CHAPTER II

MATERIALS AND METHODS

A. Materials

1. Specimens

Mucosa of tumor tissues and corresponded normal mucosa from five patients with colon carcinoma (four females and one male) were obtained by Endoscopy. The normal mucosal surface were collected from the colon area above the adjacent carcinoma. Blood samples from all patients were also collected and the sera were separated after clotted. All tissues and serum samples were immediately frozen and stored at -70 ^{o}C and -20^{o}C, respectively until used. All patient samples were collected after the informed consent at the Department of Internal Medicine, Faculty of Medicine, Chiang Mai University.

Normal sera were collected from thirty two healthy volunteers within the aged between 20-65 years at the Faculty of Associated Medical Sciences, Chiang Mai University. They were 20 females and 12 male s and composed of 11 donor with blood group B, 11 of blood group O, 8 of blood group A and 2 of blood group AB. All normal serum specimens were kept at -20^{o}C.

Informed consent were obtained from all patients and healthy volunteer donors by using forms approved by Research Ethics Committee, Faculty of Medicine, Chiang Mai University, (EC) Proforma 1 No.063/2004.

2. Instruments:

Instruments used in this study were listed as following-:

- Analytical balance A&D CF 300
- Bench-top homogenizer, Con-Totque (Eberbach Corporation, USA)
- Electrophoretic apparatus (Helena Laboratories, Beaumont, TX., USA)
- Electrophoretic Unit Hoefer mini VE (Amersham phamacial biotech. USA)
- Electrotransfer Unit V10-EBGRM (Scie-Plas Limited, UK)
- Heating block DB-101 (General Enterprises Marketing , Thailand)
- Lyophilizer (Lioalpha-10 (Telstar, Spain)
- Magnetic stirrer (Thermolyne Co., USA.)
- Microcentrifuge (Eppendorf, Germany)
- Microplate reader, Model EL 340 (Bio Tek instrument, USA)
- 96- well microplates (Linbo, Flow laboratories, Mc Lean, VA., USA)
- pH meter, Model 3560 (Beckman, USA.)
- Power supply (Major Science MP-250)
- Refigerated centrifuge 5417R (Eppendorf, Germany)
- Refrigerator (-70°C), Ultra cold (P.T.W., Thailand)
- Roller mixer SRT1 (Stuart scientific , UK)
- Stop watch
- TITAN GEL Chamber (Helena Laboratories, Beaumont, TX., USA.)
- UV-visible recording spectrophotometer UV-160 A, (Shimudzu.)
- Vortex mixture, VM-300 (Scientific Industries, New York, USA)
- Water bath, WB22 (Memert, Germany)
- X-ray film cassettes, PL-B 8x10 Inch (Okomoto Limited, Japan)

3. Chemical and Reagents

All chemicals used were analytical grade:

- Acetic acid glacial (Merck, Darmstadt, Germany)
- Acrylamide (Sigma Chemical Co., USA, No. A 8887)
- Agar (Sigma Chemical Co., USA, No. 7002)
- Agarose type V (Sigma Chemical Co., USA, No. A 3768).
- Albumin, Bovine, Fraction V (Sigma Chemical Co., USA, No. A 4503).
- Ammonium persulfate (Bio-Rad Laboratories, No. 161-0700)
- Barbitone (diethylbarbitturic acid) (BDH Chemicals, Poole England).
- Boric acid (Merck, Germany, No. K 22910765)
- Brilliant blue R -250 (Biorad Chemical Company, USA)
- Bromphenol blue (Merck, Darmstadt, Germany)
- Concanavalin A (Con A), (Sigma Chemical Co., USA, No. C7275)
- DEAE Sephacel (Sigma Chemical Co., USA, No. I6505)
- Diethanolamine : DEA (Sigma Chemical Co., USA, No.D8885)
- ECL Plus Western Blotting detection reagents (Amersham Biosciences, USA)
- Ethanol (Merck, Dermstadt, Germany)
- Glycine (Sigma Chemical Co., USA, No G8898)
- High molecular weight standard mixture for SDS gel electrophoresis (Sigma Chemical Co., USA, SDS-6H)
- Hydrochloric acid (Merck, Darmstadt, Germany)
- L-alanine (Sigma Chemical Co., USA, No.A 7627)
- L-arginine(SigmaChemicalCo.,USA,No.5006) L-Phenylalanine (L-2-amino-3-phenylpropanoic acid) (Sigma Chemical Co., USA, No P 2126)
- Lectin from *Triticum vugaris* (Wheat germ), (Sigma Chemical Co., USA, No L 9756)
- Levamisole (Sigma Chemical Co., USA, No. L 9756)
- 2-mercaptoethanol (Sigma Chemical Co,. USA No M 4125)
- Methanol (Merck, Dermstadt, Germany)

