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High-performance liquid chromatography of proteins on a ceramic hydroxyapatite with volatile buffers

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ABSTRACT

Volatile solutions were applied as eluents in the hydroxyapatite high-performance liquid chromatography (HPLC) of proteins and the elution behaviour was investigated. Four standard proteins were loaded on a ceramic hydroxyapatite column for HPLC and eluted with linear gradients of seven volatile solutions. Hydroxyapatite HPLC using ammonium hydrogencarbonate solution seems to be the best alternative although the resolution and recovery of proteins were lower than those obtained with a phosphate buffer system. The system was applied to the purification of a monoclonal antibody and of an enzyme in small amounts.

INTRODUCTION

Since the original work by Hjerten and co-workers[1,2], high-performance liquid chromatography (HPLC) on hydroxyapatite has sometimes been used for the specific separation of proteins[3–8]. However, operation with the conventional type of hydroxyapatite was difficult because of the fragility of the crystals and the difficulty of obtaining a uniform quality from batch to batch. Recently, the hydroxyapatite columns for HPLC have become commercially available, and this has led to effective methods for the purification of proteins[9–13]. Fang *et al.*[14] demonstrated the separation of proteins in very small amounts using a microbore column packed with ceramic hydroxyapatite beads with diameters as small as 2 μm.

In the purification of proteins, the samples sometimes need to be concentrated or to be desalted. Operations such as ultrafiltration or dialysis, however, may cause losses of proteins, especially with very small amounts of proteins. Volatile electrolytes are attractive in these cases, because they can be removed by freeze-drying. When desalting is performed with gel filtration chromatography, a volatile electrolyte is sometimes used to increase the ionic strength.

In hydroxyapatite chromatography, phosphate buffer is ordinarily used for elution. In this study, seven volatile solution systems were applied to the hydroxyapatite HPLC of proteins and the elution behaviour was investigated. Although these

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systems showed lower resolution and recoveries of proteins than when phosphate buffer was used, the HPLC with ammonium hydrogencarbonate gave the best separation among the volatile solutions examined.

EXPERIMENTAL

Reagents and materials

Hydrochloric acid and ammonia solution were purchased from Kokusan Kagaku (Tokyo, Japan). Other reagents were purchased from Nakarai Tesque (Kyoto, Japan). Myoglobin (equine heart), lysozyme (chicken egg), chymotrypsinogen A (bovine pancreas) and cytochrome *c* (equine heart) were obtained from Serva (Heidelberg, F.R.G.). Acetylcholinesterase (electric eel) was purchased from Sigma (St. Louis, MO, U.S.A.).

Ascites fluid containing immunoglobulin M (IgM) monoclonal antibody produced by BALB/c mouse was passed through a 0.22-μm filter (Millipore, Bedford, MA, U.S.A.) prior to HPLC separation.

Apparatus

Pentax SH-0410F (100 mm × 4.6 mm I.D., particle size 2 μm) (Asahi Optical, Tokyo, Japan) hydroxyapatite columns were used with a precolumn of hydroxyapatite (10 mm × 4.6 mm I.D., particle size 10 μm).

A Hitachi (Tokyo, Japan) HPLC system consisting of L-6210 and L6010 pumps, a dynamic mixer, a ceramic injector and an L-4000 UV detector connected to a recorder was used.

Standard chromatographic procedure

Sample proteins were introduced onto a column of hydroxyapatite and eluted with a 30-min linear gradient at a flow-rate of 0.25 ml/min. The seven volatile solution systems used were 10–500 m*M* ammonium hydrogencarbonate (pH 8.0), 10–500 m*M* ammonium formate (pH 8.0), 10–500 m*M* ammonium acetate (pH 8.0), 10–500 m*M* ethylenediamine–acetic acid (pH 8.0), 10–500 m*M* ethylenediamine–HCl (pH 8.0), 10–1000 m*M* triethanolamine–acetic acid (pH 8.0) and 10–1000 m*M* triethanolamine–HCl (pH 8.0). All separations were conducted at room temperature. The effluent was monitored by measuring the absorbance at 280 nm. After completion of each chromatographic run, the column was re-equilibrated for 16–20 min with the initial solvent before the next cycle run. All the elution buffers were prepared immediately before use.

