CHAPTER 5

RESULTS

Enzyme – linked immunosorbent assay (ELISA)

Optimization of antigen

To determine the optimal concentration of mannoprotein antigen as coating antigen in ELISA system, anti – *C. albicans* whole cell rabbit serum dilution of 1:200, 1:400, 1:800, and 1:1600 were used and the detection system was using peroxidase – conjugated swine anti – rabbit immunoglobulins (dilution 1:2,000). Four concentrations, 50, 25, 12.5, and 6.25 μg/ml of mannoprotein antigen were prepared. The result showed that the concentration at 12.5 μg/ml of mannoprotein antigen was the optimal concentration for investigation of IgG antibody (Figure 11) in this ELISA study.

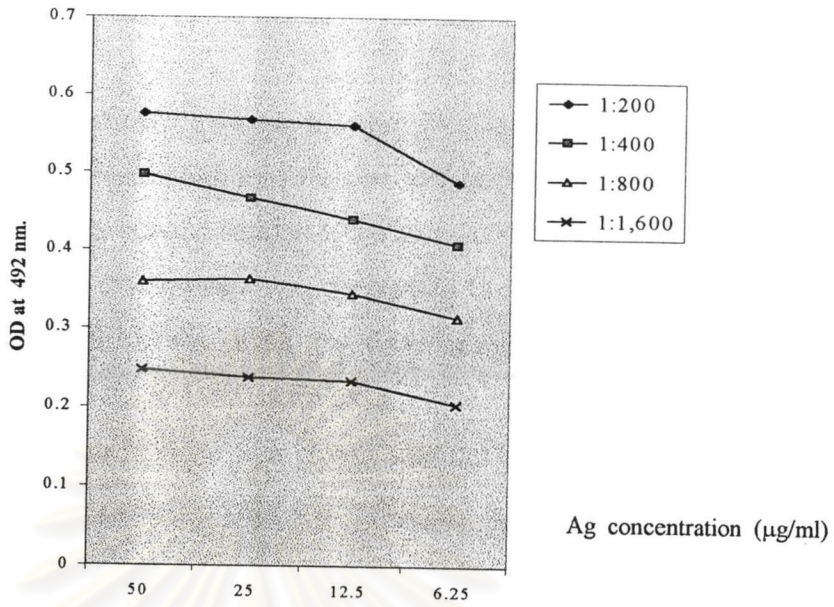


Figure 11. The mannoprotein antigen titration using rabbit serum consisting of anti – *Candida* antibody. Four different concentrations of antigen; 50, 25, 12.5, and 6.25 µ g/ml were examined for the optimal concentration for ELISA. The dilution of rabbit anti – candida serum was prepared as 1:200, 1:400,1:800, and 1:1,600.

Antibody levels to mannoprotein antigen in selected group

One hundred microliters of 12.5 µg/ml of mannoprotein was coated on a well of microtiter plate . Thirty sera from fifteen females and fifteen males (Figure 12) in the healthy individuals with the age range between 17 to 25 years old (mean = 20) were using in this experiment. Another three tested populations were fifteen sera from HIV – infected patients with oral candidiasis (HOC), five sera from systemic candidiasis patients and nine sera from other fungal infection patients. The HOC comprised of seven females and eight males (Figure 13), aged between 22 to 43 years old (mean = 30) . Five systemic candidiasis patients, aged between 3 to 60 years old (mean = 37.3) were all males (Figure 14). The other fungal infection patients consisted of five females and four males (Figure 15), aged between 4 to 70 years old (mean = 38.4) In systemic candidiasis patients, three patients were positive for *C. tropicalis,*

