THE MINI-COLUMN OF LANTHANUM TREATED HYDROXYAPATITE CERAMIC BEADS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEIN

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Abstract

Lanthanum treated hydroxyapatite (La-HAP) beads was prepared and applied for the high performance liquid chromatography (HPLC). X-ray analysis of lanthanum on La-HAP was performed. Appearance of La-HAP beads was also observed by a scanning electron microscope. After the treatment of the HAP beads with the lanthanum chloride solution, the small fluffy structure on the surface of beads and then some inside fiber-like structure were observed. The elution behavior on La-HAP mini-column (0.76 I.D. x 0.4 cm) was compared with those of HAP mini-column (0.76 I.D. x 0.4 cm). Proteins were loaded on a column and eluted by liner gradient system. The relationship between capacity factor (k') of various proteins on La-HAP column and k' on HAP was estimated through the correlation coefficient as 0.93. The elution pattern of proteins on La-HAP column was approximately similar with that on HAP, but the adsorption of proteins on La-HAP was a little stronger than on HAP. After chromatography under acidic conditions, La-HAP beads were observed by electron microscope again. Fiber-like structure remained even after fifty cycles of chromatography. The height equivalent to a theoretical plate number (HETP) was estimated with tryptophan (0.25 μ g / 5 μ l) in isocratic elution system(pH 4.0). The change of HETP by prolonged use under acidic conditions was determined. Half height width of tryptophan peak on La-HAP (approximately 0.5 mm) was persistent for 120 min and after 120 min gradually increased. While under the same conditions, HETP on HAP was remained only for about 40 min. The durability of La-HAP seemed to be as three times as larger than that of HAP. It should be studied further that the results described above simply imply whether one-third of surface area of HAP is covered by lanthanum, or the durability of this material under acidic conditions is improved actually. According to the

results of chromatography, the treatment with lanthanum chloride solution may not be homogeneous, and the improvement of processing of lanthanum coating of HAP is also remained to be studied. And then, so far studied, lanthanum-hydroxyapatite would be useful for the packing material for HPLC for separation of proteins and other biomacromolecules under fairly acidic conditions even.

1. INTRODUCTION

It is well known that hydroxyapatite is one of the components of teeth and bones, and also hydroxyapatite is the stable material under the basic conditions [1] and rather labile under acidic conditions. Accordingly, to prevent dental caries, there are various strategy tested to increase the stability even under the acidic conditions. The durability under acidic conditions is very important also for the packing material of chromatography for the separation of biological substances [6] [7] [8] [9] [10]. One of the attempts to increase the durability of hydroxyapatite under the acidic conditions was to convert it into the fluoride complex[2] [3], and the another attempt was lanthanum complex formation which were already tested in the field of the dental science [4] [5]. These results could be applicable also to the chromatographic procedure and the results obtained in the chromatographycal research could be useful for the dental sciences research vice versa, since the chromatography uses the surface interaction between biomolecules and hydroxyapatite as packing material. In this paper, it is presented a preliminary report on the packed mini-column of lanthanum treated hydroxyapatite (La-HAP), concerning with a packing material for chromatography of proteins, peptides and amino acids.

2 MATERIALS AND METHODS

2-1 Preparation of lanthanum treated hydroxyapatite beads.

La-HAP was prepared by the previously described method [5]. 1g of HAP beads (particle size 10 μ m, Asahi optical co. ltd.) was suspended in 100 ml of 0.1 % of lanthanum chloride, and stirred with magnetic stirrer for 6 hours at 80 ℃ and at around the neutral pH. After stirring, the beads were washed ten times with 50 ml portions of water respective time, and dried at 100 ℃.

2-2 X-ray diffraction analysis

X-ray diffractometer (Rigaku co. ltd.) were employed with the Nickel-filter, the CuK α radiation and scintillation counter .

2-3 Scanning electron microscope

La-HAP beads or HAP beads was spattered respectively with Pt by vacuum evaporation, and were observed with a model JSM-820 scanning electron microscope (Japan electron optics laboratory co. ltd. , Tokyo).

2-4 Column and apparatus

La-HAP beads (0.1 g) was suspended in 0.2 ml of 0.01 M sodium phosphate buffer solution and packed into a mini-column (0.76 I.D. x 0.4 cm). The construction of specific empty mini-column was shown in Fig. 1 . HAP without lanthanum treatment was also packed into a mini-column (0.76 I.D. x 0.4 cm). The HETP of the column was calculated with tryptophan as a standard test sample.

The Waters liquid chromatograph was used, which was composed the proportioning valve and the liquid chromatograph(model 600),the variable wave length UV detector(model 490) (Waters Co. Ltd., U.S.A.) and the Pantos recorder(model U-228)(Nihon Densi Co. Ltd., Tokyo).

