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H-Y 항원 유전자의 cloning에 관한 연구
Ⅰ. 친화성 크로마토그래피에 의한 H-Y 항원의 분리 정제 및 H-Y 항원 정량을 위한 화학발광 면역 분석법

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Molecular Cloning of H-Y Antigen Gene
Ⅰ. Purification of H-Y Antigen by Immunoaffinity Chromatography and Chemiluminescence Immunoassay for the Assay of H-Y Antigen

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적 요

본 실험은 H-Y 항원 유전자 크로닝을 위한 기초연구로서 H-Y 항원의 특성을 규명하기 위하여 친화성 크로마토그래피에 의하여 H-Y 항원을 분리·정제하였다. 정소 추출액을 항체가 결합된 column에 결합시킨 뒤 10% acetic acid로 용출시켰다. 용출된 분획을 모아 농축한 후 HPLC와 SDS-PAGE를 실시하여 H-Y 항원의 분자량은 약 67,000달톤 임을 알 수 있었으며 isoelectric focusing에 의하여 등전점(pI)은 5.0인 것으로 측정되었다.

H-Y 항원에 대한 단일클론항체와 표지항원으로는 H-Y항원-ABEI(aminobutylethyl isoluminol)를 사용하여 H-Y 항원 정량을 위한 화학발광면역분석법을 개발하였다.

항원항체 반응후 빛의 측정은 NaOH 존재하에서 microperoxidase/H_2O_2를 이용한 산화반응으로 실시하여 10초간 측정한 빛의 양을 적분하였다. H-Y 항원의 농도와 빛의 양과는 역비례하였으며 감도는 11.8ng/tube 정도이었다.

(Key word : H-Y antigen, immunoaffinity chromatography, antibody probe, isoelectric focusing, solid-phase chemiluminescence immunoassay)

Ⅰ. Introduction

The H-Y antigen, which was originally discovered in strains of inbred mice in which females rejected skin grafts from male (Grasser, 1972), has attracted much interest since this antigen has found to be the product of the testis determining ene on the Y chromosome and an essential inducer of primary sex (Wachtel, 1975 b). To our best knowledges, however, there is no report concerning physical and chemical properties of H-Y antigen in detail. One of the main reasons for this may be due to the difficulty of purification. We have attemped, therefore, to purify H-Y antigen using immunoaffinity column where monoclonal anti-H

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-Y antibody was coupled to Sepharose-4B.

Although numerous papers about H-Y antigen have been appeared, its exact identity has yet to be established. For this, molecular gene cloning of H-Y antigen should be done ultimately. Two approaches for the molecular cloning of H-Y antigen, that is, utilization of antibody and synthetic oligonucleotide prove can be employed. Specially for the latter, the characterization of H-Y antigen and detemination of primary structure is prerequisite. This study presented here may provide some basic data for the fully characterization of it.

Few immunoassay ststems for the detection of H-Y antigen that may be present or absent in various fluids have been developed (Farber, 1982; Farber, 1984; Brunner, 1988). They are mainly ELISA based on indirect method and two-site sandwitch method. None of them applied the principle of competitive reation using labelled antigen which might have some advantages over antigen-coated indirect method (Kemeny, 1988).

We now present here a novel immunoassay method based on solid-phase chemiluminescence immunoassay (CIA) as an alternative method. This new system, which uses H-Y antigen purified by immunoaffinity chromatography, H-Y antigen chemiluminescent tagged conjugate, and a monoclonal H-Y antibody coated-tubes as a separation method.

II. Materials and Method

According to the procedure presented in Fig. 1, all the experiments were carried out. Important procedures are as followed.

1. Preparation of Immunoaffinity Column

Monoclonal anti-H-Y antigen antibody used for this study was IgG_1 purified by Sepharose-Protein A from the ascitic fluid obtained by the injection of 2D45D4 hybridoma cell secreting the monoclonal antibody which was already identified by indirect immunofluorescence test and ELISA, respectively (Shim, 1988). This purified antibody was coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions.

