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RAPID CHROMATOGRAPHIC METHOD FOR THE ISOLATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM YEAST ENZYME CONCENTRATE

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SUMMARY

A simple method for the isolation of glucose-6-phosphate dehydrogenase (G6PDH) from yeast enzyme concentrate is described. The method is based on high-performance anion-exchange chromatography and was developed in three steps: (1) optimization of chromatographic conditions on Polyanion SI-8 μm, (2) transfer of the optimized conditions to a preparative column containing Polyanion SI-17 μm and (3) final purification on an analytical column. As the enzyme is a minor component of the crude mixture, its presence is detected by a simple enzymatic method. The purity of the final fraction is checked by polyacrylamide gel electrophoresis.

INTRODUCTION

The purification of glucose-6-phosphate dehydrogenase (G6PDH) from various sources has received attention over the past decade[1,2]. Much of the work required slow, laborious electrophoretic methods[3] or traditional soft-gel chromatography[4]. The recent availability of high-performance anion exchangers, designed for the separation of proteins, has led to significantly increased resolution and speed of purification. This work demonstrates the resolving power and the ease with which a pure enzyme can be isolated from a crude mixture.

EXPERIMENTAL

Apparatus

A Pharmacia (Uppsala, Sweden) Fast Protein Liquid Chromatography (FPLC) system was used, consisting of two P-500 pumps, a V-7 valve injector with a 10-ml Superloop, a GP-250 gradient programmer, a UV-1 monitor with an HR-10 cell, a UV-2 monitor, a FRAC-100 fraction collector and an REC-482 recorder. A pre-packed Polyanion SI HR 5/5 column (50 × 5 mm I.D.) was used for the optimization experiments and, for final purification, bulk Polyanion SI-17 μm was dry-packed into a Pharmacia HR 10/10 column (100 × 10 mm I.D.) for preparative chromatography.

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Reagents

Yeast enzyme concentrate (Cat. No. Y2875) and buffers were obtained from Sigma (St. Louis, MO, U.S.A.). All salts were purchased from Fluka (Buchs, Switzerland). Pharmacia Gradient Gel PAA 4/30 was used for polyacrylamide gel electrophoresis (PAGE) according to a standard procedure[5].

Chromatographic conditions

Optimization experiments. Column, Polyanion SI HR 5/5 (50 × 5 mm I.D.); buffer A, 50 m*M* triethanolamine (TEA) (pH 7.3); buffer B, 50 m*M* TEA (pH 7.3), 1.0 *M* sodium bromide; gradient, 20% B for 10 min, 20 to 60% B in 30 min; flow-rate, 0.5 ml/min; sample, 2.5 mg; detection, UV-1, 280 nm, 0.5 a.u.f.s.

Preparative chromatography. Column, Polyanion SI-17 μm in a Pharmacia HR 10/10 column (100 × 10 mm I.D.); buffer A, 50 m*M* TEA (pH 7.3); buffer B, 50 m*M* TEA (pH 7.3), 1.0 *M* sodium bromide; gradient, 20% B for 10 min, 20 to 60% B in 30 min; flow-rate, 2 ml/min; sample, 103 mg; detection, UV-2, 280 nm (1-mm path-length), 2.0 a.u.f.s.

Final purification. Column, Polyanion SI HR 5/5 (50 × 5 mm I.D.); buffer A, 50 m*M* TEA (pH 6.8); buffer B, 50 m*M* TEA (pH 6.8), 1.0 *M* sodium bromide; gradient, 0% B for 15 min, 0 to 100% B in 20 min; flow-rate, 0.5 ml/min; sample, diluted fraction from preparative run; detection, UV-1, 280 nm, 0.5 a.u.f.s.

