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APPENDIX I

CHEMICAL AGENTS AND INSTRUMENTS

A. Chemicals

Acrylamide (Sigma, USA)

Ammonium oxalate (Sigma, USA)

Ammonium persulphate (Sigma, USA)

Ammonium sulphate (E.Merck, W. Germany)

Bacto agar (Difco, USA)

Bacto gelatin (BBL, USA)

Bacto peptone (Difco, USA)

Bacto-urea agar base (Difco, USA)

Beef extract (Difco, USA)

Bovine serum albumin (Sigma, USA)

Brain heart infusion broth (BHIB) (Difco, USA)

Complete Freund's adjuvant (Difco, USA)

Decarboxylase media (Difco, USA)

Ethanol (E.Merck, W.Germany)

Ethylene diamine tetra acetic acid (EDTA) (E. Merk, W. Germany)

Folin-Ciocalteau phenol reagent (Sigma, USA)

Formalin (E.Merck, W.Germany)

Freund's complete adjuvant (Difco, USA)

Glacial acetic acid (CH_3COOH) (E.Merck, W.Germany)

Glycerol (BDH, England)

Glycine (Sigma, USA)

Hydrochloric acid (HCl) (E.Merck, W.Germany)

India ink (Pelikan, W.Germany)

L-arginine (Sigma, USA)

L-lysine (Sigma, USA)

L-ornithine (Sigma, USA)

Mac Conkey agar (Lab M, England)

Methanol (CH_3OH) (E.Merck, W.Germany)

Motility test medium (Gibco, USA)

Nitrocellulose paper (Bio-RAD, USA)

Non fat dry milk (Carnation, USA)

N,N-methylene bisacrylamide (Sigma, USA)

N,N,N,N-tetramethylthylenediamine (TEMED) (Sigma, USA)

OF basal medium (Difco, USA)

Potassium chloride (KCl) (E.Merck, W.Germany)

Potassium phosphate dibasic (K_2HPO_4) (E.Merck, W.Germany)

Potassium phosphate monobasic (KH_2PO_4) (E.Merck, W.Germany)

Potassium nitrate (KNO_3), (E.Merck, W.Germany)

Salmonella shigella agar (Eiken, Japan)

Silver nitrate ($AgNO_3$) (E.Merck, W.Germany)

Simmons citrate agar (Difco, USA)

Sodium carbonate (Na_2CO_3) (E.Merck, W.Germany)

Sodium chloride (NaCl) (E.Merck, W.Germany)

Sodium dihydrogen phosphate (NaH_2PO_4) (E.Merck, W.Germany)

Sodium dodecyl sulphate (SDS) (Sigma, USA)

Sodium hydroxide (NaOH) (BDH, England)

Sodium phosphate dibasic (Na_2HPO_4) (E. Merk, W. Germany)

Sulphanilic acid (Sigma, USA)

Tannic acid (E.Merck, W.Germany)

Triple sugar iron agar (TSI) (BBL, USA)

Tris (Hydroxymethyl aminomethane, Tris : $C_4H_{11}NO_3$)

(E.Merck, W.Germany)

Trypticase soy agar (BBL,USA)

Tryptone (Oxoid, England)

Tween 20 (Sigma, USA)

Tween 80 (Sigma, USA)

Xylene (BDH, England)

Zinc dust (Sigma, USA)

B. Antiserum and serum

Normal rabbit serum (Gibco, USA)

Peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins. (DAKO, Denmark)

C. Glasswares

Beaker (Pyrex, Corning, NY., U.S.A.)

Cylinder (Witeg, West Germany)

Erlenmayer flask (Pyrex, Corning, NY, U.S.A.)

Glass tube (Pyrex, Corning, NY, U.S.A.)

D. Instrument

Autoclave

Automatic pipet (EFLAB OY, Helsinki, Findland)

Beckman spectrophotometer

Eppendorf microfuge model 5412 (Beckman Instrument, Inc; Fullerton, California)

Haminton syringe 10 ul

J2-21 centrifuge, Beckman Instrument, Inc, U.S.A.

