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A Simple and Rapid Procedure for the Large Scale Purification of the recA protein of *Escherichia coli**

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A simple and rapid three-step procedure for the large scale purification of the recA protein of *Escherichia coli* is described. The method depends primarily on a single chromatographic step which is highly specific for recA protein: elution by ATP from single-stranded DNA cellulose. With this procedure, gram quantities of recA protein, greater than 99% pure, can be reproducibly prepared for biochemical and biophysical analysis.

The recA gene product of *Escherichia coli* (recA protein) is required for homologous genetic recombination and for the postreplicative repair of damage to DNA (1, 2). It is also involved in the regulation of DNA repair, mutagenesis, cell division, and prophage induction ("SOS functions") (1, 2). Because of its central role in these processes, the recA protein has become the object of intensive study. The recA protein is also a valuable reagent for the site-specific mutagenesis of DNA *in vitro* (3). Clearly, a simple and easily reproducible purification procedure for recA protein would be of value in several areas of current biochemical interest.

Several procedures for the purification of recA protein have been published (4–6), all of which involve elution from phosphocellulose or DNA-cellulose with solutions of increasing ionic strength. Although large amounts of recA protein have been prepared by these methods, the purity of the final product has been variable.

It has recently been found that, in the presence of ATP, or any of several other nucleotides, recA protein rapidly dissociates from single-stranded DNA.[1] This property has been exploited in the development of a new purification procedure for this protein that is highly selective, very simple, suitable for large scale preparations, and yields homogeneous enzyme. This procedure and its implications are the subject of this report.

EXPERIMENTAL PROCEDURES

Materials

Strain KM1842 which overproduces recA protein has been described elsewhere (4). In addition to its *recA* genotype and the *spr* and *sfi* alleles, the strain is auxotrophic for leucine, threonine, and proline, and is also *xyl*$^-$, *mtl*$^-$, *lac*$^-$, and *str*^{R}. Ardamine 2 yeast extract was obtained from Yeast Products, Patterson, NJ. Cerelose was purchased from The Saroni Sugar Co., Emeryville, CA.

Polymin P and Whatman phosphocellulose (P11) were prepared as described previously (7). Single-stranded DNA cellulose was prepared according to Alberts and Herrick (8). Brij 35 was purchased from Pierce Chemical.

Electrophoretic reagents (acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, dithiothreitol, bromophenol blue, Coomassie blue) were from Bio-Rad. Ultrapure ammonium sulfate was from Schwarz/Mann.

Single-stranded bacteriophage φX174 DNA was a gift from J. Kobori of this department. ^{3}H-labeled dUTP was purchased from Amersham. Unlabeled dUTP was purchased from Sigma.

R buffer is 20 mM Tris·HCl, 80% cation (pH 7.5), 10% (w/v) glycerol, 1 mM dithiothreitol, and 0.1 mM EDTA. P buffer contains 20 mM potassium phosphate, 50% dianion (~pH 6.8), replacing the Tris·HCl.

Methods

RecA protein was measured in three ways: dUTPase activity, a radioimmune assay, and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

dUTPase Activity—Assay of the dUTPase activity of recA protein has been described previously (4). Reaction mixtures (60 μl) contained 20 mM sodium maleate (pH 6.2), 5% (v/v) glycerol, 10 mM KCl, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM dithiothreitol, 2 mM potassium phosphate, 20% dianion (pH 6.2), 35 μM φX174 single-stranded DNA (nucleotide), 1 mM [^{3}H]dUTP (67 μCi/ml), and 2–6 μM recA protein. Reactions were carried out at 37 °C. Under these conditions the dUTPase activity is linearly dependent upon enzyme concentration, and the turnover number is 16/recA monomer/min. A unit of activity is that amount which will hydrolyze 1 μmol of dUTP/min.

Radioimmune Assay—This method, based on the method of Miles *et al.* (9) with modifications made by Stark *et al.*,[2] can detect recA protein concentrations as low as 1 ng/ml.

