CHAPTER II

MATERIALS AND METHODS

MATERIALS

1 Biological Materials

Fresh normal human sera were separated from red blood cells by centrifugation at 3,000 x g for 10 min.

Patient sera were collected at Institute of Occupational and Environment Medicine, Rajvitee Hospital, from Pb-intoxicated patients whose blood lead level was higher than 30 μg/l. The sera were preserved in ice-box and transferred to the laboratory immediately. These sera were stored at -70°C until used, normally not more than 2 months.

Human ceruloplasmin, laboratory grade (when highly purified ceruloplasmin needed, the commercial Cp was further purified with DEAE column chromatography) and other standard proteins : bovine serum albumin and human albumin were purchased from Sigma chemical company.

2 Chemicals

2.1 Chromatography

Sephadex G-25, Sephadex G-200 and blue dextran were purchased from Pharmacia Fine Chemicals. Potassium dichromate and sodium acetate were from BDH. Glacial acetic acid was from Merck and Lead acetate, analytical grade, was from Mallinckrodt Chemical Company.

2.2 Graphite furnace atomic absorption spectrophotometer (F-AAS)

Standard Pb and Cu, of atomic absorption grade, were purchased from Normex farmitaria carlo erba.

2.3 Ceruloplasmin determination

Glacial acetic acid was purchased from Merck. Sodium acetate and sodium azide were from BDH. p-phenylenediamine • 2 HCl was from Sigma.

2.4 Discontinuous Polyacrylamide Gel Electrophoresis (Disc-PAGE) and Isoelectric Focusing Poly - acrylamide Gel Electrophoresis (IEF-PAGE)

Acrylamide for Disc-PAGE was purchased from Merck. Acrylamide (Ultragrade) was from LKB. N,N'- methylene-bis-acrylamide, ampholyte pH 4-6, ammonium persulfate , N,N,N',N'- Tetraethylmethylenediamine and bromphenol blue

were from BDH. Coomassie brilliant blue R-250 was from Sigma. Glacial acetic acid, methanol and glycerol were from Merck.

2.5 Protein determination

Sodium carbonate and copper sulfate were purchased from Merck. Sodium hydroxide was from Eka Nobel Ltd. Sodium tartrate was from BDH.

2.6 Other chemicals

Tris - (hydroxymethyl)-aminomethane was purchased from Fluka. Sodium hydroxide and hydrochloric acid were from Merck. Glycine and DEAE-cellulose were from Sigma.

3 Instruments

- Fraction Collector,Pharmacia LKB Redi Frac,Pharmacia, Sweden
- Incubator,Memmert
- LYPH-Lock Freeze Dry System, model 77400, Labconnco
- Midget Electrophoresis Units, model LKB 2050, LKB producter AB, Sweden
- Power Supply , model 1000/500 , Biorad Laboratories , U.S.A.
- Spectronic 20D, Milton Roy Company
- UV-Visible recording Spectrophotometer UV-240 , Shimadzu, Eraphicord
- Hot plate, Framo - Geratetechnik, M 21/1
- Mini IEF cell, model 111, Biorad Laboratories, U.S.A.
- Muffle furnace , Thermolyne, Furnatrol II, Sybron corporation
- Graphite furnace atomic absorption spectrophotometer, SpectrAA-300, Varian

METHODS

1 Gel filtration chromatography

1.1 Sephadex G-25 column

The column (1 x 20 cm) was used for the separation of free metal from serum proteins. It was equilibrated and operated with 0.01 M acetate buffer pH 6.0 at a flow rate of 20 ml/hr. Sample size and fraction volumn were 1 ml each.

1.2 Sephadex G-200 column

The column (1.8 x 120 cm) was equilibrated and operated with 0.01 M acetate buffer pH 6.0 at a flow rate of 20 ml/hr. Sample size and fraction volumn were 2 ml each.

2 Graphite furnace atomic absorption spectrophotometer (F-AAS)

F-AAS was used for the determination of Cu and Pb concentrations. All Glasswares and storage containers were soaked overnight with 1 M HNO_3 and rinsed with deionized water before used.

