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단보 (Note)

Development of Chromatographic Downstream Processing for the Purification of Monoclonal Antibody from Ascites Fluid: Part II. Use of Single Hydroxylapatite Chromatographic Step

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생쥐 복수로부터의 단세포군 항체분리를 위한 크로마토그라피 분리정제 방법의 개발
Part II. 히드록실아파타이트 크로마토그라피 단일 단계만의 사용

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In order to obtain monoclonal antibody from ascites fluid at sufficiently high purity using a single hydroxylapatite chromatography (HA) a further optimization on its operating variables was carried out.

By adjusting the pH of the eluent, the sodium phosphate buffer, to 6.0 from 6.8 and adding $CaCl_2$ to 1 mM at the column inlet, the elution molarities (M_{elu}) for the desired monoclonal antibody and contaminating proteins can be distinguished from each other with enough resolution. Previously these two groups of proteins co-eluted at the same time at pH 6.8 and without $CaCl_2$. This single step hydroxylapatite chromatography yields the desired antibody pure enough for diagnostic use.

In Part I it was shown that very highly purified McAb could be otained by the tandem use of HA chromatography followed by GPC (7). There the use of typically known elution conditions in the first HA step resulted in co-elution of a few contaminants together with the desired McAb in the effluent from the HA column. In this second part of the study new elution conditions for the HA step were searched so that the single HA column alone, without the use of GPC column subsequent to the HA step, can give the isolated product band without the problem of co-elution with the contaminants.

Although the contaminants include host IgG, attention was focused on removal of contaminants other than host IgG because it is known that host mouse can be immunosuppressed beforehand not to produce host IgG.

With decreasing pH of the eluent in HA chromatography, the elution molarities (M_{elu}) of molecules such as proteins and tRNAs vary to different extents (1-5). For example, the elution molarity of IgM McAb is high. It is hoped that these experimental facts may contribute to separation of desired IgM McAb from contaminants with enough distinguishability.

The expreimental materials and produres used in this part are the same as those in Part I (7).

Effect of pH on purity

When pH was 5.0, the elution molarity of the desired protein IgM-0.20M (Figure 1) as compared with 0.15M-020M at pH 6.8, the exact value being dependent on other conditions such as flow velocity and salt gradient. As compared with the results for

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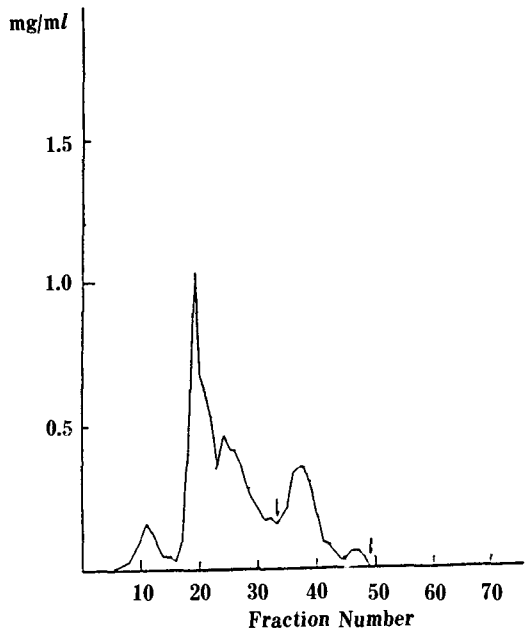


Fig. 1. Protein concentration profile from hydroxylapatite fractionation of the ascites fluid

pH 5.0; flow rate 0.2 ml/cm^2/min; gradient 5.82×10^{-3} M/cm; sample volume, 7 ml of 0.05 M NaP with 0.7 ml of ascites fluid added. Arrows indicate McAb eluting range.

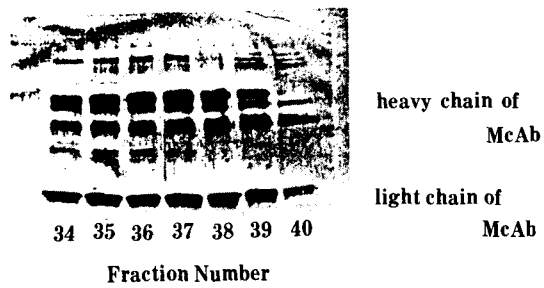


Fig. 2. Silver staining of the SDS-PAGE of the IgM McAb containing fractions from hydroxylapatite chromatography.

pH 6.8 the purity in the case of pH 5 became poorer (Figure 2). When pH was increased to 9.0, the elution molarity decreased too much and McAb coeluted with all contaminants in the early elution step. The best purity could be obtained when pH was 6.0 (Figure 3), although band dilution increased a little. The elution molarities of the contaminating components varied in a favorable direction such that relative ratio of contaminants to McAb decreased

