MATERIALS AND METHODS

2 MATERIALS AND METHODS

2.1 ARTOCARPUS SEEDS

Champedak (*Artocarpus integer*) and jackfruit (*Artocarpus heterophyllus*) belong to a common series the Cauliflori and are closely related cytotaxonomically. Figure 3 demonstrates the photographs of the two fruits. A more detail description on the champedak fruit is described in Ibrahim *et al.* (1987). The botanically characterised champedak fruits were obtained from the University Putra Malaysia. Jackfruits were purchased from a local market.

2.2 MICE

Six to eight weeks old mice (male) were used in all experiments. Inbred Balb/cj mice were obtained from the Animal House of the University of Malaya and nude mice (nu/nu) were supplied by the Institute of Medical Research, Malaysia. Figure 4 illustrates the photographs of the Balb/cj and nu/nu mice.

2.3 GENERAL MATERIALS

The materials used during the course of this study and their respective suppliers are described:

2.3.1 Fine Chemicals

All chemical used were of the highest grade available commercially. With exceptions of the following, all other chemicals were supplied by Sigma Chemical Company, USA.

A

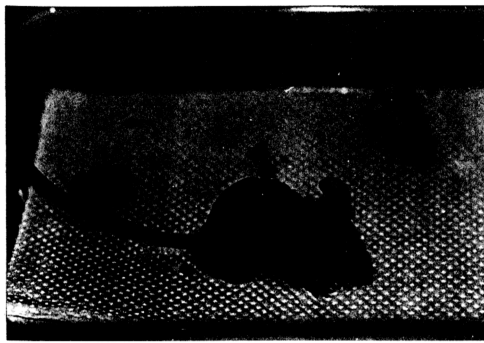
B

Figure 3. ***Artocarpus*** **Fruits**

Panel A. Photograph of the *A. integer* (1) and *A. heterophyllus* (2) fruits. The *A. heterophyllus* fruits are normally larger than *A. integer*.

Panel B. Longitudinal section through mature *A. integer* (1) and *A. heterophyllus* (2) fruits and their seeds, respectively.

(a)



(b)

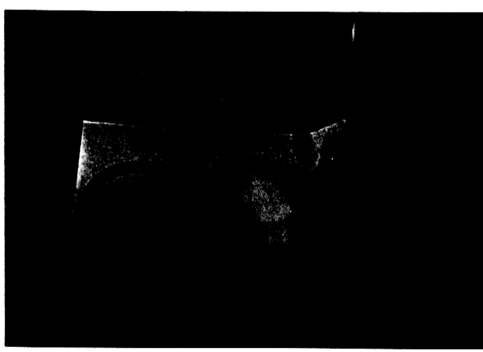


Figure 4. Mice

Photograph of (a) Nude mouse nu/nu, and (b) Inbred Balb/cj mouse.

Potassium dihydrogen phosphate, sodium azide, sodium dihydrogen phosphate, sodium hydrogen carbonate, acetic acid, hydrochloric acid, methanol and Tween-20 were obtained from Merck, Germany.

Ammonium sulphate, HEPES, dithiothreitol (DTT), glycerol and sodium acetate were purchased from ICN Biomedicals Inc., USA.

Potassium chloride and EDTA were obtained from the BDH Ltd., England.

Glutaraldehyde were purchased from Fluka, Switzerland.

Ammonium persulphate and sodium dodecyl sulphate were supplied by Bio-Rad Laboratories, USA.

Activated alkaline phosphatase was purchased from Boehringer Mannheim Biochemica, Germany.

2.3.2 Cell Culture Materials

RPMI-1640 medium and penicillin/streptomycin solution were obtained from Gibco Biocult Ltd., Scotland.

Heat inactivated foetal calf serum (FCS) and glutamine solutions were obtained from Flow Laboratories, Inc., USA.

Microtitre plates (96 wells) were purchased from Nunc, Denmark.

Glass fiber filtermat was obtained from Skatron Instruments Inc., UK.

2.3.3 Radiochemicals

[Methyl-^{3}H]thymidine with a specific activity of 2.0 Ci/mmol was purchased from Amersham International plc., England.

Polypropylene mini scintillation vials were obtained from L.I.P Ltd., England.

CytoScint scintillation cocktail was purchased from ICN Biomedicals Inc., USA.

2.3.4 Normal Human Serum Sample

Blood samples were obtained from normal healthy volunteers. Sera were collected after a centrifugation of the blood sample at 2300 x g for 10 min. Serum samples were stored at -20°C until use.

