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Purification and functional analysis of the recombinant protein isolated from *E. coli* by employing three different methods of bacterial lysis

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Abstract: In this paper, the purification of the human recombinant protein expressed in *E. coli* using the GST Gene Fusion System, by applying various methods of bacterial lysis: sonication, freeze/thaw and beadbeating, is presented. The study was an attempt to compare the properties of the proteins obtained by the sonication method, recommended by manufacturers but inaccessible for many researchers, with those obtained using two other readily available lysis methods. The data show that all purified proteins were soluble and intact with the highest protein yield being obtained *via* the freeze/thaw method. The results of functional analysis indicate that the proteins purified using the sonication and freeze/thaw methods of lysis exhibited similar DNA binding affinity, while the protein purified by beadbeating was also functional but with a lower binding affinity. The conclusion of this study is that all three lysis methods could be successfully employed for protein purification.

Keywords: protein purification, bacterial lysis, protein yield, protein-DNA interaction.

INTRODUCTION

The synthesis of recombinant proteins is essential for any biochemical study on gene control. Although *in vivo* experiments have powerful applications, most of the key molecular details of the action of a protein have to be resolved biochemically. Therefore, recombinant proteins or their important regulatory domains are synthesized and studied *in vitro*. *Escherichia coli* expression systems are the most efficient, most commonly used and inexpensive model systems for the synthesis of intact, folded recombinant proteins under 75 kDa.[1]

There are many methods that can be employed to overproduce a recombinant protein in *E. coli*. One of them is the addition of sequence tags to a protein. In this strategy,

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the DNA fragment encoding the gene of interest is cloned into a vector containing an in-frame purification tag.[1] The common purification tags fused to eukaryotic proteins expressed in *E. coli* systems are His_6, glutathione-*S*-transferase (GST), and maltose-binding protein (MBP), although many others have been successfully used.[1]

In this work, the employment of the GST Gene Fusion System (Amersham Pharmacia Biotech) for overexpression and subsequent purification of the human recombinant protein from *E. coli* is described. This fusion system is the most commonly used method since it has many advantageous characteristics: stabilizing effect on some proteins, enhancement of fusion protein expression in *E. coli* and aiding protein solubilization.[1]

Retinoic acid (RA), a derivative of vitamin A, is important for the maintenance of normal cell growth, differentiation and development.[2] One route of its action is mediated through activation of retinoid X receptors (RXRs),[3] which then bind to response elements within the promoter region of RA target genes, thus regulating their expression.[4–6]

The purification of human RXRα protein using the GST System by applying three different ways of bacterial lysis, this being one of the crucial steps during purification, is described herein. The aim was to compare the proteins obtained by the sonication method, which is recommended by manufacturer but is not available for many researchers, with those obtained by the two other lysis methods, usually on hand in many laboratories, *i.e.*, freeze/thaw and beadbeating. Lysis by sonication requires not only expensive apparatus but also lengthy optimization of the working conditions. The results of this study show that the sonication method could be successfully replaced by the other two lysis methods.

EXPERIMENTAL

Bacterial strain and plasmid DNA

The complete coding sequence of human RXRα protein cloned into pGEX-T vector was a kind gift from Dr. R. M. Evans (The Salk Institute for Biological Studies, La Jolla, California, USA). The *E. coli* strain DH5α[7] was transformed with the pGEX–T–RXRα construct.

Oligonucleotides

The following complementary oligonucleotides: RXRE F 5' GCTAGAGGTCACAGGTCAAGCTG 3' and RXRE R 5' GGCAGCTTGACCTGTGACCTCTAGC 3' were annealed in a standard reaction in order to form a double-stranded DNA probe containing the consensus binding site for RXR. The two half sites, separated by 1 base pair, are underlined and represent direct repeats of the core sequence,[8] named RXRE since it encodes the retinoid X receptor response element. This oligomer was used in all DNA binding assays presented in this paper.

Antibodies

A rabbit polyclonal antibody against peptide mapping at the amino terminus of human RXRα (D-20, sc-553) was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Protein purification procedures

The Glutathione-*S*-transferase (GST) Gene Fusion System (Amersham Pharmacia Biotech) was used in this study as an integrated system for the expression and purification of fusion proteins. This system enables fusion proteins to be purified based on the glutathione-*S*-transferase tag using the affinity matrix Glutathione Sepharose 4B. GST fusion RXRα was expressed from the pGEX-T-RXRα construct

in *E. coli* (DH5α) and purified directly from bacterial lysates using standard techniques as suggested by the manufacturer. Briefly, 50 ml overnight bacterial culture grown in ampicillin (100 μg/ml) was diluted 1:100 into 1l Luria broth[9] and grown at 37 ºC to an A_{600} = 0.6 before induction with 0.1 mM isopropylthiogalactopyranoside (IPTG) for 4 h. The cells were pelleted and resuspended in 30 ml of buffer A (100 mM NaCl, 50 mM Tris pH 8.0, 0.1 % Triton X-100, 1 mM EDTA).

