IV. RESULTS

A. Detection of immunogenic proteins in crude concentrated culture filtrate antigens

The protein components secreted from the yeast form of *P. marneffei* 391H and 302BM were separated on 10% SDS-PAGE gel and stained with CBB R-250. The protein profile demonstrated over 20 components ranging in molecular mass from 200 to 33 kDa (Figure 4) which were similar to previous study (Mekaprateep, 1995). The band of 88 kDa was the major protein secreted abundantly from *P. marneffei* 391H. Whereas, the band of 50 kDa was the major protein secreted abundantly from *P. marneffei* 302BM. When they were subcultured on BHI broth from different companies, the yield of secreted proteins and protein profiles were varied among experiments (Figure 5). Immunogenic proteins of *P. marneffei* 391H and 302BM were analyzed by immunoblot assay employing pooled sera of 10 AIDS patients infected with *P. marneffei* or rabbit antiserum against concentrated culture filtrate antigens of *P. marneffei* yeast form. At least 10 IgG-binding components that had molecular mass in rang from 200 to 30 kDa were identified. However, three major bands of 88, 54, and 50 kDa reacted intensely with pooled sera from *P. marneffei* infected patients. The results are shown in Figure 6. The IgG antibodies in pooled sera from 10 healthy subjects and pre-immune rabbit serum reacted weakly with the bands of 88 kDa and larger proteins, but did not react with the 54 and 50 kDa proteins (Figure 7).

Figure 4. Protein profiles of concentrated culture filtrate antigens from *P. marneffei* yeast-form. Culture media was BHI broth (Oxoid). Protein components were separated on SDS-PAGE gel (10%T 2.6%C) and stained with 0.1% CBB R-250. Lane 1, 3: crude proteins of 391H, and lane 2, 4: crude proteins of 302BM that cultured in different lot numbers of BHI broth.

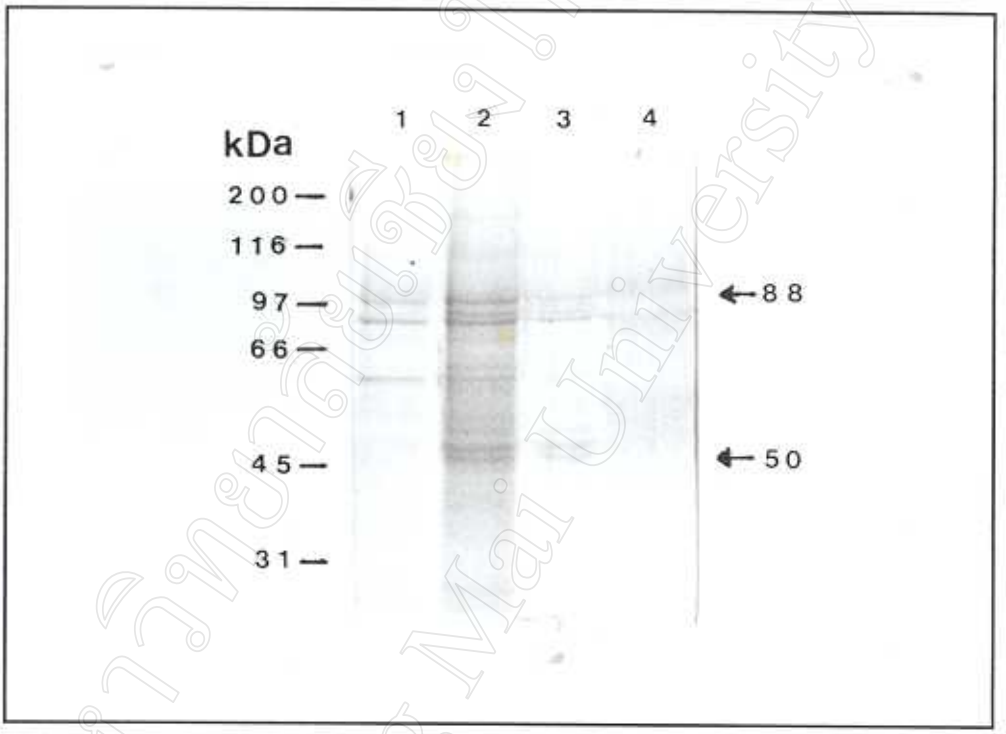

Figure 5. Protein profiles of crude concentrated antigen cultured in BHI broth from different companies (lane 1: BBL, lane 2: Oxoid, lane 3: Mast diagnostics and lane 4: Difco). The crude concentrated antigens of *P. marneffei* 391H were separated on SDS-PAGE gel (10%T 2.6%C). The gel was stained with 0.1% CBB R-250 and destained with acid methanol. The proteins were much secreted when the fungus was cultured in BHI broth from Oxoid laboratory (lane2).

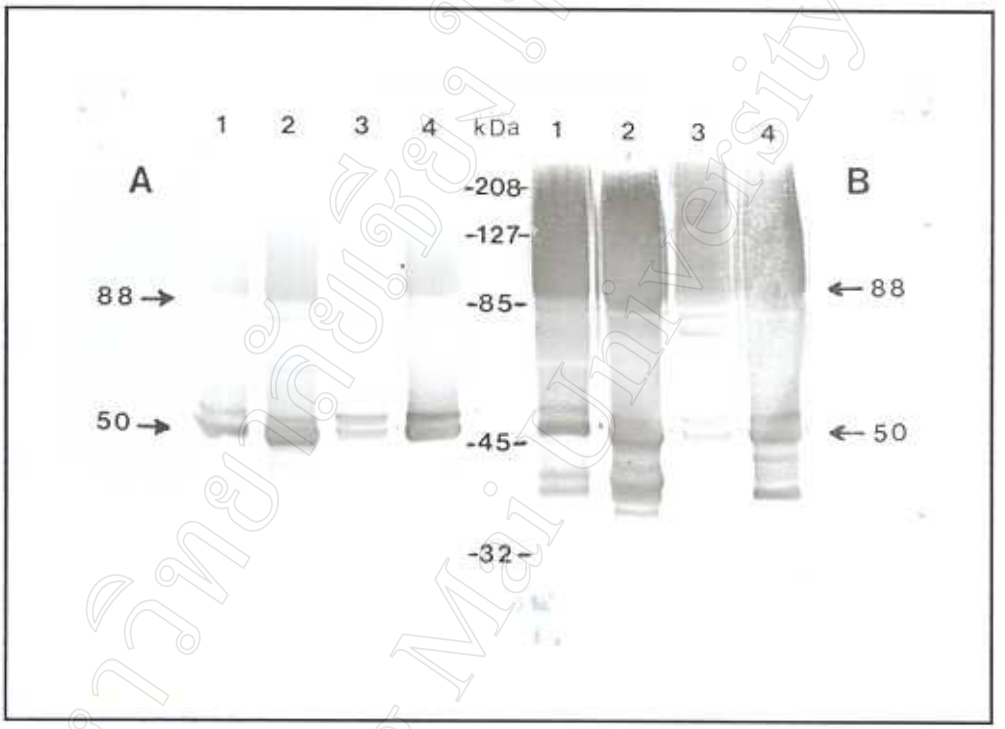

Figure 6. Immunogen profiles of concentrated culture filtrate antigens reacted with pooled sera from 10 AIDS patients with penicilliosis at serum dilution of 1:1,000 (A), and reacted with rabbit antiserum against concentrated culture filtrate antigens from *P. marneffei* yeast cells at serum dilution of 1:1,000 (B). Lane 1, 3: crude proteins of 391H and lane 2, 4: crude proteins of 302BM which were cultured in different lot numbers of BHI broth (Oxoid).

