CHROMATOGRAPHY OF PROTEIN ON HYDROXYAPATITE IMPREGNATED PAPER

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Abstract

Ceramic hydroxyapatite beads for high performance liquid chromatography (HPLC) was already reported to be a cation exchanger in type and proteins were adsorped and eluted with changing the concentration of sodium phosphate buffer solution. Hydroxyapatite crystalline impregnated paper (HAP paper) which was made of micro crystalline and paper fiber, but without sintering process was used for filtration or separation of biological macromolecule materials. A circular sheet of HAP paper was cut into a suitable size and (0.76 mm I. D.) and placed in a column for HPLC of protein. Chromatography was performed across the HAP paper sheet. One sheet of HAP paper could adsorbed about 12 μg cytochrome c. A pile of these sheets could also be used for the chromatography of protein as well as hydroxyapatite beads column. HAP paper was useful not only for the separation of protein but also for the preliminary sample clean up for the HPLC, electrophoresis or various immunochemical treatment.

1. INTRODUCTION

We reported previously on the superiority of the ceramic hydroxyapatite (HAP) beads for the chromatography of proteins[1),2),3),4)]. The elution behavier of proteins on HAP was a ion-exchange type in nature. This ceramic HAP beads were made by heat sintering[5)] of microcrystalline of hydroxyapatite. It was so efficient that, it could be lowered down to 0.1 cm, and still it could be useful for the separation of some proteins. Based on these experiences,we tried to use the hydroxyapatite impregnated papes (HAP paper)as apacking material that is as a paper-pile, for the separation of biological materials including proteins. Efficiency of HAP paper was proved to be comparative useful with that of HAP beads column.

2. MATERIALS AND METHODS

2-1 Column set up and apparatus

HAP paper was prepared by Asahi Optical Industry Co. Ltd.. Hydroxyapatite crystelline was impregnated into paper (Fig. 1). The thickness under the dry or the wet condition is about 0.125 mm, 42 g of HAP micro crystalline was impregnated in 1 m^2 of paper sheet. HAP paper was cut into circle from and was placed in the mini-chromato-pile column shown in Fig. 2.

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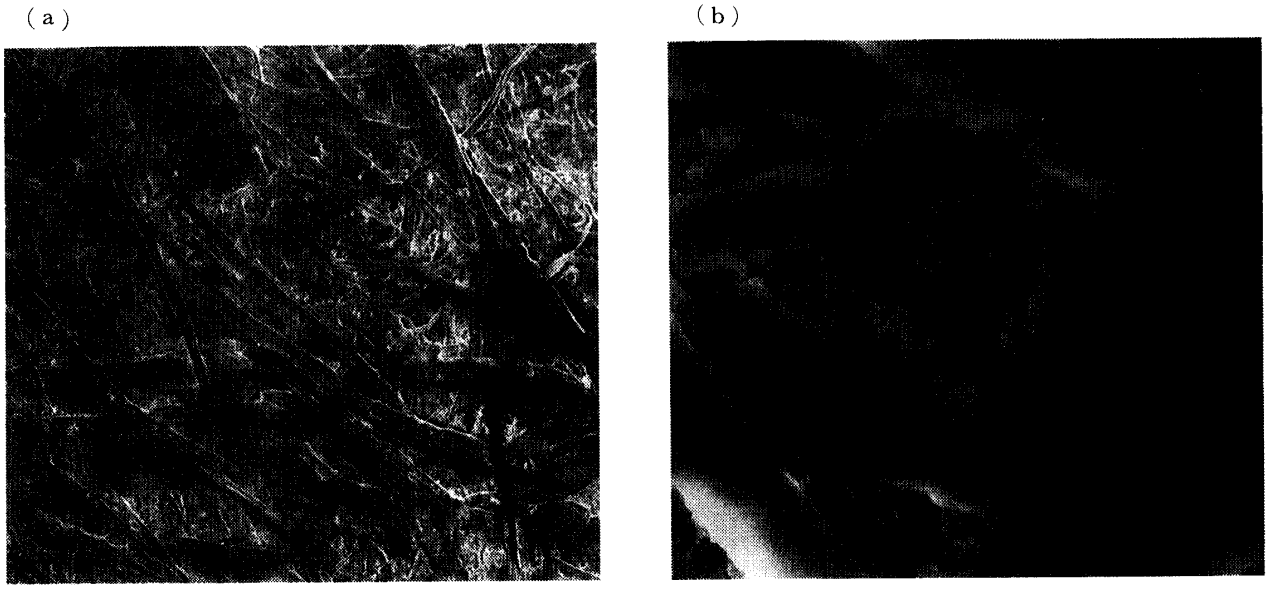

