Automated Purification of His₆-Tagged Proteins Allows Exhaustive Screening of Libraries Generated by Random Mutagenesis

BioTechniques 29:338-342 (August 2000)

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ABSTRACT

In the course of site-directed mutagenesis or directed evolution experiments, large numbers of protein variants are often generated. To characterize functional properties of individual mutant proteins in vitro, a rapid and reliable protein purification system is required. We have developed an automated method for the parallel purification of 96 different protein variants that takes about two hours. Using a 96-well format, the whole process can be performed automatically by a pipetting robot. Coupled with a suitable assay, again using a 96-well format, all variants can be functionally characterized within a few hours. The protein purification procedure described here is based on the interaction between His₆-tagged proteins and Ni-NTA-coated microplates. Typical yields are 3–8 pmol purified protein/well, which is sufficient to analyze most enzymatic activities. Using this procedure, we have purified and characterized variants of the restriction endonuclease EcoRV, which were produced in an effort to enhance the selectivity of this enzyme. For this purpose, three amino acid residues were randomized in a region known from the co-crystal structure to be located at the protein-DNA interface. From a library of about 1200 variants, predominantly single and double mutants, more than 1000 variants were purified and characterized in parallel, which corresponds to an almost complete screening of the library.

INTRODUCTION

Mutational analysis is a powerful approach to understand the relationship between protein structure and function. Creating mutants by site-directed mutagenesis normally leads to a few protein variants that can be purified and characterized by established methods. To overcome the limitations of rational protein design, random mutagenesis strategies are increasingly being used that result in large libraries of up to 10⁹–10¹⁰ different variants (1,3). To screen these libraries for variants that have the desired properties, in vivo or in vitro selection systems are used that depend on a selectable marker (4,5,11,12,16,18). Such selection procedures cannot be applied to properties that can be measured only with a purified protein preparation. To engineer such properties by random mutagenesis, a fast, efficient and reliable high-throughput system for protein expression, purification and characterization is required.

One example of such a property is the flanking sequence preference of the type II restriction endonuclease, EcoRV, which recognizes and cleaves DNA containing GATATC sequences with high accuracy (13). The majority of type II restriction endonucleases have recognition sites 4–6 bp in length, with few recognizing sequences of 8 bp (14). Thus, there is a demand for such enzymes, which in principle could be obtained by genetic manipulation of existing systems (7). We have begun to generate EcoRV variants that discriminate between differently flanked EcoRV sites by a random mutagenesis/selection

approach (8). For the in vitro characterization of these variants, purification of the proteins and removal of nonspecific nucleolytic activity are necessary. To screen many mutants, we developed a purification scheme based on the interaction between His₆-tagged proteins and Ni-NTA-coated microplates that is automatically carried out by a pipetting robot. Following the procedure described here, it is possible to analyze as many as 10⁴ different variants in an acceptable period of time. Therefore, this method suffices for many directed evolution experiments in which an almost complete analysis of a library obtained by randomization of three amino acid residues was carried out. With suitable modifications, our procedure can be applied to other enzymes that are active when expressed as His₆-tagged proteins.

MATERIALS AND METHODS

Bacterial Strains and Vectors

The plasmid vector pHis harbors the gene coding for the different His₆-tagged EcoRV variants. It is under the control of a synthetic promoter-operator sequence, a combination of two *lac*-operators and one T7-promoter, which allows for effective repression, and after induction with IPTG, for highly active transcription (19). The *E. coli* strain LK111 (λ , pLBM4422) was used for overexpression of the EcoRV proteins.

Random Mutagenesis

We have produced a library of EcoRV variants by randomization of amino

acids A181, G182 and S183. Complete randomization of these three residues would lead to 8000 different variants containing single, double and triple mutants. To preferentially obtain single and double mutants, we randomized the *EcoRV* gene at codons 181–183 using a PCR primer randomized at the three codons by the addition of a 40% mixture of all four nucleotides during oligonucleotide synthesis as described (8). Under these conditions, about 1200 single and double mutants were generated. After transformation of *E. coli*, 50 000 colonies were obtained. Sequencing of 18 different clones confirmed the success of the mutagenesis procedure. To screen a representative number of variants, 1056 different cultures were inoculated in eleven 96-well blocks.

