Preparative 2-D Electrophoresis System Purifies Recombinant Nuclear Proteins from Whole Bacterial Lysates

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Introduction

This report describes the 2-step isolation of recombinant nuclear proteins from whole bacterial cell lysates. The Rotofor® preparative isoelectric focusing cell was used in the first step of purification. Subsequent purification on a molecular weight basis was accomplished using Bio-Rad's Model 491 prep cell. These 2 techniques, used in combination, completely isolated recombinant proteins from bacterial contaminants.

The nuclear lamins are a family of structural proteins in the range of 60 to 70 kD (Figure 2, lane L) that are found in eukaryotic nuclei. Type A lamins have nearly neutral isoelectric points and are composed of lamins A and C in mammalian cells. Lamins A and C share an extensive portion of their sequence and differ only by the length of their carboxy termini (McKeon et al. 1986, Fisher et al. 1986). Type B lamins have acidic isoelectric points. Nuclear lamins have been identified as targets for human autoantibodies, but the extent to which these reactions are specific to certain autoimmune diseases has not been completely clarified (Senecal and Raymond 1992).

Further studies of the specificity of autoantibodies require an abundant source of highly purified lamins A, B, and C of human origin. For this purpose, prokaryotic expression vectors were prepared harboring individual cDNAs for human lamins A and C (McKeon et al. 1986) and B (Pollard et al. 1990). Preliminary experiments showed that the bulk of bacterial proteins have isoelectric points below pH 7. During isoelectric focusing, most bacterial proteins focus in the acidic region of the pH gradient, well separated from recombinant human lamins A and C, which focus toward the alkaline end of the pH gradient. We therefore envisaged the use of the Rotofor cell as a primary fractionation step for preparative purification of these lamins from whole bacterial lysates.

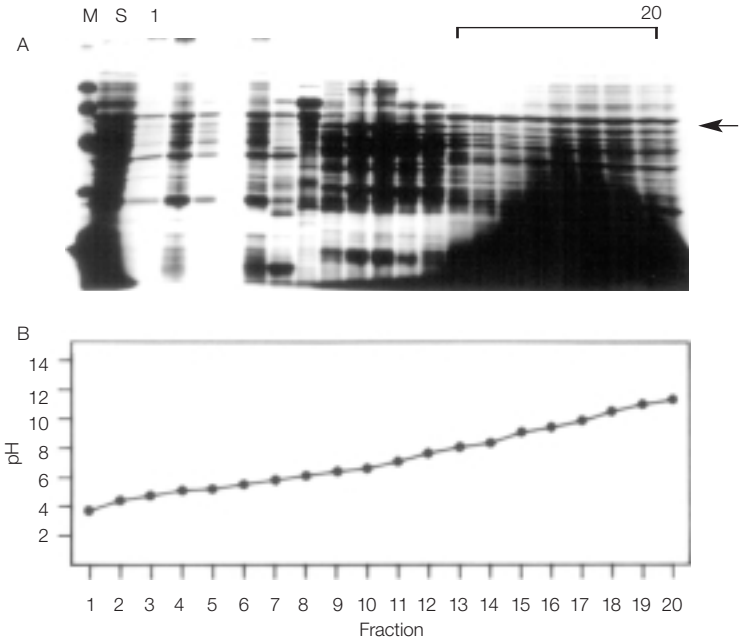

Fig. 1. Purification of recombinant human lamin C by preparative isoelectric focusing. A, 10% polyacrylamide gel of the starting material (whole bacterial lysate, lane S) and of Rotofor fractions 1–20 after staining with Coomassie Blue. The position of lamin C is indicated by the arrow. Fractions 13–20 were pooled for further purification on the Model 491 prep cell. B, pH gradient for Rotofor fractions 1–20.

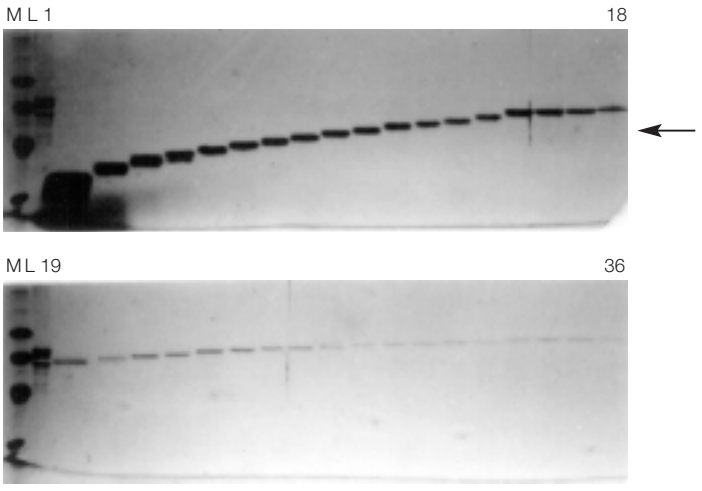

Fig. 2. Further purification of proteins. The contents of every third fraction eluted from the prep cell were analyzed on two 8% polyacrylamide gels. Lane M, molecular weight markers; lane L, rat liver nuclear lamins used as markers (lamins A, B, and C from top to bottom).

