APPENDIX

[A] Reagent preparations

I. Reagents for Alkaline phosphatase activity

1. Mg Cl_2 solution

Dissolve 20.3 gm MgCl2 * H_2O in 100 mL reagent grade water.

2. 1.0 M Diethanolamine with 0.05 mM $MgCl_2$ buffer, pH 9.8

Diluted 124 g diethanolamine (85%) with reagent grade water. Add 0.5 mL $MgCl_2$ solution and adjusted pH to 9.8 (at 37°C) with HCl.

3. 0.67M ρ-Nitrophenyl phosphate solution

Dissolve 250 mg ρ-nitrophenyl phosphate, sodium salt in 1.0 mL reagent grade water.

II. Reagent for preparation of ALP isoenzyme from patient specimens

1. Homoginized buffer: 100 nM Tris-HCl (pH 7.6) containing 100mM NaCl, 1mM $MgCl_2$, 0.02mM$ZnSO_4$

Tris	3.03	g
NaCl	5.85	g
$MgCl_26H_2O$	0.05	g
$ZnSO_47H_2O$	1.44	mg

III. Reagent for amino acid inhibition test

1. 500 mM L-Phenylalanine

Dissolved 8.26 g of L-Phenylalanine (MW 165.2) in distilled water to make 100mL of solution, mixed well. Stored at 4°C.

2. 5 mM Levamisole

Dissolved 0.1204 g of levamosole (MW 240.8) in distilled water to make 100 mL of solution, mixed well. Stored in 4°C.

3. 0.5 M L-Alanine

Dissolved g of L-Alanine (MW) in distilled water to make 100 mL of solution, mixed well. Stored in 4°C.

4. 0.1M Arginine

Dissolved g of L-Arginine (MW) in distilled water to make 100 mL of solution, mixed well. Stored in 4°C.

IV. Reagent for fraction of ALP isoenzymes by anion exchange chromatography

1. 100mM NaCl in 5 mM Tris-HCl ,pH 8.0

Dissolved 0.6057 g of Tris (MW) and 5.85g of NaCl in 900 mL of distilled water, mixed well. Adjusted pH to 8.0 with 1N HCl. Filled up with distilled water to 1,000 mL, stored in 4°C.

2. 150mM NaCl in 5 mM Tris-HCl pH 8.0

Dissolved 0.6057 g of Tris (MW) and 5.85g of NaCl in 900 mL of distilled water, mixed well. Adjusted pH to 8.0 with 1N HCl. Filled up with distilled water to 1,000 mL, stored in 4°C.

3. 300mM NaCl in 5 mM Tris-HCl pH 8.0

Dissolved 0.6057 g of Tris (MW) and 5.85g of NaCl in 900 mL of distilled water, mixed well. Adjusted pH to 8.0 with 1N HCl. Filled up with distilled water to 1,000 mL, stored in 4°C.

V. Reagent for electrophoresis on agarose gel

1. Tris-barbital-sodium barbital buffer pH 8.8-9.0

Trisma base	6.055	g
Sodium barbital	10.309	g
Barbitone	2.5788	g

Dissolved reagent in approximately 800 mL deionized distilled water, adjusted to pH 8.8 - 9.0 with HCl. Made to 1000mL with deionized distilled water and store at 4°C

2. 0.5% agar for coated plastic plate

Agar	0.125	g
Distilled water	25	mL

Boiled agar, and then left in water bath at 56°C before pour it on plastic plate about 2 mL/plate. Dired the gel at 50°C overnight.

3. 0.8% agarose

Agarose	0.2	g

Tris-barbital-sodium barbital buffer 25 mL

Bolied agarose, and then left in water bath at 56°C before pour it on the plastic plate (2) about 7 mL/plate. Dired surface of gel in 50°C for 10 minutes, and stand at room temperature for 20 minutes.

4. Staining solution

AMP	5.355	g
5-Bromo-4-cholro-3-indole phosphate p-toluidine salt	22.11	mg
Magnesium sulfate	3.07	mg
Nitroblue tetrasolium	44.89	mg
Sodium azide	0.03	g

Dissolved reagent in 30 mL deionized distilled water and stored at -20°C

VI. Reagent for electrophoresis on polyacrylamide gel.

1. Polyacrylamide gel electrophoresis for separated ALP isoenzymes

1.1 Seperating gel buffer stock (1.5M tris-HCl pH 8.8)

Dissolved 27.2 g Trisma base in approximately 100 mL deionized distilled water. Adjusted to pH 8.8 with HCl. Made to 150 mL with deionized distilled water and stored at 4 °C

