CHAPTER 4

MATERIALS AND METHODS

Study groups

1 Five sera from systemic candidiasis patients whose hemocultures were positive for *C. albicans* and *Candida* spp. from Mycology laboratory of King Chulalongkorn Memorial Hospital was studied. Their underfying disease was not HIV – infection but cancer.

2 Fifteen sera from HIV – infected with oral candidiasis patients (HOC) obtained from Mycology laboratory of King Chulalongkorn Memorial Hospital and from HIV – NAT project under Professor Praphan Phanuphak., M.D., Ph.D. The inclusion criteria for HOC were 1) anti – HIV antibody positive 2) $CD4^+$ T – lymphocyte counts ≤ 200 cells/ mm^3 and 3) white patch lesion with isolated *Candida* in the oral cavity. The lesion was confirmed as oral candidiasis by direct examination and isolation the yeast. The oral swab was collected and confirmed for blastoconidia and/or pseudohyphae by KOH – preparation. The specimen was cultured and identified.

3 Thirty sera from healthy individuals (from students of Medical Faculty and staffs in King Chulalongkorn Memorial Hospital) from sera collection in Mycology laboratory of King Chulalongkorn Memorial Hospital) were also carried out as control groups for IgG antibody determination.

4 Nine sera of other fungal infection patients from Mycology laboratory of King Chulalongkorn Memorial Hospital were studied. There were one case of pythiosis, one case of chromoblastomycosis, two cases of cryptococcosis, two cases of penicillosis and three cases of aspergillosis.

Specimen collection

Five millilitres of whole blood were collected by venepuncture. Then, each srum was separated by centrifugation and kept for enzyme - linked immunosorbent assay (ELISA) and Western blotting experiment. All sera were aliquots and stored at -20° C until used.

Candida antigens:

Candida albicans A9 (serotype B) were used for preparation of mannoprotein antigen. The antigen was kindly provided by Dr. Toshio Kanbe, Laboratory of Medical Mycology, Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya, Japan. In brief, the stationary phase cells of the A9 strain were washed in deionized water and then in 0.1 M disodium ethylenediaminetetraacetic acid (Na_2EDTA), pH 7.5. The pellet was suspended and extracted in 0.3 M, 2 – mercaptoethanol in 0.1 M Na_3EDTA, pH 9.0, for 30 minutes at 21 – 23°C with occasional mixing. The extract was dialysed against deionized water at 5 – 8°C until 2 – ME odor disappeared. The deionized water was then changed one more time and the process of dialysis was proceeded for another 4 - 6 hrs . The dialysate was lyophilized and referred as the 2 – ME extract. The 2 – ME extract was further fractionated by affinity chromatography on a concanavalin A (con A) – agarose column. Two hundred milligrams of 2 – ME was dissolved in 3 ml of phosphate buffer, pH 7.2, containing 0.15 N Sodium Chloride (NaCl) (PBS) and applied to a con A – agarose (Honen Co., Tokyo, Japan) column (20 by 120 mm). The column was then washed with 200 ml PBS (20 ml/h) until the A_{260} and A_{280} of the eluted fractions (5 ml/fraction) were negligible. The total PBS was pooled and referred as **Mannoprotein antigen** (or phosphomannoprotein antigen). It was dialysed against deionized water at 5 – 8°C, lyophilyzed and kept at 10°C until used (143).

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Enzyme – linked immunosorbent assay (ELISA)

The indirect ELISA with peroxidase system was performed (144). One hundred microliters (μl) of the optimal concentration of mannoprotein antigen in coating buffer were coated on each microtiter well plate (Nunc-immuno module, Nalge Nunc International, Denmark) and incubated at 37°C for 2 hrs. The plate was washed with five times of PBS plus tween 20 then incubated with 150 μl of 2% bovine serum albumin (BSA, Bovine serum albumin, Sigma, U.S., lot. 129H0913) in PBS plus tween 20 at 37°C for 1 h. After blocking reaction, the antigen was again washed with five times of 1x PBS plus tween 20. One hundred microliters of two fold dilution (1:100, 1:200, 1:400, 1:800, ..., 1:102,400) serum in 0.5 % BSA in 1x PBS plus tween 20 were added and incubated at 37°C for 1 h. Five times of 1x PBS plus tween 20 washing were proceeded and 100 μl (dilution 1:2,000) of peroxidase conjugate rabbit immunoglobulin of human IgG (γ-chain) (Dako, Denmark, Code no. P 0214) was added and incubated 37°C for 1 h. After five times washing, 100 μl of *o*-diphenylenediamine substrate (Dako, Denmark) in citric acid with 30% H_2O_2 was added and incubated at room temperature in dark for 10 minutes. Finally, the reaction was stopped with 100 μl of 4 N Sulfuric acid. The absorbance at 492 nm wavelenght was measured by Microelisa system reader model 311.CO (Organon Teknika, Belgium). Positive control using serum from systemic candidiasis patients were performed parallely in each experiment to evaluate the accuracy of the test (Figure 7).