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Methods

Protein Production in *E. coli*

DNA sequences coding for human lamin C were subcloned in the *Nco*I-*Bam*HI cloning site of a pET-11d translation vector bearing the bacteriophage T7 promoter (Studier et al. 1990). Plasmids were transformed into a BL21 (DE3)pLysS host (purchased from Novagen, Inc.) Bacteria were grown in Luria broth in the presence of carbenicillin at 100 µg/ml and chloramphenicol at 34 µg/ml until they reached an A_{600} of 0.6. Synthesis of the recombinant lamin C was induced by the addition of IPTG at 0.4 mM and cultures were incubated for a further 3 hr at 37°C. Bacteria were harvested by centrifugation at 6,000 x g for 15 min and pellets were processed immediately or stored at -80°C.

Isoelectric Focusing on the Rotofor Cell

A bacterial pellet equivalent to 400 ml of the induced culture was dissolved in 7 ml of 10 M urea, extensively sonicated with a tip sonifier, and ultracentrifuged at 70,000 x g for 30 min. The supernatant, containing approximately 120 mg of protein, was adjusted to 8 M urea and 1% Bio-Lyte® ampholytes (pH range 3–10) in a final volume of 55 ml. Focusing was carried out at 12 W constant power for 4.5 hr at room temperature with cooling provided by running tap water. The initial conditions were 400 V and 30 mA. At equilibrium the conditions were 1,800 V and 6.5 mA. Twenty fractions of 2.5 ml each were collected, their pH measured, and their polypeptide composition analyzed by SDS-PAGE. See Figure 1.

Preparative Gel Electrophoresis on the Model 491 Prep Cell

Rotofor fractions containing lamin C identified by Coomassie Blue staining (Figure 1A) and immunoblotting (not shown) were pooled and dialyzed extensively against 10 mM Tris-HCl, pH 7.5, and then distilled water at 4°C. Proteins were concentrated by lyophilization. The dried protein, approximately 6 mg, was dissolved in 2 ml of SDS sample buffer and applied to the Model 491 prep cell for purification. The gel composition was 7%T/2.67%C, with a height of 10 cm and a gel tube size of 28 mm. The stacking gel composition was 4% with a height of 2.1 cm. Running conditions were 40 mA constant current (180–230 V) for 8–10 hr. After elution of Bromophenol Blue tracking dye, 2.5 ml fractions were collected. The polypeptide composition of every third fraction was analyzed by SDS-PAGE. Lamin C was identified by molecular weight (Figure 2) and antibody reactivity (not shown).

Results

The Rotofor cell was extremely efficient for initial purification of lamin C from bacterial proteins. The SDS-PAGE gel shown in Figure 1A demonstrates how the Rotofor cell separated the bulk of the contaminating proteins in the lysate from the recombinant lamin C-containing fractions. A similar separation profile was obtained for recombinant lamin A (not shown) which displays the same isoelectric point as lamin C.

Figure 1A demonstrates that Rotofor fractions containing lamin C also contained higher and lower molecular weight contaminants. Therefore, further purification by molecular weight was performed using the Model 491 prep cell. The efficiency of this apparatus at separating polypeptides is clearly illustrated in Figure 2. Lamin C was recovered in a small number of fractions, completely isolated from bacterial contaminants.

The isolation of lamin C by a method using denaturation by urea and ionic detergents is not a problem for the studies we are pursuing. First, nuclear lamins do not possess any enzymatic activity that could be irreversibly lost upon denaturation. Second, lamins, like other intermediate filament proteins, can be subjected to denaturation-renaturation cycles without loss of polymerization ability. And third, very efficient extraction methods exist whereby polypeptides can be completely recovered free of SDS, and we have used such preparations in immunofluorescence competition assays where the smallest trace of SDS would have adversely affected the antibodies under study (Collard et al. 1992).

In conclusion, a combination of isoelectric focusing on the Rotofor cell and molecular weight fractionation on the Model 491 prep cell allowed the complete purification of 1.5–2.0 mg of lamin C from a complex mixture of proteins equivalent to 400 ml of whole bacterial cultures in only 2 steps and with high yield.

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