II. MATERIALS & METHODS

II.1 Preparation of Ovomucoid

Ovomucoid (OM) was prepared by the method of Linweaver and Murray (162). Hen's egg white 500 ml, was precipitated by adding 500 ml of 0.5 M trichloroacetic acid. Two volumes of acetone were slowly added and continuously stirred at room temperature. The pH of the mixture was adjusted to 3.5 with NaOH. The stirring was continued for a further 15-20 min and the OM precipitate was then separated from the filtrate by centrifugation at 3000 rpm 15 min and further dissolved in 0.02 M NaCl. One volume of cold 95% ethanol was added to the suspension at -6 °C. After adjusting the pH to 3.2 with HCl, the mixture was left standing in the freezer overnight. The precipitate formed was discarded by centrifugation. The supernatant was then precipitated by bringing the ethanol concentration up to 65% (W/V) and left standing in freezer for 2 hours. The OM was collected by centrifugation at 3000 rpm 15 min, dissolved and dialyzed in distilled water. The dialyzed solution was lyophilized and 2.05 g. of crude OM was obtained. The white powder OM in distilled water was then applied to CM-cellulose column for further purification (164).

The CM-cellulose column was equilibrated with 0.1 M acetic acid with the flow rate 25 ml/hr. The OM was eluted from the column with 0.1 M acetic acid in the first peak

fractions. OM containing solution was dialyzed and lyophilized. The retarded impurities were then washed out with 0.01 N NaOH.

II.2 Preparation of Ovomucoid-Sepharose 4B Column

Ovomucoid (OM) was conjugated to Sepharose 4B (Sigma Chemical Co. Ltd) according to the procedure described by Cuatrecases (164), Marchesi and Andrews (165). In a well ventilated hood, 20 ml of pack Sepharose 4B (washed 2-3 times with distilled water) was mixed with an equal volume of water and CNBr (3 gm) was added at once to the stirred suspension. The pH of the suspension was immediately raised to and maintained at 11 with NaOH. The reaction was completed in 8-12 min. The suspension was transferred quickly to a Buchner funnel (coarse disc) and washed under suction with cold 0.2 M $NaHCO_3$ pH 9.5. The volume of the washings was 10-15 times of the pack Sepharose. Four hundred milligrams of OM were dissolved in 20 ml of cold $NaHCO_3$. The mixture was then added to the Sepharose beads suspension immediately. The mixture was slowly stirred at 4°C for 2-3 hr, and was allowed to stand in 4°C for 16-20 hr to ensure a complete of reaction. The substituted Sepharose was then washed with a large volume of the same $NaHCO_3$ solution and cold distilled water respectively. The Sepharose beads were stored at 4°C with 0.02% NaN_3.

II.3 Extraction and Purification of Wheat Germ Agglutinin

Crude wheat germ agglutinin (WGA) was prepared according to the method of Wang et al.(166). Wheat germ (30 g.) was extracted 4-5 times with petroleum ether (150-200 ml/100g.) to remove lipids and oven dried at 45°C. The defatted material was suspended in cold distilled water and stirred at 4°C overnight and filtering the suspension through cheesecloth, the remaining precipitate was removed by centrifugation at 2000 rpm 15 min. The supernatant was then warmed at 56°C in a water bath and maintained at 56-60°C for 15 min. After rapid chilling the sample to 4°C in an ice bath, the precipitate was removd by centrifugation. The supernatant was collected, dialyzed against distilled water and lyophilized.

Further purification of crude extract by affinity chromatography was continued on OM-Sepharose 4B column. The 1.0x20 cm column was equilibrated with PBS(0.02 M phosphate buffer pH 7.0 containing 0.15 M NaCl).After the sample was applied, the column was washed with the same buffer until the absorbance at 280 nm was less than 0.02. The washed fractions were collected and did not show any detectable hemagglutinin activity assayed by hemagglutination ability of human red blood cells (Section II.7). The WGA was eluted from the column with 0.1 M acetic acid and 2.0 ml, the eluted fractions were collected and dialyzed against distilled water. The hemagglutinin positive fractions were detected, pooled and lyophilized.