- Methyl α-D-manno- pyranoside (Sigma Chemical Co,. USA, No. M6882)
- Monoclonal anti-alkaline phosphatase Clone AP-59 (Sigma Chemical Co., USA, A9549)
- Neuraminidase Type III from *Vibrio cholerae* (Sigma Chemical Co., USA, No N 7885)
- N.N'-methylene-bis-acrylamide (Sigma Chemical Co., USA, No. M 7256)
- N,N,N',N'-tetramethyl-ethylene diamine: TEMED (Sigma Chemical Co., USA, No. T 8133)
- *Pisum sativum* aggutinine (PSA), (Sigma Chemical Co., USA, No. L 5380)
- *p*- nitrophenyl phosphate substrate (Fluka, Germany)
- Polyclonal goat anti-mouse immunoglobulins/HRP (Dako Cytomation, Denmark,P0447)
- Sodium barbital (Merck, Darmstadt, Germany)
- Transfer membrane 0.45μm (Bio Trace)
- TITAN GEL Alkaline Phosphatase (HR) Kit (Helena laboratories, Beaumont, TX., USA, No. 3058)
- Tris [hydroxymethyl]-aminomethane hydrochloride (Merck, Darmstadt, Germany)
- Tween 20 (Bio Basic Science)

B. Methods

I. Method for determination of total alkaline phosphatase activity

1. Total ALP activity measured by Double-beam UV- visible spectrophotometer (Shimudzu UV 160 A)

ALP activity was determined by a double beam UV-visible spectrophotometer by using CPS kinetic mode at wavelength 405 nm. The reaction mixture containing 2.9 mL of diethanolamine (DEA) buffer, pH 9.2 and 50 μL of *p*-nitrophenyl phosphate (PNPP) substrate as incubated at 37°C for 3-5 minutes. After adding 50 μL of samples into the mixture and mixed gently, the activity of ALP was measured at 405 nm, every 2 seconds for 5 cycles. 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 progression-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 9.2 at 37°C (German Society for Clinical Chemistry., 1972; Tietz, 1982)

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 volumn of reacttion : 3.1 mL

ε = Molar absorptivity of ρ- nitrophenol at 405 nm.

= 18250 L mol^{-1} cm^{-1}

S = Sample volume in mL : 0.05 mL

B = Light path in cm: 1cm.

$$\text{ALP activity (U/L)} = \frac{\Delta A/\text{min} \times 10^3 \times 3.1 \times 10^3}{1.825 \times 10^4 \times 1 \times 0.05}$$

$$= \Delta A/\text{min} \times 3397.26$$

2. Total ALP activity measured by a microplate reader

(Bio-Tek Instruments, Inc. Laboratory Division: Model EL 340)

ALP activity of limited samples were determined in 96-well microtiter plate in a microplate reader. The reaction mixture was reduced in proportion to the usual measurement of the activity in the above procedure using a final volume of 300 μL per well. The increase of absorbance at 405 nm of ρ-nitrophenol production was measured at 37 ^{o}C. All assays were performed in triplicate tests. The ALP activity was reported as U/L using 22.7 L mol $^{-1}$ cm $^{-1}$ as the millimolar absorptivity of *p*-nitrophenol for calculation.

The activity of enzyme measured in microplate reader was calculated from the following formula :

$$\text{ALP activity (U/L)} = \frac{\Delta A/\text{min} \times 0.3 \times 10^{3}}{22.7 \times 0.001 \times 1} = \Delta A/\text{min} \times 1{,}321.59$$

3. 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 sera or fractions in both Shimudzu UV160 A spectrophotometer and microplate reader EL 340. Routine condition variation (RCV) or between run assay of ALP activity was determined by using the same control specimens as OCV and performed simultaneously with the experimental samples in each assay of ALP activity in both instruments. Precision test was evaluated from percentage of coefficient of variation (% CV) by the following formula :

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

II. Liver function test for screening patient conditions

All patients were screened for liver disease by assaying the aspartate amino transferase and alanine amino transferasase activity in their sera. Both enzyme activities in patient sera were determined by kinetic method using a reagent kits *(Trace, Australia)* in Shimudzu UV-visible spectrophotometer. The activities obtained were interpreted for free from liver disease by

comparing with reference value shown in the request form established by Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University.