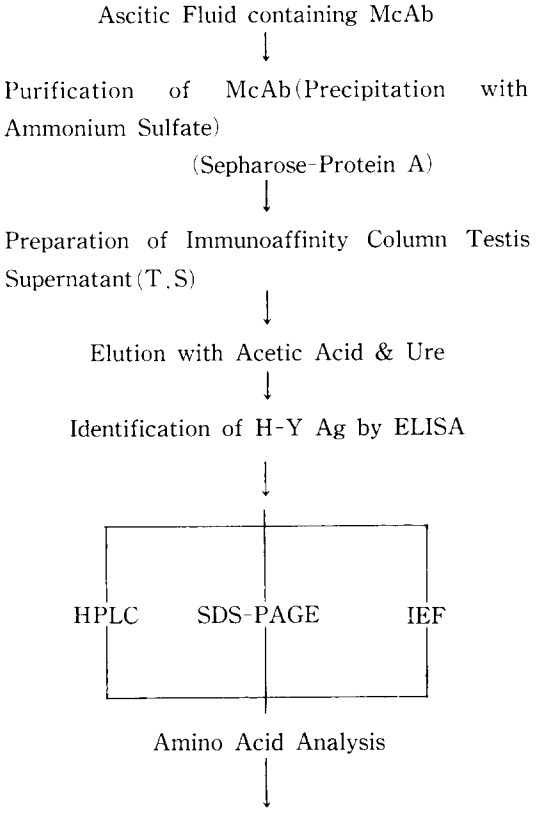

Fig. 1. Experimental procedures for the purification of H-Y antigen and development of solid-phase CIA

2. Immunoaffinity Chromatography

Testis supernatants prepared in Tris-HCl buffer (20mM, pH 7.4) were mixed with the

immunosorbent for 30min. and then were applied to the column(1.0×6.0cm). The column was washed with the Tris-HCl buffer until O. D. at 280nm reached to the zero. Both 4M urea and 10% acetic acid were used for the elution agents.

3. Characterization of Purified H-Y Antigen

Purity and molecular weight of H-Y antigen were determined by HPLC(GTi system LKB) with a column TSK 3000SW(8×300mm, LKB). Amino acid analysis was performed with amino acid analyser(ALPHA, LKB) after filtration on 0.2μm milipore according to the instructions of manual. Isoelectric focusing was also carried out by Phast System(Pharmacia, Sweden) for the determination of isoelectric point(pI).

4. Preparation of Chemiluminescence labeled Antigen and Antibody-coated Tubes

The chemiluminescent compound aminobutyl-ethyl isoluminol-hemisuccinate(ABEI-HS : LKB Wallac Oy, Filand) was conjugated to the purified H-Y antigen according to Barnard et al(Barnard, 1988).

Monoclonal antibody was suitably diluted in the coating buffer(barbital, 70mmol/l, pH 9.6) and 2000μl of this was added to each polystyrene assay tube(PTO 944 ; Luckham Ltd, UK). After an overnight incubation at 4℃, the coating buffer was aspirated and 300μl of pH 7.5 phosphate buffer was added to each tube and stored at 4℃ until required.

5. Solid-phase CIA

Add 100μl of standard(range 3μg to 11.8ng/tube) in duplicate to the assay tubes, then add 100μl of H-Y-ABEI conjugate. After the overnight incubation at 4℃, the liquid was aspirated and the tubes were washed three times with phosphate buffer. Add 200μl of 2 mol/l sodium hydoxide to each tube and incubate at 60℃ for 30min., then add 100μl of the microperoxidase solution after cooling and place it in the luminometer(M1250, LKB). Initiate the chemiluminescence reaction by rapidly injecting 100μl of diluted hydrogen peroxide with an automatic dispenser and measure the light emitted for 10sec.