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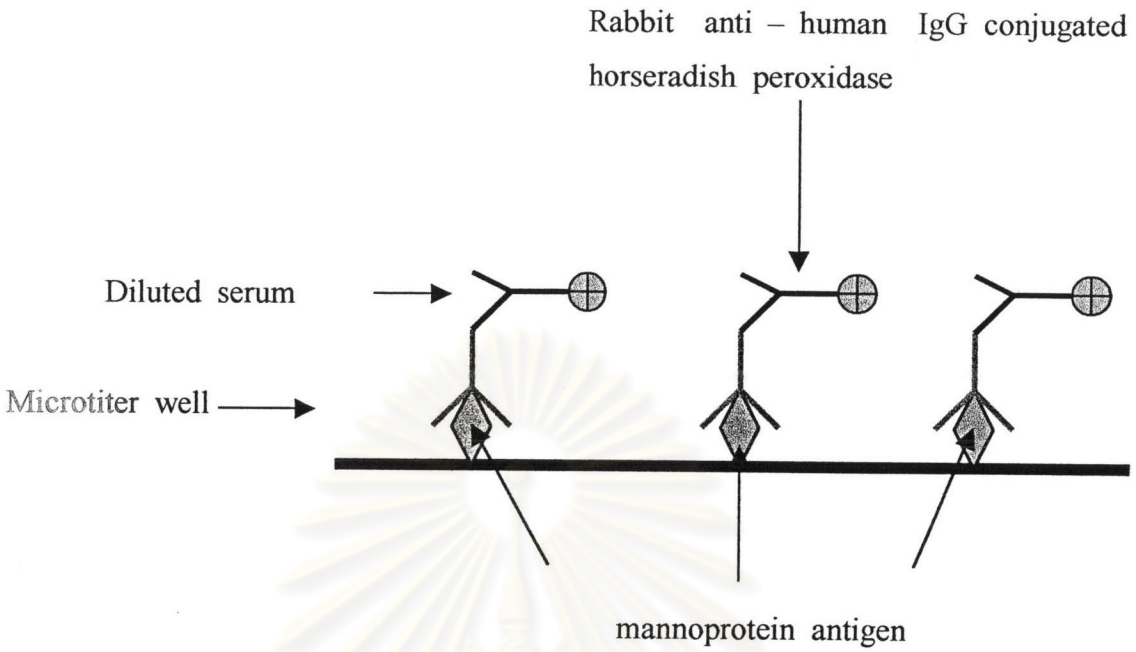

Figure 7. Principle of indirect ELISA

Procedure of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE)

SDS – PAGE was used to separate the protein molecule from mannoprotein antigen and electrophoretic transferring of proteins from the gel to immobilon membrane was carried out using a modification of the method described by Laemmi (145).

The 14% separating acrylamide gel with gentle mixing in order not to incorporate air was prepared. Immediately, the gel was pipetted into the prepared glass plate sandwich to a height of 6 cm., and was carefully overlayed with isobutanol after pouring to give a smooth interface after polymerization. Then, the gel was solidified for 45 minutes at room temperature. After gel polymerization, the overlay isobutanol was aspirated and the gel surface was washed with distilled water until clear of isobutanol.

