A New Solid-Phase Chemical DNA Sequencing Method Which Uses Streptavidin-Coated Magnetic Beads

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(Received 13 June 1995)

Abstract

To simplify the chemical DNA sequencing protocol, we developed a new solid-phase method which uses streptavidin-coated magnetic beads. This method is based on the finding that the biotinylated DNA-streptavidin complex was stable under the conditions for some chemical sequencing reactions. The 5'-biotinylated DNA generated by the polymerase chain reaction was first captured by streptavidin-coated magnetic beads and then subjected to a set of simplified chemical sequencing reactions on the beads at room temperature. Followed by the piperidine cleavage reaction, the products were resolved by gel electrophoresis, transferred onto a nylon membrane and visualized by chemiluminescent detection. As a consequence, high-quality sequencing ladders were obtained, due to complete removal of contaminating chemicals, without the time-consuming precipitation/centrifugation steps used in the conventional chemical sequencing protocol.

Key words: chemical DNA sequencing; solid-phase; magnetic beads; streptavidin; PCR; biotinylated DNA

1. Introduction

The chemical degradation method of Maxam and Gilbert¹ and the enzymatic chain termination method of Sanger et al.² are the two principal methods for DNA sequencing in use, but the enzymatic method is now overwhelmingly preferred for large-scale DNA sequencing projects because of its simplicity and speed. Nevertheless, it is still true that the chemical method has advantages over the enzymatic method in some respects. For example, DNA sequences with repetitive elements or high GC or AT regions can be more accurately determined by the chemical method since the enzymatic method frequently suffers from artifacts due to anomaly of the polymerase reaction. Although this advantage is obviously significant in DNA sequencing, the tediousness of the conventional chemical method is an obvious drawback. Thus, many efforts have been made to simplify the chemical sequencing protocol. As for principal modifications in the protocol, a solid support³⁻⁷ and microtiter dishes⁸ have been introduced to process many samples in parallel. The solid-phase method which uses anion exchange matrix⁶ is an attractive alternative, but it still has some limitations in the manipulation of multiple DNA samples in parallel. As another new solid support, we paid attention to streptavidin-coated magnetic beads which are currently used as a versatile matrix in a wide range of molecular biological applications, because the interaction between streptavidin and biotin is highly specific and stable⁹ and the magnetic beads can be easily retrieved by magnetic capture. In this study, we demonstrated that streptavidin-coated magnetic beads serve as a good solid-support of DNA during the chemical modification reactions and thereby allowed us to greatly simplify the protocol for obtaining high-quality sequencing patterns.

2. Materials and Methods

2.1. Materials

A plasmid DNA derived from pBluescript SK(-) (Stratagene, USA), which carries a fragment of mouse cDNA (Apa I-Pst I fragment of CZ-21),¹⁰ was used as a model DNA to be sequenced throughout this study. Taq DNA polymerase was purchased from Takara Shuzo Co., Ltd. (Otsu, Japan). Sources of chemicals used for the chemical sequencing reactions were as follows: Dimethyl sulfate (DMS) from Aldrich Chemical Co., Ltd., (Gillingham, Dorset, UK); potassium tetrachloropalladate, potassium permanganate and piperidine (PIP) from Kanto Chemical Co., Inc., (Tokyo, Japan); hydroxylammonium chloride from Merck, (Germany); formic acid from Wako Pure Chemical Industries (Japan); and hydrazine from Nakalai Tesque Inc., (Kyoto, Japan). Dynabeads M-280 streptavidin (10 mg/ml), streptavidin-coated magnet beads (SA-Mg), were obtained from DYNAL, Norway, and alkaline phosphatase substrates,
AttoPhos™ and CDP-Star™ were from Boehringer Mannheim (Tokyo, Japan). The biotinylated M13 reverse primer was prepared with a biotin amide (BODITETM, Pharmacia Biotech, Uppsala, Sweden) on a Pharmacia Gene Assembler Plus DNA synthesizer as in the case of non-biotinylated oligonucleotides and purified by polyacrylamide gel electrophoresis.

