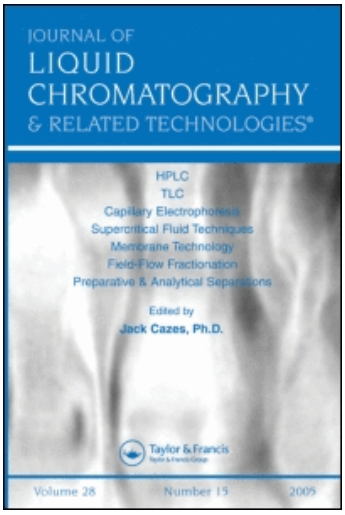

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON A HYDROXYAPATITE COLUMN

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ABSTRACT

High performance liquid chromatography on a spherical ceramic type hydroxyapatite has been applied successfully for the separation of various kinds of proteins. Twenty-one proteins of various origin, having an isoelectric point of 3.3-11.0 and a molecular weight of 11,000-190,000 daltons, were loaded on the column and eluted by linear gradient of sodium phosphate at pH 6.8. The chromatography showed good resolution and high recovery for the proteins. The analysis of the retention behavior, relation between capacity ratio and physicochemical properties of proteins, showed a tendency that the capacity ratio of protein increased with the pI value of the protein.

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INTRODUCTION

Since the original works by Tiselius et al(1,2), hydroxyapatite has been used for the specific separation of proteins(3-11). However, the convensional gel type of hydroxyapatite was inadequate for the high performance liquid chromatography(HPLC) because of the low mechanical strength, chemical durability and the time consuming elution process. Recently, a spherical ceramic type hydroxyapatite has been prepared for HPLC. In the previous paper(12), we described on the basic properties of this newly prepared hydroxyapatite column. The column showed excellent properties for the rapid separation of proteins with the complete biological activity and stability in processing.

In this paper, the more extensive application of this column to the various kinds of proteins are described. For the present study, two types of the spherical ceramic hydroxyapatite columns were used, one with smaller size particles and the other with fairly larger size particles. Retention times of proteins on the hydroxyapatite was related with the isoelectric points of proteins, and was compared with those obtained with the traditional flake-form of hydroxyapatite column.

MATERIALS AND METHODS

Proteins; Calmodulin, S100 protein and neuron-specific enolase were purified from bovine brain as described previously(13-15). Serum amine oxidase and ceruloplasmin were prepared from bovine serum by ammonium sulfate fractionation and following DEAE-Sephadex A-50 column chromatography(16). The NIG-65 protein, human myeloma immunoglobulin D, was isolated as described previously(17). Phenol oxidase was kindly donated by Dr. J. Sugiura of Oji Paper Co.(Tokyo), laccase and stellacyanin by Dr. R. Oshima of university of Tokyo. Other proteins were obtained from sources indicated in parentheses; bovine serum albumin(ICN Pharmaceutical Inc., Cleveland, Ohio), transferrin, ovalbumin, myoglobin, cytochrome c, fetuin(Sigma Chemicals Co., St.Louis,

Missouri), immunoglobulin G(Sandos Ltd., Barsel), lysozyme (Seikagaku Kogyo Ltd., Tokyo), trypsinogen and trypsin(Millipore Co., Bedford, Maryland), α-chymotrypsin and chymotrypsinogen A (Worthington Biochemicals, Freehold, New Jersey).

Reagents; Sodium dihydrogenphosphate, disodium hydrogenphosphate and calcium chloride were purchased from Wako Pure Chemicals Industries(Tokyo). Water was distilled, passed through a mixed-bed ion exchange resin, and redistilled before use.

Column; The columns(8.0 mm I.D. x 100 mm) of the hydroxyapatite of Toa nenryo Kogyo Co.-Asahi Optical Co. were used with a precolumn(8.0 mm I.D. x 30 mm) of the hydroxyapatite. The column(T) was packed with smaller particles(2-3 um) and the column(A) was packed with larger particles(6-8 um). For the comparison, KB hydroxyapatite columns(6.0 mm I.D. x 300 mm, 8 mm I.D. x 100 mm) of Koken Co. (Tokyo) were used.

