A Simple and Universal Method to Express Protein in Unfused form

Tianwen Wang[a], Aitao Li [b], Xingyuan Ma[b*], Guocheng Du[a] and Jian Chen[a]

[a]*State Key Lab of Food Science and Technology, School of Biotechnology, Jiangnan University, Wuxi 214122, Jiangsu, China;* [b]*School of Biotechnology, and Stat Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 214122, China*

Abstract: The effect of additional amino acid residue(s) fused to the N- and/or C-terminal on properties of the heterogeneously expressed protein is usually difficult to be predicted. Recombinant proteins expressed without any fused sequence should be the most desired materials for related studies, such as protein drug preparation, biochemistry investigations. Here, we report a very simple and universal method enabling the expression of protein in its unfused form between the same two restriction enzyme sites (at a higher level) if a plasmid can support the fused expression. The method provided an assessable solution for unfused expression without increase in experimental resource; the necessary material is an additional primer. The method is especially useful for making whole-cell biocatalyst in which no purification steps are required.

Keywords: Primer design, polymerase chain reaction, unfused expression, protein drug, whole-cell biocatalyst.

INTRODUCTION

Heterogeneous expression of a gene in a suitable host might be one of the most frequently involved activities in modern biology-related researches. The most convenient approach to this goal is amplifying the target gene through polymerase chain reaction (PCR) with suitable primers that are specific to the sequence, ligating between the amplified DNA fragments and plasmids with compatible ends due to the treatment with restriction enzymes whose recognition sequences have been incorporated into the primers. Since translation of an mRNA by a ribosome starts at the codon of ATG (specifying methionine), restriction enzymes whose recognition sequences containing ATG (*Nde* I and *Nco* I) are always the first one in the multiple cloning site of many available plasmid vectors. For the foreign genes in which the recognition sequences of these enzymes are present, it will be expressed as a fusion protein with additional sequences fused at their N- and/or C-terminals. The fused sequence is usually very short; and in some cases, the fused sequences are very profitable and desirable, because the fused sequences can: (1) facilitate the purification of the expressed protein (e.g. the 6 x his tag for Nicol-NTA based purification, Elastin with reversible phase transition property responding to temperature [1], and so on), (2) increase the stability of unstable proteins [2-4], increase the expression level [5] and even the enantioselectivity of an enzyme [6]. However, these examples also imply that the fused sequences will, more or less, affect the properties of the expressed proteins [7]. Therefore, it is necessary to express the foreign proteins in their unfused form, at least for a comparison purpose to determine the influence of the fused sequences in investigations related to biochemistry and cellular biology. In other cases, if a recombinant protein will be used as protein drug, the ideal form should be the unfused one.

There are several ways to express foreign proteins in their unfused forms: (1) cloning the foreign genes into the *Nde* I or *Nco* I if these restriction sites are usable; or using the enzymes that can produce ends compatible to that of *Nde* I or *Nco* I(e.g. *Bsp* HI, *Pci* I, *Fat* I). The use of this method is very limited, because it is strictly dependent on the absence of these sites in the target gene. (2) Utilization of Gate-way® technology to realize the cloning of foreign genes into site supporting unfused expression (http://www.invitrogen.com); (3) Sequence and ligation independent cloning (SLIC) methods circumventing the dependence on restriction enzymes [8]. (4) Excision of a fused protein into its unfused form by utilizing suitable vectors [9,10]. Unfortunately, the wide application of these methods is sometimes limited mainly due to the availability of experimental materials.

Here, we will introduce a different method which is very simple, universal, and highly accessible. It is a PCR-based method depending on a new pair of primer (an additional forward primer and the original reverse primer for fused expression) to realize the unfused expression. The method was exemplified with the unfused expression of an alcohol dehydrogenase (ADH) from an octane-degrading strain *Pseudomonas putida*.

MATERIALS AND METHODS

Chemicals and Reagents

Restriction enzymes (*Bam*H I, *Hin*d III) and dNTP mixture were purchased from New England Biolabs (USA). Phusion® high-fidelity polymerase was a product of Thermo Scientific (USA). Oligos as primers in PCR were synthesized from First Base Company. Kanamycin and isopropyl β-D-1-thiogalactopyranoside (IPTG) were from Sigma-

*Address correspondence to this author at the School of Biotechnology, and Stat Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, Shanghai 214122, China;
Tel:/Fax: +86 2164252257; E-mail: maxy@ecust.edu.cn

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Aldrich (USA). The substrate for enzyme activity assay was 1-(4-methylphenyl)-1,2-ethanediol from Spectra Group (USA). Other routine chemicals are commercially available products of analytical grade.