III. Preparation ALP isoenzyme from tissues

1. Preparation of Liver ALP from bovine liver tissue (Morton, 1954)

Liver ALP prepared from bovine liver was used as negative control for all experiments in this study. The preparation was carried out by homogenizing 20 g of bovine liver in 40 mL of ice cold distilled water. The homogenate was then centrifuged at 3500 rpm for 10 min in a cold room. After centrifugation, the supernatant was removed and placed in a beaker in which 20 mL of N-butanol was added and stirred for an hour at room temperature. The lowest aqueous layer containing ALP was collected after the mixture was centrifuged for 10 min at 4^{o}C and 3500 rpm, then it was added with cold acetone to the final concentration of 50% (v/v). The precipitate collected after centrifugation was dissolved with 10 mL of 0.1 mol/L Tris buffer pH 7.0. At last the liver enzyme solution was dialyzed overnight against 0.01 mol/L Tris buffer pH 7.7. Total protein and ALP activity in the dialyzed enzyme solution were measured before it was stored at 4^{o}C until used.

2. Preparation of ALP isoenzyme from patient specimens (Rambaldi *et al.*, 1997)

All tissue specimens from patients obtained from endoscopy were weighted by electrical balance. Each specimen was suspended in iced cold 25 mL /g of 100 mmol/L Tris - HCl, pH 7.6 containing 100 mmol/L NaCl, 1 mmol/L $MgCl_2$, 0.02 mmol/L and $ZnSO_4$ and homogenized by a bench top homogenizer. N-butanol, to a final concentration of 25% (v/v), was slowly added to the homogenate and the mixture was stirred at 4^{o}C overnight and then centrifuged at 9,000 g for 30 min. The aqueous layer containing ALP activity was separated from the organic phase. Cold acetone was added to the aqueous solution for the final concentration of 50%. The precipitate was collected after centrifugation and dissolved in 100 µL of 50 mmol/L Tris -HCl, pH 7.0. The enzyme solutions were partial purified by DEAE Sephacel mini column chromatography and used for further experiments.

IV. Partial purification of ALP isoenzymes from serum or enzyme solution by anion exchange chromatography (Mercer *et al.*, 1994; Karmen *et al.*, 1984)

Alkaline phosphatase isoenzymes (liver, bone and biliary etc.) were seperated by their different preferences on anion exchange column. The isoenzyme proteins were eluted from the resin by gradient concentrations of eluated buffers at different rates. Fractionation of ALP isoenzymes in sera and tissue extracts were performed on DEAE sephacel column using the below described procedure.

1. Partial purification of ALP isoenzymes

Seventy five microliters of serum or tissue extract of 75 µL was applied to the top of 6x6 column filled to a height of 1.8 cm with DEAE-Sephacel anion exchanger. The ALP isoenzymes were separated successfully from column at 25°C by step elution with 2 mL of 100 mmol/L NaCl (Buffer A), 6 mL of 150 mmol/L NaCl (Buffer B) and 3 mL of 300 mmol/L NaCl (Buffer C) per liter of 5 mmol/L Tris-HCl buffer, pH 8.0, respectively. For identification of peak fraction, each 0.5 mL of eluate was collected. The absorbance at 280 nm and ALP activity of each fraction were determined and plotted. Identification of IAP separated from column was carried out chemically and electrophoretically comparison with liver ALP previously isolated from bovine tissue with every analytical running. Eluated fractions obtained from DEAE-Sephacel column chromatography were pooled according to activity peaks and dried in the Lioalpha-10 (Telstar, Spain) and then kept at 4°C for further studies.

2. Precision of partial purification

Precision of partial purification was also performed by using 0.75 mL of five identical control serum sample subjected to column chromatography and eluted using the same procedure as previous described.

