CHAPTER II

MATERIALS AND METHODS

Materials

1. Reagents

Diethylaminoethyl-Sephadex A-50 anion exchanger with a binding capacity, 3.5 x 0.5 mEq/g. (Pharmacia, Sweden)

Carboxymethyl-Sephadex C-50 cation exchanger with a binding capacity, 4.5 x 0.5 mEq/g. (Pharmacia, Sweden)

Polyvinylpurrolidone (Arthur H. Thomas Co.)

Sepharose 4B-200 (Sigma Chemical Company)

Cyanogen Bromide (E-Merck, Germany)

Outdated plasma from Blood bank, Ramathibodi Hospital

Hemoglobin A 150 mg/ml was prepared from human red blood cells

Complete Freund's adjuvant (Difco)

Commercial anticeruloplasmin and commercial antihaptoglobin (Behringwerke)

2. Buffers

0.02 M Sodium pyrophosphate acetic acid buffer pH 7.5,

0.25 M Sodium chloride solution,

0.02 M Sodium acetate pH 5.5,

0.08 M Sodium acetate pH 5.5,

0.08 M Sodium acetate containing 0.2 M Sodium chloride pH 5.5,

0.1 M Sodium bicarbonate,

Mancini buffer pH 8.6

P-CN buffer : 0.04 M phosphate, 0.01 M KCN, pH 7.0.

P-CN buffer with 2.5 M guanidine

Phosphate buffer saline (PBS)

3.5 M Urea pH 5,

1.6 M Urea pH 7.0.

3. Animals

Eight adult rabbits weighed about 25-30 g were divided into two equal groups for immunization with ceruloplasmin and haptoglobin.

4. Equipments

Chromatography column

2.5 cm x 27.0 cm

5.0 cm x 41.0 cm

Dialyzing tubing

Disposible syringe 10 ml for packing Hb-Sepharose 4B

Methods

1. General method

Double immunodiffusion was carried out by Ouchterlony method, using 2 g purified agar (Difco) per 100 ml phosphate buffer saline pH 7.2.

Immunoelectrophoresis was carried out by the method of Scheidegger, using 1.5 g purified agar per 100 ml 0.05 M veronal buffer pH 8.6.

Radial immunodiffusion was carried out by the method of

Mancini, Carbonara and Heremans, using 2 gm% Noble agar (Difco) in Mancini buffer pH 8.0.

2. Purification of human serum protein

2.1 Ceruloplasmin

Ceruloplasmin was isolated by the method using two-stage ion exchange chromatography involving the use of DEAE and CM-Sephadex (118).

A 2.5 cm x 27.0 cm column of ion exchanger was prepared by packing a slurry of DEAE-Sephadex A-50 (2-3 gm.) preswollen in 0.02 M sodium pyrophosphate--acetic acid buffer pH 7.5.

The height of column was 18 cm this column was used in the first chromatograph but later, the modified glass tubing (1.7 x 15 cm) was used instead.

The aged plasma (250 ml) was dialyzed against 1 litre of 0.02 M sodium pyrophosphate-acetic acid buffer pH 7.5, overnight, and centrifuged to remove any precipitate. Then it was applied to the DEAE column which had already been equilibrated with the same buffer. The plasma was carefully applied and allowed to flow slowly through the column. The process was made in the dark at $4^{o}C$. A blue band appeared immediately under the gel surface after the plasma had passed through the column. The column was then washed thoroughly (40 drops/min) using about 400 ml of sodium pyrophosphate buffer pH 7.5 and the eluate was collected 5 ml per test tube, pooled

and stored frozen at -4°C. This eluate contained most of the ceruloplasmin removed. It will be later used for absorption of the prepared anticeruloplasmin. The column was then eluted (at 40 drops/min) with 150 ml of the same buffer containing 0.25 M sodium chloride. The eluate was collected at 5 ml per test tube and O.D. at 280 nm were determined. The protein peak was pooled together and dialyzed against 1 litre of 0.02 M sodium acetate buffer pH 5.5, overnight at 4°C. It was centrifuged at 2,000 rpm for 10 mins. to remove any precipitate.

