CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Specimens

Blood and saliva samples were collected from eighty-nine healthy volunteers who come for healthy check-up program, performed by the Community Service of the Faculty of Associated Medical Sciences, Chiang Mai University. They were between 19-67 years of age, 54 females and 35 males. Blood group typing and secretor status of individuals were determined and results were grouped into two groups, secretors and non-secretors. Serum and saliva samples obtained from all volunteers were aliquoted and kept at -20°C until analyzed.

Informed consents were obtained from all healthy volunteers using a form approved by Research Ethics Committee, Faculty of Associated Medical Sciences, Chiang Mai University Ref. No TE0O7BE4.

2. Instruments:

Instruments used in this study were listed as following-:

- Analytical balance (A&D CF 300.)
- Blotting module (Amersham Biosciences)
- Electrophoresis module (Amersham Biosciences)
- Power supply (Major Science MP-250)
- Roller mixer SRT1 (Stuart scientific, UK)
- Magnetic stirrer (Thermolyne Co., USA.)
- Microcentrifuge (Eppendorf, Germany)

- pH meter. Model 3560 (Beckman. USA.)
- UV-Visible recording Spectrophotometer UV 2450 (Shimadzu)
- Vortex mixture, (Scientific Industries. New York, USA.)
- Water bath (Memmert, Germany)

3. Chemical and Reagents

All chemicals (essential) used were analytical grade:

- Acetic acid glacial (Merck.Darmstadt, Germany, No.K33688063 434)
- Acrylamide (Sigma Chemical Co., USA. No. A 8887)
- 2-amino-2-methyl-1-propanol (AMP) buffer (Sigma-Aldrich, INC., USA. No. A 9199)
- Ammonium persulfate (Bio-Rad Laboratories, No. 161-0700)
- Anti A, Anti B, Anti A,B and Anti H (Chiang Mai Red Cross Division)
- AST and ALT reagent kits (Trace, Australia, No. TR 17515)
- Boric acid (Sigma Chemical Co., USA. No. B 7901)
- Brilliant blue R-250 (Biorad Chemical Company, USA.)
- 5-Bromo-4-chloro-3-indolylphosphate p-toluidine salt (Sigma Aldrich Co., USA. No. B-8503)
- Bromphenol blue (Sigma Chemical Co., USA. No. B 5525)
- Ethanol (Merck Darmstadt, Germany, No. K 32830483 402)
- Glycine (Sigma Chemical Co., USA. No. G8898)
- Hydrochloric acid (Merck Darmstadt, Germany)

- 2-mercaptoethanol (Sigma Chemical Co., USA. No. M 4125)
- Methanol (Merck Dermstadt, Germany, No. K 32975307 410)
- Mouse monoclonal anti-alkaline phosphatase Clone AP-59 (Sigma-Aldrich, INC., USA. No. A 9549)
- Neuraminidase from *Clostridium perfringens* Type V 2.5 units (Sigma Chemical Co., USA. No. N2876)
- N.N'-Methylene-bis-acrylamide (Sigma Chemical Co., USA. No.M 7256)
- N,N,N',N'-tetramethylethylene diamine: TEMED (Sigma Chemical Co., USA. No. T 8133)
- Nitro blue tetrazolium (Sigma Chemical Co., USA. No. N 6876)
- *p*- nitrophenyl phosphate substrate (Fluka, Germany, No. 71770)
- Polyclonal goat anti-mouse immunoglobulins/HRP (DakoCytomation, Denmark No. P0447)
- Tris [hydroxymethyl]-aminomethane hydrochloride (Merck, Darmstadt, Germany, No. 648311)
- Tween 20 (Fluka, Germany, No. 93773)

4. Other Materials: Other materials used in this thesis were listed as follow;

- Kodak Medical X-ray film (Eastman Kodak Company, USA. No. REF 822 5526)
- Polyvinylidene Fluoride Transfer Membrane 0.45 μm (Bio Trace,USA. No. P/N 66543)
- Film development solutions (Kodak GBX, CAT. 1900984)

B. Methods

I. Method for Determination of ABO Blood Group and Secretor Status

1. Determination of ABO Blood Grouping

(http://www.w3.whosea.org/bct/pdf/sop/13.pdf)

In principle, ABO system is the only system in which there is a reciprocal relationship between the antigen on the red cells and the naturally occurring antibodies in the serum. Routine grouping of the persons must therefore include both RBC and serum tests, each serving as check on the other. The procedure is based on the principle of agglutination of antigen positive red cells in the presence of antibody directed towards the antigen.