Apparatus; Japan Spectroscopic Co.(Tokyo) HPLC system consisted of a double plunger pump(BIP-I), Reodyne sample injector(model 7125) and a UVIDEC-100-III uv spectrophotometer(10 mm light path) connected to a recorder(Shimadzu model R-111, Shimadzu Co., Kyoto), was used. The gradient elution was performed by the gradient system which was composed from a coil mixer and two electromagnetic valves(MTV-2, Denso Sangyo Co., Tokyo.) which were controlled by a MSX microcomputer(Casio Co., Tokyo)(18).

Standard chromatographic procedure; Sample proteins(30-500 ug proteins) were introduced onto a column of the hydroxyapatite and eluted with 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 at a flow rate of 1.0 ml/min. Other than above standard elution process, shallower linear gradient system or concave or convex system were also used when necessary. All separation was conducted at room temperature. The effluent was monitored at 280 nm or 230 nm. When further analysis was necessary, the column effluent corresponding to the peak was collected into a test tube mannualy and dialyzed in a micro dialyzer. After completion of

each analytical run, the column was reequilibrated for 12-20 min with an initial solvent before the next cycle run.

Recovery of proteins; Recovery of proteins was calculated by UV absorbance at 280 nm before and after chromatography.

Estimation of isoelectric point(pI) of proteins; The pI values of proteins were measued by two-dimensional electrophoresis in the absence of denaturing agents as described previously(19), where the isoelectric focusing and the polyacrylamide gradient (4-17%) gel electrophoresis were employed for the first or second dimension, respectively. Some of the pI values of basic proteins were calculated by the microcomputer method of Manabe(20) based the amino acid compositions and Henderson & Hasselbalch equation.

RESULTS

Chromatograms of various proteins on hydroxyapatite

Twenty-one purified proteins, having an isoelectric point of 3.3-11.0 and a molecular weight of 11,000-190,000 daltons as listed in Table 1, were loaded onto a hydroxyapatite column(T) and eluted by a standard 30 min-linear gradient system. The chromatograms of proteins are shown in Fig.1. Under these conditions all the tested proteins were eluted with the recoveries of more than 90 per cent. The recovery and the retention time of each protein were summurized in Table 1. Most of the purified proteins were eluted as an almost symmetrical and sharp major peak, sometimes accompanied with some minor peaks. However, some of the so called purified proteins showed still complex elution profile; a broad peak or multiple peaks. These results would be explained by the heterogeneity of the proteins, based on the differences in contents of carbohydrates or in the micro heterogeneity in primary structue and on the conformational changes - that is partial denaturation. The following are the some of the comments on the chromatograms.

(1) Commercial trypsin was eluted into several peaks(Fig.1r). The peaks seems to be due to multiple forms of trypsin(21) and also original trypsinogen. There are no peaks of chymotrypsin in

Table 1 A list of proteins subjected to the hydroxyapatite HPLC

proteins	sources	MW	pI	R.T.(min)	R.P.(%)
a) phenol oxidase	Coriolus versicolor Que	65,000	3.3	5.0	94
b) calmodulin	bovine brain	17,000	4.0	13.2	99
c) S100 protein	bovine brain	20,900 21,000	4.2 4.5	16.5 17.0	98
d) fetuin	bovine fetal serum	48,000	3.8	13.5,15.0	96
e) neuron-specific enolase	bovine brain	100,000	4.7	14.0,16.5	100
f) serum amine oxidase	bovine serum	190,000	4.7	17.0,18.0	97
g) serum albumin	bovine serum	68,000	4.7	17.0	100
h) ovalbumin	chicken egg	45,000	5.0,5.5	14.5	93
i) myeloma immunoglobulin D	human serum	170,000	5.0-5.5	20.5	92
j) transferrin	human serum	80,000	5.5-5.9	23.0	100
k) superoxide dismutase	bovine erythrocyte	60,000	6.0	24.0	96
l) immunoglobulin G	human serum	160,000	6.0-8.5	18.0-26.0	100
m) myoglobin	equine heart	17,000	7.2	21.7	100
n) laccase	Rhus vernicifera	100,000	8.0-9.0	20.0	98
o) stellacyanin	Rhus vernicifera	23,000	9.9	17.4	95
p) α-chymotrypsin	bovine pancreas	25,000	9.8*	17.2	100
q) chymotrypsinogen A	bovine pancreas	26,000	9.9*	18.0	95
r) trypsin	bovine pancreas	23,000	9.6*	14.2	96
s) trypsinogen	bovine pancreas	24,000	9.2*	16.0	92
t) cytochrome c	equine heart	11,000	10.2,10.3*	31.2,31.8	96
u) lysozyme	chicken egg	14,000	10.7*	21.5	92