2.3.5 Serological Reagents

The following immunoglobulins were purchased from The Binding Site Limited, Birmingham, England: human IgA1, IgA2, IgD, IgM and IgE kappa paraproteins, and human IgG polyclonal protein.

2.3.6 SDS-PAGE

Pre-cast, 16.5 % (w/v) Tris-Tricine gels was purchased from Bio-Rad, USA.

2.3.7 Two Dimensional Electrophoresis

IEF immobiline dry strips (pH 3-10), ExcelGel SDS gradient 8-18, ExcelGel SDS buffer strips and Repel-Silane were purchased from Pharmacia, Sweden.

Silicone oil DC 200 was supplied by Fluka, Switzerland.

2.3.8 Chromatographic Support

Sepharose 4B and Sephadex G-100 were obtained from Pharmacia, Sweden.

2.4 STANDARD SOLUTION

Unless otherwise stated, all buffers were stored at 4°C.

2.4.1 Phosphate-Buffered Saline (PBS)

170 mM NaCl

3.4 mM KCl

10 mM Na_2HPO_4

1.8 mM KH_2PO_4 pH 7.2

(If PBS-azide, sodium azide was added at 0.10 % (w/v))

2.4.2 Citrate-Phosphate Buffer

0.1 M Citric Acid

0.2 M $Na_2HPO_4.2H_2O$

49 ml of 0.1 M citric acid solution was mixed with 51 ml of 0.2 M phosphate solution until a pH of 5.0 was obtained.

2.5 METHODS

2.5.1 Determination of Protein Concentration

Concentration of proteins was estimated using a Protein Assay ESL kit (Boehringer Mannheim, Germany) and performed according to the manufacturer's instructions using BSA as standard.

Reagents involved :

Reagent A (alkaline copper tartrate solution)

Reagent B (ascorbic acid, bathocuproine disulfonic acid solution)

100 µl of Reagent A was added to the appropriate number of semi-micro (1.5 ml) cuvettes. Sample solution and standards were added at a volume of 50 µl to the respective cuvettes and mixed. Cuvettes were allowed to stand at room temperature for at least 5 min. (Prolonged incubation up to 1 hr did not affect performance of the assay). Reagent B was added to the first cuvette, mixed briefly and the absorbance at 485 nm was measured immediately after 30 s against PBS. The last step was repeated sequentially with the remaining samples. Bovine serum albumin (BSA) was used as protein standard. A representative standard curve is shown in Figure 5.

2.5.2 Preparation of Crude Extracts of Champedak and Jackfruit Seeds

Crude extracts of champedak and jackfruit seeds were prepared as follow. The fruit seeds were dried at 37°C for 24 hr. Dried seeds were homogenised to powder, and suspended at 10 % (w/v) in PBS. The suspension was stirred at 4°C for 24 hr and centrifuged at 8000 x g for 15 min at 4°C. The supernatant was collected and subjected to ammonium sulphate precipitation (60 % (w/v) saturation). The pellet obtained after a second centrifugation was collected and dissolved in cold PBS and such crude extracts of the lectins were stored at -20°C.

2.5.3 Preparation of Sugar Affinity Columns

2.5.3.1 Activation of Sepharose Gel With Divinylsulfone

The activation of Sepharose 4B using divinylsulfone (DVS) was performed according to the method of Hermanson *et. al.* (1992) as described in Immobilised Affinity Ligand Techniques (Pirece, USA).

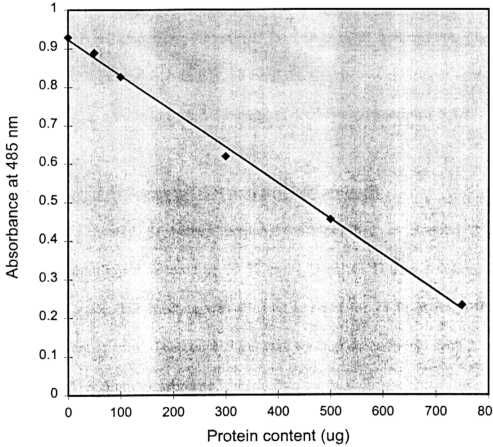


Figure 5. A representative protein standard curve as obtained by Protein Assay ESL Kit.

The standard curve was constructed with BSA content ranging from 50-500 μg. Protein content was estimated using Protein Assay ESL Kit. The method utilised a biuret-like reaction as described in Section 2.4.1. Absorbance of the complex formed was read at 485 nm.