JA.20.1 angle rotor

Light Microscope (Olympus)

LKB Apparatus 2001 Vertical Electrophoresis

LKB 2003 Slab Gel Dryer

LKB 2202 Ultrascan XL Laser Densitometer

Magnetic stirer

PHM 83 Auto cal pH meter (Radiometer, Copenhagen)

Shaker incubator (Lab-shaker, ADOLF KUHNER AG SCHWEIZ)

Soniprep 150 Ultrasonic Disintegator (MSE Scientific)

Instrument, Manor Royal, England)

Trans-Blot Electrophoretic Transfer cell (Bio-Rad Laboratories, Richmond, California)

Water bath (Julabo, West Germany)

F. Others

Microcentrifuge tube (Treff AG, Schweiz, Switerland.

Microtitration plate, V shape wells (Flow Laboratories U.S.A.)

APPENDIX II

REAGENTS, MEDIA AND PREPARATIONS

1. Reagent for maintenance of the organisms

1.1 0.85% normal saline solution (NSS)

Dissolve 0.5 gm NaCl and make up to 1 L with DW.

1.2 25% glycerol in normal saline

Dissolve 25.5 ml glycerol and make up to 100 ml with NSS

2. Media and reagent for identification of *P. pseudomallei*

2.1 Motility medium

Dissolve 20 gm motility test medium in DW 1 L, by heating, dispensing into 14x100 mm screw-cap tube and autoclave at 121 °C for 15 min.

2.2 Blood agar

Dissolve 14.85 gm trypticase soy agar in DW 250 ml and autoclave at 121 °C for 15 min. Cool the medium approximately 50 °C and add the 12.5 ml of sterile defibrinated sheep blood aseptically to the final concentration of 5%, mixed by swirling with care and pour plate.

2.3 Nutrient broth

Dissolve 1 gm beef extract, 1 gm peptone and 0.5 gm NaCl in DW 100 ml by heating, adjust to pH 7.2-7.4 and autoclave at 121 °C for 15 min.

2.4 6.5% NaCl in nutrient broth

Dissolve 6.5 gm NaCl and 0.2 gm agar in nutrient broth 100 ml, dispense and autoclave at 121 °C for 15 min.

2.5 Mac Conkey agar

Dissolve 51.5 gm Mac Conkey agar in DW 1 L by heating, autoclave at 121 °C for 15 min, allow to cool approximately at 50 °C, pour plate and allow the surface to dry before inoculation.

2.6 SS agar

Dissolve 60 gm SS agar in DW 1 L by heating, pour plate and allow the surface to dry before inoculation.

2.7 Nitrate broth

Dissolve 1 gm KNO_3 in nutrient broth 1 L and distribute into tubes containing invert Durham tube and sterilize at 115 °C for 20 min.

2.8 Urea agar (Christensen's)

Dissolve 29 gm bacto-urea agar base in DW 100 ml and filter 15 gm bacto agar and autoclave DW at 121 °C for 15 min. Add the urea solution into cool agar, then dispense and slant.

2.9 Peptone broth

Dissolve 20 gm tryptone 5 gm NaCl are in DW 1 L, then adjust to pH 7.2-7.4 and sterilize at 115 °C for 20 min.

2.10 Malonate-phenylalanine medium

Disssolve 2 gm $(NH_4)_2SO_4$, 0.6 gm K_2HPO_4, 0.4 gm KH_2PO_4, 2 gm NaCl, 3 gm sodium malonate, 2 gm. DL-phenylalanine and 1 gm yeast extract in DW 1 L by heating and filter add 12.5 ml 0.2% bromthymol blue solution to the medium and sterilize at 115 °C for 20 min.

2.11 Decarboxylase media

Dissolve 9 gm deoxycarboxylase medium base in DW 1 L and adjust to pH 6.0 divide the basal base medium into four equal parts: one part is tubed as a control; to each of the other three parts is added respectively by 1% L-arginine monohydrochloride,1% lysine dihydrochloride and 1% L-ornithine monohydrochloride. Readjust the pH of ornithine portion before the medium is sterilised. Dispend the media in screw-cap tubes and autoclave at 115 °C for 10 min.

2.12 Gelatin medium

Dissolve 15 gm bacto-gelatin in heart infusion broth 100 ml,by heating and adjust to pH 7.0. Dispend the mediumg in screw-cap tube and autoclave at 121 °C for 15 min.

2.13 Tween 80 medium

Dissolve 10 gm.peptone, 5 gm NaCl, 0.1 gm $CaCl_2.H_2O$ and 20 gm agar in DW 1 L by steaming and adjust to pH 7.4. Volume of 500 ml is sterilized in flask which is cooled to 40-50 °C. Sterilize tween

80 at 121 °C and add 5 ml of it to each flask to give a final concentration of about 1.