MW;molecular weight. pI;isoelectric point.
R.T.;retention time R.P.;recovery of proteins
*values are estimated from amino acid compositions by the microcomputer method(20).

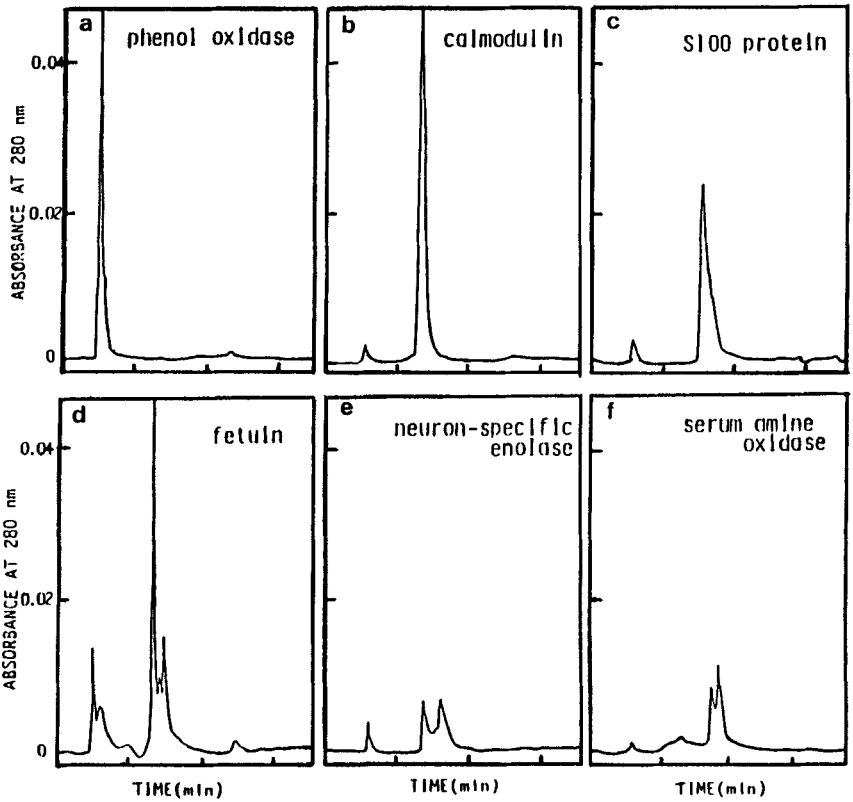

Fig.1 High performance hydroxyapatite chromatography of various proteins. The proteins were applied to the hydroxyapatite column(T) and eluted by a 30 min-linear gradient from 0.01 M sodium phosphate buffer(pH 6.8) containing 0.3 mM calcium chloride to 0.4 M sodium phosphate buffer(pH 6.8) containing 0.01 mM calcium chloride at a flow rate 1.0 ml/min. Other conditions are given under MATERIALS AND METHODS.
a;phenol oxidase, b;calmodulin, c;S100 protein, d,fetuin, e;neuron-specific enolase, f;serum amine oxidase, g;serum albumin, h;ovalbumin, i;myeloma immunoglobulin D, j;transferrin, k;superoxide dismutase, l;immunoglobulin G, m;myoglobin, n;laccase, o;stellacyanin, p;α-chymotrypsin, q;chymotrypsinogen A, r;trypsin, s;trypsinogen, t;cytochrome c, u;lysozyme.

