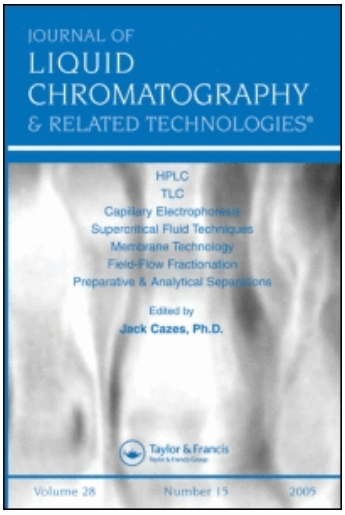


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# High Performance Liquid Chromatography of Mouse Monoclonal Antibodies on Spherical Hydroxyapatite Beads

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# HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MOUSE MONOCLONAL ANTIBODIES ON SPHERICAL HYDROXYAPATITE BEADS

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ABSTRUCT

High performance liquid chromatography (HPLC) on newly developed spherical beads of hydroxyapatite was applied for the simple purification of monoclonal antibodies (mAbs) secreted into mouse ascitic fluid. Sixteen mAbs including all four subclasses of IgG and IgM were separated successfully from serum albumin a major contaminant in the crude mAb preparation by a 30 min-linear gradient of phosphate ion concentration from 0.01M-0.3M at pH 7.2. Not only IgG mAbs but also IgM mAbs were quantitatively eluted from the column. Each antibody had a different retention time (apparent capacity factor of 2.24-4.14) in the chromatography and no relation was found between the retention time and the type of immunoglobulin (class or subclass). A monomeric form of IgM was also resolved successfully from IgM (pentamer) after its reduction with dithiothreitol; the monomer form of IgM was eluted from the column by a lower concentration of phosphate ion than was the pentamer. These results indicate that HPLC on the hydroxylapatite beads will be useful for the purification and characterization of mouse mAbs.

## INTRODUCTION

Adsorption chromatography of proteins on hydroxyapatite introduced by Tiselius et al.(1) and Hjerten (2) is sometimes a very effective process for protein purification (3-8). Inspite of its usefulness, chromatography of proteins on hydroxyapatite is not widely accepted because synthesis of a gel having a uniform quality has been difficult. Unexpected difficulties arose from the fragility of the gel such as lowering of the flow rate during chromatography, and low protein recovery prevented wide application of hydroxyapatite for protein purification. Advanced technology has eliminated,however,those faults of hydroxyapatite and has provided material suited for high performance liquid chromatography (HPLC). A shperical beads of hydroxyapatite, which has a rigid structure and uniform quality, has been synthesized by several manufacturers. Comparative studies of some of those new type of hydroxyapatite for HPLC were repoted by Kadoya et al (9).

This paper describes some application of one of the newly developed hydroxyapatite beads for HPLC for the purification and characterization of mAbs secreted into mouse ascitic fluid. The chromatographic behaviors of polyclonal serum IgG of several animals is also described.



## MATERIALS AND METHODS

Monoclonal antibodies (MAbs): MAbs produced by BALB/c mice were partially purified from ascitic fluid by precipitation with ammonium sulfate; the precipitate formed at 50% saturation of ascitic fluid with ammonium sulfate was dissolved in 10 mM phosphate buffer (pH 7.2) containing 0.15M NaCl and 0.1% $NaN_3$ to give a protein concentration of about 5-10 mg/ml. For analytical scale chromatography, the solution was diluted 20 times with 10 mM phsphate buffer (pH 7.2) and applied (200-500μ) to the column. For preparation of mAbs, up to 500 μl of the original solution containing bout 10 mg protein is applicable to the column without dialysis or dilution. The specification of mAbs used were summerized in Table 1.

Table 1. Chromatographic factors of MAb on the hydroxyapatite column

| Mab (ref) | class | Retention time (min) | Capacity factor | Resolution factor*[1] | Theorotical plates | Assmmetry factor*[2] |
|---|---|---|---|---|---|---|
| 5CD2(13) | IgG1 | 28.41±0.32*[3] (C.V=1.16%) | 2.96 | 1.38 | 5,938 | 1.44 |
| 3F10(14) | IgG1 | 23.32 | 2.24 | 1.05 | | |
| 1H2(14) | IgG1 | 27.32 | 2.78 | 1.30 | | |
| Ig-3E7(15) | IgG1 | 28.84 | 3.01 | 1.41 | | |
| OTA.2(16) | IgG1 | 30.32 | 3.21 | 1.50 | | |
| OTA.7 | IgG1 | 20.00 | 2.98 | 1.35 | | |
| 2G3(*[4]) | IgG2a | 27.92±0.4*[3] (C.V=1.61) | 2.89 | 1.35 | 3,020 | 1.58 |
| 1B7(14) | IgG2a | 29.68 | 3.12 | 1.46 | | |
| CL.5(17) | IgG2a | 37.04 | 4.14 | 1.93 | | |
| CL.6(17) | IgG2a | 28.12 | 2.91 | 1.36 | | |
| 8BE6(18) | IgG2b | 33.57±0.4 (C.V=1.28%)*[3] | 3.63 | 1.71 | 3,679 | 1.25 |
| CL.7(17) | IgG2b | 27.88 | 2.87 | 1.34 | | |
| 4CC6(18) | IgG3 | 37.18±o.61*[3] (C.V=1.65%) | 4.16 | 1.94 | 6,432 | 1.19 |
| CL.8(17) | IgG3 | 29.52 | 3.10 | 1.54 | | |
| 7BE8(18) | IgM | 29.74±0.44*[3] (C.V=1.65%) | 3.13 | 1.46 | 1,976 | 1.52 |
| 7BE8 | IgM*[5] | 24.40 | 2.39 | 1.12 | 825 | 1.32 |
| 2F11B(*[5]) | IgM | 35.04 | 3.87 | 1.81 | | |
| 2F11B | IgM*[5] | 28.04 | 2.89 | 1.35 | | |
| 26-7-11(19) | IgM | 36.20 | 4.03 | 1.88 | | |