2.14 TSI

Dissolve 54.9 gm TSI agar in DW 1 L by heating, dispense and then autoclave at 121 °C for 15 min. Cool the medium in a slanted position.

2.15 OF medium

Dissolve 9.4 gm OF basal medium in DW 1 L by heating, adjust to pH 6.8 (the medium was green), then dispense in screw-cap tube and autoclave at 121 °C for 15 min. Cool the basal medium to approximately 50 °C and add the filter-sterile carbohydrates aseptically to a final concentration of 1.

2.16 10% Lactose agar

Dissolve 5 gm peptone,4 gm beef extract, 100 gm lactose and 20 gm agar in DW 1 L by heating, adjust to pH 6.8 with 10 N NaOH, filter and then add 10 ml 0.2% bromcresol purple and dispense. The medium is sterilized at 115 °C for 20 min, and allow to cool in a slanted position.

2.17 MR-VP medium

Dissolve 7 gm peptone, 5 gm K_2PO_4 in DW 1 L by steaming, filter and adjust to pH 7.5. add 5 gm glucose and mix,then distribute in tube and autoclave at 121 °C for 15 min.

2.18 ONPG broth

Dissolve 6 gm ONPG (O-nitrophenyl-B-D-galactopyra-noside) in 0.1 M Na_2HPO_4 pH 7.5 and sterilize by filtration. Add the ONPG aseptically to the peptone water and distribute into sterile tube.

2.19 Starch agar medium

Dissolve 20 gm bacto agar in DW 300 ml by heating. Dissolve 5 gm bacto peptone and 3 gm beef extract in DW 200 ml, mix with agar solution and make up to 1 L with DW add 20 gm soluble starch into the mixture, Dissolve and autoclave at 121 °C for 15 min, then pour plates and keep in a refrigerator.

2.20 Aesculin broth Dissolve 15 gm bacto peptone 1 gm aesculin and 0.5 gm ferric ammonium citrate in DW 1 L,adjust to pH 7.0, then distribute into tube and sterilize at 115 °C for 20 min.

2.21 Citrate agar (Simmons)

Dissolve 24.29 gm Simmons citrate agar in DW 1 L by heating, dispensing into screw-cap tubes, then autoclave at 121 °C for 15 min and allow the medium to cool in a slanted position.

2.22 Reagent for Gram's stain

2.22.1 Crystal violet solution is consisted of soln A and B. Solution A is 13.87 gm crystal violet powder (99% dye content) in 200 ml 95% ethanol.

Solution B is 8 gm ammonium oxalate in 800 ml DW.

Mix soln A and B and allow to stand overnight or until the dye is completely dissolved. Filter the soln through a coarse filter paper.

2.22.2 Gram's iodine is consisted of 1 gm iodine crystal, 2 gm potassium iodide and DW 300 ml.

2.22.3 The decolorizer is 95% ethanol

2.22.4 The counterstain is 3.41 gm safranin-O, 100 ml 95% ethanol and DW 900 ml.

2.23 Reagent for flagella staining

It is consisted of 10 parts of soln A and one part of solution B, which must be freshly prepared. Soln A (mordant) is consisted of 10 ml 5% carbolic acid, 2 gm tannic acid and 10 ml saturated aluminium potassium sulphate . 12 hydrate.

Solution B (stain) is consisted of 12 gm crystal violet in 100 ml ethanol.

2.24 Oxidase test reagent

1% Tetramethyl-p-phenylenediamine dihydrochloride aqeous solution. The reagent should be colorless and be stored in a glass-stoppered bottle and protected from light, at 4 °C

2.25 3% hydrogen peroxide

Mix 10 ml 30% H_2O_2 and 90 ml DW and keep in a bottle protected from light and store in a cool place.

2.26 Nitrite test reagents

2.26.1 Solution A

Dissolve 0.8 gm sulphanilic acid in 5 N acetic acid

500 ml by gently heating.

2.26.2 Solution B

Dissolve 0.5 gm alpha-naphtylamine in 5 N acetic acid 100 ml by gently heating.

2.26.3 Zinc dust

2.27 Ehrlich's reagent

Dissolve 1 gm p-dimethylamino benzaldehyde in 95 ml absoluted ethanol and add 20 ml conc HCl, keep in a dark bottle to protect from light.