3. Total protein determination (Baines, 2001)

The amounts of protein shows maximal ultraviolet (UV) absorption at the wavelength of which was 280 nm arising from the contribution made by aromatic residue (of tryptophan, tyrosine) and to some extent from cystine groups. The intensity of absorption bears some relationship to the number of those residues presence in the protein chain. In this study, the protein concentration of fractionated ALP isoenzymes or those extracted protein solution were

determined by the characteristic absorbance at 280 nm and the formula used to calculate for protein concentrations in each isoenzyme fraction and other protein solutions was as follow :

Protein (g/L) = absorbance at 280 nm x 10

V. Identification of Chromatographic Fractions for IAP isoenzyme.

1. Chemical inhibition test .(Fishman *et al.*, 1963; Lustig and Kellen, 1971; Van Belle, 1976; Mulivor *et al.*, 1978)

There are more inhibition tests using selective inhibition by several amino acids or chemicals and drugs to show the differences among various ALP isoenzymes in serum or tissue homogenate samples (Lustig and Kellen, 1971). Principally, L-phenylalanine and levamisole are used to distinguish tissue specific ALP from those of tissue non–specific ALP isoenzymes. IAP isoenzyme is sensitively inhibited by L-phenylalanine whereas it has no effect on LAP, BAP and ALP activities from kidney tissue. In contrast, levamisole has strongly effect on inhibiting the LAP, BAP and kidney ALP but it has less effect on IAP activity (Fishman *et al.*, 1963; Lustig and Kellen, 1971; Van Belle, 1976; Mulivor *et al.*, 1978). Using these informations, the separated fraction from anion exchange column chromatography was identified and proved weather which fraction was the IAP isoenzyme.

Method of amino acid inhibition test was performed by dividing an eluated fraction into three 50 µL aliquots and placed in each cuvette appropriately labeled as control, LP (sample with L-phenylalanine inhibition) and LV (sample with levamisole inhibition) tube. After 2.95 ml of DEA buffer was added to each tube, a 50 µL of 500 mmol/L L-phenylalnine or 5 mmol/L levamisole was mixed into each corresponding cuvette. All reaction mixtures were mixed thoroughly and incubated at 37°C for 10 minutes before starting the enzymatic reaction by adding 50 µL of PNPP substrate. ALP activities sensitive to amino acid or chemical inhibitions were measured by double-beam UV spectrophotometer and the remaining activity of enzyme was calculated as the percentage of the respective control.

2. Identification of anion chromatographic fractions for IAP isoenzyme by electrophoretic method (standard method)

Electrophoretic technique is frequently used to examine the different types of ALP isoenzymes presented in serum samples (Chapman *et al.*, 1987). The separation of protein using electrophoretic technique is principally based on net negative charges on different protein molecules migrated with different rate in electrophoretic field to the anode and then separated into bands. Identifications were made by both agarose gel and polyacrylamide gel electrophoresis.

2.1 Agarose gel electrophoresis (Gonchoroff *et al.*, 1989; Miura *et al.*, 1994)

For agarose gel elctrophoresis, ALP isoenzymes were seperated on the basis of their molecular sizes and electrophoretic mobility.

An electrophoretic separation of ALP isoenzymes was carried out at 4°C for 60 minutes with a constant voltage at 200 volts. Agarose gel electrophoresis of ALP isoenzymes eluate was separated on 0.8% agarose gel prepared in this laboratory. Each lyophilized sample (4 ml origin) was dissolved with 200 µL distilled water then left standing for 30 minutes before performing electrophoresis. Ten microliters of different dissolved fractions were applied on the template slits on agarose gel and wait for 10 minutes after the last sample had been applied for absorption. After that, the template was carefully removed and the electrophoresis was carried out at 4°C for 60 minutes at 200 volts. The gel was removed from the chamber and immediately stained for 60 minutes at 45°C with 5-bromo-4-choloro-3-inodyl-phosphate p-toluidine salt and nitroblue tetrazolium. After incubation, the gel was washed in 10% (v/v) acetic acid for 5 minutes, then removed and rinsed with distilled water for 10 minutes and dried at 50-60°C. Band of each fractionated ALP isoenzyme was identified qualitatively by comparison with LAP and a commercial IAP standard.

2.2 Polyacrylamide gel electrophoresis (Chapman *et al.*, 1987; Matsushita *et al.*; 1998; Itoh *et al.*, 2002)

The 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. The 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 are fold into complexed secondary, tertiary, and quaternary structures. Their

surfaces may be hydrophilic or hydrophobic with greater or looser distribution of charge and reactive groups. In polyacrylamide gel electrophoresis at pH 9.0, the charges of ALP isoenzymes of the normal liver isoenzyme result in migrating most rapidly toward the anode while the bone and intestinal isoenzymes were progressively migrating more slowly (Chapman *et al.*, 1987).

