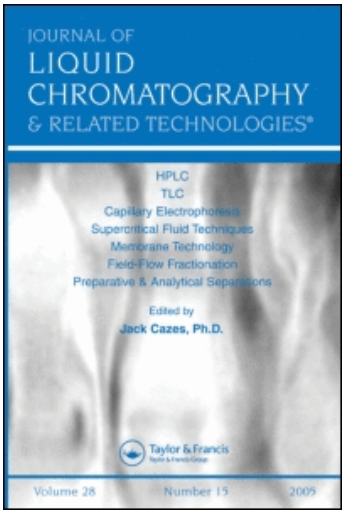

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A NEW SPHERICAL HYDROXYAPATITE FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

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ABSTRACT

Basic properties of a newly developed hydroxyapatite column and results of its application to the separation of proteins are described. The hydroxyapatite was completely spherical and porous beads in appearance by scanning electron microscopy, and showed superior properties to other types of hydroxyapatite column. The column was mechanically strong enough to show the pressure limit of 140-150 kg/cm^2. The hydroxyapatite column showed excellent mechanical and chemical stability, and was applicable to high speed and high resolution separation of proteins. Proteins are recovered in high yield after the chromatography.

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INTRODUCTION

Since the work of Tiselius and Hjerten et al(1,2), the hydroxyapatite column has been known to be a helpful tool for the purification of proteins(3-9), especially when other types of chromatographic column were not successful. Furthermore, the hydroxyapatite column can be used under solvent conditions including various additives such as glycerine, urea(10,11), nonionic detergents(12) or even some of the ionic detergents like sodium dodecyl sulfate(13). However, the properties of hydroxyapatite particles vary from batch to batch with respect to the particle size, the level of hydroxylation, or the content of calcium ions. Further, hydroxyapatite chromatography was ordinarily time consuming because of the fragility of the crystal of hydroxyapatite. Then, a higher stability of the column was required for the high performance liquid chromatography. Recently, several hydroxyapatite columns have been developed and commercialized for HPLC. In this paper, the more efficient beads of hydroxyapatite for HPLC is described. The newly developed hydroxyapatite was spherical particle in shape and porous in appearance, and were mechnically and chemically stable enough for repeated use in HPLC.

MATERIALS AND METHODS

Reagents and Proteins; Sodium dihydrogenphosphate, disodium hydrogenphosphate and calcium chloride were all guaranteed grade purchased from Wako Pure Chemical Industries (Tokyo). Water was distilled, passed through a mixed-bed ion-exchange resin, and redistilled before use. Proteins used were obtained from sources indicated in parentheses; bovine serum albumin, horse heart cytochrome c(Sigma Chemicals Co., St. Louis) and chicken egg lysozyme(Sigma Chemicals Co. or Seikagaku Kogyo Co. Ltd, Tokyo).
Column; A column (8.0 mm I.D. x 100 mm) of the hydroxyapatite was prepared from about 10 % hydoxyapatite beads slurry in 0.3 M

sodium phosphate buffer(pH 6.8) and equilibrated with the same solvent at a constant pressure of 50 kg/cm . Two types of hydroxyapatite beads(A1 and T1) differing in their particle size distribution were used.

Apparatus; Japan Spectroscopic Co.(Tokyo) HPLC system composed of a double plunger pump(BIP-I), Rheodyne model 7125 injector and a Model UVIDEC-100-III uv spectrophotometer(10 mm light path) connected to a model R-111 recorder(Simadzu Co., Kyoto), was used. The gradient elution was performed by a gradient system which was composed from a coil mixer and two electromagnetic valves MTV-2 (Denso Sangyo Co., Tokyo) controlled by a MSX microcomputer (CASIO PV-7, Casio Co., Tokyo).

Chromatographic procedure; Sample proteins were introduced onto a column of hydroxyapatite and eluted with the linear gradient from 0.01 M sodium phosphate buffer (pH 6.8) containing 0.3 mM calcium chloride to 0.3 M or 0.4 M sodium phosphate buffer (pH 6.8) containing 0.01 mM calcium chloride at a flow rate of 1.0 ml/min. All separations were conducted at room temperature. The effluent was monitored at 280 nm. After one analytical cycle run, the column was reequilibrated for 12-20 min with an initial solvent for the next analysis.

