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Ribosome display for selection of active dihydrofolate reductase mutants using immobilized methotrexate on agarose beads

Fumio Takahashi[a], Takashi Ebihara[a], Masayasu Mie[a], Yasuko Yanagida[a], Yaeta Endo[b], Eiry Kobatake[a], Masuo Aizawa[a,*]

[a] *Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta, Midoriku, Yokohama 226-8501, Japan*

[b] *Department of Applied Chemistry, Faculty of Engineering, Ehime University, Matsuyama 790-8577, Japan*

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Abstract Ribosome display was applied to the selection of an enzyme. As a model, we selected and amplified the dihydrofolate reductase (DHFR) gene by ribosome display utilizing a wheat germ cell-free protein synthesis system based on binding affinity to its substrate analog, methotrexate, immobilized on agarose beads. After three rounds of selection, the DHFR gene could be effectively selected and preferentially amplified from a small proportion in a mixture also containing competitive genes. Active enzymes were expressed and amplified and by sequence analysis, four mutants of DHFR were identified. These mutants showed as much activity as the wild-type enzyme.

Key words: Ribosome display; Dihydrofolate reductase; In vitro protein synthesis; Active enzyme mutant

1. Introduction

Phage display [1,2] has been a widely available method to obtain proteins that have binding affinity to target molecules. Recently, selections of proteins that show catalytic activity by this method were also performed [3,4]. Thus, creation of novel proteins with various functions by evolutionary molecular engineering has been desired. However, in phage display, some problems have been indicated, such as complicated manipulations by alternation of in vitro and in vivo, difficulty of expression of toxic proteins for host bacteria, limitation of DNA fragment size enclosable in phage particle, and limitation of the available amount of libraries. Therefore, to avoid these problems, development of consistent in vitro systems using the cell-free protein synthesis system has been desired and some methods have been proposed [5,6].

Ribosome display [7] is one of such in vitro methods based on a cell-free transcription and translation system. In this method, correspondence of genotype and phenotype is realized by formation of a polysome complex that consists of mRNA, ribosomes, and nascent polypeptides. This method has been developed as an efficient tool to obtain single chain antibodies that show binding affinity to immobilized target antigen [8], and so far, various antibodies with high affinity have been selected and evolved by this method from mouse [9] or human [10] libraries. However, some attempts to obtain other functional proteins by this method have been scarcely carried out [11]. Although selection and directed evolution of enzymes by an emulsion method was performed, selectable enzyme functions by this method are limited only to DNA methylase activity [12] and DNA polymerase activity [13].

Here, we attempted to select and evolve enzymes using its substrate analog by ribosome display. Although the selection of enzyme by this method was performed [14], creation of novel enzyme has not been carried out yet. We attempted to select dihydrofolate reductase (DHFR) using methotrexate (MTX) immobilized on agarose beads as a model. The scheme of this study is shown in Fig. 1. In the selection step, we used cycloheximide to stop the translation reaction and to form a stable and selectable polysome complex. Only the complex containing the DHFR gene could bind to MTX. Then, selected complexes were dissociated by elimination of magnesium ion with ethylenediamine tetraacetic acid to yield mRNA.

2. Materials and methods

2.1. Plasmid construction and preparation of mRNA

All restriction enzymes were purchased from TaKaRa. In this study, we used two kinds of mRNAs. To introduce a common sequence downstream of these mRNAs, synthetic oligonucleotides 5′-TTGGACTCTTGGCGGTCTGCAGGCATGCA-3′ and 5′-AGCTTGCATGCCTGCAGACCGCCAAGAGTCCAAATAA-3′ were annealed to each other and ligated into *Bst*XI- and *Hin*dIII-digested sites of pSP65-DHFR, which encodes a DHFR gene under the control of an SP6 promoter [15]. The resulting plasmid, pSP65II-DHFR, has a common annealing site of rear primer for PCR. The luciferase gene of *Renilla* (R Luc) was derived from pRL-CMV (Toyo Ink Co.) with *Nhe*I and *Cla*I digestion, and ligated into *Sma*I- and *Bst*XI-digested sites of pSP65II-DHFR replacing the DHFR gene to produce pSP65II-R Luc. pSP65II-DHFR and pSP65II-R Luc were linearized with *Hin*dIII digestion for termination following in vitro transcription. MEGAscript SP6 (Ambion) was used for in vitro transcription of DHFR and R Luc genes. The reaction mixture was containing $m^7G(5')ppp(5')G$ (Ambion) to produce capped mRNA.

