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Development of Chromatographic Downstream Processing for The Purification of Monoclonal Antibody from Ascites Fluid: Part I. Tandem Use of Hydroxylapatite Chromatography and Gel Permeation Chromatography

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복수로부터의 단세포군 항체의 정제를 위한 크로마토그래프 분리 정제 시스템의 개발

— I. 히드록실 아파타이트 크로마토그라프와 겔 여과 크로마토그라프로 이루어진 2단계 연속공정 사용 —

안익성·최차용

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A sequential system composed of hydroxylapatite chromatography and gel permeation chromatography was developed to purify the IgM type monoclonal antibody against the colon cancer cell SC-1 from the ascitic fluid of mice injected with the murine hybridoma CH07E02.

In the hydroxylapatite chromatographic step the band dilution could be reduced by controlling the gradient and flow rate of the eluent, the sodium phospate buffer, the optimum values for these variables being 5.82×10^{-3}M/cm and 0.2 ml/cm^2/min, respectively.

A degree of purity better than 99.99% as judged from silverstaining of the SDS-PAGE bands, was obtained by adding the gel permeation chromatographic step in tandem.

Mouse ascitic fluid generated by the injection of hybridoma cells contains numerous proteins, such as albumin and transferrin, in addition to the intended product, i.e. the monoclonal antibody (McAb) produced by the hybridoma cells. For numerous applications in basic research and medicine the separation of the McAb from the rest of the proteins in the ascitic fluid is highly desirable.

Stanker (1) recently reported on the use of HPLC with hydroxylapatite as the only gel material for the successful purification of McAb directly from the ascites fluids from immunosuppressed mice. The reports that followed (2,3) indicated the possibility of large dilution and low purity with the particular hydroxylapatite HPLC under the column conditions employed.

In this work attempts were made to reduce the band dilution by changing the column operation conditions for the hydroxylapatite chromatographic (HA) step and, to get a purity higher than 99.99% for the ascitic fluid from non-immunosuppressed mice, a gel permeation chromatographic step (GPC) was added in tandem to the HA step.

Key words: downstream processing, monoclonal antibody, two-stage continuous process, ascites fluid, hydroxylapatite chromatography, gel permeation chromatography, murine hybridoma

Materials and Methods

Monoclonal antibodies

The hybridoma cell, CH07E02, producing the IgM type monoclonal antibody against the colon cancer cell, SC-1, was used in this study (5). Ascites fluids were generated by injection of $0.5\text{-}1.0 \times 10^7$ hybridoma cells into pristane-treated BALB/c mice.

Treatment of ascites fluids

Ascites fluids were centrifuged at 20,000 × g for 5 minutes in order to remove fibrin clots and stored at -20 °C prior to chromatographic separation on HA columns.

Hydroxylapatite chromatography

0.7 m*l* of ascites fluid was diluted in 6.3 m*l* of 0.05M sodium phosphate (NaP) buffer (pH 6.8) and the mixture was applied to a K 9/15 column (Pharmacia, Uppsala, Sweden) packed with hydroxylapatite (HTP grade, Bio-Rad Laboratories, Richmond, CA) hydrated in 0.01M NaP buffer to make the final bed volume of 5.5 m*l*. The unbound contaminants were washed out with same buffer. The bound proteins were eluted with 0.05M to 0.3M or 0.5M linear phosphate gradients (pH 6.8) at room temperature. The flow rate was controlled with a peristaltic pump. Column was regenerated by washing with 3 bed volumes of 0.5M sodium phosphate buffer followed by 3 bed volumes of 1N NaCl. The final wash was done with 6 bed volumes of 0.01M sodium phosphate buffer.

Gel permeation chromatography

Following HA chromatography all fractions having McAb activity were pooled (7.3 m*l*) and used as sample for the following step. The sample was applied to a K 26/100 column (Pharmacia) packed with Sephadex G200 (Pharmacia) hydrated in 0.1M sodium phosphate buffer to give a final bed volume of 400 m*l*. The flow rate was controlled at 2 m*l*/cm^2/hr and 5 m*l* fractions were collected. Column was regenerated by washing with 1 bed volume of 0.1M sodium phosphate buffer.

Antibody assay and protein assay

Antibody activity in the column fractions and the ascites fluids was determined using an enzyme-linked immunosorbent assay (ELISA) (5). Protein concentration in the column fractions was assayed according to Lowry method (6).

SDS-PAGE

Column fractions were analyzed by SDS-PAGE on 10% gels according to Laemmli (7). Protein bands were stained with Coomassie blue or silver.

Results and Discussion

Identification of McAb class

The culture supernatant of the CH07E02 hybridoma was added to the first two columns of wells from left on the 96-well plate. The two control fluids, i.e. the McCoy cell culture fluids and the P3U1 culture fluids, were each added to the next two columns in sequence. The top three rows of wells were preadsorbed with anti-IgA, anti-IgG, and anti-IgM in this order.

As shown in Fig. 1 the first two wells from left on the third row exhibits the color reaction, which indicates that the antibody is of IgM class.