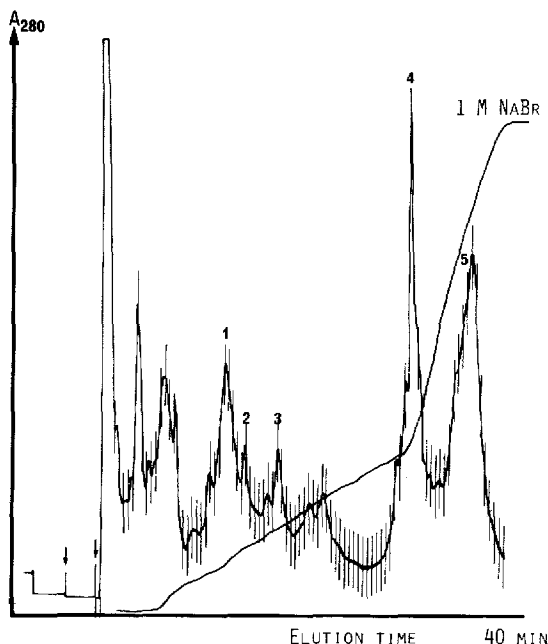

Fig. 1. Separation of yeast enzyme concentrate on Polyanion SI HR 5/5. Chromatographic conditions as in *Optimization experiments*, except for the gradient, which was 0 to 25% B in 30 min, 25 to 100% B in 10 min. Peaks: (1) superoxide dismutase; (2) gluconate-6-PDH; (3) alcohol-DH; (4) glucose-6-PDH; (5) β-glucosidase.