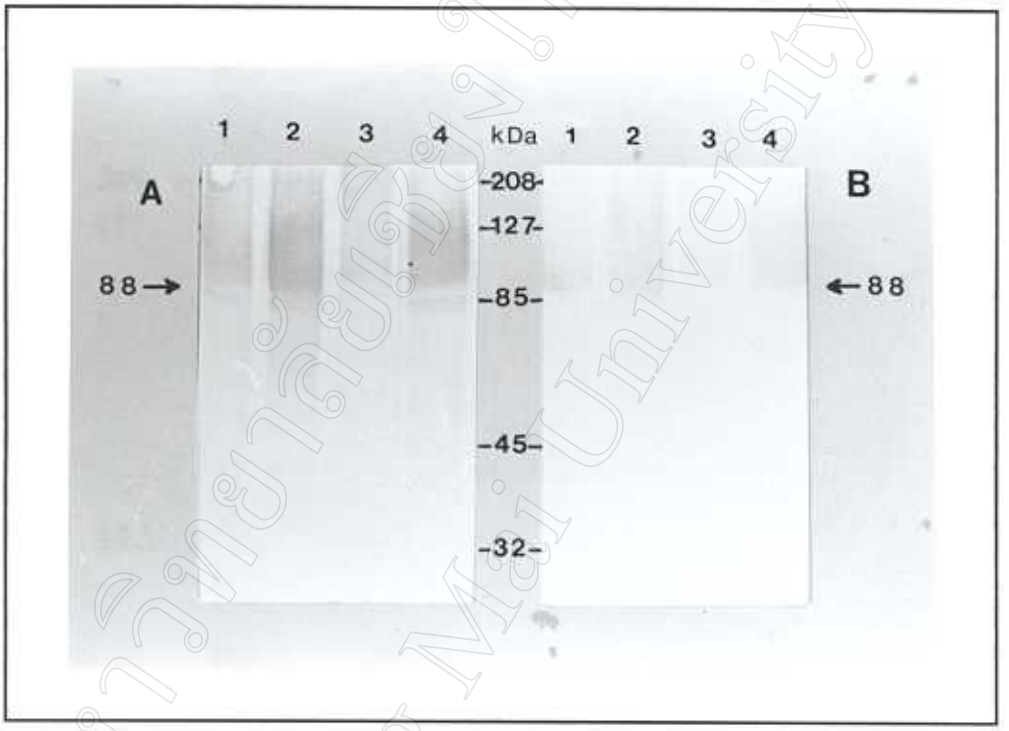

Figure 7. Immunogen profiles of concentrated culture filtrate antigens reacted with pooled sera of 10 healthy subjects at serum dilution of 1:100 (A), and pre-immune rabbit serum at dilution of 1:100 (B). The crude proteins and the pattern of loading were the same as in Figure 6 (lane 1, 3: crude proteins of 391H and lane 2, 4: crude proteins of 302BM).

B. Partial purification of 88 and 50 kDa protein antigens

The major immunogenic proteins (88 and 50 kDa) were separated by preparative gel electrophoresis using Prep-cell Model 491 (BIO-RAD) and Mini-protean II preparative apparatus (BIO-RAD). The Prep-cell Model 491 was conducted to purify the protein of 88 kDa from *P. marneffei* 391H. This protein could be seen in the elution buffer fractions number 160 to 210 (Figure 8 A, B) after precipitating with cold acetone overnight at -20°C and drying by incubating at 37°C for 5 h or by using speed vacuum for 2 h. The pellet was further analyzed with SDS-PAGE and stained with CBB-R250. The fractions containing 88 kDa protein were pooled and concentrated by using ultrafiltration and centricon device. When the protein was confirmed for it's antigenicity by Western blot analysis, the antigenicity could be identified but with some detected lower molecular mass antigens (Figures 9, 10).

The 50 kDa protein was much secreted in culture medium from *P. marneffei* 302BM yeast cells. This protein could not be purified by using Prep cell (Model 491) apparatus because of the presence of other protein with closely molecular weight. They were always eluted together and could be seen on the 10% resolving gel following with CBB R-250 staining. The 50 kDa protein needed to be separated by preparative gel electrophoresis using Mini-Protean II apparatus (BIO-RAD) with preparative comb (1.0 mm or 1.5 mm of thickness). The band of 50 kDa was cut with a scalpel. The cut gels (about 20 gels) were pooled, minced and then the protein was eluted from the gels with electro-elutor. The eluted protein was concentrated with cold acetone and allowed to dry by incubating at 37°C for 5 h or by using speed vacuum for 2 h. When it's molecular weight, and antigenicity were confirmed by comparing with crude culture filtrate antigens, only single band of the 50 kDa was identified (Figures 11,12).

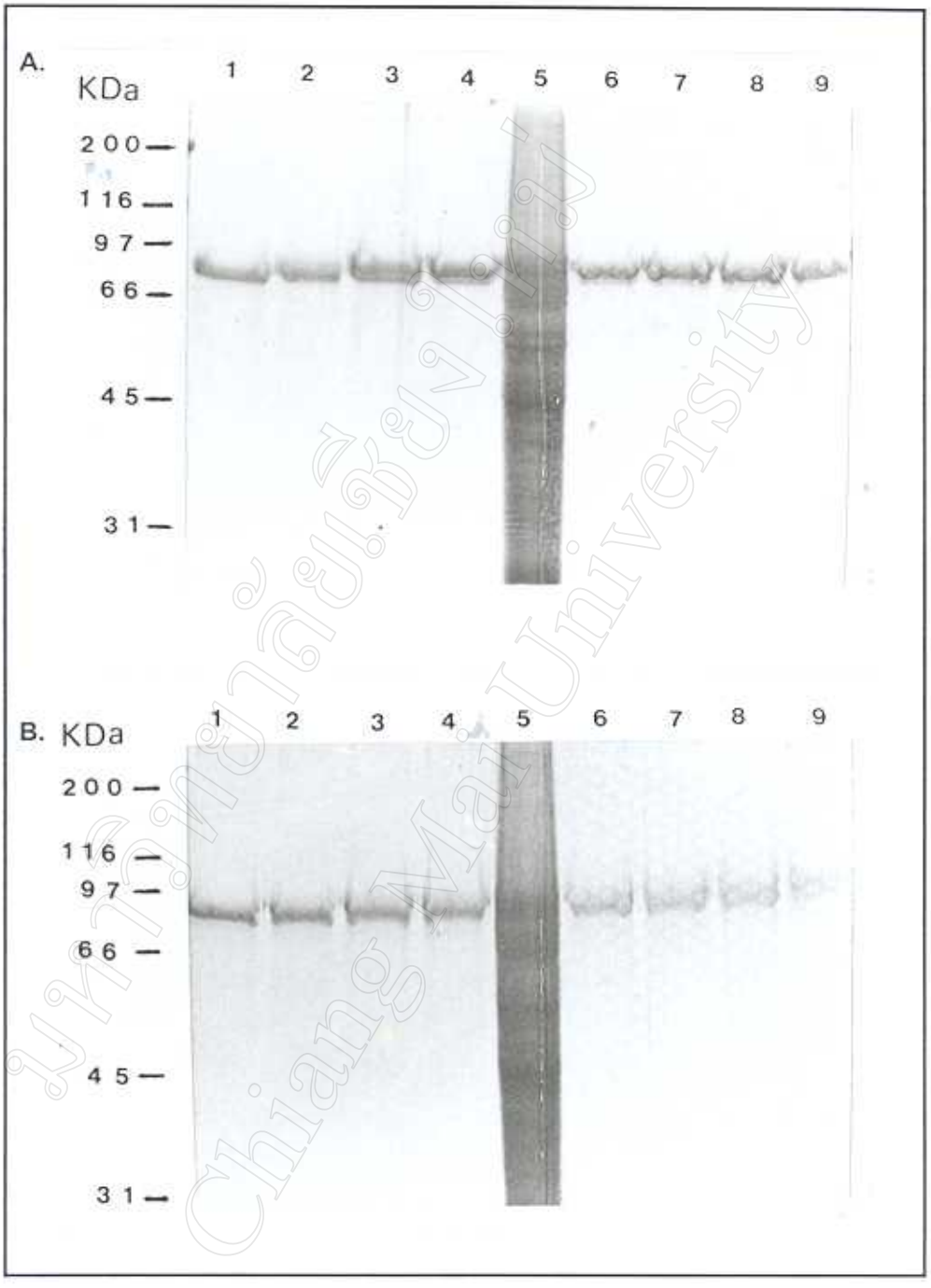

Figure 8. Elution profiles and SDS-PAGE analysis of aliquots from Prep-cell fractions of 140-175 (A) and fractions of 180-215 (B) which contain the 88 kDa protein. After concentrating the protein, it was separated on SDS-PAGE gels (10%T 2.6%C) and stained with 0.1% CBB-R250. A) lane 1-4: fractions number of 140, 145, 150 and 155, lane 5: 10 μl of crude proteins of P.M. 391H, and lane 6-9: fractions number of 160, 165, 170, 175. B) lane 1-4: fractions number of 180, 185, 190, 195, lane 5: 10 μl of crude proteins of P.M. 391H, and lane 6-9: fractions number of 200, 205, 210, 215.