Effect of flow rate and phosphate gradient on band dilution in HA chromatography

For the case of linear concentration gradient the gradient can be given by:

$$\text{grad} = \left(\frac{M}{V}\right)\left(\frac{V_o}{V_t}\right)\cdot S \tag{1}$$

where M is the difference in the molarity of phosphate between the initial and final buffers; V, the

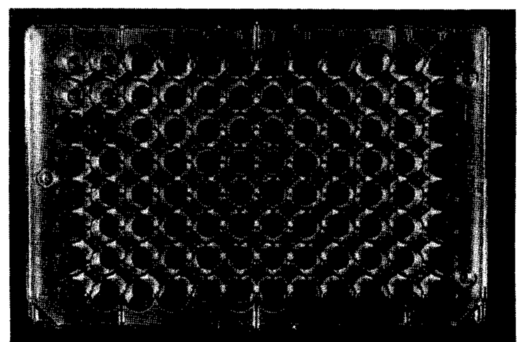


Fig. 1. Test on the Ig class of the McAb produced by the hybridoma, CH07E02, against the colon cancer cell, SC-1.
The top three rows were coated with anti-IgA (row A), anti-IgG (row B), and anti-IgM (row C) respectively.

Table 1. Examples of Various Elution Conditions Used for the Hydroxylapatite Column Chromatography

Run Number	7	8	9	10	11[a]
Flow Rate(ml/cm²/min)	0.2	0.2	0.2	0.2	0.2
Gradient(M/cm) × 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 $CaCl_2$.

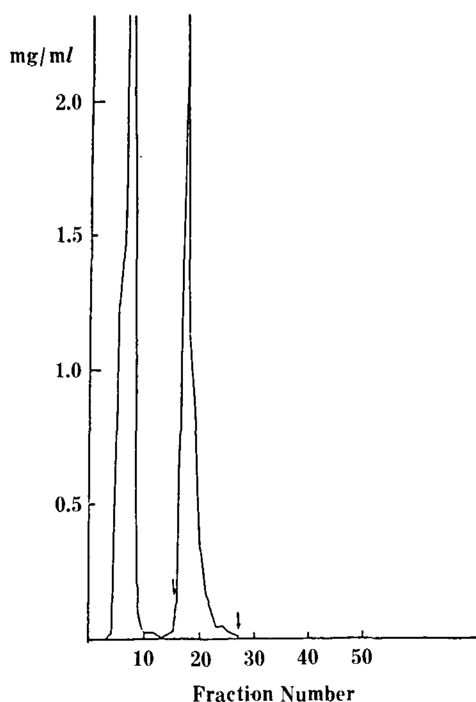
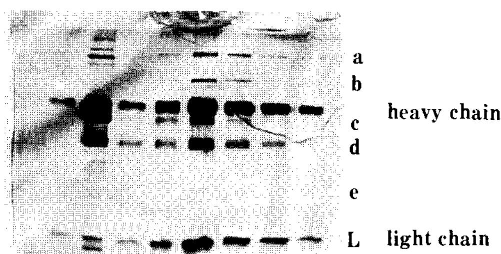


Fig. 2. Protein concentration profile of the fractions eluting from the HA column.
pH = 6.8, flow rate = 0.2 ml/cm²/min, gradient = 5.82 × 10^{-3}M/cm. Arrows indicate the region where McAb activity is detected.



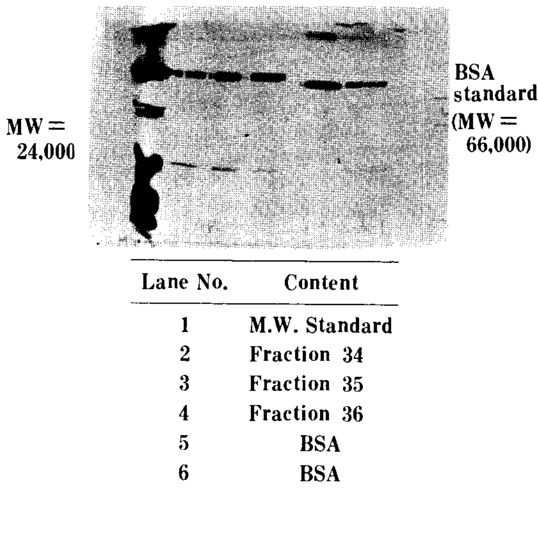
Lane No.	Content
1	GPC Fraction
2	ascitic fluid
3	Fraction 23
4	Fraction 24
5	Fraction 25
6	Fraction 26
7	Fraction 27
8	Fraction 28

Fig. 3. SDS-PAGE of the fractions showing the IgM McAb activity.
The gel was silver-stamed
Lanes are numbered from left.

total volume of the buffer; S, the cross-sectional area of the column; and V_o and V_t, the outer and total volumes of the column, respectively. If S and V are given in cm² and cm³ respectively, gradient represents the increase in phosphate molarity per centimeter of column.