1.2 Stacking gel buffer stock (0.5M Tris-HCl pH 6.8)

Dissolved 6 g Trisma base in approximately 60 mL deionized distilled water. Adjusted to pH 6.8 with HCl. Made to 100 mL with deionized distilled water and store at 4 °C

1.3 Sample buffer

Deionized distilled water	4.8	mL
0.5M tris-HCl, pH 6.8	1.0	mL
Glycerol	2.0	mL
0.5% bromphenol- blue	0.2	mL

Total volume 8.0 mL mixed and stored at -20°C

1.4 30% Acrylamide stock solution

Acrylamide	146.0	g
N'N'-bis-methylene-acrylamide (Bis)	4.0	g

Dissolved in about 350 mL deionized distilled water then adjust to 500 ml with deionized distilled water. Filter and store at 4°C in the dark.

1.5 20% Triton X-100

Dissolved Triton X100 20 g made to 100mL with deionized distilled water.

1.6 0.375 M Tris - boric acid buffer pH 9.0

Dissolved 15.5 g boric acid with deionized water 100 mL. Dissolved 45.5 g of Trisma base in 900 mL of deionized distilled water. Adjusted to pH 9.0 with boric acid solution. Made to 1000 mL with deionized distilled water and stored at 4°C.

1.7 Staining solution

3-indoxyl phosphate p-toluidine salt		20	mg
Nitroblue tetrazolium	10	mg	
2-amino-2-methyl-1,3-propanediol (AMP)		4.47	g
Magnesium sulfate		6	g

Dissolved in deionized distilled water approximately 30 mL, adjust pH to 10.1. Made to 50 mL with deionized distilled water.

1.8 10% ammonium persulfate

Dissolved 0.1 g of ammonium persulfate with 1 mL of deionized distilled water.

2. SDS-PAGE

2.1 Sample buffer (SDS-reducing buffer : 0.125M Tris HCl, 4% SDS, 20% v/v glycerol, 0.02%bromphenol blue, pH 6.8)

Stacking gel buffer	2.5	mL
SDS	0.8	g
Glycerol	2	mL

Made to 10 mL with deionized distilled water. Added 50 µL beta-mercaptoethanol to 950 µL of sample buffer prior to use. Mixed aqual volume of sample buffer, and heat 95°C for 5 minutes.

2.2 10x Electrode (running) buffer pH 8.3

Tris base	30.3	g
Glycine	144.0	g
SDS	10.0	g

Dissolved and adjust to 1,000mL with deionized distilled water. Do not adjust pH with acid or base.

2.3 1x Electrode (running) buffer pH 8.3

To make 1 liter of 1x electrophoresis buffer (0.025M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3), diluted 100 mL of 10x electrode buffer with 900mL deionized water.

2.4 Protein staining (Coomassie Blue)

2.4.1 Coomassie Blue staining solution

Coomassie Billiant Blue R250	0.125	g
Methanol	200	mL
Acetic acid	35	mL

Mixed and adjusted volume to 500 mL with deionized distilled water. Stored at room temperature.

2.4.2 Destain solution I

Methanol	200	mL
Acetic acid	35	mL

Mixed and adjusted volume to 500 mL with deionized distilled water. Stored at room temperature.

2.4.3 Destain solution II

Acetic acid	70	mL
Methanol	50	mL

Mixed and adjusted volume to 500 mL with deionized distilled water. Stored in room temperature.

VII. Reagent for lectin precipitation test

1. 3g/L wheat germ agglutinin (WGA)

   Dissolved weat germ agglutinine 10 mg in distilled water 3.33 mL. Stroed at -20°C

2. 6 g/L concanavalin A (Con A)

   Dissolved Concanavalin A 0.03 g in distilled water 5 ml. Stored at -20°C

3. 1g/L *Pisum sativum* agglutinin (Pea)

   Dissolved Pea 5 mg in distilled water 5 mL. Stored at -20°C

VII. Reagent for Concanavalin A column

1. $NaHCO_3$ 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.

$NaHCO_3$	2.10	g
NaCl	8.19	g
$MnCl_2$	0.20	g
$MgCl_2$	0.20	g
$CaCl_2$	0.11	g
Na N3	0.1	g

Dissolved in about 800 mL deionized distilled water then adjust pH to 8.0 ml. Added deionized distilled water to 1000mL. Store at 2-8°C.

2. 0.1 mol/L α-methylmannoside in $NaHCO_3$ 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.