2.1 Sample preparation

Sample containing high protein but low metal concentration, such as serum, was completely hydrolyzed before determining metal concentration by F-AAS. The

following hydrolyzing process was as described by Ohchi (Ohchi *et al*, 1985). One milliliter of serum was dried and carbonized in crucible on a hot plate at 150°C for 3-4 hrs. and subsequently ashed at 500°C for 24 hrs. in a muffle furnace. At the middle of the ashing process, the crucible was allowed to cool down, and 10μl of conc.HNO_3 were added. When the process was completed, the ash was dissolved in 1 ml of 0.02 M HNO_3 , and 16 μl of the solution was thereafter injected to F-AAS for metal concentration determination.

Sample containing low protein concentration (<2 mg/ml), such as fractions from column chromatography, could be injected to the F-AAS directly without prior subjected to the ashing process.

2.2 Spectrophotometer setting

A spectrophotometer was operated at 283.3 nm and 324.8 nm for Pb and Cu determination, respectively, in the peak height mode with a 0.5 nm slit width and internal standards were included. The sample size for injection was 16 μl, 0.1 M H_3PO_4 and palladium were used as emulsifiers for Pb and Cu determination, respectively. The graphite - furnace heating variables and gas flow rate were shown in the following table.

STEP No.	TEMPERATURE (°C)	TIME (sec)	GAS FLOW (l/min)
1	85	5.0	3.0
2	120	30.0	3.0
3	500	5.0	3.0
4	500	10.0	3.0
5	500	2.0	0.0
6	2100	0.8	0.0
7	2100	2.0	0.0
8	2600	5.0	3.0

3 Ceruloplasmin Determination (Wolf,P. *et al*,1973)

Ceruloplasmin concentration was determined for its oxidase activity, rate of oxidation of p-phenylenediamine at 37°C, at pH 6.0. The rate of appearance of the purple oxidation product (Wurster's red), which has an absorption peak at 520-530 nm was measured spectrophotometrically.

3.1 Reagents

a) 0.1 M acetate buffer, pH 6.0

Add 10 ml of 0.1 M acetic acid (0.57 ml glacial acid plus water to 100 ml) to 200 ml 0.1 M sodium acetate (1.36 g

$CH_3COONa \cdot 3H_2O$ in 100 ml water), the pH obtained was 6.00

b) 0.1% sodium azide, in 0.1 M acetate buffer, pH 6.00

c) 0.25% p-phenylenediamine·2HCl, in 0.1 M acetate buffer, pH 6.00

12.5 mg p-phenylenediamine·2HCl was dissolved in 3.0 ml acetate buffer pH 6.00, the pH was adjusted to 6.00 by adding 1.0 N NaoH. The final volume was adjusted to 5.0 ml. This reagent was prepared immediately before used or less than 2 hours if kept in the dark.

3.2 Sample Preparation and Calculation

The followings were set up in cuvet with 1.0 cm light path. For the blank, 1.0 ml azide was incubated with 1.0 ml of buffer and 1.0 ml of PPD reagent and for the test, 2.0 ml of buffer was incubated with 1.0 ml of PPD reagent. After that, the cuvet was place in compartment and allowed for 5 minutes, then 0.1 ml of serum was added to each tube. Finally, the absorbance of the test was read against the blank a 530 nm at exactly 10 minutes and again at 40 minutes.