Scanning electron microscopy; The hydroxyapatite beads were dried under vacuum and subjected to sputtering coating with Au/Pd. The microstructure of the samples was observed with a Model S-430 scanning electron microscope(Hitachi Ltd., Tokyo).

RESULTS AND DISCUSSIONS

Observation of various hydroxyapatite by scanning electron microscopy

The various kinds of hydroxyapatite preparations are now supplied on a commercial basis for liquid chromatography. Figure 1 shows the appearance of those various hydroxyapatite beads by scanning electron microscopy. The observation was made at two

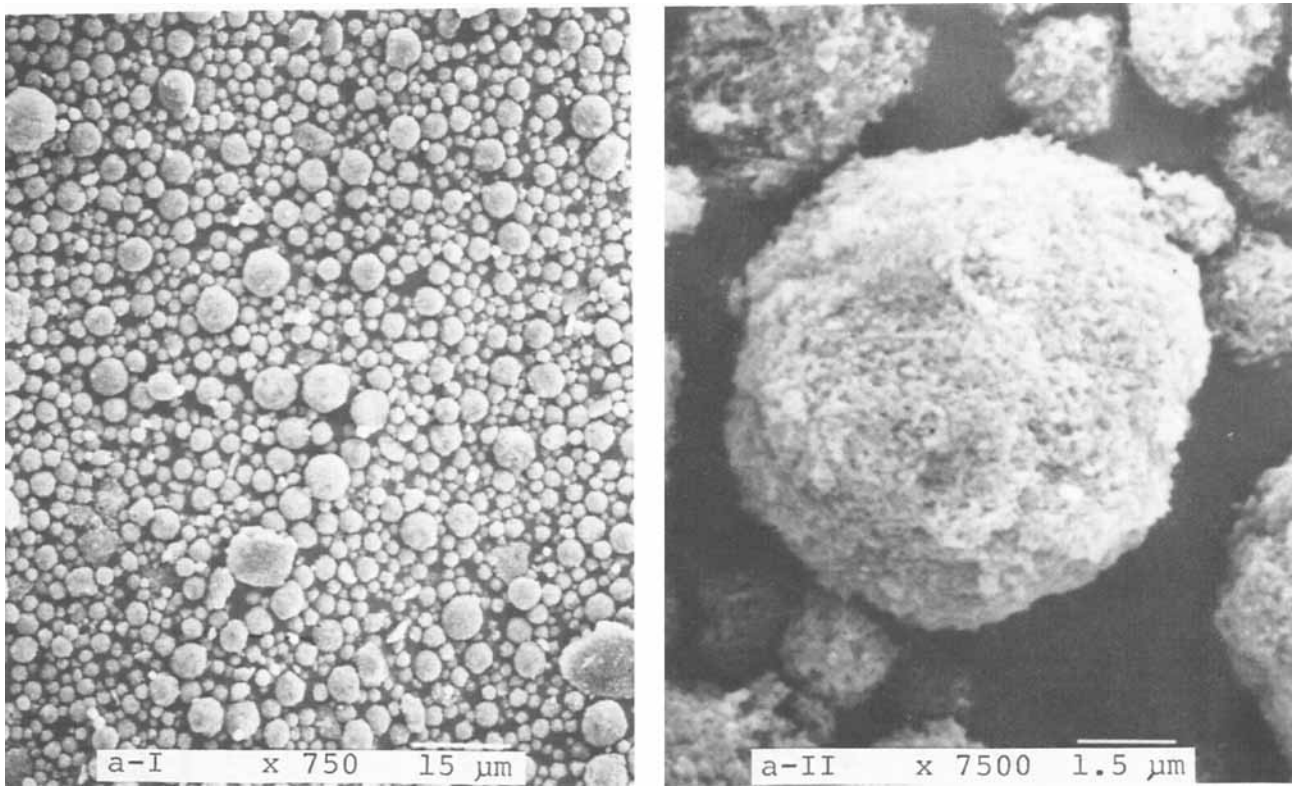

Fig.1 Scanning electron photomicrograph of various hydroxyapatites. (a)Toa Nenryo Kogyo and Asahi Optical, (b)Bio-Rad, (c)Mitui Toatsu, (d)British Drug House. Magnification was indicated at the bottom of respective photographs.