α-methylmannoside	19.41	g
$NaHCO_3$	2.10	g
NaCl	8.19	g
$MnCl_2$	0.20	g
$MgCl_2$	0.20	g
$CaCl_2$	0.20	g
NaN3	0.1	g

Dissolved in about 800 mL deionized distilled water then adjust pH to 8.0 ml. Added deionized distilled water to 1000mL. Store at 2-8°C.

VIII. Reagent and buffer for Western bolt analysis

1. Electrotransfer of the separated protein into the blotting paper

1.1 10x Transfer buffer

Tris base	30.3	g
Glycine	144	g

Dissolved with deionized distilled water approximately 800 mL, adjusted pH to 8.3 and filled deionized distilled water to volume 1000 mL

1.2 1x Transfer buffer

To make 5 liter of 1x transfer buffer pH 8.3, diluted 500 mL of 10x with 3500 mL deionized water. Added 1000 mL of methanol. Strore at room temperature.

2. Labeling of the transferred proteins with antibodies

2.1 10x TBS-Tween buffer pH 7.5

Tris base	24.2	g
NaCl	80	g

Dissolved with deionized distilled water approximately 800 mL, adjusted pH to 7.5 and filled deionized distilled water to volume 1000 mL. After that added 10 mL of Tween-20, mixed and stored at 4°C

2.2. 1x TBS-Tween buffer pH 7.5

To make 1 liter of 1x TBS-Tween buffer pH 7.5, diluted 100 mL of 10x TBS-Tween buffer pH 7.5 with 900mL deionized water

2.3 Blocking buffer (2.5% skimmed milk in 1x TBS-Tween buffer pH 7.5)

Dissolved 2.5 g of skimmed milk in 1x TBS-Tween buffer pH 7.5.

[B] Chromatography data

1. Results of separated intestinal alkaline phosphatase by gel filtration chromatography

Figure 29 showed the pattern of partial purification of IAP standard on Sepharore - 4B chromatography. Separation yielded 2 protein peaks at fraction No. 3-7 and No.12-21. Protein eluates containing ALP activity in each protein peak. The first protein peaks were collected as P1 pooled fraction. The second protein peaks were collected as P2 pooled fraction. Both P1 and P2 pooled fraction were subjected to DEAE-Sephacel column chromatography to identified the position of P1 and P2 elution profiles.