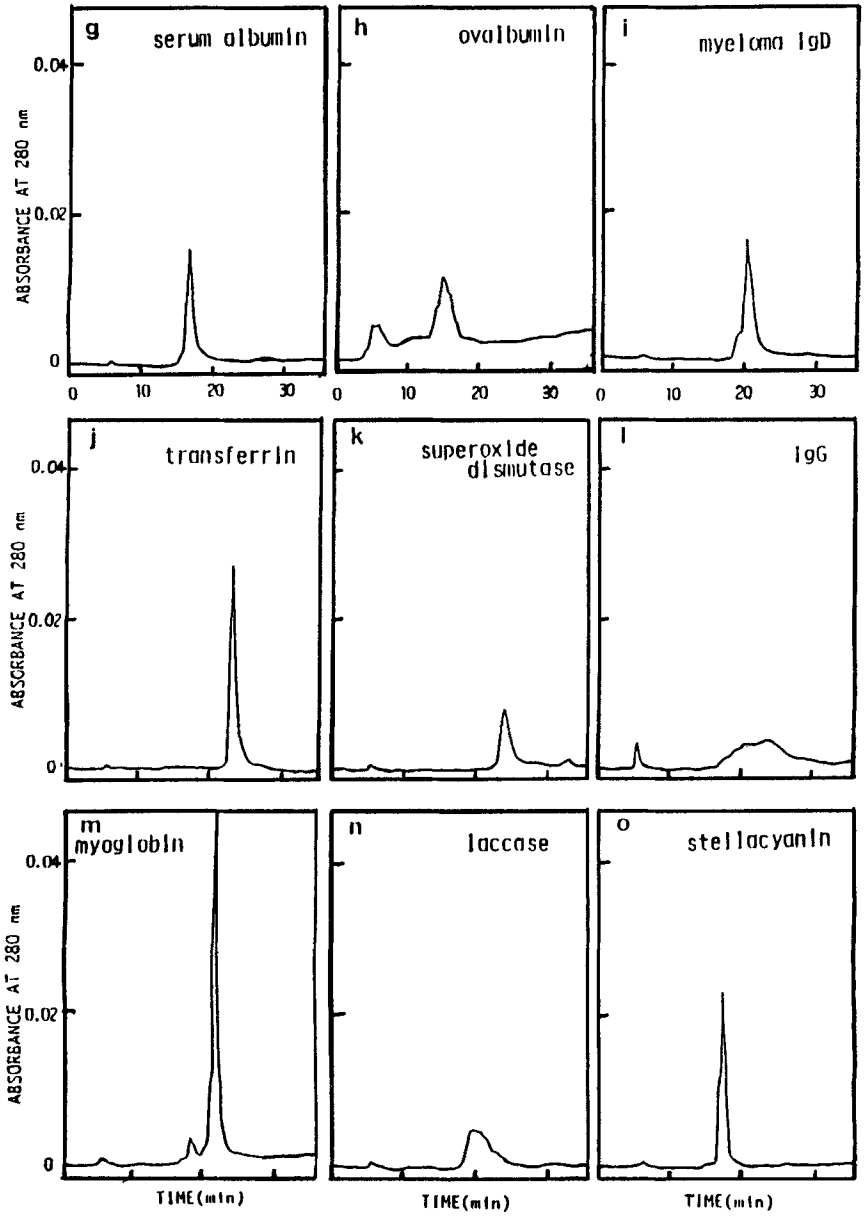

Fig. 1 (continued)