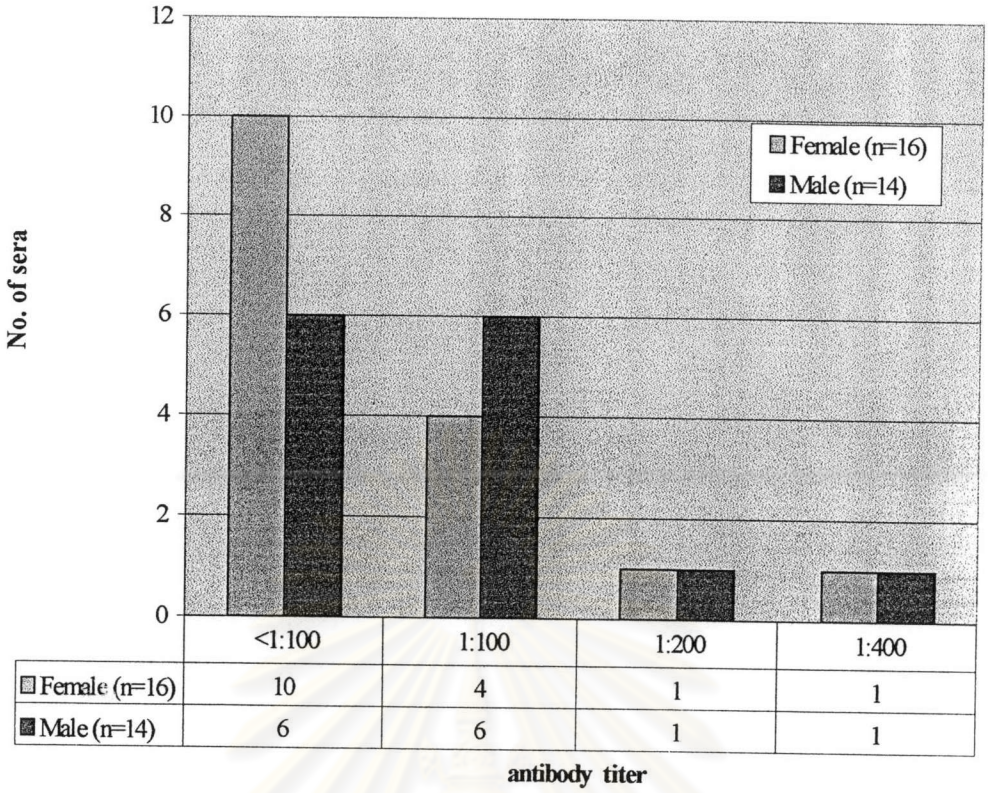


Figure 12. The antibody titers of IgG antibody against mannoprotein antigen in healthy individuals (n=30) , age