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Evaluation of ammonium acetate as a volatile buffer for high-performance hydrophobic-interaction chromatography

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ABSTRACT

Hydrophobic-interaction chromatography (HIC) is a widely used technique for the separation of proteins without denaturation. In HIC, although, ammonium sulphate or sodium sulphate buffer is generally used as an eluent, volatile buffers such as ammonium acetate and ammonium formate seem to be advantageous in order to simplify the subsequent procedures including desalting. Therefore, the applicability of ammonium acetate buffer was evaluated, as a representative of volatile buffers for HIC, with respect to effects on the retention and peak broadening of proteins. Several proteins were successfully separated under the optimized conditions using volatile ammonium acetate buffer.

INTRODUCTION

In hydrophobic-interaction chromatography (HIC), a salt gradient elution of ammonium sulphate or sodium sulphate is widely used[1–5]. However, with such non-volatile buffers, extensive desalting procedures are usually required after the separation. On the other hand, volatile buffers such as ammonium acetate and ammonium formate seem to be advantageous in order to simplify the subsequent procedures including desalting. This simplification would be especially helpful for the preparative separation and purification of proteins, which are also a practically important aspect of HIC. Hitherto, volatile buffers have often been used in reversed-phase and ion-exchange chromatography[6,7]. However, they have not been used in HIC except in one instance[8]. In a previous study[9], volatile ammonium acetate, ammonium formate and ammonium carbonate buffers were evaluated in comparison with non-volatile ammonium sulphate buffer for the separation of proteins by HIC on a macroporous polymer having butyl groups, Polyspher BUTYL. Although proteins showed weaker retention in volatile buffers than in ammonium sulphate buffer, the separation of proteins was successfully achieved with volatile buffers. In this work,

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ammonium acetate buffer was chosen as a representative of volatile buffers and the influence of pH and salt concentration on the chromatographic behaviour of proteins was investigated in detail using Polyspher BUTYL.

EXPERIMENTAL

Reagents

Analytical-reagent grade ammonium acetate was obtained from Kanto Chemical (Tokyo, Japan). Water was purified with a Milli-Q system (Millipore, Bedford, MA, U.S.A.). Ovalbumin from hen egg white, myoglobin from horse skeletal muscle, haemoglobin from bovine erythrocyte and α-chymotrypsinogen A from bovine pancreas were purchased from Sigma (St. Louis, MO, U.S.A.). Bovine serum albumin (BSA), cytochrome *c* from horse heart, lysozyme from hen egg white and ribonuclease from bovine pancreas were obtained from E. Merck (Darmstadt, F.R.G.). These proteins were used without further purification, and were dissolved in 0.1 *M* ammonium acetate (pH 7.0) to make stock solutions (10 mg/ml).

High-performance liquid chromatographic apparatus

The gradient liquid chromatograph consisted of an L-6000 and an L-6200 intelligent pump (Hitachi, Tokyo, Japan) equipped with a high-pressure gradient programmer controlled by an internal CRT computer, a Hitachi L-4000 UV detector and a Hitachi D-2000 data processor. Generally, a 10-μl aliquot of the stock solution was injected through a Rheodyne Model 7125 sample injector with a 20-μl loop. The proteins were separated on Cica-Merck Polyspher BUTYL (10 μm) column (100 mm × 7.8 mm I.D.) (Kanto Chemical) by a 30-min linear gradient elution with a decreasing concentration of ammonium acetate (1.0–4.0 *M* to 0.1 *M*) at a flow-rate of 1.0 ml/min and were detected at 280 nm.

RESULTS AND DISCUSSION

Effect of salt concentration

The gradient elution with a decreasing concentration of ammonium acetate was performed in 30 min. The initial salt concentration was changed and the effects on retention times and peak shapes of various proteins were evaluated. The final concentration of ammonium acetate and pH were fixed at 0.1 *M* and 7.0, respectively, throughout this work. Fig. 1 shows the relationships between the initial concentration and the retention of proteins. Naturally, the retention became stronger with increase in the initial salt concentration. Although haemoglobin and ovalbumin showed broad peaks with higher initial concentrations, the peak shapes of α-chymotrypsinogen A and lysozyme were improved when higher initial concentrations were used.

Effect of pH

The effect of pH on the retention of proteins was examined by gradient elution from 2.0 to 0.1 *M* ammonium acetate. The results are summarized in Fig. 2. Generally, the retentions seemed to become stronger with increase in pH, except for the acidic proteins ovalbumin and BSA. The retention of ovalbumin (p*I* 4.6–5.0) and BSA (p*I* 4.7–4.9) was greatest at pH 5 with the broadest peaks. In contrast, haemoglobin and