100 ml of settled Sepharose 4B gel was washed with 1 L water in a sintered glass funnel and suction-dried to a wet cake. The gel was suspended in 100 ml 0.5 M sodium carbonate and the suspension was stirred. 10 ml of DVS was added slowly in drop-wise with a constant stirring over a period of 15 min. After addition was completed, the gel suspension was stirred for 1 hr at room temperature. The activated gel was washed extensively with water until the filtrate was no longer acidic. The gel was then used to couple to ligands (sugars) as described in Section 2.5.3.2.

2.5.3.2 Coupling of Sugars to DVS-activated Sepharose 4B

DVS-activated Sepharose prepared as described in Section 2.2.4.1 was suspended in an equal volume of 20 % (w/v) D-gal or D-man solutions in 0.5 M sodium carbonate. The mixture was allowed to react for 24 hr at room temperature with a constant stirring. The coupled gel was washed successively with 2 L of water and 2 L 0.5 M sodium bicarbonate.

In a fume hood, the gel was suspended in 100 ml 0.5 M sodium bicarbonate containing 5 ml 2-mercaptoethanol. Excess vinyl reactive groups were blocked by stirring the mixture at room temperature for 2 hr. The gel was washed with 2 L each of water and PBS. Finally, 10 ml of these settled sugar affinity gel was packed into a column and used to isolate lectins (Section 2.5.4). The unused gel was stored in 0.02 % (w/v) sodium azide at 4°C.

2.5.4 Purification of Lectins

Lectins with different affinities were purified from the crude extracts by using the respective affinity chromatographies.

2.5.4.1 Purification of Galactose-binding Lectin

Galactose-binding lectins were purified from the crude extracts by affinity chromatography on a Sepharose-Gal Column (prepared as in Section 2.5.2).

1 ml of crude extract was loaded into the immobilised Gal-Sepharose affinity column (10 ml bed volume) at room temperature. Fractions of 1.0 ml were collected and their absorbance were measured at 280 nm using a Shimadzu Spectrophotometer. The column was washed extensively with PBS until the Abs_{280} reached the base line and the bound protein was eluted out with 0.8 M D-galactose in PBS. After elution, the column was washed with PBS before it was used again. The bound fractions with high absorbance were pooled and dialysed extensively against PBS. The isolated galactose-binding lectin was stored at -20°C in aliquots.

2.5.4.2 Purification of Mannose-Binding Lectin

The crude extract was applied into an immobilised Gal-Sepharose affinity column and washed with PBS until the absorbance at 280 nm reached the baseline. To ensure that the effluent was completely free from galactose-reactive lectins, the unbound protein was applied to a second Gal-Sepharose column and again washed with PBS. Fractions not retained by this second Gal-Sepharose column were then subjected to immobilised Man-Sepharose affinity separation. Desorption of the mannose-binding lectins from the column was performed using 0.8 M D-mannose in PBS. The mannose-binding lectin was pooled , extensively dialysed against PBS, and stored at -20°C in aliquots.

2.5.5 Preparation of Lectin M-Affinity Column

Cyanogen bromide activated gel (Sigma, USA) was used in the preparation of lectin M-affinity column. 5 g of activated gel was swollen in ice-cold distilled water overnight. The swollen gel was washed successively with 2 L ice-cold water and 1 L 0.1 M sodium bicarbonate, pH 8.5. After suction-dried under vacuum to a moist cake, 100 mg of lectin M in 25 ml PBS containing 0.1 M mannose was added. The gel suspension was stirred at 4°C for 20 hr.

After coupling, the reaction mixture was filtered and washed with 200 ml 1.0 M NaCl and subsequently with 200 ml water. The filtrate was collected to determine the amount of unreacted lectin by measuring the absorbance at 280 nm. To mask the excess reactive groups on lectin M-Sepharose 4B, the gel was stirred with 100 ml of 1.0 M ethanolamine, pH 9.0 for 1 hr at room temperature.

Finally, the lectin M-Sepharose 4B was successively washed with 2 L of distilled water, 1.0 M NaCl, distilled water and PBS. The gel was then packed into mini columns (0.8 x 4 cm) and used in affinity chromatography (Section 2.5.6). The unused gel was stored at 4°C in PBS containing 0.1 % (w/v) sodium azide.

2.5.6 Lectin M-Sepharose Chromatography

Separation of glycoproteins and normal human serum on lectin M-Sepharose affinity columns was performed at room temperature. 2.5 ml of the immobilised lectin in a 0.8 x 4 cm mini column was used for each chromatography. Eluents were added to the column in 1 ml aliquots and allowed to flow through by gravity feed at a flow rate of approximately 0.2 ml/min. The column was washed extensively with PBS until

the absorbance at 280 nm reached the base line. Bound protein was eluted with 0.1 M methyl-α-mannopyranose in PBS, pooled, and dialysed against PBS.