ranged 17 – 25 years old.

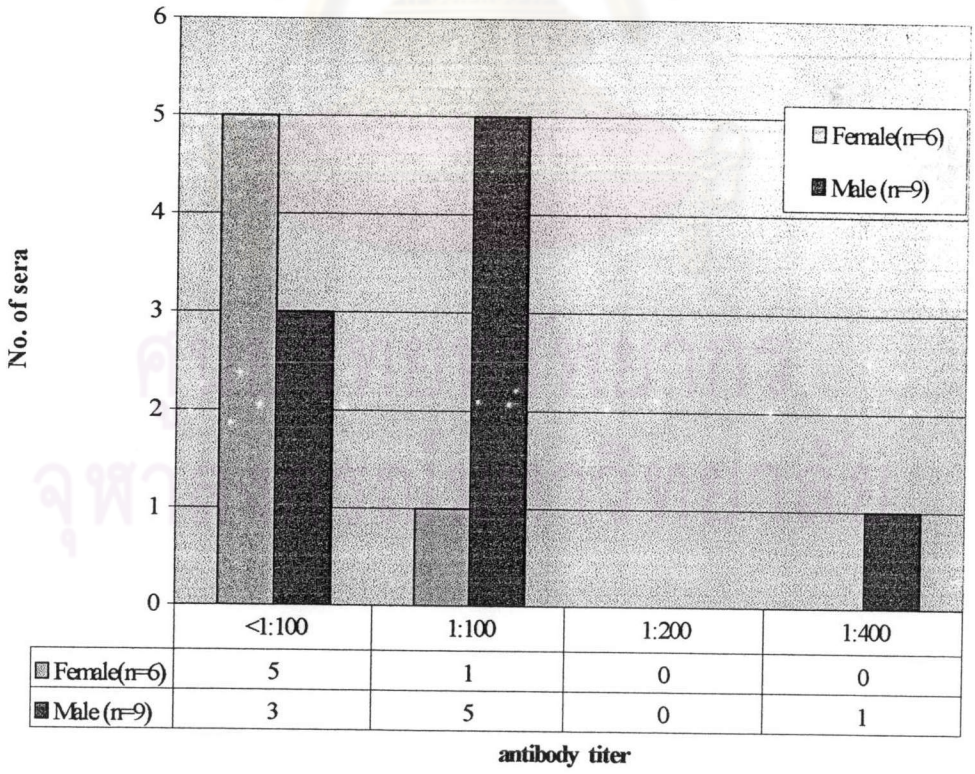
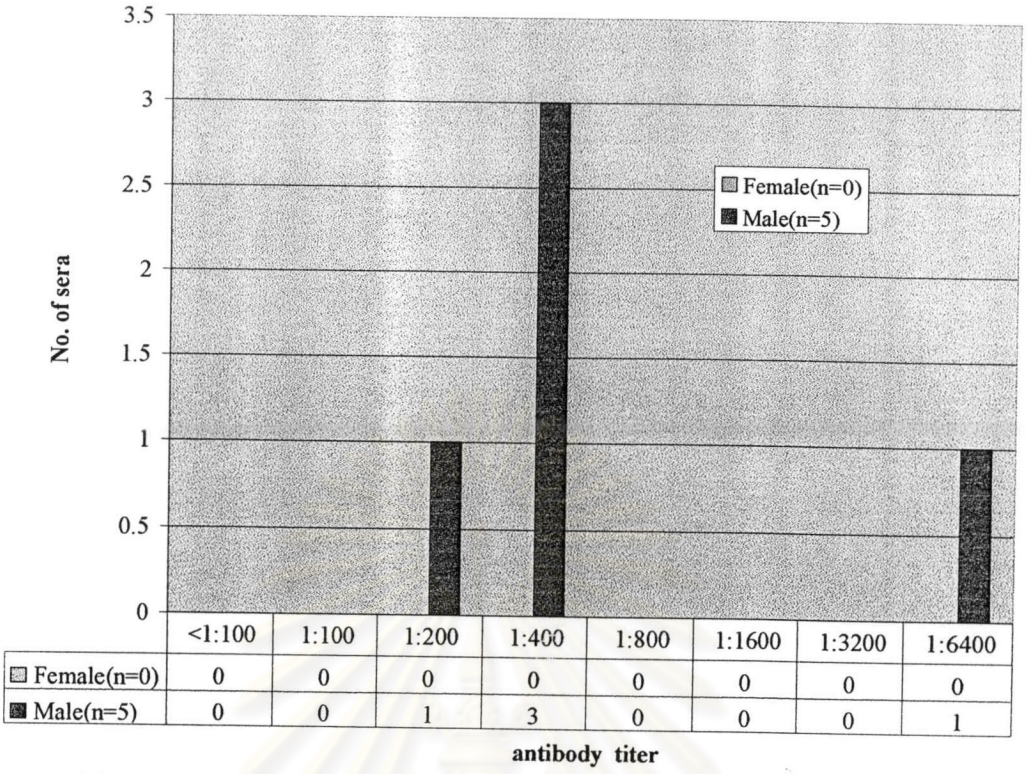
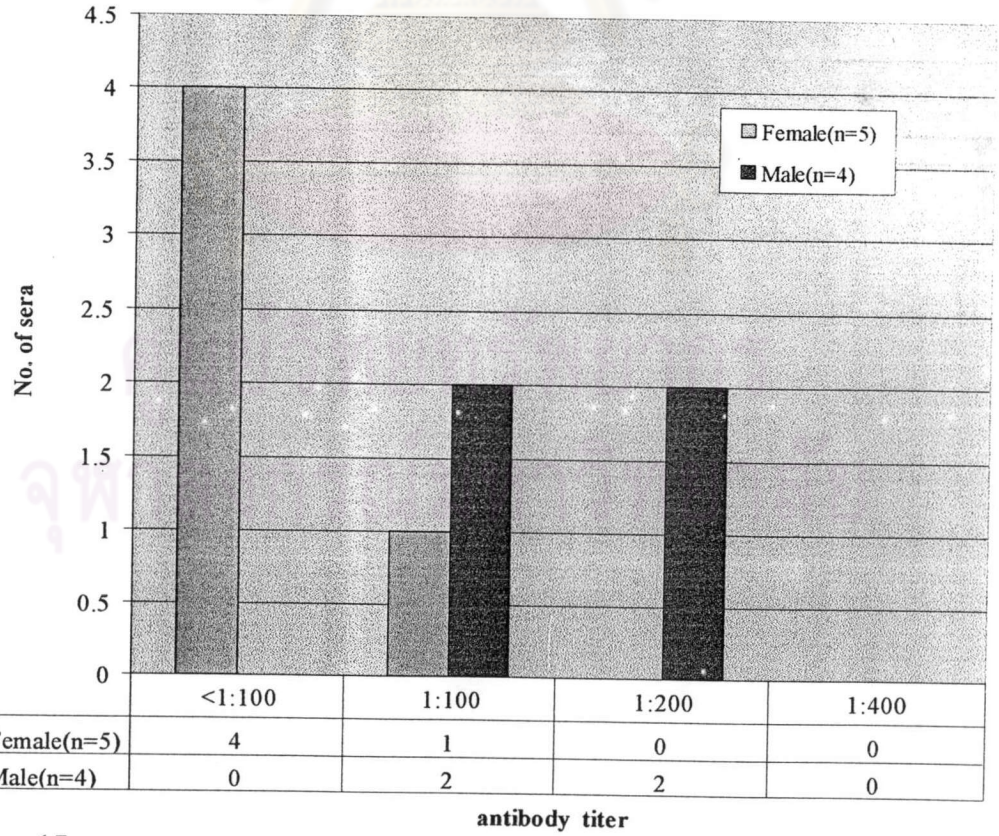