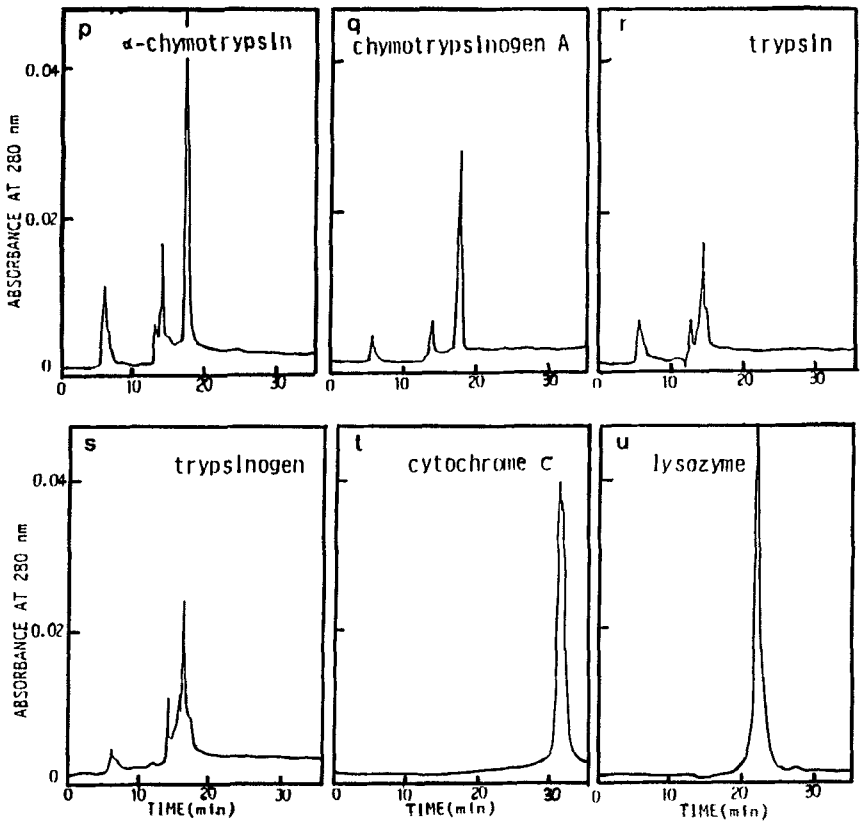

Fig. 1 (continued)

the result of Fig.1h, but there are peaks of chymotrypsin by activity analysis as described in the chromatogram of Fig.1p. In the chromatogram of commercial chymotrypsinogen A(Fig.1q), there showed same contaminant peaks which exactly corresponded with those of trypsin.

(2) It is known that bovine brain S100 protein contains several molecular species which have highly homologous amino acid sequences(22). The hydroxyapatite HPLC showed the multiple shoulders(Fig.1c). It suggested that the potentialty for the

separation of those molecules with shallower gradient and slower elution rate.

(3) Laccase, a blue-copper proteins containing the high content of carbohydrates(23), was eluted into a broad peaks(Fig.1n).

(4) Ovalbumin was eluted into the broad peaks with shoulders (Fig.1h). The proteins has been known to be heterogenous in sugar chains and in their content of phosphate group(24).

(5) Fetuin, a glycoprotein whose content of carbohydrates was about 20 per cent, was eluted into one major peak at a retention time of 13.5 min and some peaks(Fig.1d). It has been known that the proteins shows the heterogeneity that is attributed to the content of sialic acid(25). However, in other type of HPLC, these kinds of separation have not been observed so far.

(6) Polyclonal immunoglobulin G showed very broad peak(Fig.1l). On the other hand, monoclonal myeloma immunoglobulin D showed rather sharp peak(Fig.1i) and monoclonal antibodies showed sharp peaks with different retention times(26).

Retention behavior on hydroxyapatite

Relationship between retention times and physicochemical properties of proteins were investigated. Figure 2 shows the plots of the capacity ratio(k') values of proteins against pI values of the proteins. The k' values were obtained using the column(T) under the condition of a standard 30 min-linear gradient elution method tentatively. The result shows a tendency that the retention times of the proteins increased with the pI values of the proteins. The plots suggested somewhat linear relationship between the k' values and pI values of the proteins, but the correlation coefficient was 0.55. The correlation in proteins having pI values lower than 8.5 was especially good; the correlation coefficient was 0.89. On the other hand, in the range of pI values higher than 8.0 except cytochrome c, the same kind of relation was observed that the correlation coefficient was 0.88. The similar type of plotting of k' values versus molecular weights of the proteins are shown in Fig.3. The results