Factors were calculated by the elution profiles obtained by the 30-min gradient of the phosphate buffer as described in the text. *[1]:resolution from contaminating lubumin peak(22.6min). *[2]:calculated at 10% adsorption level of peak height. *[3]:SD and C.V were calculated from results of 4 different runs. *[4]:not published. *[5]: IgM monomer.

Ascites fluid which did not contain a specific mAb was obtained by the i.p. injection of tumor cells (SP2/o). Ascitic fluid was also treated with ammonium sulfate as described above and chromatographed as a reference mAb-free ascites protein.

Serum immunogloblin G: Human IgG (Cohn's fraction II) was a product of Sigma Chemical Co., St.Louis, Mo. Mouse IgG (Cohn's Fr.II) was purchased from Miles, Ltd., Rehovot, Israel. Rabbit IgG was purified from the serum by precipitation with ammonium sulfate and DEAE-cellulose column chromatography as described by Fahey and Terry (10).

Apparatus: Shimazu LC 4A liquid chromatograph (Shimazu Co., Kyoto, Japan) equipped with a gradient system and u.v. detector (SPD-2AS, 1cm light pass) was used.

Column: A packed column (0.75x10 cm) of hydroxyapatite was a product of Toa Nenryo Kogyo K.K. (Tokyo, Japan). Number of theoretical plates (NTP) of column determined by the isocratic elution of lysozyme (20 µg) with 0.12M phosphate buffer(pH 6.8) at the flow rate of 0.5 ml/min was about 4,000 (retention time 18 min): capacity factor(k') and assymmetry factor at 10% absorption level ($Af_{10}$) of the protein was 1.54 and 2.8 rerpectively. By the elution profile of lysozyme obtained with the gradient elution system used in this experiments, an apparent NTP, k' and $Af_{10}$ was calculated as 18,000, 3.58(retention time 33 min) and 1.3 respectively. The hydroxyapatite is composed of completely spherical porous particles with 0.2-3µm diameter as revealed by scanning electron microscopy. The physical and chemical properties of the gel used in this experiments were described previously by Kadoya et.al.(9) in comparison with several other hydroxyapatite columns for HPLC use.

Preparation of phosphate buffer: Sodium phosphate, monobasic and dibasic (analytical grade) were obtained from Wako Pure Chemical Industries, Ltd.,Tokyo, Japan. Phosphate buffers (0.01M and 0.3M, pH 7.2) were prepared by titrating the dibasic solution with the corresponding concentrations of monobasic solution. To 1 liter each of these buffers, 1.0 ml of 1M calcium chloride and 1 gram of sodium azide were added. After gradual addition of calcium chloride, the buffers were left to stand for

at least 24 hr. The resulting precipitate (calcium phosphate) was then removed by filtration with a membrane filter of 0.3 μm pore size (Millipore menbrane filter, Type PH). The remaining calcium ions concentration in these phosphate buffers were estimated to be about 30 and 10 μM, respectively and the ion prevented deterioration of the column.

Chromatographic procedures: About 200 μg protein in 200-500 μl of 10 mM phosphate buffer prepared as above was introduced onto the column, which had been equilibrated with 10 mM phosphate buffer(pH 7.2) saturated with $CaCl_2$. When there was any turbidity, the sample solution was clarified by filtration through a membrane filter of 0.45 μm pore size (Millipore SJHV004NS) prior to the chromatographic run. The column was eluted at a constant flow rate of 0.5 ml per min at room temperature by use of a 30-min linear gradient from 0.01 to 0.3M phosphate buffer (pH.7.2) saturated with $CaCl_2$. At the end of each run, the column was washed for 10 min with the 0.3M phosphate buffer and then re-equilibrated with the 10 mM phosphate buffer for the next run. The pressure of the column was 20 $Kg/cm^2$ when eluted with the first buffer at the flow rate of 0.5 ml per min. In the final buffer, the pressure rose to 30 $kg/cm^2$. On some occasions, the eluate from the column was manually collected for the electrophoresis in polyacrylamide gel and determination of protein content.