Strains, Plasmids, and Culture Media

Escherichia coli strains DH5α™ (Invitrogen, USA) and T7™ (New England Biolabs, USA) were used as the host for cloning and expression. Plasmid pET28a(+) (Novagen, Germany) was used as expression vector. The plasmid used for cloning and expression in this research was from Novagen. For plasmid propagation, the LB medium was used (peptone 10 g/L, yeast extract 5g/L and NaCl 5g/L), while for expression, the medium was a richer one (1%, V/V) (glycerol, 15g/L, peptone 15g/L, yeast extract 4g/L, NaCl 2g/L, KH_2PO_4 58Mm, $MgSO_4$ 2mM, pH6.0).

Molecular Cloning and Expression

DNA manipulations and transformations were carried out according to standard procedures [11]. Briefly, the gene of an alcohol dehydrogenase (accession no.: AJ245436) was amplified by PCR with the following primers (FusedF: 5'-cgcGGATCCatgtacgactatataatcgttggtg-3'; FusedR: 5'-cgcGTCGACttacatgcagacagctatcatggc-3'; the underlined part was the recognition site for *Bam*H I and *Hin*d III.) The template was the DNA sample extracted with a plasmid miniprep kit from Qiagen (USA). The thermocycling parameters were as follows: 98°C 3min, 98°C 10sec, 60°C 15sec, 72°C 1min, 30 cycles. A final extension step for 5min was added to ensure the completeness of the PCR products. After digestion of the PCR products and the pET28a (+), ligation with Quick Ligation® kit from NEB, transformation and identification with colony PCR, the desired recombinants were identified and confirmed with commercial DNA sequencing. The recombinant plasmid was transformed into *E. coli* T7™ electro-competent cells.

Construction of the recombinant plasmid that could be used for unfused expression of ADH was achieved by amplifying the gene with another newly synthesized primer UnfusedF (5'-cgcGGATCCTaaTaaAAGGAGatataatgtacgac tatataatcgttggt-3', the underlined part was the recognition site for *Bam*H I) and the previous FusedR (see above). The amplification and the subsequent steps were the same as described previously.

The recombinant strain was cultured in 2mL autoclaved LB at 37°C with shaking 250rpm overnight, and then inoculated into 50mL expression medium. When optical density reached 0.7, IPTG was added to an initial concentration of 0.5mM. The cultivation lasted for another 6hrs at 30°C. Kanamycin was added at a concentration of 50 μg mL^{-1} when the strain contained the empty or recombinant plasmid.

Analytical Methods

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to check the expression of the enzyme (12.5%). Enzymatic activity assay was carried out with the substrate 1-(4-methylphenyl)-1,2-ethanediol. The concentration was determined with reverse phase HPLC (Shimadzu Prominence) equipped with Poroshell 120 EC-C18 column (2.7μm, 4.6X150mm, Agilent, USA). The substrate had a retention time of 5.7min when washed with acetonitrile-water (4:6) at a flow rate of 0.4ml/min (25°C). One unit was defined as the amount of enzyme causing decrease of 1μmol substrate.

RESULTS AND DISCUSSION

The Design of the Primer for Unfused Expression of ADH

The structure of the forward primer used in subcloning ADH for unfused expression was shown in (Fig. **1**), and the map of the recombinant plasmid was shown in (Fig. **2**). At