The harvested bacterial cells were then divided into three aliquots in order to perform three different methods of lysis:

– sonication (bacteria were pulsed for 3 × 45 s intervals)

– freeze/thaw (liquid nitrogen / 37 ºC water bath cycles were repeated 10 times)[10]

– beadbeating (bacterial cells were mixed with 0.3 g of 0.1 mm inexpensive zirconium beads (Biospec Product, Inc., Bartlesville) and lysed using a Beadbeater (modified sand machine) three times for 1 min.[11]

Subsequently, all three lysates were treated in the same manner, *i.e.*, the purification of the fusion proteins was continued according to the instructions of the manufacturer.

Protein concentrations were determined by the method of Bradford,[12] with BSA as the standard. The purified proteins, together with molecular weight standards (Fermentas), were run on a 10 % SDS-polyacrylamide resolving gel using a Bio Rad minigel electrophoresis apparatus and stained with Coomassie Brilliant Blue.

Western blot analysis

After separation by SDS-PAGE, the protein samples were electrotransferred to a nitrocellulose membrane (Amersham-Pharmacia Biotech). The membrane was subjected to Western blot analysis[13] using polyclonal rabbit antibody against human RXRα and then goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma) as a secondary antibody. Immunoreactive bands were detected using BCIP/NBT reagents as described by the manufacturer (Promega).

Radioactive gel retardation assay

For the radioactive gel retardation assay, the annealed oligonucleotides were labeled by filling in the overhanging 5' ends using the Klenow fragment *E. coli* DNA polymerase (USB) in the presence of [α-^{32}P]dCTP.

The indicated amounts (see figure legend) of purified RXRα were incubated with 1 ng of ^{32}P-labeled oligonucleotide in a binding buffer containing 10 mM Hepes pH 7.9, 12 mM KCl, 0.25 mM EDTA, 0.25 mM DTT, 3 % glycerol and 1 μg of poly(dI-dC) in a total volume of 25 μl. Incubation was performed at 37 ºC for 30 min. The DNA–protein complexes were resolved on a 6 % non-denaturating polyacrylamide gel. The gel was dried and subjected to autoradiography at –70 ºC.

Non-radioactive gel retardation assay

The non-radioactive gel retardation assay was performed using a DIG gel shift kit (Roche Molecular Biochemicals). The probe was 3' end-labeled with digoxigenin-11-ddUTP and terminal transferase. The binding reaction was carried out as described above for the radioactive gel retardation assay and run onto a native polyacrylamide gel. Following the electrophoretic separation, the oligonucleotide–protein complexes were electroblotted onto a BM nylon membrane (Roche Molecular Biochemicals). The DIG-labeled probe was subsequently detected by an enzyme immunoassay using anti-DIG antibody, Fab-fragments coupled to alkaline phosphatase and CSPD chemiluminescent substrate. The chemiluminescent signals were recorded by exposure to an X-ray film.

RESULTS AND DISCUSSION

Purification of the recombinant human RXRα protein

The recombinant human RXRα protein was overexpressed in *E. coli* and purified using GST Gene Fusion System by employing three different methods of bacterial lysis. The aim was to compare the sonication method, which is recommended

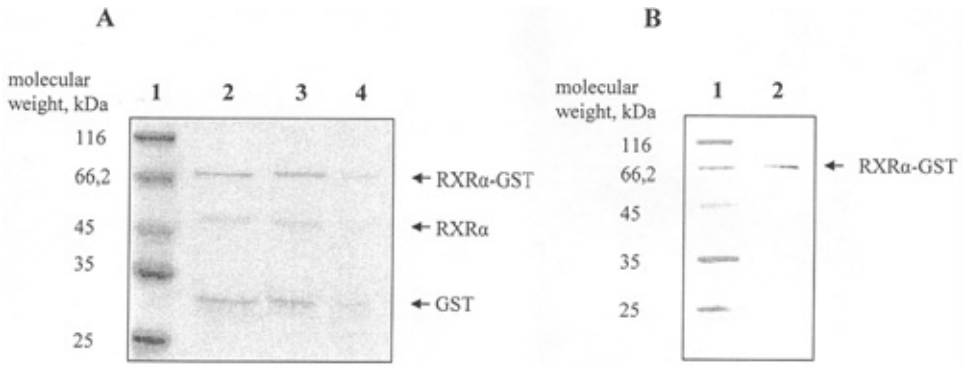

Fig. 1. A. Coomassie-stained 10 % SDS-PAGE: line 1 – Molecular weight standards; lines 2, 3, 4 – 10 μl aliquots of purified RXRα obtained from the three different methods of bacterial lysis (sonication, freeze/thaw, beadbeating, respectively). Arrows indicate GST fusion RXRα protein as well as distinct forms of both proteins. B. Western blot analysis of purified RXRα: line 1 – Ponceau S stain of molecular weight marker; line 2 – 2 μg of purified RXRα (sonication lysis method) detected using anti-RXRα antibody. Arrow indicates GST fusion RXRα protein.