2.28 1 N HCl

2.29 10% $FeCl_3$ solution

2.30 Nessler's reagent

Dissolve 5 gm Potassium iodide in 5 ml freshly DW, add cold saturated mercuric chloride solution until a slight precipitate remain permanently after thorough shaking, then add 40 ml 9 N-NaOH and dilute to 100 ml with DW. The solution is allowed to stand for 24 hrs.

2.31 Lead acetate paper-strip

Cut a filter paper into strip 5-10 mm width and 50-60 mm length, and impregnate with the 1% lead acetate soln and dry at 50-60 °C, store in a tightly-closed container.

2.32 Methyl red reagent

Dissolve 0.1 gm methyl red in 300 ml 95% ethanol and make to 500 ml with DW.

2.33 Reagent for VP test

Reagent A is consisted of alpha naphthol 5 g in 100 ml 95% ethanol.

Reagent B is consisted of 40 gm KOH in 100 ml DW.

2.34 Lugol's iodine

Dissolve 5 gm iodine and 10 gm potassium iodide in DW 10 ml and make to 1 L with DW dilute the soln to 1:5 with DW before use.

2.35 Benedict's reagent (double strength)

Dissolve 173 gm sodium citrate and 100 gm Na_2CO_3 (anhydrous) in DW 300 ml by heating and filter dissolve 17.3 gm $CuSO_4.5H_2O$ by heating in DW 50 ml and pour slowly into the first solution with constant stiring. The solution is cooled and make up to a final volume of 500 ml with DW.

3. Media and reagents for preparation of sonic extract antigen

3.1 Brain heart infusion broth (BHIB)

Dissolve 37 gm BHIB in 1 L of DW by heating, pour aliquots (50 ml) into 125 ml flasks and autoclave at 121 °C for 15 min.

4. Reagent for protein determination (Modified Lowry method)

4.1 Reagent A

Dissolve 2 gm Na_2CO_3, 0.4 gm NaOH, 0.16 gm disodium tartate and 1 gm SDS (Sodium dodecyl sulfate) 100 ml of DW.

4.2 Reagent B

Dissolve 4 gm copper sulfate in 100 ml of DW

4.3 Reagent C (alkaline copper reagent)

Mix 100 parts of reagent A with 1 part of reagent B, The reagent must be freshly prepared.

4.4 Folin-Ciocalteu phenol reagent

Phenol reagent is diluted 1:1 with DW for use with one day.

4.5 Bovine serum albumin (BSA) standard (1 mg/ml)

Dissolve 0.1 gm BSA and make up to 100 ml with DW.

4.6 Standard curve of protein concentrations

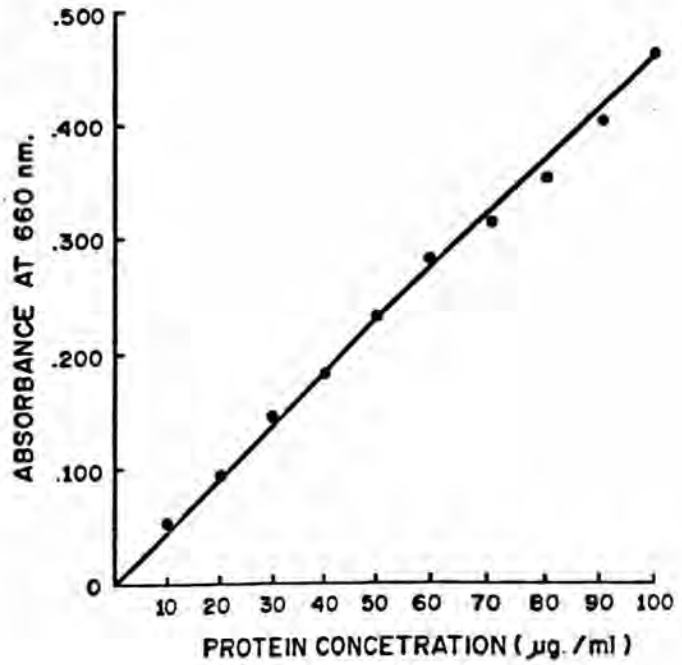

Fig 29 The standard curve of protein concentrations ranging from 10-100 ug of BSA is determeined by modified Lowry method. Plot optical densities against these concentration. Each polt was the average of duplicate determinations.