2.2. Preparation of 5'-biotinylated DNA captured on streptavidin-coated magnetic beads

Preparation of biotinylated DNAs captured on SA-Mg through a biotin group located at a single 5'-end was conducted essentially as described by Hultman et al. Briefly, the polymerase chain reaction (PCR) was carried out on a DNA Thermal Cycler™ (Perkin-Elmer Cetus, Norwalk, CT, USA) in 40 μl with 1 ng of a plasmid, 4 pmole each of the biotinylated M13 reverse primer (5'-biotin-CAGGAAACAGCTATGACC-3') and the M13 forward primer (5'-GGTGTAAACAGACCAGGCGT-3'), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM each of 4 dNTPs, 0.001% autoclaved gelatin, and 2.5 units of Taq DNA polymerase. PCR cycle conditions were as follows: Denaturation, 94°C for 30 sec; annealing, 55°C for 30 sec; extension, 72°C for 30 sec (25 cycles). The last extension was at 72°C for 5 min. After the PCR, 10 μl of 5 M NaCl was added and then biotinylated DNAs (bioDNA) in the PCR mixture were captured onto 400 μg of SA-Mg prewashed with binding buffer containing 1 mM AttoPhos, 100 mM diethanolamine-HCl (pH 10.0) and 1 mM MgCl₂ for 10 min. The resultant DNAs retrieved from SA-Mg were washed with H₂O and then treated with 30 μl of 1 M PIP at 90°C for 30 min. The resultant PIP solution was recovered from the SA-Mg suspension and then PIP was removed from the sequencing sample by repeated evaporation with SpeedVac (AES-2000; Savant Instruments Inc., Farmingdale, NY, USA).

2.4. Visualization of sequencing patterns

After the chemical reactions, DNA samples were solubilized in 3 μl of dye solution (80% formamide, 10 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue) followed by heat denaturation, and then 1-2 μl of the samples were applied to a 6% polyacrylamide gel (0.35 mm in thickness) containing 7 M urea, 50 mM Tris-borate (pH 8.3), 0.5 mM EDTA. After electrophoresis, DNA fragments were electrophoretically transferred onto a positively charged nylon membrane and immobilized by UV irradiation. For visualization of the sequencing ladders, the membrane was semi-automatically processed with Gel/Membrane Processor RP 1000 (Hoefer Scientific Instruments, San Francisco, CA, USA) using a chemiluminescent detection kit (Sequencing high; TOYOBO, Osaka, Japan) except for use of CDP-Star as a chemiluminescent phosphatase substrate. The phosphatase reaction was routinely allowed to proceed for 1 hr, and then the membrane was exposed to X-ray film for 5-10 min.

2.5. Quantitation of biotinylated DNA on the membrane

After PIP treatment following the chemical modification reactions, the resultant DNAs retrieved from SA-Mg were suspended in 10 μl of 0.1 N NaOH for 10 min at room temperature. Each of the resultant DNAs (2 μl) was spotted onto a positively charged nylon membrane and fixed by UV irradiation. The amounts of biotinylated DNAs spotted onto the membrane were measured by alkaline phosphatase-mediated detection method as described in 2.4. except for using a fluorogenic substrate, AttoPhos. The membrane was manually processed at room temperature using a detection kit from TOYOBO and finally incubated in a fluorogenic substrate solution containing 1 mM AttoPhos, 100 mM diethanolamine-HCl (pH 10.0) and 1 mM MgCl₂ for 10 min. The fluorescent signals were quantitatively detected with a fluorescent gel/membrane scanner (FluorImager; Molecular Dynamics, Sunnyvale, CA, USA).

3. Results and Discussion

3.1. Stability of SA-Mg/bioDNA complex under the conditions for chemical sequencing reactions

In the conventional chemical sequencing protocol, preparation of a DNA sample tagged only at the single
end of a single strand is tedious step. However, the emergence of PCR technology has made it easy to tag at the single 5'-end by using a 5'-labeled primer. Therefore, we tried to design a solid-phase protocol using SA-Mg so as to use DNA samples tagged with biotin by PCR as a starting material. Nevertheless, biotinylated DNA fragments prepared by other methods (e.g., filling-in reaction with DNA polymerase and tailing reaction with terminal deoxynucleotidyl transferase) can be also treated in the same way. When we performed PCR with relatively low concentrations of primers (0.1 μM), the PCR products could be readily captured by SA-Mg without first removing the remaining free biotinylated primer. The reduction in the primer concentration also contributed, to some extent, to the suppression of the formation of spurious products during PCR. Thus, the preparation of DNA samples ready for the chemical sequencing reactions could be greatly simplified by PCR and the use of SA-Mg.