A column of CM-Sephadex (5.0 x 20 cm) equilibrated in 0.02 M sodium acetate buffer pH 5.5 was made.

The ceruloplasmin rich protein obtained from DEAE-Sephadex chromatography was chromatographed through CM-Sephadex column. Then washed with 500 ml of 0.02 M sodium acetate buffer pH 5.5, at a flow rate of 20 drops per minute. The bound ceruloplasmin was then washed with 0.08 M sodium acetate buffer pH 5.5 to remove the contaminants. Finally the ceruloplasmin was eluted with 100 ml of the same buffer (0.08 M sodium acetate pH 5.5) containing 0.2 M sodium chloride at a flow rate of 30 drops per minute. The eluate was collected 5 ml in each test tube and O.D. at 280 nm were measured. The presence of ceruloplasmin and other serum proteins were detected by Ouchterlony technic. Fractions contain pure ceruloplasmin were concentrated. Alpha-globulin from this protein concentrate, as obtained after agar gel electrophoresis, was at first used for the production of anti ceruloplasmin. Later instead

of this agar gel electrophoresis, the purified ceruloplasmin was subjected to a second chromatography in CM-Sephadex as just described. It is aimed at getting a more purified ceruloplasmin, and then used for further immunization.

2.2 Haptoglobins

The purification of Hp was based on the fact that Hp can bind Hb stoichiometrically (59) and Hp was recovered from Hb bound to Sepharose

Preparation of Sepharose hemoglobin conjugate:

Six grams of packed Sepharose 4B-200 were washed and suspended in 100 ml of distilled water, at 10°C. And then powdered cyanogen bromide 200 mg was added to Sepharose-4B suspension and the reaction was allowed to proceed for 6 mins. with continuous strirring. The pH was kept at 10.5 - 11.5 by 5 M sodium hydroxide as needed. The slurry was then rapidly washed over a coarse sinthered-glass filter with 500 ml of cold distilled water, followed by 500 ml of cold 0.1 M sodium bicarbonate. The Sepharose was activated with CNBr and ready to be attached with Hb. Fifty ml of a stroma-free hemolysate (containing 150 mg/ml of Hb) was then added to the activated agarose. The mixture was stirred gently for 2 hours at room temperature and then left at 4°C overnight. The conjugated Sepharose was washed in succession with 2 litre of P-CN buffer, and 400 ml of P-CN buffer containing 2.5 M Urea. Finally, the Sepharose:Hb was washed

exhaustively in the P-CN buffer, using about 1 litre. This conjugate of Sepharose : Hb was stable for several weeks, at $4^{o}C$.

Binding of haptoglobin to the solid matrix.

In order to minimize the non-specific adhearance of other proteins to Sepharose, 5 M sodium chloride 12.5 ml was mixed with 50 ml of pooled serum having the combined phenotype of haptoglobins. The mixture was poured into the conjugate (Sepharose:Hb) and mixed by gentle stirring with a magnetic strirrer for 30 minutes. Prior to elution, the loaded Sepharose was washed with 100 ml of 1.6 M Urea, pH 7.0 to remove serum protein other than haptoglobin. The Sepharose: Hb attached with haptoglobin was then packed into a 10 ml syringe which serve as a small column. It was then washed thoroughly with P-CN buffer until there was no trace of protein coming out. To detect the traces of protein, the liquid was read O.D. at 280 nm until O.D. is lower than 0.01.

The packed Sepharose was then stirred for 30 minutes with 5 ml of eluting fluid, containing 3.5 M Urea pH 5. The eluate which is rich in haptoglobin fraction was collected in test tubes with the aid of syringe. The elution from the same batch of Sepharose as described above were repeated 4 more times. All the process were carried out at room temperature. The eluate was dialyzed in PBS and concentrated with PVP. These fractions were detected for haptoglobin by Ouchterlony technic and immunoelectrophoresis.