The 4% stacking acrylamide gel was prepared. The comb was placed between the glass plate – sandwich and the gel solution was added immediately to the top of the glass plate for 20 mintues at room temperature. After polymerization, the comb was removed by gently pulling in a vertical direction. Then, the wells were washed with electrophoretic buffer and any small fragments of polyacrylamide gel and unpolymerized monomers were removed. Thereafter, the glass plate – sandwich was placed in the electrophoretic tank (Mini – Protean II cell, Bio – Rad, U.S.). The electrophoretic buffer was then added to the tank. The one hundred and twenty microliters of mannoprotein antigen (protein concentration = 14.28 μg/ml) and eight microliters of marker (Prestained SDS – PAGE Standards, Broad Range. Bio – Rad, CA. Cat.no. 161 – 0318.) were loaded and the gel was electrophoresed with a constant volt of 100 volts (Power supply model 200/2.0, Bio – Rad, U.S.) in stacking gel and resolving gel until the marker dye appeared nearly the end of the gel. When electrophoresis was completed, the gel was cut into two parts. One part was stained with 0.25% Coommassie brilliant blue R and destained with destaining solution. The other piece was soaked in blotting buffer for further Western blot experiment.

Procedure for staining of the gel (Coomassie brilliant blue R)

Coomassie brilliant blue R: After electrophoresis or electrophoretic transferring, the gel was fixed in fixative solution for 40 minutes and then stained 20 minutes in 0.25% Coomassie brilliant blue R (Coomassie Brilliant Blue R – 250., Bio – Rad, CA., cat.no. 161 – 0400). To observe the band, the gel was destained until the destaining solution was clear. The destaining reaction was stoped by adding distilled water.

Blotting

Following electrophoresis, the gel was equilibrated in the blotting buffer. The immobilon membrane (Millipore) was wet for thirty seconds in absolute methanol (Methanol, analytical grade, ACS. Scharlau, Spain.Me 0316.). Later, the membrane was soaked in the blotting buffer less than 5 minutes. This step was important to insure proper binding of the protein to the membrane. Two pieces of filter pad were soaked in blotting buffer. After equilibration, one of the soaked filter pad was placed on the stand of the blotting machine (Trans – Blot SD Semi – Dry, Bio – Rad, U.S., cat.no. 170 – 3940) and a piece of saturated immobilon membrane was placed on the top of the pad (Figure 8A). The equilibrated gel was gently placed on the top to the membrane and finally, another equilibrated filter pad was gently placed on the top of the gel (Figure 8B). This process was to expel any air bubbles. After completing the sandwich, the semidry tank was covered with its lid (Figure 8C). Electrophoretic Transfer Cell was performed at 10 mA (Power Pac200, Bio – Rad, U.S.) for 45 minutes. After transfering, the membrane was removed and air dried less than 3 hours at room temperature or 1 h. at 37 °C. The immobilon membrane was kept at 4°C in a plastic bag until used.

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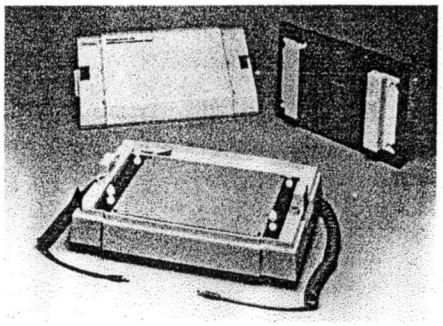

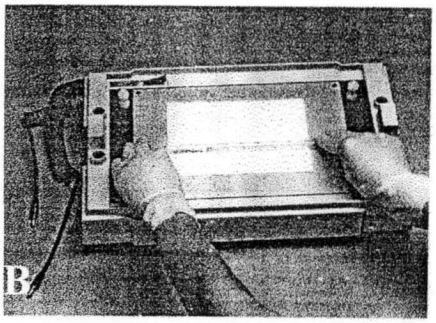

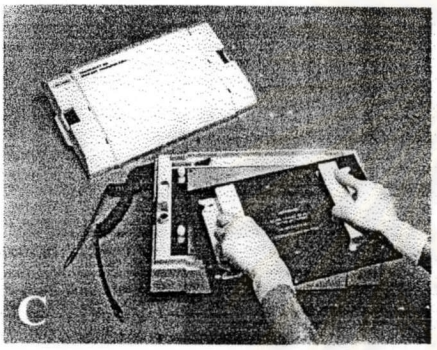

Figure 8. To transfer protein from gel to immobilon membrane by using Electrophoretic Transfer Cell (Bio – Rad, US.)
A : the semi – dry blot machine.
B : expelling air – bubble.
C : closing the system.