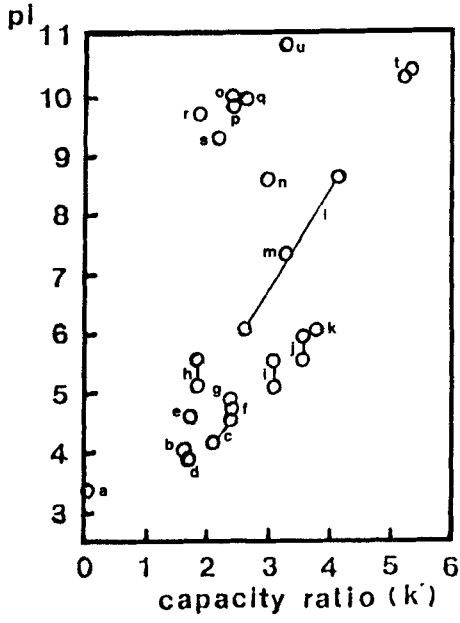

Fig.2 Plots of the capacity ratios(k') against isoelectric points of the proteins.
a;phenol oxidase, b;calmodulin, c;S100 protein, d,fetuin, e;neuron-specific enolase, f;serum amine oxidase, g;serum albumin, h;ovalbumin, i;myeloma immunoglobulin D, j;transferrin, k;superoxide dismutase, l;immunoglobulin G, m;myoglobin, n;laccase, o;stellacyanin, p;α-chymotrypsin, q;chymotrypsinogen A, r;trypsin, s;trypsinogen, t;cytochrome c, u;lysozyme.

showed negative correlation, the coefficients was -0.22. From these studies on relationship between k' and the physicochemical properties of proteins, it is suggested that the major mode of separation of proteins on hydroxyapatite would be ion-exchange type in nature rather than hydrophobic type.

Effect of particle size on elution patterns

The separation of proteins on two types of the column with differnt particle sizes was compared. The distribution of

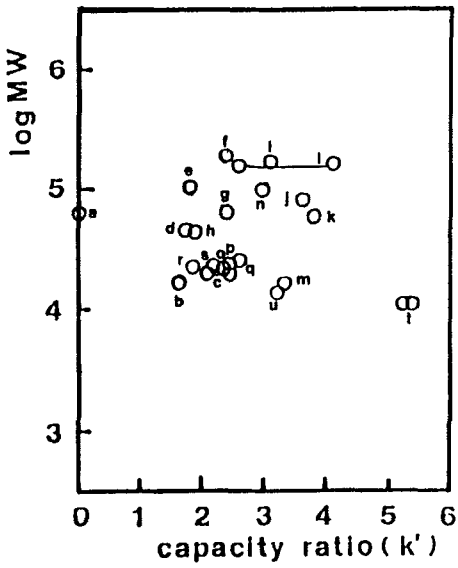

Fig.3 Plots of the capacity ratios(k') against molecular weights of the proteins.
a;phenol oxidase, b;calmodulin, c;S100 protein, d,fetuin, e;neuron-specific enolase, f;serum amine oxidase, g;serum albumin, h;ovalbumin, i;myeloma immunoglobulin D, j;transferrin, k;superoxide dismutase, l;immunoglobulin G, m;myoglobin, n;laccase, o;stellacyanin, p;α-chymotrypsin, q;chymotrypsinogen A, r;trypsin, s;trypsinogen, t;cytochrome c, u;lysozyme.

particle sizes were 0.2-3.0 um(column(T)) and 1.0-6.0 um (column(A)), and the tentative theoretical plate numbers in the present gradient system were calculated as 3700 and 1000 with lysozyme, respectively. The elution profiles of cytochrome c, stellacyanin and serum amine oxidase on these columns were shown in Fig.4, respectively. The resolution on column(T) was higher than that on the column(A), which was due to the smaller size of particles of the column(T), although basic elution patterns were same. The results suggested that even 3 cm length of column(T) would be possible for practical use.

