# III. MATERIALS AND METHODS

## A. Preparation of crude culture filtrate proteins

*Penicillium marneffei* 391H and 302BM were clinical isolates from Chiang Mai University Hospital. The organisms were kept on Sabouraud's dextrose agar (SDA) at 25°C. They were subcultured on Brain heart infusion (BHI) agar slants and grown at 37°C for 4 days to obtain the yeast form. Three milliliters of normal saline solution with 0.05% tween 80 was added to each slant. The yeast cells were scraped from the surface of media by using sterile loop and transferred into 250 ml erlenmeyer flask containing 50 ml of BHI broth. The cultures were incubated in an orbital shaker bath (37°C, 140 rev $min^{-1}$) for 2 days. Subsequently, 25 ml of the 2-day-culture broth was used to inoculate 500 ml of BHI broth in a 2 liters erlenmeyer flask and was incubated under the same condition for 4 days. The culture was harvested by addition of merthiolate solution at a final concentration of 0.01% and allowed to stand at room temperature (RT) for 24 h, and then filtered twice through filter papers (Whatman no.1). Thereafter, the volume of culture filtrate was measured. The enzyme inhibitors (50 mM iodoacetate, 50 mM phenylmethanosulfonyl fluoride and 500 mM EDTA) were added to it to give final concentrations of 10 μM, 0.1 mM and 1 mM, respectively. Thereafter, it was passed through the filter membrane (pore size of 0.45 μm), and was concentrated by precipitating the protein in 70% ammonium sulfate, as described previously (Mekaprateep, 1995). The protein pellet were resuspended with phosphate buffer saline (pH 7.2) for a few volume and then dialyzed at 4°C against 0.1% NaCl solution and deionized water by using dialysis bag that has pore size about 12,000-14,000 daltons. Subsequently, it was concentrated approximately 50X of the original volume by using ultrafiltration (YM 10) and the concentration of proteins was determined by dye-binding method. The concentrated proteins were stored in small aliquots at -20°C until used.

Proteins concentration of crude concentrated antigens from *P. marneffei* 391H and 302BM was determined by dye-binding method that proceeded with dye reagent from BIO-RAD (California, USA). The method was carried out with standard procedure

for microtiter plates assay using various concentrations of BSA (0.5, 1, 2, 3, 4, and 5 μg) to set the standard curve. Samples of 10 μl were added to 200 μl diluted dye reagent (concentrated dye reagent 1 part: $ddH_2O$ 4 parts) in each well, made for triplicate. After 5-60 min color developing time at RT, the mixture was measured for color intensity at O.D. 595 nm that lonely diluted dye reagent served as blank. Following, the graph was plotted between concentrations of BSA and O.D. values. The concentrations of culture filtrate proteins from *P.marneffei* 391H and 302BM calculated from standard curve were 3.7 and 3.0 mg/ml, respectively (Figure 1).