2.2. Selection of DHFR gene by ribosome display

The wheat germ extract system (Promega) was used for in vitro translation. The reaction mixture was 100 μl containing 50 μl wheat germ extract, 8 μl Complete Amino Acid Mixture (Promega), 60 mM potassium acetate, 2 μl RNase inhibitor (Promega), and 2 μg purified mRNA. After incubation for 8 min at 25°C, 900 μl ice-cold stop buffer (50 mM Tris–HOAc pH 7.5, 10 mM $MgCl_2$, 1 μg ml^{-1} cycloheximide) was added, and the mixture was centrifuged at 272 000 × *g* for 40 min at 4°C. Then, the pellets were suspended in 100 μl stop buffer and further centrifuged at 10 000 × *g* for 5 min at 4°C. After

*Corresponding author. Fax: (81)-45-924 5779.
E-mail address: maizawa@bio.titech.ac.jp (M. Aizawa).

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removing the insoluble component, 5 μl MTX-agarose (Sigma) beads was added to the supernatant and the mixture was gently rotated for 2 h at 4°C. After five times washing with 300 μl stop buffer, elution buffer (50 mM Tris–HOAc pH 7.5, 20 mM EDTA, 150 mM NaCl) was added to the beads, and the mixture was kept for 10 min at 4°C. The supernatant was purified by extraction with phenol/chloroform and then with chloroform, and RNA was precipitated with ethanol.

Purified RNA was reverse-transcribed to cDNA using Superscript II (Gibco BRL). Obtained cDNA was amplified by PCR using Pyrobest DNA Polymerase (TaKaRa), and the reaction was for 5 min at 98°C, followed by 25 cycles of 10 s at 98°C, 30 s at 65°C, 90 s at 72°C. Common primers for genes of DHFR and R Luc containing SP6 promoter sequence were SP6D-2 (5′-GATTTAGGTGACACTATAGAATACACGGAATTCGAGCTCGCCC-3′), and rear primer was DHFRR-1 (5′-TGCATGCCTGCAGACCGCCAAGAGT-3′). Amplified cDNA was then purified with Wizard PCR Preps DNA Purification System (Promega). mRNA synthesis for the next selection was performed in the same manipulation with the first preparation of mRNA using DNA fragment amplified by PCR instead of linearized plasmid. Synthesized mRNA was purified by agarose gel electrophoresis before next translation and selection.

2.3. DHFR activity assay using selected DNA pool

Amplified DNA was transcribed to mRNA, and translated in the same conditions for 2 h without purification by electrophoresis. DHFR activity was determined by measuring the successive decrease of absorbance of NADPH at 340 nm in a DHFR enzyme reaction at 30°C. The reaction mixture contained 50 mM potassium phosphate pH 7.0, 60 μM NADPH, 12 mM 2-mercaptoethanol, 200 μM dihydrofolic acid. The reaction volume was 3 ml containing 30 μl of translation mixture.

2.4. Sequencing of amplified DHFR gene

After three rounds of selection, the obtained DHFR gene was digested with *Sac*I and *Pst*I, and then ligated into the similarly digested sites of pBluescript II. After transformation of the *E. coli* JM109 strain, cloned plasmids were purified and sequenced with a DNA analysis system (Beckman; CEQ2000XL).

