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ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MEMBRANE-BOUND PROTEIN CYTOCHROME P-450

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SUMMARY

The multiple forms of cytochrome P-450 were separated on anion-exchange (DEAE-5PW) and cation-exchange (SP-5PW) columns The detergent, glycerol, buffer, and salt for gradient elution in the mobile phase affected resolution The best detergent was Emulgen 911, with an optimum concentration of 0 4% for both columns Buffer with 20% glycerol was more effective than that with 10% Bands spread at low concentrations of Emulgen and glycerol Elution with sodium acetate and that with sodium chloride gave different profiles

INTRODUCTION

Cytochrome P-450 (P-450) is a membrane-bound protein that catalyses the oxidation of many compounds, including steroids, fatty acids and xenobiotics [1, 2] Multiple forms of P-450 are present in liver microsomes [3, 4] Different forms of liver microsomal P-450 from rats have been purified using diethylaminoethyl (DEAE) or carboxymethyl (CM) ion-exchange chromatography [5—8] We separated P-450 by high-performance liquid chromatography (HPLC) using a SynChropak AX-300 anion-exchange column [9, 10]. Bansal et al [11] have also analysed P-450 by HPLC using our method The column support of SynChropak AX-300 is made of a silica-based material, so basic buffers cannot be used This column is used at a high pressure but the support makes it physically unstable

Recently, new DEAE-5PW and SP-5PW columns, which are polymer-based ion-exchange HPLC columns, have become commercially available [12, 13] Polymer gel is stable both chemically and physically, and is used at a lower pressure than silica-based materials. It can be washed with alkaline or acidic

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solution when the gel is stained DEAE-5PW is an anion-exchange column with a base of hydrophilic polymer with large pores (1000 Å), SP-5PW is a cation-exchange column containing sulphopropyl (SP) groups, based on the same polymer These HPLC columns give good resolutionof soluble protein [12, 13] and membrane-bound proteins [14] The resolution of P-450 isozymes by HPLC using an ion-exchange column is greatly affected by the detergent, buffer, concentrations of glycerol and detergent, and pore size of the column [15] Here, we investigate how the chromatographic conditions for these two columns affect resolution of the multiple forms of P-450

EXPERIMENTAL

Columns and apparatus

The DEAE-5PW anion-exchange column (74 × 7 5 mm I D , particle size 10 μm) and the SP-5PW cation-exchange column (74 × 7 5 mm I D.) were obtained from Toyo Soda (Tokyo, Japan). We used an Altex Model 100 gradient HPLC apparatus (Berkeley, CA, U S A) for chromatography, and haemoprotein was monitored at 417 nm with a spectrophotometer (UV-8, Toyo Soda)

Preparation and solubilization of microsomes

Male Sprague—Dawley rats weighing 250—300 g (Nippon Clea, Japan) were given phenobarbital (PB, 80 mg/kg, dissolved in saline) or 3-methylcholanthrene (MC, 40 mg/kg, dissolved in corn oil) intraperitoneally daily, the PB for four days and the MC for three days. They were starved overnight and then killed by decapitation Liver microsomes were prepared using the usual method [15] The specific contents of P-450 in the microsomes of untreated rats, rats given PB and rats given MC were 0 70, 1 69 and 1 40 nmol/mg.

The microsomal preparation (1 8 ml, 30 mg/ml protein) was solubilized by adding 0 45 ml of 10% sodium cholate (adjusted to pH 7 5) and 0.45 ml of 10% Emulgen 913 (Kao Chemicals, Tokyo, Japan). When this solution was not clear, it was centrifuged at 100 000 *g* for 30 min before being examined by HPLC

Chromatography

Ion-exchange chromatography was done using the DEAE-5PW and SP-5PW columns Solubilized microsomes induced with PB (100 μl, containing 2 mg of protein) were injected into the HPLC apparatus equipped with the DEAE-5PW column The pass-through fraction of microsomes induced with MC from a DEAE-5PW column was collected and concentrated using an ultrafiltration membrane (UK-50, Toyo Roshi, Tokyo, Japan). This solution (200 μl, containing 0 2 mg of protein) was then put into an SP-5PW column. Haemoproteins were separated at 20—25°C by linear gradient elution with the two buffers described in detail in the legends to Figs 1—7 at a flow-rate of 1 ml/min monitored at 417 nm. Emulgen 911 was kindly supplied by Kao Chemicals, Lubrol was from Sigma (St Louis, MO, U S A) and Triton X-100 from Wako Pure Chemical Industries (Osaka, Japan)