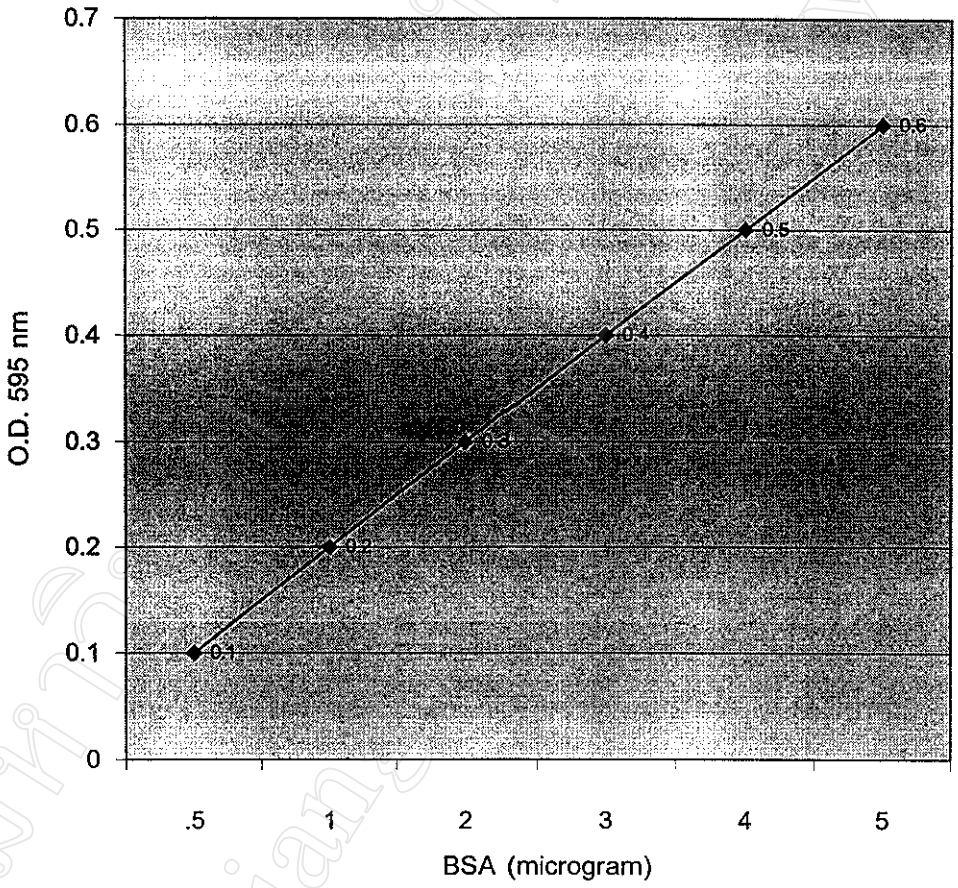


**Figure 1.** Standard curve for protein concentration determination using bovine serum albumin as standard

**B. Detection of immunogenic protein in crude concentrated culture filtrate antigens**

The procedure was conducted in three stages: 1) protein components in crude concentrated antigens were separated on polyacrylamide gel electrophoresis (PAGE) in the presence of a reducing agent and sodium dodecyl sulfate (SDS); 2) the resolved protein bands were electrophoretically blotted onto nitrocellulose membranes ; 3) the antigens fixed on blotted nitrocellulose membrane were identified by Western blotting technique.

**1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)**

Vertical SDS-PAGE was done by using the discontinuous buffer system of Laemmli with 10 % polyacrylamide (Laemmli, 1970), which was able to separate the proteins in the range of 15-200 kDa.

1.1 Preparation of slab gels

The clean glass plates (7 x 10 cm and 8 x 10 cm) were assembled with 0.75 mm spacers in the gel casting stand. The separating gel monomer solution for 2 gels was prepared. The solution contained 10 % acrylamide, 0.1% SDS and 375 mM Tris-HCl (pH 8.8). It was poured into the gel cassette, using a Pasteur pipette, to about 0.7 cm above the level which will be occupied by the well-forming combs. Isopropanol was immediately overlaid and the gels were allowed to polymerized for 1-2 h at RT. After pouring off the alcohol, the area above the polymerized gel was dried with filter papers. Five milliliters of the stacking gel monomer solution containing 4% acrylamide, 0.1% SDS and 125 mM Tris-HCl (pH 6.8) was laid over the separating gel and the combs were inserted carefully. The gels were allowed to polymerize at RT for at least 30 min. Thereafter, the combs were removed gently. All wells were blotted carefully with filter papers to remove resting fluid and air bubbles. The gels were ready for sample application.

1.2 Preparation of the samples

Samples were solubilized in equal volume of 2X sample buffer, containing 62.5 mM Tris-HCl (pH6.8), 5% SDS,10% glycerol, 5% 2-$\beta$-mercaptoethanol and 0.01%

bromphenol blue, and denatured at 100°C for 3 min. The volume loaded per well was 10 µl (for10 lane comb). The concentration of proteins was about 7.5 µg/ well.

1.3 Electrophoresis

After loading the samples and standard protein markers, the gel cassettes were installed in the electrophoretic apparatus. The electrode reservoir buffer solution (0.1% SDS, 192 mM glycine and 25 mM Tris-HCl) was added into the inner and outer chambers. Electrophoresis was carried out with a constant voltage of 100 V (per 2 gels) for approximately 1 h 45 min. The bromphenol blue tracking dye was observed until it ran from the bottom of the gels into the buffer for about 25-30 min, the electrophoresis was then stopped. The gels were removed from the glass plates for staining or electro-transferring to nitrocellulose membranes.

