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Use of Rotofor Preparative Isoelectrofocusing Cell in Protein Purification Procedure

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ABSTRACT

The Rotofor cell is a preparative isoelectric focusing (IEF) apparatus, in which IEF is performed entirely in free solution. Electrofocusing in Rotofor cell has been described as well-suited for use at any stage of a purification scheme. However, it has some important limitations in resolving complex mixtures of proteins. This paper describes the advantages and disadvantages of using the Rotofor cell in purification protocols.

Index Entries: Rotofor; superoxide dismutase; 6-phosphogluconate dehydrogenase.

INTRODUCTION

The purification of proteins has become an important part of many of the more interesting biological problems. The difficulty of the purification process is increased when a preparative amount of a highly purified protein is needed. In our laboratory, protein purification is an important part of the research *(1–5)*. When routinely attempting preparative protein purification, we have tried to use the Rotofor cell. The Rotofor cell is a preparative isoelectric focusing (IEF) apparatus, in which IEF is performed entirely in free solution *(6)*. Although electrofocusing in Rotofor cell has been described as well-suited for use at any stage of a purification scheme *(7,8)*, it has some important limitations in resolving complex mixtures of proteins.

This paper describes the advantages and disadvantages of using the Rotofor cell in purification protocols. To test the capabilities of the Rotofor

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cell, we used soluble and mitochondrial fractions of rat brain homo-genates and two commercial soluble enzymes, superoxide dismutase (SOD) and 6-phosphogluconate dehydrogenase (6PGDH). The results show that, although the purification of large quantities of partially purified proteins can be achieved in a single step, proteins of complex mixtures do not focus tightly, and the same proteins can be seen throughout many fractions.

MATERIALS AND METHODS

The Rotofor unit (BioRad, Hercules, CA) was prerun with distilled water prior to loading the sample. The sample (1–11 mg proteins) was mixed with ampholyte (BioRad) and injected through the ports of the sample chamber. The power supply was set to 12 W constant power. The run was stopped when the voltage reached a constant value.

Ampholytes were removed from focused proteins by bringing the concentration of NaCl in the fractions to 1.0 *M*. Fractions were then extensively dialyzed (1:100 vol) using 12,000 MCO dialysis tubing. After dialysis, aliquots from each Rotofor fraction were dried using a speed-vacuum centrifuge, and loaded onto a 12% polyacrylamide gel *(9)*. Proteins were visualized with Coomassie brilliant blue G staining.

The activity of SOD (bovine erythrocytes, Sigma, St. Louis, MO) was assayed by the method of McCord and Fridovich *(10)*. 6-phosphogluconate dehydrogenase (human erythrocytes, Sigma) was assayed by the method of Sapag-Hagar et al. *(11)*. The molecular size of proteins in gel was determined using a computer-controlled scanner (Molecular Dynamics, Sunnyvaley CA).

RESULTS AND DISCUSSION

It has been proposed that the Rotofor cell can be integrated in any stage of a purification scheme *(7,8)*. In our laboratory, we have tried to use the Rotofor in our purification procedures. We have used the Rotofor cell as a means of separating complex mixtures of proteins (soluble and mitochondrial proteins), and as a final step of protein purification. In our study, initial attempts to fractionate mitochondrial proteins with the Rotofor cell were unsuccessful because of considerable precipitation of proteins inside the focusing chamber. This problem was partially solved using 8 *M* urea, CHAPS (a nonionic detergent), glycerol, and the prefocusing of the pH gradient before loading the protein. However, when working with soluble proteins (105,000*g* supernatant proteins), no precipitation was observed, not only because this supernatant contains soluble proteins, but also because of the high concentration of detergent (1% Tween-20), glycerol (20% w/v), and urea (8 *M*) used to solubilize and maintain the proteins in