Fig. 1 Scanning electron microphotograph of hydroxyaptite microcrystalline impregnated paper (HAP paper). (a) Appearance of HAP paper. Magnification was 10^2 folds at microphotography level. (b) Appearance of microcrystalline among paper fiber. HAP paper was impregnated 42 g of HAP into 1 m^2 of paper sheet.

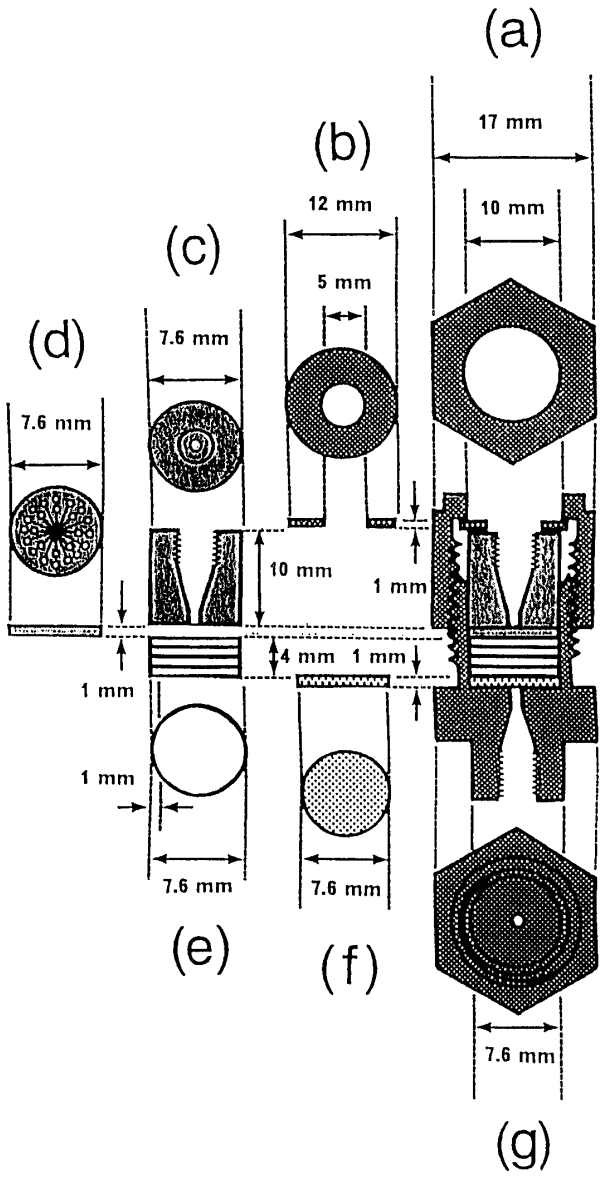

Fig. 2 Construction of the mini-chromato-pile column. Hydroxyapatite paper (diameter 0.96 cm) (e) was placed on a stainless steel support (f) in column joint (g). HAP paper was settled with filter of tetrafluoroethylene copolymer (d). Filer (d) was pressed by a spacer made from halt-cut union of tetrafluoroethylene copolymer (c), a stainless steal washer (b) and a stainless steel the (a). Half-cut union was curved to fit into nut inside of column joint (g). This column was available to one sheet piie of up to HAP paper or ten sheets pile or more.

