A NEW CERAMIC TYPE SPHERICAL FLUOROAPATITE BEADS FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF NATURAL PROTEINS

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Abstract The chromatographical behaviors of proteins on FAP column are described. Firstly the elution patterns from FAP column were approximately same as those from HAP column. The recovery estimated with cytochrome c and others were approximately 100 % but the less recovery was estimated for denatured proteins. The pH dependancy of protein adsorption on FAP was determined. All of proteins including very acidic proteins were adsorbed at pH 5 and were eluted at pH 8.

Key words Fluoroapatite, high performance liquid chromatography, protein

1 INTRODUCTION

In the previous paper⁽¹⁾⁽²⁾⁽³⁾⁽⁴⁾ we described on the superiority of the new ceramic type hydroxyapatite (HAP) for high performance liquid chromatography (HPLC) of proteins, and also we described on the new ceramic type fluoroapatite (FAP) which was very promising for HPLC at even lower pH conditions from the physico-chemical property of this material. In this paper, elution patterns of proteins on FAP are described, and were compared with those on HAP. The elution patterns of proteins on FAP were approximately the same as the elution patterns on HAP in various respects.

2 MATERIALS AND METHODS

2-1 COLUMN AND APPARATUS Fluoroapatite column was 7.5 mm I.D. x 100 mm (particle size, 10 μ m) column of PENTAX ceramic fluoroapatite (Asahi optical co. ltd.). The hydroxyapatite column was 7.5 mm I.D. x 100 mm (particle size, 10 μ m) column of PENTAX ceramic hydroxyapatite (Asahi optical co. ltd.) The Hitachi liquid chromatograph, a model 655 system was used.

2-2 REAGENT NaH_2PO_4 , Na_2HPO_4 , and NaN_3 (chromatography grade) were purchased from Merck (Darmstadt, Germany). Water should be purified by distiller passing through a mixed-bed ion-exchange resin and the redistiller with glass apparatus, before use.

2-3 PROTEINS

Calmodulin and s-100 protein were purified in this laboratory⁽⁵⁾⁽⁶⁾⁽⁷⁾. Serum albumin (6x, crystallized, National Biochemicals co., USA). Ovalbumin, transferrin (Fe free), myoglobin, trypsinogen, trypsin, α -chymotrypsin and cytochrome c (SIGMA chemicals co., USA). Immunoglobulin G (Sandz ltd., Switzerland). Lysozyme (Seikagaku Kogyo co. ltd., Japan). Other properties and notes of proteins were listed in TABLE 1.

2-4 STANDARD CHROMATOGRAPHIC PROCEDURE

The chromatography was programmed for 30 min liner gradient system from 0.01 M sodium phosphate buffer solution (pH 6.8) containing 3mM NaN_3 to 0.40 M sodium phosphate buffer solution (pH 6.8) containing 3mM NaN_3 at a flow rate of 1.0 ml/min.

3 RESULTS AND DISCUSSION

3-1 ELUTION PATTERNS OF PROTEINS BY FAP COLUMN

Chromatographic procedure was performed as described in the standard procedure. Some of the typical chromatograms with purified proteins were shown in Fig. 1 (a,b,c,d and e). The retention volume and peak width were estimated and the relationship between capacity factor (k') and theoretical plate number were plotted as in Fig. 2. In the cases of transferrin, Myoglobin, cytochrome c and lysozyme, the relationship were liner. The theoretical plate number of α -chymotrypsin was 1670. If the relationship between the theoretical plate number of pure proteins and k' were liner, the theoretical plate number of pure α -chymotrypsin must be 2500. The theoretical plate numbers by isocratic procedure were approximately 400.

Effect of pH of eluent on the capacity factor (k') was observed. Serum albumin, cytochrome c or lysozyme was applied on the column, and it was eluted by isocratic procedure with various buffer solution with various pH values or the phosphate solution with various basic pH values, which might have scarce buffering action. Fig. 3 shows the relation between k' of proteins and pH of eluent. These proteins

were not adsorbed in 0.1 M sodium phosphate solution (pH 11 and pH 12). Generally proteins were adsorbed strongly in low pH eluents and weakly in high pH eluent. Elution behavior of protein on FAP was seemed to depend on the pH value of eluent. Acidic proteins were adsorbed in 0.001M sodium phosphate buffer solution (pH 6.0).