In this study gradient was determined by controlling V and the experimental conditions used in the several example runs which will be further referred to in PART II are summarized in Table 1. The sharpest IgM peak was obtained when flow rate and gradient were 0.2 ml/cm²/min and 5.8 × 10^{-3} M/cm respectively (Fig. 2). Increasing the gradient results in the more steep change in the ionic strength of the buffer across the bound protein band. With decreasing flow rate the response time of protein to unit change of ionic strength of buffer per unit gel bed volume increases. The decrease in the number of fractions containing the McAb activity is thought to be caused by these reasons. It was impossible to get a gradient higher than 5.8 × 10^{-3}M/cm due to the mechanical limit on the experimental apparatuses available. Anyhow the still further increase in the gradient will result in the overlap of the two peaks, and this will give poorer resolution and lower degree of purity.

As the experimental mice used in this study were not immunosuppressed the heavy chain of the host mouse IgG coexists in the sample as shown in the SDS-PAGE (Fig. 3). The band "d" in this figure is the IgG heavy chain made by the host mouse. One can notice 4 more contaminating bands. If the intended McAb from our hybridoma had been of the IgG type the host IgG could not have been detected as a separate band, causing a misjudgement. This kind of host mouse IgG production can be prevented via immunosuppression. Our tandem system was developed to be useful for samples even from nonimmunosuppressed mouse and is asserted to



Lane No.	Content
1	M.W. Standard
2	Fraction 34
3	Fraction 35
4	Fraction 36
5	BSA
6	BSA

Fig. 4. SDS-PAGE of the fractions from the second stage, i.e. the gel permeation chromatography, showing the IgM type McAb activity.
Lanes are numbered from left.

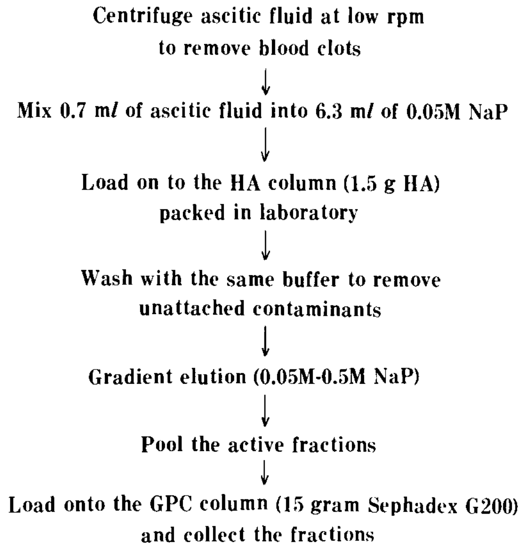


Fig. 5. The schematic diagram showing the overall purification procedure.

have some practical significance. Other than this coexisting host mouse IgG one can consider that HA chromatography alone can give highly purified McAb, as can be judged from this result. There was almost no influence of gradient on the degree of purity.

Therefore, considering the column operation time and dilution, a flow rate of 0.2 $ml/cm^2/min$ and a gradient of $5.8 \times 10^{-3}M/cm$ are chosen as the optimal operating conditions.

Gel Permeation Chromatography

In order to get the very highly purified McAb the gel permeation chromatography was added downstream from the HA chromatographic step.

There is a limit on the amount of sample volume that can be applied onto the gel permeation chromatography(8). The sample dilution at the HA chromatographic step can have an undesirable effect on the sample volume to be applied to the subsequent gel permeation chromatographic (GPC) step. All McAb-containing fractions from HA chromatography were pooled to give a total volume of 7.31 ml and applied to the gel permeation chromatographic step. This sample volume corresponded to 2% of gel bed volume and contained 3.25mg of proteins. At the exit from GPC the fractions were found to have been diluted 8 times on the average during the passage through the coumn, which was accepted as a very satisfactory result. As shown in the electrophoresis (Fig. 4) contaminants are removed completely. Approximately 3 μg of proteins was loaded into each well and, considering the detection limit of silver staining to be 1 ng, the purity is presumed to be at least 99.997%.

The overall purification procedure is depicted in Fig. 5. A more detailed explanation can be found elsewhere(9)

요 약

생쥐 하이브리도마 세포를 주사한 생쥐의 복수로부터 대장암에 대한 단세포군 항체 IgM을 분리 정제해 내기 위하여 히드록실 아파타이트 크로마토그라피와 겔 여과 크로마토그라피를 연속적으로 사용하는 2단계 분리 정제 시스템을 개발하였다.

히드록실 아파타이트 크로마토그라피 단계에서는 단백질 밴드의 회석현상을 탈착제인 인산 나트륨 완충액의 농도구배와 유속을 제어함에 의하여 줄일 수 있었는 바 이들 변수들의 최적치는 $5.82 \times 10^{-3}M/cm$ 및 $0.2\ ml/cm^2/min$으로 각각 나타났다.

겔 여과 크로마토그라피를 제 2단계로 사용함으로써 SDS-PAGE 밴드들의 은착색으로 판단된 바 99.99% 이상의 순도를 갖는 단세포군 항체 단백질을

분리 정제해 낼 수 있었다.

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