The chromatograph was not for micro column system, so the dead space were fairly large comparing with the column so that it required the longer time to substitute the buffer system up to 10 min .

2-5 Chemical analysis of La-HAP

The quantitation of calcium was performed by the atomic absorption spectrochemical analysis [11]. Lanthanum content was measured by atomic absorption spectrochemical analysis [12], where nitrous-oxide acetylene flame was used. Phosphorus content was estimated by the colorimetric method with vanadomolybdenum [13].

2-6 Reagents and proteins

Sodium dihydrogen phosphate (guaranteed grade), disodium hydrogen phosphate dihydrate (guaranteed grade), calcium chloride (guaranteed grade) and water (high performance liquid chromatography grade) were purchased from Wako Pure Chemical Industries ltd. (Osaka, Japan). Tryptphan was purchased from Kyowa Hakko Kogyo co. ltd. (Tokyo, Japan)

Serum albumin, ovalbumin, transferrin (Fe ion free) myoglobin, trypsin, trypsinogen, cytochrome c and lysozyme were purchased from SIGMA Chemicals co.(USA). Concanavalin A was purchased from Pharmacia-LKB Biotech AB. Immunoglobulin G (for intravenous administration grade) was the product of Sandoz ltd. (Switzerland).

PTH-arginine, PTH-glycine, PTH-glutamic acid, PTH-histidine, and tryptophane were purchased from Wako Pure Chemical Industries ltd. (Osaka, Japan). Angiotensine I and insulin were purchased from Wako Pure Chemical Industries ltd. (Osaka, Japan).

For the sample solution, 2mg of protein or peptide was dissolved in 1ml of initial eluent or 0.1mg of amino acid was dissolved in 1ml of initial eluent. These sample solutions were kept in a deep freezer until use.

2-7 Standard procedures for chromatography

Two type of gradient elution program (pH 6.0 and pH 6.8)---(A) and (B)--- and three type isocratic elution system ---(C),(D) and (E)--- were adopted. The various volume of the sample solution of 5 μ l～50 μ l was loaded on the column, so that, amounts of protein loaded were various in the range of 10g μ ～ 100 μ g. Procedure (A)--- Gradient elution (pH 6.8): The chromatography was started with 1 min equilibration with initial eluent and followed by the 10 min gradient elution which was programmed with 5 min liner gradient from initial eluent of 0.001 M sodium phosphate buffer solution (pH 6.8) containing 0.30 mM calcium chloride to final eluent of 0.24 M sodium phosphate buffer solution (pH 6.8) containing 0.12 mM calcium chloride and further 5 min elution by isocratic elution with final solution at a flow rate of 1.0 ml/min at room temperature. The whole elution process was monitored at 280 nm in the absorbance range 0.04 full scale. Then it returned to the initial elution conditions. It took about 14 min for mini-column to change from