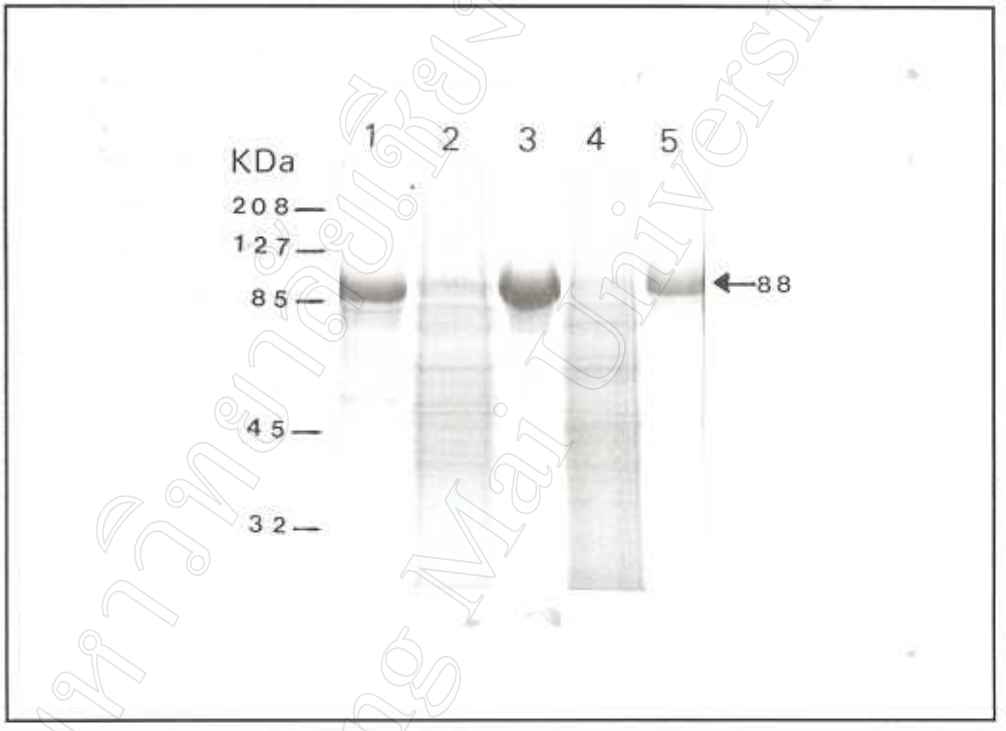

Figure 9. Protein profiles of concentrated proteins obtained from the pooled fractions containing 88 kDa protein from Prep cell Model 491 were analyzed on SDS-PAGE gel (10%T 2.6%C) and stained with 0.1% CBB-R250. Lane 1: pooled fractions number of 160-174, lane 2, 4: crude proteins of 391H, lane 3: pooled fractions number of 175-195, and lane 5: pooled fractions number of 196-210.

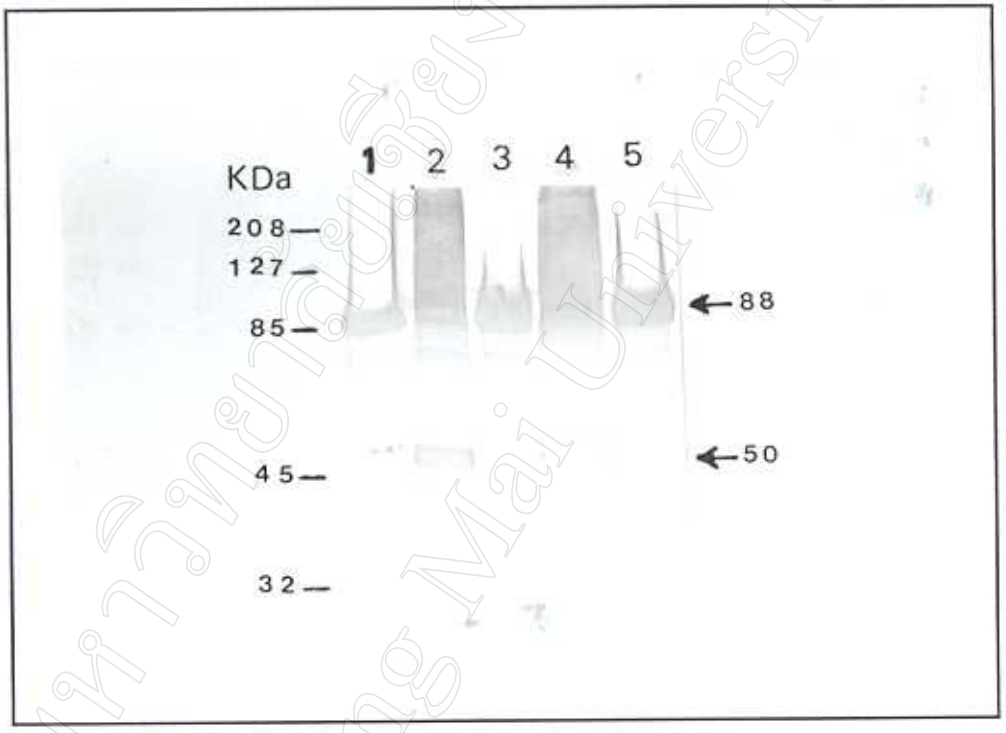

Figure 10. The immunogenicity of concentrated proteins from pooled fractions were confirmed by using SDS-PAGE and Western blot analysis. They were analyzed on SDS-PAGE gel (10%T 2.6%C) and transferred onto nitrocellulose membrane. The transferred proteins were reacted with rabbit antiserum at serum dilution of 1:1,000. The loading pattern was the same as Figure 9 (lane 1: pooled fractions of 160-174, lane 2, 4: crude proteins of 391H, lane 3: pooled fractions of 175-195, and lane 5: pooled fractions of 196-210).

Figure 11. SDS-PAGE and Coomassie blue staining of aliquots from electro-eluted protein from the pooled cut gels. Lane 1 and lane 3: cut band of 50 kDa from 302BM, lane 2: cut band of 88 kDa from 391H, lane 4: cut band of 88 kDa from 302BM, and lane 5: crude proteins of 391H. In addition, the concentrated protein of pooled fractions 160-174 (lane 6) and 175-195 (lane 7), containing 88 kDa protein, prepared from another experiment were run comparatively.

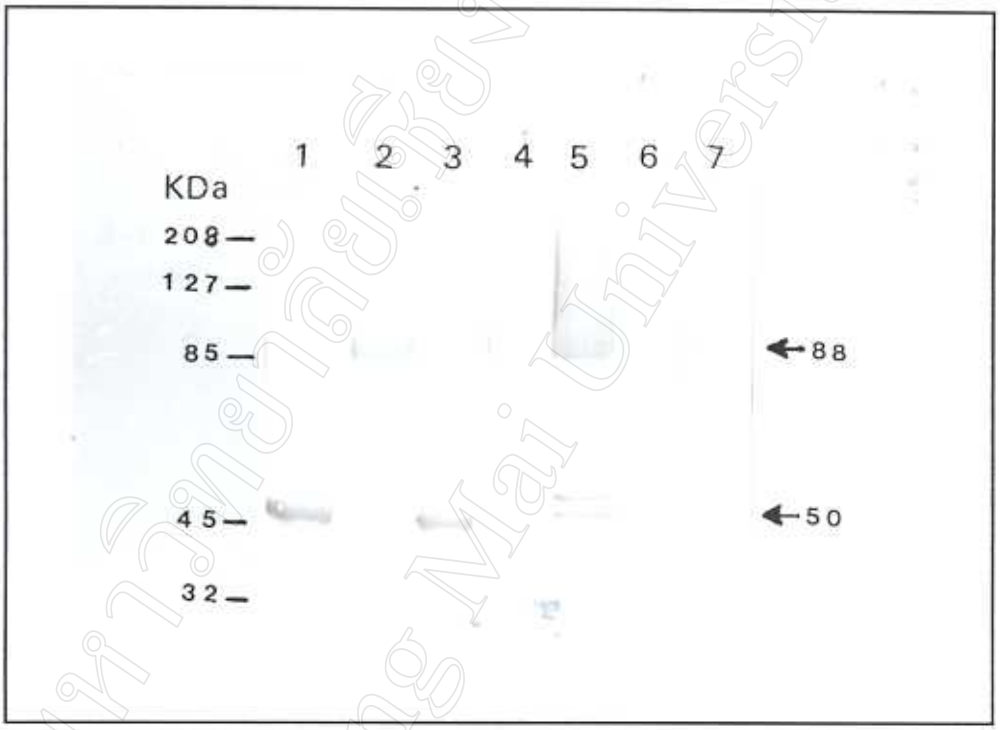

Figure 12. Immunogen profiles of concentrated proteins eluted from cut gel which were analyzed on SDS-PAGE gel (10%T 2.6%C) and transferred onto nitrocellulose membrane. The membrane was probed with pooled sera of 10 AIDS patients with penicilliosis at serum dilution of 1:1,000. Sample loading pattern and running conditions were the same as Figure 11 (lane1 and lane 3: cut band of 50 kDa from 302 BM, lane 2: cut band of 88 kDa from 391H, lane 4: cut band of 88 kDa from 302BM, lane 5: crude proteins of 391H, lane 6: concentrated protein from pooled fractions of 160-174, and lane 7: pooled fractions of 175-195).

