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Isolation of Differentially Expressed Proteins by Protein Subtraction

Doo Yeon Kim, Su-jin Cho, Soo Kyung Koo, Younghoon Lee[1], and Cheol O Joe*

Department of Biological Sciences, [1]Department of Chemistry, Korea Advanced Institute of Science and Technology, Taejon 305-701, Korea

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A prototype to isolate differentially expressed proteins (Pds) by protein subtraction was presented. Two sets of protein mixture A and (A-Pd) were prepared. The mixture A contained bovine serum albumin, ovalbumin, α-lactalbumin, trypsinogen and carbonic anhydrase while the mixture (A-Pd) was the same as the mixture A except that one of the proteins acting as a differentially expressed proteins was lacking. Rabbit antisera were raised against the mixture A. Proteins in the mixture (A-Pd) were then coupled to CNBr- activated Sepharose 4B in order to prepare the affinity column. The antisera were applied to the affinity column and the pass-through exclusively contained the antibodies that specifically reacted with the differentially expressed proteins. After three cycles of successive affinity chromatography, the differentially expressed proteins from the mixture A was isolated following by immunoprecipitation. The SDS-PAGE analysis demonstrated that the differentially expressed proteins was effectively isolated by this method of "protein subtraction." In the elaboration of the protein subtraction, the experimental procedures were adapted to isolate a recombinant gene product from *E. coli* cells. The human poly (ADP-ribose) polymerase was isolated by the protein subtraction from *E. coli* cells expressing the exogeneous poly (ADP-ribose) polymerase gene.

In many instances of biological studies, it is necessary to identify the cellular proteins that are differentially expressed in quantitative or qualitative way. Cells may express proteins that differ in their time of expression and locations. Differentially expressed proteins might also be observed among the cells under different conditions. Unless the differentially expressed proteins (Pds) are isolated or selected by relatively easy screening procedures, one has to analyze the whole cellular proteins to identify them. For example, heat shock proteins in *E. coli* cells were identified on a two dimensional polyacrylamide gel by comparing protein spots from *E. coli* cell extracts before and after a shift in temperature (Yamamori et al., 1978; Neidhardt et al., 1981). The major shortcoming of this type of procedure is that the purification of Pds in sufficient amount is very difficult and laborious. Another approach is to detect Pds by means of their antigenicity (Kaetzel et al., 1989; Nakayama et al., 1995), but antibodies against Pds are not readily available in most cases. In the present study, we describe a prototype procedure for the protein subtraction which permits a direct selection of Pds from the two defined protein mixtures. The procedure was applied to the isolation of a recombinant gene product from *E. coli* cells.

* To whom correspondence should be addressed.
Tel: 82-42-869-4017, Fax: 82-42-869-2610

Materials and Methods

Reagents

[^{35}S]-methionine (>1,000 Ci/mmol) was obtained from Amersham (UK). Isopropyl-β-D-thiogalactoside (IPTG), Freund's complete and incomplete adjuvant and phenylmethylsulfonyl fluoride (PMSF)-inactivated trypsinogen were purchased from Sigma Chemical Company (USA). All other chemicals were of analytical grade.

Preparation of E. coli cell extracts

Human poly(ADP-ribose) polymerase gene (Kurosaki, et al., 1987) was cloned into the expression vector, pGEM7Zf(+), and transformed to the *E. coli* strain BL21(DE3). The expression of the cloned gene was induced by the addition of IPTG. The cells were grown to mid-log phase in 50 ml of LB media at 37℃ with or without the addition of 2 mM IPTG and were collected by centrifugation at 700 x g for 10 min at 4℃. The cell pellets were disrupted by sonication in 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM PMSF and centrifuged at 1,000 x g for 10 min at 4℃. The supernatant was used as *E. coli* cell extracts. In order to label cellular proteins, [^{35}S]-methionine (100 μCi/ml) was added to the culture media and incubated for 3 h.