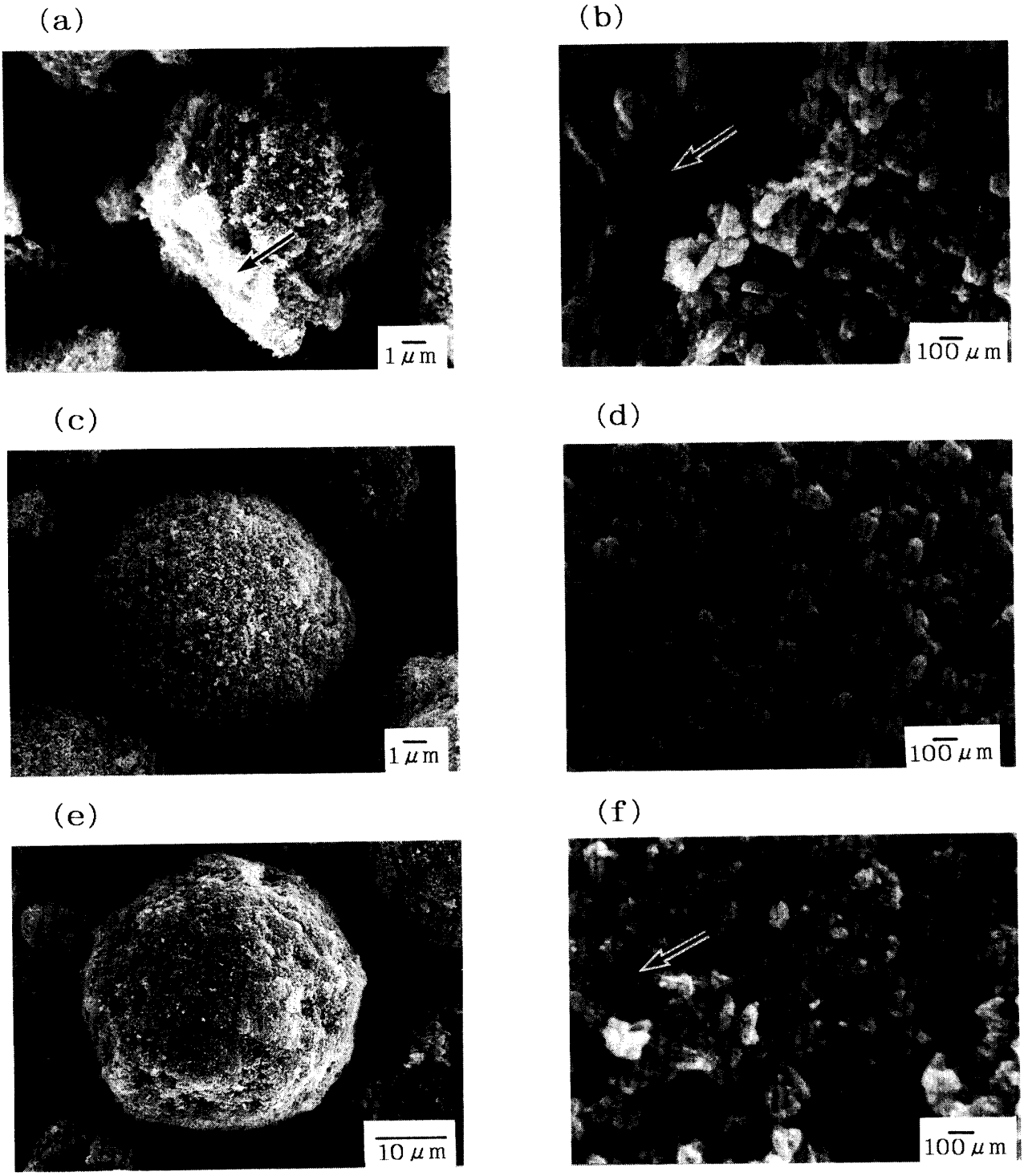

Fig. 1 Scanning electron microphotograph of lanthanum treated hydroxyapatite (La-HAP) beads and hyd roxyapatite (HAP) beads. (a) Appearance of La-HAP before use. Magnification was 5 x 10^3 folds at the microphotograph level. The scale indicate 1 μ m length in actual size. (b) The surface texture of La-HAP before use. Magnification was 5 x 10^4 folds at the microphotograph level. The scale indicate 1 μ m length in actual size. (c) Appearance of HAP before use. Magnification was 6 x 10^3 folds at the microphotograph level. The scale indicate 1 μ m length in actual size. (d) The surface texture of HAP before use. Magnification was 5 x 10^4 folds at the microphotograph level. The scale indicate 1 μ m length in actual size. (e) Appearance of La-HAP after 50 cycles of chromatography. Magnification was 5 x 10^3 folds at the microphotograph level. The scale indicate 1 μ m length in actual size.
(f) Appearance of La-HAP after 50 cycles of chromatography. magnification was 5 x 10^4 folds at the microphotograph level. The scale indicate 1 μ m length in actual size.

final eluent to initial eluent. So, respective over-all analytical cycle required 25 min.

Procedure (B)--- Gradient elution (pH 6.0): The chromatography was performed with 10 min gradient which was programmed with 5 min liner gradient from initial eluent of 0.001 M sodium dihydrogen phosphate solution (pH 6.0) containing 0.30 mM calcium chloride to final eluent of 0.24 M sodium phosphate buffer solution (pH 6.0) containing 0.12 mM calcium chloride and 5 min of further isocratic elution. Other conditions were the same as in above described gradient elution (pH 6.0).

Procedure (C)--- Isocratic elution (pH 6.0): The chromatography was performed with 0.001 M sodium phosphate solution (pH 6.0) containing 0.30 mM calcium chloride. Other conditions were the same as in above Procedure (B).

Procedure (D)--- Isocratic elution (pH 6.0): The chromatography was performed with 0.400 M sodium phosphate buffer solution (pH 6.0) containing 0.12 mM calcium chloride. Other conditions were the same as in above Procedure (B).

was performed with 0.400 M sodium phosphate buffer solution (pH 4.0) containing 0.12 mM calcium chloride. Other conditions were the same as in above Procedure (B).