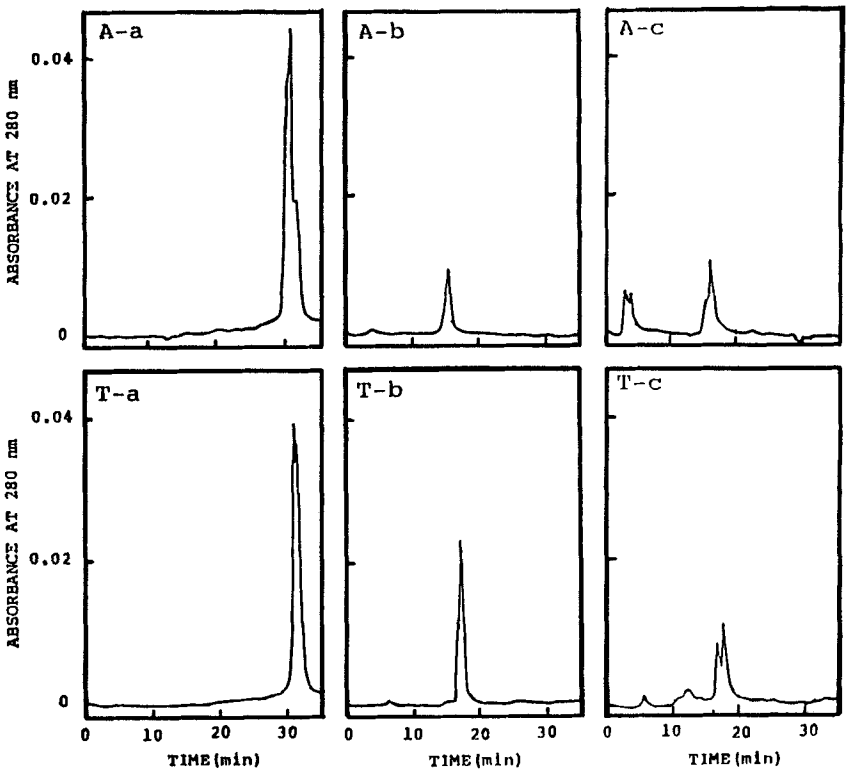

Fig.4 Comparison of HPLC on two types of the hydroxyapatite columns with different particle sizes. The proteins were applied to the column(T) and the column(A), respectively, and eluted by a standard 30 min-linear gradient system.
A-a;cytochrome c on column(A), T-a;cytochrome c on column(T), A-b;stellacyanin on column(A), T-b;stellacyanin on column(T), A-c;serum amine oxidase on column(A), T-c;serum amine oxidase on column(T).

Ceramic spherical hydroxyapatite vs crystalline flake-form hydroxyapatite

The elution pattern obtained using two different particle type of hydroxyapatite columns were compared. Figure 5 and 6 indicated the comparison of retention behavior on the ceramic beads of hydroxyapatite and on traditional crystalline flakes of