Relationship between the isoelectric point (pI) of protein and k' at pH 6.0, pH 6.8 and pH 8.0, indicated in Fig. 4 (a), (b) and (c) respectively. The elution order of protein was the same as that at pH 6.0, pH 6.8 or pH 8.0. Correlation coefficient between pI and k' at pH 6.0 was 0.77, and the slope of calculated line is 0.92. Correlation coefficient between pI and k' at pH 6.8 was 0.83, and the slope of calculated line is 1.24. Correlation coefficient between pI and k' at pH 8.0 was 0.87, and the slope of calculated line is 1.36. The slopes of the calculated lines were compared. The slope was increasing (0.92 $\rightarrow$ 1.24 $\rightarrow$ 1.36) in higher pH eluent. The correlation coefficient was also compared. The value was increasing (0.77 $\rightarrow$ 0.82 $\rightarrow$ 0.87) in higher pH eluent. Gradient elution of protein by higher pH eluent was useful to make good correlation. Basic proteins were adsorbed stronger than acidic proteins in various pH eluents. A protein in low pH eluent adsorbed strongly.

The relation between molecular weight and k' was not correlative. The mechanism of elution by FAP is looks like a cation exchanger. But FAP can elute both acidic proteins and basic proteins. FAP was amphoteric type packing material.

3-2 COMPARISON OF CAPACITY FACTOR (k') ON FLUPROAPATITE (FAP) AND CERAMIC HYDROXYAPATITE (HAP)

Basic proteins adsorbed strongly on FAP, can be eluted by the elevated concentration of phosphate. The elution of proteins on FAP was almost the same as HAP, seems to be a cation exchanger⁽¹⁰⁾⁽¹¹⁾⁽¹²⁾. FAP column was compared with HAP column. Adsorption degree of proteins was measured as k' . The elution of protein listed in Table 1 on HAP were performed according to standard chromatographic procedure of FAP. Fig. 4 shows the relation between the k' by FAP and the k' by HAP. The correlation coefficient is 0.98, and the slope of the calculated line is 1.30. The value of k' on FAP is correlative with the value of k' on HAP. So FAP have the same elution mechanism as HAP. Hydroxyl group of HAP was not important with the elution. According to the slope of the calculated line, FAP is slightly stronger than HAP in adsorption of proteins. But the difference between FAP and HAP is nonsense, because the difference is seemed to occur by the different condition in producing the apatite.

3-3 RECOVERY OF PROTEINS

Basic proteins were adsorbed stronger than acidic proteins. Cytochrome c (10 μ g/ 10 μ l) was loaded without column; the sample injector connected directly to the detector. The peak area of cytochrome c without column was assumed as 100 %. Then FAP column was connected between the sample injector and the detector, cytochrome c was applied on the column. The peak area was compared with that without column. The repeated recovery estimation of cytochrome c showed 98 % 108 %. The carry over peak was also estimated but no peaks were observed. Recovery of another proteins were 100 % and there were no carry over peak.

3-4 ELUTION OF DENATURATED PROTEIN

Partly denaturated cytochrome c was eluted and recovery was measured by the same method as natural cytochrome c. The recovery was decreased to 48 %, and carry over peak was observed (Fig. 6). Peak area of denaturated cytochrome c appeared and be decreasing in every 6 cycles after injection. The sum of peak areas of partly denaturated cytochrome c in 7 cycles was 100 % of applied quantity.

3-5 CHROMATOGRAPHY OF IgG

The elution pattern of polyclonal IgG was shown in Fig. 7 (a). Polyclonal IgG was eluted to be a wide peak. Rechromatography pattern of the peak head fraction of polyclonal IgG were shown in Fig. 7 (b). Rechromatography pattern of the peak tail fraction of polyclonal IgG were shown in Fig. 7 (c). The chromatography of FAP was reproducible and able to separate proteins. The peak width of half height of polyclonal IgG was more than six times wide than that of transferrin (Fig. 1 a). Immunoglobulin G has more than six heterogenous molecules at least.