by the manufacturer for larger scale cultures (but for many researchers not always accessible), with two other lysis methods usually on hand in many laboratories, *i.e.*, freeze/thaw and beadbeating. The sonication method requires expensive apparatus and optimization of the working conditions. Inadequate sonication conditions may lead to protein denaturation and loss of its function. For example, over-sonication or excessive frothing may denature the fusion protein and prevent its binding to the Glutathione Sepharose matrix. In addition, over-sonication can lead to co-purification of bacterial proteins with the GST fusion protein. On the other hand, insufficient sonication may lead to reduced protein yield.

After purification, the proteins were eluted in the same volume and the determined concentrations of the isolated proteins were as follows: lysis by sonication 200 ng/μl, by the freeze/thaw method 400 ng/μl and by beadbeating lysis 250 ng/μl. Therefore, the highest amount of purified RXRα protein was obtained by applying the freeze/thaw method. All three samples of isolated proteins were then run on an SDS-polyacrylamide gel (Fig. 1A). This analysis demonstrated that each purification give rise to the expected pattern of bands (in accordance with the molecular weight standards, as indicated by the arrows). It is important to mention that, since the presence of the GST domain in the fusion protein does not interfere with the subsequent analyses performed in this study,[14] proteolytic cleavage was not applied to separate the RXRα protein.

In addition, Fig. 1A also illustrates that all purified proteins obtained by the three different methods of bacterial lysis were soluble and intact.

Western blot analysis of purified RXRα

In order to additionally confirm the presence of RXRα protein in the purified samples, Western blot analysis was performed (Fig. 1B). Thus, by using anti RXRα

antibody in the immunoreaction with the sample from the sonication lysis, the 80 kDa protein which corresponds to the GST fusion RXRα protein was identified (data with other two samples are not shown).

DNA binding assays

To examine the DNA binding ability of the purified RXRα proteins, two types of gel retardation assays were performed. For this purpose, radioactively (^{32}P-) and non-radioactively (DIG-) labeled oligomer RXRE containing the consensus binding site for RXRα were used.

Radioactive gel retardation assay

As shown in Fig. 2, in the reactions with the ^{32}P-labeled RXRE oligomer, band shifts were observed with all three protein samples used. The strongest binding was detected with the 400 ng of RXRα purified *via* sonication lysis method (Fig. 2, line 2), whereas the same amount of protein purified using the freeze/thaw method resulted in a slightly lower intensity of the complex (Fig. 2, line 5). The same quantity of protein isolated by the beadbeating method failed to produce a visible complex (data not shown), therefore, it was decided to increase the amount

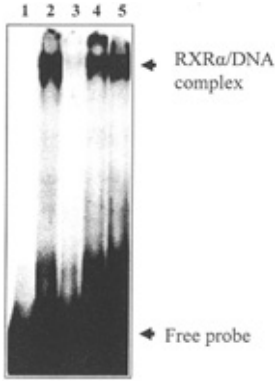

Fig. 2. Gel retardation assay. The DNA-binding activity of purified RXRα was assayed by using a ^{32}P-labeled probe containing the consensus binding site for RXR. The free probe is at the bottom of the gel, whereas the slower-migrating RXRα/DNA complexes are near the top of the gel (indicated by the arrows): line 1 – Free probe; line 2 – Purified RXRα (using the sonication lysis method) 400 ng; lines 3 and 4 – Purified RXRα (using the beadbeating lysis method) 500 ng and 750 ng, respectively; line 5 – Purified RXRα (using the freeze/thaw lysis method) 400 ng.

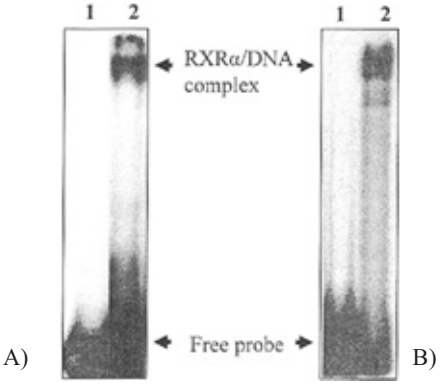

Fig. 3. Radioactive and non-radioactive gel retardation assays. The DNA-binding activity of 750 ng RXRα purified using the beadbeating lysis method was assayed with ^{32}P-labeled (A) and DIG-labeled (B) RXRE probe. The free probe is at the bottom of the gel, whereas the slower-migrating RXRα/DNA complexes are near the top of the gel, as indicated by the arrows.