2.5. In vitro synthesis and purification of DHFR enzyme

Identified DHFR mutant genes were introduced to pEU-DHFR, a plasmid for in vitro expression of DHFR (unpublished). The region between *Van*91I and *Psh*AI restriction sites in pEU-DHFR was substituted with the corresponding mutational region in each DHFR mutant gene. In vitro transcription and in vitro translation using these plasmids were performed according to previously reported methods [16], and synthesized DHFR mutants were purified with an affinity column filled with MTX-agarose. The reaction mixtures were applied into the column and synthesized DHFR was bound to MTX. Then, unbound and non-specifically bound components were washed out by loading the column buffer (50 mM Tris–acetate pH 8.2) and the washing buffer (50 mM Tris–acetate pH 8.2, 1000 mM potassium chloride) into the column, respectively. Finally, specifically bound DHFR proteins to MTX-agarose beads were collected with the elution buffer (50 mM Tris–acetate pH 9.3, 4 mg ml^{-1} dihydrofolic acid).

2.6. Activity assay of DHFR mutants

Activity of purified DHFR mutants was analyzed by successive increase of absorbance of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma) at 560 nm in a DHFR enzyme reaction. The reaction was performed in the conditions of 50 mM Tris–HCl pH 7.5, 200 μM dihydrofolic acid, 200 μM NADPH, 0.1 mg ml^{-1} MTT, 400 ng ml^{-1} enzyme at 24°C.

3. Results

3.1. Selection of DHFR gene with MTX-agarose

First, we confirmed whether DHFR gene could be selected with MTX-agarose. Only DHFR mRNA was translated in vitro with the wheat germ extract protein synthesis system. After selection with MTX-agarose, collected RNA was amplified by reverse transcription (RT)-PCR and analyzed by agarose gel electrophoresis (Fig. 2). When an equal volume of H_2O was added to translation reaction mixture instead of

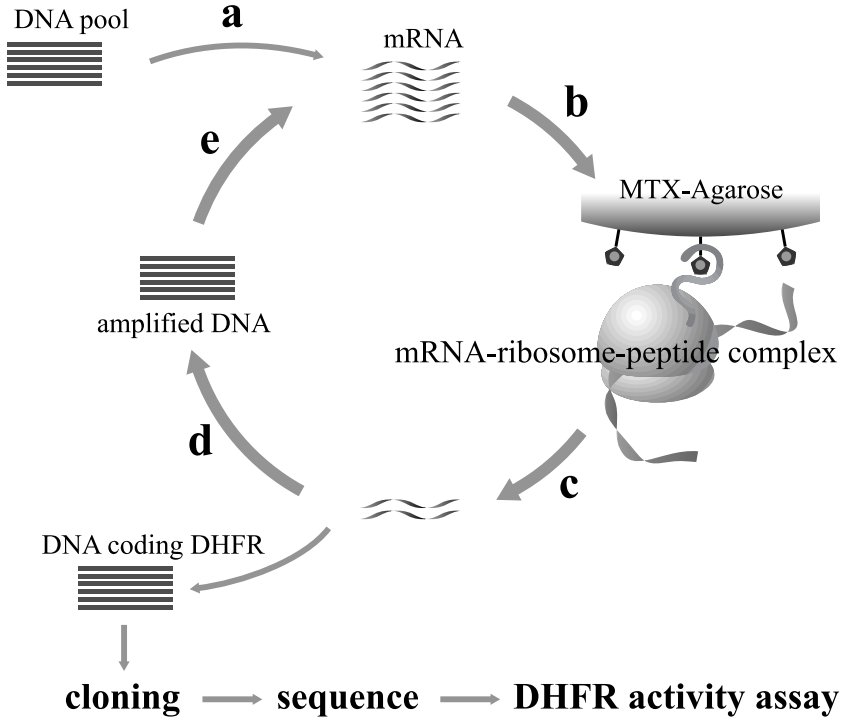

Fig. 1. Scheme of ribosome display system to obtain active DHFR enzyme. a: DHFR gene was mixed with competitive gene in various ratios as a DNA pool, and then transcribed in vitro. b: Yielded mRNA was translated in vitro with wheat germ cell-free protein synthesis system. Only the polysome complexes displaying DHFR enzyme were trapped by MTX-agarose. c: Selected complexes were dissociated to collect mRNA. d: Collected mRNA was amplified with RT-PCR. e: Yielded DNA was transcribed for the next selection. After three rounds of selection, amplified DHFR genes were cloned and sequenced, and specific activities of identified DHFR mutants were assayed.