For the chromatography, Waters chromatograph was used which was composed from a model 600 E system cotroller, a model 600 F multisolvent delivery system, a model 490 programmable multiwavelength detector and U-228 Pantos recorder.

2-2 Reagent

Sodium dihydrogen phosphtae monohydrate (garanteed grade), disodium hydrogen phosphate dihydrate (garanteed grade), calcium chloride (garanteed grade), sodium chloride (garnteed grade), sodium azide (cemical grade) and water (HPLC grade) were perchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan).

Acrylamide (electophoresis grade), N, N'-methylenebisacrylamide (electrophoresis grade), glycine (guarantee grade), tris (hydroxylmethyl)- aminomethane (guarantee grade) and ammonium persulfate (guarantee grade) were perchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). N, N, N', N'-tetramethylethylenediamine (electrophoresis grade) and Coomassie Brilliant Blue R-250 (electrophoresis grade) were perchased from Nakarai Chemicals (Kyoto, Japan). Ampholines (pH 5 ~ 7, pH 9 ~ 11) were perchased from Pharmacia-LKB (Sweden). Water for electrophoresis should be purified by distillation, after passing through a mixed-bed ion-exchange resin.

2-3 Proteins

Serum albumin, cytochrome c and lysozyme were perchased from SIGMA chemicals co. (USA). Immunoglobulin G was donated from Sandoz ltd. (Switzerland).

Serum was separated from human blood cells by cetrifugation (10001 × g, 10 min) after 60 min incubation at room temperature.

2-4 Standard chromatographic procedure

Gradient elution was programmed for 5 min liner increasing gradient from 0.001 M sodium dihydrogen phosphate solution (pH 6.0) containing 0.30 mM calcium chloride to 0.400 M sodium phosphate buffer solution (pH 6.8) containing 0.12 mM calcium chloride at a flow rate of 1.0 ml/min. The pressure drop is upto 1 kgf/cm^2. The loaded sample amount was 6 μg ~ 20 μg protein in 3 μl ~ 5 μl of 0.001 M sodium dihydrogen solution (pH 6.0) containing 0.30 mM calcium chloride. The effluent was monitored at 280 nm. After every analytical cycle, the column was re-equilibrated for 1 min with the initial solvent.

Dead volume was calculated by tne measurement of weight of water filled in the stainless steel tubing between injection port through the column to detector. The dead volumn of column (8 sheet, height 1 mm) was 200 μl. The dead volume between injection port to detector was 400 μl. Capacity factor (k') was calculated Vm = 0.2, retension volume ; Vr. $k' = (Vr-Vm)/Vm$

2-5 Filtration of serum on HAP paper

Treatment of serum was performed on two type of HAP paper. HAP paper (small pore) and HAP paper (Large pore). Small pore type was made HAP impregnated into random fablic. Large pore type was made HAP impregnated into non-woven fablic.

Filtration of serum protein was performed on double layer of HAP paper (diameter 2.2 cm). HAP paper was placed on a filter holder (LP-25, Toyo Roshi inc.) and equilibrated 5 mM sodium phosphate buffer solution (pH 8.0). The pH was various depending on the purpoise of the treatment, but, here, the condition of pH 8.0 was used for the removal of phosphic lipids.

Serum was diluted with the same volume of 5 mM sodium phosphate buffer solution (pH 8.0) and 0.5 ml of the diluted sample was applied on HAP papers. Proteins were washed with 0.5 of 5 mM sodium phosphate buffer solution (pH 8.0).