Figure 13. The antibody titers of IgG antibody against mannoprotein antigen in HOC (n=15), age ranged 22 – 43 years old.



	<1:100	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400
Female(n=0)	0	0	0	0	0	0	0	0
Male(n=5)	0	0	1	3	0	0	0	1

Figure 14. The antibody titers of IgG antibody against mannoprotein antigen in systemic candidiasis patients (n=5), age ranged 3 – 60 years old.



	<1:100	1:100	1:200	1:400
Female(n=5)	4	1	0	0
Male(n=4)	0	2	2	0

Figure 15. The antibody titers of IgG antibody against mannoprotein antigen in other fungal infection patients (n=9), age ranged 4 - 70 years old.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE)

Characterization of antigenic component of mannoprotein antigen

The protein profile of the antigenic components was separated by SDS – PAGE. The gel stained with Coomassie brilliant blue R was shown in Figure 16. Twelve protein components were observed. Their molecular weights ranged from 22.5 to 52 kDa; the most intense bands were observed at 46.5, 38, 29, and 22.5 kDa.

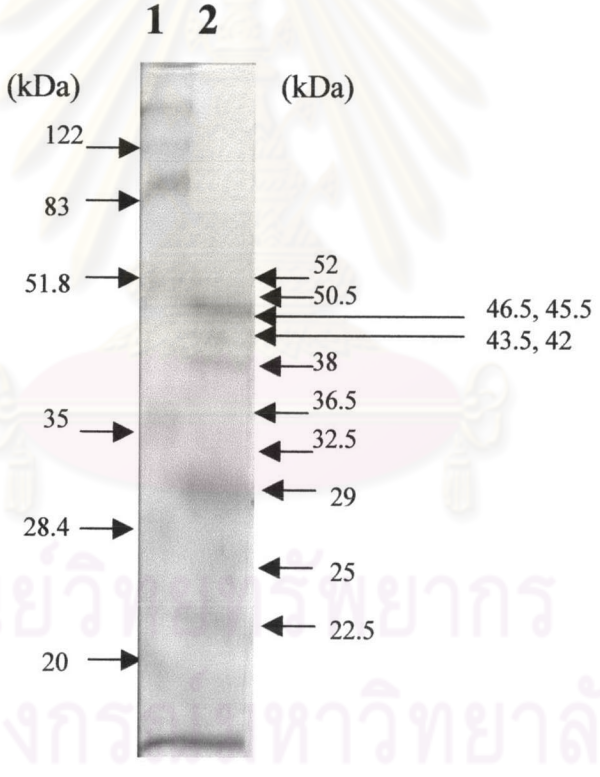


Figure 16. SDS – PAGE of mannoprotein antigen. Mannoprotein antigen was separated by electrophoresis in a 14% polyacrylamide gel and then stained with Coomassie brilliant blue R. The position of molecular weight of marker are shown on the lane 1: β - galactosidase, 122 kDa; bovine serum albumin, 83 kDa; ovalbumin, 51.8 kDa; carbonic anhydrase, 35 kDa; soybean trypsin inhibitor, 28.4 kDa; and lysozyme, 20 kDa. The numbers on the lane 2 indicate the molecular weight (kDa) of protein components of mannoprotein antigen.

The protein components in the gel were transferred to an immobilon membrane. To confirm the efficiency of transferring, one strip of the membrane was stained with amido black and that gel was stained with Coomassie brilliant blue R. Same bands were detected on the membrane and twelve bands on the membrane were observed. Comparing the protein markers, the size of the bands responsed to the size on the gel (Figure 16, 17). This verified test was done in every experiments. After transferring the protein onto the membrane, immunoblot was performed as described in the materials and methods. The strip was reacted with each individual serum to identify the immunoglobulin G antibody pattern.

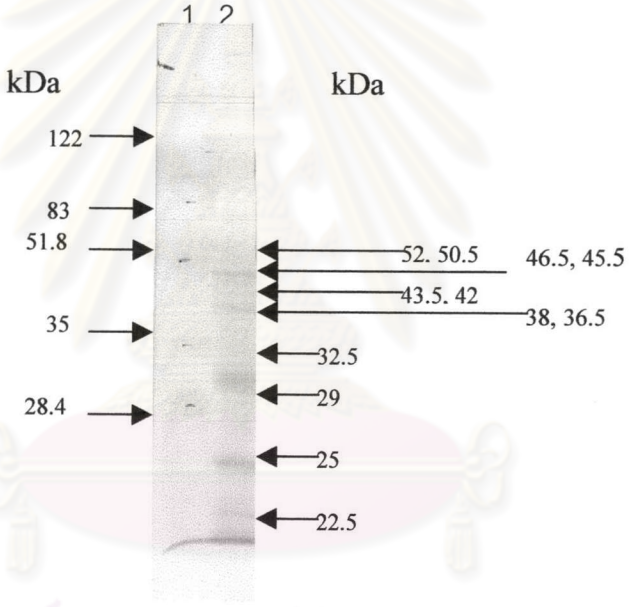


Figure 17. The protein components on the membrane after blotting. After separating by SDS – PAGE, protein components were transferred onto the immobilon membrane then stained with amido black. The position of molecular weight (kDa) marker are shown on lane 1 : β - galactosidase, 122 kDa; bovine serum albumin, 83 kDa; ovalbumin, 51.8 kD; carbonic anhydrase, 35 kDa and soybean trypsin inhibitor, 28.4 kDa. The numbers on lane 2 indicate the molecular weight (kDa) of the protein components of mannoprotein antigen.

Immunobloting

Optimization of rabbit anti - human IgG conjugated with peroxidase.

Before Western blot was performed, the optimization of the rabbit anti – human IgG conjugated with horseradish peroxidase was examined. The conjugated reagent was diluted to 1:200, 1:500, 1:1,000 and 1:1,500. One of the sera from systemic candidiasis patients which showed 1:400 titer from ELISA was selected. As the criteria mentioned in the materials and methods, this serum was diluted to 1:40 for this test. The result revealed that the clearest band was observed on the membrane using 1:1,000 conjugated peroxidase dilution. To provide more sensitivity to the Western blot, one more dilution of the conjugated reagent, 1:500 was decided to use in this study (Figure 18).

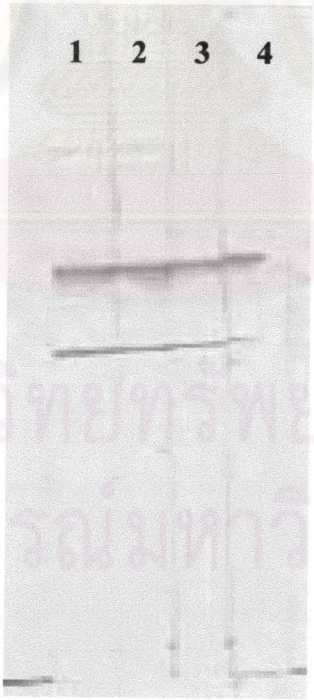


Figure 18. Optimization of rabbit anti – human IgG conjugated with horseradish peroxidase. The blotting membrane was reacted with diluted systemic candidiasis patient's sera (1:40), and then incubated with different concentration of rabbit anti – human IgG conjuagted with horseradish peroxidase, 1:200, 1:500, 1:1,000 and 1:1,500, respectively. (Lane 1, 2, 3 and 4, respectively)

The patterns of human IgG antibody against mannoprotein antigen in healthy individuals, HOC, systemic candidiasis patients and other fungal infection patients.