Recovery of P-450 and protein

The recovery of protein from DEAE-5PW was evaluated by injecting 0 5 mg of semi-purified P-450 (PB-4 induced mainly with PB, as described in detail under Results and discussion) into a DEAE-5PW column equilibrated with 0 02 *M* Tris—acetate buffer (pH 7 5) containing 20% glycerol and 0.4% Emulgen 911 After the injection, the column was washed with the same buffer at a flow-rate of 1 ml/min for 15 min, and P-450 was eluted with 0.02 *M* Tris—acetate buffer (pH 7 5) containing 1 *M* sodium acetate, 20% glycerol and 0.4% Emulgen 911 The amount of protein in the eluate containing Emulgen was measured by a modification of the method of Dulley and Grieve [16], and the amount of P-450 was estimated spectrally by the method of Omura and Sato [17]

Recovery of the protein and P-450 from the SP-5PW column was evaluated by the same procedure The P-450 fraction (1 0 mg of protein) that was not adsorbed on the DEAE-5PW column was injected into an SP-5PW column equilibrated with 0 02 *M* sodium phosphate buffer (pH 6.5) containing 20% glycerol and 0 4% Emulgen 911 Elution was done with 0 02 *M* sodium phosphate buffer (pH 6 5) containing 0 5 *M* sodium chloride, 20% glycerol and 0 4% Emulgen 911

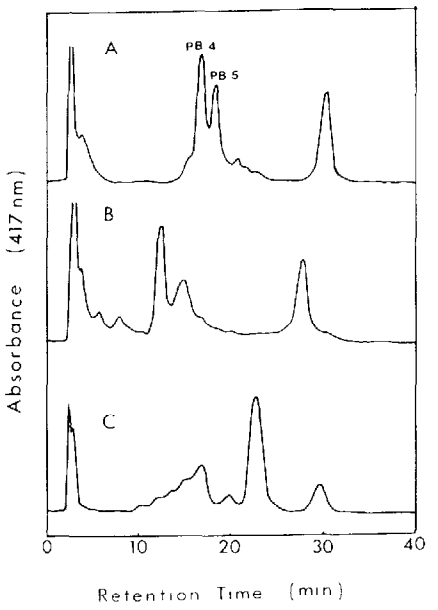
RESULTS AND DISCUSSION

DEAE-5PW

We named the major forms of P-450 induced by PB (Fig 1A) PB-4 and PB-5. They corresponded to the P-450 PB-4 and P-450 PB-5 reported by Waxman and Walsh [6, 18], and to the P-450b and P-450e of Ryan and co-workers [19, 20], judged by their chromatographic behaviour, molecular weight and catalytic properties [21]

Efffects of buffer, salt and pH Chromatography with DEAE-5PW was done using Tris buffer (pH 7 5) or phosphate buffer (pH 7 5) as the mobile phase (Fig 1A and B) The resulting profiles were not very different, and both buffers gave good separation of PB-4 and PB-5 The phosphate buffer eluted P-450 earlier than the Tris buffer When the gradient was of sodium chloride instead of sodium acetate (Fig 1C), PB-4 and PB-5 were not separated and the peak was small Soluble proteins and other membrane-bound proteins have been separated using Tris—hydrochloric acid buffer and sodium chloride for elution [12, 14], but we found sodium acetate to be more effective for the separation of PB-4 and PB-5 Chromatography was also done using Tris—acetate buffer of pH 7 0, 7 5 and 8 2 P-450 was eluted later at higher pH but PB-4 and PB-5 were not resolved at pH 8 2.