2. Determination of ABH Secretion

(http://www.matcmadison.edu/is/hhps/mlt/mljensen/BloodBlank/Lab_Manual/determinig_secretor_.htm)

In principle approximately 80% of the population has the secretor (Se) gene. These people secrete water-soluble blood group substances in their saliva and other body fluids. Group A secretes A substance and a small amount of H, group B secretes B (and H) substance, group O secretes H substance only, and group AB secretes A, B, and a small amount of H.

To determine if an individual is a secretor, the principle of agglutination inhibition is utilized, where the presence of agglutination means a negative test, and no agglutination is interpreted as a positive result.

Part I - Antibody Neutralization:

Saliva is mixed with commercial antiserum (Anti-A, Anti-B or Anti-H) and allowed to incubate briefly. If the person is a secretor, the soluble blood group antigens in the saliva will react with and neutralize the antibodies in the commercial antiserum. It is necessary, however, to dilute the commercial antiserum so that its antibody titer more closely matches the antigen level in the saliva.

Part II - Agglutination Inhibition:

When commercial RBC of the appropriate blood group are then added to the test mixture, there should be no free antibody to agglutinate them if the person is a secretor, because the antibodies have already reacted with the blood group antigens in the saliva. The reaction will be negative for agglutination, but is interpreted as positive for secretor status.

If an individual is a non-secretor, there will be no blood group antigens in the saliva; the antibodies in the antiserum will not be neutralized and will be free to react when the test cells are added. Therefore, agglutination is a negative test for secretor status. Detail protocols for determination of blood group and ABH secretion were shown in Appendix B.

II. Preparation of ALP Isoenzyme from Tissues for Use as Electrophoresis Control ALP isoenzymes (Bianes, 2001)

Tissues from different sources were sectioned and weighed by electrical balance. Bovine liver and human placenta (donated by The PHPT, a joined research group at the Faculty of Associated Medical Sciences, Chiang Mai University) was used as raw material for preparation of liver and placental ALP. The preparation was carried out by homogenizing of 40 g of tissue in 80 ml of ice cold distilled water. The homogenate was then centrifuged for 15 min at 4^{O} C and 3500 rpm by using Warring blender. After centrifugation, the lowest aqueous layer containing ALP protein was removed and placed in a beaker in which 40 ml of N-butanol was added and stirred for an hour at room temperature. The supernatant obtained, after the mixture was centrifuged for 15 min at 4° C and 3500 rpm, was then added with cold acetone to the final volume of 50% (v/v). The precipitate collected after centrifugation was dissolved with 20 ml of 0.1 mol/L Tris buffer pH 7.0. At last, the control enzyme solutions were dialyzed overnight against 0.01 mol/L Tris buffer pH 7.7. Total ALP activity in the dialyzed enzyme solutions were measured before it was stored at 4° C until used.

III. Screening of Patient Conditions to Rule out Liver Disease

All serum samples were screened for liver disease by assaying for liver enzymes, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity. Enzyme activities in all sera were determined by a kinetic method using a reagent kits (Trace, Australia) in a Shimadzu UV 2450 Spectrophotometer. The activities obtained were interpreted for free from liver disease by comparing with

reference values shown in the insert document of the reagent kit and reference values on the request form of Maharaj Nakorn Chiang Mai Hospital.