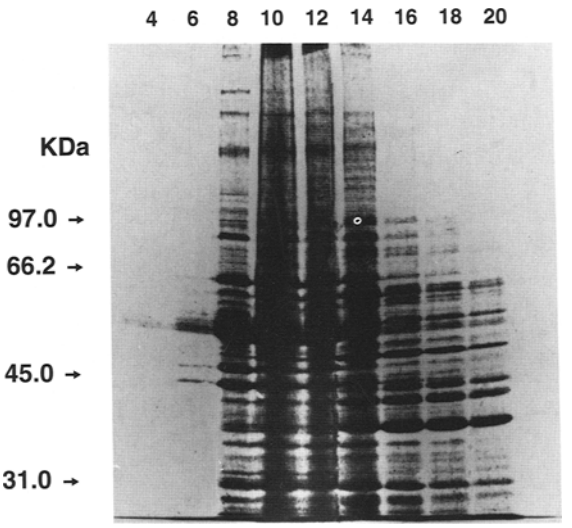

Fig. 1. Coomassie blue-stained SDS-gel of the 105,000g supernatant from rat brain homogenate fractionated in Rotofor cell. The pH range in the Rotofor was 3.0–10.0: The 105,000*g* supernatant was dialyzed against Tris-HCl 10 m*M*, pH 7.4, 20% glycerol, 1% Tween-20. The focusing medium containing 2.5% ampholyte, 8 *M* urea and 20% glycerol was prefocused without protein for 1 h. Eight mL of the protein sample, containing 11.0 mg protein was injected into the Rotofor chamber. The sample was made 8 *M* urea before injection. Numbers on the top indicate Rotofor fraction.

solution during focusing. Although none of these additives affected the performance of the Rotofor cell, long periods of dialysis were required to minimize the concentration of urea and Tween-20. In addition, urea may denature proteins, and the detergent may not be compatible with the protein of interest. The most relevant limitation was that the proteins do not focus, and therefore, the same protein can be seen throughout many fractions (Figure 1). This problem remained even after a prefocusing step.

The ideal sample run in the Rotofor cell would contain only the protein mixture, water, and ampholytes. When working with partially purified enzymes, this was possible because both SOD and 6PGDH are soluble enzymes, so that there was no precipitation of proteins in the cell. Figure 2, lane 2, shows a SDS-PAGE analysis of commercial SOD prior to purification, using the Rotofor. Besides SOD, other contaminant proteins are present. Their molecular masses were 61,000, 44,000, and 14,000 Da Lanes 3–8 show Rotofor fractions 5–10 of SOD. A single 15,000-Da band, which corresponds to SOD and was substantially free of protein contaminants, was visible on a Coomassie blue stained gel. Figure 3 indicates that SOD was localized in Rotofor fractions 3–10, covering a pH range of 3.13–6.66, with fraction 7 (pH 5.4) containing the greatest part of the protein (32.5%). Ninety-four percent of the total SOD activity was recovered.