II.4 Preparation of Collacolia Mucoid

Collocalia mucoid (CM) was extracted from edible bird's nest of swiflets genus Collocalia by the method of Howe et al. (167). The bird nest bought from a local drug store was ground into fine powder with a waring blender. Ground material weighing 40 g was soaked in 500 ml of distilled water overnight, the pH of the suspension was adjusted to 11 with 1 N NaOH, and the material was thoroughly homogenized in a warring blender for 15 min. The mixture was warmed at 65°C in a water bath with occasional shakings for 1 hr. The soupy suspension was centrifuged at 2000 rpm for 20 min, the supernatant was decanted and the precipitate was stored in freezer for further extraction. The pooled supernatant were dialyzed against distilled water at 4°C overnight and finally lyophilized. About 2.0 g. of white fluffy mucin was obtained from 40 g of dry bird's nest. The partially purified mucin was designated as Collocalia mucoid and used throughout this study.

II.5 Extraction and Purification of Lectin from Pisum sativum

The lectin from Pisum sativum (PSA) was prepared according to the method described by Trowbridge (168). Pea seeds (220 g.) obtained from a local market were washed 2-3 times with distilled water. The seeds were ground in a waring blender. The souppy slerry was stirred with 1.5 l of distilled water at 4°C overnight. The extract was adjusted

to pH 4.6 with 5 N HCl and was centrifuged at 2500 rpm 12 min to collect the supernatant. Solid $(NH_4)_2SO_4$ was added to the solution to give a final concentration 60% (W/V). The pellet was collected by centrifugation at 3000 rmp 20 min, dissolved in 120 ml distilled water and dialyzed at 4°C against phosphate buffer (0.01 M pH 7.2) containing 0.15 M NaCl (PBS) and then lyophilized. The crude lectin was applied to a Sephadex G-100 column (2.5x30 cm). The column was equilibrated with PBS at a flow rate of 60 ml/hr. The impurities were washed from the column with PBS. The fractions collected did not show any detectable hemagglutinin activity. The PSA was then eluted with 0.2 M glucose in PBS and 15.0 ml per fractions was collected. The absorbance at 280 nm of the eluates was determined. The hemagglutinin-positive fractions were pooled and dialyzed against PBS.

II.6 Preparation of Lentil Lectin

The lentil lectin, LcH, used in this study was kindly obtained from Kangvantrakul, the Department of Biochemistry, Chiang Mai Medical school. LcH was locally prepared from lentil seeds were soaked in distilled water at 4°C overnight and homogenated. The supernatant was precipitated by adding $(NH_4)_2SO_4$ up to its two-third saturation. The obtained centrifugal pellet was dissolved in and dialyzed with 0.05 M Tris-HCl buffer pH 8.1. The further purification was done on Sephadex G-100 affinity chromatography and eluted with 0.1 M glycine-HCl buffer pH 2.0.

II.7 Assay of Hemagglutination (HA) Activity

In wells of a microtiter plate (U shape, 96 wells, NUNC), a two-fold serial dilution of hemagglutinin samples to be tested for HA titer was made in 50 µl of buffer solution (pH 7.2-7.4). Fifty microlitres of a 0.4% suspension of human red blood cells (group O, washed 2-3 times with PBS) were added. The plated was hand-shaken until well mixed. After incubation for 1½ hours at suitable temperature, the HA activity was visually assayed. The suitable incubation temperature for influenza-virus hemagglutinin was 4°C and for plant lectins was 37°C. The reading of HA titer^{-1} was recognized as "positive" when at least 75% of red blood cells agglutinated and as "negative" when less than 75% of total red blood cells agglutinated.

The buffer used for horse shoe crab lectin-HA assay was 0.05 M Tris-HCl buffer pH 7.4 containing 0.01M NaCl and 0.01 M $CaCl_2$, and that for the HA assay of influenza virus hemagglutinin, wheat germ agglutinin, lentil lectin and pea lectin was 0.1 M phosphate buffer pH 7.2 containing 0.15 M NaCl.