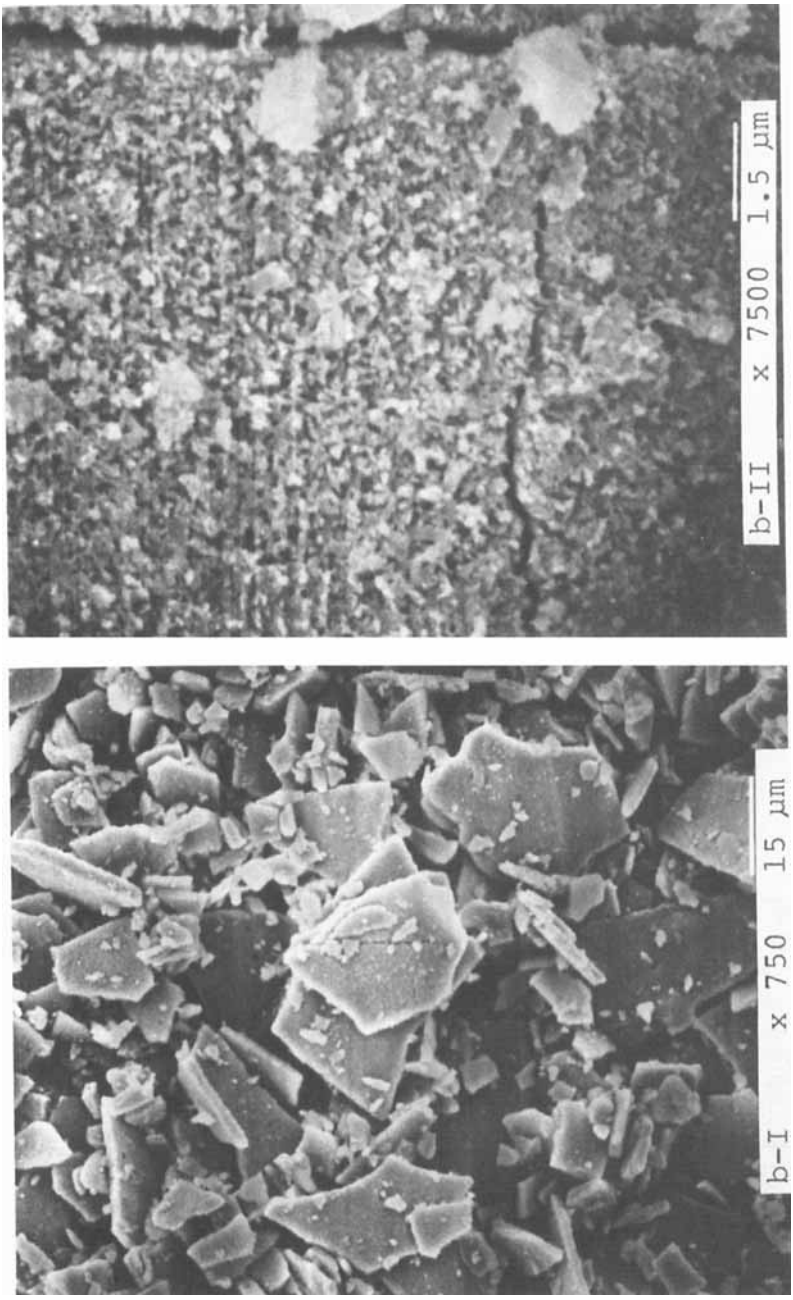

Fig. 1b

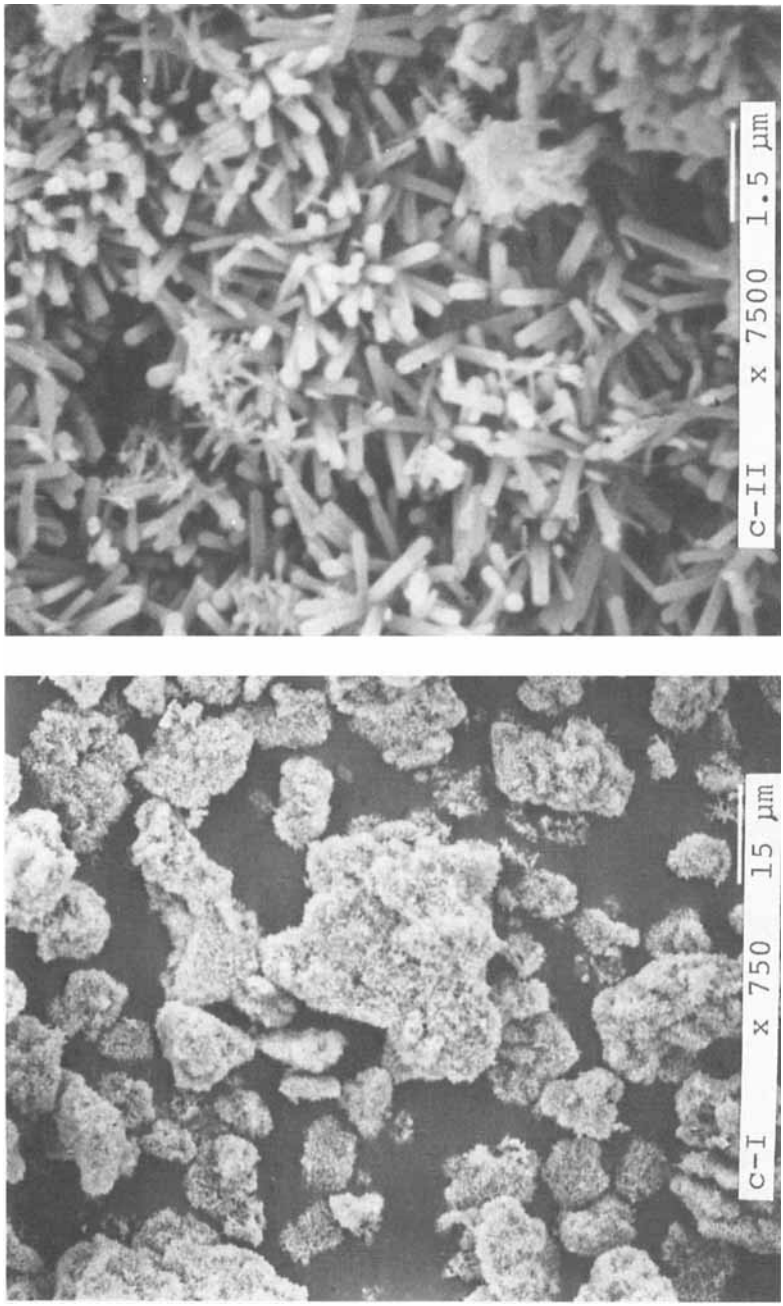

Fig. 1c

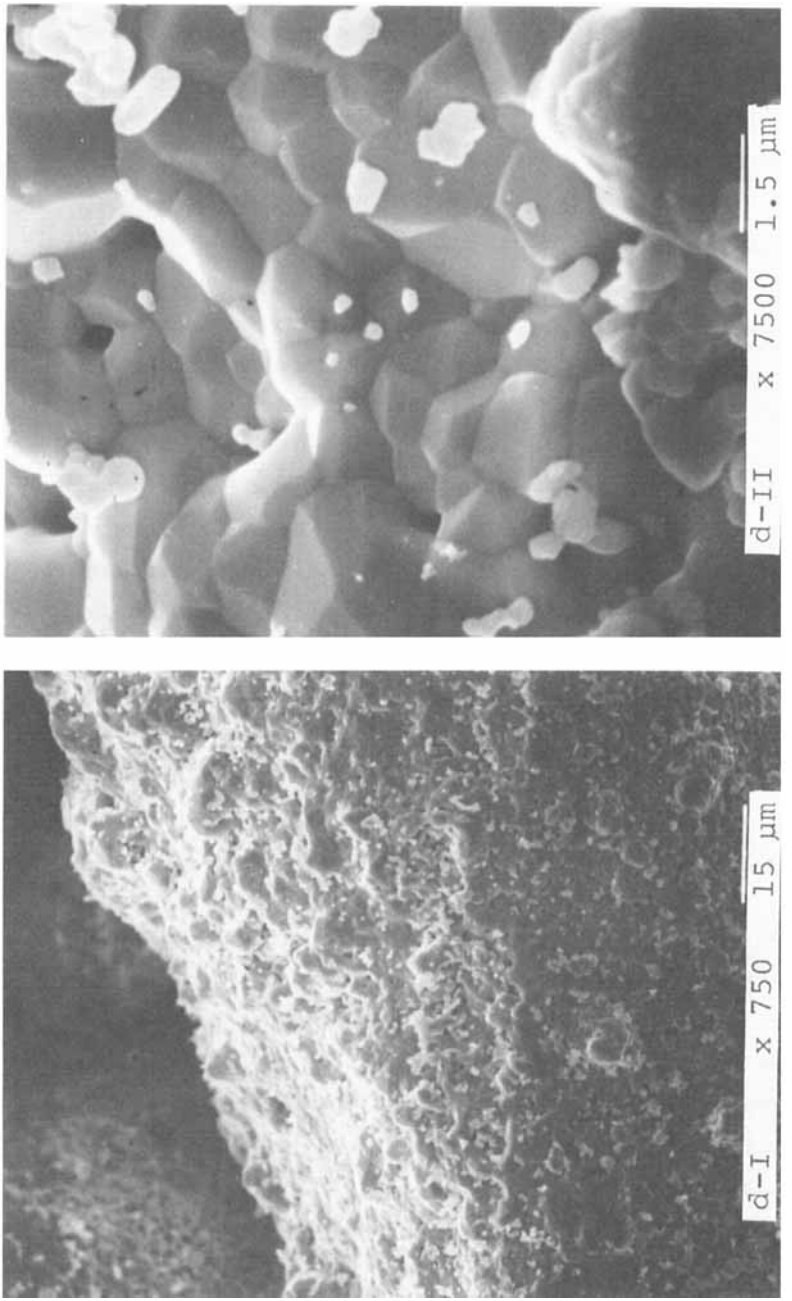

Fig. 1d

magnifications, 750 fold and 7500 fold. The former gives the particle size and its distribution, and the latter shows the surface texture of those particles. These observations show that the hydroxyapatite beads are in various forms, but they may be classified into four groups.

In the first group, where the hydroxyapatite of Toa Nenryo Kogyo Co. Ltd-Asahi Optical Co. Ltd is involved, the particles are all spherical and the size of particles is very small, although they still have rather broad distributions(Fig.1a-I). The surface of particles seems to be porous and to be formed from many whiskers(Fig.1a-II).