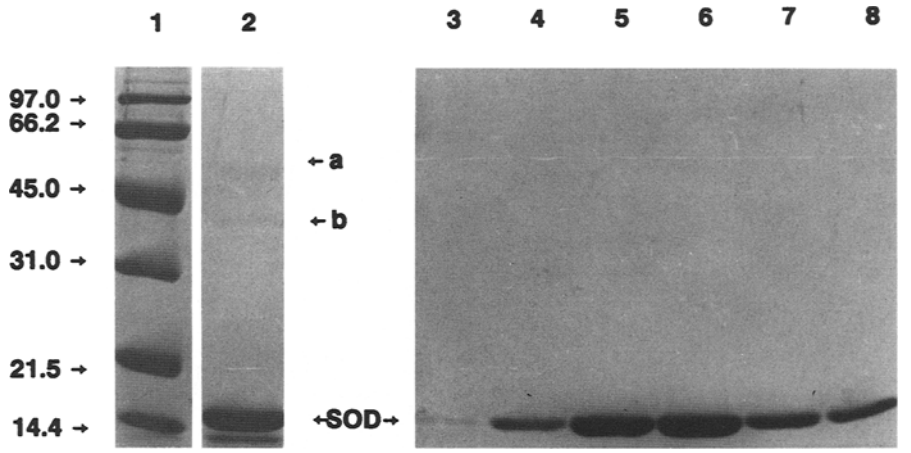

Fig. 2. SDS-PAGE analysis of SOD. One mg of partially purified SOD was dissolved in 50 mL distilled water prior to addition of 1.5 mL ampholyte. Ampholytes were removed from focused proteins by bringing the concentration of NaCl in the fractions to 1.0 *M*. Fractions containing SOD were then extensively dialyzed (1:100 vol) against several changes of 0.005 *M* potassium phosphate, pH 7.8, using 12,000 MCO dialysis tubing. Lane 1: mol wt standards; lane 2: partially purified SOD prior to purification using Rotofor cell; a: 61,000 Da, b: 44,000 Da; lanes 3–8: Rotofor fractions containing SOD activity: lane 3, fraction 10; lane 8, fraction 5.

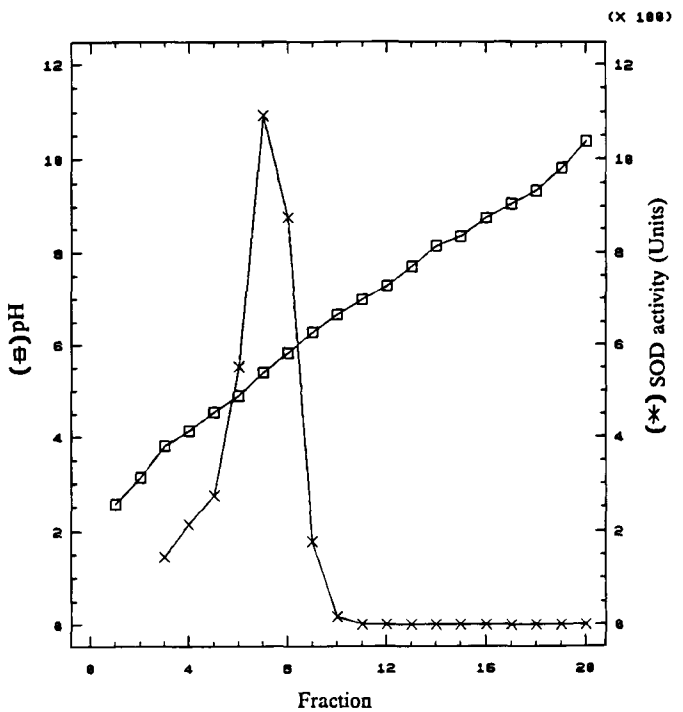

Fig. 3. Analysis of 20 Rotofor fractions by pH and SOD activity. The pH range in the Rotofor was 3–10. pH of the fractions and SOD activity were determined as described in the text.