II.8 Assay of Hemagglutination Inhibition (HAI) Activity

II.8.1 WGA

a. The serum samples to be tested were diluted to 1:8 in 0.1 M phosphate buffer pH 7.2 containing 0.15 M NaCl (PBS). A two-fold serial dilution of 50 µl of each samples

were made in 50 µl of 0.9% NaCl in wells of a microtiter plate. Fifty microlitres of WGA (2 HA in PBS) were added to each well. After well-mixing, the plate was incubated at room temperature for 1 hr. Fifty microlitres of 0.4% human red blood cells suspension (group O) were added. The HAI titer^{-1} was read after incubation for 1½ hours.

b. In the second experiment, another group of human serum samples were diluted to 1:10 in 0.9% NaCl Each of the samples was heated in a water bath at 80°C for 1 hr and 56°C 30 min for comparison. Further steps of HAI titer^{-1} assay were done as the same as in part a.

II.8.2 PSA

Serum samples were diluted to 1:10 with 0.5 M phosphate buffer pH 7.0 in 0.15 M NaCl and heated in water bath 80°C, 1 hr. A two-fold serial dilution of each heated sample (50µl) was made in 50 µl of PBS in wells of microiter plate. Fifty microlitres of PSA (2HA in PBS) were added to each well. After well-mixing the plate was incubated at 4°C for one hour and 50 µl of 0.4% suspension of human red cells, group O, was then added. The HAI titer^{-1} was read after incubation for 1½ hr.

II.8.3 LcH

The serum samples were diluted to 1:10 in PBS, were then heated in a water bath at 80°C for 1 hr. Fifty microlitres of the treated samples were two-fold serially diluted in 50 µl of PBS in a microtiter plate. After adding

50 µl of LcH (2HA in PBS), the plate was gently shaken for a moment and left standing in room temperature for 2 hr. In another condition, each sample was incubated with LcH in 4°C overnight (12hr). The reading of HAI titer^{-1} was done by using 50 µl of 0.4% cell suspension and incubation at room temperature for 4½ hours. The reading of HAI titer^{-1} was visually determined as previously described.

II.8.4 IFV

The serum samples were first diluted to 1:10 in 0.9% NaCl and each sample was heated in a water bath at 80°C for 1 hr and 56°C 30 min for comparison. After cooling to room temperature, the samples were further diluted to 1:50 with 0.9% NaCl and used as tested samples. In wells of a microtiter plate, a two-fold serial dilution of 50 µl of tested sample was made in 50 µl of PBS. Fifty microlitres of influenza virus solution (2HA in PBS) were added and the plate was hand shaken for a moment. After incubation at 4°C overnight, 50 µl of red cell suspension (0.4%,group O) was added to each well. The plate was left standing at 4°C for 1½ hours, the HAI titer^{-1} was then visually assayed.

II.8.5 Horse shoe crab lectin from Tachypleus gigas Hemolymph.

The serum samples were diluted to 1:10 in 0.05 M Tris-HCl buffer pH 7.8 (T7.8) containing 0.01 M $CaCl_2$ and heated in a water bath at 80°C for 1 hr. A two fold serial dilution of each sample was made in 50 µl of T 7.8 in wells

of a microtitre plate. Fifty microliters of horse shoe crab lectin (2HA in T 7.8, 0.01 M $CaCl_2$) were added to each well. After mixing by hand shaking, the plate was left standing at room temperature for 2 hr. Fifty microlitres of 0.4% suspension of red blood cell group O were then added to the plate. The mixture was incubated at room temperature for 1 hr. before the determination of HAI $titer^{-1}$.

II.8.6 Effect of Neuraminidase on the Specific of WGA and Influenza Virus Hemagglutinin

On Red Blood Cells and WGA-Hemagglutination

a. Human red blood cells, group O, 0.4 % Suspension in 0.1 M phosphate buffer pH 7.2 containing 0.15M NaCl(PBS), 500 µl, was incubated with 10 U of neuraminidase (N'ase) enzyme at 37°C for 15 min. The cell suspension was washed twice with the same buffer and then was brought for the HA test using WGA (7 HA, 50 µl in PBS). Affer 1 hr incubation at 37°C with treated red-cells the WGA-HA $titer^{-1}$ was observed N'ase untreated red cells were the positive control of the agglutination

On Collocalia mucoid and WGA-Hemagglutination

b. One hundred microlitres of CM (10 mg/ml) was incubated with 10 U and 20 U of N'ase in 0.1 M acetate buffer pH 5.6 at 37°C. Affer 30 min incubation the treated CM solution 50 µl was two-fold serially diluted with 50 µl

PBS in a microtiter plate and the WGA (2HA, 50 µl in PBS) was added. The plate was incubated at 4°C for 15 min. A 50 µl, 0.4% suspension of formalinized red cells, group O was added to each well. Affer 1 hr incubation at 4°C, the HAI titer^{-1} of CM was read. The native CM was compared as the positive HA inhibitor.