The blotting membrane was incubated with two different dilutions of each sera from thirty healthy individuals, from fifteen HOC, from five systemic candidiasis patients and from nine other fungal infection patients. For example, serum from one of healthy individual showed the titre 1:100 from ELISA, in this Western blot, this serum will be prepared as titre 1:10, and also 1: 5 (Figure 19). The patterns of protein components was recognized by healthy individuals, HOC, systemic candidiasis patients and other fungal infection patients could be categorised into 15, 12, 5 and 9 patterns, respectively. To simplify the results, each pattern was drawn out as diagram shown in Figure 20.

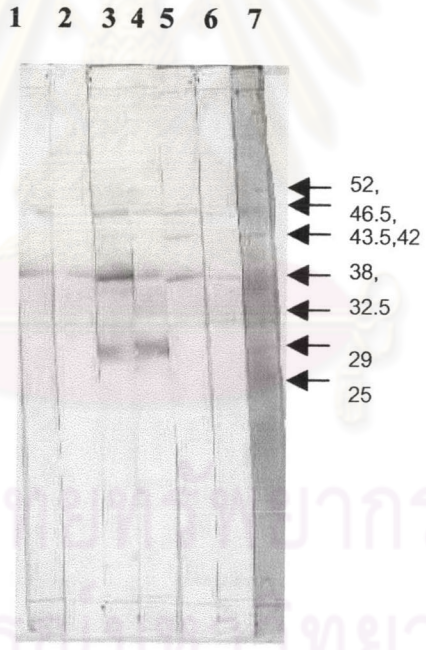


Figure 19. Immunoblot of mannoprotein antigen was recognized by human IgG. Mannoprotein antigen was separated by SDS – PAGE and was transferred to an immobilon membrane and incubated with human serum: serum from healthy host no. 1 and 2 (lane 1-2 and 5-6), serum from systemic candidiasis patient (lane 3 – 4) and lane 7 : marker are shown. The numbers on the right indicate the molecular weight (kDa) of the protein components were recognized.