Gel preparation

The separating gel concentration was 7.5% acrylamide consisting of 2.25 mL of 30% acrylamide solution, 2.25 mL of 1.5M Tris-HCl buffer, pH8.8, 450 μL of 20% Triton X-100 , 35μL of 10% Ammonium persulfate , 5 μL of TEMED and 4.05 mL of deionized distilled water. The solution was mixed thoroughly before pouring into the vertical gel appratus (Amersham phamacial biotech) until reached the desired level. The gel was then allowed to polymerize for 40 minutes at room temperature. After that, the 4% stacking gel consisting of 300 μL of 30% acrylamide solution, 500 μL of 0.5 M Tris HCl buffer (pH 6.8), 100 mL of 20% Triton X-100, 6 μL of 10% Ammonium sulphate , 3 μL of TEMED and 1.1 mL of deionized distilled water was poured on the top of the seperating gel and the comb was inserted into the stacking gel then allowed to polymerize for 30 minutes.

Sample preparation

Serum or reconstituted fractions separated from anion exchange column chromatography were applied on the native polyacrylamide gel. For loading, 15 μL of serum or reconstitued eluated fraction was mixed with 15 μL of native sample buffer. After the working buffer was filled in the chamber, 20 μL of sample mixture was loaded on each well of the stacking gel by using a submerged technique before the apparatus was plugged for starting the electrophoresis run.

Electrophoresis

The polyacylamide gel electrophoresis was carried out at 4° C, initially at 30 volts for 10 minutes and then at 90 volts for 70 minutes at constant voltage mode with using 0.375 mol/L Tris-boric acid buffer, pH 9.0 to 9.5 as electrophoresis buffer. The ALP activity was detected by staining the gel in solution containing nitroblue tetrasolium and 5-bromo-4-choloro-3-inodyl phosphate at 50°C for 2 hour.

VI. Study of IAP properties

1. Heat inactivation (Farley *et al.*, 1993)

The ALP isoenzymes are rendered inactive at different temperatures. The most remarkable property of ntestinal ALP isoenzyme is its pronounced stability to heat at temperature of 65°C secondarily to placental ALP which is more stable for an hour or more without loss of activity (Neale *et al.*, 1965; Chang *et al.*, 1980; Goldstein *et al.*, 1982). In contrast the liver, bone and kidney ALP isoenzymes are rapidly inactivated under these conditions. However, the intestinal ALPs are somewhat more thermostable than liver, bone and kidney ALPs. It has also been shown that liver ALP is slightly through significantly, more thermostable than bone ALP.

The procedure for heat inactivation assay was modified from Farley *et al.*,1993; Moss *et al.*, 1994; Henderson *et al.*, 1994. In this study, the eluated fraction was 100 μL collected into three different aliqouts appropriately labeled as control, 52°C and 65°C. The control aliquot was left at room temperature, while the 52°C and the 65°C aliquots were incubated separately for exactly 1 hour in each corresponded waterbath. After the aliquots were removed from the waterbath, they were cooled in iced water. The ALP activities in eluated fractions of heat and unheat control immediately were determined in DEA buffer, pH 9.3 in double-beam UV spectrophotometer using previous described method. The rate of heat inactivation of isoenzyme was calculated in the percentage of activity comparison with the unheat control kept at room temperature.

2 .Amino acid inhibition test

Tissue-specific property of IAP is enzymes can be exhibited by amino acid inhibition test. Various low molecular weight of amino acids show different inhibition of the different ALPs. Placental, placental like and intestinal ALPs are more sensitive to the inhibition with L-phenyalanine than liver, bone and kidney ALPs whereas the liver, bone and kidney ALPs are more sensitive to the inhibition with levamisole. The property of IAP which may be influented by different amino acids other than L-phenylalanine and levamisole were observed. The selected amino acids, 0.05 mol/L of L-arginine and L-alanine have been shown to inhibit the activity of IAP (Lustig and Kallen, 1971,modified) and was used instead of L-phentlalanine or levamisole in the inhibition test. Results of the inhibition test were compared with the

corresponding control using distilled water instead of amino acid in double-beam UV spectrophotometer. The remaining activity of enzyme was calculated as the percentage of the respective control.