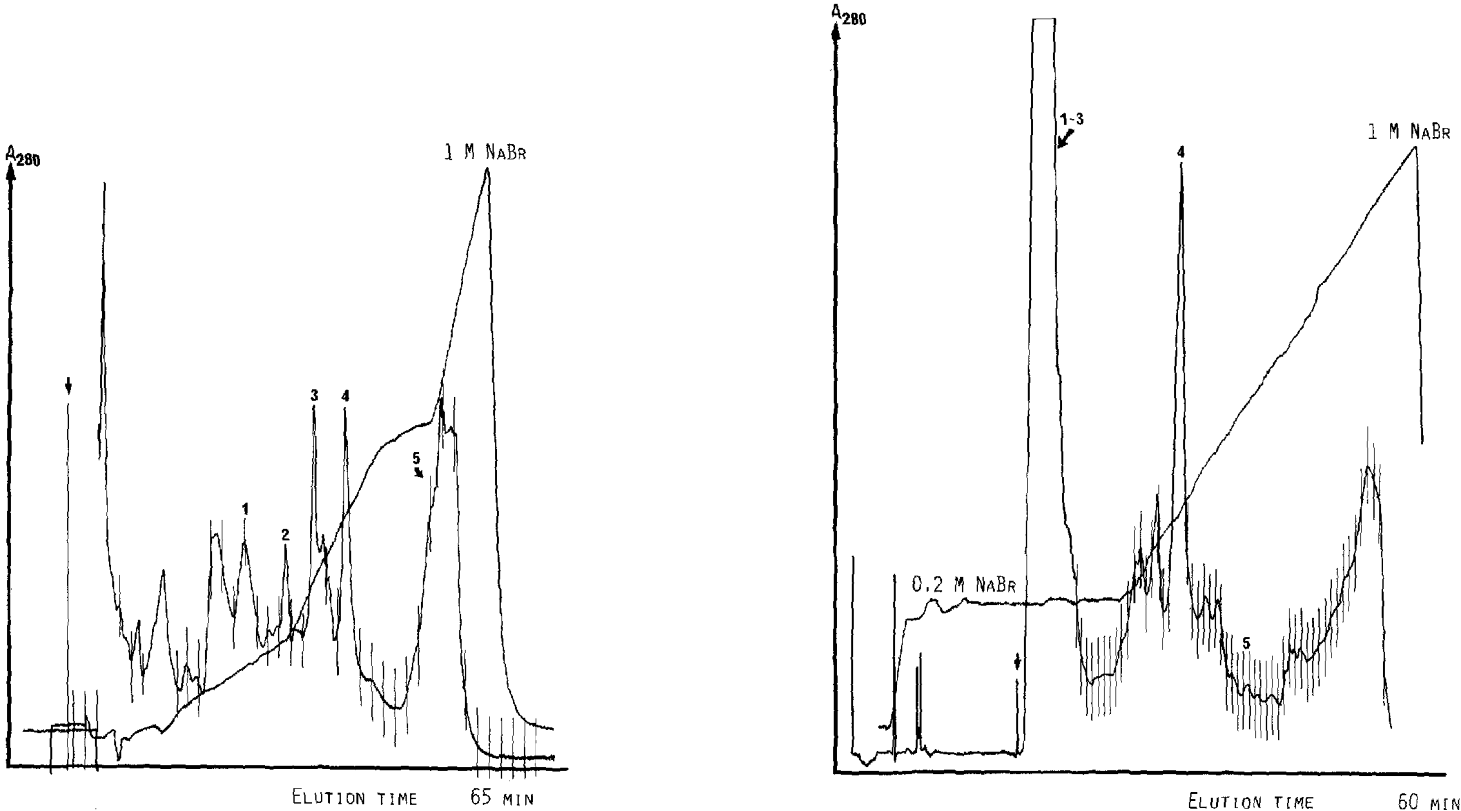

Fig. 2. Separation of yeast enzyme concentrate on Polyanion SI HR 5/5. Chromatographic conditions as in *Optimization experiments*, except for the gradient, which was 0 to 5% B in 5 min, 5 to 15% B in 25 min, 15 to 40% B in 15 min, 40 to 45% B in 10 min, 45 to 100% B in 10 min. Peaks as in Fig. 1.

Fig. 3. Optimized separation of G6PDH activity. Conditions as in *Optimization experiments*. Peaks as in Fig. 1.

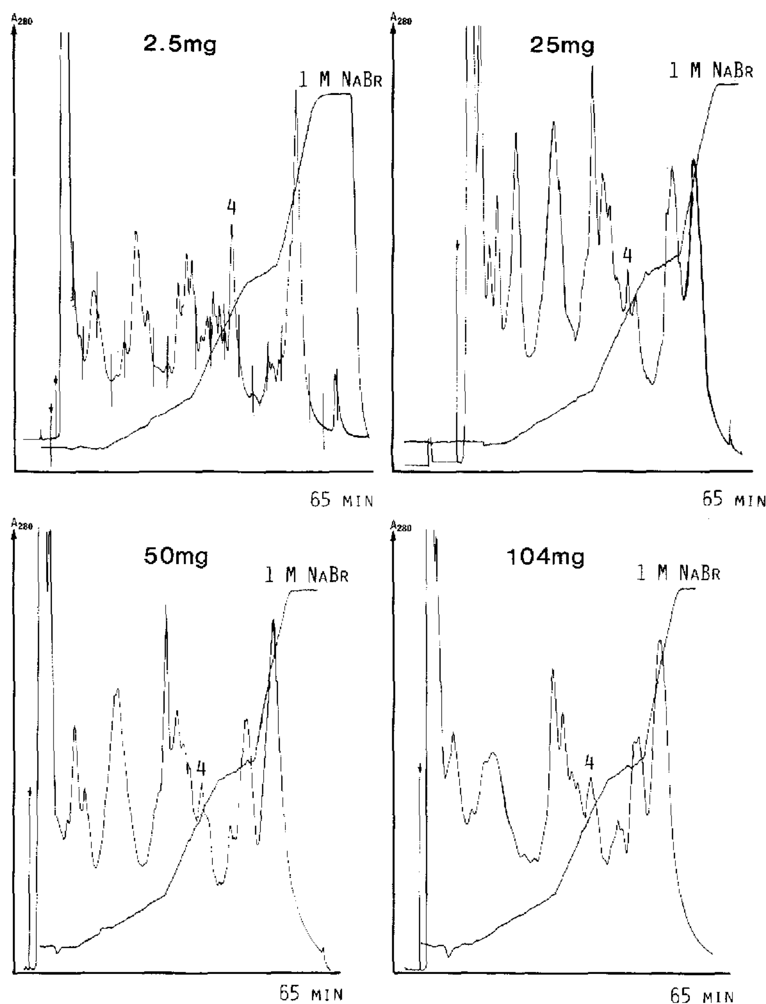

Fig. 4. Load capacity study on Polyanion SI HR 5/5. Chromatographic conditions as in Fig. 2, except the flow-rate was increased to 2 ml/min.

The fraction from *Preparative chromatography* was diluted from 6 ml to about 10 ml in order to obtain the same ionic strength as the starting buffer.

The probability of G6PDH and impurities having the same charge and therefore the same elution ionic strength at two different pH values is slight. In the last step, the pH was varied so that possible impurities mixed with the main fraction could be removed.