In the second group, where the Bio-Rad hydroxyapatite is involved, the material appears as the crystalline flakes, and the size distribution is broad(Fig.1b-I). The surface texture looks like to be granulated(Fig.1b-II). The similar forms are observed on the hydroxyapatites of Koken Co. Ltd. and of Central Glass Inc. AN type, but the Central Glass's one shows some whiskers on the crystal surface.

In the third group, where the hydroxyapatite of Mitsui Toatsu Co., Wako Pure Chemicals and of Central Glass Inc. BN type are involved, the appearance of particles looks like irregular shaped granulated lumps. The size is fairly large but the distribution itself is rather narrow(Fig.1c-I). The surface appears to be formed from small rods something like sugar confects(Fig.1c-II).

The hydroxyapatite of British Drug House has different appearance from other hydroxyapatites. The particle size is very large(Fig.1d-I). The surface structure is smooth and looks like the melted surface(Fig.1d-II). This can be classified into the fourth group.

Flow rate-pressure relation of the spherical hydroxyapatite

The relation between flow rate and pressure drop observed on a 0.8 cm I.D. x 10 cm column is shown in Fig.3. The pressure of

the A1 column remained low with increasing flow rate; the pressure was 9 kg/cm^2 even at a flow rate of 7 ml/min. In the T1 column, the pressure increased linearly to 100 kg/cm^2 with increasing flow rate to 2.5 ml/min. Further increase in flow rate caused sudden increase in back pressure (Fig. 3) indicating that the pressure limit of this column was 140-150 kg/cm^2. These results suggest that the spherical hydroxyapatite has sufficent mechanical strength in a practical use for HPLC.