IV. Method for Determination of Total Alkaline Phosphatase Activity

1. Measurement of Total ALP Activity by a Kinetic Method Using Double-beam UV 2450 Spectrophotometer (Tietz, 1982)

Serum ALP activity was determined by a double beam UV 2450, UV-Visible Spectrophotometer using a kinetic mode. The reaction mixture containing 2.7 ml of 2-amino-2-methyl-1-propanol (AMP) buffer, pH 10.3 and 200 μL of *p*-nitrophenyl phosphate (PNPP) substrate were incubated at 37°C for 3-5 minutes in the UV 2450 Spectrophotometer. After adding 100 μL of sample into the mixture and mixed gently, the activity of ALP was measured at 405 nm, for 120 seconds. The rate of increased absorbance against time or ΔA/ min and total ALP activity were calculated, using data obtained from a linear portion of reaction progress-curve. One unit of activity was defined as the quantity of enzyme catalyzing the hydrolysis of 1 μmol of PNPP substrate per minute under pH 10.3 at 37°C

Calculations

The activity of alkaline phosphatase was calculated from the following formula:

$$\text{ALP activity (U/L)} = \frac{\Delta A/\text{min} \times 10^3 \times V \times 10^3}{\varepsilon \times S \times B}$$

Where A/min = Absorbance change per minute at 405 nm.

10^3 = Conversion of ml to L

V = Total volume of reaction: 3.0 mL

10^3 = Conversion of mole to micromole

ε = Molar absorptivity of *p*-nitrophenol at 405 nm.

= 18,750 L mol^{-1} cm^{-1}

S = Sample volume in ml: 0.10 ml

B = Light path in cm: 1 cm

Therefore:

$$\text{ALP activity (U/L)} = \frac{\Delta A/\text{min} \times 10^3 \times 3.0 \times 10^3}{1.875 \times 10^4 \times 0.10 \times 1}$$

$$= \Delta \text{ A/min} \times 1600$$

2. Quality Control of Total ALP Activity Determination

Optimal condition variation (OCV) or within run assay of ALP activity determination was performed in 20 identical aliquots of control serum by a Shimadzu UV 2450 spectrophotometer. Routine condition variation (RCV) or between run assay of ALP activity was determined by using the same control specimen as OCV and performed simultaneously with the experimental samples in each assay of ALP activity. Precision test was evaluated from percentage of coefficient of variation (%CV) by the following formula:

$$\%CV = \frac{SD \times 100}{\text{Mean}}$$

V. Method for Separation of ALP Isoenzymes by Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoretic technique is frequently used to examine the different types of ALP isoenzymes presented in serum samples. The separation of protein using electrophoretic technique is principally based on net negative charges on different protein molecules migrated with different rate in electrophoresis field to the anode and then separated into bands. Identifications were made using 6% polyacrylamide gel electrophoresis containing Triton X-100.

Protocol for Polyacrylamide Gel Electrophoresis (Chapman *et al*., 1987; Itoh *et al*., 2002; Matsushita *et al*., 1998)

Separation of protein molecules on polyacrylamide gel is based on the rate of migration of native protein through a sieving medium related to their shapes and molecular size. Electrophoresis performed on native gel followed by staining for enzymatic activity is a commonly used technique for the qualitative analysis of ALP isoenzymes in serum. In native form, the enzymes fold into complex secondary, tertiary, and quaternary structures. Their surfaces may be hydrophilic or hydrophobic, with greater or looser distribution of charge and reactive group. In polyacrylamide gel electrophoresis at pH 9.0, the charge of ALP isoenzymes result in the normal, liver isoenzyme migrating most rapidly toward the anode, with bone and intestinal isoenzymes migrating progressively more slowly (Chapman *et al*., 1987)

Sample preparation

Serum or reconstituted fractions separated from anion exchange column chromatography were applied on the native polyacrylamide gel (6% PAGE containing Triton X-100). For loading, 15 μL of serum was mixed with 15 μL of native sample

buffer. After the working buffer was filled in the chambers, a 20 μL of sample mixture was loaded on each well of the stacking gel (using a submerged technique) before the apparatus was plugged and starting the electrophoretic run.

Electrophoresis

The polyacylamide gel electrophoresis was carried out at 4°C, initially at 30 volts for 10 min and thereafter at 120 volts for 120 min at constant voltage mode using 0.375 mol/L Tris-boric acid buffer, pH 9.0 to 9.5 as electrophoresis buffer. ALP activity was detected by incubating the gel at 55°C in solution containing nitroblue tetrasolium and 5-bromo-4-choloro-3-indolylphosphate p-toluidine salt for 30 min.