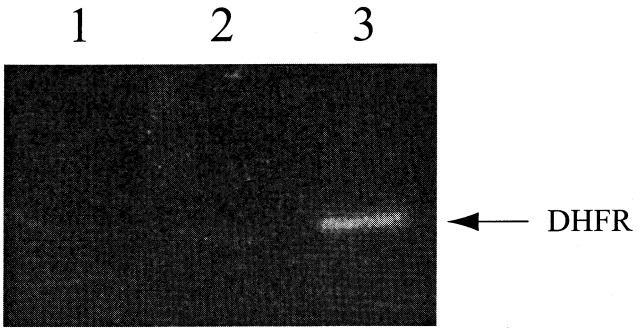

Fig. 2. Selection and amplification of DHFR gene with MTX-agarose. Lane 1: equal volume of H_2O was added to translation reaction mixture instead of mRNA and translated for 5 min; lane 2: mRNA of DHFR was translated for 0 min; lane 3: mRNA of DHFR was translated for 5 min.

DHFR mRNA, no band was observed at the expected size as DHFR gene. When translation mixture containing mRNA was not incubated, a faint band was observed. This shows that no DHFR mRNA was adsorbed on MTX-agarose. In contrast with these samples, a clear band was observed when DHFR mRNA was translated. This result shows that the DHFR gene could be selected by interaction between DHFR proteins displayed on ribosomes and MTX.

3.2. Preferential amplification of DHFR gene

We attempted to select and amplify a small amount of DHFR gene preferentially in the presence of competitive gene, R Luc. mRNAs of DHFR and R Luc were mixed with various mixture ratios. Total amounts of mRNA were the same in all samples. Each mRNA mixture was treated according to the described methods. We repeated the selection for three rounds. After each round of selection, PCR-amplified DNA samples were analyzed by agarose gel electrophoresis (Fig. 3). After the first selection, a faint band of DHFR amplified from 10^{-3} dilution was detected. Repeating selection rounds to the third selection, DHFR gene was further amplified and a band of DHFR amplified from 10^{-4} dilution could be observed.

3.3. Amplification of DHFR activity

After three rounds of selection, yielded DNA samples were transcribed and translated in vitro, and their DHFR activities were measured to confirm that genes of active DHFR were amplified (Fig. 4). In samples whose mixture ratios of DHFR to R Luc before selection were 10^{-1}, 10^{-2}, and 10^{-3}, DHFR activities were amplified through three rounds of selection. However, in sample 4, DHFR activity was not detected even after selection. Although relative DHFR activity does not represent the existence ratio of DHFR gene actually, we can understand that the gene of active DHFR was surely amplified.

Table 1
Sequence analysis of amplified DHFR gene

Kind of mutation	Mutation
Group I	
Substitution of amino acid residue	$Arg^{98} \rightarrow His$
	$His^{124} \rightarrow Tyr$
	$Trp^{30} \rightarrow Cys$, $Met^{92} \rightarrow Ile$
	$Glu^{120} \rightarrow Val$
Group II	
Frame shift	downstream of Asn^{34}
	downstream of Ala^{107}
Group III	
Appearance of stop codon	$Pro^{21} \rightarrow Thr$, $Gly^{56} \rightarrow stop$
	$Trp^{133} \rightarrow stop$
	$Trp^{22} \rightarrow stop$

After three rounds of selection, amplified DNA from 10^{-3} ratio was cloned and sequenced. Identified nine DHFR mutants of 48 analyzed DHFR clones were divided according to kinds of mutation and listed.