On Collocalia mucoid and IFV-HA Inhibition

c. A 50 µl solution of CM (10 mg/ml) was incubated with N'ase (20 U) in 0.1 M acetate buffer pH 5.6 at 37°C. After 30 min incubation the enzyme mixture was warmed in a water bath at 56°C for 15 min, then 50 µl of treated CM samples was two-fold serially diluted with 50 µl PBS in a microtiter plate. A 50 µl suspension of IFV (2HA, 50 µl) was added to each well. After 15 min incubation of the microtiter plate at 4°C, 50 µl suspension of formalinized red cells was added to each well. After well-mixing and 1 hr incubation at 4°C, the HAI titer^{-1} of CM was read. The HA inhibitory activity of native CM was performed as the control.

II.9 Preparation of Formalinized Erythrocytes

Human erythrocytes group O were washed 3 times with 0.1 M phosphate buffer pH 7.2 containing 0.15 M NaCl (PBS). The cells were then suspended at a concentration of 8.0% in PBS and an equal volume of formalin (3% in PBS) was added. The mixture was incubated at 37°C for 16 hr with moderate

shaking. The cells were then washed 5 times with PBS and kept in 4°C in 0.9% NaCl. (170)

II.10 Purification of Horse shoe Crab Lectin (TGA)

II.10.1 The hemolymph (60 ml) of horse shoe crab Tachypheus gigas was obtained by cardiac puncture from the four alive animals, pooled and freezed. The hemolymph was thawed at room temperature then the clot was removed by centrifugation at 3000 rpm 30 min. The clear supernatant was collected. The HA assay and protein determination of crude hemolymph was measured. The next step of crude hemolymph was measured. The further purification was made by incubation of packed formalinized red blood cells (FRBC) with 6.0 ml of hemolymph at 4°C for 2 hr with moderately occasional shaking. The mixture was then washed 3 times with 10 vol of 0.05 M Tris-HCl buffer pH 7.8 containing 0.01 M $CaCl_2$. Elution of the adsorbed hemagglutinin was accomplished by incubating the cells with 4.0 ml Tris-HCl buffer pH 7.8 containing 0.1 M NaCl and 0.01 M $CaCl_2$ (3 times, 10 min each). The eluated mixture was collected by centrifugation at 3000 rpm 20 min. The content of lectin was assayed as HA titer by hemagglutination titration,(171).

II.10.2 To remove the remaining bound lectin on the eluted FRBC, neuraminidase was employed to hydrolyze glycosidic acids existing on the red cell surface. The cells were further washed with Tris-HCl buffer 0.05 M pH 7.4

containing 0.1 M NaCl. The packed cells (2 ml) were then suspended in 0.05 M sodium acetate buffer pH 5.0 containing 500 U of neuraminidase. The mixture was incubated at 40°C in a shaking water bath for 17 hr. The supernatant was then collected and its HA titer^{-1} was found to be 256. Five millilitres of supernatant was applied to the Sephadex G-200 column (1.4x70 cm) previously equilibrated with 0.05 M Tris-HCl buffer pH 7.8 containing 0.1 M $CaCl_2$ and 0.1 M NaCl and then eluted with the same buffer. The flow rate was 6.0 ml/hr. Fractions, 2.0 ml/tube, were collected. The optical density of each fraction was measured at 280 nm. Their lectin activities were assayed and the lectin positive fractions were pooled and concentrated.

II.11 Horse Radish Peroxidase-Labelled Horse Shoe Crab lectin

Horse shoe crab lectin was conjugated to horse radish peroxidase (HRP) by the technique of Nakane and Kawaoi (172) and Murensen et al.(171) with few modifications.

1. HRP 5.0 g was dissolved in 1.0 ml freshly made 0.3 M sodium bicarbonate pH 8.1 (RZ=1.6)
2. To the above solution 0.1 ml of 1 % FDNB in an absolute ethanol was added and mixed for 1 hr at room temperature.
3. One millilitre of 0.08 M $NaIO_4$ in distilled water was added and gently mixed for 30 min at room temperature.