3 RESULTS AND DISCUSSION

3-1 Stability of lanthanum attached on La-HAP

Stability of La-HAP through the chromatography was observed by scanning electron microscope. The appearance of La-HAP was shown in Fig. 1,(a)low magnification and (b)high magnification. La-HAP was covered with fluffy surface structure. This fluffy structure looked like to be mechanically fragile, and it could be removed easily by mechanical stirring processing and the spherical appearance was improved rather after fifty cycles of chromatography (Fig.1,(e)). But so called fibrous structure which was located rather inside of the beads and observed by higher magnification (directed by arrow head mark in Fig.1 (b), was durable and persisted as in Fig. 1 (f) after fifty cycles of chromatography even---the arrow head mark indicate the fibulas structure(Fig.1,(f)).

Surface area of HAP beads and porosity was estimated, based on the previous results, as follows [3]. The average radius of pore : r (cm) = 7 x 10^{-8} , the volume of pore; V(cm^3/g) = 0.115, the surface area of beads; S(cm^2/g), r = 3(V/S). 7 x 10^{-8} = 3 (0.115 / S). S = 4.92

Table 1 Content of beads

	Ca^{2+}(%)	P(%)	La^{3+}(%)	Ca / P
HAP particles (before use)	39.6	18.2	-	1.68
HAP particles (after use)	39.1	18.4	-	1.64
La-HAP particles (before use)	34.4	17.2	4.3	1.55
La-HAP particles (after use)	33.9	17.4	3.9	1.51

The results of atomic adsorption spectrochemical analysis was shown in Table 1. Calcium content of La-HAP had decreased 0.5 % through fifty cycles of chromatography, and same results with HAP. The calcium content of La-HAP was always smaller than that of HAP, though. The lanthanum content had decreased by

0.36 % through chromatography. The phosphorus content had increased 0.4 % through chromatography. This result could be interpreted possibly in either two way. First possibility is that a small amount of positive components of La-HAP like Ca or La were dissolved away during packing and during chromatography, and the gray paste-like impurity materials have deposited on the column head through the chromatography. Second possibility is that phosphorus compound such as phosphoprotein of pyrogen happened to accumulated on the surface of La-HAP.

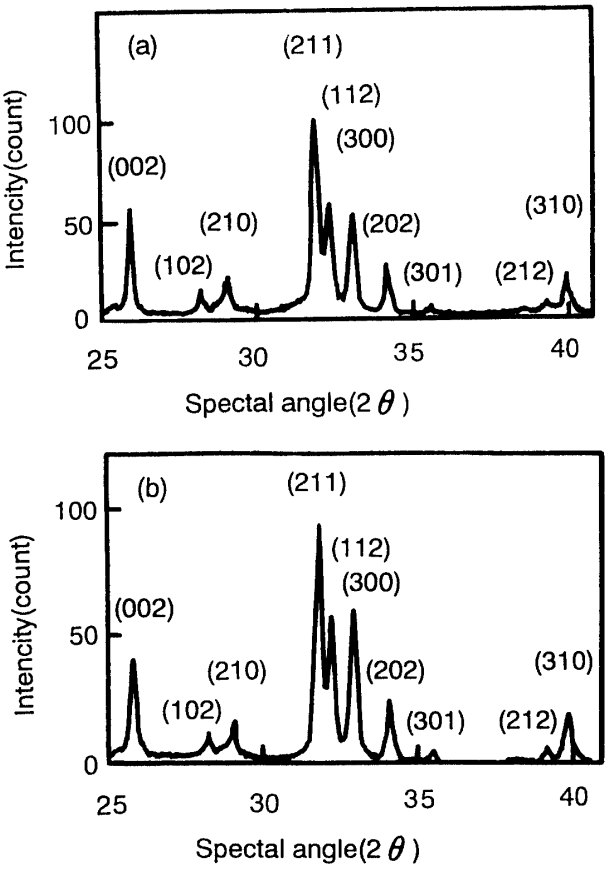

Fig.2 X-ray diffraction pattern of lanthanum treated hydroxyapatite(La-HAP) and hydroxyapatite (HAP). (a) X-ray diffraction pattern of La-HAP before use. (b) X-ray diffraction pattern of lanthanum after fifty cycles of chromatography. The ordinate is relative intensity of the plane (211),assumed use as one hundred counts before use. The abscissa is spectral angle. The numbers in the bracket were the Miller indices of respective diffraction peaks.

Lanthanum of La-HAP was also observed by X-ray diffraction. Fig. 2 shows the diffraction patterns of La-HAP beads before and after chromatography. X-ray diffraction pattern shows the plane form of hexagonal system, either for La-HAP or HAP. And diffract angles of X-ray diffraction patterns of HAP and La-HAP remained same after many cycles of chromatography.