2.5.7 Molecular Weight Determination by Gel Filtration Chromatography

Mannose-binding lectin isolated by sugar affinity chromatography was subjected to gel filtration on a Sephadex G-100 column (1.5 x 100 cm). Elution was carried out using 0.1 M acetic acid at 20 ml/hr at 4°C, and fractions of 2.0 ml were collected. To determine the molecular weight of the lectin, the column was calibrated with a gel filtration calibration kit (Pharmacia, Sweden), consisting of ribonuclease A (13.7 k), chymotrypsinogen A (25.0 k), ovalbumin (43.0 k), and albumin (67.0 k). The protein markers calibration curve obtained is shown in Figure 6.

2.5.8 SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was carried out using pre-cast, 16.5 % (w/v) Tris-Tricine gels. Samples were mixed with equal volumes of buffer consisting of 8 M urea, 2 % (w/v) SDS, 4 mM iodoacetamide and 5 mM bromophenol blue dissolved in Tris-HCl pH 7.4 and boiled for 2 min. Reduction of samples was performed by addition of 2 % (w/v) dithiothreitol. Gels were stained with 0.025 % (w/v) Coomassie Brilliant Blue R-250 in methanol, acetic acid and water [4:1:5 (v/v)].

2.5.9 Double Diffusion.

Double diffusion was performed in 1 % (w/v) agarose prepared in PBS. Approximately 8 ml of warm agarose solution was pipetted onto each microscope

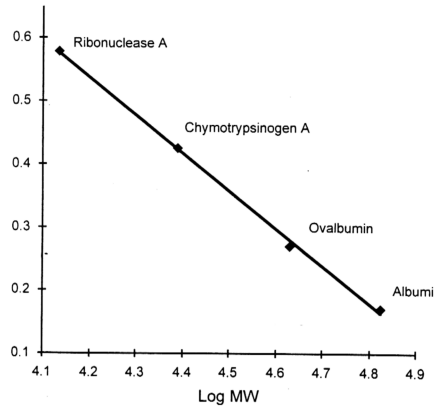


Figure 6. Calibration Curve for the Determination of Molecular Weight Using Sephadex G-100 Gel Filtration Chromatogrphy

The calibration curve of Sephadex G-100 gel filtration column was plotted on a semi-logarithmic scale. The protein markers consisted of ribonuclease A (13.7 k), chymotrypsinogen A (25.0 k), ovalbumin (43.0 k), and albumin (67.0 k). Elution was carried out at a flow rate of 20 ml/hr with 0.1 M acetic acid and 2 ml fractions were collected. Absorbance at 280 nm was measured and K_{av} for each protein marker and lectin were calculated. The molecular weight of the markers (in log) were plotted against their K_{av}.

slide placed on a levelling table (Shandon, England) and allowed to solidify. The gels were punched using a 7-well gel punch (LKB 6808A, Sweden). A Pasteur pipette connected to a vacuum pump was used to suck out the agar plugs.

Dilutions of the human serum, immunoglobulins, lectins and glycoproteins were prepared in PBS. Fifteen microlitre of the reagents were dispensed into each well. The slides were placed in a humid chamber and incubated overnight at room temperature for the immunoprecipitin lines to be visible. Unprecipitated excess proteins in the gel were removed by extensive dialysis against PBS. The washed gels were Coomassie stained as described in Section 2.5.8.

2.5.10 Competitive HRP-Binding Assay

The specificity of mannose-binding lectins was studied by analysing the effect of glycoproteins, sugars and other substances on interaction of the lectin with horseradish peroxidase (HRP). Microtitre plates were coated with 18.5 μg/ml of lectin and incubated overnight at 4°C. Plates were washed extensively with PBS containing 0.05 % (v/v) tween-20 (PBS-tween) and added with HRP (amount that gave 50% of the maximum colour yield) in the presence of sugars or other substances. Blanks were prepared by addition of 100 μl of PBS, and controls consisted of 50 μl of HRP and 50 μl of PBS. The plates were incubated for 2 hr at room temperature and washed with PBS-tween. The amount of HRP bound was estimated using 0.4 mg/ml o-phenylenediamine and 0.012 % (v/v) H_2O_2 in 0.05 M phosphate-citrate buffer, pH 5.0. Absorbance values were read using an SLT Labinstrument ELISA reader at a wavelength of 492 nm. Percentage of inhibition were expressed as follows:-

$$\frac{\text{Absorbance of control - Absorbance of test}}{\text{Absorbance of control}} \times 100\ \%$$

2.5.11 Immunoelectrophoresis.

Immunoelectrophoresis was performed using a Hydragel IEP kit from Sebia, France.