1.4 Coomassie brilliant blue staining

Protein bands were stained by soaking (with agitation) for 30 min with Coomassie brilliant blue R-250 solution (0.1% CBB R-250 in 40% MeOH, 10% acetic acid and distilled water). Excess stain and background were removed from the gels by agitating in acid methanol solution (40% MeOH, 10% acetic acid and distilled water) for about 1-3 h.

1.5 Gel drying

The stained gels, they were soaked overnight in distilled water. The gels were placed on filter paper (Whatman no.1), which was cut larger than the gel's size. They were dried by using Slab Gel Dryer (SAVANT, BIO-RAD) at 65°C for 1 h 30 min.

1.6 Molecular mass determination

The molecular mass could be determined by comparing the electrophoretic mobility of any unknown component with standard protein markers. The standard curve was obtained when a relative mobility of the migrated standard proteins distances was plotted against logarithmic values of their molecular mass (Figure 2). The standard proteins used in the present study were Kaleidoscope Prestained Standards (BIO-RAD) containing: myosin (208 kDa), β-galactosidase (127 kDa), bovine serum albumin (85 kDa), carbonic anhydrase (45 kDa), soybean trypsin inhibitor (32 kDa), lysozyme (18 kDa), and aprotinin (7 kDa).

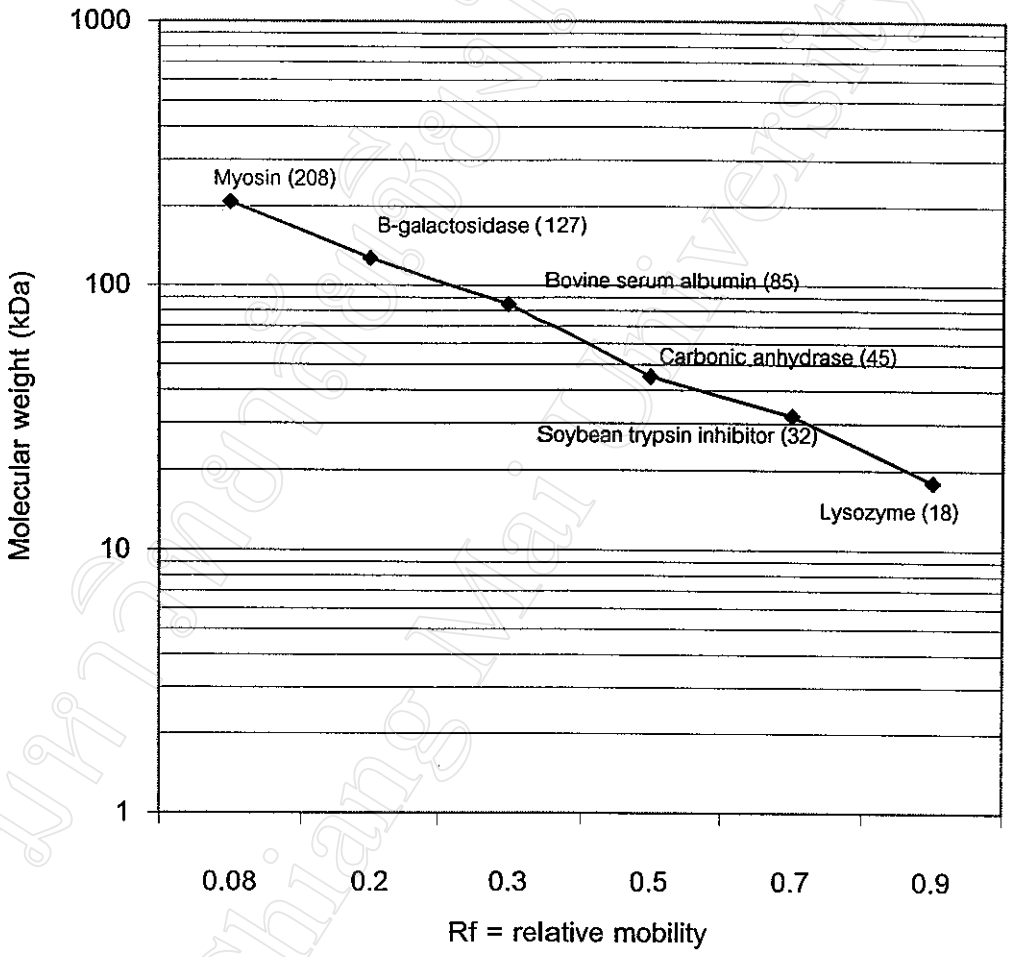


**Figure 2.** Standard curve used for molecular weight estimation of various extracellular proteins of *P. marneffei*

**2. Electrotransferring**

2.1 Transfer proteins from gel to nitrocellulose membrane