3-2 Chromatograms of proteins, peptides and amino acids on La-HAP mini-column

Proteins were applied on La-HAP mini-column, and eluted by standard gradient procedure (A). Chromatograms of respective protein were shown in Fig. 3 (a ~ j). Retention of protein was calculated as capacity factors (k') and summarized in Table 2. Dead volume of column and of tubing from injection port to detector was 0.65 ml measured with injection of 25 μ l of 3 mM sodium azid. Concanavalin A (Fig. 3a), serum albumin (Fig. 3b) and ovalbumin (Fig. 3c) were adsorbed on La-HAP column in the initial eluent. But, in the case of transferrin, depending on the conditions of sample application, the various results were observed. When pH value of sample was 6.8 (Procedure (A)), more than 45% of transferrin was not adsorbed(Fig. 3(d)). At pH 6.0 (Procedure (B)), 6.5 % of trnsferrin was not adsorbed. Transferrin sample in distilled water, still 0.3% could not absorbed.Immunoglobulin G(Fig. 3(e)) and myoglobin (Fig. 3(f)) were adsorbed completely, but trypsinogen (Fig. 3(g)) and trypsin (Fig. 3(h)) were not adsorbed completely. Cytochrome c(Fig. 3(i)) and lysozyme (Fig. 3(j)) were adsorbed and could not be eluted by 5 min. linear gradient. And there were some carry over peaks in the