2.5.11.1 Stock Solutions

All solutions were stored at room temperature.

1.	Tris Barbital buffer	: Tris	: 60 mM
		Barbital	: 10 mM
		Sodium Barbital	: 50 mM
		Sodium Azide	: 0.01 % (w/v)
2.	Staining Solution	: Amidoblack	: 0.5 % (w/v)
		Acetic Acid	: 5% (v/v)
		Distilled Water	: 95% (v/v)

2.5.11.2 Preparation of Samples and Electrophoresis

1 µl of control serum (normal human sera, mixed with bromophenol blue) was applied into the wells of a pre-cast gel and electrophoresed in Tris Barbital buffer for about 40 min at a constant voltage of 100 V.

Upon completion of the electrophoresis, troughs were filled with 35µl of purified lectins, anti-human whole serum and immunoglobulins. The gel was placed

on a wet thick filter paper in an incubation box and was left to diffuse for 24 hr at room temperature.

2.5.11.3 Staining and Destaining the Gels

After 24 hr incubation, the gel was washed in 0.15 M NaCl for 20 min. The gel was then placed on a flat surface and the saline on the gel was soaked up using filter papers. In order to remove all the non-reacted proteins from the gel, the above processes were repeated.

The gel was then dried with hot air (< 80°C) and immersed in the staining solution. After 5 min staining, the gel was destained in two successive baths of 5 % acetic acid until the background was completely clear. Finally, a filter paper was used to soak up excess liquid on the gel surface and the gel was dried under hot air < 80°C.

2.5.12 Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was performed using the Multiphor II System (Pharmacia, Sweden). Immobiline Dry Strips pH 4-7 were used in the first dimension and ExcelGel precast SDS-gradient 8-18 gels were used in the second dimension run.

2.5.12.1 Standard Solutions

All chemicals used were at the highest purity. Double distilled water was used.

1.	Rehydration solution	: Urea	: 8 M
		DTT	: 0.2 % (w/v)
		Pharmalyte 3-10	: 0.5 % (v/v)

		Triton X-100	: 0.5 % (v/v)
		Orange G	: 0.002 % (w/v)
2.	Sample buffer	: Urea	: 9 M
		DTT	: 1 % (w/v)
		Pharmalyte 3-10	: 2 % (v/v)
		Triton X-100	: 0.5 % (v/v)
		Bromophenol Blue (BPB)	: 0.025 % (w/v)
3.	Equilibrium Solution	: 0.5 M Tris-HCl, pH 6.8	: 10 % (v/v)
		Urea	: 6 M
		Glycerol	: 30 % (v/v)
		SDS	: 1 % (w/v)

Equilibrium solutions for the first and second equilibration steps respectively contained 0.5 % (w/v) DTT and 4.5 % (w/v) iodoacetamide plus a few grains of BPB.

2.5.12.2 Rehydration of Immobiline Dry Strip

The dry strips were placed in the cassette with the gel side up facing the U-frame which was previously treated with Repel-Silane. The cassette was filled to the top with 25 ml of rehydration solution and left to stand overnight.

2.5.12.3 First Dimension Run

The apparatus for the first dimension run was set up. The rehydrated strips were placed into the apparatus and samples were loaded. The samples were prepared in sample buffer in a ratio of 1:4. Running conditions were as below:

Phase	Voltage	mA	W	Time(hr)	Vh
1	300	1	5	6	1800
2	300	1	5	5	9500
3	3500	1	5	5.5	19250
Total				16.5	30550

Subsequently, the strips were immediately used to run in the second dimension or wrapped in plastic foil and stored at -80°C.

2.5.12.4. Second Dimension Run

The immobilised strips were equilibrated in the first and second equilibration solution for 10 min each. The strips were drained on a piece of filter paper for 3 min, and placed, with the gel side down, on the ExcelGel. The assembly of the ExcelGel for the second dimension run was started while the strips were equilibrating and draining. The running conditions were as shown below:

Step	Voltage	mA	W	Time(min)
1	600	20	30	30[1]
2	600	50	30	5[2]
3	600	50	30	70[3]

[1] *When the BPB dye has moved 1-2 mm from Immobiline DryStrip, the strip and the application pieces were removed*

[2] *When the front has moved a further 2 mm, the cathodic buffer strip was moved forward to cover the area of removed Immobiline DryStrip by 1-2 mm.*

[3] *When the BPB front has just reached the anodic buffer strip, electrophoresis was continued for 5 min and then stopped. The buffer strips were removed.*

The gel was removed from the cooling bed and stained (Section 2.5.13) or subject to Western blotting (Section 2.5.14).

