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Note

High-performance hydroxyapatite chromatography of proteins

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Hydroxyapatite chromatography has been employed extensively for the purification of proteins since Tiselius *et al.*[1] developed a method of preparing coarse-grained hydroxyapatite crystals in 1956. It has been applied successfully to a wide range of proteins. Furthermore, high-performance columns have been developed during the last few years[2–8] and rapid separations have become possible. However, it seems that some problems still remain to be solved. For example, the column stability is insufficient and the resolution is not as high as that obtained by other modes of high-performance liquid chromatography (HPLC).

A new high-performance hydroxyapatite column has recently become commercially available under the trade-name of TSK gel HA-1000 (Toyo Soda, Tokyo, Japan). We have evaluated this column for protein separation and the results are now described.

EXPERIMENTAL

All chromatographic measurements were performed at 25°C with a system consisting of a Model CCPM pump and a Model UV-8000 variable-wavelength UV detector operated at 280 nm (Toyo Soda). Proteins were usually separated with a 30-min linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8) at a flow-rate of 1 ml/min on a 75 mm × 7.5 mm I.D. stainless-steel column. A guard column of 10 mm × 6 mm I.D. was also used in series. The packing materials were spherical macroporous microparticulate hydroxyapatite, as shown in Fig. 1. The particle diameter was about 5 μm. The average pore size and the specific surface area, determined by mercury porosimetry, were *ca.* 750 Å and 30 m^2/g, respectively. The protein adsorption capacity, determined by a dynamic method with bovine serum albumin in 10 m*M* sodium phosphate (pH 6.8), was about 20 mg/ml. The X-ray pattern displayed only the narrow peaks characteristic of hydroxyapatite.

The recovery of proteins was determined from the areas of eluted peaks. As controls, we used the areas obtained when the column was replaced by an empty 1 mm I.D. stainless-steel tube of 1 ml total inner volume and the elution was performed isocratically at the eluent compositions at which each protein was eluted in gradient elution. Amounts of 50 μg of each protein were injected. The peak areas were measured with a data processor Model CP-8000 (Toyo Soda).

Bovine serum albumin was obtained from Wako (Osaka, Japan) and oval-

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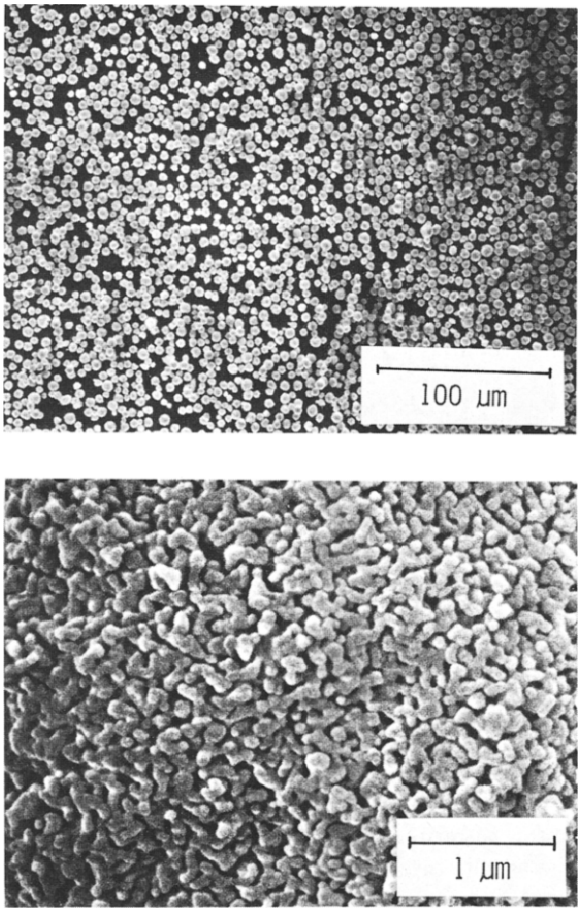

Fig. 1. Scanning electron photomicrographs of TSK gel HA-1000.