5. Reagent for immunization

5.1 Complete Freund's adjuvant

5.2 Xylene

6. Reagent and control sera for indirect haemagglutination test (IHA)

6.1 Formalin treatment of sheep red blood cells

6.1.1 Sheep red blood cell (SRBC)

Collect sheep blood in an equal volume of Alsever's solution and store for 3-5 days at 4 °C before use.

6.1.2 Normal saline solution (NSS)

Dissolve 9 gm NaCl in DW 1 L.

6.1.3 Formalin solution 7.5%

Dilute 18.7 ml 37% formaldehyde solution with 81.3 ml NSS.

6.2 Tannic acid treatment

6.2.1 Phosphate buffer saline (PBS) pH 7.2

Dissolve 4.672 gm $Na_2HPO_4 . 2H_2O$, 1.53 gm KH_2PO_4 and 6.75 gm NaCl in DW 1 L.

6.2.2 Phosphate buffered saline (PBS) pH 6.4

Dissolve 4.673 gm $Na_2HPO_4.2H_2O$, 6.63 gm KH_2PO_4 and 4.5 gm NaCl in DW 1 L.

6.2.3 Tannic solution (1:40,000)

Dissolve 2.5 mg tannic acid in 100 ml PBS pH 7.2, the solution must be freshly prepared.

6.3 Diluent (1% PBS-Rabbit serum)

Inactivated normal rabbit serum at 56 °C for 30 min and absorb with an equal volume of packed formalin treated SRBC for 10 min at room temperature. After centrifugation dilute the absorbed normal rabbit serum to 1% soln in PBS pH 6.4.

This diluted serum keep at -20 °C until use.

6.4 Positive control serum

A patient's serum with melioidosis from Chulalongkorn hospital

7. Reagent for preparation of globulin

7.1 Ammonium sulfate, saturated solution

Dissolve 1 kg $(NH_4)_2SO_4$ completely in DW 100 ml by heating, filter, allow to stand overnight at room temperature and adjust to pH 7.0 with ammonium hydroxide soln.

7.2 0.2 M $Na_2HPO_4.12H_2O$

Dissolve 71.63 gm $Na_2HPO_4.12H_2O$ and make to 1 L with DW.

7.3 0.2 M $NaH_2PO_4.2H_2O$

Dissolve 7.8 gm $NaH_2PO_4.2H_2O$ and make to 1 L with DW.

7.4 0.01 M Phosphate buffer pH 7.2

Mix 5.6 ml 0.2 M $NaH_2PO_4.2H_2O$ and 14.75 ml 0.2 M $Na_2HPO_4.12H_2O$, adjust to pH 7.2 and make up to 1 L with DW.

8. Reagent for SDS-PAGE

8.1 Stock acrylamide

Dissolve 30 gm acrylamide and 0.8 gm N, N - methylene bisacrylamide and make up to 100 ml with DW, then filter and store in the dark bottle at 4 °C

8.2 Tris-HCl buffer 1.5 M, pH 8.8

Dissolve 36.3 gm Tris in 50 ml of 1 N HCl, adjust to pH 8.8 with HCl and make up to 100 ml with DDW.

8.3 Tris-HCl buffer 0.5 M, pH 6.8

Dissolve 3.03 gm Tris in 40 ml of 1 N HCl, adjust to pH 6.8 with MCE and make up to 50 ml with DDW.

8.4 0.2 M EDTA

Dissolve 0.37 gm EDTA and make up to 50 ml with DDW.

8.5 10% SDS (Sodium dodecyl sulfate)

Dissolve 1 gm SDS and make up to 10 ml with DDW.

8.6 N,N,N,N-tetramethylethylenediamine (TEMED)

8.7 10% Ammonium persulphate solution

Dissolve 0.1 mg ammonium persulphate in DDW 1 ml. This reagent must be freshly prepared before use.

8.8 Sample buffer 5X concentration

Dissolve 0.378 gm Tris 0.5 gm SDS, 5 ml glycerol, 2.5 ml 2-mercaptoethanol and 0.005 gm bromphenol blue and adjust to pH 6.8 with conc HCl and make to 10 ml with DDW.

8.9 Electrode buffer (pH 8.3)

Dissolve 15.15 gm Tris 72 gm glycine and 5 gm SDS and make up to 5 L with DDW.

9. Reagent for silver stain

9.1 Fixatives (50% methanol in 12% acetic acid)

Mix 500 ml of absolute methanol with 120 ml of glacial acetic acid and make to 1 L with DDW.