Effect of detergents and glycerol Fig. 2 shows the effects of the four detergents, Emulgen 911, Emulgen 913, Lubrol and Triton X-100. Emulgen 911 and 913 are both commonly used in the solubilization and chromatographic separation of P-450, but we found that only Emulgen 911 gave satisfactory resolution With the two other detergents, PB-4 and PB-5 could not be identified, because peaks were braod and low Resolution was incomplete at low concentrations of Emulgen 911 (0 05, 0 1 and 0 2%), the optimum concentration was 0 4%, and concentrations higher than that gave the same degree of



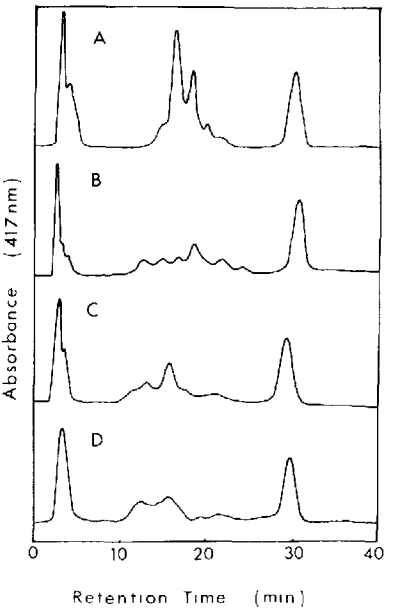


Fig 1 Effects of buffer and salt Chromatography was done at a flow-rate of 1 0 ml/min, taking 40 min, with a linear gradient of sodium acetate from 0 to 0 4 *M* in 0 02 *M* Tris—acetate buffer, pH 7 5 (A), in 0 02 *M* sodium phosphate buffer, pH 7 5 (B) or with a linear gradient of sodium chloride from 0 to 0 4 *M* in 0 02 *M* Tris—hydrochloric acid buffer, pH 7 5 (C) Each buffer contained 20% glycerol and 0 4% Emulgen 911

Fig 2 Effects of detergents Chromatography was done using 0 02 *M* Tris—acetate buffer (pH 7 5) containing 20% glycerol and 0 4% Emulgen 911 (A), 0 4% Emulgen 913 (B), 0 4% Lubrol (C) or 0 4% Triton X-100 (D) Other conditions were as in Fig 1A

resolution (Fig 3). DEAE-5PW required a higher concentration of Emulgen than either the conventional methods or the SynChropak AX-300. The polymer-based material in DEAE-5PW may be more hydrophobic than the polysaccharide matrix of the usual ion exchangers or the silica-based supports of SynChropak AX-300. Increases in the concentration of detergent decrease hydrophobic interaction between the protein and the support Glycerol is added to the buffer during both the preparation of P-450 and the chromatographic separation to stabilize this protein [22] However, glycerol increased the viscosity of the buffer, thus increasing the column pressure. We tried using 10 and 20% concentrations of glycerol in the buffer, P-450 was not resolved at 10%, and the peak was low

SP-5PW

The MC-1 shown in Fig 4A was the high-spin form and corresponded to the P-450d of Botelho et al [23] and Ryan et al [24] (see also ref 21)

Effect of buffer, salt and pH The fraction of microsomes not adsorbed on DEAE-5PW (the MC-PT fraction of Fig 6C) was put on an SP-5PW column and chromatographed using a phosphate buffer (pH 6 5) or Tris buffer (pH 6.5) (Fig 4A and C) The linear gradient was of sodium chloride (Fig 4A) or sodium acetate (Fig 4B) Although the three profiles shown in Fig. 4 are not very different, the sharp ghost peak in Fig 4C appeared before the two peaks