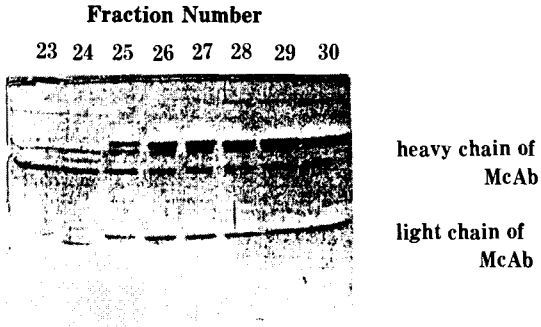


Fig. 3. SDS-PAGE of IgM McAb containing fractions from hydorxylapatite chromatography that is stained with Coomassie Blue.

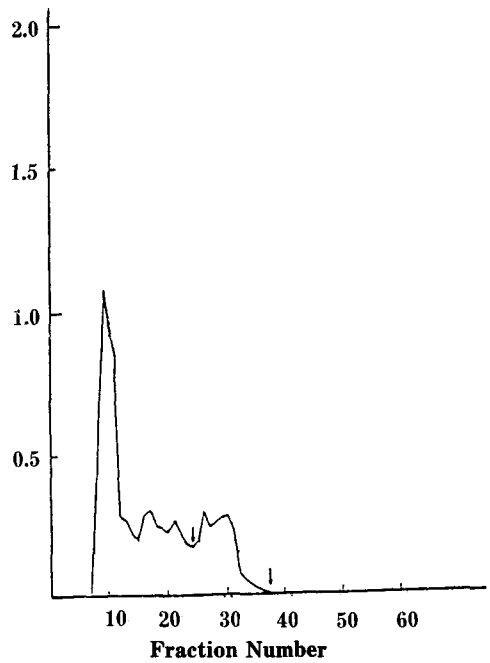
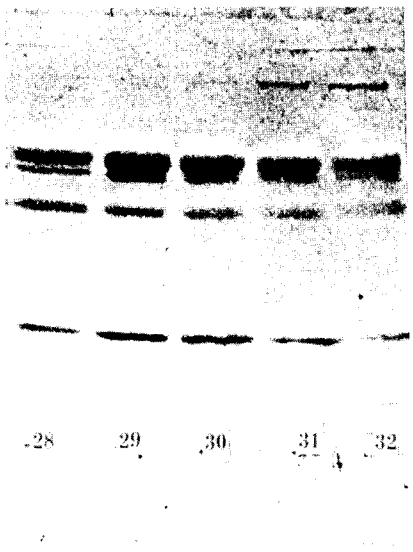


Fig. 4. Protein concentration profile from hydroxylapatite chromatography of ascites fluid.

pH 6.0; flow rate 0.2 ml/cm^2/min; gradient, 5.82×10^{-3} M/cm; sample volume, 7 ml of 0.05 M NaP with 0.7 ml ascites fluid added. Arrows indicate the McAb eluting range.

quite substantially (Figure 4). When pH was 5.5, the difference between elution molarities of McAb and contaminants decreased and purity became poorer than pH 6.8 (Fig. 5), although there was a substantial decrease in band dilution (Fig. 6). Data at pH 6.5 were very similar to those at pH 6.8 (data not shown).



Fraction Number

Fig. 5. SDS-PAGE of the IgM McAb containing fractions from hydroxylapatite chromatography, that is stained with Coomassie Blue.

It could be concluded that the best pH value for the highest purity was 6.0, although it accompanied a little more dilution.

Effect of trace element $CaCl_2$ on purity.

Divalent ions such as Ca^{2+} in the eluent make binding between HA and acidic proteins much stronger, and basic proteins never bind HA at concentration levels down to 1 mM (6). In a preliminary test the IgM McAb in this study was found to be an acidic protein. If there exist some basic proteins among the contaminating components coeluting with IgM McAb, the product purity can be much enhanced by adding $CaCl_2$.

Combining these observations pH of the eluent was set at 6.0 and $CaCl_2$ was also included in the eluent at 1 mM. Taking the case without $CaCl_2$ as control, a little increase in purity and a decrease in dilution could be noted (Fig. 7 and 8).

Thus, using the finally chosen values 6.0, 0.2 m*l*/cm^2/min, and 5.82×10^{-3}M/cm for pH, flow rate, and gradient, respectively, together with1 mM $CaCl_2$ the McAb product of high purity and low dilution could be obtained with a HA chromatography step alone.