For calculation :

Ceruloplasmin Units = (A at 40 min - A at 10 min) x 10^3

4 Discontinuous polyacrylamide gel electrophoresis (Disc-PAGE)

4.1 Compositions and Stock Solutions

a) 30% Acrylamide, 0.9% Bis-acrylamide

29.1 g of acrylamide and 0.9 g of bis-acrylamide was dissolved in water, the volume was adjusted to 100 ml, filtered and stored in the dark bottle at 4°C.

b) 0.05 M Tris-glycine Electrode Buffer,pH 8.3

6.0 g of Tris and 28.8 g of glycine were dissolved in 800 ml of distilled water, pH was adjusted to 8.2 with 1 N HCl and the volume was made up to 1 l with distilled water.

c) 3.0 M Tris-HCl, pH 8.9

36.6 g of Tris, 0.3 ml of TEMED, and 15 ml of 6 N HCl were mixed, pH was adjusted to 8.9 with 1 N HCl. The final volume was made up to 100 ml with distilled water.

d) 0.5 M Tris-HCl, pH 6.7

5.98 g of Tris and 0.46 ml of TEMED were dissolved in 90 ml of distilled water, pH was adjusted to 6.7 with 1 N HCl. The volume was made up to 100 ml with distilled water.

e) 10% Ammonium Persulfate Solution

0.1 g of ammonium persulfate was dissolved in 1 ml of distilled water, prepared immediately before used.

f) Sample Buffer

20 ml of glycerol, 10 ml of stock solution (d) and 20 ml of distilled water were mixed well with 1 ml of 0.1% of bromophenol blue.

g) Protein Staining Solution

0.5 g of coomassie blue R-250 was dissolved in 100 ml of methanol, stirred for one hour and filtered, and then the solution was mixed with 100 ml of 20% acetic acid.

h) Protein Destaining Solution

First Destaining Solution :

The solution contained 10% acetic acid and 50% methanol.

Second Destaining Solution :

The solution contained 7% acetic acid and 5% methanol.

4.2 Preparation of Disc-PAGE

The separating gel (10 x 6 x 0.075 cm) containing 7.5% acrylamide, and stacking gel (10 x 2 x 0.075 cm) were

polymerized between two glass plates (10 x 8 cm) of Midget Electrophoresis Unit. The gels were prepared from stock solutions, as follow.

Solutions	Separating gel 7.5% T (ml)	Stacking gel 3% T (ml)
30% Acrylamide solution	5.00	1.00
3.0 M Tris-HCl stock solution,pH 8.9	2.50	-
0.5 M Tris-HCl stock solution,pH 6.7	-	1.25
Distilled water	12.50	7.75
10% Ammonium persulfate (μl)	150.00	150.00
Total volume (ml)	20.00	10.00

4.3 Sample Preparation

Protein sample was suspended in sample buffer to give approximate 2.5 to 5 mg/ml concentrations, about 4μl for electrophoresis application. In case of very diluted protein solution,eg.,fractions collected from Sephadex G-200

column, 100μl of each fraction was lyophilized with freeze dry system and redissolved in 10μl of 0.01 M acetate buffer pH 6.0. Two μl of this solution was mixed with 2μl of sample buffer and finally applied to polyacrylamide gel.

4.4 Electrophoresis Run

The eletrophoresis was carried out vertically at 4°C in midget electrophoresis unit with a constant current (20 mA per gel) until the tracking dye came down to 0.5 cm from the bottom of the gel (usually 30-45 min). The power supply was then turned off, the gel was removed from glass plates and stained immediately.

4.5 Protein Staining and Destaining

After the electrophoresis operation, the gel was stained for proteins in the staining solution (solution g) for overnight and then destained with first destaining solution for 1 hr and finally soaked in second destaining solution.

5 Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE)

5.1 Stock solutions

a) Monomer concentration

24.25% (w/v) acrylamide

0.75% (w/v) bis (N,N'-methylene-bis-acrylamide)

24.25 g acrylamide and 0.75 g bis were dissolved in water, to give a final volume of 100 ml, the solution was filtered through a quanlitative filter paper No.4, Whatman. This solution was stored up to 1 month, if protected from light at 4°C.

b) 10% (w/v) Ammonium persulfate

100 mg ammonium persulfate was dissolved in 1 ml water and prepared immediately before used.

c) 25% Glycerol (w/v)

12.5 ml of glycerol (87 %, w/v) and 37.5 ml of distilled water were mixed well together.

d) TEMED (N,N,N',N'-tetraethymethylenediamine)

Pure distilled TEMED was from the bottle. Store cool, dry, and protected from light.

e) Protein staining solution

The solution contained 27% ethanol, 10% acetic acid, 0.04% coomassie blue R-250 and 0.5% $CuSO_4$, was prepared by dissolving $CuSO_4$ in water before adding the alcohol.

f) Protein destaining solution

First destaining solution :

The solution containing 12% ethanol, 7% acetic acid and 0.5% $CuSO_4$, was prepared by dissolving $CuSO_4$ in water before adding the alcohol.