VI. Method for Identification of ALP isoenzymes by Using Biochemical Characterization of the IAP Isoform Molecule on PAGE Containing Triton X-100

To identify the different types of ALP isoenzymes migrated on PAGE, the desialylation of the different ALP isoenzymes were performed to differentiate the IAP isoforms (Neuraminidase treatment) from those of other ALP isoenzymes.

In principle, the mobility of ALP isoenzymes in electrophoretic field affected markedly by the sialylation of sialic acid (or neuraminic acid) which mostly attached to the end of carbohydrate side chain of the ALP isoenzyme molecules. The IAP isoenzyme by nature has no sialylation on the carbohydrate side chain. Therefore, this is a useful characteristic of this isoenzyme to differentiate itself from those of other isoenzymes separated by electrophoresis technique. For separation, the ALP isoenzymes in serum and controls were treated with neuraminidase to eliminate the charge of sialic acid before electrophoresis (Miura *et al.*, 1994). Retard of electrophoresis band compared with its control without treating with neuraminidase

demonstrating the presence of sialic acid in the glycosylated enzyme. The procedure was described as followed.

Neuraminidase from *Clostridium perfringen* (C-Neu, 0.025 U/ml) was mixed with serum in polypropylene tubes. Then a 25 μL of serum sample was mixed with 5 μL of neuraminidase and incubated in 37°C water bath for 1 hour. After cooling, the neuraminidase treated sample was electrophoresis on polyacrylamide gel comparing with that untreated control. Interpretation of the migrated isoenzyme bands was made by comparing the migration of unknowns with the mobility of LAP & IAP isoenzyme treated the same way as the testing specimens and separated on the same gel of each electrophoresis run.

VII. Method Use to Confirm the Presence of IAP Isoforms in Serum and Molecular Weight Determination: Western Blot Analysis

Western blot is a technique for identification and quantitation of protein. The technique consists of four step procedures *i.e.* separation of polypeptides by SDS-PAGE, electrotransfer of separated proteins from the gel into the blotting paper, labeling of the transferred proteins by antibodies conjugated with the enzyme and detecting of the labeling enzyme signal. The details of procedure used in this experiment were as followed.

1. Separation of protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is the most widely used technique for quantification of complex mixtures of protein (Laemmeli, 1970). It is a technique that denatures the protein to nullify structural effects on mobility by SDS, denaturant, allowing separation on a ratio of a charge to mass basis. They also separate subunits from multimeric protein

by 2-mercaptoethanol, which is a reducing agent to distrupt any disulfide bonds through reduction. SDS is an anionic detergent. It denatures protein by binding to the protein chain with its negative charge molecule, dodecyl sulfate group. Proteins separation on a SDS-PAGE depends on their molecular size (Walker, 2002).

In this study, the separating gel was 12% concentration containing 8 mL of 30% acrylamide solution, 5 mL of 1.5 M Tris-HCl buffer pH8.8, 100 μL of 10% ammonium persulfate, 20 μL of TEMED and 6.94 mL of deionized water mixed and filled into gel sandwich until reaching the desired level, the gel was allowed to polymerize for 40 min at room temperature. The 4% stacking gel concentration composed of 665 μL of 30% acrylamide solution, 1.25 mL of 0.5 M Tris HC1 buffer, pH 6.8, 100 μL of 10% ammonium persulphate, 20 μL of TEMED and 1.1 mL of deionized water. All reagents were mixed and filled on the top of seperating gel. After that, the combs were inserted into stacking gel and allowed gel to polymerize for 30 min at room temperature.

Sample preparation

Serum were loaded on wells in denature form. For loading, 15 μL of serum was mixed with 15 μL of SDS reducing sample buffer (See Appendix A, III, 2.1). Samples were heat at 95°C for 5 min. After the electrophoresis working buffers (See Appendix A, III, 2.3) were filled in the tank, 20 μL of sample mixture was loaded to each well of gel and electrophoresis was carried on.

Electrophoresis

Separation of isoenzymes on SDS-PAGE was performed at a constant voltage of 190 V for 45 min. The position of tracking dye which monitors the progress of the run was checked after 5 min. Electrophoresis was carried on until the tracking dye reached the bottom of the gel. After electrophoresis, the gel was removed for staining or transferring the enzyme to the membrane. The gel was stained with Coomassie blue (Appendix A, III, 2.4.1) overnight. Destaining of the gel was performed by immerging the gel in the destaining solution I (Appendix A, III, 2.4.2) for 30 min and destaining II (Appendix A, III, 2.4.3) until the background of the gel was clear.