C. Further purification of partially purified proteins 88 kDa and 50 kDa

The partially purified proteins, 88 kDa and 50 kDa, were subjected for further purification by using two-dimensional gel electrophoresis technique. The proteins could be separated by their differences of isoelectric focusing in the first dimension and by their differences of sizes in the second dimension SDS-PAGE. Their pI values were obtained by comparing with the pattern of 2-D SDS-PAGE standard proteins separated on 12.5% polyacrylamide gel in the Mini-Protean II cell and then stained with 0.1% CBB-R250 or Silver (Figure 13 A, B). A 2-D SDS-PAGE standard contained the proteins that have pI values in rage from 4.5 to 8.5 and molecular weights in range from 17,500 to 76,000 daltons. The standards were consisted of hen egg white conalbumin type I (MW; 76 KDa, pI values; 6.0, 6.3, 6.6), bovine serum albumin (BSA) (MW; 66.2 kDa, pI values; 5.4, 5.6), bovine muscle actin (MW; 43 kDa, pI values; 5.0, 5.1), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (MW; 36 kDa, pI values; 8.3, 8.5), bovine carbonic anhydrase (MW; 31 kDa, pI values 5.9, 6.0), soybean trypsin inhibitor (MW; 21.5 kDa, pI value; 4.5), and equine myoglobin (MW; 17.5 kDa, pI value; 7.0). However, for all of experiments the standard protein spots could not be seen entirely and the protein spots of conalbumin were always shifted to the basic part (Figure 13 A, B).

The length of first dimension gel was approximately 7 cm. The migrated distances of each standard protein from the basic to acidic part were 0-1 cm for conalbumin, 2-3.5 cm for BSA, 4-5 cm for actin, 0-1cm for GAPDH, and 6.5 cm for trypsin inhibitor (Figure 13 A, B). As a result, the pI values of the 88 kDa and 50 kDa were determined by comparing their migration distances with the standard proteins. In addition, the gels of samples and standards were matching on two-criteria. One is absolute spot position and the other is relative spot position and intensities. The migrated distance of 88 kDa protein was streaked from 3-7cm (Figure 14 A) and 4.5-7.0 cm for 50 kDa (Figure14 B). However, highly intensity of the 88 kDa protein ranged from 4.5-6.5 cm (7.5 cm length of gel) and of the 50 kDa protein ranging from 5.0-7.0 cm (7.5 cm length of gel). Consequently, the pI values of the 88 kDa protein were estimated to be 4.5 to less than 5.6, between trypsin inhibitor (pI 4.5) and BSA (pI 5.4, 5.6). For 50 kDa protein, the pI values were estimated to be 4.5 to less than 5.1 between trypsin inhibitor (pI 4.5) and actin (pI 5.0, 5.1). Subsequently, the Western blot analysis was carried out to confirm anitgenicity by probing with pooled sera from

10 AIDS patients infected *P. marneffei*. The result showed that the antigenicity of both proteins, 88 kDa and 50 kDa, were retained (Figure 15). In addition, the crude concentrated culture filtrate proteins of *P. marneffei* (391H and 302BM) yeast-form were also separated on two-dimensional gel electrophoresis and Western blot analysis. Twenty-five microliters of the samples were loaded on the first dimension gel (4%T 5.4%C), and the gel was then placed onto the second dimension slab gel (12.5%T 2.67%C). The second dimension gel was stained for proteins with 0.1% CBB-R250 and the other gel was tested for the antigenicity by Western blot analysis probing with pooled sera from 10 AIDS patients infected with *P. marneffei*. The protein profiles of crude culture filtrate antigens from *P. marneffei* 391H and 302BM seen on second dimension gel were similar (Figure 16 A, B). From basic to acidic end, the 88kDa protein intensity ranged from 4.5-7.5 cm and 5.5-7.0 cm (7.5 cm length of gel) for the 50 kDa protein of *P. marneffei* 391H (Figure 16 A). From *P. marneffei* 302BM, the 88 kDa protein intensity ranged from 3-5.5 cm and 4.5-7 cm (7.5 cm length of gel) for the 50 kDa (Figure 16 B). However, the immunogen profiles of the interesting proteins from both isolates of *P. marneffei* separated by the two-dimensional gel electrophoresis were marked similar (Figure 17 A, B) and corresponding with the positions of protein spots in Figure 16 A and B, respectively.