2.5.13 Silver Staining

Staining of gels with silver was performed according to the method of Heukeshoven and Dernick (1988), with some modifications. 250 ml of solution was needed per ExcelGel for every step.

2.5.13.1 Stock Solutions

All solutions were stored at room temperature. Formaldehyde and glutaraldehyde were added immediately before use.

1.	Fixing solution	: Ethanol	: 40 % (v/v)
		Acetic Acid	: 10 % (v/v)
2.	Incubation solution	: Ethanol	: 30 % (v/v)
		CH_3COONa	: 0.5 M
		Glutaraldehyde (25 %)	: 0.52 % (v/v)
		$Na_2S_2O_2 \cdot 5H_2O$	: 8mM
3.	Silver solution	: Silver nitrate	: 0.1 % (w/v)
		Formaldehyde (37 %)	: 0.02 % (v/v)
4.	Developing solution	: Na_2CO_3	: 2.5 % (w/v)
		Formaldehyde (37 %)	: 0.01 % (v/v)
5.	Stop solution	: EDTA-$Na_2 \cdot 2H_2O$	: 40 mM

2.5.13.2 Staining Procedures

The gel was immersed in fixing solution for 30 min to precipitate the proteins and allow SDS to diffuse out. The gel was then placed in incubation solution for another 30 min and washed with distilled water for three times in 15 min. The silver reaction was carried out by putting the gel in silver solution for 40 min.

Subsequently, the gel was rinsed with 50 ml of developing solution and placed in a fresh developing solution for another 15 min. When the protein bands were clearly seen, the reaction was stopped by placing the gel in stop solution for 5 - 10 min. Finally, the gel was washed with distilled water and preserved in 10 % (v/v) glycerol.

2.5.14 Western Blotting

Western blotting was performed using the Multiphor II Novablot Unit (Pharmacia, Sweden).

2.5.14.1 Stock Solutions

All chemicals used were at the highest purity. All solutions were kept at room temperature.

1.	Transfer Buffer:	Tris	: 25 mM
		Glycine	: 192 mM
		Methanol	: 20 % (v/v)
		SDS	: 0.1 % (v/v)
2.	Tris Buffered Saline (TBS):	Tris	: 100 mM
		NaCl	: 0.9 %

The pH was adjusted to 7.5 using concentrated HCl. If TBS-Tween (TBST), 0.1 % (v/v) Tween-20 was added.

3. Triethanolamine Buffer, pH 7.6:

Triethanolamine	: 50 mM
NaCl	: 150 mM
$MgCl_2$	: 1 mM
$ZnCl_2$	: 0.1 mM
Glycine	: 10 mM
Sodium azide	: 0.1% (w/v)

4. Sodium Carbonate Buffer, 1 M, pH 9.8:

The pH of the 1 M $NaHCO_3$ solution was adjusted to 9.8 by adding 1 M Na_2CO_3 solution.

2.5.14.2 Conjugation of Lectin M to Alkaline Phosphatase

Pre-activated alkaline phosphatase (AP) purchased from Boehringer Mannheim was used in the preparation of AP-conjugated lectin M. The procedure of conjugation was carried out according to the manufacturer's instructions.

The pH of lectin M solution (2 ml) was adjusted to pH 9.8 with 1 M sodium carbonate buffer. The lectin was then pipetted into a vessel containing 0.1 ml activated AP. The mixture was incubated overnight at 4°C.

To stop the reaction, 20 µl of 2 M triethanolamine, pH 8.0 was added to the incubation solution and mixed. Subsequently, 40 µl of ice-cold 0.2 M sodium borohydride which was prepared immediately prior to use was pipetted into the mixture, and incubated for 30 min at 4°C. 5 µl of 2 M triethanolamine solution was further added and incubation was continued for another 2 hr at 4°C.

The conjugate was stabilised by the addition of 10 µl of 1 M glycine, pH 7.6 and followed by a brief mix. The conjugate was placed in a dialysis tube (boiled water treated) and allowed to dialysed extensively with triethanolamine buffer.

To stabilise the conjugate for storage, BSA of 10 mg/ml and sodium azide of 1 mg/ml was added. The conjugate was then aliquoted and stored at-70°C.