After electrophoresis, the proteins on gels were electrophoretically transferred to nitrocellulose membranes by the method of Towbin (Towbin et al,1979). Nitrocellulose membrane (0.45 μm of pore size Hybond-C Amersham, Buckinghamshire,UK) and filter papers (Whatman no.1) were cut into pieces at the same size as of the gel. One piece of nitrocellulose membrane, 8 pieces of filter papers and 2 pieces of support pad were needed for each gel. The membrane, filter papers and support pads were soaked with cold transfer buffer for 15-20 min. The membranes were especially slowly slid at 45°. The gels removed from the glass plates were also immersed in cold transfer buffer around 10 min, for desalting. All materials prepared above were tightly assembled as shown in Figure 3. All air bubbles were carefully rolled out in each step. The assembled sandwiches and ice unit were installed in transfer apparatus (Mini Trans-blot Electrophoretic Transfer cells, BIO-RAD). Chilled transfer buffer (25 mM Tris base pH 8.3, 192 mM glycine containing 20% methanol) was filled in the chamber. Transfer was done under a constant voltage of 110 V for 45-50 min at 4°C (initial) to 25°C (ending). After transfer, the transblotted nitrocellulose membranes were washed with PBS for 30 min. If the molecular mass standard did not pre-stain, they were cut from the sample blots into a strip and stained with Amido black solution in order to confirm efficiency of blotting and serve as standard markers.

2.2 Amido black staining

The nitrocellulose strips of protein markers were stained with Amido black solution (0.1% Amido black 10B in acid-ethanol solution containing 25% absolute ethanol, 10% acetic acid and distilled water) for 1 min. Excess stain and background were removed from the blots by agitating in acid-ethanol solution for about 15-30 min.

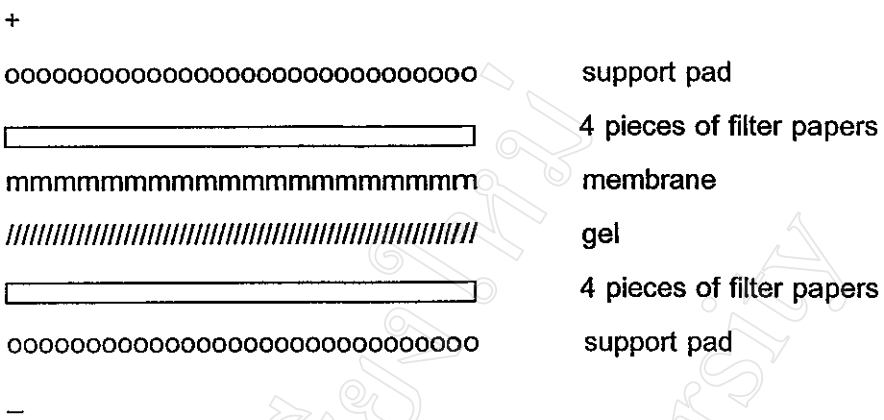


**Figure 3.** Assembly pattern of the materials used in electrotransferring of protein from acrylamide gel to nitrocellulose membrane.