3. Reactivity with IAP monoclonal antibody

To examine the antigenicity of ALPs in serum and tissue extracted enzyme containing ALP isoenzymes in the reaction with anti IAP antibody (monoclonal anti IAP), ALP isoenzymes in serum of normal controls and patients with colon cancer were treated with anti IAP antibody in polypropylene tubes. The experiment was performed by incubating the mixture of 60 μL of serum (or IAP standard and LAP control) and 3 μL of anti IAP (1:2000 : v/v) for 1 hour at room temperature. After incubation, the reactivity of IAP with monoclonal anti IAP was detected by electrophoresis on agarose or polyacrylamide gel using the method described earlier. The band with treated monoclonal antibody (MoAb) reaction was compared with that of untreated control (Fujimori-Arai *et al.*, 1991-Modified).

VII. Biochemical characterizations of The IAP isoforms

1. Sialylation (Neuraminidase treatment)

This experiment performed to show the molecular structure of carbohydrate side chain of the IAP isoenzyme. Mobility of ALP isoenzymes in electrophoretic field affected markedly by sialylation of sialic acid (or neuraminic acid) which mostly attached to the end of carbohydrate side chain of the isoenzyme molecule. ALP isoenzymes in serum of normal controls and patients with colon cancer were treated with neuraminidase to eliminate the charge of sialic acid before electrophoresis (Miura *et al.*, 1994). Retard of electrophoretic band compared with that control without treating with neuraminidase demonstrating the presence of sialic acid in the glycosylated enzyme. The procedure was described as follow.

5 μL of neuraminidase from *Vibrio cholerae* (V-Neu) or *Clostridium perfringen* (C-Neu) was mixed with 25 μL of serum or tissue homogenate in polypropylene tubes. The mixture was incubated in 37°C waterbath for 1 hour. After cooling, the neuraminidase treated sample was electrophoresed on agarose gel comparing with that untreated control. Interpretation of the migrated isoenzyme bands was made by comparing with LAP & IAP treated the same way as the testing specimens and separated on the same gel of each electrophoretic run .

2. Lectin precipitation of sugar moieties of the ALP isoenzymes.

Several lectins have been used to characterize the internal sugars of the carbohydrate site chains of ALP isoenzymes. The lectins used in this experiment were 3g/L wheat germ agglutinin (WGA), 6 g/L concanavalin A (Con A) and 1g/L *Pisum sativum* agglutinin (PSA)(Farley *et al.*, 1993; 1994). Each lectin was prepared as an aqueous solution in distilled water before used. In polypropylene tube, four 200μL aliquots of IAP (or LAP) fractions was mixed with 20 μL of 20% Triton-X 100. All reaction tubes were incubated at 37oC for 30 minutes. After incubation, one aliquot was added with 150 μL of distilled water, and the other aliquots were added with 150 μL of different lectin solutions respectively. All tubes were thoroughly mixed and incubated at 37°C for 30 minutes. After that, the mixtures were centrifuged at 1000 x g for 15 minutes. The supernatant was assayed in triplicate for remaining activities using microplate method for determining total ALP activity. Lectin insensitive ALP activity was determined as the difference between the lectin-treated samples and the distilled water-treated control.

3. Treatment with Phosphatidylinositol phospholipase C (PI-PLC)

The membrane anchoring ALPs in serum of normal controls and patients with colon cancer were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) to remove the lipid moieties in the ALPs molecule. The mixture of 100μL of serum and 10 μL of PI-PLC (50mU/mL) were mixed and incubated at room temperature for 3 hours. After that, the PI-PLC-treated serum was further separated on Concanavalin A column compared with its untreated-serum and standard ALP isoenzyme (Miura *et al.*, 1989).

4. Affinity chromatography: Concanavalin A column chromatography

Lectin are another class of ligands that have been used for direct detection of clinical analytes by affinity chromatography. The lectins are non-immune system proteins that have ability to recognize and bind certain types of carbohydrate residue. These ligands commonly are used in the isolation of many carbohydrate-containing compounds, such as polysaccharides, glycoproteins, and glycolipids. The Concanavalin A binds to α-D-manose and α-D-glucose residue of the glycol-molecule (Cummings *et al.*, 1982, Koyama *et al.*, 1987, Hage, 1999).

In this experiment, 75 μL of serum, tissue extracted or sample treated with PI-PLC was applied to the top of 6x6 column filled to a hight of 1.8 cm with Concanavalin A Sepharose 4B.

