRII and aaRSFII-aaRSRI was performed on pAC-d to produce pAC-*proK*-mutRNA-*glnS′*-*p*AcFRSD286R (pAC-f).

Tandem tRNA gene cassettes were constructed by isocaudamer BglII and BamHI. The mutRNA from plasmid pAC-f was digested with enzymes BglII and AvaI and ligated into pAC-f digested with enzymes BamHI and AvaI to afford pAC-2*proK*-mutRNA-*glnS′*-*p*AcFRSD286R, which consists of two identical tRNA operons, each encoding one tRNA gene under the control of the *proK* promoter and terminator. Using the same method as described above, plasmids pAC-3*proK*-mutRNA-*glnS′*-*p*AcFRSD286R (pAC-g) and pAC-5*proK*-mutRNA-*glnS′*-*p*AcFRSD286R (pAC-h) containing three and five identical tRNA operons were generated. To create the dual *glnS′* version of *p*AcFRSD286R, the *p*AcFRSD286R gene was amplified from pAC-f using PCR with the primers glnS′F and glnS′R, digested with NdeI and SpeI, and ligated into pAC-h vector to create pAC-5*proK*-mutRNA-2*glnS′*-*p*AcFRSD286R (pAC-*p*AcF). All the constructed plasmids were confirmed by sequencing.

2.3. Chloramphenicol acetyltransferase assay of the optimized suppressor tRNA/aaRS pair and its copy number

The gene for chloramphenicol acetyltransferase (CAT), with an amber codon in place of Asp^{112} [14], was cloned into pBR322 to create the plasmid pBR-CAT. The assay was carried out by transforming the pACM series of vectors into competent *E. coli* DH10B cells harboring the pBR-CAT plasmid. The transformed cells were picked and grown overnight at 37 °C in LB medium with ampicillin (Amp, 100 μg/mL) and tetracycline (Tet, 25 μg/mL). Cells from 1 mL of this culture were inoculated into 100 mL of minimal medium containing 1% glycerol and 0.3 mM leucine (GMML) [24]. Various concentrations of chloramphenicol (Cm) were added, and the Cm tolerance of *E. coli* DH10B was recorded in the presence and absence of 1 mM *p*AcF, respectively.

2.4. E. coli BL21(DE3) growth curve

pAC-*p*AcF was transformed into *E. coli* BL21(DE3). pACM was used as a control. One colony from each transformation was grown overnight to saturation at 37 °C in LB medium with Tet. The cells were inoculated into 100 mL of GMML at 37 °C. OD_{600} was monitored at 1 h intervals over a period of 16 h. Specific growth rates (μ) were determined when the cells appeared to be in maximum growth [25].

2.5. Expression and purification of mutant uricase

pAC-*p*AcF was cotransformed with pET24a-UOX mutants (Material S1) into *E. coli* BL21(DE3). As control, pET24a-UOXWT (Material S1) was similarly cotransformed into *E. coli* BL21(DE3) with pAC-*p*AcF, so cultures could be grown under identical