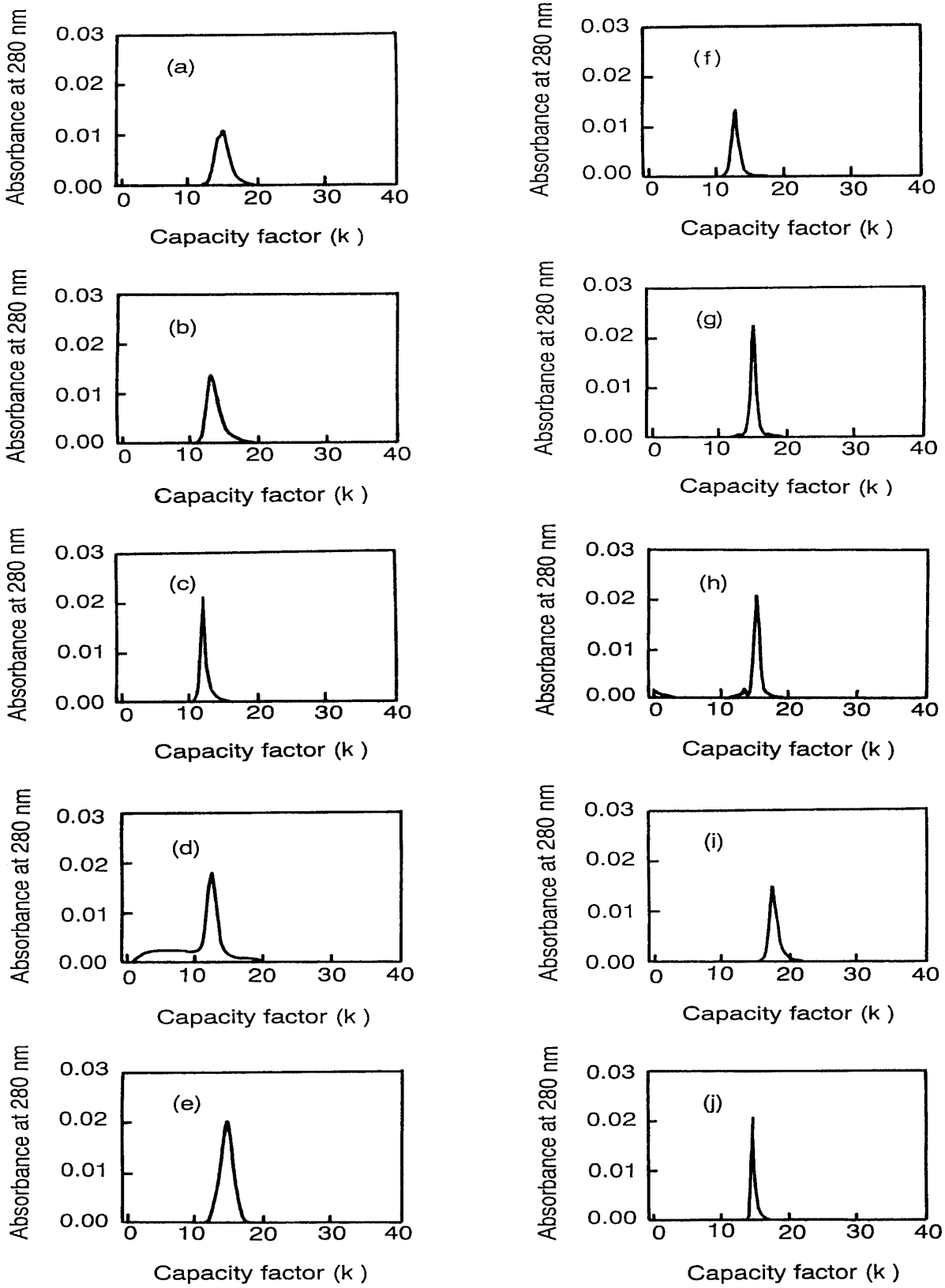

Fig. 3 Chromatograms of Proteins on lanthanum treated hydroxyapatite. Protein was applied and eluted by standard chromatographic procedure (A). Chromatogram shown in the figure was corrected by the subtraction of blank elution pattern. Ordinate is absorbance at 280 nm. Abscissa is retention of protein as capacity factor (k'). (a) concanavalin A, (b) serum albumin, (c) ovalbumin, (d) transferrin, (e) immunoglobulin G, (f) myoglobin, (g) trypsinogen, (h) TPCK trypsin, (i) cytochrome c and (j) lysozyme. Other conditions are given under MATERIALS AND METHODS.

Table 2 Retention time and capacity factor (k') of various proteins

Proteins	pI	MW	Capacity factor (k')	
			La-HAP	HAP
1 PTH-glutamic acid	4.25	264	0.00	0.00
2 PTH-methionine	7.0	266	0.00	0.00
3 PTH-glycine	7.0	192	0.00	0.00
4 tryptophane	7.0	204	0.00	0.00
5 PTH-arginine	12.48	327	0.00	0.00
6 insulin	5.3-5.4	5807	0.00	0.00
7 angiotensin I	7.4	1296	0.16	0.13
8 calmodurin (4 Ca^{++})	4.0	16700	14.65	13.51
9 serum albumin	4.7	68000	12.37	11.71
10 ovalbumin	5.0, 5.5	45000	11.46	11.02
11 transferrin (Fe free)	5.9	80000	12.16	11.88
12 immunoglobulin G	7.0	148000-170000	14.34	14.20
13 myoglobin	7.2	17000	12.88	11.34
14 trypsinogen	9.2	24000	14.31	13.22
15 trypsin	9.6	23000	16.60	13.49
16 cytochrome c	10.2, 10.3	11000	17.23	14.98
17 lysozyme	10.7	14000	14.40	12.89

chromatograms even after several cycles of chromatography, in the standard Procedure (A). Proteins could be eluted by 5 min. liner gradient on HAP. But on La-HAP some of the proteins could not be eluted by 5 min liner gradient.

Chromatogram of the mixture of some of proteins is shown in Fig.4.

Peptides and amino acids were applied on La-HAP mini-column, and eluted by standard isocratic procedure (C). Chromatograms of respective peptide, amino acid were shown in Fig. 5 (a) ~(c). Retention of peptide or amino acid was calculated to k'. Peptides and amino acids were not adsorbed (k'=0) on either La-HAP or HAP.

Relation between capacity factor (k') on La-HAP column and k' on HAP column were observed (Table 2). The correlation of k's of protein on La-HAP and those on HAP was shown in Fig. 6. Correlation coefficient was 0.93. The slope of the line in Fig. 6 was 0.73 . So the elution behavior of proteins on La-HAP was a cation exchanger in type and similar as in HAP.

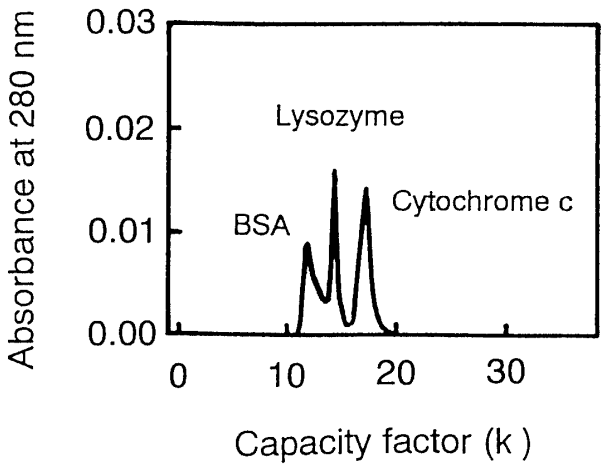

Fig. 4 Chromatograms of Protein Mixture on lanthanum treated hydroxyapatite chromatography. Protein mixture (serum albumin, lysozyme, cytochrome c) was applied and eluted by standard chromatographic procedure (A). Chromatogram shown in the figure was corrected by the subtraction of blank elution pattern. Ordinate is absorbance at 280 nm. Abscissa is retention of protein as capacity factor (k'). The peaks were indicated by numbers orderly. Other conditions are given under MATERIALS AND METHODS.