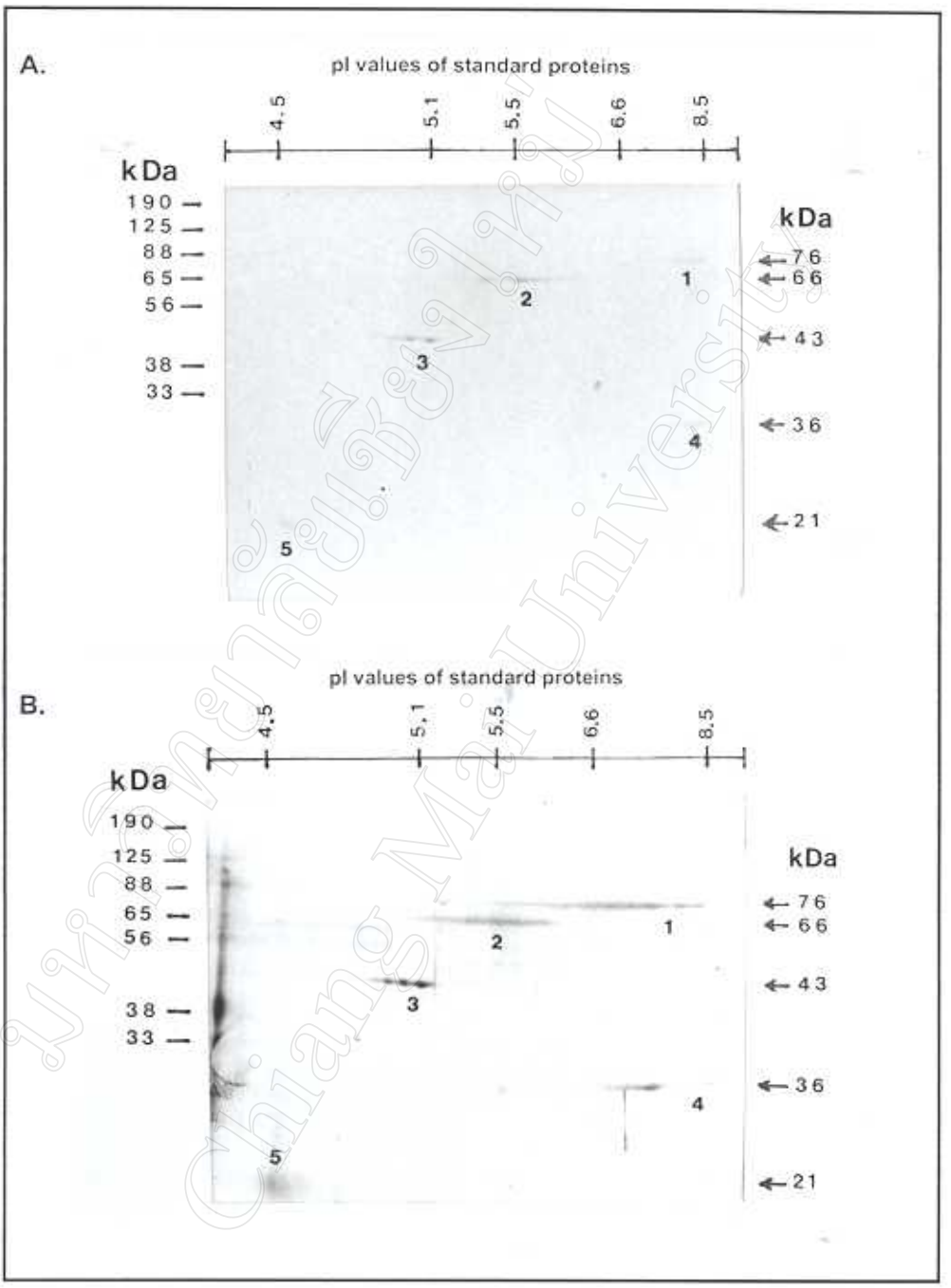

Figure 13. Two-dimensional electrophoretic protein pattern of 2-D SDS-PAGE standard that was separated in the Mini-Protean II cell. Five microliters of the standards were run on IEF gel (4%T 5.4%C) at 750 V 5 hr, and followed with second dimension slab gel (12.5%T 2.6%C) at 150 V 1 hr. The second dimension gel was stained with 0.1% CBB R-250 (A), and silver (B). The standard protein spots are as follows: 1) conalbumin (76 kDa; pI 6.0, 6.3, 6.6), 2) albumin (66 kDa; pI 5.4, 5.6), 3) actin (43 kDa; pI 5.0, 5.1), 4) GAPDH (36 kDa; pI 8.3, 8.4), and 5) Trypsin inhibitor (21.5 kDa; pI 4.5). Their migration distances from basic to acidic part were 0-1 cm, 2-3.5 cm, 4-5 cm, 0-1 cm and 6.5 cm, respectively.

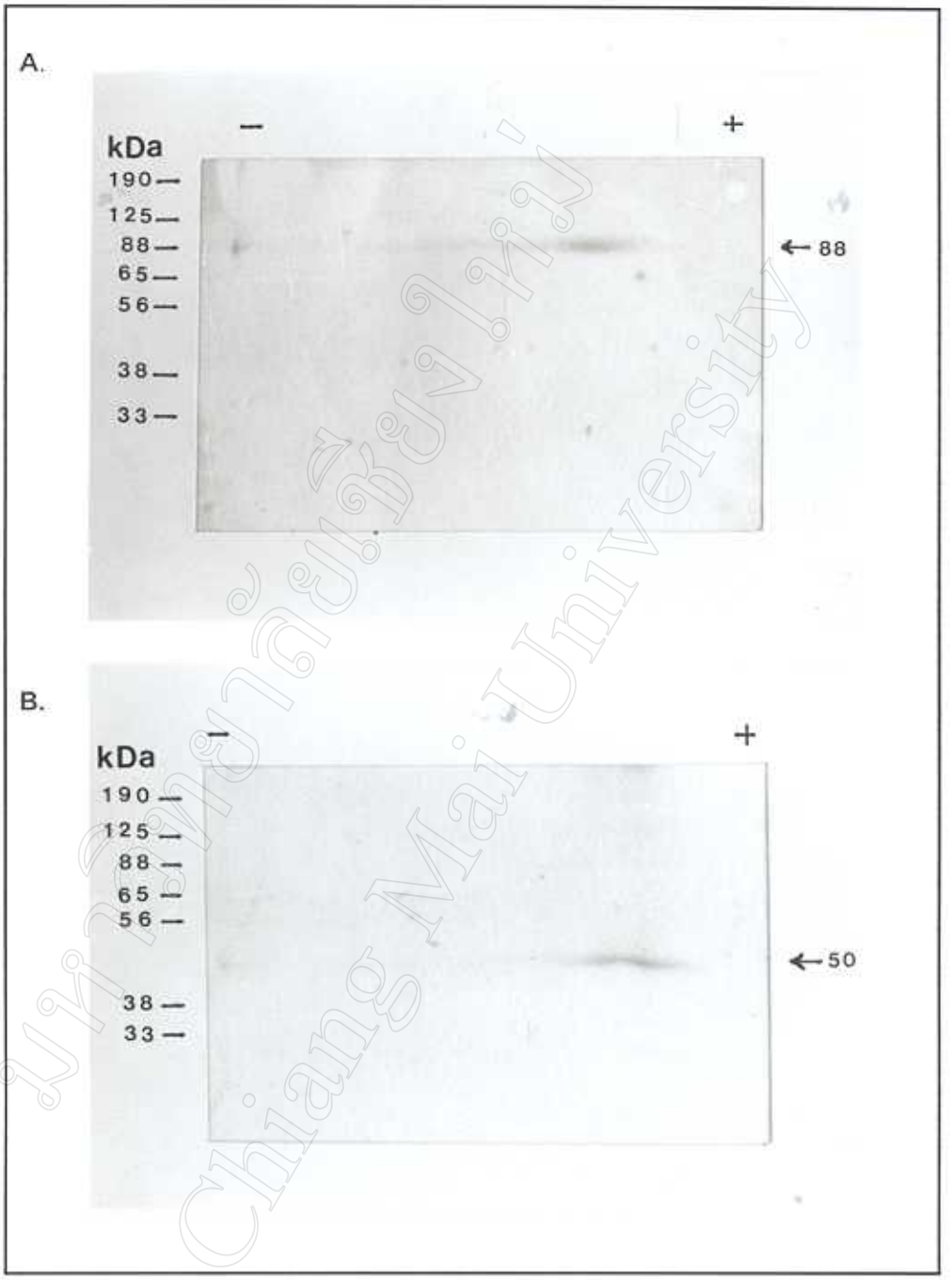

Figure 14. Two-dimensional electrophoretic protein pattern of partially purified proteins [88 kDa (A), and 50kDa (B)] that were separated in Mini Protean II cell. Twenty microliters of each partially purified protein (88kDa and 50 kDa) was loaded onto IEF gel and followed with second dimension gel as described in Figure 13. From basic to acidic end, the 88 kDa protein (A) was streaked from 3-7cm (7.5 cm length of gel), but the high intensity of protein was at 4.5-6.5 cm. The pI values of this protein was approximately $\leq$4.5 to 5.6 when it's migration distance was comparative with standard proteins. The 50 kDa protein (B) was streaked from 4.5-7.0 cm (7.5 cm length of gel), but the protein intensity was at 4.5-6.5 cm. The pI value of this protein was approximately $\leq$4.5 to 5.1.

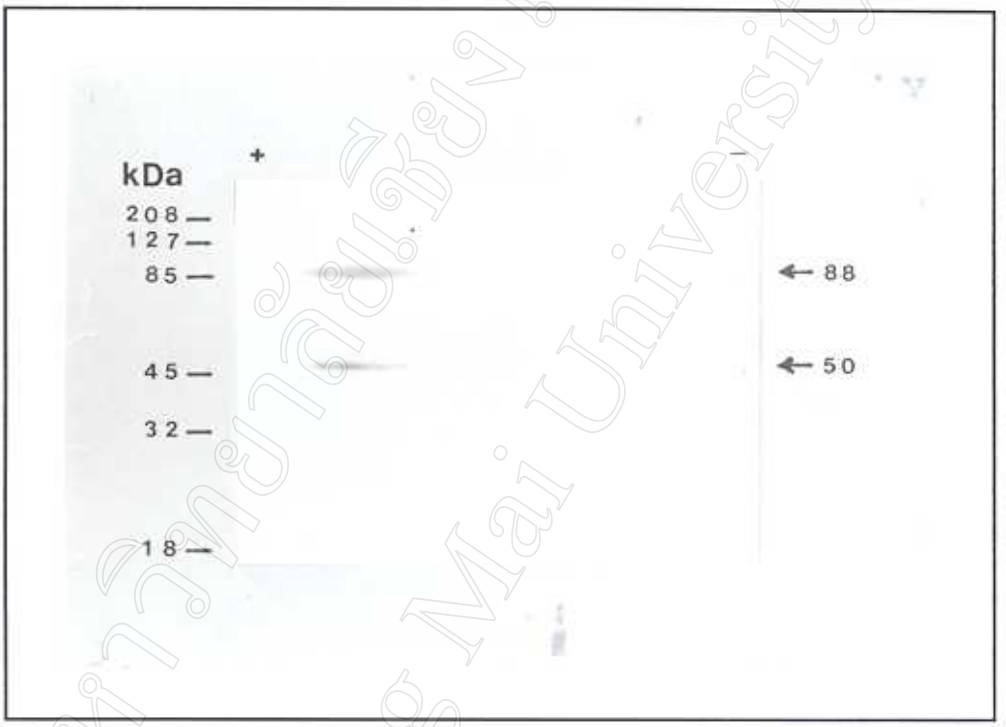

Figure 15. Immunogenic profiles of partially purified proteins (88 kDa and 50 kDa) that were reacted with pooled sera from 10 AIDS patients infected with *P. marneffei* after separating on two-dimensional gel electrophoresis and transferring onto nitrocellulose membrane. Ten microliters of each protein were mixed before loading onto IEF gel. Highly immunogenic reactivities of both proteins were nearby acidic part.