2. Electrotransfer of separated ALP isoenzyme from the gel into the blotting paper (Korolainen *et al*., 2002)

While the gel was running, PVDF membrane (8 x 6 cm in size) was prepared for blotting by soaking the membrane in absolute methanol for 1 min followed by deionized distilled water for 5 min and transferring buffer for 1 min. After that, PVDF membrane, two pieces of fiber pad and two filter papers were soaked in transfer buffer. As the electrophoresis was terminated (bromphenol blue tracking dye reached the bottom of the gel), the gel was removed for transfer. The following items were assembled in order to start blotting, from the black side of the cassette: fiber pad, filter paper, gel, membrane, filter paper, fiber pad, and the red cassette clamp, respectively. The cassettes were placed in the transfer tank, the black side was closed to the negative electrode and the buffer was filled until reaching the maximum filled lines. Electroblotting was performed by applying 25 volts at constant voltage for 3 hours. After transfer, the blotted membrane was placed in plastic box

and 5% skim milk in 1X TBS – Tween buffer pH 7.5 (Appendix A, IV, 2c) was added. Then, the membrane was incubated for overnight to block non-specific binding side.

3. Labeling of the transferred ALP isoenzyme by antibodies conjugated with the enzyme (Immunodetection)

After the blocked buffer was poured off and drained, the blotted membrane was incubated for 1 hour at room temperature with 6 mL of primary antibody (mouse anti IAP) mixture which previously diluted with 5% skim milk in 1X TBS-Tween pH 7.5 (ratio of 1:1000 v/v). Then the membrane was rinsed with 6 changes of 1X TBS-Tween pH 7.5 for 10 min in each change while shaking. After rinsing, 6 mL of secondary antibody (anti-mouse immunoglobulin) diluted with 5% skim milk in 1X TBS-Tween pH 7.5 in the ratio of 1:1000 (v/v) was added and the membrane was further incubated on the shaker for 1 hour at room temperature. At the end of reaction time with secondary antibody, the membrane was rinsed 6 times with 1X TBS -Tween pH 7.5 for 10 min in each change while shaking on a shaker.

4. Detection of the labeling ALP isoenzyme signal

Detection solution A and B (ECL) were mixed together in a ratio of 1:1 (2mL solution A plus 2mL solution B) before used. The final volume of detection reagent required is 0.1 mL/cm^2 of the membrane. After the excess washed buffer was drained, the membranes were placed protein side up on a piece of cling film. The mixed detection reagent was then spread on the membrane which was further incubated at room temperature for another 1 min and drained. The blotted enzyme was placed side down on a fresh piece of cling film, wrapped up and gently smoothed out the air bubbles. The wrapped blotted membrane was placed, protein side up, in an X-ray film cassette. The visualization of labeling isoenzyme was performed in dark

by placing the sheet of autoradiography film on top of the membrane. The cassette was closed and the film was exposed for 5 min or according to the optimized exposed time. For more exposure, the first film was removed out and replaced with a second sheet of unexposed film. At the end of exposure, the film was developed in developing solution for 1 min, washed in distilled water for 1 min, fixed in a fixing solution for 5 min, washed in distilled water for 5 min and finally it was let drying at room temperature.

VIII. Determination of IAP Isoform Activity and Ratios

Electrophoretic membranes with enzyme activity staining or the autoradiodetection membranes were scanned by using the Scion image program (Scion Corporation, USA) equipped on a PC. The ALP isoenzyme activity including the IAP isoforms separated on the PAGE were calculated by area normalization comparing with the total ALP activity determined previously by a kinetic method in UV 2450 Spectrophotometer. For the Western blot membranes, the area of each IAP isoform was also determined by the same program. The activities of two isoforms were calulated as an intensity ratio of HIAP/NIAP (See Appendix C).

IX. Statistic Analysis

Difference between mean of the interested groups of experiments were made by using the Wilcoxson rank sum test (Dawson-Saunders & Trapp, 1990). The probability at $p<0.05$ was considered significant difference.