Measurement of acetylcholinesterase activity

Acetylcholinesterase activity was measured by a fluorimetric method using acetylthiocholine iodide (Wako, Osaka, Japan) as a substrate and 7-diethylamino-3-(4′-maleimidylphenyl)-4-methylcoumarin (Molecular Probes, Eugene, OR, U.S.A.) as a fluorescence reagent.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

The eluate from the column was lyophilized and analysed by SDS-PAGE (10–15% polyacrylamide gradient gel) in the presence of a reductant using the Phast System (Pharmacia, Uppsala, Sweden).

RESULTS AND DISCUSSION

Seven volatile solutions were applied to hydroxyapatite HPLC for the elution of proteins and the elution behaviours were investigated using four purified proteins, myoglobin, chymotrypsinogen A, lysozyme and cytochrome *c*.

The protein mixture was loaded on the column and eluted with a 30-min linear gradient. The chromatograms are shown in Fig. 1. For comparison, a chromatogram obtained with sodium phosphate buffer is shown in Fig. 1A. Compared with the use of phosphate buffer, HPLC using the volatile solutions gave lower resolution and recoveries of proteins. The proteins were not eluted with triethanolamine systems and

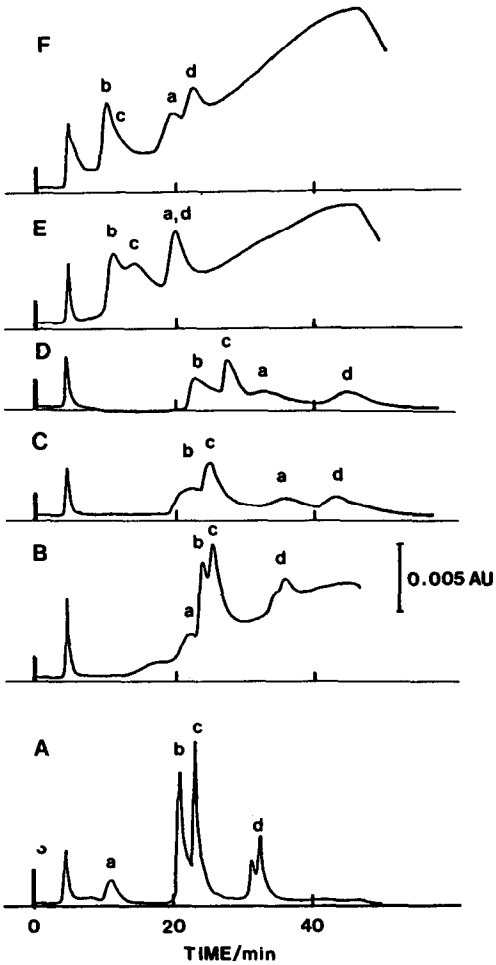

Fig. 1. Hydroxyapatite HPLC of a protein mixture (1.5–3 μg of each protein) using various volatile solutions for elution: (A) sodium phosphate (pH 8.0) (10–250 m*M*); (B) ammonium hydrogencarbonate (pH 8.0) (10–500 m*M*); (C) ammonium formate (pH 8.0) (10–500 m*M*); (D) ammonium acetate (pH 8.0) (10–500 m*M*); (E) ethylenediamine–acetic acid (pH 8.0) (10–500 m*M*); (F) ethylenediamine–HCl (pH 8.0) (10–500 m*M*). Peaks: a = myoglobin; b = lysozyme; c = chymotrypsinogen A; d = cytochrome *c*.