Although the streptavidin/biotin complex is known to be stable under various harsh conditions, it was uncertain whether or not SA-Mg/bioDNA complex could tolerate the chemical sequencing reactions, although biotin group itself is known to be stable during the chemical sequencing reactions. Thus, we first examined the stability of the SA-Mg/bioDNA complex under the conditions used for the chemical sequencing method. Table 1 lists the chemical reactions examined and the relative amounts of the remaining bioDNA on SA-Mg after the respective chemical reactions. Since the PIP reaction was found to destroy the SA-Mg/bioDNA complex completely (data not shown), we used this reaction for quantitative recovery of the remaining bioDNA on SA-Mg after the chemical reactions. Although T- and C (hydroxylamine)-specific reactions included a preceding denaturation step, the loss of bioDNA during denaturation was only about 10% of the initially bound bioDNA when bioDNA was denatured with 0.1 M NaOH at room temperature, not by boiling. The results indicated that SA-Mag/bioDNA complex was resistant to the chemical reactions for G, A, C (hydroxylamine), and T. In contrast, the treatments with 1 M NaOH at 60°C (A>C reaction) with 66% formic acid at room temperature (A+G reaction), and with 50% hydrazine in the presence or absence of salt at room temperature (T+C or C reactions) were found to dissociate the bioDNA from SA-Mg almost completely.

### 3.2. Solid-phase chemical sequencing protocol and its performance

Based on the findings described above, we selected G, A, T, and C (hydroxylamine) reactions as standard solid-phase chemical sequencing reactions because these reactions can be carried out directly using the SA-Mg/bioDNA complex at room temperature. Although it is possible to use SA-Mg in a post-reaction step for circumvention of tedious precipitation/centrifugation steps after the reactions, it requires some additional purification steps of 5'-biotinylated PCR products prior to the chemical reactions. PIP treatment at 90°C for 30 min completely cleaved the bond between streptavidin and the biotin group, and thus the modified bioDNAs to be analyzed were released from SA-Mg into the PIP solution. Since the chemical agents used for the nucleotide modification reactions could be efficiently removed by repeated washes of SA-Mg carrying bioDNA prior to the PIP reaction, no residual chemicals remained with the bioDNA after removal of PIP by evaporation. Thus, the use of SA-Mg as a solid support allowed us to purify and recover the chemical sequencing samples.
Figure 1. Flowchart of the steps used in the solid-phase chemical sequencing protocol. Abbreviations used in this figure are as follows: DMS, dimethyl sulfate; 2-ME, 2-mercaptoethanol; RT, room temperature; PIP, piperidine.
cent detection system for visualization of the sequencing pattern. Figure 2 shows a typical sequencing gel electrophoretic pattern of samples generated by this solid-phase chemical method. Since G, A, T, and C-specific reactions were selected, the gel pattern looks like that obtained by the enzymatic method and therefore could be analyzed with existing sequence-reading software for the enzymatic method. It was impressive that dyes (xylene cyanol and bromophenol blue) entered and migrated as sharp bands in the sequencing gel while the sequencing sample prepared by the conventional protocol usually shows blurry dye migration patterns, indicating that the use of SA-Mg as a solid support minimized contaminating chemicals in the sequencing sample. Therefore, deterioration of the resolution of the sequencing pattern caused by contaminating salt and modification reagents, which sometimes happens with the conventional chemical method, is very unlikely to occur when using this solid-phase method.

T- and C-specific reactions with KMnO4 and NH2OH, respectively, are known to depend, to some extent, on the secondary structure of the sample DNA, which might hinder accurate sequence reading by this solid-phase method. However, we have not observed any missing T and C bands using this method so far although the T and C band intensities varied slightly depending on their positions in some cases (Fig. 2). Conceivably, the secondary structure problem in T- and C-specific reactions appeared not to be so serious in most cases.

3.3. Concluding remarks

Using SA-Mg as a solid support, we developed a reliable and efficient chemical sequencing protocol. As long as PCR generates a specific product with no or few spurious products, this method could reproducibly give us high-quality sequencing ladders. If PCR is refractory to a DNA fragment of interest, other enzymatic labeling reactions can be applicable to tag it with biotin. Since the solid-phase enzymatic sequencing protocol with SA-Mg has already achieved great popularity, automation of this solid-phase protocol is actively being pursued. Therefore, it is very hopeful that an automated device suitable for both the solid-phase chemical and enzymatic sequencing protocols will emerge in the future.

Although the biotin moiety was used not only for anchoring DNA on SA-Mg but also for visualization of the sequencing ladders in this study, other tags (e.g., digoxigenine and fluorophores) must be also acceptable for detection of DNA bands. Thus, as a simple logical extension, development of a solid-phase chemical sequencing method using fluorescent tag, which is suitable for automated DNA sequencers, is now underway.
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