9.2 10% Ethanol in 5% acetic acid

Mix 105.26 ml 95% Etanol with 50 ml of glacial acetic acid and make to 1 L with DDW.

9.3 0.0034 M Potassium dichromate in 0.0032 N nitric acid

Mix 1 gm Potassium dichromate with 63.02 ml HNO_3 and make to 1 L with DDW.

9.4 0.012 M silver nitrate

Dissolve 0.41 gm silver nitrate in DDW 200 ml.

9.5 Image developer (0.28 M sodium carbonate solution containing 0.05% formalin)

Dissolve 29.7 gm Na_2CO_3 and 0.5 ml commercial formalin (37%) and make to 1 L with DDW.

9.6 1% Acetic acid

Dissolve 10 ml glacial acetic acid and make to 1 L with DDW.

10. Standard curve for determination of molecular weight of components by SDS-PAGE

The protein standard molecular weight markers are myosin, rabbit muscle (M.W. 205,000), beta-galactosidase Escherichia coli (M.W. 116,000), phosphorylase - B from rabbit muscle (M.W. 97,400)

bovine serum albumin (M.W. 66,000), egg albumin (M.W. 45,000), carbonic anhydrase (M.W. 29,000), beta - galacto globulin from bovine milk (M.W. 18,400) and lysozyme from egg white (M.W. 14,300). They are electrophoresed on same gel with unknown specimen.

Plot the molecular weight and the relative mobility of these commercial protein standard on a semilogarithmic scale as shown in Fig 30 and Fig 31.