Chromatographic performance of the spherical hydroxyapatite

The performance of the various types of hydroxyapatite columns were evaluated by eluting lysozyme with the standard linear gradient elution (Materials and Methods). From the chromatograms, "theoretical plate numbers" were calculated as a tentative parameter for the column resolution, although the precise estimation of this parameter requires the chromatography under an isocratic elution condition. The values are summarized in Table 1. As Compared with the other types of hydroxyapatite (14), the new spherical hydroxyapatite column showed higher theoretical plate number. This can be attributed by the small particle size and spherical shape of this hydroxyapatite. The details of particle size distribution of the spherical hydroxyapatite, estimated by multiple image analysis under electron microscope, is shown in Fig. 2.

Stability of the hydroxyapatite column

The stability of the column were tested under two chromatographic conditions; in the presence or in the absence of calcium chloride (0.3 mM to 0.01 mM) in the phosphate buffer eluant. After a hundred repeated cycles of the chromatography at flow rate of 1.0 ml/min (40 kg/cm^2), the beads did not show any changes in size distribution and in appearance as obseved by scanning electron microscopy. Even after the application of 100 kg/cm , no changes occured in the particles size. Figure 4 shows the elution patterns of proteins obtained at the 3rd(Fig.4a),

Table 1 Comparison of various types of hydroxyapatite columns for HPLC

hydroxyapatite	TPN* (10 cm column) 60 min grad.	30 min grad.	Particle Sizes (um)	Ca/P
Toa Nenryo-Asahi Optical T1	19,000	3,700	0.2 - 3	1.67
Toa Nenryo-Asahi Optical A1	3,200	1,000	1.0 - 6	1.67
Bio-Rad	4,200	2,000	2 x 7 - 19 x 27**	1.53
Mitsui Toatsu	730	-----	4 x 7 - 40 x 70**	1.50
Koken KB	900	250	5 x 7 - 35 x 50**	1.52

* Theoretical plate numbers. The numbers were calculated with lysozyme.
** Values of (breadth x length)

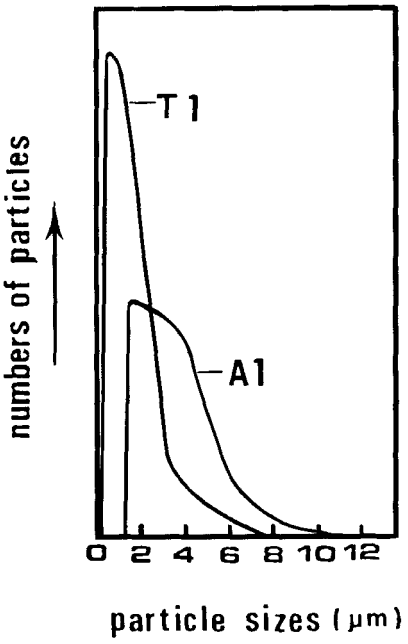

Fig.2 Distribution of particle size of the spherical hydroxyapatite.

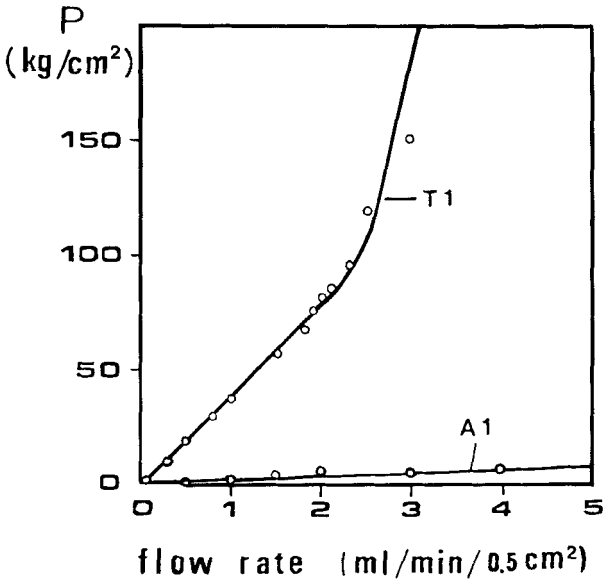

Fig.3 Reletion between flow rate and the pressure of the hydroxyapatite column. The test was performed on a 0.8 cm I.D. x 10 cm column with 0.01 M sodium phosphate buffer (pH 6.8) containing 0.3 mM calcium chloride.