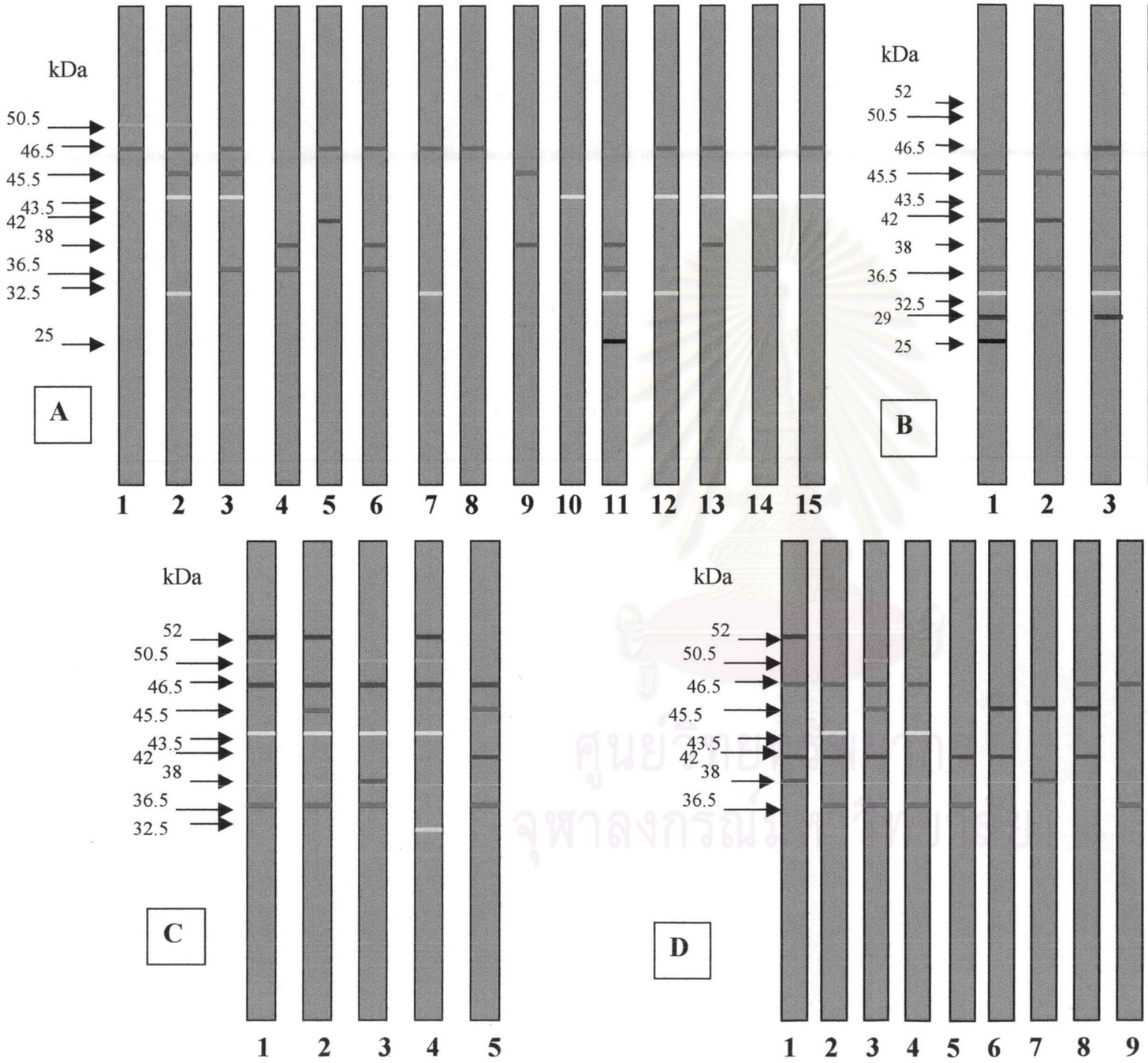


Figure 20. The antibody patterns of Healthy individuals, HOC, Systemic candidiasis patients and other fungal infection patients.
A: patterns of healthy individuals
B: HOC
C: Systemic candidiasis patients
D: Other fungal infection patients

The antibody profiles in all four groups were explained as followed :

1. Healthy individuals : eight in thirty sera reacted no band with the protein components whereas the other twenty – two sera, individual sera reacted to 1 – 5 bands among all twelve separated bands (Figure 20A). All these eight sera demostrated the IgG titre <1:100. The sizes of the detected components were 50.5, 46.5, 45.5, 43.5, 42, 38, 36.5, 32.5 and 25 kDa. They were categorized into fifteen patterns. Eleven patterns (no. 1-11) was found in individual serum while the four left (no. 12-15) was found in two individuals, two individuals, three individuals and four individuals, respectively. The common sizes were 46.5 kDa (81.8%), 43.5 kDa (57.1%), 38 kDa (31.8%) and 36.5 kDa (31.8%) (Figure 21, 22).

2. HOC : fifteen individual serum reacted to 3 – 6 bands among all twelve separated bands of this mannoprotein antigen (Figure 20B). The sizes of the detected components were 52, 50.5, 46.5, 45.5, 43.5, 42, 38, 36.5, 32.5, 29 and 25 kDa. They were categorized into twelve patterns. Ten patterns (no. 1 - 11) was found in individual serum whereas the two left (no. 11 and 12) was found in two sera and three sera. The common sizes were the 46.5 kDa (80%), 45.5 kDa (53.3%), 43.5 kDa (46.7%), 42 kDa (46.7%), and 36.5 kDa (86.7%) (Figure 21, 22).

3. Systemic candidiasis patients : five sera demostrated each individual pattern, regardless the species of the isolated *Candida*. The anti – *candida* components among these groups were 52, 50.5, 46.5, 45.5, 43.5, 42, 38, 36.5 and 32.5 kDa (Figure 20C). Some of these components were regularly observed with rather high frequency as followed, 52 kDa (60%), 50.5 kDa (60%), 46.5 kDa (100%), 43.5 kDa (80%) and 36.5 kDa (80%) (Figure 21, 22).

4. Other fungal infection patients : each of nine detectable patterns belonged to individual patient. The protein components among these sera were 52, 50.5,

46.5, 45.5, 43.5, 42, 38 and 36.5 kDa (Figure 20D). Some of these components were regularly observed with rather high frequency as followed 46.5 kDa (66.7%), 45.5 kDa (44.4%), 42 kDa (66.7%) and 36.5 kDa (55.6%) (Figure 22).