Electrophoretic Assay—Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as described (10). Gels were stained in a solution containing 25% (v/v) 2-propanol, 10% (v/v) acetic acid, and 250 μg/ml of Coomassie brilliant blue R-250 for 4 h at 37 °C. They were destained in 10% (v/v) 2 propanol, 10% (v/v) acetic acid for 2.5 h at 37 °C and then immediately scanned spectrophotometrically using a Helena Laboratories "Quick Scan Jr." equipped with an integrator.

Protein concentrations were determined by the Coomassie method of Bradford (11) or by measurement of A_{280}. The $E^{1\%}_{280}$ for recA protein was taken as 5.16.[3]

RESULTS

Purification of recA Protein—The recA protein was purified from cells (KM1842) containing high levels of the protein as a consequence of a *spr* mutation in the *lexA* gene, a multicopy plasmid carrying the *recA* gene (12), and treatment with nalidixic acid. Under normal growth conditions this

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[2] Van der Zeijst, B. A. M., Noyes, B. E., Mirault, M.-E., Parker, B., Osterhaus, A. D. M. E., Swyryd, E. A., Bleumink, N., Horzinek, M. C., and Stark, G. R., as cited by C. Paoletti, manuscript in preparation.

[3] G. M. Weinstock, K. McEntee, and I. R. Lehman, manuscript in preparation.

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strain produces 200,000–300,000 molecules of recA protein/cell before induction as determined by the radioimmune assay. Throughout the purification, recA protein was monitored by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Figs. 1 and 2).

Cells were grown at 37 °C in 300 liters of AZ broth, which contained, per liter: 10 g of K_2HPO_4, 1.85 g of KH_2PO_4, 10 g of Ardamine Z yeast extract, 10 mg of thiamine, 50 mg of thymine, 10 g of Cerelose; with the pH maintained between 7.1 and 7.3 by addition of NaOH. Cells were grown to an A_{595} of about 1, treated with nalidixic acid (40 μg/ml) for 90 min, and then harvested in a Sharples continuous flow centrifuge. The cell paste was suspended in cold 50 mM Tris·HCl, 80% cation (pH 7.5), 10% sucrose to an A_{595} of ~400 and then quickly frozen in liquid nitrogen. The frozen cell paste was stored at −20 °C.

The procedure for lysis and selective extraction of recA protein from polymin P described previously (4) was followed with minor modifications. To 300 g of frozen cell paste was added 200 ml of 25% (w/v) sucrose, 0.25 M Tris·HCl, 80% cation (pH 7.5). The cells were allowed to thaw at 4 °C with occasional stirring. All subsequent steps were carried out at 4 °C. Once thawed, 96 ml of 5 mg/ml of lysozyme in 0.25 M Tris·HCl, 80% cation (pH 7.5), was added, followed by 160 ml of 25 mM EDTA, and finally 800 ml of 1% Brij-35 in 50 mM Tris·HCl, 80% cation (pH 7.5), 2 mM dithiothreitol. The suspension was stirred for 30 min after each addition. The resulting lysate was centrifuged for 90 min at 13,000 rpm in a Beckman JA-14 rotor. The slightly turbid supernatant (1420 ml) is fraction I.

Polymin P (10% (v/v), pH 7.5) was added slowly to fraction I to a final concentration of 0.5%. The suspension was stirred for 30 min and then centrifuged for 15 min at 9000 rpm in a Beckman JA-14 rotor. The pellets were resuspended in 360 ml of R buffer containing 150 mM ammonium sulfate with the aid of a Waring Blendor. The suspension was stirred for 30 min and centrifuged as before. The resulting pellets were resuspended in 280 ml of R buffer containing 300 mM ammonium sulfate, and again stirred and centrifuged. RecA protein appeared in the supernatant fraction. After re-extracting the pellets with 140 ml of the same solution, the supernatants were combined and solid ammonium sulfate (0.28 g/ml) was added. The suspension was kept on ice for 4–12 h. The precipitate was collected by centrifugation at 13,000 rpm for 20 min, dissolved in 100 ml of P buffer containing 200 mM NaCl, and dialyzed for 8 h against 18 liters of the same buffer to generate Fraction II (158 ml).