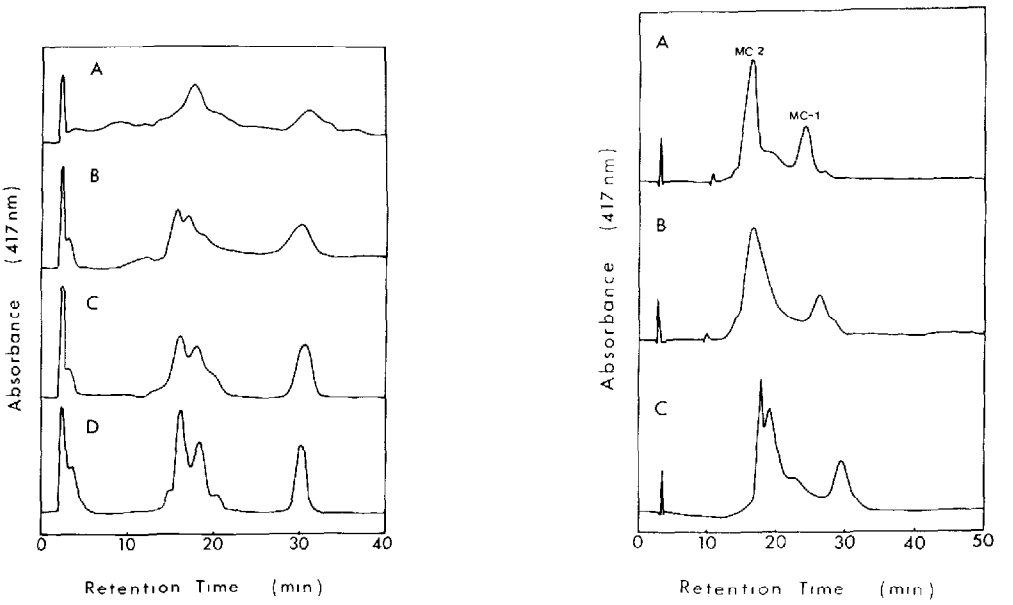


Fig 3 Effects of the concentration of Emulgen 911 Chromatography was done using 0 02 *M* Tris—acetate buffer containing 20% glycerol and 0 05% (A), 0 1% (B), 0 2% (C) or 0 4% (D) Emulgen 911 Other conditions were as in Fig 1A

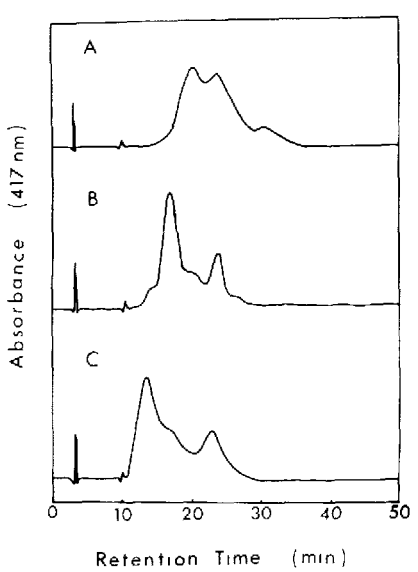
Fig 4 Effects of buffer and salt Chromatography was done at a flow-rate of 1 0 ml/min, taking 50 min, with a linear gradient of sodium chloride from 0 to 0 5 *M* in 0 02 *M* sodium phosphate buffer, pH 6 5 (A), with a linear gradient of sodium acetate from 0 to 0 5 *M* in 0 02 *M* sodium phosphate buffer, pH 6 5 (B) or with a linear gradient of sodium acetate from 0 to 0 5 *M* in 0 02 *M* Tris—acetate buffer, pH 6 5 (C) Each buffer contained 20% glycerol and 0.4% Emulgen 911

derived from P-450 (which we named MC-1 and MC-2) Tris buffer was not suitable with SP-5PW Elution of P-450 with sodium chloride was more efficient than with sodium acetate (Fig 4A) Kato et al. [13] separated soluble proteins with the same buffer using sodium chloride Fig 5 shows the effects of the pH of the buffer MC-2 was eluted earlier with higher pH. The MC-2 peak was broad at pH 7 0, but MC-1 and MC-2 were separated

Effect of detergents and glycerol As with DEAE-5PW, we tried four kinds of detergents Triton X-100 an Emulgen 911 gave good resolution, the other two detergents did not As with DEAE-5PW, buffer containing 0 4% Emulgen 911 or Triton X-100 was the most effective, but at concentrations of 0.2% or less, broadening of the peaks occurred As with DEAE-5PW, 20% glycerol gave better results than 10% glycerol Glycerol decreases hydrophobic interaction At the lower concentration of glycerol, separation was poor

HPLC profile of multiple forms of P-450

Fig. 6 shows chromatographic profiles of solubilized microsomes that were not induced and of those induced with PB or MC Chromatography was done at a flow-rate of 1 0 ml/min with a linear salt gradient prepared with buffer A (pH 7 5, 0.02 *M* Tris—acetate containing 20% glycerol and 0 4% Emulgen 911) and buffer B (pH 7 5, 1 0 *M* sodium acetate added to buffer A) Buffer B