### 3. Immunogenic protein detection

Before the blots could be processed for antigen detection, they had to be blocked by soaking with blocking buffer (5% non fat dry milk in PBS pH 7.2) overnight at 4°C for saturation of free binding sites. After the blots were washed 2 times for 10 min with PBS-T (PBS pH 7.2 containing 0.05% tween-20) by slowly agitation at RT, they were incubated with diluted pooled patient's sera or rabbit anti-*P. marneffei* antiserum (dilution 1: 1000 in PBS-T) and healthy subject's sera or pre-immune rabbit serum (dilution 1:100 in PBS-T). The blots were washed in PBS-T 4 times for 20 min. Subsequently, the blots were exposed to diluted horseradish peroxidase conjugated goat anti-human IgG or anti-rabbit IgG (BIO-RAD) (dilution 1: 2000 in blocking buffer, without sodium azide) for 1 h at RT with slowly agitation and washed in PBS-T 4 times for 20 min. The antigen-antibody complexes were visualized by staining for peroxidase activity with 4-chloro-1-naphthol (Sigma) as substrate. This substrate solution (0.1 ml of stock solution of 30 mg 4-chloro-1-naphthol / ml ethanol in10 ml of 50 mM Tris buffer pH 7.6 and 10 μl of 30% $H_2O_2$) was prepared fresh. The color reaction was stopped by washing with PBS after maximum incubation time of 10 min and allowed to air dry.

**C. Protein purification procedure**

In this study, major immunogenic proteins (88 kDa and 50 kDa) could be separated from crude concentrated proteins by preparative gel electrophoresis using Model 491 Prep cell, BIO-RAD) or Mini-Protean II apparatus (BIO-RAD) with preparative comb (1.0 mm or 1.5 mm of thickness). The separated interesting proteins were further purification by using two-dimensional gel electrophoresis (Mini-Protean II 2-D cell, BIO-RAD). The details of each method are as follows.

**1. Prep-cell preparative gel electrophoresis**

This procedure could purify specific proteins on the basis of size separation from complex mixture by continuous-elution electrophoresis. During a run, proteins were electrophoresed vertically through a cylindrical sieving gel. As individual bands migrated off the bottom of the gel, they passed directly into an elution chamber consisting of a thin frit and should be trapped within the chamber by dialysis membrane (molecular weight cut off 6,000 daltons), directly underneath the elution frit. Whereas, the proteins that have molecular mass less than 6,000 daltons could pass the membrane directly into lower buffer. The retained proteins were washed with elution buffer and were also drawn inward to an elution tube in the center of the cooling core, and out to the peristaltic pump, UV monitor and fraction collector.

1.1 Casting the preparative gel in the gel tube assembly

The clean gel tube assembly (28 mm ID) was assembled with casting stand and adjusted the level of casting stand by using level legs. Thereafter, cooling core was inserted in the gel tube assembly where the center pin on the casting stand was inserted in the elution tube of cooling core. This assembly set was pre-cooled in an ice box and connected to the circulating pump (speed 90 ml/min for 1–2 h). The separating gel monomer solution was prepared by using 7.5% polyacrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.05% ammonium persulfate and 0.05% TEMED. This solution was poured into the gel tube at about 9.5 cm height and immediately overlaid with distilled water about 2-3 cm height. The gel was allowed to polymerize at RT for 1-2 h. After gel polymerization, the water overlay was carefully aspirated from the gel surface by using a syringe and rubber tube. Ten milliliters of the stacking gel monomer solution containing 4% polyacrylamide, 0.125M Tris-HCl pH 6.8, 0.1% SDS, 0.05% ammonium persulfate and 0.1% TEMED was laid over the separating gel about 2.5-3

cm of highness and immediately overlaid with 2-3 ml of distilled water. The stacking gel was allowed to polymerize for 1-2 h. The gel tube assembly containing the gel was removed from the casting stand.

1.2 Assembly of the elution chamber with the gel tube

The soaked support frit was inserted into the base of elution chamber and the dialysis membrane was placed on it, and the elution frit was then placed on the membrane. The gel tube assembly containing the gel was placed on the elution chamber base, tighten by aligning the four screws with the holes in the elution chamber base.