of this protein in subsequent binding reactions. As shown in Fig. 2, line 3, the addition of 500 ng resulted in the appearance of a very faint complex, and, as expected, the intensity of the DNA–protein complex increased further with increasing amount (750 ng) of protein employed for the binding reaction (line 4). The intensity of this complex was similar to that obtained using 400 ng protein obtained by the freeze/thaw method of lysis (compare lines 4 and 5 in Fig. 2). Thus, the results of DNA binding analysis show that the recombinant human RXRα proteins purified from the three different bacterial lysates are all functional. The protein purified using the sonication lysis method had the strongest binding affinity to the RXRE DNA oligomer, nevertheless by applying the other two lysis methods, purified RXRα proteins were obtained which could also be used for DNA binding studies.

Non-radioactive gel retardation assay

In order to compare the sensitivity of the radioactive gel retardation assay with that of the non-radioactive one, the binding ability of the purified RXRα protein was tested in the reaction with DIG-labeled RXRE DNA oligomer. For this purpose, the protein sample isolated *via* the beadbeating method was used because it showed the weakest binding affinity in the previous assay. As shown in Fig. 3A and 3B (lines 2), by applying the same amount of RXRα protein (750 ng) in binding reactions with the ^{32}P- and DIG-labeled RXRE oligomers, similar shifted complexes were detected. With this experiment, it was additionally confirmed that the RXRα protein purified using the beadbeating method of lysis, although having a low binding ability, could be effectively used for protein–DNA interaction studies.

The results of this experiment also suggested that the sensitivity of the non-isotopic gel retardation technique is comparable to that of the radioactive one. It is important to point out that, together with other advantages, non-radioactively labeled probes, are safe and stabile for long periods without loss of activity. The non-radioactive gel retardation assay can be the method of choice in the absence of a permanent supply of radioisotopes.

In conclusion, the purification of the human RXRα protein from *E. coli* using the GST Gene Fusion System by applying various methods of bacterial lysis, *i.e.*, sonication, freeze/thaw and beadbeating, is presented in this paper. The data show that all the purified proteins were soluble and intact with the highest yield of protein being obtained *via* the freeze/thaw method. Furthermore, the results of functional analysis indicate that the proteins purified using the sonication and freeze/thaw lysis methods exhibited a similar DNA binding affinity, while the protein purified by beadbeating was also functional but with a lower binding ability. Therefore, it was concluded that the sonication method could be successfully replaced by either of the two other lysis methods, which are readily available in many laboratories.

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pGEX-T vector was the kind gift from Dr R. M. Evans (The Salk Institute for Biological Studies, La Jolla, California, USA).

ИЗВОД

ПРЕЧИШЋАВАЊЕ И ФУНКЦИОНАЛНА АНАЛИЗА РЕКОМБИНАНТНОГ ПРОТЕИНА ИЗОЛОВАНОГ ИЗ *E. coli* КОРИШЋЕЊЕМ ТРИ РАЗЛИЧИТЕ МЕТОДЕ ЛИЗЕ БАКТЕРИЈА

МАРИЈА МОЈСИН, ГОРДАНА НИКЧЕВИЋ, НАТАША КОВАЧЕВИЋ ГРУЈИЧИЋ, ТИЈАНА САВИЋ, ИСИДОРА ПЕТРОВИЋ и МИЛЕНА СТЕВАНОВИЋ

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У овом раду приказано је пречишћавање хуманог рекомбинантног протеина експримираног у *E. coli*. Рекомбинантни протеин, фузионисан са глутатион-*S*-трансферазним доменом (GST), изолован је применом три различите методе бактеријске лизе: сонификовање, замрзавање/одмрзавање и разбијање бактерија куглицама. Произвођач GST фузионог система препоручује бактеријску лизу сонификовањем, али ова метода је често недоступна истраживачима јер захтева скупу опрему и дуготрајну оптимизацију услова. Циљ нашег рада је био да утврдимо да ли се коришћењем и друге две методе, лако применљиве у свакој лабораторији, могу добити протеини одговарајућих карактеристика. Наши резултати показују да све три методе лизе омогућавају изоловање солубилних и интактних протеина, а да је највећи принос добијен применом методе замрзавања/одмрзавања бактерија. На основу функционалне анализе закључили смо да протеини добијени методом сонификовања и поновљеног замрзавања/одмрзавања показују висок афинитет за специфично везивање за ДНК. Рекомбинантни протеин добијен применом методе разбијања бактерија куглицама показује смањен афинитет за везивање за ДНК али се, такође, може користити у анализама интеракција протеина и ДНК. Закључак наших истраживања је да се све три методе лизе бактерија могу успешно применити за изоловање рекомбинантних протеина.

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