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Short Communication

Procedure for refolding and purification of recombinant proteins from *Escherichia coli* inclusion bodies using a strong anion exchanger

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Abstract

Using *Escherichia coli* system expressing papilloma virus HPV16 E7MS2 fusion protein as a model system, a novel procedure was applied to solubilize, purify and refold recombinant proteins from *E. coli* inclusion bodies. The necessity to reactivate proteins at low protein concentrations (owing to their tendency to aggregate at high concentrations) was overcome by solubilization of inclusion bodies in alkaline solution and immobilization of proteins on a strong and resistant anion exchanger. This procedure has an inherent advantage of combining refolding and purification procedures in one step. The solubilization of the fusion protein in an alkaline reagent with the use of an anion exchanger resulted in considerable purification of the recombinant protein at a fairly high concentration. The protein was soluble under mild conditions and reacted with antibodies against the "native" papilloma virus.

1. Introduction

An advantage of protein engineering is the ability to use prokaryotic organisms to overexpress mutant proteins. Many proteins produced in *Escherichia coli* accumulate in inclusion bodies (IB) [1]. A great part of the protein contained within these inclusion bodies is in a denatured form; dimers and higher-molecular-mass multimers may be present. Inclusion bodies are largely resistant to proteolysis, and entrapment of expressed proteins into IBs may protect them from further degradation. Solubilization of IB under denaturing conditions, denaturation and reduction, with a subsequent renaturation procedure to promote the formation of the correct intramolecular disulphide bonds and a native conformation of fusion proteins, are usually required [2–4].

Correct folding, formation of native disulphide bonds and the proper association of different domains are strongly dependent on the renaturing conditions used. A common observation has been that the final yield of renatured protein decreases, sometimes drastically, with increasing concentration of the solubilized protein undergoing renaturation, caused mainly by hydrophobic interactions of the denatured peptide chains [5,6].

Using an *E. coli* system expressing papilloma

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virus HPV16 E7MS2 fusion protein as a model system, we have overcome the necessity to reactivate proteins at low protein concentrations (owing to their tendency to aggregate at high concentrations) by solubilization of IB in alkaline solution and immobilizing the proteins on a strong anion exchanger. The fusion protein HPV16 E7MS2 contains the whole amino acid sequence of E7 protein of human papillomavirus type 16 except for the first eight amino acids fused to 100 N-terminal amino acids of the bacteriophage MS2 polymerase [7]. Because there is no method available to grow papillomaviruses *in vitro*, the preparation of a recombinant protein is one of the few possibilities for obtaining this protein and for studying the antibody response directed towards this oncoprotein. Moreover, the protein is necessary for preparing hyperimmune polyclonal and monoclonal antibodies to detect this protein in HPV16 transformed cells or vaccinia virus recombinants expressing HPV16 E7.

2. Experimental

2.1. Preparation of soluble and insoluble cell fractions

All procedures were carried out at 4°C unless indicated otherwise. *E. coli* cells containing overexpressed fusion protein HPV16 E7MS2 were harvested by centrifugation. A 1-g amount of cell pellets was resuspended with 3 ml of buffer A (10 m*M* Tris–10 m*M* NaCl–10 m*M* dithiothreitol–1 m*M* phenylmethylsulphonyl fluoride, pH 7.5) and lysed by sonication on ice. Soluble and insoluble cell lysates were collected by centrifugation (30 min at 15 000 *g* and 4°C). Sediment was resuspended with 5 ml of buffer B (50 m*M* Tris–0.1% sodium desoxycholate–0.02% lysozyme–1 m*M* EDTA, pH 7) and incubated for 1 h. The suspension was centrifuged and resuspended with 9 ml of buffer A. The resulting suspension of washed inclusion bodies was stored at −80°C until further analysis.

2.2. Solubilization of inclusion bodies

To solubilize the IB we employed three commonly used solubilization agents: 8 *M* urea, 1% sodium dodecyl sulphate (SDS) and 0.01 *M* NaOH.