Preparation of antibodies

Antisera were raised in rabbits by initial subcutaneous immunization with 5 mg of protein mixture A containing equal amount of bovine serum albumin (BSA), ovalbumin (OVA), α-lactalbumin (LAA), trypsinogen (TRP) and carbonic anhydrase (CA) or with the extracts from *E. coli* cells induced to produce poly (ADP-ribose) polymerase. Booster injection of antigens was given at 4 weeks after primary injection and at two week intervals thereafter. Rabbit antisera were collected 5 days after the last injection. Immunoglobulins were precipitated from the immune sera with 50% saturation of ammonium sulfate and dialyzed against 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.5% (v/v) Tween-20.

Preparation of affinity column

Protein mixture (A-Pd) was prepared by omitting one protein from the mixture A. *E. coli* cell extracts (human poly (ADP-ribose) polymerase was not induced) were dialyzed against 1 l of 100 mM H_2CO_3 buffer (pH 8.3) containing 500 mM NaCl for 12 h at 4℃. Protein mixture (A-Pd) or the cell extracts containing 10 mg of protein was added to 300 mg of CNBr-activated Sepharose 4B and stirred gently for 3 h. Each antigen-Sepharose preparation was then packed into a column (0.5×2 cm) and the column was equilibrated with 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM NaCl and 0.5% (v/v) Tween-20.

Isolation of differentially expressed proteins

The antibodies (~10 mg) against protein mixture A or *E. coli* cell extracts were adjusted to 10 mM Tris-HCl (pH 7.4) and 0.5% (v/v) Tween-20 and were loaded to the corresponding antigen-Sepharose column respectively. The unbound antibodies passed through the column were collected (flow rate, 1 ml/h) and then reapplied onto the same column which were washed with 30 ml of 100 mM glycine buffer (pH 2.2). This subtraction procedure was repeated in cycles. Appropriate number of cycles was chosen after measuring the titers of antibodies against the antigens by ELISA after each subtraction cycle. Antibodies thus purified was used to immunoprecipitate the Pds.

In a model experiment, antibodies against the Pds were isolated after three cycles of the subtraction. Aliquots of antibody solution (1 mg of protein) were incubated with 20 mg of CNBr-activated Sepharose 4B. Each 1 ml of the reaction buffer for immunoprecipitation contains 20 mg of antibody-Sepharose preparation in 10 mM Tris-HCl buffer (pH 7.4) containing 0.5% (v/v) Tween-20. Each antibody-Sepharose preparation was then incubated with 1 mg of protein mixture A for 30 min at 4℃. The suspension was centrifuged, the supernatant was discarded and the pellet was washed three times with 10 mM Tris-HCl (pH 7.4) buffer containing 0.5% (v/v) Tween-20. The bound proteins were eluted from the complex by the addition of 100 µl of 100 mM Na-acetate buffer (pH 3.0). The eluted proteins, which are Pds, were analyzed by 10% SDS-PAGE. The protein bands were visualized by staining with 0.1% Coomassie Blue R-250 solution.

In the next experiment, we attempted to isolate a protein from living cells by using the procedure of protein subtraction. The human poly (ADP-ribose) polymerase was chosen as a Pd and the antibodies against the human poly (ADP-ribose) polymerase were prepared after five cycles of subtraction. The antibody solution (contains 1 mg protein) was incubated for 30 min at 4℃ with ^{35}S-methionine labeled cell extracts (100 µCi/ml) from *E. coli* cells induced to produce the cloned poly (ADP-ribose) polymerase. To the 3 ml of sample mixture, was added 10 µg of Protein A resin. After incubation for 30 min at 4℃ with gentle agitation, the suspension was centrifuged to precipitate the antibody-Pd complex bound to Protein A resin. The pellet was washed three times with 1 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 0.5% (v/v) Tween-20. To the sample pellet, was added 50 µl of SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.75), 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. The sample mixture was centrifuged and the proteins in the supernatant were analyzed by 10% SDS-PAGE. The purity of human poly (ADP-ribose) polymerase isolated from *E. coli* cells by the protein