But, the adsorption of proteins on La-HAP was somewhat stronger than that on HAP.

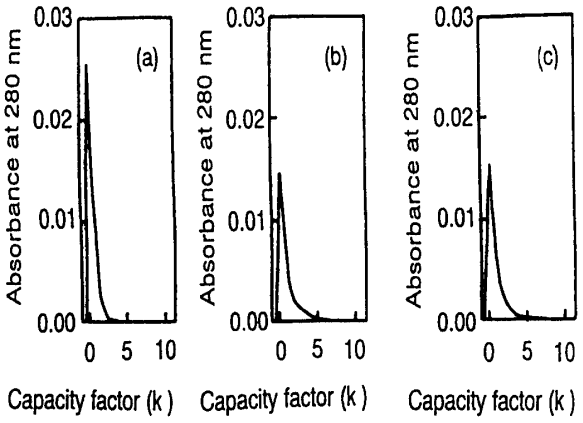

Fig. 5 Chromatograms of peptides and amino acids on lanthanum treated hydroxyapatite chromatography . Peptides or amino acids were eluted by the standard chromatographic procedure (C). (k) tryptophan, (l) insulin and (m) angiotensin 1. Other conditions are given under MATERIALS AND METHODS.

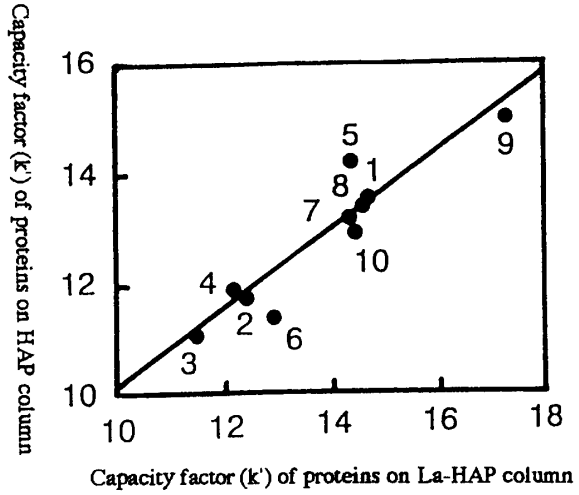

Fig. 6 Relationship between capacity factor (k') of proteins on lanthanum treated hydroxyapatite (La-HAP) column and k' of proteins on hydroxyapatite (HAP) column. Protein was loaded and eluted by standard chromatographic procedure. Numbers in the figure refer to the sample numbers in Table 1. Other conditions are given under MATERIALS AND METHODS.

3-3 Durability of La-HAP in the eluent (pH 4.0) solution

Durability of La-HAP column under the acidic conditions was tested with 0.400 M sodium dihydrogen phosphate solution (pH 4.0) at various intervals, through the elution profile of tryptophan (2.5 μ g/ 5 μ l). Fig. 7 shows chromatograms of tryptophan. The peak shape on La-HAP was not changed by the exposure of the material against pH4 condition for 180 min.