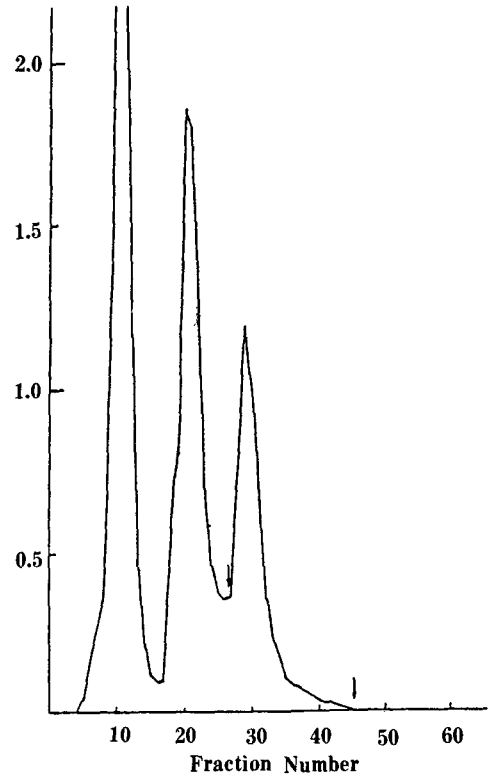
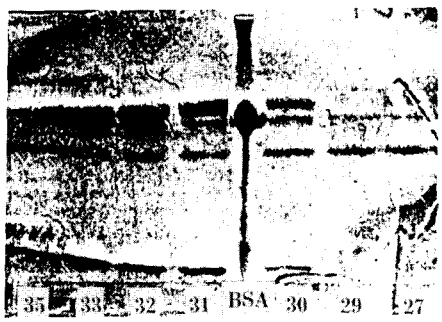


Fig. 6. Protein concentration profile from hydroxylapatite fractionation of ascites fluid.
pH 5.5; flow rate 0.2 m*l*/cm^2/min; gradient, 5.8×10^{-3} M/cm; sample volume, 7 m*l* of 0.05 M NaP with 0.7 m*l* of ascites fluid added. Arrows indicate the McAb eluting range.



Fraction Number

Fig. 7. SDS-PAGE of the IgM McAb containing fractions from hydroxylapatite chromatography, that is stained with Coomassie Blue.

Recently culture of hybridoma in an *in vitro* bioreactor with serum free media or low serum media is practiced on a routine basis and this

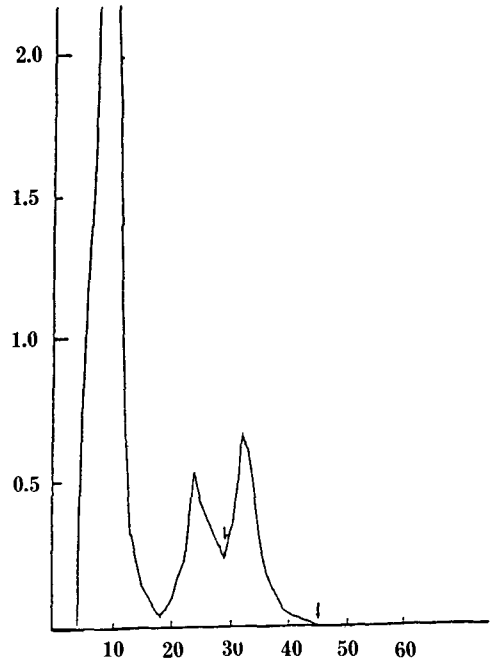


Fig. 8. Protein concentration profile from hydroxylapatite fractions of ascites fluid.
pH 6.0; flow rate 0.2 ml/cm^2/min; gradient 5.8×10^{-3} M/cm. Arrows indicate the McAb eluting range. The elution buffer contains 1 mM $CaCl_2$.

Table 1. Examples of various elution conditions used for the hydroxylapatite column chromatogrphy

Run Number	7	8	9	10	11[a]
Flow Rate (ml/cm^2/min)	0.2	0.2	0.2	0.2	0.2
Gradient (M/cm)$\times 10^3$	5.82	5.82	5.82	5.82	5.82
pH	5.0	6.0	5.5	6.5	6.0

[a]: eluent contains 1 mM $CaCl_2$

makes the single step procedure reported here a more feasible reality, because culture supernatants from bioreactor with serum-free media or low serum media have low levels of contaminating proteins.

HA is autoclavable and applicable to separation of any calss of antibody. It can also be applied to HPLC and easily scaled up.

Because immunoaffinity chromatography needs antigens, that are usually difficult to obtain, and protein coupling to a substrate surface, and protein A chromatography is applicable to only a few classes of antibody, HA chromatography can be claimed to have merits of its own. Almost every separation procedure included GPC at a later stage in order to get a very highly purified McAb product for therapeutic use. HA chromatography may not need desalting and buffer exchange because eluents may not be harmful to man.

The operating conditions of the HA chromatography runs are given in Part I (7) and reproduced here as Table 1.

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