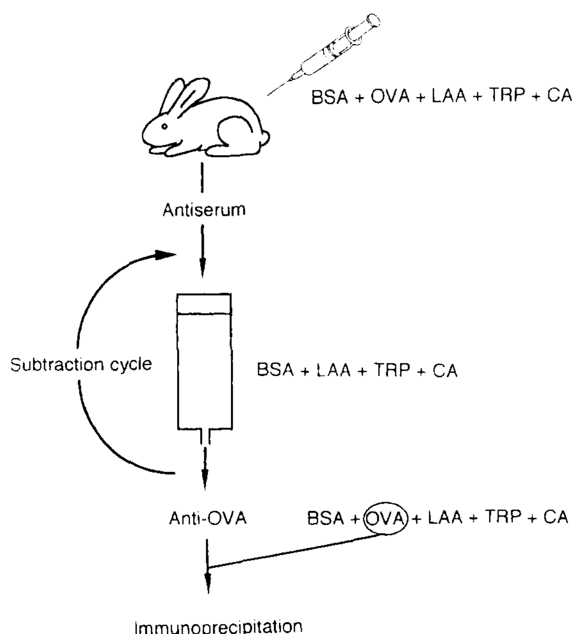

Fig. 1. Schematic representation of protein subtraction procedure to isolate Pds from two sets of protein mixture. Protein mixture A comprises of BSA, OVA, LAA, TRP and CA. Protein mixture (A-Pd) has the same composition as the mixture A except that the protein chosen as a Pd is omitted. LAA, α-lactalbumin; TRP, trypsinogen; CA, carbonic anhydrase; OVA, ovalbumin; BSA, bovine serum albumin; Pd, differentially expressed proteins.