SDS-polyacrylamide gel electrophoresis: Protein eluted from the column was analyzed by electrophoresis in 10% polyacrylamide gel containing 0.1% sodium dodecylsulfate (SDS) at pH 8.6 as described by Lemmli (11). Prior to the electrophoretic run, eluates were treated with 1% SDS at 50 °C for 30 min. After electrophoresis, protein in the gel was stained with Coomassie brilliant blue R-250 (12).

## RESULTS

Fig.1(a-e) shows typical elution profiles of mAbs partially purified from ascitic fluid by precipitation with 50% ammonium sulfate; in Fig.1-f, there shows a chromatogram of ascitic

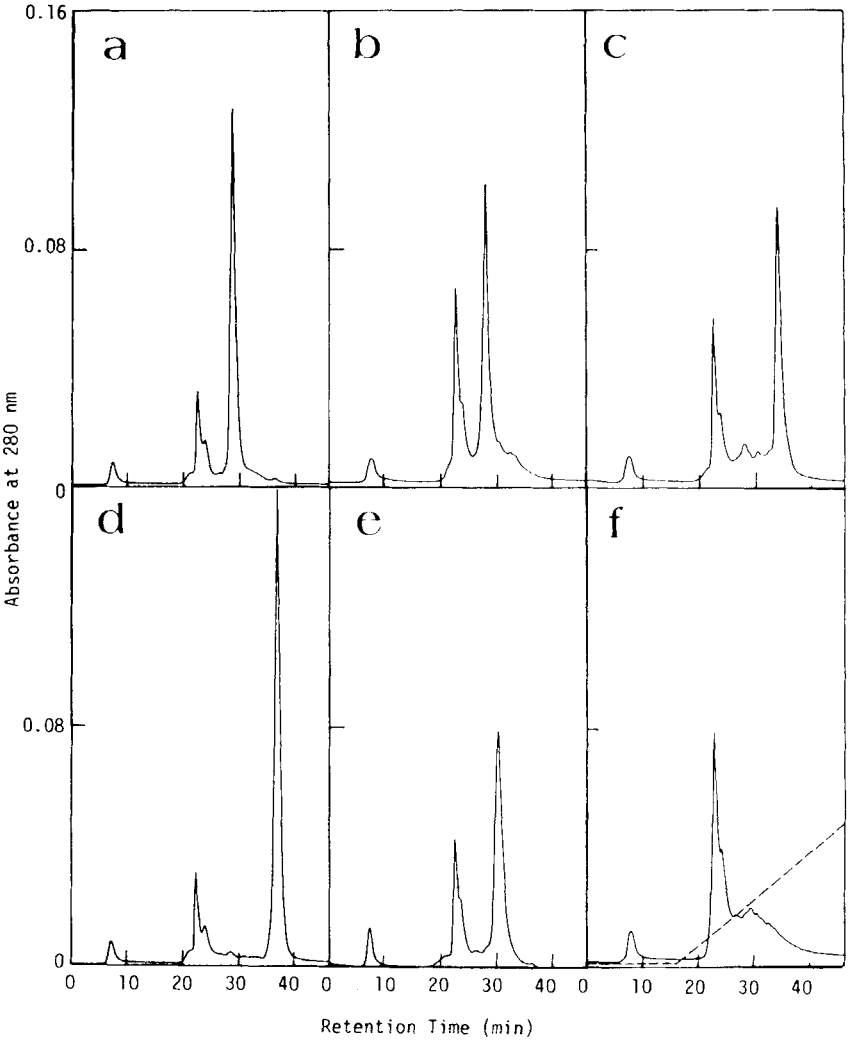


Fig1. High performance liquid chromatography of mouse monoclonal antibodies on hydroxyapatite gel.
Partially purified monoclonal antibodies prepared from ascites fluid as described in "Materials and Methods" were applied to the column of hydroxylapatite (0.75x10cm) and eluted by a 30-min linear gradient of phosphate ion concentration (0.01-0.3M) at pH 7.2 containing $CaCl_2$ with a flow rate of 0.5 ml/min.
a:5CD2(IgG1), b:2G3(IgG2a), c:8BE6(IgG2b), d:4CC6(IgG3), e:7BE8(IgM),
f:monoclonal antibody free ascites protein (control). The hachet line on (f) indicates the gradient profile of phosphate ion concentration(0.01-0.3M).