2.5.14.3 Protein Transfer

The sample (normal human serum) was electrophoresed at 2-D as described in Section 2.5.13. The gels and pre-cut blotting papers (the same size as gel or slightly smaller) were equilibrated in transfer buffer for 5 min in separate containers. Nine sheets of saturated blotting paper was then placed on the Novablot electrode (anode) of the transfer unit and a pipette was rolled over the sheets to remove trapped air. Nitrocellulose membrane of the size of the gel (or smaller) was cut and soaked with transfer buffer for 2-5 min. The soaked membrane was placed onto the stack of blotting paper.

The plastic backing of the gel was removed by using a film remover and the gel was placed carefully on the membrane. Another nine sheets of blotting paper saturated with transfer buffer was layered on top of the gel. After connecting to the Novablot anode electrode, the transfer of protein was carried out by applying 0.8 mA/cm^2 of current and a voltage not exceeding 50 volt for 1 hr. When the electroblotting was completed, the membrane was removed from the apparatus and detection of protein was carried out as described in Section 2.5.14.4.

2.5.14.4 Protein Detection

The membrane was washed 3 times for 10 min each with TBS-Tween (TBST). To block the non-specific protein binding-sites, the blot was immersed in 3 % (w/v) of BSA in TBS for 2 hr. The 3 times washing steps were repeated and the membrane was incubated in AP-conjugated-lectin M solution at the appropriate dilution for 2 hr at room temperature. After another round of washing, the membrane was developed as described in Section 2.5.14.5.

2.5.14.5 Chromogenic Visualisation of Alkaline Phosphatase

The substrate used for AP visualisation was BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) purchased from Boehringer Mannheim, Germany. To prepare the staining solution, 2 BCIP/NBT ready-to-use tablets was dissolved in 20 ml of redistilled water. The membrane was placed into the staining solution until the blue bands showed adequate intensity. The reaction was stopped by washing the membrane with distilled water. The membrane was then dried on paper towels and stored under light protection for documentation.

2.5.15 Mitogenic Study

All techniques were carried out under aseptical condition.

2.5.15.1 Tissue Culture Medium

The composition of RPMI-1640 medium is described in Table 4. For complete tissue culture medium, the RPMI-1640 was supplemented with 10 % (v/v) foetal calf serum (heat inactivated), 2 mM fresh L-glutamine, penicillin (10^5 I.U./litre) and

Table 4. Composition of RPMI-1640 Medium.

Inorganic salt	mg/litre
$Ca(NO_3) \cdot 4H_2O$	100
KCl	400
$MgSO_4$	48.84
NaCl	6000
$NaHCO_3$	2000
Na_2HPO_4	800
Amino Acids	
L-Arginine	200
L-Asparagine	50
L-Aspartic acid	20
L-Cystine•2H2O	65
L-Glutamic acid	20
Glycine	10
L-Histidine	15
L-Hydroxyproline	20
L-Isoleucine	50
L-Leucine	50
L-Lycine•HCl	40
L-Methionine	15
L-Phenylalanine	15
L-Proline	20
L-Serine	30
L-Threonine	20
L-Tryptophan	5
$L\text{-Tyrosine} \cdot 2Na \cdot 2H_2O$	29
L-Valine	20
Vitamins	
Biotin	0.20
D-Ca Panthothenate	0.25
Choline Chloride	3.00
Folic Acid	1.00
i-Inositol	35.00
Niacinamide	1.00
Para-aminobenzoic Acid	1.00
Pyridoxine HCl	1.00
Riboflavin	0.20
Thiamine HCl	1.00
Vitamin B_{12}	0.005
Other Components	
D-Glucose	2000
Glutathione (reduced)	1.0
Phenol Red	5.0

streptomycin (100 mg/litre). L-glutamine, penicillin/streptomycin solutions were kept at -20°C as 100 X stock.

2.5.15.2 Preparation of Mitogen and Sugars

Purified lectins and sugars were filtered (0.22 μm) immediately before used and diluted to different concentrations using sterile PBS. The protein concentrations were determined as described in Section 2.5.1.