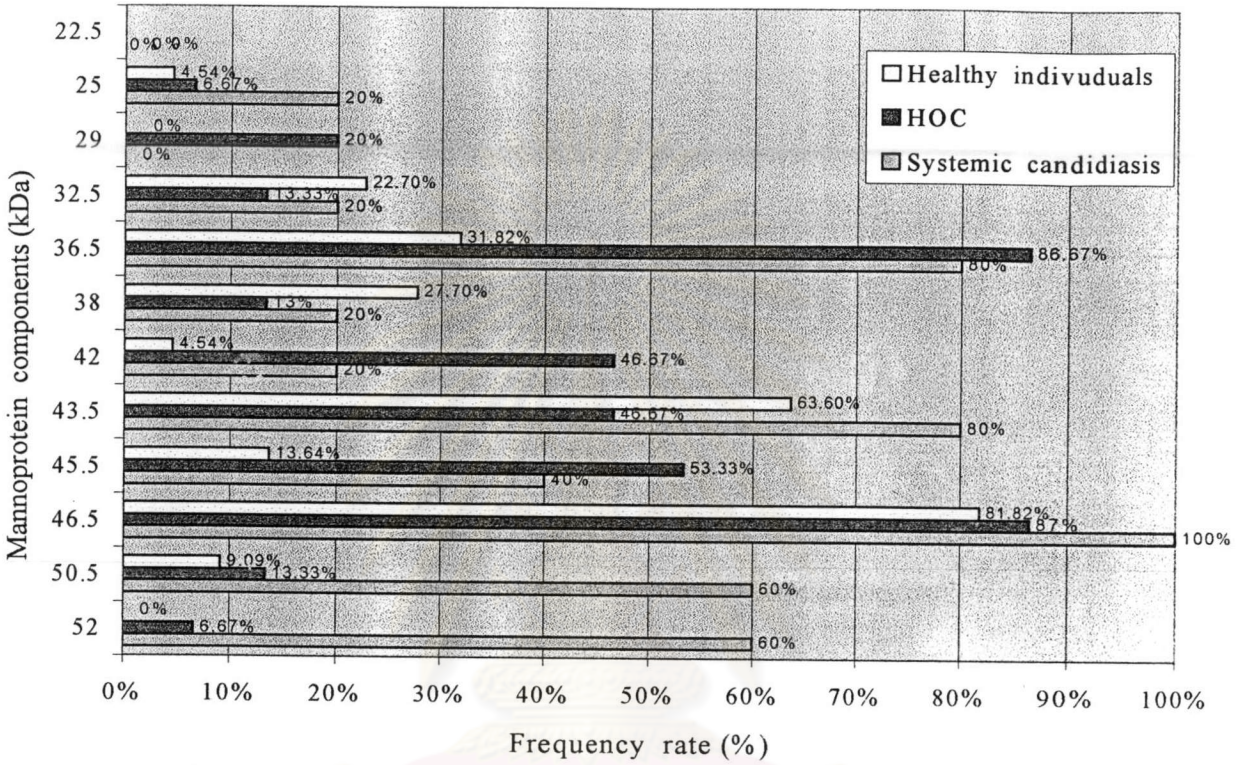


Figure 21. IgG immune response of 22 healthy individuals, 15 HOC and 5 systemic candidiasis patients against mannoprotein antigen as revealed by Western blotting analysis.

Table 8. Characterization of mannoprotein antigen was recognized by human sera IgG antibody.

Group (%)	**52 kDa**	**50.5 kDa**	46.5 kDa	45.5 kDa	43.5 kDa	42 kDa	38 kDa	36.5 kDa	32.5 kDa	**29 kDa**	25 kDa	22.5 kDa
Healthy host n = 22	**0**	**9.09**	81.8	13.6	63.6	4.54	27.7	31.8	22.7	**0**	4.54	0
HOC n = 15	**6.67**	**13.3**	86.7	53.3	46.7	40	13.3	86.7	13.3	**20**	6.67	0
Systemic candidiasis n = 5	**60**	**60**	100	40	80	20	20	80	20	**0**	20	0
Fungal infectious n = 9	**11.1**	**11.1**	66.7	44.4	22.2	66.7	22.2	55.6	0	**0**	0	0

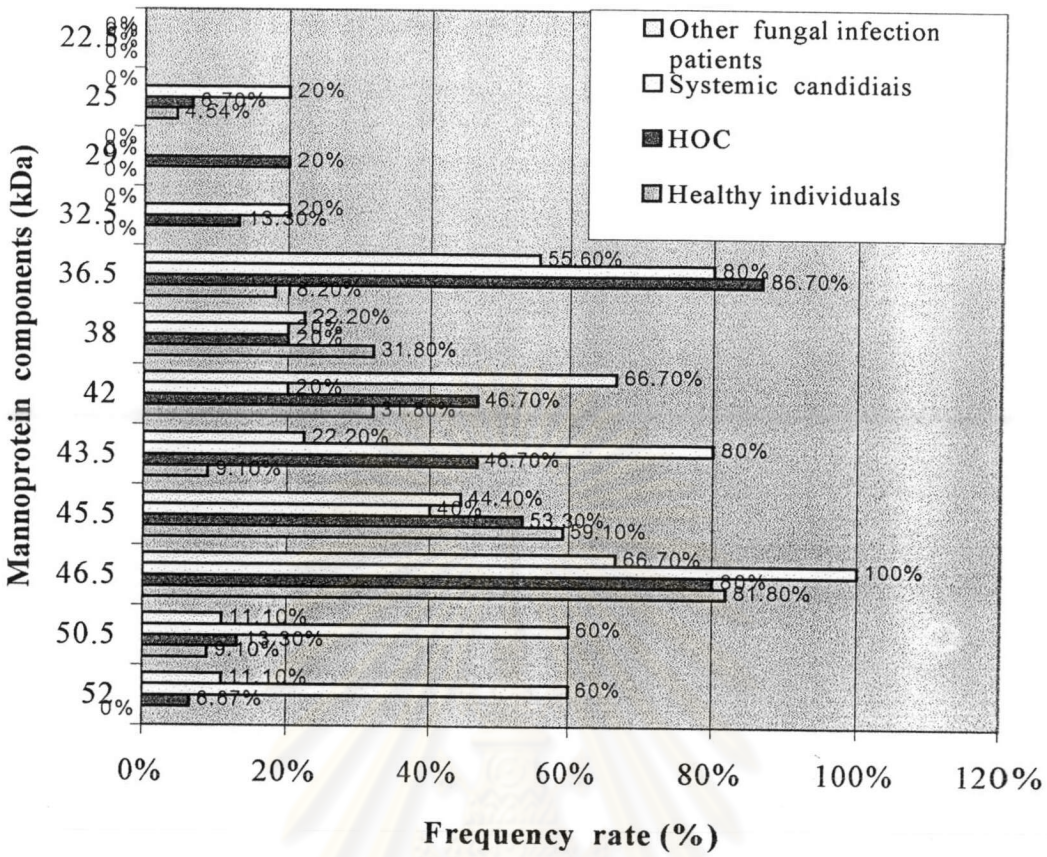


Figure 22. IgG immune response of 22 healthy individuals, 15 HOC, 5 systemic candidiasis patient, and 9 other fungal infection patients against mannoprotein antigen as revealed by Western blotting analysis.