proteins obtained from mAb-free ascitic fluid by the same method. It is noted that a protein peak emerged 23 min after injection of sample is commonly seen all the chromatograms even in the preparation of mAb-free ascitic fluid. Upon the electrophoretic analysis (data not shown), this common 23-min peak contained serum albumin as a major component. Beside serum albumin, several non-immunoglobulin proteins were also seen in the peak. In this connection, we have observed that mouse serum albumin and transferrin to be contained in those crude mAb preparations have similar retention time of about 23 min under the same chromatographic condition. It was also proved that polyclonal IgG in ascitic fluid has been eluted as broad and irregular protein peak(s) after the elution with the common 23-min peak by analyzing ptotein in the broad peak with electreophresis on polyacrylamide gel. Such broad and irregular elution of polyclonal IgG from the hydroxyapatite column was confirmed by subjecting purified serum polyclonal IgG on the column (see Fig.5). The 2nd sharp and symmetrical peak which were only seen in those chromatograms (a) to (e) corresponds to the elution of mAb. Such evident protein peak was never seen in the chromatogram (f) which is demonstrating elution profile of mAb-free ascitic proteins. Retention time of the mAb peak seems to be different from each other. The results shown in Fig.2, which demonstrates the separation of an equimixture of 5 different monoclonal antibodies shown in Fig.1a-e, confirmed such difference in retention time of each mAb. In order to detect any relation between the retention time and types of immunoglobulin, 12 additional mAbs were analyzed under the same conditions. Elution profiles (data not shown) of these monoclonal antibodies were also similar to those profiles shown in Fig.1 a-e; a mAb emerged from the column as a sharp symmetrical peak having $Af_{10}$ of 1.2-1.6 after elution of common albumin peak(23-min peak). Retention time and capacity factor of all the mAbs by the hydroxyapatite column chromatography are summerized in Table 1. NTP, $Af_{10}$ and resolution factor between mAb peak and contaminating albumin peak(common 23 min peak) were calculated for several mAb peak and cited also in the table. Retention time obtained for each mAbs is quite reproducible. It is evident that each mAb has

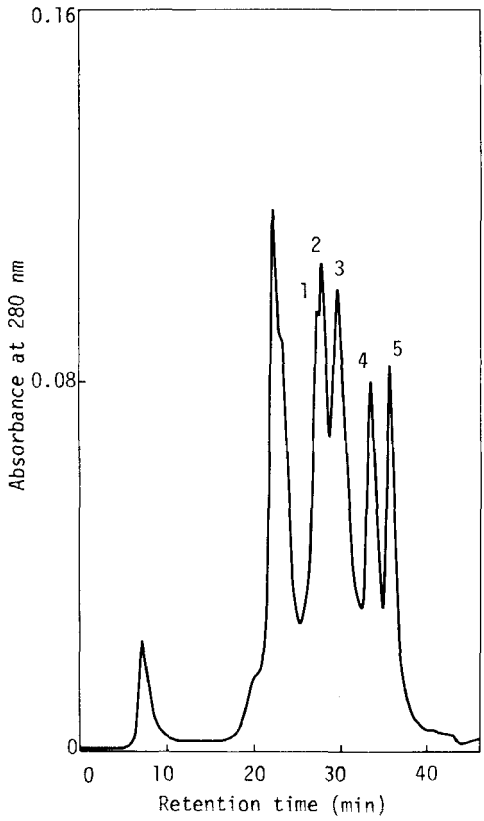


Fig.2. High performance liquid chromatography of a mixtur of monoclonal antibodies.
An equimixture of monoclonal antibodies showed in Fig.1 was applied to the hydroxyapatite column and eluted as described in the regend for Fig1. The protein peaks 1-5 correspond to the monoclonal antibodies shown in Fig.1a-e.

different retention time by the chromatography, even in the same subclasses, and no relation has been found between the retention time and type of immunoglobulins (classes or subclasses). Among the mAbs tested, CL.5 has the largest retention time of 37.04 min and 3F10 has the smallest retention

time of 23.32 min.. The retention time of 3F10 is exceptionally small as compared to other mAbs and almost identical to that of mouse albumin. Therefore purification of this mAb by this elution system was difficult.

NTP of 37,00 calculated by 8BE6 seems too small when compared it to the value(18,000) which was alculated with lysozyme having a similar retention time with the mAb. Since molecular weight of IgG molecule is about 10 times larger than that of lysozyme, such difference in NTP is considared to be the differnce in the diffusion constant of these molecules in the column. Infact the peak width at the half-height(0.4min) of lysozyme is about 3 times narrower than that of 8BE6 (1.2min) and other mAbs(1.12-1.5min).

We have usually applied 20-100 μg of crude mAb preparation for semi-micro analysis of mAbs but the column is applicable for the preparation of mAbs in mg quantity. Use of 45-min gradient with the flow rate of 1ml/min was recomended for such large scale preparation. Upon this modified method, we have succeeded in obtaining several to 5mg of purified mAbs at once. So far as tested, maximum loadability of the column seems to be 10 mg for the crude mAbs preparation.