The column was rinsed with 5mL of $NaHCO_3$ buffer (25 mmol/L pH 8.0), containing, per liter, 0.14 mol of NaCl, 1 mmol of $MnCl_2$, 1 mmol of $MgCl_2$, 1 mmol of $CaCl_2$, and 0.1 g of azide. After that, ALPs isoenzymes were eluted with 50 mL of 0.1 mol/L α-methylmannoside in the same buffer. Two mililitres of each fractions were collected for determination of protein at 280 nm and ALP activity.

VIII. 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 polypepetides 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 (Page and Thorpe, 2002). 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. SDS-PAGE 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 form multimeric protein by 2-mercaptoethanol, which is a reducing agent to disrupt any disulfide bonds through reduction. SDS is an anionic detergent. It denatures protein by binding to the protein chain with its negative charge molecule, dodecylsulfate group. Proteins separation on a SDS-PAGE depend 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 1.5M Tris-HCl buffer pH8.8, 100 μL of 10% ammonium persulpfate , 10 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 polymerized for 40 minutes at room temperature. The 4% stacking gel concentration composed of 665 μL of 30% acrylamide solution, 1.25 mL of 0.5 M Tris HCl buffer, pH 6.8, 25 μL of 10% ammonium sulphate, 5 μL of TEMED and 1.1 mL of deionized water. All reagents were mixed and

filled on the top of separating gel. After that, the combs were inserted into stacking gel and allowed gel to polymerized for 30 minutes

Sample preparation

Serum or reconstituted enzyme fractions from anion exchanged chromatography were loaded on wells in denature from. For loading, 15 μL of serum or reconstituted fraction was mixed with 15 μL of SDS reducing sample buffer. Samples were heat at 95oC for 5 minutes. After the electrophoresis working buffer were filled in the tank , 20 μL of sample mixture was loaded to each well of gel and electrophoresis was carried on.

Electrophoresis

Seaparation of isoenzymes on SDS-PAGE was performed at a constant voltage at 190 V for 45 minutes. The position of tracking dye which monitor the progress of the run was check after 5 minutes. Electrophoresis was carried out 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 Billiant blue R-250 overnight. Destaining of the gel was preformed by immerging the gel in the destaining solution I for 30 minutes and destaining II until the background of the gel was clear.

2. Electrotransfer of separated ALP isoenzyme from the gel into the blotting paper (Page and Thorpe, 2002)

While the gel was running, nitrocellulose membrane (8 x6 cm in size) was prepared for blotting by soaking the membrane in methanol. After that, nitrocellulose 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 cassette was 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 preformed by applying 30 volts at constant voltage for overnight and the water cooled base of tank was rinsed thoroughly with water to prevent overheating effects of the system. After transfer, the blotted membranes were removed for detection.

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

The blotted membrane was placed in plastic tube and 3 mL of 2.5% skim milk in TBS - Tween , pH 7.8 was added. Then, the membrane was incubated on a shaker at room temperature for 1 hour to block non specific binding side. After the blocked buffer was poured off and drained, the blotted membrane was incubated for 1 hour at room temperature with 3 mL of primary antibody (mouse anti IAP) mixture which previously diluted with 2.5% skim milk in TBS-Tween, pH 7.8 (ratio of 1:1000, v/v). Then the membrane was rinsed with 6 changes of TBS-Tween, pH 7.8 for 20 minutes while shaking. After rinsing, 3 mL of secondary antibody (anti-mouse immunoglobulin) diluted with 2.5% skim milk in TBS-Tween, pH 7.8 in the ratio of 1:2000 (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 TBS -Tween, pH 7.8 for 20 minutes while shaking on a shaker.

4. Detection of the labeling ALP isoenzyme signal

Detection solution A and B (ECL) was mixed together in a ratio of 40:1 (2 mL solution A + 50 µL 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 was 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 5 minutes 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 60 seconds 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 minutes, washed in distilled water for 1 minute, fixed in a fixing solution for 5 minutes, washed in distilled water for 5 minutes and finally it was let drying at room temperature.

IX. Statistic Evaluation

Analytical data of ALP activity and protein determination were reported as average of duplicate test (Mean ± SD). Comparison of two set of results was performed by Student t-test using Microsoft Excel 98 on a Microsoft Windows 2000 in personal computer. A value of $p < 0.05$ was concluded significant.