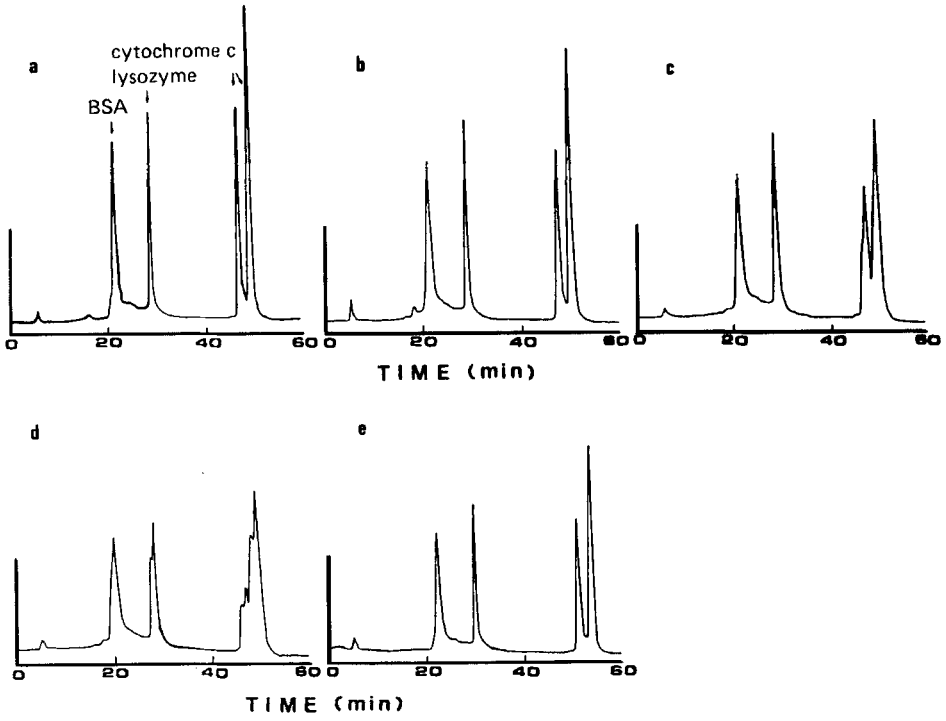

Fig.4 Stability and restoration of the hydroxyapatite column in HPLC of proteins. Bovine serum albumin, chicken egg lysozyme and horse heart cytochrome c were applied to the column and eluted by 60 min-linear gradient from 0.01 M sodium phosphate buffer containing 0.3 mM calcium chloride to 0.3 M sodium phosphate buffer containing 0.01 mM calcium chloride.

56th(Fig.4b), 78th(Fig.4c) and 83rd(Fig.4d) chromatography cycles, which was performed using the elution buffer containing calcium chloride. The results showed good reproducibility indicating excellent stability of the column. The relative standard deviations of the retention time were very small. After the 83rd cycles, however, it began to show the double peaks; one appeared at 0.5 - 1.0 min earlier than the other regular peak in any proteins. Such a phenomenon was also observed even in the

silica or chemically modified silica after repeated cycles of chromatography. This kind of double peaks could be restored by repacking of the column or by taking out small amount of packing material from the top of the column and refilled with the new packing material. In the case of hydroxyapatite, the same kind of treatment has been also successful as shown in Fig.4e which shows the results after the restoration mentioned above.

Without addition of calcium chloride in the elution buffer, the changes in peak shape have already been observed after 15 cycles. This could be also restored by the treatment mentioned above.

Recovery of proteins

The recovery of proteins from two types (T1, A1) of column was estimated by measuring uv absorbance at 280 nm before and after chromatography. In the repeated estimation with appropriate amount of proteins, the average recovery was 90-100 per cent.