1.3 Assembly of upper and lower buffer chambers

The upper buffer reservoir was carefully placed on the gel tube assembly and seated it firmly. The elution buffer feedline (white cap) from the elution buffer reservoir was connected to the elution port (white port) on the elution chamber cap, while the cooling buffer feedline (clear cap) was connected to the cooling buffer port on the elution chamber cap (clear port). The cooling buffer line (clear cap) from the upper electrophoresis reservoir was connected to one of two clear ports on the cooling core to form a small loop and the second cooling buffer line on the cooling core was connected to cooling pump. The elution tubing was connected to the white elution buffer outlet in the center of the cooling core. The electrode reservoir buffer solution (0.1% SDS, 192 mM glycine and 25 mM Tris-HCl) was filled into the upper electrophoresis buffer reservoir (500-750 ml) and the elution buffer reservoir (900 ml). Before running the cell, air bubbles in the elution chamber must be purged from the elution chamber by using a 20 ml syringe attached to the elution buffer outlet tubing and the buffer was gently pulled through the chamber and pushed (not air) back into the elution chamber. Thereafter, the lower buffer reservoir was filled into the lower buffer tank to cover at least the height of gel and then the whole gel tube assembly was placed into the lower buffer reservoir. The elution buffer outlet tubing was attached to the elution pump, set speed to 1 ml/min. The tubing from the cooling core and the tubing from the cooling valve on the lower buffer chamber were connected to the buffer re-circulation pump providing a flow rate of ~100 ml/min. Pre-run electrophoresis was performed at 200 V for a few minutes to check whether system completely and to purge air bubbles from the lines. All materials were settled at room temperature overnight.

1.4 Samples preparation and running condition

The later day, 3 ml of sample was solubilized in 2X sample buffer containing 62.5 mM Tris-HCl pH 6.8, 10% glycerol, 5% 2-β-mercaptoethanol and 0.01% bromphenol blue for an equal volume and denatured at 100°C for 3 min. The concentration of interested protein should be approximately 1-2 mg of total protein load. The sample solution was gently loaded on the stacking gel surface using a 10 ml syringe with a long needle. Electrophoresis was carried out with a constant voltage of 250 V for approximately 3-4 h in cold room until the bromphenol blue tracking dye ran out of the gel through the elution buffer outlet tubing.

1.5 Fraction collection and analysis

The elution from gel electrophoresis was collected with fraction collector immediately after the tracking dye ran out of the gel. In this study, about 250 fractions were collected. The volume for each fraction was set at 2.5 ml / fraction. The fractions containing the protein of interest were analyzed by SDS-PAGE. Every fifth fraction was screened for interesting proteins. Each fraction was concentrated by acetone precipitation (Ortiz et al, 1996) (300 µl of solution: 875 µl of cold acetone) at -20°C overnight and centrifuged at 4°C 13,000 rpm for 10 min. The pellet was washed with cold acetone once more to reduce SDS which retained in buffer solution. Finally, the protein pellet was dried with speed vacuum at RT for 1-2 h or allowed to dry in the 37°C incubator for 5-6 h. When the fractions containing protein of interest were identified, they were pooled and concentrated by using ultrafiltration and centricon (MW. cut off 10,000 daltons, Amicon). This concentrated protein was confirmed purity, intensity and it's antigenicity by SDS-PAGE / Western blot analysis. This partially purified protein was used for further analysis.

**2. Mini-protein II preparative electrophoresis**

The SDS-PAGE with 10% polyacrylamide was prepared as described in B1 (page 21) except the single lane comb was replaced the comb with multiple lanes. The thickness of comb and the spacer was 1.0 or 1.5 mm. The volume of loading sample was about 100 µl and 150 µl, respectively. The bands of interested proteins were cut with a scalpel after the gel was stained with 0.1% CBB R-250. About 20 gels were performed in the same manner to get enough amount of one interesting protein.

The cut gels were pooled and minced with a 5ml syringe. Thereafter, the proteins were eluted from the minced materials with electro-eluter (BIO-RAD) containing the membrane cap with the molecular weight cut off of 12,000-15,000 daltons. The eluted samples were collected, pooled and concentrated by acetone precipitation (Ortiz et al, 1996). The protein intensity, purity and it's antigenicity were analyzed by SDS-PAGE and Western blotting. These proteins were used for further analysis.

### *3.* Two-dimensional gel electrophoresis

This procedure was conducted to purify protein obtained from Prep-cell preparative or Mini-protein II preparative gel electrophoresis. The proteins could be separated by their differences of isoelectric focusing (IEF) in the first dimension gel and by their differences of size in the second dimension gel (SDS-PAGE). In the first dimension, proteins would be migrated from cathode to anode through polyacrylamide tube gel that contained pH gradients. The proteins would stop at the pH equivalent to their pI values. If the sample is a mixture of proteins of different pI, they could be seen as separated spots of the same size in the second dimension gel. The details of the procedure are as follows.