2.5.15.3 Isolation of Suspension cells from Mouse Organ

Murine cells suspension was isolated from mouse organ. The mouse was sacrificed by cervical dislocation and the organ (thymus/spleen) was removed into petri dish containing complete RPMI medium. After a brief wash, the medium was discarded. 10 ml of medium was replaced and a cut was made on the organ by using scissors to released the cells. The remaining part of the organ was then teased and squeezed gently using forceps until cells leave the thymus. This cells suspension was filtered through a mesh filter (Millipore) to eliminate the large debris. Subsequently, the suspension was centrifuged at 2000 rpm for 5 min. The pellet was resuspended in 5 ml of tissue culture medium and ready for counting (Section 2.5.15.6)

2.5.15.4 Preparation of Murine B Cells Suspension

Murine B cells were isolated from the nude mouse (nu/nu) spleen cell suspension which was prepared as described in Section 2.5.15.3. To eliminate the vast majority of macrophages, the spleen cell suspension were incubated in a plastic petri dish at 37°C for a period of 1-2 hr (Rosenwasser, 1986). The petri dish was then

washed twice with warm complete RPMI medium. The non-adherent cells taken from the washing were pooled, washed and used as a source of enriched B lymphocytes.

2.5.15.5 Preparation of Murine T Cells Suspension

Cell suspension prepared from thymus Balb/cj mouse as described in Section 2.5.15.3 was used as an enriched T cells source, without any further purification.

2.5.15.6 Cell Counting

The counting chamber of a Neubauer haemocytometer and coverslip were cleaned, and dried thoroughly. The coverslip was layered across the ruled counting area and pressed down firmly onto the support on either side. 10 µl of cell suspension was added to equal volume of trypan blue solution on top of a parafilm and mixed. The mixture was introduced onto the ruled counting area under the coverslip, and let to fill the space by capillary action. The cells were allowed to settle for 1 min and observed under a microscope. The number of cells in the middle large square were counted. This was repeated at the duplicate side of the haemocytometer and the average of these two counts were taken. Cell concentration in original suspension was taken as number in the counting squares x 2 (dilution fold) x 10^4/ml.

2.5.15.7 Determination of cell viability by Trypan Blue Exclusion

Cell viability was determined by mixing equal volumes of cell suspension and trypan blue solution. Number of live (unstained) and dead (stained) cells were estimated by counting in a Neubauer haemocytometer as described in Section 2.5.15.6.

2.5.15.8 Proliferation Assay

Proliferation response of the lymphocytes was measured by [^{3}H]-thymidine uptake. Cultures having 5 x 10^5 cells per well of the 96 well flat-bottom microtitre plate were established in triplicates in a final volume of 0.2 ml of the complete RPMI 1640 in the absence and presence of increasing concentration of each lectin.

The cells were incubated for 72 hr at 37°C in a humidified atmosphere of 5 % CO_2 and 95 % air. The cultures were pulsed by adding 1 μCi of [^{3}H]-thymidine during the last 18-24 hr prior to harvest. The incorporation of [^{3}H]-thymidine was determined by harvesting the cells onto glass fiber filtermats with the aid of a semi-automatic Micro96 Cell Harvester (Skatron, Model 11055) and the radioactivity was counted with a liquid scintillation counter (Section 2.5.15.9).

2.5.15.9 Liquid Scintillation Counting

Filters were punched into mini plastic scintillation vials containing 3 ml of CytoScint scintillation cocktail. Incorporation of [^{3}H]-thymidine was counted in a Tri-Carb Liquid Scintillation Analyzer (Model 2500TR, Packard, USA) and expressed as counts per minute (cpm) ± standard deviation (SD).

2.5.16 Effect of Lectin On the Secretion of Immunoglobulins by B Lymphocytes

2.5.16.1 Stimulation of Ig secretion by B Lymphocytes using lectin

B cells prepared as described in Section 2.4.16.5 were diluted into a concentration of 2.5 x 10^6 cells/well with complete RPMI medium and cultured in a microtitre plate in the presence and absence of lectins. The supernatants were

collected at different times (1 - 7 days) and assayed for immunoglobulin production by the antibody capture enzyme-linked immunosorbent assay (Section 2.5.16.2).

2.5.16.2 Antibody Capture Enzyme-Linked Immunosorbent Assay (ELISA)

The antibody capture ELISA was carried out in microtitre plates coated with anti-mouse IgG /IgM (overnight). Supernatants were added to the wells and incubated for 2 hr. Plates were washed extensively with PBS-Tween (0.05%) after every incubation step. The amount of bound immunoglobulin was detected by the addition of AP-conjugated anti-mouse IgG/IgM antibody. 1mg/ml of 4-nitrophenyl phosphate in 1 M diethanolamine buffer, pH 9.8 containing 0.5 mM $MgCl_2$ was used as a substrate for AP and the yellow reaction product was measured at 405 nm. All experiments were done at least three times.