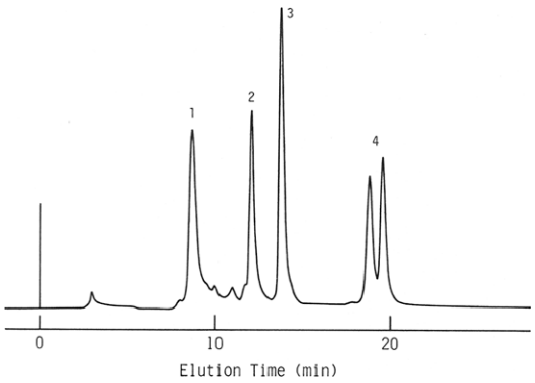

Fig. 2. Separation of a protein mixture on TSK gel HA-1000. Peaks: 1 = bovine serum albumin; 2 = ribonuclease A; 3 = α-chymotrypsinogen A; 4 = cytochrome *c*.

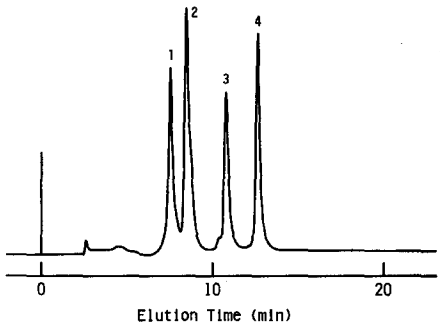

Fig. 3. Separation of a protein mixture on TSK gel HA-1000. Peaks: 1 = ovalbumin; 2 = α-lactoalbumin; 3 = myoglobin; 4 = lysozyme.

bumin from Seikagaki (Tokyo, Japan). All other proteins were obtained from Sigma (St. Louis, MO, U.S.A.).

RESULTS AND DISCUSSION

Figs. 2 and 3 show separations of protein mixtures. The proteins were separated in 10–20 min with high resolution. It may be concluded from these results that chromatography on the new hydroxyapatite has a resolution comparable to that of other modes of HPLC.

The column stability was evaluated by separating a protein mixture repeatedly at intervals of about 35 min. The guard column was repacked with new hydroxyapatite after every 30 experiments. The first and 120th chromatograms are shown in Fig. 4. There was almost no change in the patterns obtained, suggesting that the TSK gel HA-1000 column is stable and the results obtained are reproducible. When the guard column was used without repacking, however, shoulders appeared before the regular peaks after 35–40 experiments. Once such shoulders had appeared, the column could not be regenerated by wsashing it with solvents like aqueous sodium hydroxide solution.

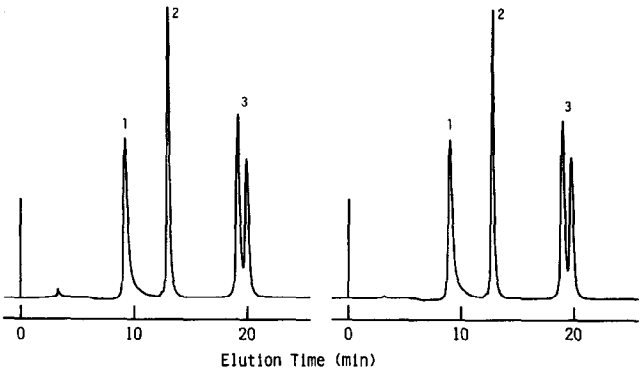

Fig. 4. First (left) and 120th (right) chromatograms of a protein mixture obtained on TSK gel HA-1000. Peaks: 1 = bovine serum albumin; 2 = lysozyme; 3 = cytochrome *c*.

TABLE I

RECOVERY OF PROTEINS FROM TSK gel HA-1000

Protein	*Recovery (%)*	*Protein*	*Recovery (%)*
Transferrin	100	Trypsinogen	91
Bovine serum albumin	91	Myoglobin	99
Ovalbumin	101	Lysozyme	96
α-Chymotrypsinogen A	100	Ribonuclease A	100
α-Chymotrypsin	99	Cytochrome *c*	100

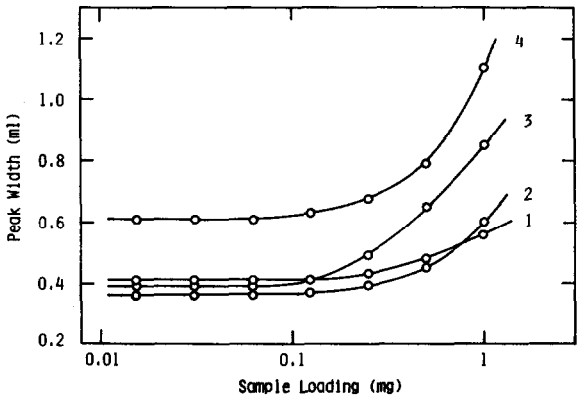

Fig. 5. Dependence of the peak width on sample load in the separation of proteins on TSK gel HA-1000. Samples: 1 = α-chymotrypsinogen A; 2 = lysozyme; 3 = ribonuclease A; 4 = myoglobin.