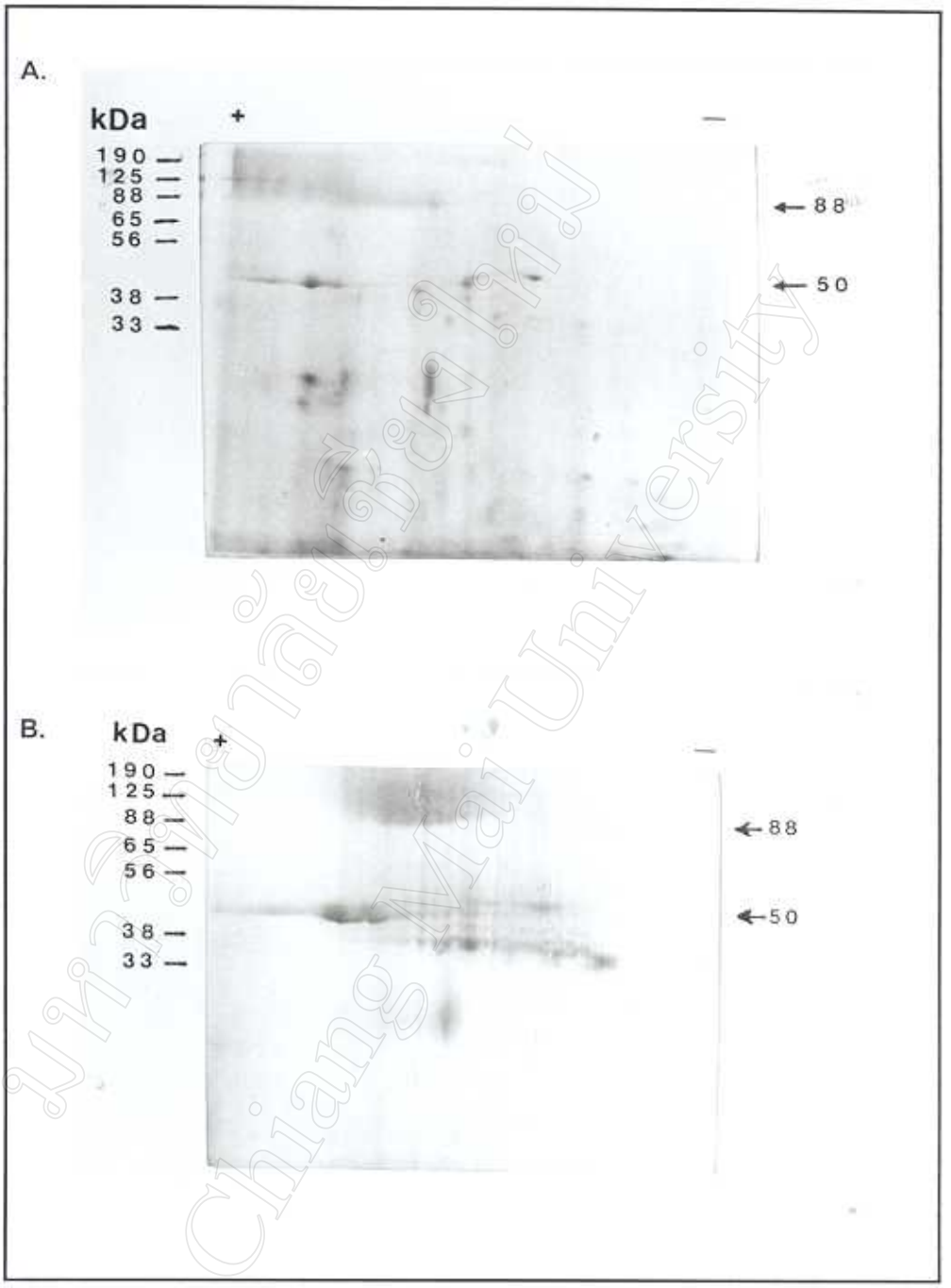

Figure 16. Two-dimensional electrophoretic protein profiles of crude concentrated culture filtrate proteins from *P. marneffei* 391H (A) and *P. marneffei* 302BM (B) which were separated in Mini-Protean II cell. Twenty-five microliters of crude proteins were loaded onto IEF gel (4%T 5.4%C) run at 750 V 5 h, and followed by polyacrylamide slab gel (12.5%T 2.6%C) at 150 V 1 h. The various protein spots were seen after the slab gel was stained with CBB-R250. The intensity of protein (88 kDa) was streaked nearby acidic part from 4.5-7.5 cm. The 50 kDa protein spots were also streaked from 5.5-7.0 cm. The positions of protein spots on two gels (A and B) were similar and the positions of proteins (88 kDa and 50 kDa) corresponded to the sites of the partially purified proteins in Figure 14 A, B. The pI values of these proteins were approximately $\leq$ 4.5 to 5.1 when their migration distances were compared with standard proteins.