Recovery of mAbs from the hydroxyapatite column: To determin the recovery of mAb from the column, several purified mAbs (0.5-1mg) were chromatographed to compare the values of $A_{280}$nm before and after the re-chromatography. So far as tested, not only IgG mAbs(OTA.2, CL.5 and 5CD2) but also IgM mAb (26-7-1) has been eluted almost quantitativery from the column. The actual protein recovery of each mAbs is as follow ; 98.8% for OTA.2, 96.8% for CL.5,100.9% for 5CD2 and 97.6% for 26-7-1, respectively. Incidentally, all the mAbs re-chromatographed were eluted as a sharp symmetrical protein peak with identical retention times cited in Table 1. Considering stability of immunoglobulin molecule and good protein revovery from the column, denaturation of mAbs during chromatographic process should not be considered. In fact, binding activity of CL.5 on encephalitis virus has been quantitatively eluted from the column when the binding activity has been determined by enzyme linked immno-assay. In this connection, it is worthy to note that recovery of proteins from

the column were almost quantitative when 25 different proteins were eluted under similar chromatographic conditions herein used(20).

Rechromatography of IgM immunoglobulins after reduction: It is known that the pentamer form of IgM (MW:900,000) dissociates into monomer form (Mw: 180,000) by releasing so-called J peptide (Mw: 15,000) upon mild reduction of pentamer form at neutral pH(13). We analyzed this structual change in IgM by the hydroxyapatite column chromatography. A once purified pentamer form of IgM (7BE8 see Fig 1-e) was rechromatographed after incubation for 30 min at 37 °C with or without dithiothreotol(DTT; 10 mM). The resulting chromatograms are shown in Fig.3 a and b. As expected, pentamer form of IgM was eluted from the column with correct retention time of 29.76 min as shown in Fig.3-a, whereas reduced IgM was eluted from the column with the retention time of 24 min(Fig.3-b). Another monoclonal IgM (2F11B) showed a similar change in retention time (Table 1). Such change in retention time was not observed when IgG mAbs had been reduced (data not shown). A small protein peak of undissociated pentamer form of IgM or more likely a part of polyclonal IgG in ascitic fluid, has been eluted from the column but the amount is negligible. The large absorption peak appered at the break-through fraction of Fig.3-b is due to the elution of reductant (DTT). Because of low content of J-peptide in IgM (1.3% of whole molecule), we could not detect elution of the peptide by the chromatography. The chromatography on hydroxyapatite clearly demonstrated the structual change of IgM upon the reduction. Thus, the chromatography will be useful for obtaining the monomeric form of monoclonal IgM.

Polyacrylamide gel electrophoresis of monoclonal antibodies: Fig.4 shows electrophoretic analysis of several mAbs on polyacrylamide gel before and after the chromatography. A monoclonal antibody 4CC6 having large retention time has been purified extensively. Other antibodies are also purified effectively but they contained some other contaminant proteins although the amount of the contaminants is small. Mab 7BE8 (IgM) was found to contain a small amounts of IgG upon the electrophoresis (see lane 7) even after purified by the

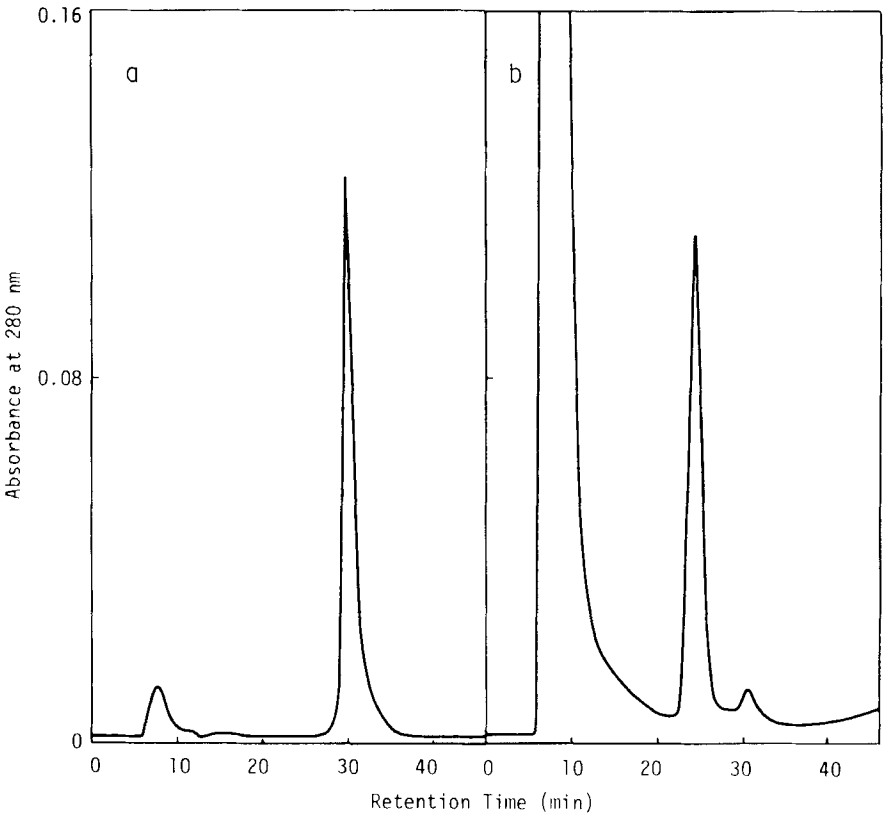


Fig.3. Rechromatography of immunoglobulin M.
Purified IgM(7BE8) as shown in Fig.1-e was rechromatographed after incubation at 37 °C without reductant(a) or with reductant (dithiothreitol) (b).