A 1-ml volume of the IB suspension was slowly added to 10 ml of stirred solubilization agent and the stirring was continued for 1 h. The resulting slightly cloudy solutions were centrifuged at 15 000 *g* for 40 min at 12°C. Sediments and supernatants were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

2.3. Refolding of solubilized recombinant protein

Inclusion body proteins solubilized in the respective agents were dialysed against 50 m*M* Tris–HCl (pH 8.0) (containing 0.01% of mercaptoethanol) at concentrations of about 10 μg/ml in a refrigerator. Dialysates were centrifuged and supernatants applied to a Mono Q column.

Inclusion body proteins solubilized in 0.01 *M* NaOH were either dialysed or also applied directly (without previous dialysis) to a Mono Q 5/5 column equilibrated in the starting buffer [20 m*M* Tris (pH 8.0)] and eluted with a gradient of NaCl.

2.4. Protein analyses

Recombinant proteins were analysed by SDS-PAGE [8,9] and immunoblot analysis [10]. Polyacrylamide gels (exponential gradient, 5–15% of acrylamide) were prepared and the gels were stained with Coomassie Brilliant Blue or with ammoniacal silver according to Sammson *et al.* [11]. Standard proteins transferred to a nitrocellulose sheet were stained with colloidal silver [12].

2.5. Chromatographic purification of viral recombinant proteins

Solubilized, either refolded by dialysis or without dialysis in 0.01 *M* NaOH, recombinant viral

proteins were loaded (about 3 mg of proteins) on an HR 5/5 Mono Q column (Pharmacia) pre-equilibrated with the same buffer. The fractions were eluted at a flow-rate of 1.0 ml/min with a linear gradient from 0 to 0.5 *M* NaCl in 30 min in the same buffer. Chromatographic fractions of 1.0 ml were collected and tested by dot-blotting and by SDS-PAGE and immunoblotting for the presence of recombinant proteins.

3. Results and discussion

The protein composition in isolated inclusion bodies is illustrated in Fig. 1, lane 5. The main contaminants presented on the electropherogram slightly above the recombinant fusion protein HPV16 E7MS2 were almost insoluble in 8 *M* urea and in 0.01 *M* NaOH, whereas solubilization of IB in 1% SDS resulted in dissolution of almost all proteins present in IB. As the recombinant protein was basically completely dissolved in all three solubilization reagents tested, only the use of first two reagents resulted in some degree of purification without lowering the yield of the recombinant protein.

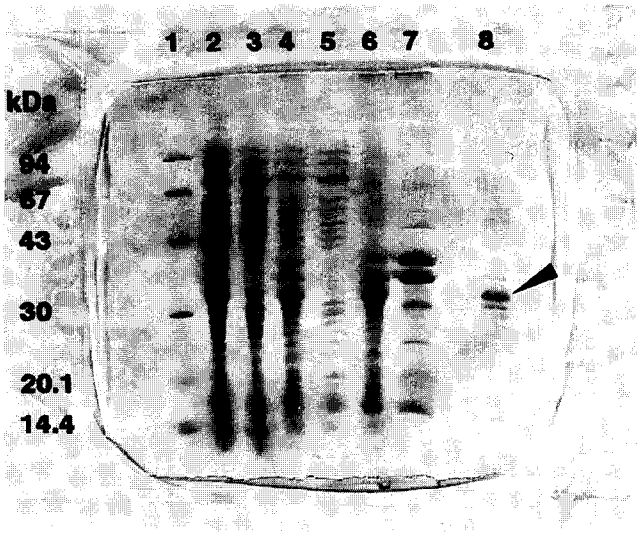

Fig. 1. SDS-PAGE of reduced samples with an exponential gradient, 5–15%, and Coomassie Brilliant Blue staining. Lanes: 1 = low-molecular-mass standard (Pharmacia) (M_r 94 000, 67 000, 43 000, 30 000, 20 100 and 14 400); 2 = total bacterial lysate; 3 = supernatant of bacterial cells after sonication; 4 = sediment of bacterial cells after sonication; 5 = supernatant of sediment (4) treated with 50 m*M* Tris–0.1% sodium desoxycholate–0.02% lysozyme, 1 m*M* EDTA (pH 7); 6 = isolated inclusion bodies (IB); 7 = IB solubilized in 0.01% NaOH (sediment); 8 = IB solubilized in 0.01% NaOH (supernatant). The arrow shows the position of the recombinant protein identified on Western blotting with polyclonal antibodies specific for HPV16 E7 protein.