2-6 Two-dimensional electrophresis of protein

Two-dimensional electrophresis was performed with micro slab gel[6]. Isoelectric focusing gels were polymerized with 0.20 % of Ampholines (pH 3.5 ~ 10) and 0.05 % of Ampholines (pH 3.5 ~ 5). The sample was applied on the acidic side. The electrophoresis were performed at a constant current of 0.1 mA/tube until the voltage up to 300 V and then at a constant voltage of 300 V for 120 min. After isoelectric focusing the capillary gel was transferred on the top of a 4 to 17% liner gradient slab gel. The gel was stained in 0.025% Coomassie Brilliant Blue R-250 in 50% (v/v) methanol - 7 % (v/v) acetic acid.

3. RESULT AND DISCCUSION

3-1 Chromatogram of protein

Chromatography was performed under the standard chromatographic procedure. Fig. 3 shows the chromatogram of protein on HAP paper column. Proteins were applied and eluted by the standard chromatographic procedure. Fig. 3 (a) shows a chromatogram of cytochronme c (12 μg/3 μl) on one sheet of HAP paper. Fig. 3 (b) shows a chromatogram of cytochrome c (20 μg/5 μl) on a pile of ten sheets of HAP paper. Cytochrome c was adsorbed on a ten sheets pile and also on a one sheet pile in initial eluent and eluted by standard chromatographic procedure. Fig. 3 (c) shows a chromatogram of lysozyme (20 μg/5 μl) on one sheet of HAP paper. Fig. 3 (d) shows a chromatogram of lysozyme (20 μg/5 μl) on ten sheets of HAP paper. Lysozyme 20 μg was partially adsorbed on one sheet of HAP paper. And lysozyyme 20 μg was all adsorbed on ten sheets of HAP paper. Fig. 3 (e) shows a chromatogram of serum albumin (12 μg/3 μl) on one sheet of HAP paper. Serum albumin 12 μg was partially adsorbed on one sheet of HAP paper. Fig. 3 (f) shows a chromatogram of serum albumin (20 μg/5 μl) on ten sheets of HAP paper. Serum albumin 20 μg was also partially adsorbed on ten sheets of HAP paper. Although acidic proteins were adsorbed comparatively weak, adsorbed part of protein was not eluted in 1000 column volume of initial eluent. And it took more than ten sheets for adsorption of all 12 μg of serum albumin. Basic proteins were adsorped strongly on HAP paper. Although, the isoelectric point (pI) of lysozyme was 10.7 and pI of cytochrome c was 10.2 - 10.3, lysozyme was weakly adsorped than cytochrome c. This phenomenom also observed on HAP beads[1,2,3,4]or on FAP beads[7,8,9,10]. We think that lysozyme molecule was compact, and charge interaction on HAP was weak. We had already reported about the chromatography of natural proteins and denaturated proteins on HAP or FAP[11]. Random coil type protein seemed to have larger interaction than ligid type of proteins on HAP or FAP. HAP or FAP can separate proteins mainly by the difference of charge, and by the the difference of structure of molecule. HAP paper could be used for HPLC of protein as HAP beads.

3-2 Comparison with HAP beabs columns

The specifications of HAP beads were already described[1,2,3,4,5]. HAP paper was used also in chromatoraphy of proteins in the similar ways as HAP beads. HAP paper