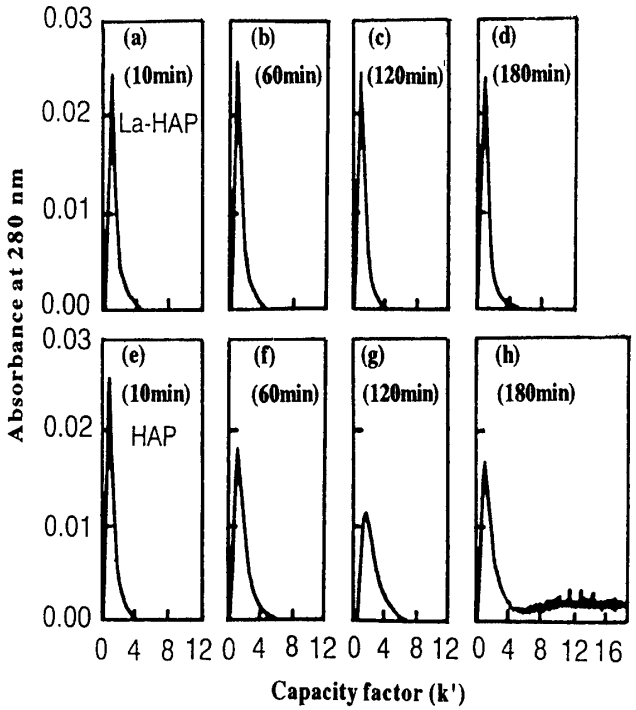

Fig. 7 Chromatograms of tryptophan on lanthanum treated hydroxyapatite (La-HAP) mini-column (0.76 I.D. x 0.4 cm) or hydroxyapatite (HAP) mini-column (0.76 I.D. x 0.4 cm). Tryptophan (2.5 μ g / 5 μ l) was eluted by isocratic elution with 0.4 M sodium dihydrogen phosphate solution (pH 4.0). (a) Chromatogram of tryptophan on La-HAP after 10 min exposure in eluent at a flow rate 1 ml/min. (b) Chromatogram of tryptophan on La-HAP after 60 min exposure in eluent at a flow rate 1 ml/min. (c) Chromatogram of tryptophan on La-HAP after 120 min exposure in eluent at a flow rate 1 ml/min. (d) Chromatogram of tryptophan on La-HAP after 180 min exposure in eluent at a flow rate 1 ml/min. (e) Chromatogram of tryptophan on La-HAP after 10 min exposure in eluent at a flow rate 1 ml/min. (f) Chromatogram of tryptophan on La-HAP after 60 min exposure in eluent at a flow rate 1 ml/min. (g) Chromatogram of tryptophan on La-HAP after 120 min exposure in eluent at a flow rate 1 ml/min. (h) Chromatogram of tryptophan on La-HAP after 180 min exposure in eluent at a flow rate 1 ml/min.

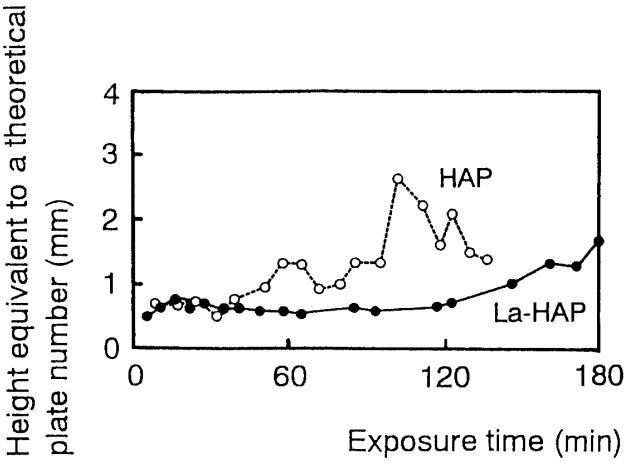

Fig. 8 Comparison with height equivalent to a theoretical plate number of Tryptophan on lanthanum treated hydroxyapatite (La-HAP) mini-column (0.76 I.D. x 0.4 cm) and hydroxyapatite (HAP) mini-column (0.76 I.D. x 0.4 cm). Tryptophan (2.5 μg / 5 μl) was eluted by isocratic elution with 0.4 M sodium dihydrogen phosphate solution (pH 4.0). The abscissa is exposure time of beads in 0.4 M sodium dihydrogen phosphate (pH 4.0) at a flow rate 1 ml/min. The ordinate is height equivalent to a theoretical plate number on mini-column. Solid circle (●) indicate the plots of HETP on La-HAP. Open circle (○) indicate the plots of HETP on HAP.

hours. While the peak shape on HAP was changed even after 60 min exposure and some of the error peaks were observed after 150 min exposure. The change of HETP was calculated and shown in Fig. 8 . HETP on La-HAP column were retained as the beginning at least up to 120 min under the acidic elution conditions. On the contrary, HETP on HAP column were gradually increased and then ghost peaks began to appear after 150 min. After series of experiments, it was turned out that the column head of HAP column was lost up to 0.1 cm, but in La-HAP column practically no decrease of column height. La-HAP was three times more durable than HAP under acidic conditions.

4. CONCLUSION

La-HAP was prepared for the packing material of chromatography of biomolecules. And the elution behavior of proteins, peptides and amino acids on La-HAP were investigated. Elution profiles of protein on La-HAP were practically same as that on HAP, so that the elution mechanism would be ion exchange in type. But, adsorption of proteins were comparatively a little stronger on La-HAP than on HAP. While some of peptides which were adsorbed on HAP or La-HAP. Amino acids were not adsorbed on either La-HAP or HAP. The durability of La-HAP under acidic conditions was much larger. The stability of lanthanum on La-HAP was stable through chromatography. The observation by electron microscope suggested, that there would be on the crystalline part of hydroxyapatite and no change at lanthanum modified part. X-ray diffraction pattern showed that La-HAP and HAP have no recognizable differences, although the behavior of protein adsorption were different.

For the preparation of La-HAP beads, we have to study more on improvement and development on the application method for other biomaterials.

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