4. One milliitre of 0.2 M glycerol in distilled water was mixed gently for 1 hr at room temperature.
5. The solution was dialyzed against 1 litre of 0.1 M sodium bicarbonate buffer pH 9.5 at 4°C (RZ=0.5). The buffer was changed three times.
6. The TGA 2 mg (300 µl, HA titer^{-1} = 32) was added to the HRP-aldehyde solution (1.8 ml about 3.0 mg of protein). The mixture was mixed gently for 3 hr at room temperature.
7. $NaBH_4$ (3.0 mg) was dialyzed at 4°C against 0.5 M Tris-HCl buffer pH 7.8(T 7.4) containing 0.1 M NaCl and 0.1 M $CaCl_2$
8. The mixture was dialyzed at 4°C against 0.5 M Tris-HCl buffer pH 7.8 (T 7.4) containing 0.1 M NaCl and 0.1 M $CaCl_2$.
9. The sample(2.0 ml) was applied to the Sephadex G-200 column (1.4x70 cm), previously equilibrated with T 7.4 buffer. The column was eluted with T 7.8 with the flow rate of 6.0 ml/hr, fraction were collected (2.0 ml/tube). The absorbance of the solution was read at 280 nm and 403 nm respectively.

II.12 Staining Procedure of Tissue Section With TGA-HRP Conjugates.

The tissue specimens for this experiment were breast cancer tissues and cervix cancer tissue obtained from Department of Pathology, Faculty of Medicine, Chiang Mai University. The tissue was fixed with 10% formalin for 24hr and was embedded in a single paraffin block using a routine procedure. After cutting into 3 µm with microtome, the section were fixed on slides by warming in an incubator at 60°C min,the staining procedurewas done as following steps (171, 173, 174, 175, 176, 177):

1. Deparaffinization in xylene 15 min.
2. Three rinsing in distilled water.
3. Rehydration in 95% ethanol 10 min.
4. Three rinsing in distilled water.
5. Incubation in H_2O_2/methanol(1:5) 15 min,2 changes.
6. Rinsed in distilled water and 0.05 Tris-HCl buffer pH 7.4 containing 0.01 M $CaCl_2$ and 0.05 M NaCl (THB-Ca^{2+}).
7. Incubation in TGA-HRP solution 25µl/each specimen (25 µg/ml in THB-Ca^{2+}) at room temperature in moist chamber for 2 hr.
8. Five rinsing in THB-Ca^{2+}.
9. Incubation in DAB-solution (Stock solution: 25 mg dimethylaminobenzaldehyde in 1.0 ml ethyleneglycol monoethylether ; Working solution : 50 µl of stock solution and 50µl of 30 % H_2O_2 in 350µl of THB-Ca^{2+}) in moist chamber in the dark.
10. Two rinsing in THB-Ca^{2+}.

11. Counterstaining in hematoxylin, dehydration in 95% ethanol and clearing in xylene.
12. Mounting in permount.
13. Negative control experiments were
    a. Incubation THB-Ca^{+2} instead of THA-HRP solution
    b. Neuraminidase treatment was done before incubation with HRP-TGA solution. Neuraminidase 500U in 0.1 M acetate buffer pH 5.0 containing 0.04 M $CaCl_2$, was added on the slide and warm at 37 °C 12 hr in moist chamber).

All the stained slides were be observed and photographed under a microscope.

II.13 Determination of Sialic Acid

II.13.1 Thiobarbituric Acid (TAB) Method

The TAB method described by Aminoff (46) and Warren (45) was employed for the assay of sialic acids. This method was more sensitive and can distinguish free sialic acids from the bound ones.