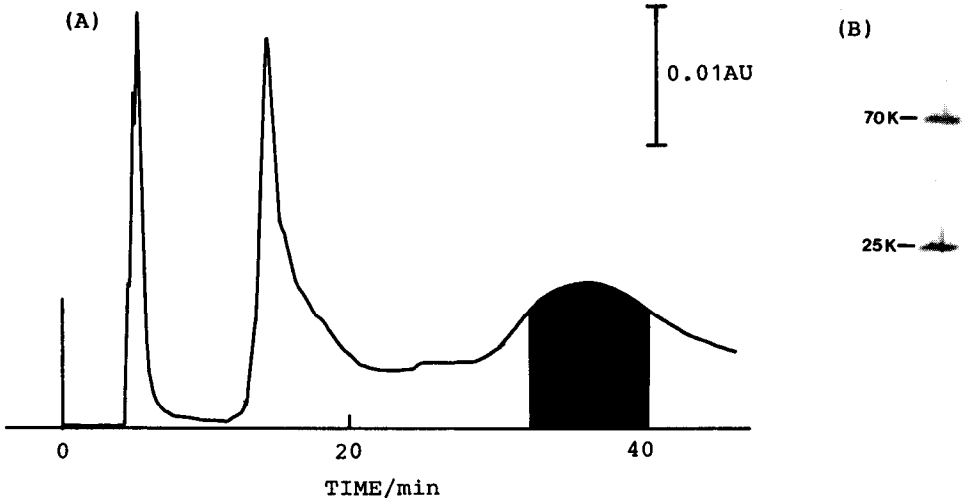

Fig. 2. Purification of IgM monoclonal antibody from mouse ascites fluid by hydroxyapatite HPLC using ammonium hydrogencarbonate solution for elution. Mouse ascites fluid (5 μl) was loaded onto the column and eluted by a 30-min linear gradient of ammonium hydrogencarbonate (pH 8.0) from 10 to 1000 m*M*. The column effluent in the shaded portion in A was collected and analysed by SDS-PAGE (B). K = kilodalton.

in the other systems the protein recoveries, calculated from the areas of the eluted peaks, were 40–80%, whereas with the phosphate buffer system the recovery was more than 90%. In the systems in Fig. 1C–F, myoglobin was retained on the column more tightly than in the other systems (Fig. 1A and B). Also, cytochrome *c* was eluted as one broad peak (Fig. 1C–F), whereas it was eluted as two peaks with phosphate buffer (Fig. 1A) or ammonium hydrogencarbonate (Fig. 1B). The reason for this difference in retention behaviour is not clear, but the solutions might affect the structures of these proteins. Among the systems tested, ammonium hydrogencarbonate seems to be the best alternative. This solution also has the advantage that the base and the acid are equally volatile and the residue after lyophilization is neither strongly acidic nor alkaline.

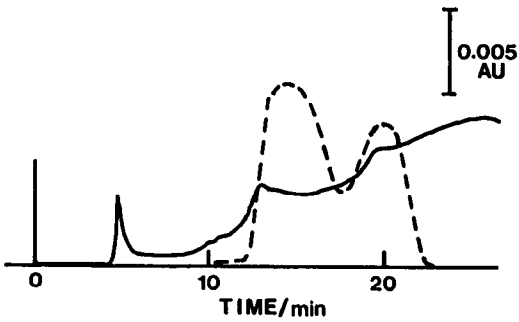

Fig. 3. Purification of acetylcholinesterase by hydroxyapatite HPLC using ammonium hydrogencarbonate solution for elution. A commercial sample of acetylcholinesterase (*ca.* 500 ng) was aplied to the column and eluted by a 20-min linear gradient of ammonium hydrogencarbonate (pH 8.0) from 10 to 500 m*M*. The broken line represents the activity of acetylcholinesterase.

IgM monoclonal antibody was purified from ascites fluid on the hydroxyapatite column using ammonium hydrogencarbonate solution for elution. A 5-μl volume of ascites fluid was applied to the column and eluted with a 30-min linear gradient of ammonium hydrogencarbonate from 10 to 1000 m*M*. The effluent was fractionated and lyophilized, and then analysed by SDS-PAGE and enzyme-linked immunosorbent assay (ELISA). The results are shown in Fig. 2. IgM monoclonal antibody was eluted as a broad peak (Fig. 2A), which preserved the immunoreactivity. The results of SDS-PAGE (Fig. 2B) suggested that the antibody was highly purified. Fig. 3 shows the separation of a commercial crude sample (*ca.* 500 ng) of acetylcholinesterase by hydroxyapatite HPLC with ammonium hydrogencarbonate. The effluent from the column was collected and lyophilized, and the activity was measured. Acetylcholinesterase activity was found in two peaks eluting at *ca.* 14 min and *ca.* 20 min. The recovery of the activity was about 70%.

In the purification of very small amounts of proteins, the concentration or desalting step may cause losses of proteins. Hydroxyapatite HPLC of proteins using volatile solutions for elution appears to be a useful tool for the purification of very small amounts of proteins.

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