Chromatography of solubilized proteins in 0.01 *M* NaOH is shown in Fig. 2. To refold the bound proteins (immobilized and thus prevented from any intermolecular interactions and aggregation), the column with bound proteins was washed with the starting buffer containing 1% dithiothreitol (DTT) at pH 7.5, *i.e.*, below the pH at which the two thiol groups in DTT can ionize and become negatively charged. Binding of DTT to the anion exchanger was thus avoided. The recombinant protein could then be eluted by increasing the salt concentration in the elution buffer at a fairly high concentration of the protein (nearly 1 mg/ml) and with very good resolution. Virtually identical separations were obtained using samples of recombinant proteins solubilized in 8 *M* urea and 0.01 *M* NaOH dialysed against 50 m*M* Tris–HCl (pH 8.0).

The results indicate that combining the

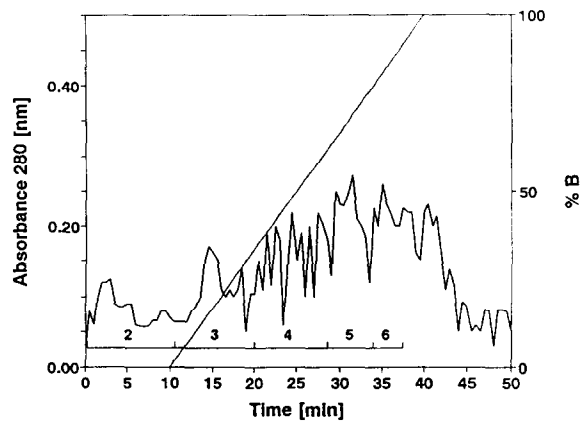

Fig. 2. Chromatography of inclusion body proteins solubilized in 0.01 *M* NaOH. Column, Mono Q HR 5/5; buffer A, 20 m*M* Tris–HCl (pH 8); buffer B, 0.5 *M* NaCl in buffer A; gradient, 100% buffer A to 100% buffer B in 30 min (dotted line); flow-rate, 1 ml/min; detection, absorbance at 280 nm (solid line). Maximum immunoreactivity on dot-blots with polyclonal antibodies specific for HPV16 E7 protein was found in fraction 5.

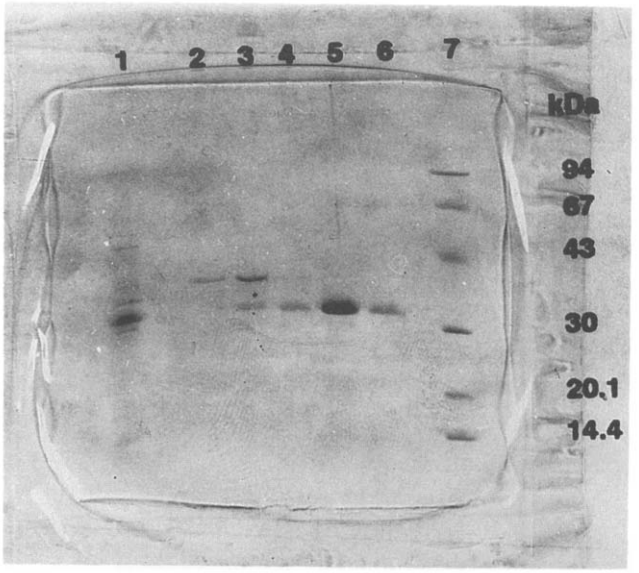

Fig. 3. SDS-PAGE of chromatographic fractions. Conditions as in Fig. 1. Lanes: 1 = injected sample; 2–6 = chromatographic fractions indicated in Fig. 2; 7 = low-molecular-mass standard.

solubilization in an alkaline reagent with the use of an anion exchanger may result in considerable purification of the recombinant protein and at a satisfactorily high concentration. This procedure has the inherent advantage of combining refolding and purification procedures in one step.

4. Acknowledgements

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