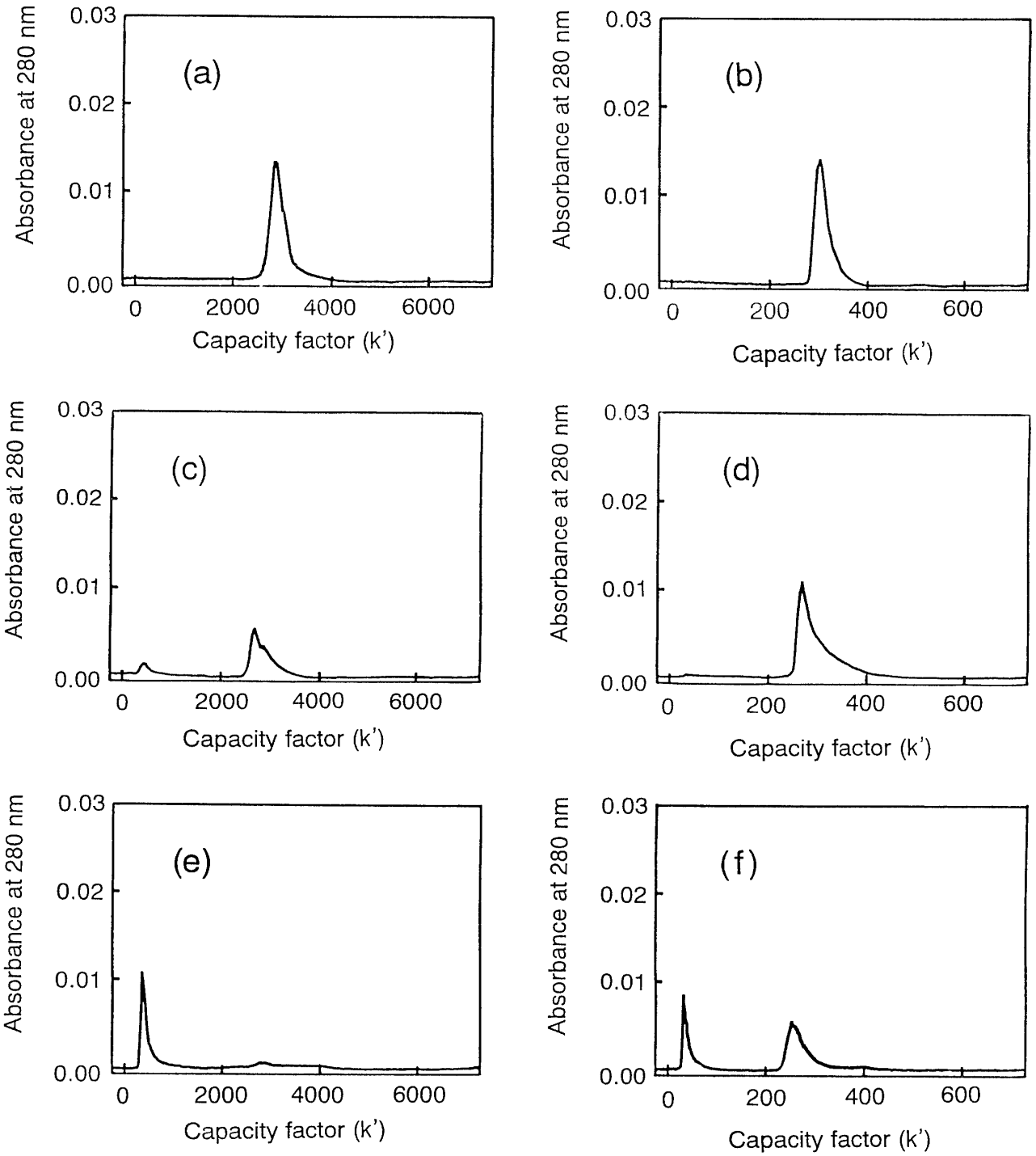

Fig. 3 High performance hydroxyapatite chromatography of protein on HAP paper. Protein was applied and eluted by standard chromatographic procedure. (a) Chromatogram of cytochrome c on one sheet of HAP paper. (b) Chromatogram of cytochrome c on ten sheets of HAP paper. (c) Chromatogram of lysozyme on one sheet of HAP paper. (d) Chromatogram of lysozyme on ten sheets of HAP paper. (e) Chromatogram of serum albumin on one sheet of HAP paper. (f) Chromatogram of serum albumin on ten sheets of HAP paper. Chromatogram shown in figure was corrected by the subtraction of blank elution pattern. Ordinate is absorbance at 280 nm. Abscisa is retention of protein as capacity factor (k'). Other condit ions are given under MATERIALS AND METHODS.

was observed with scanning electron microscope. Fig. 1 shows some of the photographs of different magnifications of HAP paper. Hydroxyapatite microcrystalline was held among paper fibers. Acording to the content of HAP in HAP paper (42 g/m^2), height

of 1 sheet of HAP paper was approximately equivalent to 0.070 mm HAP particle.