Protein Expression and Purification

E. coli cells were grown in a 96-well block in a volume of 1 mL LB medium for 12 h at 37°C. Two hundred microliters were transferred to 1.5 mL LB medium in a 96-well block and incubated for 3 h at 37°C. Expression of the variants was induced by adding 1 mM IPTG (final concentration). After a 3-h incubation at 37°C, cells were harvest-

ed by centrifugation at 2000× *g* for 15 min. Pellets were resuspended in 250 µL lysis buffer [30 mM potassium phosphate (pH 7.5), 0.1 mM DTE, 0.01% (w/v) lubrol, 500 mM NaCl, 20 mM imidazole, 100 µg/mL BSA, 1 mg/mL lysozyme] and vortexed for 15 min at room temperature. The lysate (200 µL) was transferred to each well of a Ni-NTA HisSorb Plate (Qiagen, Hilden, Germany) and incubated at least for 1 h at room temperature. During the incubation, the plates were continually vortexed. Plates were washed with 2 × 200 µL lysis buffer. To elute the His₆-tagged protein, 40 µL elution buffer [30 mM potassium phosphate (pH 7.5), 0.1 mM DTE, 0.01 % (w/v) lubrol, 200 mM imidazole] were added. The whole procedure (starting from the transfer of the *E. coli* lysates) was carried out by an automated pipetting system (BIOROBOT 9600; Qiagen). The remaining 800 µL of the 1 mL pre-cultures were harvested by centrifugation, and the cell pellets were stored at -70°C. They were later used to prepare DNA from mutants of interest using a standard DNA plasmid preparation or by PCR. To guarantee continuous experiments, the cultures were prepared in advance and stored at -70°C.

Plasmid Cleavage Assay with Enzymes Purified on Ni-NTA-Coated Microplates

The eluate (15 µL) of the Ni-NTA-coated microplates were added to 100 ng prelinearized plasmid pATRV(II) dissolved in 5 µL cleavage buffer [20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, 100 µg/mL BSA). The remaining eluate was diluted 1:10 with elution buffer, and 15 µL were used for a second cleavage experiment. After incubating for 30 and 60 min at 37°C, the reaction was stopped by adding 10 µL fivefold gel loading buffer containing 250 mM EDTA and analyzed by agarose gel electrophoresis. Cleavage products were visualized by ethidium bromide staining. This parallel assay allows the characterization of 96 different variants within 3 h. The complete assay before the electrophoretic analysis was carried out by an automated pipetting system (BIOROBOT 9600).

Plasmid Cleavage Assay with Enzymes Purified in Preparative Amounts on Ni-NTA Agarose Beads

To examine variants of special interest in detail, a purification on a preparative scale was carried out. For this purpose, expression of the *EcoRV* mutants was induced in 3 mL LB medium at a cell density of A₆₀₀ = 1 by adding 1 mM IPTG (final concentration). The His₆-tagged mutants were purified using Ni-NTA magnetic agarose beads (5% suspension) (Qiagen). Harvested cells were resuspended in 600 µL binding buffer [30 mM potassium phosphate (pH 7.5), 0.1 mM DTE, 0.01 % (w/v) lubrol, 500 mM NaCl, 20 mM imidazole] and after sonication (2 × 30 s on ice), cell debris was removed by centrifugation, and 200 µL supernatant was transferred to a 96-well microplate. Agarose bead suspension (20 µL) was applied to the supernatant and vortexed on a minishaker (IKA, Staufen, Germany) for 30 min at 4°C. The microplate was then placed on a 96-well magnet (Qiagen) for 1 min, and the supernatant was removed with a pipet. The beads were washed twice with binding buffer. To elute the His₆-tagged proteins, 50 µL elution buffer [30 mM potassium phosphate (pH 7.5), 0.1 mM