chromatography. This does not neccessarily mean the heterogeneity of the clone used because ascites fluid contained a small amounts of nonspecific polyclonal antibodies as revealed in Fig.1-f. Therefore, contaminating IgG in the eluate of IgM was considered to be a part of such nonspecific polyclonal IgG present in ascitic fluid. The same things would happened with other IgG mAbs purified by this chromatographic method. Immunospecific affinity

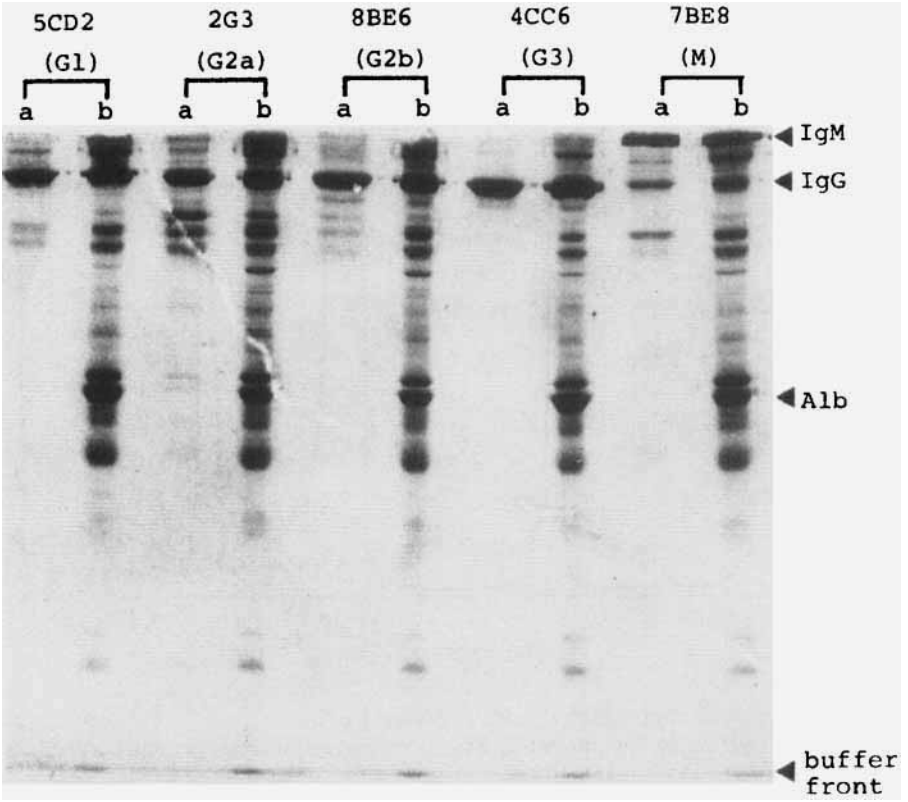


Fig.4. Polyacrylamide gel electrophorersis of monoclonal antibodies purified by hydroxyapatite gel.
Monoclonal antibodies before and after purification by the chromatography on hydroxyapatite(see Fig.1) were analyzed by 10% polyacrylamide gel containing 1% SDS. a: after chromatography, b: befor chromatography. Samples were treated with 1% SDS at 50 °C for 45 min prior to the electrophoretic run.

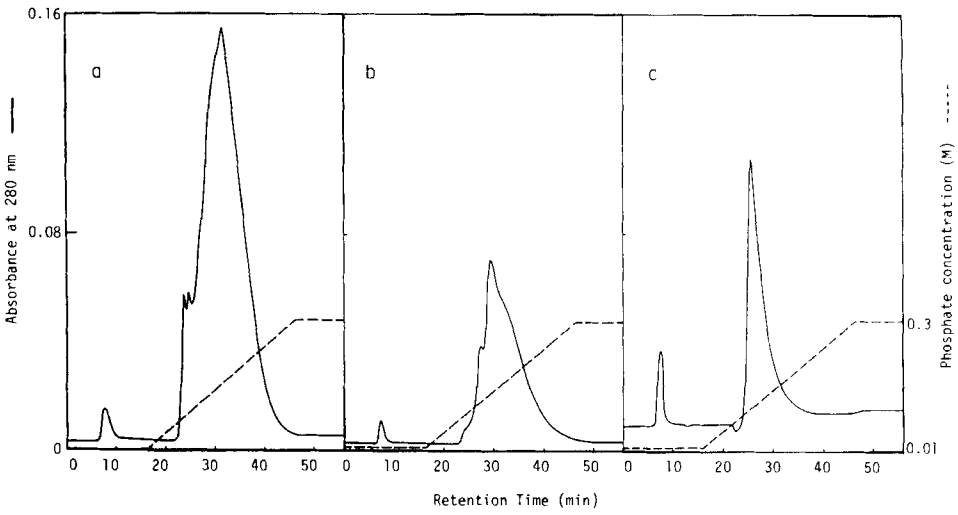


Fig.5. High performance liquid chromatography on hydroxyapatite gel of purified immunoglobulin G from several animals. a: mouse serum IgG (Cohn's Fr.II), b: human serum IgG (Cohn's Fr.II), c: rabbit serum IgG.



chromatography shoud be used for the complete removal of such polyclonal immunoglobulins from a monoclonal antibody.