The chromatograms of protein on HAP paper were compared with the chromatograms of protein on HAP column packed with HAP beads. Fig. 4 (a) shows a chromatogram of proteins on 0.76 I. D. × 0.4 cm column packed with HAP beads. Serum albumin, lysozyme and cytochrome c were all adsorbed and elutede by standard chromatographic procedure. Fig. 4 (b) shows a chromatogram of proteins on 0.76 I. D.× 0.1 cm HAP column. A past of serum albumin was not adsorped before gradient elution on HAP column, and the majority was eluted on HAP paper column. Acidic proteins were adsorbed comparatively weak on HAP paper and on HAP beads. Shortened column was scarcely adsorbed acidic proteins. So efficiency of HAP paper was thought to be the same as that of HAP particle. In the case of removal of acicic protein such as albumin, HAP paper was suitable, because of easy handling and permiability.

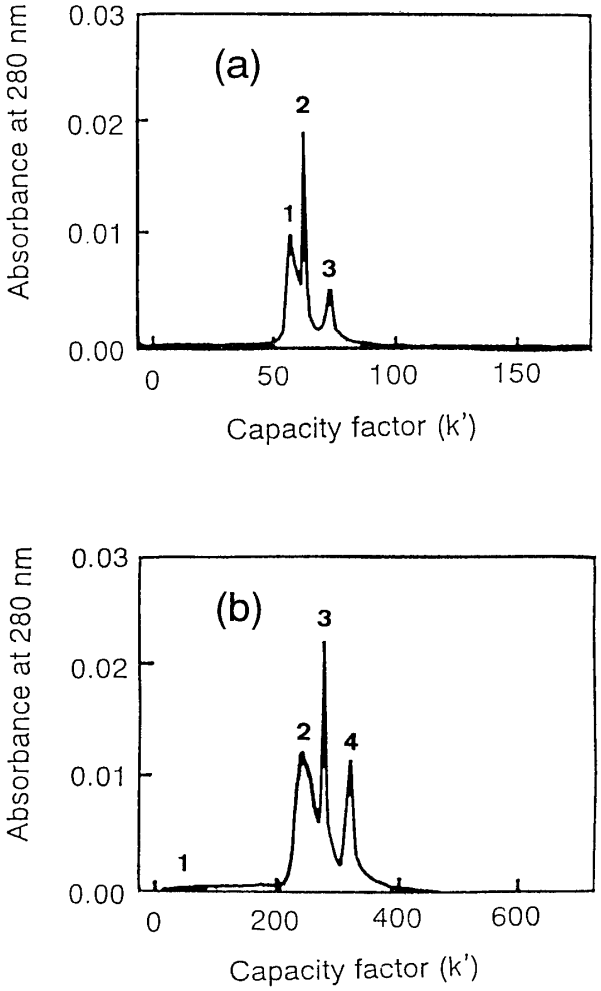

Fig. 4 High performance hydroxyapatite chromatography of protein on HAP mini-column. Protein mixture applied and eluted by standard chromatographi procedure. Peak was numbered orderly. (a) Chromatogram of proteins on HAP column (0.76 I. D.×0.4 cm). Peak 1 was serum albumin, peak 2 was lysozyme and peak 3 was cytochrome c. (b) Chromatogram of proteins on HAP column (0.76 I. D. × 0.1 cm). Peak 1 and peak 2 were serum albumin, peak 3 was lysozyme and peak 4 was cytochrome c. Chromatogram shown in figure was corrected by the subtraction of blank elution pattern. Ordinate is absorbance at 280 nm. Abscisa is retention of protein as capacity factor (k').