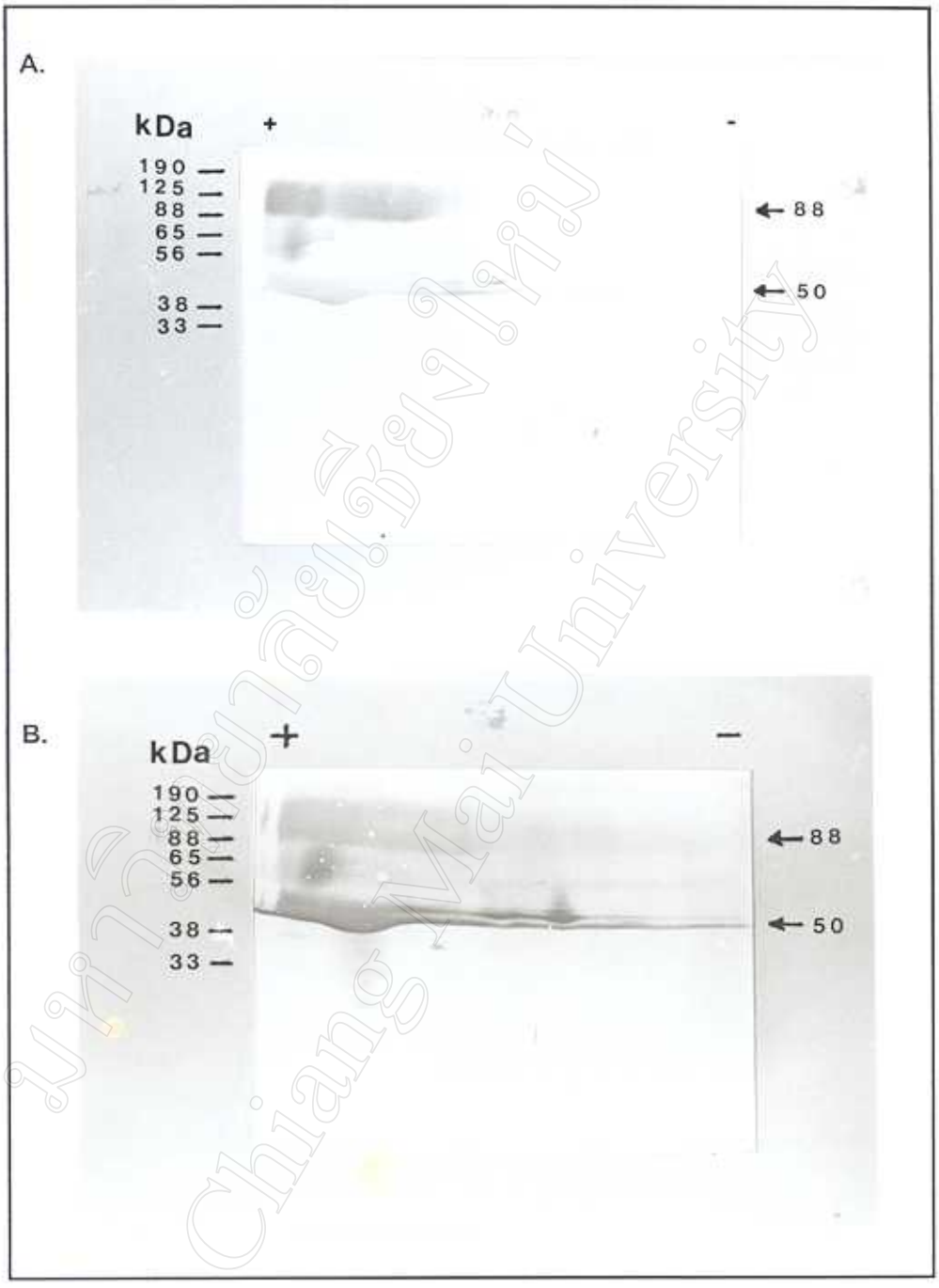

Figure 17. Western blot analysis of crude proteins of *P.marneffei* 391H (A), and 302BM (B) that were separated by the two-dimensional gel electrophoresis. After separating and transferring the crude proteins (15 µl) onto nitrocellulose membrane as described under materials and methods, the membrane was reacted with pooled sera of 10 AIDS patients with penicilliosis at serum dilution of 1:1,000. The positions of highly immunogenic proteins (88 kDa and 50 kDa) of *P. marneffei* [391H (A) and 302BM (B)] were similar and their immunoreactivity corresponding the sites of protein spots on the CBB R-250 stained gel in Figure 16 A, B.

D. Glycoprotein detection on nitrocellulose membrane

After partially purified proteins (88 kDa and 50 kDa) were separated by the two-dimensional gel electrophoresis, the proteins located on second dimension gel were transferred onto nitrocellulose membrane. The transferred proteins were detected for glycoprotein by using Immun-blot kit (BIO-RAD laboratory). The sensitivity of the test was 25 ng when ovalbumin (45kDa) was used as the positive control (Figure 18). The spot of 88 kDa protein was strongly positive and the 50 kDa protein was weakly positive, although they were streaked (Figure 19). In addition, the partially purified proteins (88 kDa and 50 kDa) from the Prep-cell and from the eluted gel cut were also detected for glycoprotein on the membrane after separating and transferring from SDS-PAGE gel (10%T 2.67%C) as shown in Figure 18; lane 7 and 8, respectively. These results indicated that the 88 kDa protein was a large glycoprotein with high carbohydrate moiety, where as the 50 kDa protein had very low carbohydrate moiety.

E. Lectin blotting

The lectins used in this study were concanavalin A (Con A) and wheat germ agglutinin (WGA) conjugated-horseradish peroxidase (HRP). The proteins from second dimension gel were transferred onto nitrocellulose membrane and reacted with these lectins. The signal was detected by adding chromogenic substrate (4-chloro-1-naphthol and H_2O_2). The 88 kDa protein gave strongly positive signal (dark purple color) with Con A, a characteristic indicative of a mannoprotein (Figure 20). In contrast, the 50 kDa protein was weakly positive indicated that this protein consisted of a few mannoprotein (Figure 20). Both proteins of 88 kDa and 50 kDa were not reacted with wheat germ agglutinin (data not shown), suggesting that these proteins did not have N-acetylglucosamine or N-acetylneuraminic acid residues. Furthermore, the crude concentrated proteins from three isolates of *P. marneffei* (527, 747, and 827) were also analyzed with SDS-PAGE (10%T 2.6%C) and lectin blotting technique. The results were correlated with the previous experiments with purified 88 and 50 kDa proteins. The Con A could react with separated proteins ranging from 31 to 116 kDa with high intensity at the 88 kDa band and very low signal at the with 50 kDa band (Figure 21). Wheat germ agglutinin could react weakly with proteins of low molecular weight ranging from 39 to 66 kDa including the 50 kDa band (Figure 22). This result contrasted with the previous experiment.

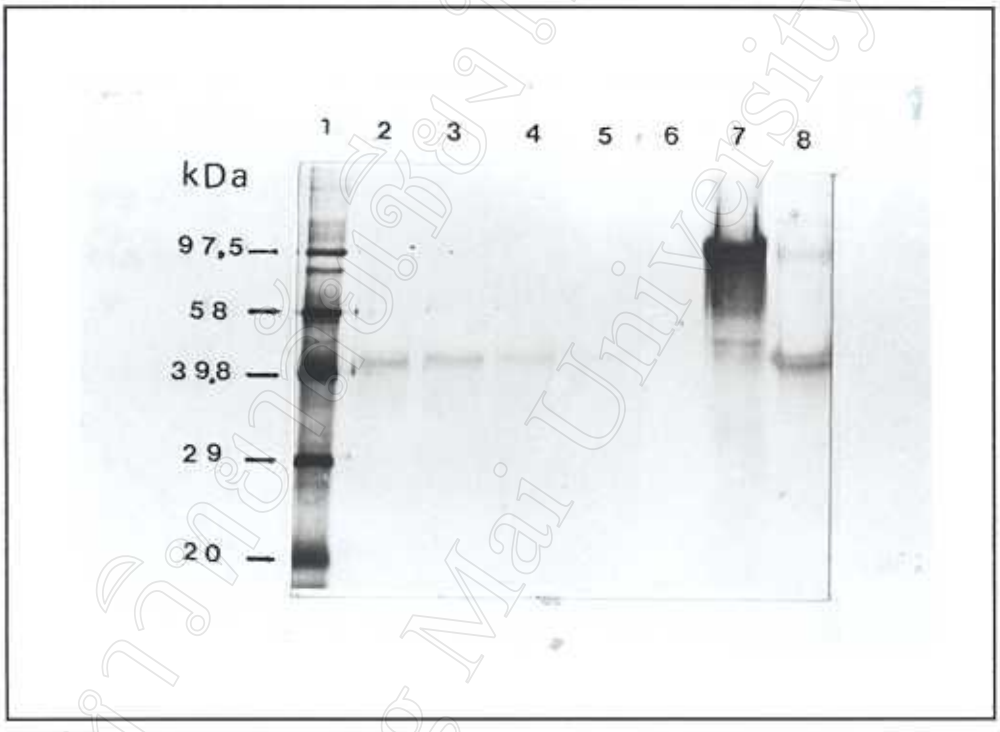

Figure 18. Sensitivity test of Immun-blot kit (BIO-RAD laboratory) for glycoprotein detection on the membrane was done after loading the various concentrations of ovalbumin (45 kDa) on one dimension SDS-PAGE (10%T 2.6%C) and transferring the proteins onto nitrocellulose membrane. The glycoproteins on the membrane were detected as described in materials and methods. Lane 1: biotinylated markers (5 μl), lane 2-5: ovalbumin of 200, 100, 50, and 25 ng, respectively, lane 6: β-glactosidase (127 kDa) from *E.coli* 200 ng (negative control), lane 7: partially purified 88 kDa protein (10 μl), and lane 8: partially purified 50 kDa protein (10 μl).

Figure 19. Glycoprotein detection of two-dimensional electrophoretic protein profiles of partially purifiled proteins (88 kDa and 50 kDa) on the membrane after separating and transferring as described in materials and methods. The 88 kDa and 50 kDa proteins (10 µl of each) were mixed before loading onto IEF gel run at 550 V 6h, and followed with second dimension slab gel (10%T 2.6%C) run at 100 V 1 h 30 min. The sugar residues were detected as described by manufacture. The 88 kDa protein was strongly positive, and 50 kDa was weakly positive at the acidic part.