3.1 First dimensional gel electrophoresis

3.1.1 Casting the tube gels

The casting tube (1.3 cm x 8.5 cm) was sealed one end with several layers of Parafilm. The capillary tubes (1 mm x 7 cm) were filled into the casting tube in the manner that the blue stripped end of the capillary tubes was at the open end of the casting tube. These must be kept vertical with plasticine. Ten milliters of first dimension gel monomer solution containing 9.2 M urea, 4% acrylamide, 2% triton X-100, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, and 0.1% TEMED were prepared by mixing all ingredients except the last two reagents, which were the polymerization catalysts. The vessel was placed in warm water ($\leq$ 45°C) with swirling to dissolve the urea. After the monomer solution was degassed for 15 min, the APS and TEMED were added, swirled gently and briefly. Thereafter, a 10 ml syringe was filled with this solution, a needle was attached and inserted into the casting tube. The capillary tubes were filled with the gel solution from

the bottom. The monomer solution was carefully delivered to about ¾ of the length of the tubes. The gel was allowed to polymerize at RT for 1-2 h. After polymerization, the Parafilm was removed slowly from the bottom of casting tube, and the batch of capillary tubes were pushed out of the casting tube from the top part. The blue stripped end of the tubes were attached to the sample reservoirs with the flexible tubing connectors after they were wiped the extra acrylamide off and the bottom of the tube gel was rinsed with lower buffer (10 mM $H_3PO_4$).

3.1.2 Electrode preparation

A gel tube with reservoir or a stopper was inserted into each of the 16 positions in the tube adaptor. Each sample reservoir was filled with degassed upper buffer chamber electrolyte (100 mM NaOH) by using a 25 µl Hamilton syringe expelled any air bubbles from the capillary space in the neck of the sample reservoir and in the flexible tubing connector. The tube gel adaptor was placed in the lower buffer tank containing a stir bar (1 inch length). The lower tank was filled with approximately 800 ml of lower buffer (10 mM $H_3PO_4$) to reach the maximum blue line level of the glass gel tubes. If air bubbles were trapped at the bottom of each tube, they could be removed using a Pasteur pipette with a curved tip. The degassed upper buffer (approximately 60 ml) was added into the upper chamber to the level slightly above the sample reservoirs but below the top of the plastic bar. The tank was placed on a magnetic stirring plate, and stirred slowly. The tube gels were pre-electrophoresed by running at 200 V for 10 min, 300 V 15 min, and 400 V for 15 min.

3.1.3 Sample preparation and loading

The 25 µl of sample was solubilized with an equal volume of first dimension sample buffer containing 9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholyte, and 0.4% Bio-Lyte 3/10 ampholyte, and then incubated at RT for 10-15 min. The 2.5 µl of IEF marker (BIO-RAD) was added to the sample solution before loading. After pre-electrophoresis, both of the buffer in lower and upper chambers were discarded. Thereafter, the gel tubes and the sample reservoir were filled with fresh degassed upper buffer and all air bubbles were removed with 25 µl Hamilton syringe. The sample was drawn up into 25 µl Hamilton syringe and applied directly onto the first dimension tube gel. The sample was carefully overlaid with sample overlay buffer (25 µl) and followed with upper chamber buffer until filling

the sample reservoir. Fresh degassed upper buffer was added into the upper chamber and fresh-degassed lower buffer was added into the lower chamber as described above.

3.1.4 Running conditions

Electrophoresis was conducted at 500 V 10 min and increased to 750 V 5 h (alternative conditions, 550 V for 6 h) with continuous stirring to prevent heating which can occur in the capillary tubes.