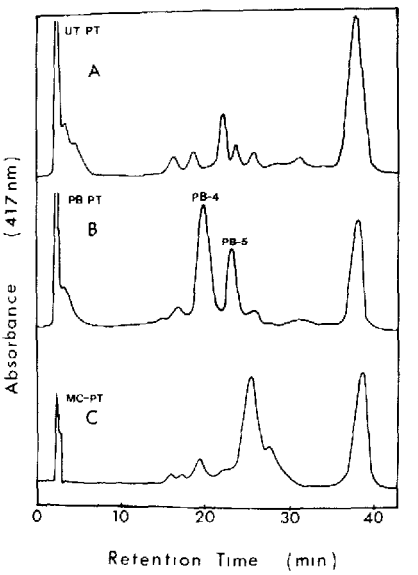


Fig 5 Effects of the pH of the buffer Chromatography was done using sodium phosphate buffer of pH 6 0 (A), 6 5 (B) or 7 0 (C) Other conditions were as in Fig 4A

Fig 6 Chromatographic profiles of solubilized microsomes not induced (A), induced with PB (B) or induced with MC (C) Chromatography was done at a flow-rate of 1 0 ml/min with a 60-min linear gradient of sodium acetate from 0 to 0 4 *M* in 0 02 *M* Tris—acetate buffer (pH 7 5) containing 20% glycerol and 0 4% Emulgen 911

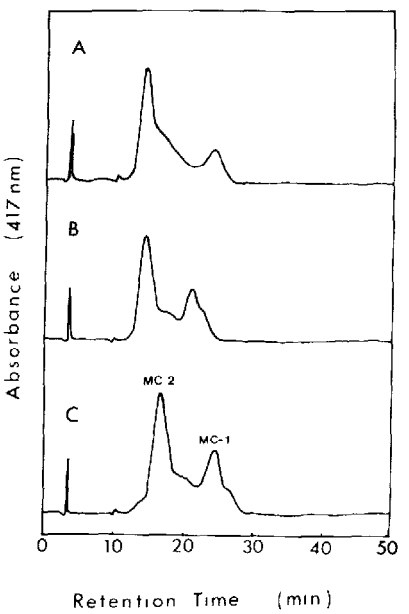


Fig 7 Chromatographic profiles of the non-adsorbed fraction of uninduced (A), PB-induced (B) or MC-induced (C) microsomes on DEAE-5PW Chromatography was done at a flow-rate of 1 ml/min, taking 50 min, with a linear gradient of sodium chloride from 0 to 0 5 *M* in 0 02 *M* sodium phosphate buffer (pH 6 5) containing 20% glycerol and 0 4% Emulgen 911

was added to buffer A linearly over 60 min to reach 40% Eight peaks were seen (Fig. 6A) and the last peak was identified as cytochrome b_5 from spectral evidence. The new peaks shown in Fig. 6B and C were P-450, newly induced with PB and MC By comparison of these profiles, the induced forms of P-450 were easily detected Each of the pass-through fractions (designated UT-PT, PB-PT and MC-PT) that could not be resolved by DEAE-5PW was concentrated and put on an SP-5PW column, the results are shown in Fig. 7. Each gave two peaks with a linear gradient of sodium chloride (0—0 5 *M*) using 0.02 *M* phosphate buffer (pH 6.5) containing 20% glycerol and 0.4% Emulgen 911 at a flow-rate of 1 ml/min, taking 50 min.

Recovery

Recovery of the semi-purified form of PB-4 from DEAE-5PW was 86% for protein and 92% for P-450 Recovery of protein and P-450 from SP-5PW was 66 and 72%, respectively.

CONCLUSIONS

As shown in Fig. 6, uninduced solubilized hepatic microsomes and those induced with PB and MC produced six, four and four fractions of P-450, respectively Fractions not adsorbed on DEAE-5PW were further resolved into two peaks by SP-5PW and were found to be P-450 We could separate seven kinds of P-450 from the uninduced microsomes and five kinds each from microsomes induced with PB and MC using these two ion-exchange columns. In chromatography using DEAE-5PW and SP-5PW, good resolution and reproducibility were maintained even after 100 injections, and recovery of P-450 was still satisfactory

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