III. Results and Discussion

1. Purification of H-Y Antigen by Immunoaffinity Chromatography

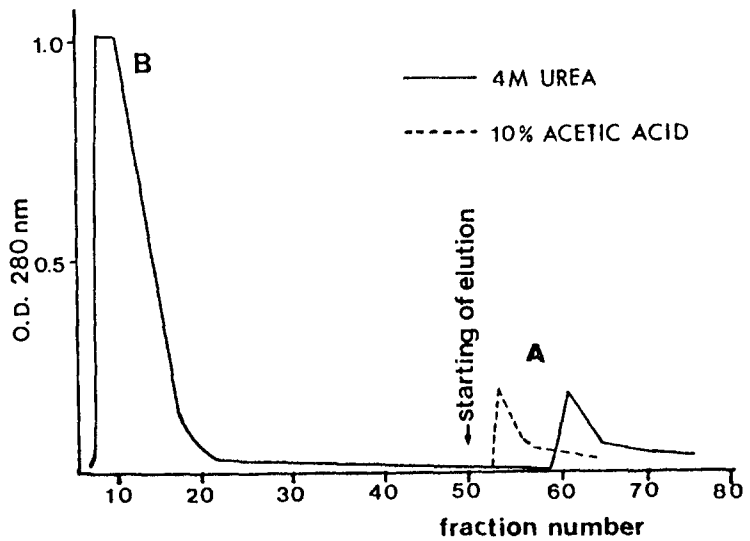

Fig. 2. Immunoaffinity chromatography of H-Y antigen

When the immunoaffinity column after absorption of H-Y antigen was eluted with 4M urea and 10% acetic acid, the fractions which may contain H-Y antigen were obtained(Fig. 2). These fractions were subjected to the further analysis. We got the same results when testis supernatants of bovine and rat were applied to the column(data not shown). This result indicate that the antibody used for this study may cross react with them of bovine and rat.

2. Characterization of Purified H-Y Antigen

The purified H-Y antigen was identified by ELISA procedure using antigen coated-tube and rabbit anti-mouse IgG-HRP conjugate. As seen in Fig. 3, the eluted fractions reacted with monoclonal H-Y antibody.

Molecular weight determined by HPLC(Fig. 4) were found to be approximately 67,000, which agreed with the other results(Bradley,

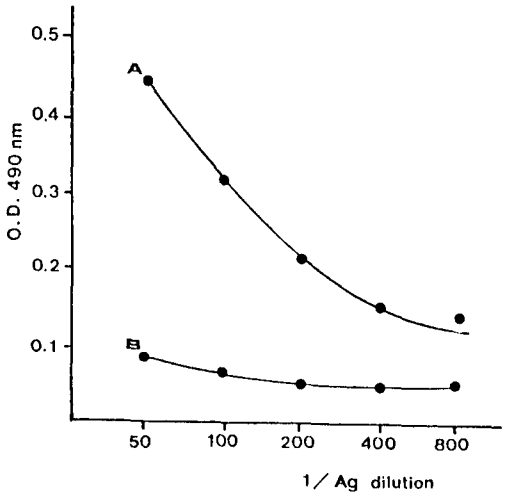

Fig. 3. Detection of H-Y antigen by ELISA using H-Y antigen-coated tubes
A : Eluted fraction, 2.3μg/ml
B : Washed fraction, 14μg/ml