Purify immunoglobulin G antibody (IgG) from pooled serum by Hitrap rProtein A

To verify the true positive bands from the Western blot, the IgG of pooled tested sera was trapped and then reacted again with all the protein components. The solutions which were passed the column were kept to measure the absorbance at wavelenght 280 nm. (Table 9).

Table 9. The optical density value at wavelenght 280 nm. of solutions in each tubes.

No. of tube	Optical density (OD) value at wavelenght 280 nm.	
1	2.940	Flow through solution
2	2.241	
3	0.406	
4	0.311	
5	0.080	
6	0.030	Washing solution
7	0.009	
8	0.078	
9	0.054	
10	2.216	Eluting solution
11	0.186	
12	0.182	
13	0.056	
14	0.012	Washing solution
15	0.002	

We plotted graph by using the optical density (OD) of the consecutive fraction or tube in the table 9. The fraction number 1-5 were named as "flow through solution", no. 6 – 9 and 14 – 15 as "washing solution", and no. 10 – 13 as "eluting solution". Flow through solution contained pooled serum which was first passed column and mixed with washing buffer, eluting solution consisted of purified immunoglobulin G antibody mixed with eluting buffer. After that, flow through solution and eluting solution were desalted in order to concentrate the solution. The final volume of each solution was approximately 2 ml and kept at 4°C until used.

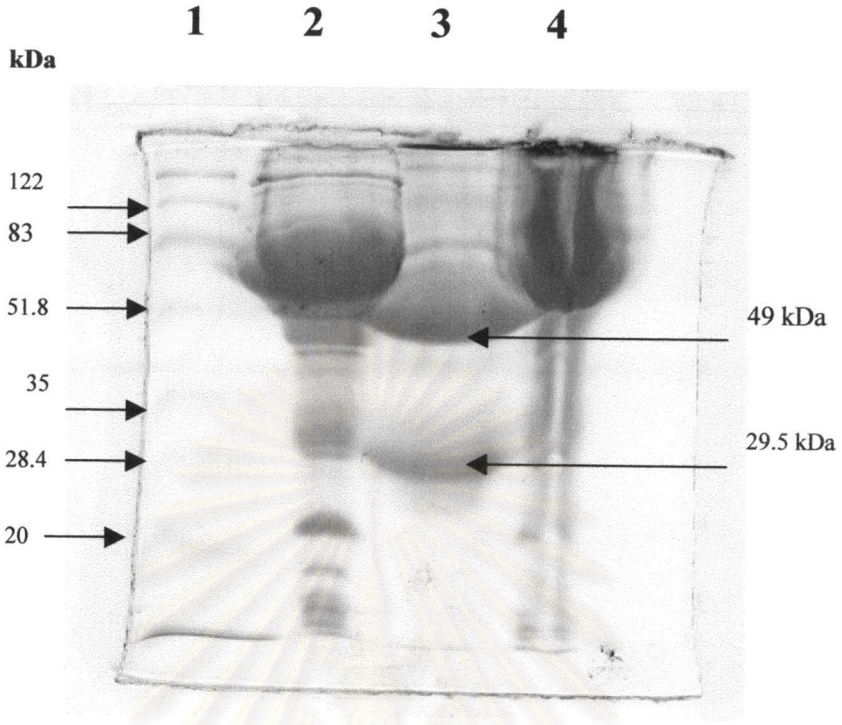


Figure 23. SDS – PAGE of purification of immunoglobulin G of human sera from pooled sera on Hitrap rProtein A Sepharose, with a 95% recovery of highly purified immunoglobulin G antibody.

After separating, the gel was stained with Coomassie brilliant blue R. (Figure 23). In lane 2, two bands of protein components were appeared, 49 and 29.5 kDa, respectively. And then, we confirmed true positive bands. Immunoglobulin G antibody compared with pooled sera and flow through solution to verify the bands by using Western blot analysis. The result showed the same pattern. (Figure 24, lane 1 and lane 2). For lane 3, we can see some bands were appeared from flow through solution because the flow through solution has immunoglobulin G antibody high levels more than the limiting of column could trap all.

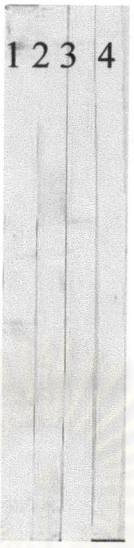


Figure 24. Immunoblot of mannoprotein antigen was recognized by IgG antibody and human sera. Lane 1 = pooled sera, Lane 2 = Purified immunoglobulin G antibody (eluting buffer), Lane 3 = flow through solution, and Lane 4 = negative control.