4 CONCLUSION

FAP works like a cation a exchanger. Proteins were adsorbed strongly on FAP in acidic eluent, and weakly in basic eluent. The relationship between capacity factor (k') of proteins and isoelectric point (pI) was determined and compared with that of ceramic spherical hydroxyapatite (HAP). Elution of proteins by FAP was the same as that by HAP. Recovery of proteins on FAP was approximately 100 %. Carry over peak was not observed for natural form of proteins, but the denaturated proteins were adsorbed firmly on the column and eluted gradually by repeated elution. Recovery of partly denaturated proteins were less than 100 % and carry over peaks appeared after injection of sample. Denaturated protein was adsorbed strongly, because of changing of structure. FAP or HAP can be washed with NaOH solution. Proteins can be separated by the difference of structure on FAP. These properties would be useful for the purification of renaturation proteins.

Fig. 1 High performance fluoroapatite chromatography of protein. Proteins was applied and eluted by standard chromatographic procedure (a) transferrin, (b) myoglobin, (c) α -chymotrypsin, (d) cytochrome c, (e) lysozyme. Other conditions are given under MATERIALS AND METHODS. Chromatograms shown in figure were corrected by the subtraction of blank elution pattern. Abscissa is retention of protein as capacity factor (k'). Left ordinate is absorbance at 280 nm. Right ordinate is phosphate concentration of gradient.

Fig. 2 Relationship between the theoretical plate number and capacity factor (k') of proteins on FAP column. Ordinate is the theoretical plate number per 10 cm. Abscissa is the capacity factor (k') of protein. Proteins were eluted by standard chromatographic procedure, plotted as closed circle. Proteins eluted by isocratic procedure (0.1 M sodium phosphate buffer solution) were plotted as open circle. The solid line was supposed to be the plots of the pure proteins by the standard chromatographic procedure. The broken line was supposed to be the plots of the pure proteins by the isocratic procedure.

Table 1 List of proteins applied on FAP column

proteins	pI	MW
1 calmodulin	4.0	17000
2 concanavalin A	4.2	25800
3 protein G	4.2	35000
4 s-100a	4.5	10400
5 serum albumin	4.7	68000
6 ovalbumin	5.5, 5.9	45000
7 transferrin	5.9	80000
8 immunoglobulin G	5.8-8.5	148000-170000
9 myoglobin	7.2	17000
10 trypsinogen	9.2	24000
11 trypsin	9.6	23000
12 α -chymotrypsin	9.8	25000
13 cytochrome c	10.2(reduced)	11000
	10.3(oxidized)	11000
14 lysozyme	10.7	14000

Fig. 3 Effect of pH of eluent on the capacity ratio (k') of proteins. Numbers in the circle refer to the sample numbers in the table 1. Proteins were eluted by isocratic procedure (0.1 M sodium phosphate solution) at various pH values. Ordinate indicate the pH value of the eluent. Abscissa indicate the capacity factor (k') of proteins.

Fig. 4 Relationship between the isoelectric point and capacity ratio (k') of proteins on FAP column. Numbers in the figure refer to the sample numbers in the table 1. (a) Relationship between the isoelectric point and capacity ratio (k') of proteins by gradient procedure at pH 6. (b) Relationship between the isoelectric point and capacity ratio (k') of proteins by standard chromatographic procedure. (c) Relationship between the isoelectric point and capacity ratio (k') of proteins by gradient procedure at pH 8.

Fig. 5 Relationship between the capacity ratio (k') of proteins on FAP column and k' of HAP column. Numbers in the figure refer to the sample numbers in the table 1. Proteins were eluted by standard chromatographic procedure.

Fig. 6 Recovery of denatured protein. Vertical axis is the percentage of recovery. The peak area of cytochrome c without column assumed as 100 %. Partially denatured cytochrome c (100ug/100ul) was applied and eluted by standard chromatographic procedure. Horizontal axis is the cycle of the chromatography. After second cycle, recovery was calculated by the peak area of carry over peak.

CYCLES OF CHROMATOGRAPHY

Fig.7 High performance fluoroapatite chromatography of immunoglobulin G by standard chromatographic procedure. (a) Polyclonal immunoglobulin G from pooled human serum (100 μ g/50 μ l). (b) Rechromatography of acidic side of immunoglobulin G. Acidic side of immunoglobulin G was collected as the fraction of at the peak head of polyclonal immunoglobulin G. (c) Rechromatography of basic side of immunoglobulin G. Basic side of immunoglobulin G was collected as the fraction of at the peak tail of polyclonal immunoglobulin G

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