Figure 1. *EcoRV* activity of 96 protein preparations produced in parallel using the automated protein purification procedure representing a random sample of screened variants. Shown are ethidium bromide-stained 2% agarose gels of plasmid pATRV(II) cleavage reactions. Each protein preparation was tested at two different concentrations (undiluted in one lane and 1:10 diluted in the next lane). Lanes 1–48: variants 1–24; lanes 49–96: variants 25–48; lanes 97–144: variants 49–72; lanes 145–192: variants 73–96. ↓ denotes the lanes with the λ × *EcoRV* standard (i.e., λ-DNA completely digested with *EcoRV*). The area bordered by the red box is enlarged in Figure 2. Under the conditions used, a variant with wild-type activity would completely cleave linearized pATRV(II) into three fragments when assayed undiluted and partially when diluted 1:10. Note that all preparations are free of nonspecific exo- and endonucleolytic activity. The accumulation of one of the two cleavage intermediates (see Figure 2) is suggestive of an enhanced selectivity for AT flanked *EcoRV* sites.

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DTE, 0.01 % (w/v) lubrol, 500 mM NaCl, 200 mM imidazole] were added, and the eluates were collected after placing the microplate on the magnet for 1 min.

Protein preparations contained as much as 90% pure *EcoRV* and were free of contaminating nuclease activity determined by co-incubation with a supercoiled preparation of pUC8 for 2 h (data not shown). Typical yields were 5 μ g in 50 μ L (corresponding to 1–2 μ M) starting from a 3-mL culture. For the DNA cleavage kinetics, prelinearized plasmid was digested with appropriate dilutions of *EcoRV* variants in the standard cleavage buffer at 37°C. After defined time intervals, 15- μ L aliquots were withdrawn, the reaction stopped with 5 μ L fivefold gel loading buffer containing 250 mM EDTA and then analyzed by agarose gel electrophoresis.

RESULTS AND DISCUSSION

We are engaged in a protein-engineering project concerning the restriction endonuclease *EcoRV*. This enzyme recognizes the sequence GATATC and cleaves dsDNA within this sequence with little preference for flanking se-

quences (15). Obtaining *EcoRV* variants with a preference for sites flanked for example by A...T would be the first step towards the generation of a new restriction enzyme with an 8-bp recognition site, for which there is a considerable demand, because only a few such “rare cutters” are available from natural sources.

It is known from the structure of the *EcoRV*-DNA complex that amino acid residues no. 181–183 are located at the DNA-protein interface, in close proximity to the DNA upstream and downstream of the recognition sequence (6,20). We had demonstrated previously that exchanging Ala181 for other amino acid residues leads to *EcoRV* variants with an enhanced selectivity (15). We expected that it should be possible to produce even more selective variants by including positions no. 182 and 183 in the mutagenesis scheme. This results in several thousand variants, which must be produced and analyzed. We have therefore developed a protocol for the parallel culture of individual *E. coli* clones, as well as the parallel isolation and the functional characterization of the *EcoRV* variants from these clones. As described in the Materials and Methods section, this protocol makes use of a pipetting robot and 96-well microplates.

To estimate the yields of the purified proteins, cleavage experiments with λ -DNA were performed with a wild-type enzyme as described previously (9). Specific activities of 0.5 U/ μ L were measured, corresponding to 3–5 pmol His₆-tagged protein/well on the basis of the known specific activity of *EcoRV*, in which 1 U is defined as the activity that completely cleaves 1 μ g λ -DNA in 1 h. This is within the capacity of the plates, 2–10 pmol, according to the supplier's manual (Qiagen). To determine the activity and selectivity of the *EcoRV* variants in the protein preparations, cleavage experiments with plasmid DNA were carried out immediately after purification (Figures 1 and 2). Specific *EcoRV* activity was detected by the occurrence of three defined products in cleavage experiments with prelinearized pATRV(II) that contains two *EcoRV* sites. The absence of a smear in the stained gels shows that the preparations do not contain a detectable contamination by non-specific endo- or exonucleases.