Enzyme test

A 10-μl volume of each fraction was mixed with 10 μl of the staining reaction mixture on a micro-titration plate. The staining reaction mixture contained the following: 25 ml of 0.2 M Tris–HCl (pH 8.0); 10 mg of glucose-6-phosphate (Na_2 salt), final concentration in the reaction mixture 0.6 mM; 5 ml of 0.2 M magnesium

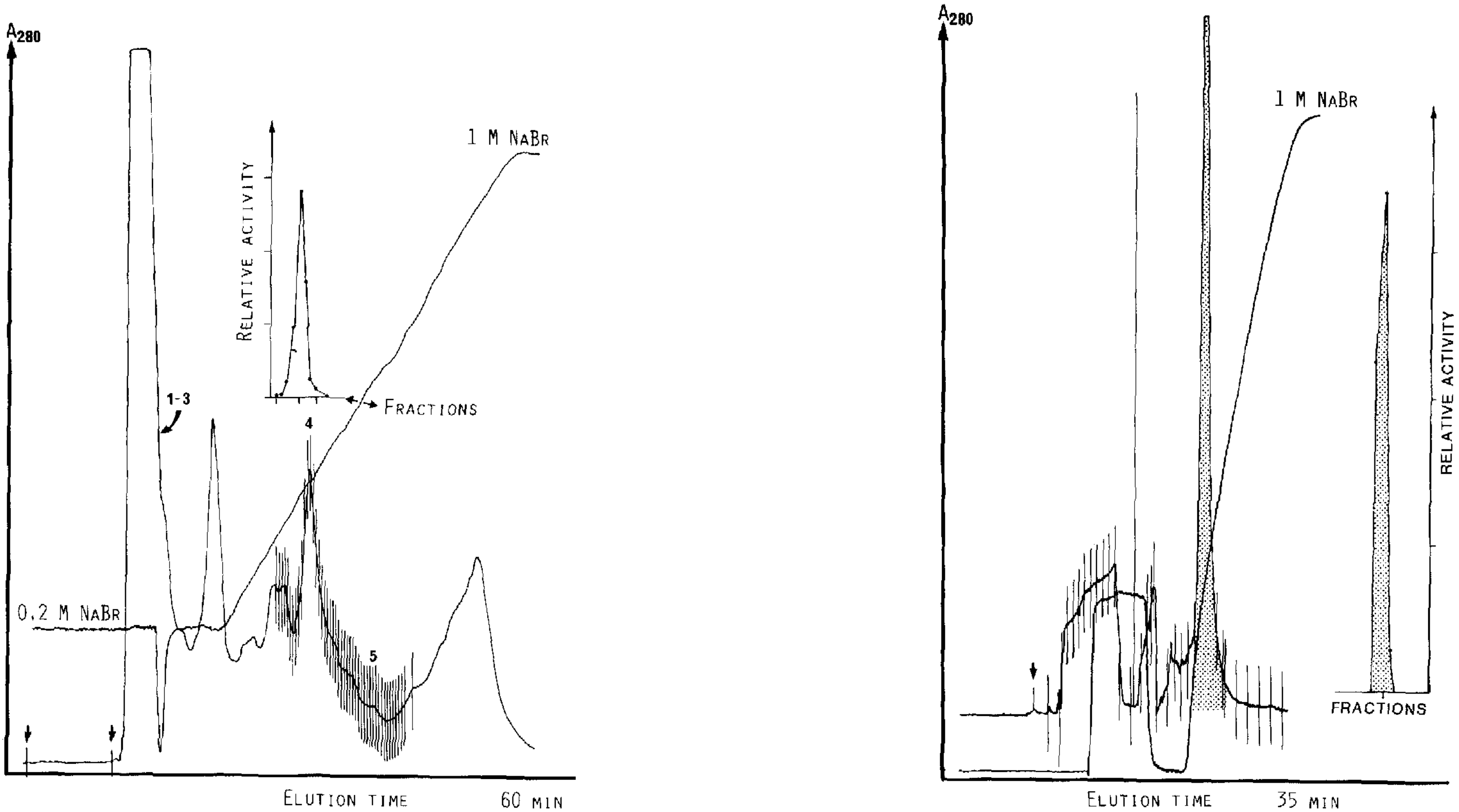

Fig. 5. Preparative separation of G6PDH from yeast enzyme concentrate. Chromatographic conditions as in *Preparative chromatography*. Peaks as in Fig. 1.