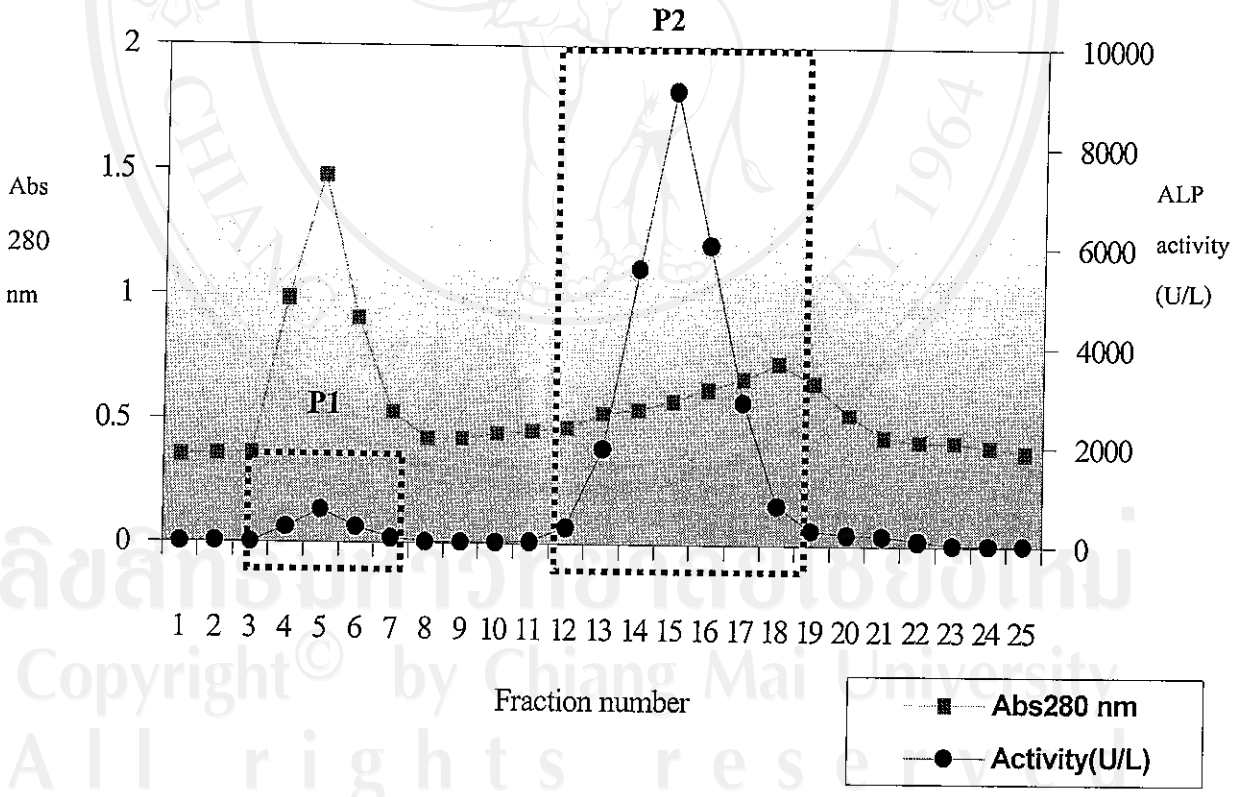

Figure 29. Pattern of partial purification of IAP standard on Sepharose 4B (gel filtration) column chromatography. P = pooled fraction

2. Results of separated intestinal alkaline phosphatase by anion-exchangechromatography

Pool fractions of IAP standard separated by gel filtration chromatography (P1,P2) were separated by anion-exchange chromatography. P1 activity peak between fraction 23- 27 (30A), P2 activity peak between fraction 3-7 was NIAP. (Figure30B),

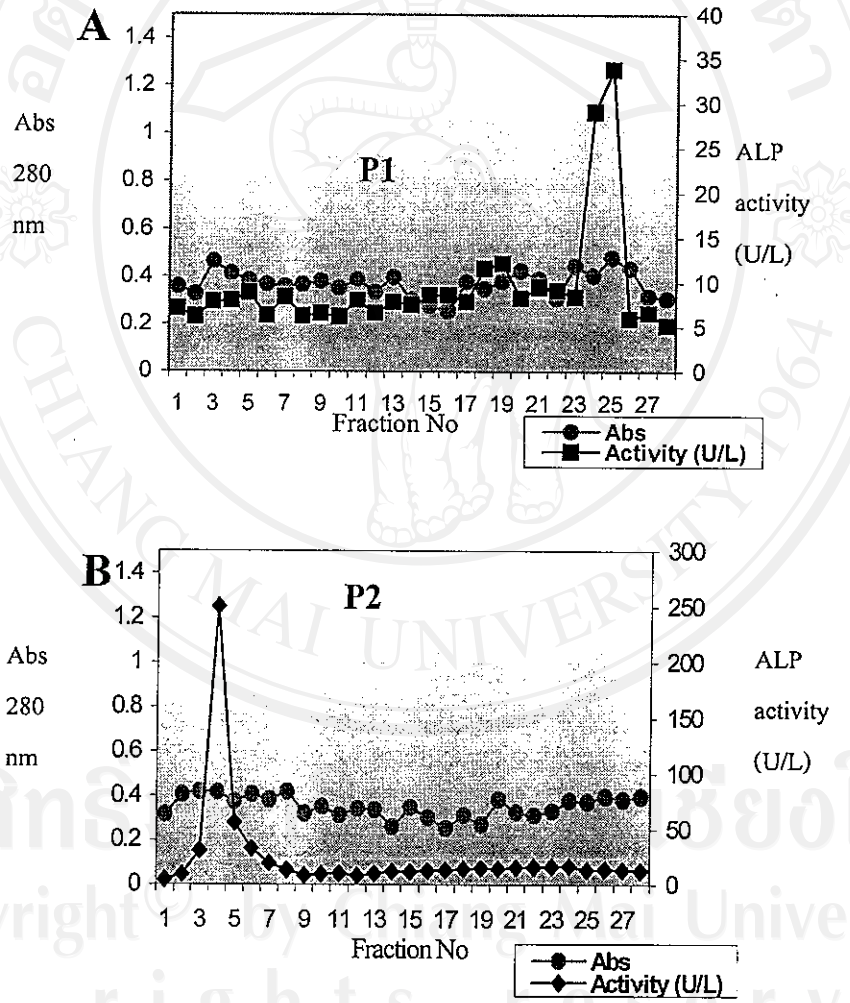

Figure 30. Pattern of partial purification of IAP standard eluted from sepharose 4-B column by DEAE sephacel anion exchange chromatography.

A : Pooled solution (P1) from figure 30.