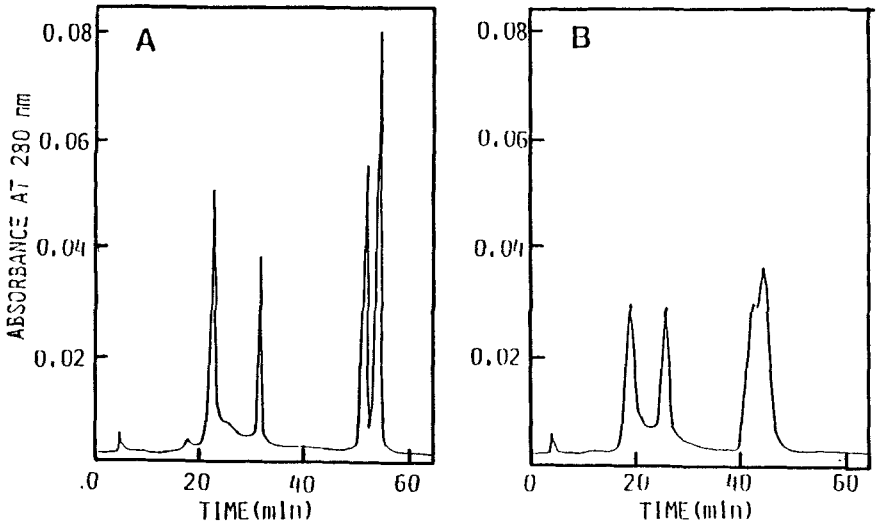

Fig.5 Comparison of the HPLC on a ceramic spherical hydroxyapatite column with that on a crystalline flake-form hydroxyapatite column. The sample of protein mixture(bovine serum albumin, lysozyme, cytochrome c) was applied to the column(T) and KB column, respectively, and eluted by a 60-min linear gradient from 0.01 M sodium phosphate buffer(pH 6.8) containing 0.3 mM calcium chloride to 0.3 M sodium phosphate buffer(pH 6.8) containing 0.01 mM calcium chloride at a flow rate of 1.0 ml/min. (A) column(T), (B) KB column

hydroxyapatite(KB column). Figure 5 shows the chromatograms of a protein mixture on these two different types of hydroxyapatite column, respectively. The comparison of the k' on these columns are shown in Fig.6. The correlation coefficient was 0.84. The chromatography on the spherical hydroxyapatite column shows basically the same retention behavior as that on the flake form hydroxyapatite. However, the resolution of HPLC on spherical hydroxyapatite was higher than that on the crystalline flake-form hydroxyapatite that the tentative theoretical plate numbers calculated with the lysozyme peak were 19,000 on column(T) and 900 on KB column, respectively.

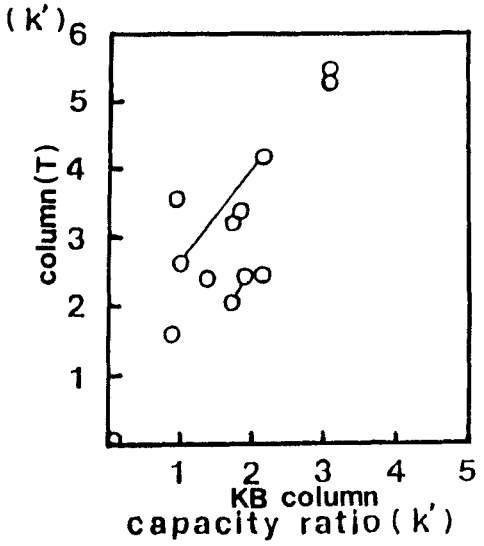

Fig.6 Comparison of the capacity ratios(k') of HPLC on a ceramic spherical hydroxyapatite column(column(T)) with those on a crystalline flake-form hydroxyapatite column(KB column).

This can be attributed by the spherical shape of the hydroxyapatite.

DISCUSSIONS

The hydroxyapatite HPLC on a column of newly developed ceramic hydroxyapatite beads was applied to various type of proteins. The HPLC always showed good resolution and the high recovery of proteins, higher than 90 per cent. The protein which has a prosthetic group such as metalic ion was eluted also as the sharp peaks without losing any biological activity, although the minor peaks or shouders were accompanied in many cases. The recent developments of hydroxyapatite column have enabled the separation of proteins in high efficiency without denaturation. In this paper, the retention behavior was described concerning with relationship between pI and k'. Bernardi and Kawasaki

suggested the separation mechanism with P sites and C sites of the hydroxyapatite for the specific binding sites (6,27). In the present study, it is observed that there is a tendency that k' values on hydroxyapatite chromatography increase with the pI values of the proteins(Fig.2). In ion-exchange HPLC, we already indicated approximately linear relationship between pI and k' in the previous paper(28,29). Comparing the hydroxyapatite chromatography with the anion or cation-exchange chromatography, the proteins of wide range of pI were retained on the hydroxyapatite column. For example, the acidic proteins such as calmodulin(pI 4.0) were retained on the hydroxyapatite column in the neutral buffer system, whereas such proteins were not retained on the cation-exchange column under the same conditions. In this sense, the retention mechanism of hydroxyapatite chromatography is likely to be same as that of ion exchange chromatography with multi-ionic residues or that of mixed-bed ion exchange chromatography or bimodal ion-exchange chromatography. The difference of hydroxyapatite chromatography from the regular ion exchange chroamtography is the use of phosphate buffer for hydroxyapatite chroamtography, which could be replaced by fluoride or parchorate.

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