3.2 Second dimensional gel electrophoresis

The second dimension slab gels were carried out according to the instruction manual of the Mini-Protein II as described above while the first dimension tube gels were running. The stacking gel was cast to a maximum level of 5 mm by pushing the 2-D comb (thickness of 1 mm) about halfway in. After the first dimension electrophoresis was completed, each gel was removed from the tube with tube gel ejector attached to a 1.0 ml syringe containing electrophoresis buffer or distilled water. The tube gel was extruded onto a piece of Parafilm and wetted it slightly with SDS sample equilibration buffer. It was carefully slid from the film between the glass plates and laid onto the slab gel. The tube gel was then covered with 200 µl of SDS sample buffer, and allowed to settle down for up to 10 min. After equilibration, the running buffer was overlaid and also filled the electrode buffer reservoirs. Electrophoresis was carried out with a constant voltage of 100 V for approximately 1 h 30 min. The electrophoresis was stopped when the dye indicator ran to approximately 1 mm from the bottom of the gel. The slab gels can be stained with CBB R-250 or silver, and sometimes can be transferred onto nitrocellulose membrane for immunoblotting as described above or for other chemical analysis.

**D. Glycoprotein detection**

Sugar residues in glycoconjugates were detected by Immun-blot kit (Bio-Rad Laboratory). After the protein was transferred from 2-D gel to nitrocellulose membrane, the membrane was washed with PBS (9 mM sodium phosphate, 27 mM sodium chloride, pH 7.2) at RT for 10 min (x2, 5 min each) with gentle agitation. The nitrocellulose membrane was incubated with fresh 10 ml of 10 mM sodium periodate in

sodium acetate/EDTA buffer (100 mM sodium acetate / 5 mM EDTA) in the dark (cover dish with aluminum foil) at RT for 20 min with gentle agitation. After that, the membrane was washed for 12 min (x3, 4 min each) with PBS and it was immersed in fresh prepared biotinylation solution containing 2 µl hydrazide solution in 10 ml sodium acetate/EDTA buffer and then incubated at RT for 60 min with gentle agitation. The membrane was washed 3 times (4 min each) with 10 ml TBS (50 mM Tris, 27 mM sodium chloride, pH 7.2). The membrane was incubated overnight at 4 °C in blocking solution (0.5 g of vial C in 100 ml TBS) to saturate additional protein-binding sites, and washed with 10 ml TBS 3 times for 4 min of each. It was then incubated with 5 µl Streptravidin–alkaline phosphatase conjugate solution in10 ml TBS at RT for 60 min with gentle agitation. The membrane was washed with 10 ml TBS 3 times for 4 min of each and subsequently incubated with color development solution, containing 50 µl NBT and 37.5 µl BCIP in 10 ml of color development buffer (1.21 g Tris, 1.01 g $MgCl_2.6\ H_2O$, 0.58 g NaCl and 90 ml $ddH_2O$), at RT without agitation for development to desired intensity (3-60 min). Later, the development was stopped by rinsing the membrane several times in $ddH_2O$ and allowed to air dry.

**E. Lectin blotting**

Glycoproteins on nitrocellulose membrane were determined for their carbohydrate components by using lectin blotting. These lectins were concanavalin A (Sigma) which reacts with α-mannose and α-glucose and wheat germ agglutinin (Sigma) which reacts with N-acetylglucosamine and N-acetylneuraminic acid (Rosenberg IM, 1996) that had been conjugated with enzyme horseradish peroxidase. The glycoprotein binding membrane was reacted with conjugated lectin. If it has carbohydrate residues that can bind with each lectin, the dark purple color would be appeared after the membrane was immersed in chromogenic substrate (4-chloro-1-naphthol and $H_2O_2$) for few min. This procedure is as follows:

After separating and transferring proteins from 2-D gel onto nitrocellulose membrane, the membrane was incubated overnight with blocking solution (5% BSA in TTBS, pH 7.5) and washed 2 times with TTBS containing 0.05% tween 20, 150 mM NaCl and 10 mM Tris-HCl. To detect their carbohydrate components, the membrane was incubated with lectin-conjugated enzyme peroxidase dilution 1:2000 in TTBS at RT

with gentle agitation for 1 h, and then washed the membrane 4 times for 5 min of each. The fresh chromogenic substrate was prepared containing 2 ml of stock chromogen (60 mg 4-chloro-1-naphthol in 20 ml ice cold methanol ) and 10 ml of TBS (0.2 M NaCl, 20 mM Tris-HCl, pH 7.5). Before using this solution, 6 µl of 30% $H_2O_2$ was added and then it was used to incubate with the membrane for 10 min. After that, the membrane was washed 5 times for 10 min of each with distilled water, and then allowed to air dry.