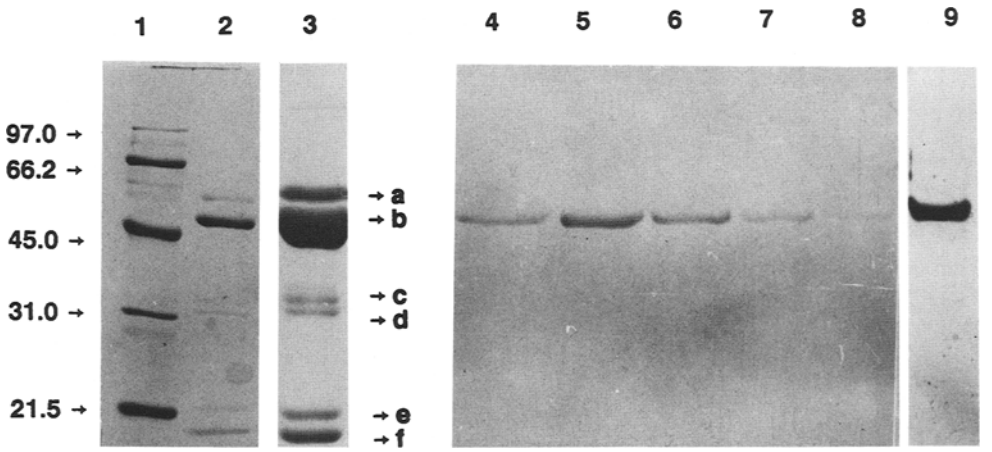

Fig. 4. SDS-PAGE analysis of 6PGDH. The sample containing 1 mg of 6PGDH (28 U) was resuspended in a minimum volume of Tris-HCl 10 m*M*, pH 7.4, dialyzed in this buffer. The dialyzed sample was mixed with water and ampholyte and loaded into the sample chamber. Fractions containing 6PGDH were then extensively dialyzed (1 : 100 volumes) against several changes of Tris-HCl 10 m*M*, pH 7.4, using 12,000 MCO dialysis tubing. Lane 1: mol wt standards; lanes 2–3: partially purified 6PGDH prior to purification using Rotofor cell; lanes 4–8: Rotofor fractions 5, 7, 9, 11, and 13 containing 6PGDH activity. These fractions were pooled and loaded onto a 10% acrylamide gel (lane 9). Proteins were visualized by Coomassie brilliant blue G staining.

In the case of 6PGDH, the preparation was heavily contaminated. It had five contaminant bands prior to purification with Rotofor (Fig. 4, lanes 2 and 3). The molecular masses of these proteins were 61,000 (glucose-6-phosphate dehydrogenase), 36,000, 34,000, 20,000, and 18,000 Da. After the focusing was completed, SDS-PAGE analysis of Rotofor fractions 5–13 revealed that 6PGDH-containing fractions were substantially free of protein contaminants (Fig. 4, lanes 4–9). Although the activity of the protein shows a maximum in fraction 7, pH 5.85 (Fig. 5), 6PGDH focuses across the range of pH 5.55–6.51. The recovery of activity was only 55%. In this case, to maximize recovery, several factors must be tested, such as keeping the temperature of the chamber at lower temperature, adding particular ionic species (divalent cations), and so on; all of them can affect the performance of the Rotofor cell.

The Rotofor is particularly effective in resolving separation of proteins in the last stage of purification, in which a preparative amount of soluble protein is needed. However, to obtain high purity of enzyme and high recovery of the activity, the enzyme must be soluble and stable in water. In this case, the Rotofor cell provides reagent levels of purified, biologically active enzymes in free solution, permitting structural and functional assays to be completed. However, if the protein of interest is not soluble in water, or its activity is not maintained when dissolved in water, the Rotofor cell does not provide an effective method for generating purified protein preparations. In addition, Rotofor cell may not be effective in com-

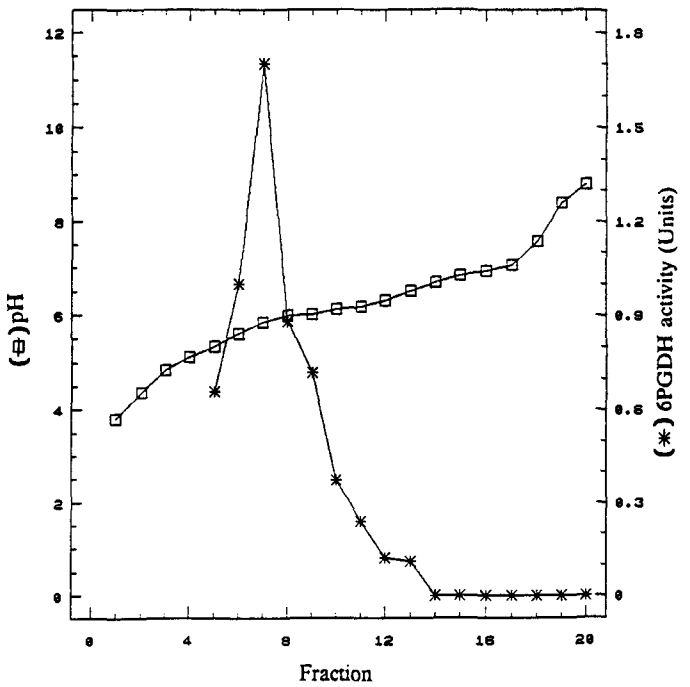

Fig. 5. Analysis of 20 Rotofor fractions by pH and 6PGDH activity. The pH range in the Rotofor was 5.0–7.0. Activity of 6PGDH is expressed as units.

plex mixtures, because, in the free solution of Rotofor cell, proteins do not focus, and, therefore, the proteins appear throughout many fractions.

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