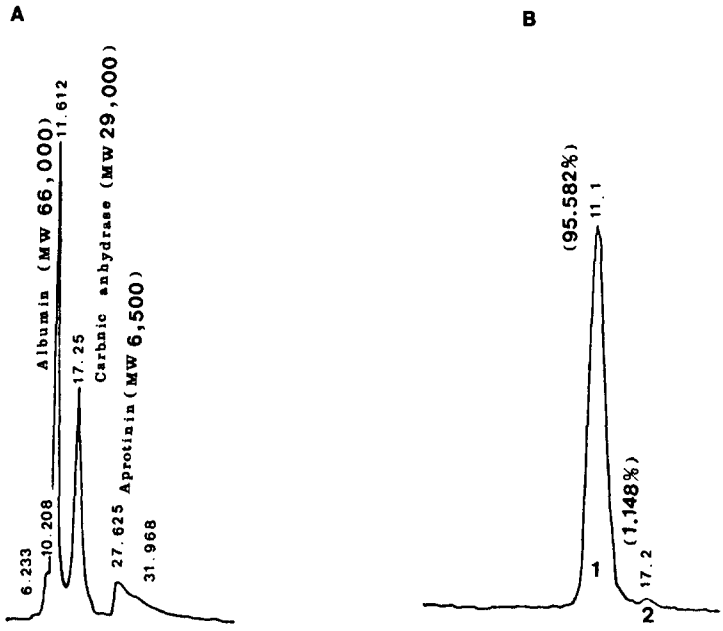

Fig. 4. HPLC elution profiles of several samples
A : Protein standard marker
B : Fraction A of Fig. 2 eluted with 10% acetic acid

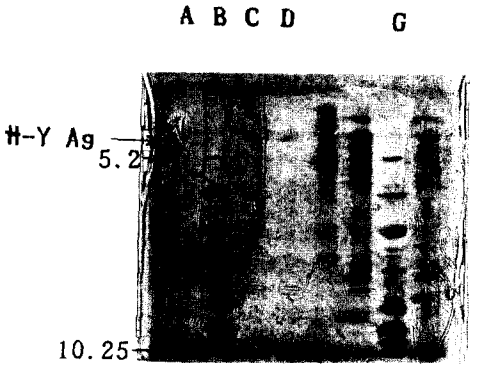

Fig. 5. Isoelectric focusing of H-Y Ag
A, C, D : Purified H-Y Ag
B, G : Standard marker

1987; Hall, 1981). The purity of fraction eluted with 10% acetic acid was about 98.5% (Fig. 4 and Fig. 5). According to the result of isoelectric focusing in Fig. 5, isoelectric point looks about pH 5.0.

The predominant amino acids were aspartic acid(9.8%) and leucine(8.7%) whereas others less than 5% according to the results of amino acid analysis(data not shown).

3. Solid-phase CIA

Antibody dilution curve was made with and without antigen to determine the appropriate dilution of antibody for the standard curve(Fig. 6). Fig. 7 shows a typical standard curve for H-Y antigen as determined solid-phase CIA. The minimum concentration of H-Y antigen that