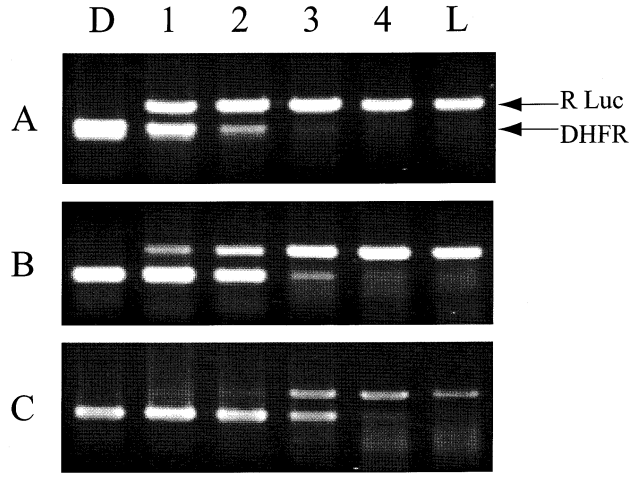

Fig. 3. Preferential amplification of DHFR gene from a mixture with competitive R Luc gene by three rounds of selection. In lanes 1–4, the ratios of DHFR to R Luc before selection were 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}, respectively. Lane D and L are control samples consisting of only DHFR and R Luc. A–C show samples after the first, second, and third selection, respectively.

3.4. DHFR activities of obtained mutants

After three rounds of selection, sequences of amplified DHFR gene were analyzed (Table 1). In 48 analyzed DHFR clones, nine DHFR mutants were identified. Among these mutants, four clones were DHFR variants whose amino acid residues were substituted. Then, DHFR activities of four identified mutants were analyzed by assaying translation reaction mixtures using equal an amount of each DHFR variant mRNA (Fig. 5A). All of these mutants showed as much ac-

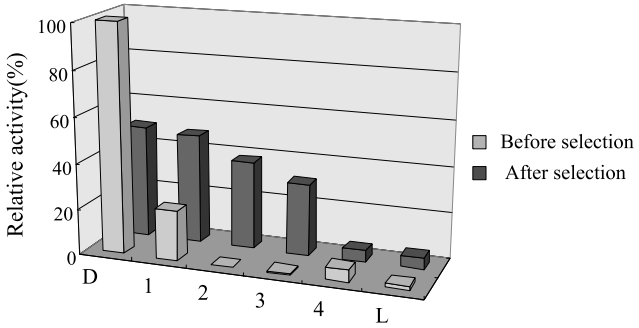

Fig. 4. DHFR activity assay. Amplified DNA samples were transcribed, and 1 μg of yielded mRNA was translated for 2 h. mRNA mixtures before selection were analyzed as controls. DHFR activity was evaluated by absorbance change of NADPH at 340 nm in DHFR enzyme reaction. DHFR activity of each sample was represented as relative value to 100% of sample D before selection. Each value is the average of three similar experiments. Sample numbers correspond with Fig. 3.