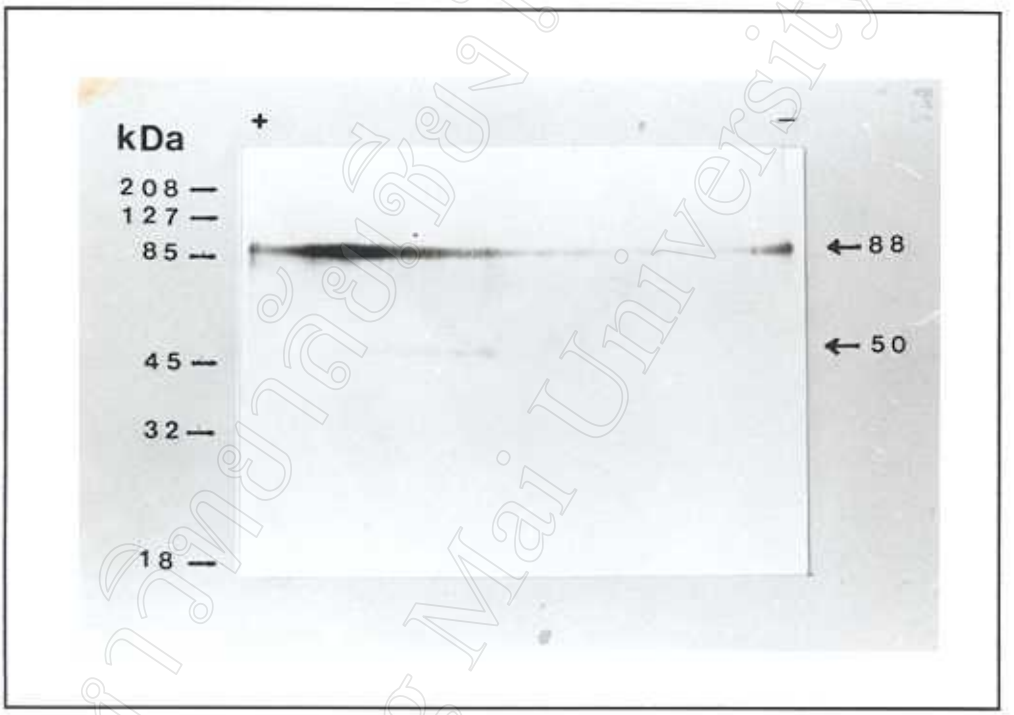

Figure 20. Concanavalin A blotting of partially purified proteins (88 kDa and 50 kDa) on the membrane after the proteins were separated and transferred as described in Figure 19. The proteins on membrane were reacted with Con A conjugated-HRP at a dilution of 1:2,000 and the signal was detected by incubating with chromogenic substrate (4-chloro-1-naphthol and H_2O_2). The 88 kDa protein was strongly positive, but the 50 kDa protein was weakly positive. The intensity of signal was at the acidic part.

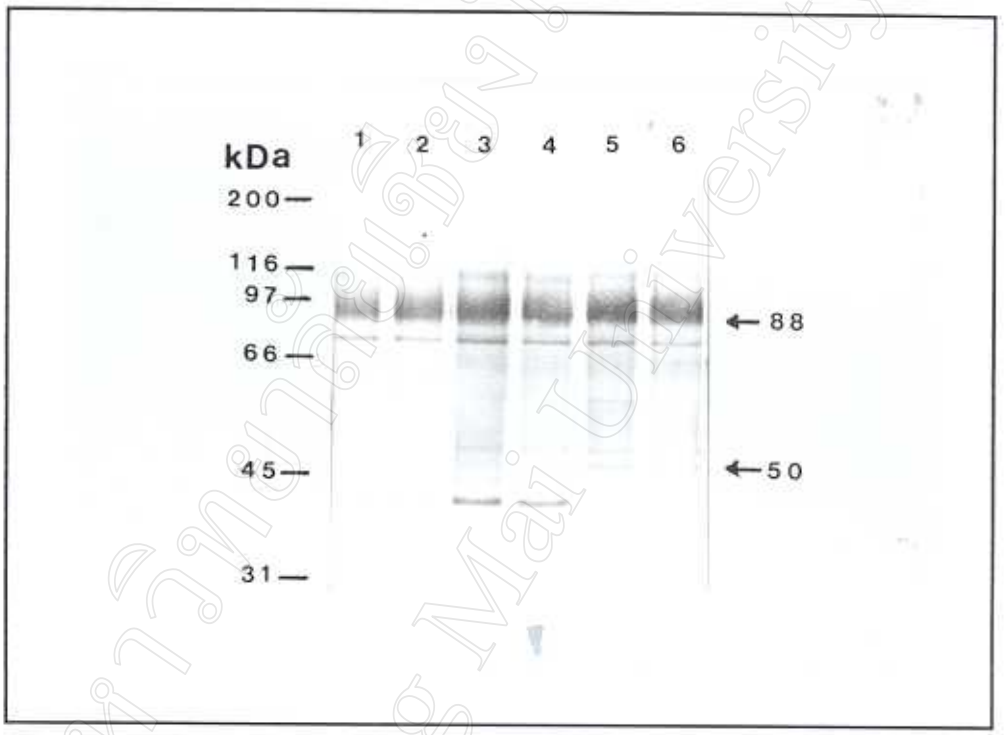

Figure 21. Concanavalin A blotting of crude concentrated proteins from three isolates of *P. mameffei* (lane 1, 2: isolate 527; lane 3, 4: isolate 747; lane 5, 6: isolate 827) on the membrane. Two concentrations of each (1:20 and 1:30, respectively) were separated on one dimension SDS-PAGE (10%T 2.6%C) run at 100 V for 2 h. The separated proteins were transferred onto nitrocellulose membrane and the proteins on membrane were reacted with Con A-conjugated HRP at a dilution of 1:2,000. Signaling was detected with 4-chloro-1-naphthol and H_2O_2. Con A could react with the proteins ranging from 30-116 kDa with intense signal at 88 kDa protein.

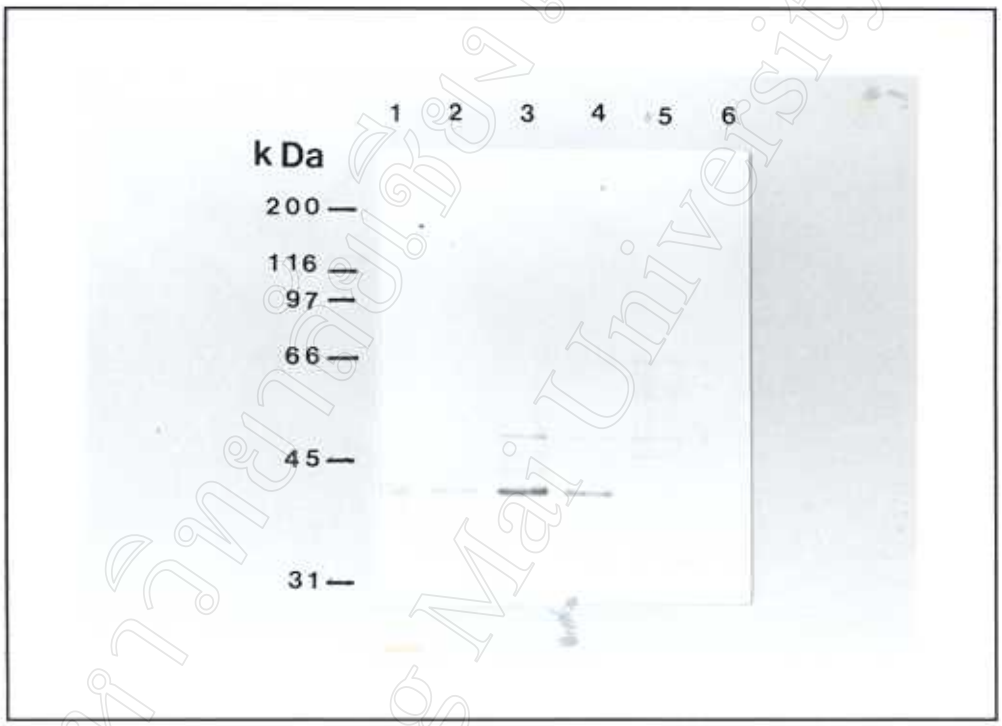

Figure 22. Wheat germ agglutinin blotting of crude concentrated proteins from three isolates of *P. marneffei* (lane 1,2: isolate 527; lane 3,4: isolate 747; lane 5,6: isolate 827) on the membrane after the proteins were separated and transferred as described in materials and methods. The loading pattern and concentrations of each crude protein were the same as in Figure 21. The proteins on membrane were reacted with wheat germ agglutinin-conjugated HRP at a dilution of 1:2,000. The wheat germ agglutinin could react with some proteins of three isolates of *P. marneffei* ranging from 39 to 66 kDa with intense signal at 39 kDa protein, but not react with the higher molecular weight proteins.