Procedure of Western blotting

Prior to use, the membrane was cut into strips (wide about 0.4 cm.) and each strip was blocked in 3% non – fat dry milk (instant non – fat milk powder, Mission milk) in 1xTBS, pH 7.5 with 0.05% Tween -20 and shaked for 1 h. at room temperature. The strip was incubated at 4° C overnight with two concentrations of serum : diluted 10 times less than the titre from ELISA and diluted 2 fold dilution less than above. Thereafter, the strip was 4 times washed in 1% non – fat dry milk in 1xTBS, pH 7.5 (10 minutes per 1 time) and was incubated with the optimal dilution of peroxidase conjugated rabbit anti – human IgG (Dako, Denmark, Code no. P 0214), shaked for 2 hrs. at room temperature and then washed as above. The excess unbound antiserum conjugated was washed.

After washing, the strip was soaked in a mixture of substrate [3, 3' – Diaminobenzidine (3,3',4,4' – Tetraaminobiphenyl) Tetrahydrochloride, Sigma., U.S.,

lot 94H3677.] and 30% H_2O_2. Furthermore, it was incubated at room temperature (in the dark) for 30 minutes. The reaction was stopped by rinsing with distilled water. The membrane was dried and stored in dark to prevent fading of the pattern prior to photograph.

Procedure for staining of immobilon membrane (amido black)

After transferring, the blotted immobilon membrane was stained for 10 mintues in amido black solution and then destained with destaining solution until background turned clear.

Procedure for purified IgG antibody

In this study, we used HiTrap rProtein A (Amercham pharmacia biotech, code no.17-5079-01) to exclude false positive bands from the Western blot. In brief, pooled serum was passed through a 0.45 micron filter. The column was equilibrated with 5 column volumes of binding buffer (0.02 M Sodium phosphate, pH 7.0). Then, serum was applied and the column was washed approximately 5 column volumes of binding buffer again to remove unbound contaminants until the A_{280} (by Bio – Rad SmartSpec spectrophotometer, Bio – Rad, U.S.) was negligible (Figure 9A). Strong elution conditions are required to release bound material. Later, IgG was eluted with 5 column volumes of elution buffer (1M Acetic acid, pH 3.0) (Figure 9B). Since the elution conditions are quite harsh, it may be necessary to transfer eluted fractions immediately to more mild conditions by collecting them in a "neutralising" solvent (a few drops of 1 M Tris – HCl, pH 9.0), so the final pH of the fractions will be approximately neutral. Immediately, the column was re- equilibrated with 5 –

10 column volumes of binding buffer until the A_{280} was negligible. Column was preserved with 25% ethanol (use approximately 5 column volumes for packed media) and stored at 4 – 8 °C until used (Figure 9C).

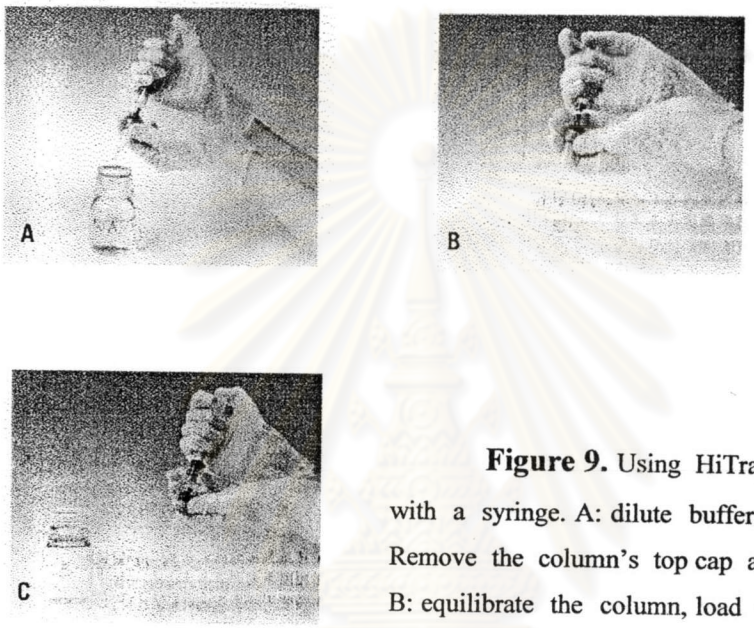

Figure 9. Using HiTrap rProtein A Sepharose with a syringe. A: dilute buffers and prepare sample. Remove the column's top cap and twist off the end. B: equilibrate the column, load the sample and begin collecting fractions. C: wash, elute and collect fractions.