Chromatography of polyclonal serum IgGs: In comparison with the monoclonal antibody, we have chromatographed IgG purified from human, mouse and rabbit serum. The results are shown in Fig.5 a-c. Unlike the mAbs shown in Fig.1a-e, polyclonal IgG from serum was eluted from the column as broad irregular shape. It must be emphasized that polyclonal IgG of these animals began to elicit at about 0.06M phosphate concentration (about 23 min after injection of the sample) and its elution has completed when phosphate concentration reached to 0.3M. From the results, it is estimated that most of mAbs would have been eluted from the column in such range of phosphate ion concentrations. In fact, all the mAbs are eluted from the column between such phasphate ion concentrations (see Table 1).

## DISCUSSION

Use of HPLC on spherical beads of hydroxyapatite gave exellent result for the purification of monoclonal antobodies from ascitic fluid in sub-mg to several mg quantity. Both protein and antigen binding activity from the column are seemed to be very high. This method is as simple as affinity chromatography on Protein A (22,23) which have been widely used for facile purification of mAbs. Inspite of its simplicity and usefulness, purification of mouse monoclonal IgG1 and IgM are sometimes difficult by protein A method(24,25) because they have low or no affinity on protein A. Hydroxyapatite method seems to give more consistent results over Protein A method for facile purification of mouse mAbs since all subclass IgG and IgM were adsorbed on the column and eluted quantitatively from the column at neutral pH without using caotropic ion such as KSCN or low pH buffer which were occasionally used for the elution of IgGs from protein A column. The hydroxyapatite column of the same manufacturer has been successfully used for the purification of myeloma IgD and IgA from human serumn by Okuyama et al.(20) with similar chromatoraphic conditions used here. Their findings accompanied with our results described here, indicates that the hydroxyapatite column would be generally appicable for a facile purification of mAbs from various animals. Unfortunately the chromatographic condition used in this study is not suited for purification of such mAbs as 3F10 having close retention time to that of albumin, a majour contaminant in the crude preparation. Although, considering from the elution pattern of polyclonal mouse IgG shown in Fig.5, population of such IgG molrcule having similar retention time with albumin would be small. Some modification such as changing pH of the eluent or using more shallow phosphate gradient, may improve separation of such extraordinal mAbs from contaminant proteins.

Polyclonal IgG purified from serum were eluted as wide spread irregular shapes from the column. Such diversed elution of polyclonal IgG from hydroxyapatite has been demonstrated by early work of Hjerten(2). More recently, using hydroxyapatite for HPLC, Okuyamaa et al.(20) and Kawasaki et al.(26) are also repoting such broad elution of IgG. Such diversed elution of IgG from the

hydroxyapatite column should be attributed to the polymorphism of immunoglobulin molecule. Among the serum immunoglobulin G tested, rabbit IgG has been eluted relatively narrow range of phosphate ion concentration as compared to human and mouse serum IgG. This may indicates narrower polymorphism of IgG molecule in the species and might have some relation to the fact that IgG subclass is not known in the animal.

Bernardi and Kawasaki (27) and Gorbunoff (28-30) reported that interaction between protein and hydroxyapatite is essentially electrostatic, and that difference in surface charge of individual proteins is a crucial factor for determining retention property of proteins on the gel. Recently, Okuyama et.al.(20) reported that good relationships between retention times of proteins on the chromatography and isoelectric point(pI) of proteins; a protein having a higher pI value is strongly adsorbed on hydroxyapatite. Based on the finding, they proposed zwitter ion type retention mechanism. From this point of view, differnce in retention time obtained for each mAbs even in the same subclass of IgG refer to difference in pI of each mAb molecule. In this connection, it is worth to note that wide distribution of retention time observed in mouse mAbs belonging to IgG1 is compatible with the findings of Haward and Virell(31); who reported isoelectric points of human IgG1 molecule distributed in wide pH range compared to other subclasses of IgG.

No significant change in flow rate (pressure) and apparent NTPs of 18,000 of lysozyme befor and after this experiments (more than 50 runs) indicates that deterioration of hydroxyapatite gel is negligibly small, if any. In a systematic experiment, lowring of NTP of the column befor and after consequative runs(100 times) of lysozyme by isocratic elution with 0.12 M phosphate buffer(pH 6.8) was also unchanged(Ishikawa,H. personal communication). In this connection, Kadoya et al.(9) have noticed deterioration of the column after 85 runs of protein mixture (gradient elution) but it was restored by washing the gel with 0.1N NaOH. Their finding is variable for re-generation of deteriorated column.