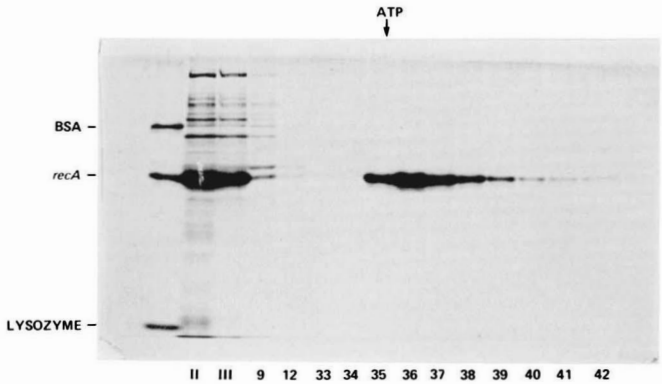


FIG. 1. **Purification of recA protein by ATP elution from single-stranded DNA-cellulose.** Fractions of recA protein were analyzed by electrophoresis on a polyacrylamide gel (11%) in the presence of sodium dodecyl sulfate. Enzyme fractions II and III are run in *lanes 2* and *3*. Other numbers refer to column fractions (14 ml) generated during chromatography on single-stranded DNA-cellulose. Fractions 9 and 12 represent part of the flow-through. The *arrow* indicates the fraction in which ATP is first detected. *BSA*, bovine serum albumin.

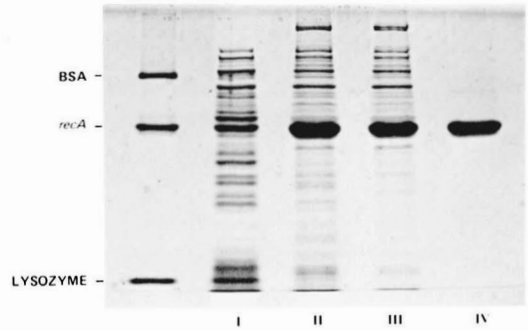


FIG. 2. **Sodium dodecyl sulfate-gel electrophoresis of purification fractions.** Fractions I, II, III, and IV were electrophoresed on an 11% polyacrylamide gel in the presence of sodium dodecyl sulfate as described under "Methods." The *final lane* contains 25 μg of Fraction IV. *BSA*, bovine serum albumin.

Fraction II was applied to a phosphocellulose column (6.3 × 7.4 cm) equilibrated with P buffer containing 200 mM NaCl. The flow-through fraction was dialyzed into P buffer to generate fraction III (205 ml, 5.1 mg of protein/ml, based on A_{280}).

Part of fraction III (25 ml) was loaded onto a single-stranded DNA cellulose column (3.8 × 12.5 cm) equilibrated with P buffer. The column was then washed in succession with 10 ml of P buffer, 2 column volumes of P buffer containing 50 mM NaCl, and then 1 column volume of P buffer containing 50 mM NaCl and 1 mM ATP, followed by the same buffer without ATP. RecA protein was quantitatively eluted by the P buffer containing ATP. Peak fractions were located by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 1) and pooled. RecA protein binds ATP tightly and precipitation with ammonium sulfate is required to remove it. Solid ammonium sulfate (0.28 g/ml) was added to the pooled peak fractions. The suspension was kept on ice for 4–12 h, centrifuged, resuspended in R buffer containing 0.28 g/ml of ammonium sulfate, and centrifuged again. The pellet was dissolved in R buffer and dialyzed extensively into R buffer to generate fraction IV (13 ml) (Table I). The ratio, A_{280}/A_{260} was 1.67 and the protein concentration was 4.03 mg/ml.

Fraction IV contained no detectable endo- or exonuclease activities as determined by its inability to convert ^{3}H-labeled single-stranded or double-stranded DNA to an acid-soluble form or to convert superhelical DNA (φX RF I) to a relaxed form (data not shown). Both fractions III and IV could be stored for at least 3 months at −80 °C with no loss of any of the activities associated with recA protein.

The phosphocellulose step results in only a slight purification, but is necessary to remove polymin P which interferes with DNA-cellulose chromatography. As illustrated in Fig. 1, recA protein is eluted rapidly and specifically from DNA-cellulose by 1 mM ATP. Identical results have been obtained with 0.5 mM ATP (data not shown). In the absence of ATP, recA protein is eluted from single-stranded DNA-cellulose at 140–180 mM NaCl and contains several contaminants not present following elution with ATP.