Protein Assay

The concentration of protein was determined by using commercial kit, Bio-Rad Protein Assay (Bio-rad, US). In brief, standard protein, BSA (Sigma cat. no. P7656 Lot: 093H6158) concentration of 0.1, 0.2, 0.4, 0.8, and 1 mg/ml were used for preparing the standard curve. The optimal density of each concentration was measured at 595 nm. wavelength by UV – 160 A (UV visible recording Spectrophotometer, Shimadzu, Japan). The ten – fold dilution of mannoprotein antigen was determined for the protein amount by using this standard curve. The results are shown in Table 7.

Table 7. OD. at wavelenght 595 nm. of protein standard and mannoprotein antigen was measured by spectrophotometer.

Protein concentration (mg/ml)	Optical density (OD) value at wavelenght 595 nm.
Protein standard	
0.1	0.038
0.2	0.155
0.4	0.323
0.8	0.643
1.0	0.724
mannoprotein antigen diluted 1:10	0.518

From the Table 7, we plotted a standard curve from the five dilutions of protein standard and read the protein concentration of the samples from standard curve (Figure 10).

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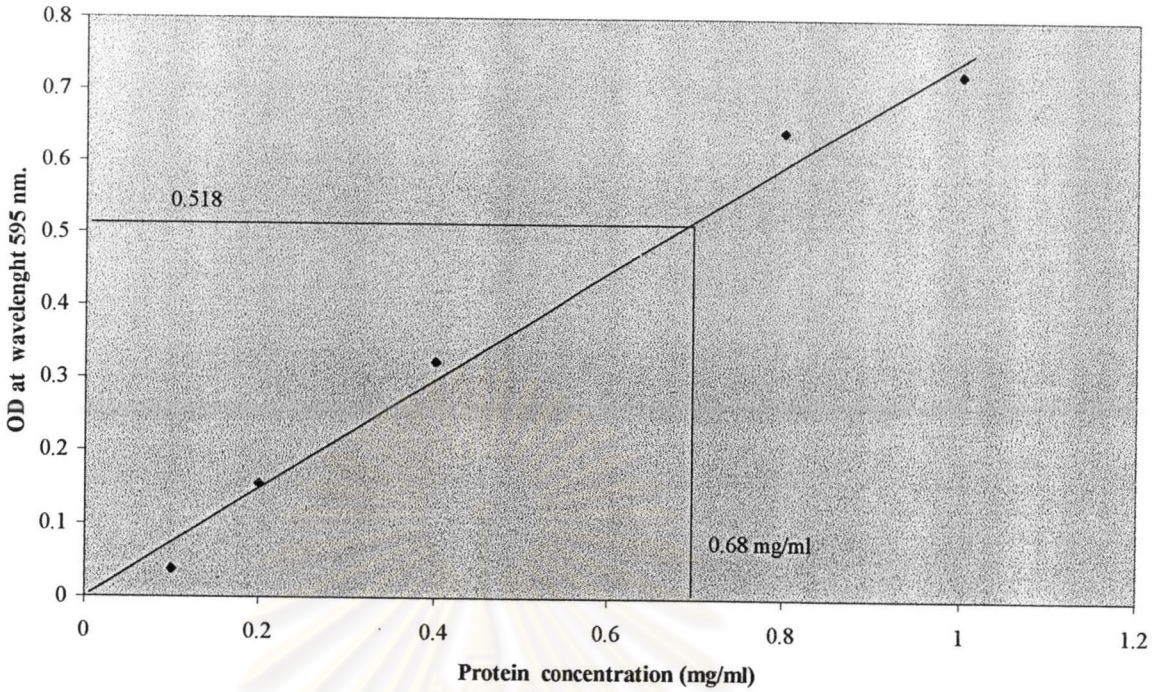

Figure 10. Standard curve of protein assay. The OD of both protein standards and mannoprotein antigen were read at wavelenght 595 nm..

From the Table 7, the optical density of mannoprotein antigen is 0.518. Which protein concentration of mannoprotein antigen is equal to 0.68 mg/ml. from standard curve (Figure 10). In this study, the protein concentration of mannoprotein antigen is 6.8 mg/ml.

Statistical Analysis

Frequency and mode were used to assess the statistical significance of the variables and parameters under study (146, 147).