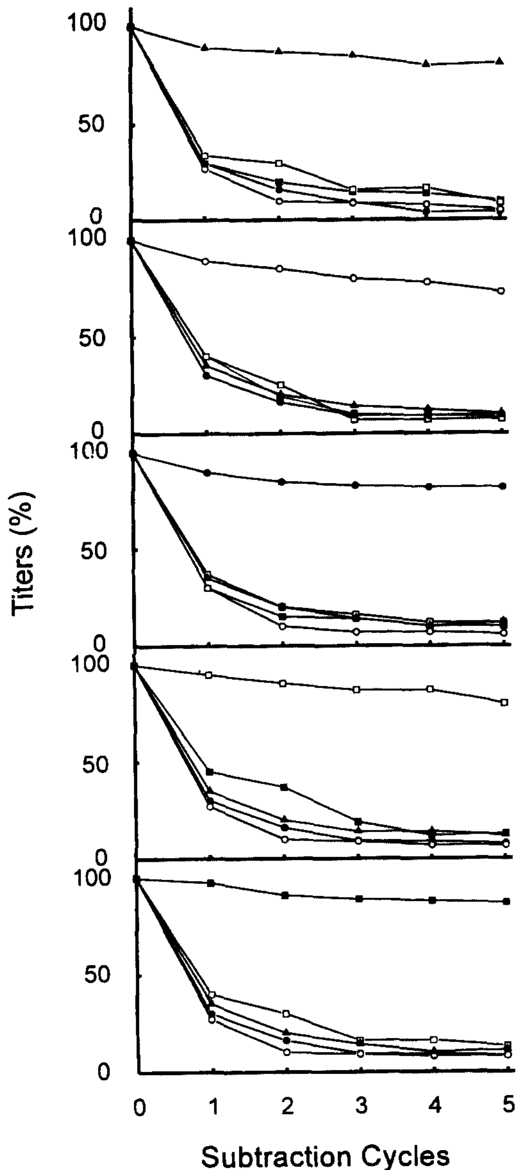

Fig. 2. Efficiency of protein subtraction. Rabbit antisera against protein mixture A were applied onto each affinity column in which proteins in the mixture (A-Pd) were crosslinked to the column matrix. The specificities of antibodies against each antigen protein were measured by ELISA after the subtraction. The concentrations of antibodies specific to each antigen protein were expressed as percent titer. The titer of antibodies prior to the subtraction was regarded as 100 percent. The α-lactalbumin, trypsinogen, carbonic anhydrase, ovalbumin or bovine serum albumin was chosen as a differentially expressed proteins in (A), (B), (C), (D) or in (E) respectively. ▲, α-lactalbumin; ○, trypsinogen; ●, carbonic anhydrase; □, ovalbumin; ■, bovine serum albumin.

subtraction was examined by SDS-PAGE and autoradiography.

Results

The strategy of isolating Pds by protein subtraction was illustrated in Fig. 1. Two sets of protein mixtures, namely A and (A-Pd) were prepared for a model experiment. Protein mixture A was composed of bovine serum albumin (BSA), ovalbumin (OVA), α-lactalbumin (LAA), trypsinogen (TRP) and carbonic anhydrase (CA). The mixture (A-Pd) was prepared by taking out one protein as a Pd from the mixture A. Rabbit antibodies against the mixture A were passed through the affinity column in which the proteins of (A-Pd) were immobilized on Sepharose 4B. The unbound antibodies were eluted and reapplied onto the affinity column. This procedure was repeated in cycles.

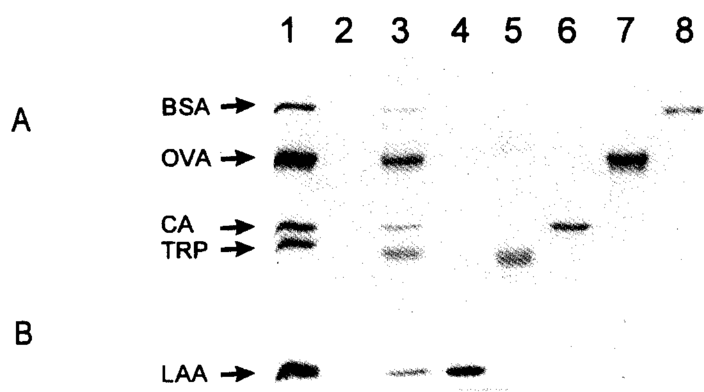

Fig. 3. Isolation of differentially expressed proteins by protein subtraction. Each differential protein in protein mixture A was isolated by immunoprecipitation using the subtracted antibodies. The five distinctive proteins in the mixture A were analyzed in 10% SDS-PAGE (lane 1). Preimmune sera did not react with any proteins in the mixture A (lane 2) but the immune serum bound to all the proteins in the mixture A (lane 3). The antibodies against each differential protein were subtractively purified by using the affinity column in which the following proteins were immobilized: lane 4, protein mixture (A-LAA); lane 5, protein mixture (A-TRP); lane 6, protein mixture (A-CA); lane 7, protein mixture (A-OVA); lane 8, protein mixture (A-BSA) was crosslinked to Sepharose 4B.

Efficiency of the protein subtraction was shown in Fig. 2. The specificities of the antibodies against the proteins in the mixture A were measured after each run of the subtraction cycle. The antibody concentrations were determined by ELISA and expressed as percent of titers. The data show that the titers of subtractively purified antibodies against the Pds were enriched as the subtraction cycles were repeated. Thus this protocol allows the isolation of respective antibodies against the Pds. The titers of specific antibodies beyond three cycles of subtraction remained virtually the same high level while the titers of nonspecific antibodies were substantially low. Fig. 3 shows the SDS-PAGE profiles of each Pd isolated by protein subtraction method. The immune sera before the protein subtraction reacted with all the five proteins (lane 3) while the subtracted antibodies reacted only with the corresponding Pds (lanes 4-8).