Although there is a substantial increase in specific activity as judged by assays of UTPase activity (Table I), the specific elution of recA protein from DNA-cellulose by ATP is best illustrated by comparison of the sodium dodecyl sulfate-polyacrylamide gel patterns obtained before and after this step (Fig. 2). Fraction IV is 99.4% pure as determined by quantitative scanning of sodium dodecyl sulfate-polyacrylamide gels.

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TABLE I

Purification of recA protein from E. coli KM1842

Step	Total protein[a]	Total recA protein[b]	recA protein[c]	Yield of recA protein	dUTPase activity	Specific activity[d]	Specific activity[d]
	g	*g*	*% of total*	*%*	*units*	*units/mg protein*	*units/mg recA protein*
I. Extract	17.8	3.86	10.6				
II. Ammonium sulfate	2.8	1.43	24.7	37.0			
III. Phosphocellulose	2.2	1.26	27	32.6	615	0.27	1.01
IV. Single-stranded DNA cellulose[e]	0.43	0.89	(100)	23.1	468	1.09	1.09

[a] Measured by the Coomassie staining assay. This assay yields values for recA protein that are lower by a factor of 0.48 than those obtained by A_{280} measurements.

[b] Measured by the radioimmune assay, standardized with pure recA protein, whose concentration was determined by absorption at A_{280}. Note the discrepancy with the determination by Coomassie staining for fraction IV.

[c] Values for total recA protein were decreased by a factor of 0.48 to agree with the Coomassie staining assay for fraction IV.

[d] These values are based on protein concentrations measured by Coomassie staining.

[e] Only 12% of fraction III was applied to the single-stranded DNA cellulose column. The yield of enzyme was adjusted accordingly.

The one impurity that is evident in every preparation migrates with the β subunit of *E. coli* RNA polymerase and has a molecular weight of approximately 150,000. This impurity copurifies with recA protein through at least six different chromatographic steps, including DEAE-cellulose, hydroxylapatite, blue dextran-Sepharose, and Sepharose 6B. No enzymatic activity has thus far been associated with this protein.

Two other impurities, with molecular weights of approximately 60,000 and 110,000, are occasionally present in trace amounts. When necessary, these contaminants can be removed by chromatography on blue dextran-Sepharose. Fraction IV is applied to a column of blue dextran-Sepharose (1 mg of recA protein/ml) in R buffer. Both recA protein and the impurities are adsorbed under these conditions. About 30% of the bound recA protein is recovered by elution with R buffer containing 200 mM NaCl, and another 40% can be eluted with R buffer containing 2 M NaCl. The two impurities appear only in the second fraction.

DISCUSSION

The purification procedure described here can easily be scaled up to produce gram quantities of pure recA protein for biochemical and biophysical analysis. Only three steps are involved, one batch and two chromatographic steps, neither of which involves gradient elution. The recA protein purified by this procedure can catalyze all of the reactions associated with this enzyme, including strand annealing and assimilation, ATP and UTP hydrolysis, and proteolysis of phage λ repressor (4–6).

The procedure depends upon a highly specific interaction of nucleotides with the enzyme. That ATP reduces the affinity of recA protein for single-stranded DNA is probably an important feature of the mechanism of action of this protein in DNA annealing and assimilation reactions. However, release of recA protein from single-stranded DNA can be achieved with ADP, UDP, and dTTP, none of which are hydrolyzed by recA protein.[1] The single-stranded DNA cellulose column contains little or no Mg^{2+}, which further argues against a requirement for ATP hydrolysis for elution of the recA protein. A more plausible explanation is that binding of ATP induces a conformational change in recA protein which alters its affinity for single-stranded DNA. Unlike nonspecific elution which involves an increase in ionic strength to weaken binding, most, if not all of the recA protein that is eluted by ATP binds this nucleotide and is likely to be fully active. We have used only ATP in the purification procedure; other nucleotides that bind to recA protein should be equally effective.

The apparent specificity of the ATP elution of recA protein from single-stranded DNA cellulose suggests that this procedure should be useful in the isolation of recA-like proteins from other sources.

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