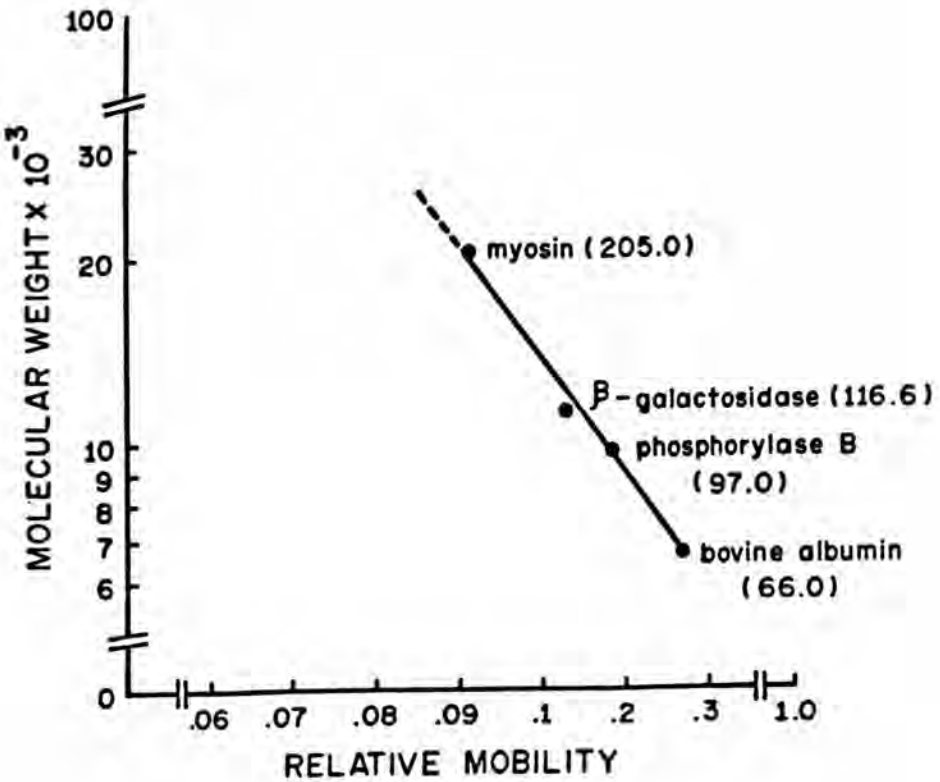

Fig 30 A standard curve for high molecular weight estimation by SDS-PAGE

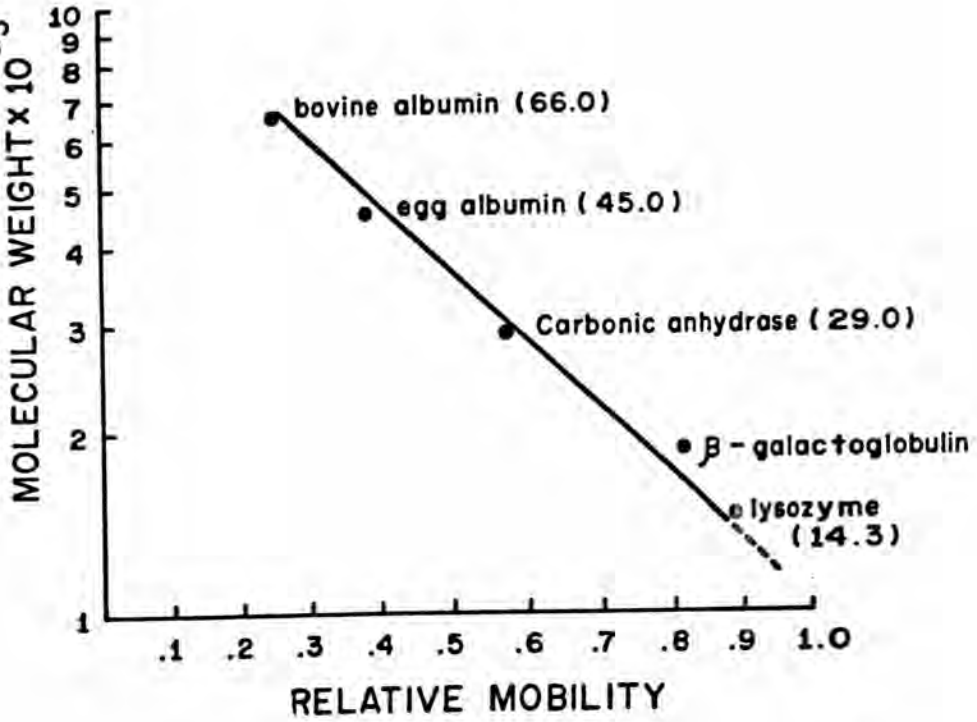

Fig 31 A standard curve for low molecular weight estimation by SDS-PAGE.

11. Reagent for dry gel

Mix 400 ml methanol with 50 ml glycerol and make to 1 L with DDW.

12. Reagent for Immunoblot

12.1 Towbin buffer (20 mM Trizma-base, 150 mM glycine and 20% methanol, pH 8.3)

Dissolve 12.12 gm Trizma-base, 57.6 gm glycine and 800 ml methanol with DDW, adjust to pH 8.3 and make up to 4 L.

12.2 Phosphate buffer saline pH 7.4 (PBS pH 7.4)

Dissolve 8 gm NaCl, 0.2 gm KCl, 1.15 gm Na_2HPO_4 and 0.2 gm KH_2PO_4 adjust to pH 7.4 and make up to 1 L with DDW.

12.3 0.1% Tween 20 in phosphate buffer saline (0.1% PBS/T)

Dissolve 1 ml Tween 20 and make up to 1 L with PBS pH 7.4

12.4 5% Non fat dry milk in 0.1% PBS/T

Dissolve 5 gm Non-fat dry milk and make up to 100 ml with 0.1% PBS/T and filter. Prepare the solution before use.

12.5 Peroxidase conjugated swine antirabbit Ig

12.6 1 M Tris-HCl pH 7.4

Dissolve 12.12 gm Trizma-base in 1 N HCl, adjust to pH 7.4 with HCl and make up to 100 ml with DW.

12.7 Substrate solution

Add 10 ml of 4 chloro-1-naphtol in methanol (3 mg/ml) to 50 ml 1 M Tris HCl pH 7.4, mix vigorously and add 20 ul 30% H_2O_2. The solution must be freshly prepared.

13. Reagent for India ink staining

13.1 0.3% Tween 20 in phosphate buffer saline (0.3% PBS/T)

Dissolve 8.7 gm NaCl in 40.5 ml 0.2 Ml Na_2HPO_4 and 7.5 ml 0.2 M NaH_2PO_4, add 3 ml Tween 20 and make up to 1 L with DW.

13.2 Pelikan fount India drawing ink for fountain pen

Bibliography

Mrs. Sudaluck Chantarachada was bone October 14, 1957 Petchaburi, Thailand. She graduated with the Bachelor degree of Science in Medical Technology from Faculty of Medicine, Chulalongkorn University in 1980. She is a scientist of the bacteriology unit, Department of Microbiology, Faculty of Medicine, Chulalongkorn University.