3-3 An amount of serum protein adsorbed on HAP paper

Adsorption capacity of protein was estimated with immunoglobulin G (IgG). HAP papers (double layered, diameter 2.0 cm) were placed on a filer, and equibliated 50 ml of 1 mM sodium dihydrogen phosphate solution (pH 6.0) containing 0.30 mM calcium chloride. IgG (1.94 mg/2.3 ml) was applied and washed 16 ml of 1 mM sodium dihydrogen phosphate solution (pH 6.0) containing 0.30 mM calcium chloride. These proteins on HAP were eluted with 5.15 ml of 400 mM disodium hydrogen phosphate solution (pH 9.0) containing 0.12 mM calcium chloride. Protein content in 18.3 ml of applying was 1.903 mg. And protein content in 5.15 ml of eluent was 1.009 mg. Adsorption capacity was calculated to be 40 $\mu g/cm^2$. Recovery was 93.8 %.

Adsoption of proteins in human serum was studied for observation of change before and after filtration with HAP paper in the condition (pH 8.0) which was stronger eluent than the condition (pH 6.0). Proteins were investigated by two-dimensional micro electrophoresis. Fig. 5 (a) shows the pattern of electrophoresis of serum protein before

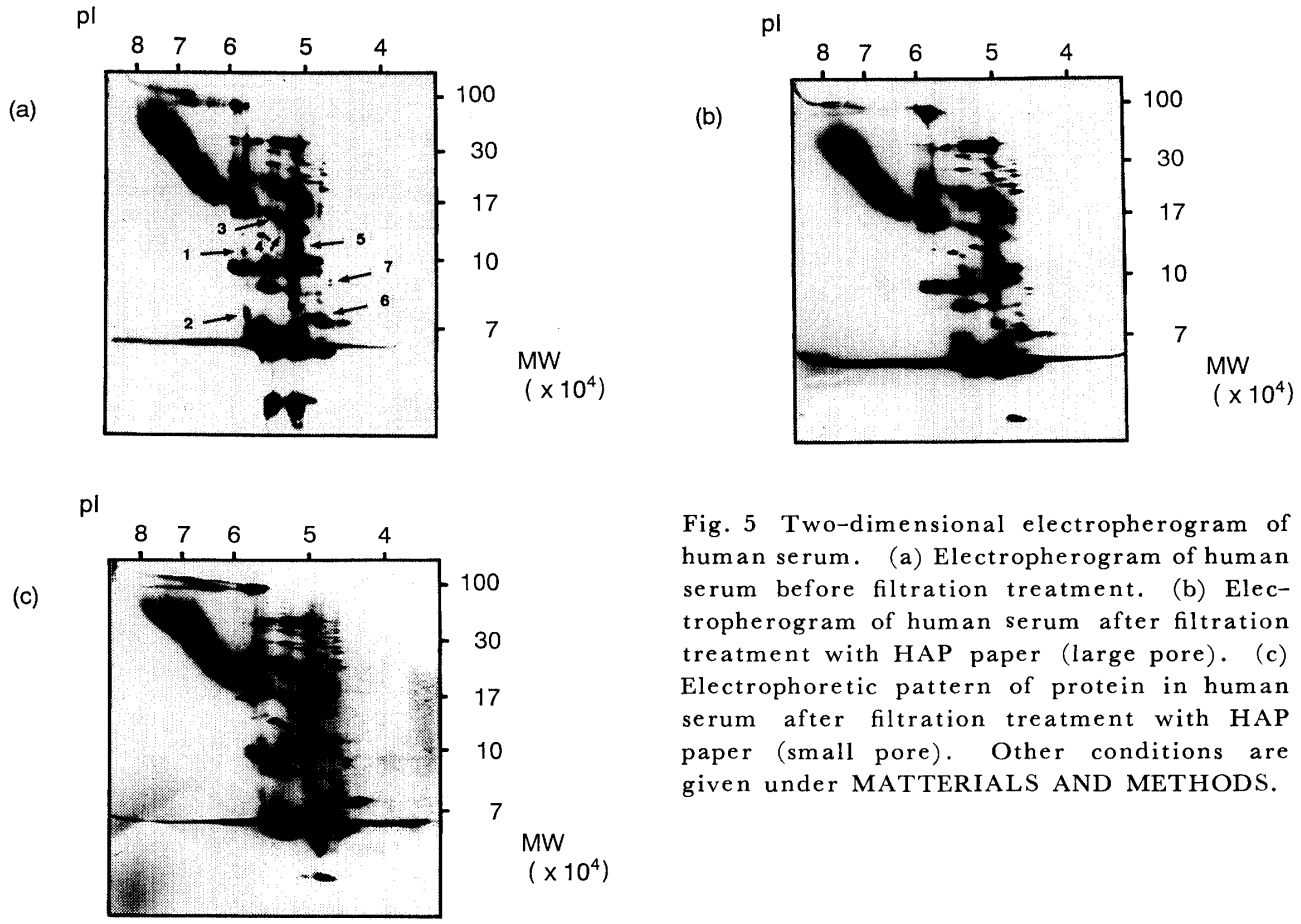

Fig. 5 Two-dimensional electropherogram of human serum. (a) Electropherogram of human serum before filtration treatment. (b) Electropherogram of human serum after filtration treatment with HAP paper (large pore). (c) Electrophoretic pattern of protein in human serum after filtration treatment with HAP paper (small pore). Other conditions are given under MATTERIALS AND METHODS.

filtratration. Fig. 5 (b) shows the pattern of electrophoresis of serum protein after filtration with HAP paper of large pore type. Fig. 5 (c) shows the pattern of electrophoresis of serum protein after filtration with HAP paper of small pore type. Approximately protein pattern did not change. Small changings, which observed were the proteins numbered 1, 2, 3 and 5 (HDL) in the figure, were actually becreased after filtration treatment. The proteins numbered 4 and 7 were increased after filtration treatment. The protein numbered 6 was shifted its