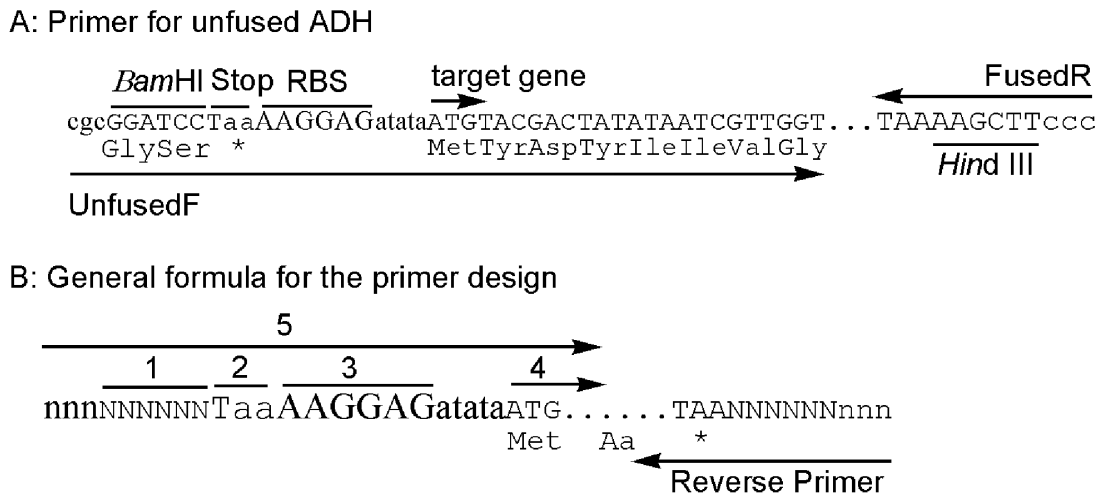

Figure 1. The structure of the primer for unfused expression

A: The primer for unfused ADH

The primer (UnfusedF) was 50bp, including 3bp as protective bases for high efficiency digestion (cgc), the *Bam*HI site (GGATCC), a stop codon (taa), the RBS (aaggaga) from pET series plasmid, 25bp identical to the encoding sequence of ADH. The combined use of unfusedF and FusedR in PCR amplification and cloning into pET28a(+), supporting the expression of the ADH as an unfused protein.

B: The general formula for the primer design in forced termination of translation. The primer(5) contains a suitable restriction enzyme recognition site (1), a stop codon(2), RBS(3), sequence specific to the target gene(4).

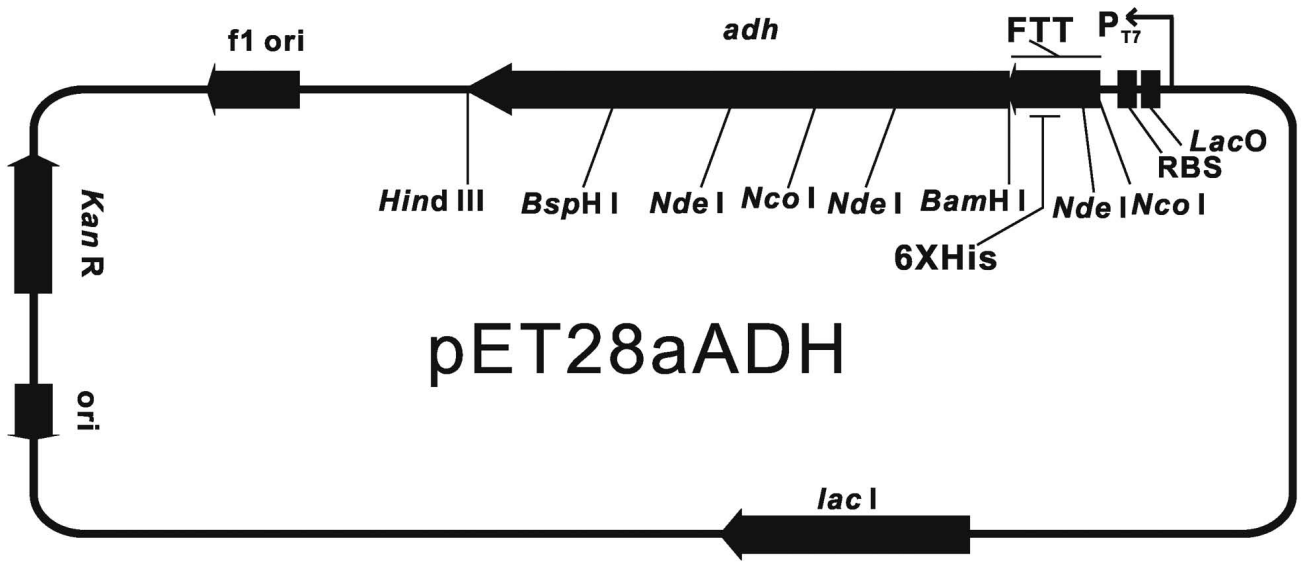

Figure 2. The map of the recombinant plasmid expressing unfused ADH
The recombinant plasmid for unfused ADH expression was constructed by inserting the PCR product amplified from primers unfusedF and fusedR between the *Bam* HI and *Hin*d III site, which was previously used for fused expression of the ADH. Forced translation termination enabled the ADH was expressed as a protein without any additional sequences to its both ends.

its 3'-end, the sequence was identical to the sequence of the ADH. It was responsible for the binding onto the templates for successful amplification in PCR. At its 5'-end, it was the *Bam*H I site which had been used for the cloning of the ADH. After this site, there was the preferential stop codon (TAA) of *E.coli* leading to a forced termination of translation (FTT). It was notable that the stop codon added after the *Bam*H I site should be in-frame with the translation initiated by the binding of a ribosome at the ribosome binding site (RBS) located at the upstream of the multiple cloning sites. Adjacent to the stop codon, there was another RBS sequence, which could support an independent binding of another ribosome. The following sequence was from the frame of pET series plasmid vectors.