Loadability

Various amounts of lysozyme were loaded on the column, and the peak shape and protein recovery were examined. The results of the chromatography are shown in Fig.5, and the relation between amounts of the loading protein and height equivalent of a theoretical plate is shown in Fig.6. Up to 50 mg of lysozyme could be adsorbed on the 0.8 cm I.D. column and chromatographed by the subsequent elution. However, with an increasing amount of proteins the tailing of peaks increased. In the ordinary ranges of protein amount, the recovery was within a level of 90 - 100 per cent and no ghost peaks were observed in the successive elution pattern.

CONCLUSION

The newly developed hydroxyapatite beads show superior characteristics than hydroxyapatite previously reported for

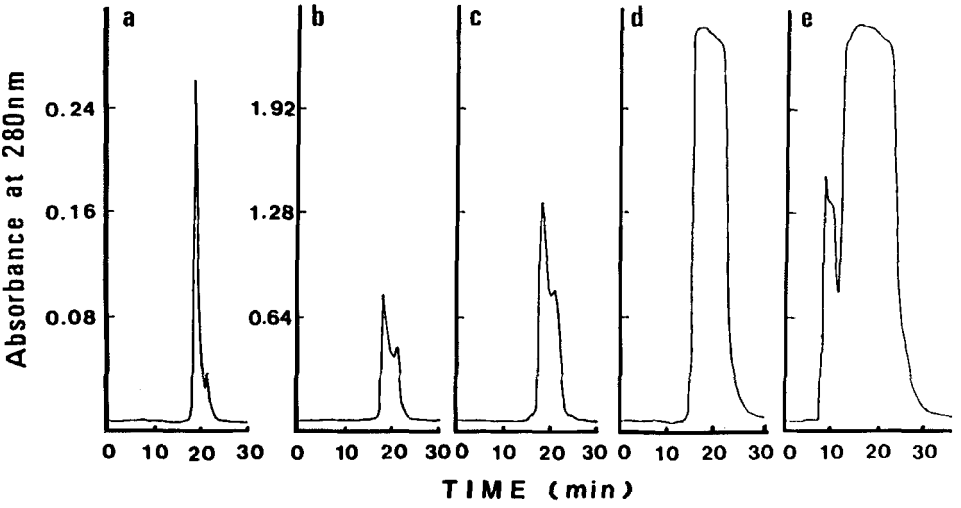

Fig.5 Loadability of the hydroxyapatite column. Various amounts of chicken egg lysozyme were applied to the column and eluted by 30 min-linear gradient from 0.01 M sodium phosphate buffer (pH 6.8) containing 0.3 mM calcium chloride to 0.4 M sodium phosphate buffer (pH 6.8) containing 0.01 mM calcium chloride. 400 ug(a), 3.2 mg(b), 10 mg(c), 50 mg(d), 100 mg(e) of lysozyme were applied ,respectively.

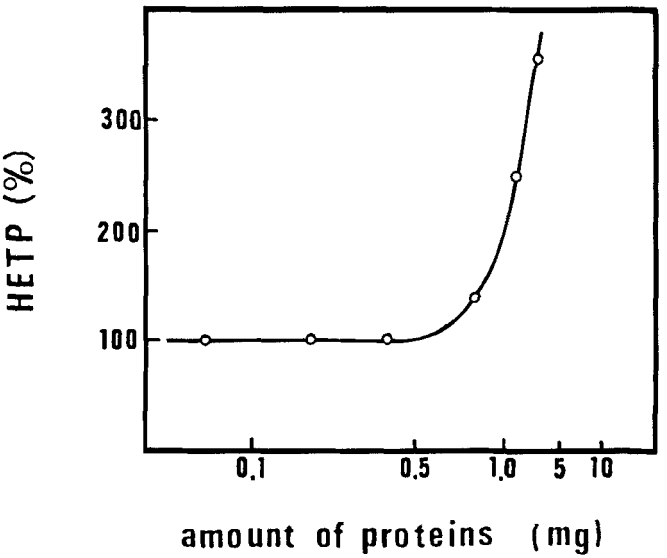

Fig.6 Dependence of height equivalent of theoretical plate on sample loading. Conditons were as in the legend to Fig.5.

liquid chromatography. It is almost spherical and porous in type, and has sufficient mechanical strength for HPLC. The hydroxyapatite column is quite stable mechanically and chemically and shows extremely high resolution in separation of proteins.

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