Fig. 6. Final purification of G6PDH on a Polyanion SI HR 5/5 column. Chromatographic conditions as in *Final purification*; 85% of the activity was found in the shadowed area.

chloride; 5 mg of Na_2-NADP in 1 ml of water; 7.5 mg of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) in 1.5 ml of water; 5 mg of (N-methylphenazinium methylsulphate (PMS) in 1 ml of water; and 25 ml 2% agarose solution.

Staining methods for G6PDH, gluconate-6-PDH, and alcohol-6-DH may be found in ref. 6, the assay procedure for superoxide dismutase in ref. 7 and that for β-glucosidase in ref. 8.

RESULTS AND DISCUSSION

Chromatographic conditions were optimized for the isolation of the G6PDH activity on an analytical Polyanion SI HR 5/5 column. The small size of the column allowed minimal use of crude extract during the search for optimal conditions. Several gradient shapes were tested (Figs. 1–8). The chromatographic conditions listed under *Optimization experiments* (see Fig. 3) were found to be optimal and used for

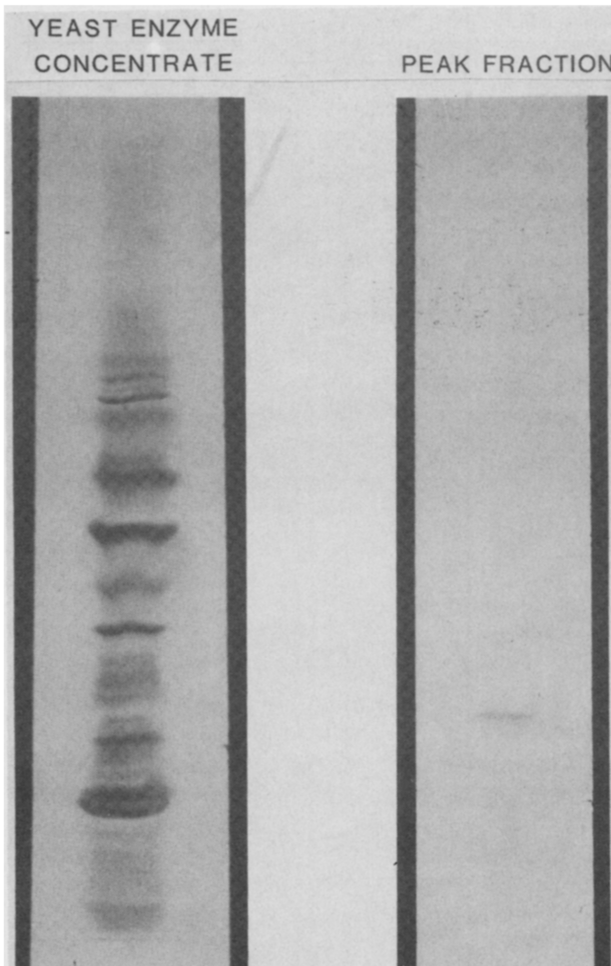

Fig. 7. PAGE of the purified fraction from Fig. 6.

preparative chromatography. The load capacity of the preparative column was tested, and 103 mg was found to be the maximal load allowable before a significant loss in resolution occurred (Fig. 4).

The loading of sample volumes up to 10 ml was facilitated by the use of a 10-ml sample loop (Superloop). The preparative chromatogram is shown in Fig. 5. Peak 4 was collected and purified further on the analytical column. As can be seen in Fig. 6, the analytical column was not overloaded. If larger amounts of purified G6PDH are needed, chromatograms on the preparative column can be repeated and the fractions pooled before injection onto the analytical column.

The purity achieved by the final chromatogram was tested by PAGE (Fig. 7), which showed the G6PDH to be free from several other impurities. The recovery of G6PDH in the last two steps was 79%.

CONCLUSIONS

Polyanion SI provides an adequate resolving power for the separation of G6PDH from a crude yeast mixture. As Polyanion SI is available in two particle sizes, the scale-up called only for an increase in the flow-rate. For valuable preparations Polyanion SI HR 5/5 has the advantage that optimization requires relatively little material. Once the conditions have been established, large samples can be fractionated on the HR 10/10 column. This obviates the risk of low recoveries when less than optimal conditions are used for preparative chromatography. By varying the gradient profile, one can optimize the separation of a number of enzymes from yeast enzyme concentrate (Fig. 1).

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