site. The difference between the pattern of proteins by small pore type and the pattern of proteins by large pore type were also observed. The proteins numbered 1, 2, 5 (HDL) and acidic IgG were decreased less by large pore type thanby small pore type. According to the immunochemical study[12], a protein was lipoprotein. The phosphate groupe interaction might be on HAP paper. Other proteins were not changed.

Erythrocytes were not adsorped in 0.85 % sodium chloride solution on HAP paper, and were not passed through the HAP paper, and could be washed away easily by pipet jet wash.

4. CONCLUSION

HAP crystalline impregnated paper (HAP paper) could be used for chromatography of proteins. The chromatograms on HAP paper were approximately the same pattern as the ceramic HAP beads. All of serum proteins could be adsorbed on HAP column (0.76 I. D. × 10 cm). Adsorption of protein was proportionally decreased when column

height was decreased from 0.4 cm to 0.1 cm. HAP paper could be used for HPLC as insimilar manner as HAP particle. The thickness of HAP paper was 0.125 mm, and the column height of HAP paper was equivalent to 0.070 mm of HAP particles. Basic proteins such as immunoglobulins were absorded on HAP or on FAP comparatively stronger than acidic proteins. Immunoglobulin G was adsorbed 40 $\mu g/cm^2$ HAP paper. According to this rate 1 ml of serum (approximately 70 mg proteins/1 ml) should be adsorbed on 1750 cm^2 of HAP paper. HAP paper did not change the proteins patterns of serum at the analysis with electrophoresis. This fact suggest that this chromatographic process at pH 8.0 might be useful for the very rapid and simplified separation procedure for serum from whole blood. HAP paper and pH condition change was useful for concentration of proteins on account of high efficiency and good permiability. HAP paper should be effective for concentration of diluted proteins or trap filer of proteins. HAP paper was also effective for quick separation of basic protein, especially of immunoglobulins.

5. REFERENCE

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