As demonstrated by Okuyama et al.(20) the column is applicable for purification of proteins other than immunoglobulins

describes here. We also used the column for purification of proteins in snake venom (unpublished). Through the experiment, the hydroxyapatite column is rather suited for chromatography of basic proteins than that of acidic proteins.

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REFERENCES

1. Tiselius,A., Hjerten,S. and Levin,O. (1956) Arch. Biochem. Biophys., 65, 132-155.
2. Hjerten,S. (1955) Biochem. Biophys. Acta., 31, 216-235
3. Berbardi,G. (1971) In Methods in Enzymology XXII (Jakoby,W.B. eds.), Academic Press, New York, pp.325-339.
4. Bernardi,G. (1973) In Methods in Enzymology XXVII (Hirs,C.H.W. and Timasheff,S.N. eds), Academic Press, New York, pp.471-479.
5. Moss,B. and Rosenblum,E.N. (1972) J. Biol. chem., 247, 5194-5189.
6. Hirata,H. Kambe,T. and Kagawa,Y. (1984) J. Biol. chem., 259, 10653-10655.
7. Dolganov,G.M. Chestukhin,A.V. and Shemyakin,M.F. (1981) Eur.J.Biochem., 114, 247-254.
8. Yeh, M-F. and Trela,J.M. (1976) J. Biol. Chem., 251, 3134-3139.
9. Kadoya,T., Isobe,T., Ebihara,M., Ogawa,T., Sumita,M., Kuwahara,H., Kobayashi,A., Ishikawa,H. and Okuyama,T. (1986) J. Liquid Chromatogr., 9, 3543-3557
10. Fahey,J.L. and Elizabeth,W.T., (1978) In "Handbook of Experimental Immunology, Chapter 7". (Weir,D.M., ed.), Blackwell Scientific Publications, Oxford.
11. Leamlli,U.K., (1970) Nature, 270, 680-685.
12. Fairbanks,G., Steck,T.L. and Wallch,D.F.H. (1971) Biochemistry, 10, 2106-2617.
13. Elias,J.M., Chiba,J., Shevach,E.M. and Godfrey,H.P. (1985) Lab.Investigation, 52, 270-277.

14. Sato,H., Ito,A., Chiba,J. and Sato,Y. (1984) Infect.Immunity, 46, 422-428.
15. Furuta,T., Nishizawa,T., Chiba,J. and Hamada,S. (1983) Infect.Immunity, 41, 72-875.
16. Chiba,J., Kajii,H., Kawamura,M. Ohi,K., Morooka,N. and Ueno,Y. (1985) Proc.Jpn.Assoc.Mycotoxicol, 21, 28-29 (in Japanease)
17. Yamamoto,K., Hashimoto,K., Chiba,J. and Shimizu,B. (1985) J.Virol., 55, 840-842.
18. Chiba,J., Chused,T.M., Leiserson,W.M., Zweig,S.E. and Shevach,E.M.(1983) J.Immunol.Methods, 63, 247-261.
19. Tadakuma.K., (1981) Transplantation Proceeding, 13, 958-962.
20. Okuyama,T., Kadoya,T., Ogawa,T. and Kuwahara,H. (1986) Proceedings of the 3rd Meeting on Liquid Chromatography. pp.1-4. The Division of Liquid chromatography, The Japan society for Analytical Chemistry.(in Japanese:summary in inglish)
21. Koshland,M.E. (1975) In Advance in Immunology Vol.20, (Dixon,F.J. and Kunkel,H.G. eds.) Academic Press, New York, London, pp.4-69.
22. Hjelm,H., Hlelm.K. and Sjoquist,J. (1972) FEBS Lett. 28, 73-76.
23. Goodswaard,J., Van Der Donk,J.A., Noordzij,A., Van Dam,R.H. and Verman J.-P. (1978) Scand.J. Immunol., 8, 21-28.
24. Kronvall,G., Grey,H.M. and Williams JR.R.C.(1970) J.Immunol., 105, 116-1123.
25. Ey,P.L., Prowse,S.J. and Jenkin,C.R. (1978) Immunochemistry, 5, 429-436.
26. Kawasaki,T., Takahashi,S. and Ikeda,K. (1985) Eur. J. Biochem., 152, 361-371.
27. Bernardi,G. and Kawasaki,T., (1968) Biochem.Biophys.Acta, 160, 301-10.
28. Gorbunoff,M.J. (1984) Anal.Biochem., 136, 425-432.
29. Gorbunoff,M.J. (1984) Anal.Biochem., 136, 433-439.
30. Gorbunoff,M.J. (1984) Anal.Biochem., 136, 440-445.
31. Haward,A. and Virella,G. (1969) Protides of the Biological Fluid, 17, 449-453.