Here, the distance between the introduced stop codon TAA to the first codon of the ADH was 14 base pair. Theoretically, the forced termination of translation initiated at the site designed in pET28a (+) would not render the target gene untranslated, because the start codon in ADH could be recognized by the bound ribosome. Therefore, the main function of the newly introduced RBS sequence would be the independent recruitment of another ribosome, increasing the chance of being translated of an mRNA. It might be very important for foreign genes that were less efficiently expressed in *E.coli*. However, the translation of an mRNA was rather complicated. It could not be merely affected by the number of RBSs. That might be the reason why a doubled expression level was not observed in the SDS-PAGE (Fig. **3**). It was reported that the first 30~50 amino acid residues determining the rate of an mRNA being translated [12]; and for a gene, if a certain codon was adopted to specify an amino acid residue, it tended to use the same codon for the subsequent identical amino acid residues [13]. These discoveries could, to some extent, explain why the expression level was different when a protein was expressed as fused or unfused one.

When the ADH gene was amplified with FusedF and FusedR, the product could be cloned into between the *Bam* HI and *Hin*d III site. The sequence fused to the ADH protein was a small peptide with a length of 34 amino acid residues (MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGS), leading to a 3.5kDa difference in molecular weight of the fused and unfused ADH (Fig. **3**).

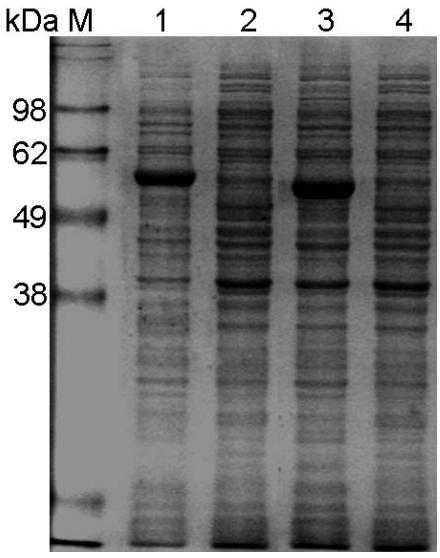

Figure 3. SDS-PAGE analysis of the fused and unfused ADH
The difference of molecular weight between the unfused and fused ADH was calculated as 3.5kDa. As shown in the figure, the unfused ran faster than the fused one did.
Lane M: protein marker, Seeblue^{TM} plus 2 from Invitrogen.
Lane 1: lysate of whole cell expressing unfused ADH
Lane 2: control for unfused ADH
Lane 3: lysate of whole cell expressing fused ADH
Lane 4: control for fused ADH

Table 1. Comparison of Performance of Fused and Unfused ADH in Biotransformation

Whole Cell Biocatalyst	Cell conc. (dcw, g/L)	Sub. Conc. (mM)	Conv. rate (%)		SA[a] (U/g.dcw)	ee (%)
Fused ADH	2	2	48.86	57.59[b]	16.28	>99
Unfused ADH	2	10	38.21	57.93[c]	63.69	>99

SA: specific activity
a: The data of conversion rate and specific activity were based on the analysis of samples taken at 30min.
b & c: the data were based on the analysis of samples taken at 13h and 12h when the *ee* reached >99%.

Activity Comparison

A comparison of the specific activities of the fused and unfused ADH was carried out with the diol as substrate (Table **1**). Results showed that when the ADH was expressed as an unfused protein, its specific activity on the diol was increased by about 4 folds. Their behaviors in the kinetic resolution of the substrate were also compared. The same enantiomeric excess value (99%) could be reached in a comparatively shorter time (12hrs), even when the ratio of substrate to catalyst (mM substrate: g/L catalyst) was higher. These data clearly showed that the unfused ADH was more active than its fused counterpart.

CONCLUSION

A very simple and effective PCR-based method to express protein in unfused form was developed, making it possible to express a recombinant protein in a form without any additional sequence attached to its N- and/or C-terminal. Except for a piece of newly synthesized oligo, there were no requirements for other new experimental resources (such as plasmids, and other molecular reagents). The method has been tested with the successful subcloning of an ADH. However, the method reported here could be universally used to express a protein in its unfused form when such a protein was highly expected. It was of great importance for experiments, such as biochemistry and cell biological investigations, protein drug preparation, in which the ideal protein material should be the unfused one. It is equally useful in biocatalysis in which whole cells expressing the desired enzymes are directly used as the biocatalyst, as demonstrated here.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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