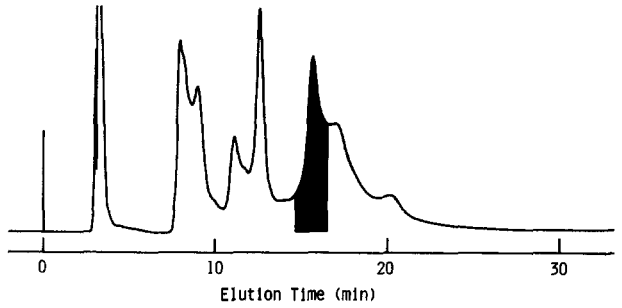

Fig. 6. Purification of lactate dehydrogenase by high-performance hydroxyapatite chromatography on TSK gel HA-1000.

The recovery of proteins is summarized in Table I. All proteins tested were recovered almost quantitatively. The recovery of enzymatic activity was also high, more than 90%, as described later.

The loading capacity was evaluated by separating pure proteins individually using various sample loads. The peak width is plotted against the sample load in Fig.

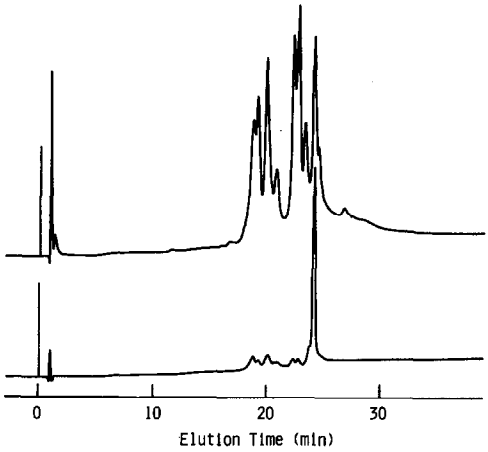

Fig. 7. Purity test of lactate dehydrogenase fraction by reversed-phase chromatography. Crude lactate dehydrogenase sample (upper curve) and the fraction in Fig. 6 (lower curve) were separated on TSK gel Phenyl-5PW RP (75 mm × 4.6 mm I.D.) with a 2-min linear gradient from 5 to 20% acetonitrile followed by a 48-min linear gradient from 20 to 80% acetonitrile in 0.05% trifluoroacetic acid at a flow-rate of 1 ml/min at 25°C and detected at 220 nm.

5. It remained constant at loads up to 0.1–0.2 mg, and then increased with further increase in the sample load. Accordingly, the maximum sample load resulting in the highest resolution is 0.1–0.2 mg. However, this amount is for pure samples and larger amounts (up to 1–2 mg) of crude samples could be applied without decrease in resolution. Furthermore, if a slight decrease in resolution is acceptable, more samples can be applied.

Fig. 6 shows an application of high-performance hydroxyapatite chromatography on TSK gel HA-1000 to protein purification. A commercial crude sample of lactate dehydrogenase (1 mg) was separated. Lactate dehydrogenase activity was found in the peak eluted at *ca.* 15 min and the column effluent in the shaded portion

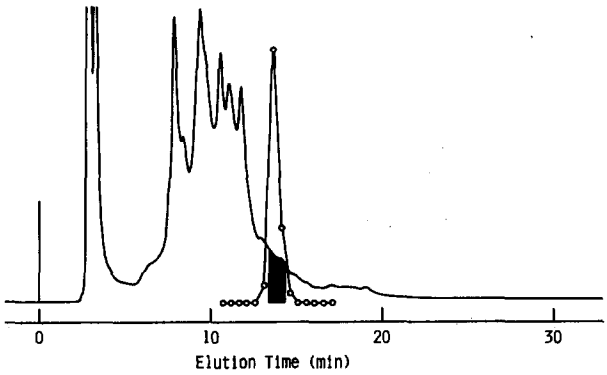

Fig. 8. Purification of glucose-6-phosphate dehydrogenase from yeast extract by high-performance hydroxyapatite chromatography on TSK gel HA-1000. The open circles represent the glucose-6-phosphate dehydrogenase activity.