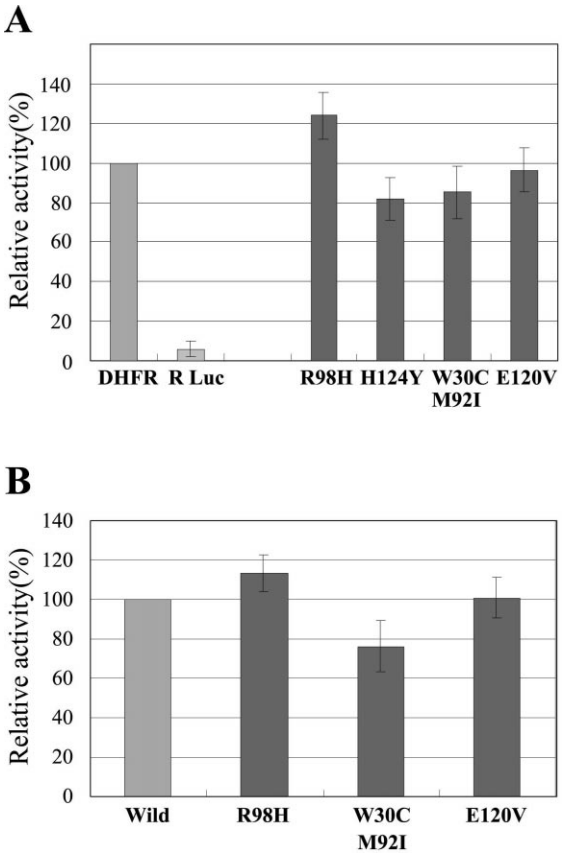

Fig. 5. DHFR activity assay of obtained DHFR variants. A: The genes of DHFR variants identified by sequence analysis were transcribed and translated in vitro, and DHFR activities of reaction mixtures were analyzed. As controls, genes of DHFR and R Luc that encode correct sequences were analyzed similarly. Each value is the average of five similar experiments and shows relative activity to wild-type DHFR. B: DHFR activities of purified mutants were analyzed.

tivity as wild-type DHFR enzyme. Then, we tried to analyze these mutants with purified enzyme. We succeeded to purify three of these mutants completely confirmed by SDS–PAGE (data not shown). Each mutant showed the same result (Fig. 5B).

4. Discussion

It is necessary for selection of genes to form a polysome complex, which retains the linkage of genotype and phenotype, consisting of mRNA, ribosomes and proteins. A long time translation probably should not contribute to efficiency of selection because complete DHFR proteins are released from ribosomes by termination of translation, and inhibit the binding of polysome to MTX competitively. Therefore, to optimize the translation reaction time, we investigated the selection with varied reaction time and analyzed intensities of DNA band. Although the selection efficiency gradually rose with the extension of translation time up to 8 min, improvement of selection efficiency was no longer observed at 10 min of translation time (data not shown). Therefore, we determined that optimal translation time was 8 min and it was applied for further experiments.

We succeeded to amplify the DHFR gene selectively from a small proportion by repeating the selection rounds. Although R Luc does not have binding affinity to MTX, R Luc gene was also amplified (Fig. 3). It was probably prone to adsorb non-specifically to MTX-agarose since the R Luc gene also formed a large polysome complex during the translation reaction. This result suggested a high background level in screening from library. However, it should be resolved by repeating selection rounds (Fig. 3C), and it seems possible to select the DHFR gene from a smaller proportion by repeating more selection rounds and optimizing selection conditions.

After three rounds of selection, DHFR activity of control sample decreased to about 50% in comparison with that of before selection (Fig. 4). This decrease was probably not only due to amplification of incorrect sequences by point mutations but also residual products in PCR reaction. This problem might be solved by agarose gel purification of mRNA before in vitro translation and DHFR enzyme reaction.

We identified four DHFR mutants (Table 1). Although other mutants had incomplete amino acid sequences, they probably showed a little affinity to MTX because the N-terminal fragment of DHFR could bind to its substrate [17]. Washing in such conditions that only strongly binding mutants remain on MTX, it is possible to remove those incomplete DHFR mutants.

In this study, the mutant of DHFR, which had as much enzyme activity as wild-type, could be obtained by ribosome display using MTX, substrate analog, immobilized on agarose beads (Fig. 5). We think this method is available to a primary screening of active enzyme from library. It may be possible to evolve enzyme activity further by selecting repeatedly, optimizing efficiency of one-round selection, and increasing mutation rate. Our selection is based on binding affinity of enzyme and substrate. In the future, however, it will be needed to select enzyme based on catalytic activity for extending applications of ribosome display. This methodology was attempted in phage display in recent years [18–20], and it must be possible to apply to ribosome display. Facilitation and automation of this complicated process are also necessary for improving ribosome display. Overcoming such problems, ribosome display will be really practical technology and evolutionary molecular engineering will be advanced.

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