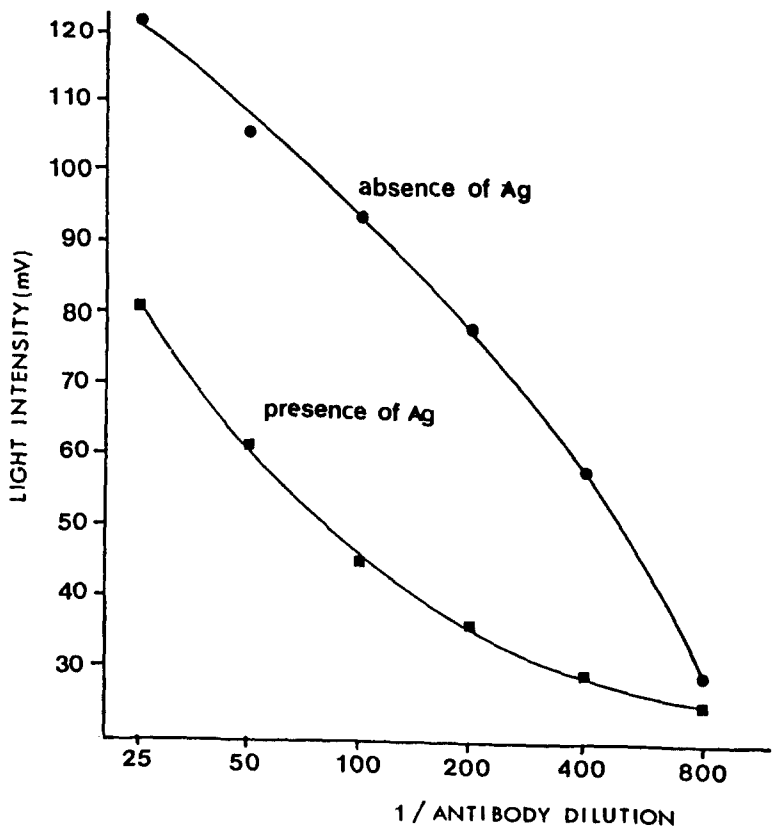

Fig. 6. Antibody dilution curve of monoclonal H-Y antibody on solid-phase CIA

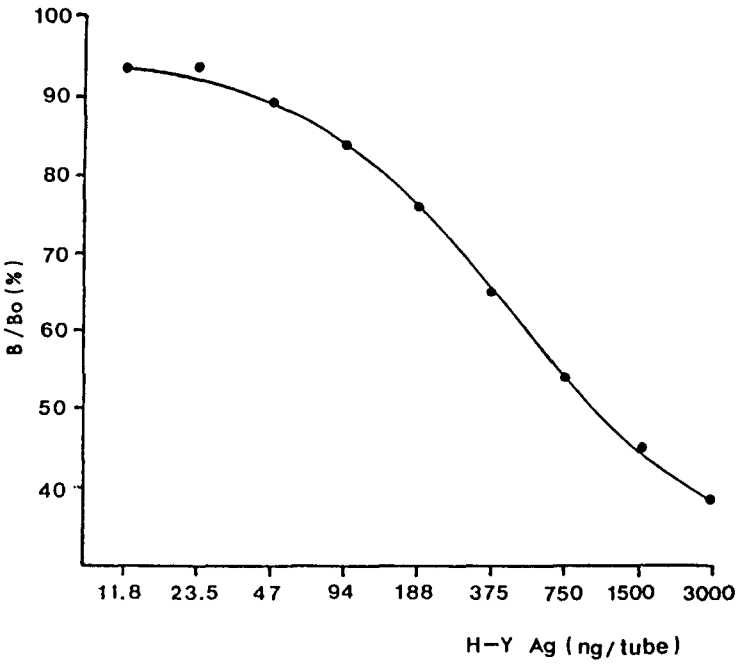

Fig. 7. Standard curve for H-Y antigen on solid-phase CIA

could be significantly distinguished from zero (mean-2SD), calculated from three standard curves, was about 11.8ng/tube. This sensitivity is much better than others(Farber, 1982; Farber, 1984; Brunner, 1988). This new method based on chemiluminescence could be used in clinical practice and basic research. However, more evaluations should be done in order to be accepted as an alternatvie method. These are under investigation.

IV. SUMMARY

In order to purify H-Y antigen, immunoaf finity chromatography using monoclonal H-Y antibody was perfomed. When testis super natant were applied to that column the fraction containing H-Y antigen was eluted by 10% acetic acid. This fraction eluted was subjected to the determination of molecular weight and purity by HPLC and SDS-PAGE, and amino acid analysis. Molecular weight was found to be approximately 67,000 and aspartic acid and leucine residues were the most abundant amino acids.

Solid-phase chemiluminescence immunoassay for the assay of H-Y antigen using purified H -Y antigen were developed. For this, and IgG fraction of monoclonal antibodies to H-Y antigen was passively adsorbed onto the walls of plastic tube. The labelled antigen was H-Y antigen-aminobutylethyl isoluminol. After reac tion, luminescence reaction was initiated in the presence of NaOH by oxidation with micropeF oxidase/H_2O_2 and the signal intergrated for 10 second. The light yield was inversely propor tional to the concentration of H-Y antigen in the standard. This method may be useful in the

direct tests for circulating H-Y antigen in body fluids such as blood, urine, and blastocoelic fluid.

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