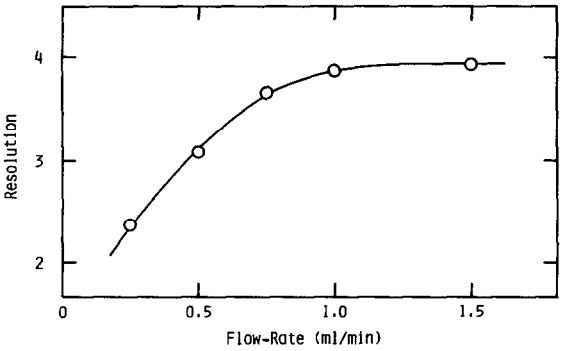

Fig. 9. Dependence of the resolution on flow-rate in high-performance hydroxyapatite chromatography on TSK gel HA-1000. A mixture of ribonuclease A and α-chymotrypsinogen A was separated with a 60-min linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8) at various flow-rates. The resolution was calculated from the peak widths and elution volumes of the two proteins.

in Fig. 6 was collected. The recovery of enzymatic activity in this fraction was 93%. The purity of the fraction was examined by reversed-phase chromatography (Fig. 7). One main peak and several small peaks were observed. Because the main peak was identified as lactate dehydrogenase from the elution position of a commercial pure sample, it can be said that lactate dehydrogenase was highly purified.

Fig. 8 shows another example of protein purification. Yeast extract (1 mg) was separated. The column effluent was fractionated and the 0.5-ml fractions were examined for glucose-6-phosphate dehydrogenase activity. The total recovery of glucose-6-phosphate dehydrogenase activity was 96%, and 89% of the applied activity was recovered in two fractions. The degree of purification for the two fractions was 29-fold.

The effects of operational variables were also studied in order to determine the optimum conditions for high-performance hydroxyapatite chromatography on TSK gel HA-1000. Fig. 9 shows the dependence of the resolution on flow-rate at a constant

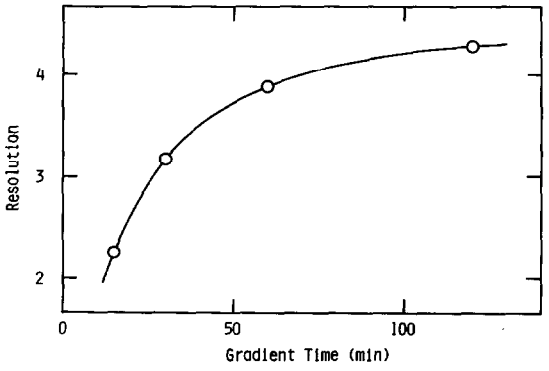

Fig. 10. Dependence of the resolution on gradient time in high-performance hydroxyapatite chromatography on TSK gel HA-1000. A mixture of ribonuclease A and α-chymotrypsinogen A was separated with a linear gradient from 10 to 500 m*M* sodium phosphate (pH 6.8) at a flow-rate of 1 ml/min. The gradient time was varied between 15 and 120 min. Resolutions calculated as in Fig. 9.

gradient time. The resolution increased with increasing flow-rate up to about 0.8 ml/min, then became almost constant. Because an increase in flow-rate results in dilution of the sample during separation, too high flow-rates are disadvantageous. Consequently, flow-rates of 0.8–1.0 ml/min seem to be a good choice. Fig. 10 shows the dependence of resolution on gradient time at a constant flow-rate. Higher resolution was obtained at longer gradient times although the effect became insignificant at gradient times longer than 60 min. Because longer gradient times result in longer separation times and greater dilution of the sample, it is disadvantageous to employ extremely long gradient times. Gradient times around 60 min, which correspond to an increase in sodium phosphate concentration of about 8 m*M*/min, seem to be a good compromise. The effect of eluent pH was investigated by separating a protein mixture with a 30-min linear gradient from 10 to 500 m*M* sodium phosphate at pH 5.8, 6.8 and 7.8. Proteins were more strongly retained at lower pH, but the selectivity was little changed. The peak width and peak interval were also dependent on the eluent pH. Although both became broader as the eluent pH decreased, the resolution was almost unchanged at pH 5.8–7.8.

As demonstrated above, the new macroporous spherical microparticulate hydroxyapatite, TSK gel HA-1000, was very useful for the separation of proteins. Proteins could be separated rapidly with high resolution and recovery. In addition, the column was very stable and the reproducibility of separation was excellent.

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