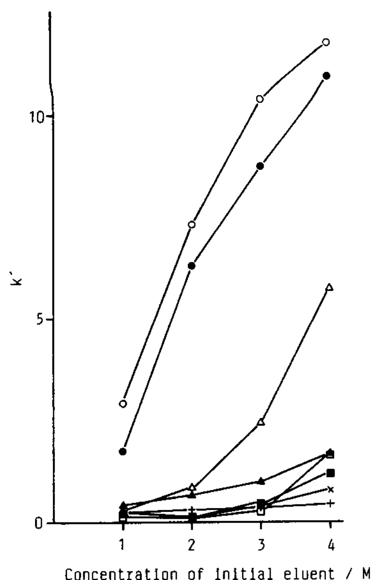

Fig. 1. Effect of concentration of initial eluent (eluent A, ammonium acetate buffer, pH 7.0) on capacity factor (k') of proteins. Eluent B, 0.1 *M* ammonium acetate (pH 7.0); linear gradient, from 0% to 100% eluent B in 30 min; column, Polyspher BUTYL (10 μm) (100 mm × 7.8 mm I.D.); column temperature, ambient; flow-rate, 1.0 ml/min; detection, UV (280 nm). ○ = α-Chymotrypsinogen A; ● = lysozyme; △ = haemoglobin; ▲ = ribonuclease; □ = BSA; ■ = ovalbumin; × = myoglobin; + = cytochrome *c*.

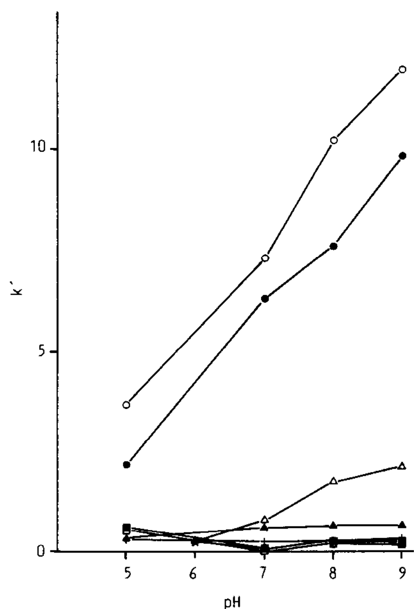

Fig. 2. Effect of pH on capacity factor (k') of proteins. Eluent A, 2.0 *M* ammonium acetate; eluent B, 0.1 *M* ammonium acetate. Other conditions and symbols as in Fig. 1.

basic proteins, α-chymotrypsinogen A (p*I* 9.2–9.3) and lysozyme (p*I* 11.0–11.4), were most strongly retained at pH 9. In HIC, it is generally considered that proteins are most strongly retained at pH values near their p*I* values. The results in this work showed agreement with this, though the results also suggest that the peak broadening is greatest at pH values near the p*I* values of proteins.

Optimization of conditions

Based on the above results, the conditions were optimized for the HIC separation of four proteins, cytochrome *c*, haemoglobin, lysozyme and α-chymotrypsinogen A. The chromatogram obtained is shown in Fig. 3. The results suggest that HIC separation using volatile ammonium acetate buffer can be successfully performed if the initial concentration and pH are properly selected with respect to the p*I* values of the proteins to be separated. Miller *et al.*[8] evaluated the application of several buffers in HIC and size-exclusion chromatography, including ammonium sulphate and volatile ammonium acetate buffer. Unlike our results obtained on Polyspher BUTYL, they concluded that ammonium acetate buffer cannot be used as an eluent for HIC on a column with an ether-bonded stationary phase because proteins are not retained. This discrepancy in the applicability of ammonium acetate as an eluent for HIC suggests that the effectiveness of volatile buffers is limited to situations where both the stationary phase and the proteins have some hydrophobic character. In fact, the retention of proteins with lower concentrations of ammonium acetate buffer was very weak also in this work because of insufficient hydrophobicity of the proteins, although ammonium acetate was found to be usable for HIC with a high initial concentration in gradient elution. In combination with more hydrophobic stationary phases than that

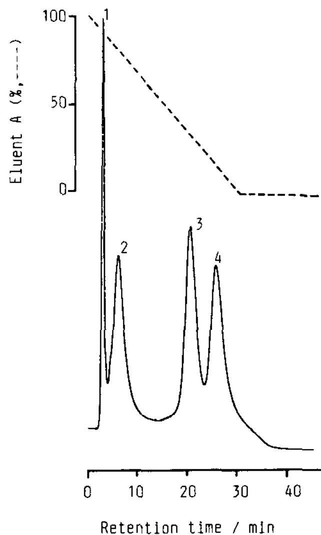

Fig. 3. Separation of proteins by HIC using ammonium acetate as the eluent. Eluent A, 2.0 *M* ammonium acetate (pH 8.0); eluent B, 0.1 *M* ammonium acetate (pH 8.0); other conditions as in Fig. 1. Peaks: 1 = cytochrome *c* (40 μg); 2 = haemoglobin (100 μg); 3 = lysozyme (20 μg); 4 = α-chymotrypsinogen A (40 μg).

employed here, HIC using volatile buffers should find wide applicability and become a practically useful tool for the preparative separation and purification of proteins.

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