To compare specific activities of the protein variants differing by more than one order of magnitude, the cleavage experiments were carried out with two different dilutions of the protein preparation. The cleavage data show that about

Figure 2. Cleavage analysis of *EcoRV* variants. (A) Selectivity of different *EcoRV* variants (enlargement of Figure 1, lanes 23–32, 71–80). The preference of different *EcoRV* variants for one or the other *EcoRV* site present in pATRV(II) will lead to different intermediates during the cleavage reaction. For example, a preference for the AT flanked site will lead to the accumulation of the intermediate labeled red and green. Variant 12 (lanes 23 and 24) displays only a slight preference; variants 39 and 40 (lanes 77–80) strongly prefer the AT flanked *EcoRV* site of pATRV(II), while variant 36 (lanes 71 and 72) is inactive. ↓ denotes the lanes with the λ *EcoRV* standard. (B) Schematic overview of the different cleavage intermediates and products of pATRV(II).

Table 1. DNA Cleavage Specificities of 1056 Preparations Containing *EcoRV* Variants Obtained by Random Mutagenesis

<i>EcoRV</i> Specificity	No Activity	Wild-Type⁺ Specificity	Extended⁺⁺ Specificity
Total Numbers	438	412	206
%	41	39	20
*no preference for either site			
++more than 5-fold preference for one or the other site			

40% of the protein variants examined had no cleavage activity (Figure 1 and Table 1), a result that is not surprising because the mutated region is quite close to a loop that is important for substrate recognition of *EcoRV* (17,20); 40% had wild-type activity. To test the reproducibility of our protocol, the wild-type enzyme was purified and analyzed by cleavage experiments several times, with similar results concerning yield and catalytic activity. Furthermore, relative

cleavage activities of wild-type and mutant enzymes were comparable to those determined for preparations obtained by alternative methods (8).

pATRV(II) harbors two differently flanked *EcoRV* sites (GGATATCG and AGATATCT), which are cleaved with identical rates by the *EcoRV* wild-type enzyme (8). Variants with an enhanced selectivity lead to cleavage patterns different from that of the wild-type enzyme (Figure 2); 20% of the active

variants displayed an altered preference (Table 1). In contrast to the *EcoRV* wild-type enzyme, these variants favor an AT flanked GATATC site. We have sequenced some of the genes of the variants that display a preference for the AT flanked site, produced the variants in preparative amounts and characterized them in detail. Figure 3 shows, as one example, the kinetics of pATRV-(II) cleavage by the variant G182R/S183P. In agreement with the results of the cleavage experiments carried out with protein obtained by the automated purification procedure, this variant shows an almost 20-fold preference for AT flanked *EcoRV* sites. The increase in selectivity obtained in one round of random mutagenesis is comparable to those obtained by site-directed mutagenesis (15).

In conclusion, we have developed a high-throughput protein purification protocol to characterize the activity and

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Figure 3. Kinetics of cleavage of 5 mM linear pATR(VII) by 1 nM wild-type *EcoRV* (A) and 8 nM G182R/S183P variant (B) with respect to time in minutes. The wild-type enzyme shows no preference for one of the differently flanked sites of the substrate. In contrast, the G182R/S183P variant strongly prefers the AGATATCT over the GGATATCC site. While the AT flanked site is cleaved after 1 min, the GC flanked site disappears only after 15 min. $\downarrow$ denotes the lanes with the $\lambda \times EcoRV$ standard.

specificity of enzymes in vitro. The time-limiting step of this procedure is the pipetting rate of the robot and the duration of the electrophoretic analysis. Further improvement could be achieved with a multichannel robot and a parallel analysis of the cleavage reactions by capillary electrophoresis (2). In addition, the automated protein purification could be combined with a colony-picking robot (10), leading to fully automated clone management.

Randomizing a whole gene normally leads to much higher clone numbers than we generated in our experiment. Even with the high-throughput system described here, it is not possible to screen more than 10^5 variants in a reasonable period of time. However, even in these experiments, an increase in the number of clones screened will directly increase the probability of obtaining the desired variant. This is of particular interest because it was recently reported that under certain circumstances even small libraries ($<10^5$) contain variants with the desired properties (21).

ACKNOWLEDGMENTS

This work has been supported by grants from the Deutsche Forschungsgemeinschaft, the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie, the European Union and the Fonds der Chemischen Industrie.

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Received 20 December 1999; accepted 14 March 2000.

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