To apply this procedure to a living system, we designed an experiment to isolate a recombinant gene product from *E. coli* cells. The antisera were collected from rabbits injected with *E. coli* cell extracts containing the human poly (ADP-ribose) polymerase. The cellular proteins obtained from *E. coli* cells which were not induced to produce the poly (ADP-ribose) polymerase, were crosslinked to CNBr-activated Sepharose

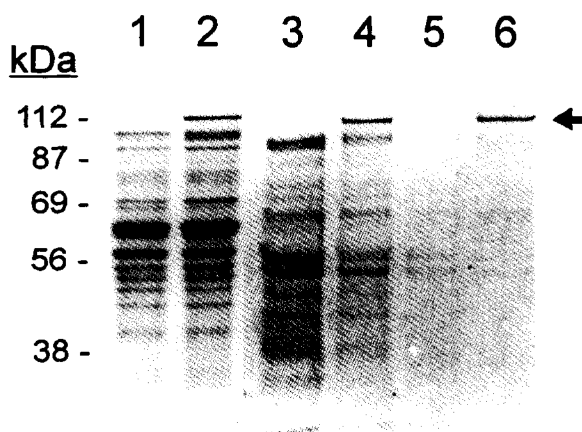

Fig. 4. Isolation of recombinant gene product from *E. coli* cells by protein subtraction. Proteins of *E. coli* cells were metabolically labeled with [^{35}S]methionine. After the induction of poly(ADP-ribose) polymerase by the addition of 2 mM IPTG, protein subtraction was performed to isolate the antibodies against the cloned poly(ADP-ribose) polymerase. The subtracted antibodies were used to immunoprecipitate the radiolabeled proteins of *E. coli* cells. Cellular proteins from *E. coli* cells before (lane 1) and after IPTG induction (lane 2) were analyzed in 10% SDS-PAGE followed by autoradiography. The rabbit antisera against the cell extracts after IPTG induction were collected to immunoprecipitate the radiolabeled proteins of control cells (lane 3) or proteins of cells producing poly (ADP-ribose) polymerase (lane 4). The subtracted antisera were reacted with proteins of control cells for the immunoprecipitation (lane 5), or with those producing poly (ADP-ribose) polymerase (lane 6). Arrow head indicates poly (ADP-ribose) polymerase.

4B. The antibodies against the Pd, which is human poly (ADP-ribose) polymerase expressed in *E. coli* cells, were subtractively isolated. The subtracted antibodies were reacted with proteins of control cells for the immunoprecipitation (Fig. 4, lane 5), or with those producing poly (ADP-ribose) polymerase (Fig. 4, lane 6). The data show the poly(ADP-ribose) polymerase was successfully isolated from *E. coli* cells after the successive subtraction cycles.

Discussion

The Pds of biological interest have been generally identified on the basis of their relative abundance. Attempts have been made to identify Pds on the basis of their antigenicity (Beug, et al., 1979; Kornfeld et al., 1983). For example, Beug et al. (1979) have adapted immunoadsorption procedure to purify specific antisera. By using the antisera against the specific antigens, they were able to detect the differentiation-specific surface antigens on transformed chicken hematopoietic cells. In the extension of these antibody based methods, we developed a new method of isolating Pds by proteins subtraction. The Pds were successfully isolated in a defined system as well as in a living system using *E. coli* cells. Our results suggest that this method might permit a direct detection and purification of proteins that are differentially expressed in certain cell types or under certain biological conditions. The efficiency of this protocol mainly depends on the enrichment step at which the specific antibodies against the Pds are subtractively purified. Any possible appearance of false-positive Pds due to the insufficient subtraction could be avoided by increasing the number of subtraction cycles (Fig. 2).

The major advantage of this method is that this procedure leads to a simple purification of Pds under nondenaturing conditions. It should be emphasized that this procedure can identify Pds not only by their relative abundance but also by the change of their antigenicity. Therefore this method can be used as a general method for isolating Pds that differ in their abundance or antigenicity between the experimental and control cells.

Acknowledgments

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