3. Preparation of antisera.

Purified proteins at a concentration of about 1 mg/ml (ceruloplasmin and haptoglobin) were emulified with the equal volume of complete Freund's adjuvant. The emulsion was immunized into the rabbits foot-pad (0.1 ml per each foot-pad) and immunization was repeated subcutaneously with 0.2 ml of similar suspension, at one week interval for about 1 month. Test bleedings were made weekly. Antiserum which produce intense band with the intended protein, with minimum of antibodies to other proteins were collected. Traces of antibodies directed against IgG and other serum proteins were removed by absorptions with purified IgG obtained by the method of J.S. Baumstark et al (2), and other proteins.

In case of anticeruloplasmin, antisera to other proteins were removed by eluate obtained from DEAE chromatography which is rich in all serum proteins except ceruloplasmin.

The obtained antisera were tested by immunoelectrophoresis and Ouchterlony technic to make sure that the monospecific antisera was employed.

4. Preparation of Immunodiffusion plate.

The titer of antisera were tested before making immunodiffusion plate, by using 1.5% Noble agar in Mancini buffer mixed with antisera at the suitable dilution such as 1:10, 1:20, or 1:30.

The final dilutions of antiserum in the immunodiffusion

plate were predetermined by using different dilutions of human serum, against different dilutions of antiserum. The highest dilution of both components which produced proper intensity and size of immunodiffusion is selected for the further determination of such protein. They depend to some extent upon the size, spacing, and depth of holes. The final dilution of anticeruloplasmin and antihaptoglobin were 1:7 and 1:8 respectively when undiluted human serum was used as antigen. The immune plate of anticeruloplasmin and antihaptoglobin was then prepared as follows: 2% Noble agar in Mancini buffer was dissolved by heating in a boiling water-bath. In the case of antiseruloplasmin plate, 5.25 ml of the hot agar solution was allowed to cool to 50°C on a water-bath and was thoroughly mixed with 1ml of prepared anticeruloplasmin which is diluted by 0.75 ml of buffer and already warm to 50°C. Antihaptoglobin plate was prepared in the same manner as anticeruloplasmin plate using 5.25 ml of 2% agar solution, 0.87 ml of antihaptoglobin and 0.88 ml of buffer. And then the antisera-agar mixture was poured on the plastic plate which place on a level surface. The solution spread uniformly over the surface of plate and was allowed to solidified completely. Holes of uniform size (1 mm diameter) were made, 24 holes per plate, 3 mm apart from each hole.

5. Serum specimens.

Sera from 200 patients with liver diseases (hepatitis,

cirrhosis of liver and carcinoma of liver), and 100 plasma with hemoglobinopathies were obtained from Ramathibodi Hospital. Those 100 patients having hemoglobinopatnies were all confirmed by electrophoresis of their Hb. This group included Hb H disease, Hb E disease, AE Bart's Disease, β/E thalassemia and β trait.

One hundred normal sera from donors were obtained from Blood Bank, Ramathibodi Hospital and used as standard containing "100%" ceruloplasmin and haptoglobin. One ml amounts of serum from each donor were pooled, dispensed in the bottles, and stored at -20°C.

Preparation of Standard Serum for ceruloplasmin and haptoglobin determination.

Equal volumes of one hundred specimens of normal human serum from donors were pooled and mixed together. This pooled serum was used as a 100% standard. Another standard was obtained by diluting the pool with Mancini buffer to 1:2, 1:4, and 1:8 respectively. Then the standard values were 100%, 50%, 25% and 12.5% and these were used as standards for both anticeruloplasmin and antihaptoglobin determination.

6. Determination of Ceruloplasmin and Haptoglobin in patients' sera.

Seven mcl. of undiluted sera (standards and all the patients' sera) were filled into the holes of immuno-plate. The plates were

left in a moist chamber at room temperature, overnight. The diameter of immunoprecipitin rings were measured with a measuring microscope. All the sera which gave a diameter of immunoprecipitin larger than 10 mm. were diluted to 1 2 dilution and repeated the determination again.

The concentration of respective protein in unknown specimens were calculated according to method of Mancini which is based on the fact that log area of diffusion correlate with the concentration of the protein.