Reagents

: Periodate, 0.2 M in 9 M H_3PO_4
: Sodium arsenite, 10% (W/V) in 0.5 M-Na_2SO_4 - 0.1 N H_2SO_4
: Thiobarbituric acid, 0.6% (W/V) in 0.5 M-Na_2SO_4 (TBA solution)
: Acid-butanol, 5% (V/V) of 12 N HCl in butane-1-ol
: Standard N-acetylneuraminic acid, 1 mg/ml

Procedure

1. The standard, blank and sample solutions were prepared in a total volume of 0.1 ml
2. Sodium periodate solution, 0.1 ml was added to the test solution and mixed at room temperature for 20 min.
3. Sodium arsenite solution, 1.0 ml was then added to remove excess sodium periodate.
4. The mixture was heated with TBA solution (3.0 ml) in boiling water bath for 15 min, then cooled to room temperature.
5. The pink color was extracted from the solution by adding acid butanol in an equal volume. The solution was mixed and centrifuged. The upper phase of the solution was removed and its colour intensity was measured by spectophotometer at 549 nm.
6. A standard curve made from 1 mg/ml crystalline N-acetylneuraminic acid (5 µg-40 µg/tube).

b. Modified direct Ehrlich Method

The method was modified from that previously reported (178). The method is less sensitive compared to the TBA method but more sensitive to both free and bound sialic acid. For the serum samples, free sialic acid will be liberated by H_2SO_4 and then total remaining protein will be seperated by precipitation with trichloroacetic acid (TCA) to get rid of turbidity and interfering tryptophan containing proteins.

Reagents

: 0.2 N H_2SO_4

: 10 % Trichloroacetic acid (TCA)

: Ehrlich reagent 5 gm of p-dimethylamino-beazaldehyde in a mixture of 50 ml of 12 N HCl and 50 ml of distilled water.

: Standard N-acetylneuraminic acid, 1 mg/ml

Procedure

1. Two hundred microlitres of human serum were mixed with 0.2 ml H_2SO_4 and incubated in a water bath at 80°C for 1 hr.
2. One millilitre of TCA was added and the solution was mixed and centrifuged at 3,000 rpm 15 min. The supernatant (0.5 ml) was withdrawn for an analysis.
3. Two millilitres of distilled water were added to the supernatant, then 0.1 ml of Ehrlich reagent was added. The solution was heated in a boiling water bath for exactly 30 min.
4. The blue-violet solution was obtained. The reaction was stopped by cooling the sample tubes. The mixture was centrifuged if necessary. The absorbance of the colour was measured by spectrophotometer at 565 nm.
5. A crystalline N-acetylneuraminic acid (50 µg-200 µg/tube) was used as the standard and 0.2 N H_2SO_4 - 10% TCA as the blank. Sialic acid content of the samples was determined from the linear standard curve.

II.14 Protein Determination

II.14.1 Lowry Method

Reagents

: Reagent A;2 g of Na_2CO_3 in 0.1 N NaOH solution.

: Reagent B; 0.5 g of $CuSO_4 \cdot 5H_2O$ in 1% sodium potassium tartrate solution.

: Reagent C; a fresh prepared of 50 ml reagent A and 1.0 ml reagent B .

: Folin-phenol reagent 1 N.

: Standard BSA 1 mg/ml

Procedure

1. An aliquot of 0.1 ml standard BSA (10-100 µg/ml) and the unknown samples was added to 3 ml of the reagent C. The mixture was incubated for 10 min.
2. A 0.3 ml of Folin-phenol reagent was added to the above solution and was incubated for 30 min.
3. The colour intensity of the standard BSA and the unknown samples were measured by spectrophotometer at 750 nm.
4. The standard curve was constructed and the protein concentration of the samples were then determined from the standard curve.

II.14.2 Protein-Dye Binding Method (Coomassie Brilliant Blue Method) (180)

Reagents

: Standard BSA 1 mg/ml

: Coomassie brilliant blue G-250(Sigma) solution; 100 g of the dye was dissolved in 50 ml of 95% ethanol and 85% (W/V) of H_3PO_4 (50 ml) was added. The resulting solution was diluted to final volume of 1 litre.

Procedure

1. Standard BSA (1 mg/ml) was added to 6 duplicated test tube; 0,10,20,40,60,80 µg/tube.
2. Sample solutions to be tested were prepared and were made up to 100 µl with distilled water.
3. The dye solution 5.0 ml was added to the above solutions. The mixture was then mixed and left standing at room temperature for 2 min.
4. The colour intensity as optical density was measured by spectrophotometer at 595 nm.
5. The standard curve of BSA was constructed The samples were then determined from the standard curve.