Second destaining solution :

The solution contained 25% ethanol and 7% acetic acid.

5.2 Preparation of IEF-PAGE

A gel mixture containing 5% acrylamide, 0.15% bisacrylamide, 5% glycerol, 2% ampholyte (pH range 4-6), 0.072% (v/v) TEMED and 0.03% ammonium persulfate was polymerized in the space between a glass plate which attached to gel support film and casting tray. The gel solution was prepared as described in the following table.

Solution	Volume (ml)
Distilled water	2.75
Monomer concentrate	1.00
25% (w/v) glycerol	1.00
Ampholyte 4-6 (40%)	0.25
10% (w/v) Ammonium persulfate (μl)	15
TEMED (μl)	3
Total volume	5.00

Approximately, 2μl of sample containing 5 mg/ml of protein concentration was applied to the gel.

5.3 IEF Operation

Electrofocusing was carried out horizontally under constant voltage in a stepwise increase of 100 v for 15 min, 200 v for 15 min and 450 v for 1 hr.

5.4 Staining and Destaining

After electrofocusing, the gel which attached to gel support film was soaked in the staining solution overnight. Then the gel was immersed in two or three 200 ml changes of first destaining solution until the background

was nearly clear. After that it was transferred to the second destaining solution.

6 Determination of Proteins

6.1 Absorbance at 280 nm (A280)

Absorbance was commonly used for generating a protein elution profile after column chromatography, using 0.01 M acetate buffer pH 6.0 for blank.

6.2 Lowry Assay

Protein concentration was determined according to Lowry method (Lowry *et al.*, 1951) with bovine serum albumin as standard. A standard curve was prepared from BSA solution containing 10 to 100 μg protein in a final volume of 0.1 ml. Three milliliters of the solution containing 1:1:100 of 0.5% $CuSo_4 \cdot 5H_2O$: 1% sodium potassium tartrate : 2% Na_2CO_3 anhydrous in 0.1 N NaOH was added to 0.1 ml protein sample, mixed well and left for 10 min. Phenol reagent was then added and mixed. After 30 min the absorbance at 750 nm was measured comparing with standard curve of BSA.

7 Pb-binding assay

One milliliter of sample (serum or 0.6 mg/ml of partial purified ceruloplasmin) was incubated with or without lead acetate (the concentration varied in each

experiment) in 0.01 M acetate buffer pH 6.0, at 37°C for 30 min. The solution was loaded on Sephadex G-25 column (1x20 cm) equilibrated and eluted with about 14 ml of the same buffer while maintaining the flow rate at 20 ml/hr. The protein fractions were separated from free metal and determined for metal concentration by F-AAS.

8 Purification of ceruloplasmin

DEAE-cellulose Column

DEAE-cellulose (50 g) was swollen in water (100 ml) and transferred to 0.5 N HCl. The resin was washed several times with distilled water until pH = 7.0 before suspended in 0.5 N NaOH. The resin was rewashed with distilled water until pH was about 7 and finally with 0.2 M acetate buffer pH 5.0 until pH was about 5.0. The resin was packed into a column of 2.0 x 15 cm and equilibrated with 0.01 M acetate buffer, pH 5.0 at the flow rate of 20 ml/hr.

The Cp was loaded onto the column and washed with 0.01 M acetate buffer pH 5.0 until A_{280} was negligible. Cp was thereafter removed with 0.5 M NaCl in 0.01 M acetate buffer, pH 5.0. Fraction of 3 ml were collected.