B : Pooled solution (P2) from figure 30

[C] Surgical pathology report

P1 Female Age: 52 Years

DIAGNOSIS:

1. Sigmoid colon, resection-T67700P10315M81413

   ADENOCARCINOMA, WELL DIFFERENTIATED

   Subserosal invasion, lymphatic (+), venous (not seen)

   Proximal margin: FREE; Distal margin: FREE

   Mesocolic lymp nodes, dissection:

   NO METASTATIC DEPOSITS SEEN, N=0/7

2. Proximal margin, resection-

   NO MALIGNANCY SEEN

3. Distal margin, resection-

   NO MALIGNANCY SEEN

Macroscopic Description: The specimens are submitted in 3 container.

1. A resected segment of sigmoid colon, 21x5x4 cm, with sutures marked at both side of the resected margins. There is a infiltrative mass, 4x3 cm, at 9.5 cm from the lower margin. The serosal surface is smooth and glistening. The mesocolic nodes are small, 0.3-0.6 cm in diameters, with soft consistency. Representative sections are taken and submitted together as A: proximal margin; B: distal margin; CDE: longitudinal sections of the tumor mass; FG: cross sections of the lesion; H: vascular margin; IJ: mesocolic nodes.
2. Labeled " proximal margin " is a small compressed strip of colonic wall measuring 2 x 1 x 0.5 cm. It is submitted entirely as K
3. Labeled " distal margin " is a small compressed strip of colonic wall measuring 2.5 x 1.5 x 0.7 cm. It is submitted entirely as L.

Microscopic Description: Sections of the colonic lesion show invasive growths of well differentiated adenocarcinoma with inflamed desmoplastic stroma. The cancer directly invades

through the muscular wall into the subserosal fat tissue with lymphatic invasions. However, the mesocolic nodes, total 7 are all negative for metastatic deposits. The cancer forms various-sized tubular structures lined by tall columnar cells with bizarre vesicular nuclei bearing prominent nucleoli. The vascular margin is negative as well as both sides of resected margin including the separately submitted colonic walls , K and L.

P2 Female Age: 69 Years

DIAGNOSIS:

Rectum, 8x, biopsy. T68000P107030M81423

ADENOCARCINOMA. 6/8

MODERATELY DIFFERENTIATED

Macroscopic Description:

The specimens are eight pieces of white tissue measuring 0.2 cm in average diameters. They are submitted together as A 4, B 4.

Microscopic Description :

Sections show invasive growths of moderately differentiated adenocarcinoma with mildly inflamed desmoplastic stroma and lymphatic invasions. The cancer displays wide spectra of growth patterns from tubular, tubuloparpillary and cribiform structures lined by tall columnar cells with hyperchromatic bizarre nuclei. Cancer necrosis is also prominent.

P3 Female Age: 63 Years

DIAGNOSIS

Ascending colon, right half colectomy- T67200P103151M81423

ADENOCARCINOMA

MODERATELY DIFFERENTIATED

Subserosal invasion, lymphatic (+), venous (+)

Mesocolic nodes: METASTATIC ADENOCARCINOMA, n = 3/3

Proximal margin : FREE; Distal margin : FREE

Vascular margin : NO TUMOR THROMBUS

Metastatic deposits in the apical node with perinodal invasions

Omentum : NO METASTATIC DEPOSITS SEEN

TUBULAR ADENOMAS, 2X

LOW GRADE DYSPLASIA

Macroscopic Description :

The specimen is an en bloc segment of right half colectomy measuring 6x2 cm for the ileum, 26x6.5 cm for the colon and 4.5x0.6 cm for the unremarkable vermiform appendix. The cecum is dilated , 14 cm, containing an ulcerated lesion, 5x7.5 cm, at 11 cm from the distal margin. There are two pedunculated polyps, 1.3 and 1.0 cm in diameters, at 6 and 12 cm from the IC valve. The mesocolic nodes are palpable with hard consistency. Representative sections are taken and submitted together as follow: A = ileal margin; B = colonic margin; C = appendicle tip; D-F/G-I = step longitudinal sections of ulcerating lesion from the lower to upper side; JK/LM = cross sections of lesion at the medial and lateral sides; NO/P = the polyps at 6 and 12 cm from the IC valve; Q-R = sections from the attached omentum, 23x8x1 cm.

Microscopic Description:

Sections show invasive growths of moderately differentiated adenocarcinoma with desmoplastic stroma. The cancer directly invades through the muscular wall into the fibrotic subserosa. The serosal surface free. There are prominent lymphatic invasions, including

metastatic deposits in the mesocolic nodes (3/3), without definitive venous invasion and vascular margin is free. The cancer from solid nestes, with occasionally observed glandular lumina containing mucous material, and tubular structures lined by bizarre columnar/ polygonal cells with clear/ vasculated cytoplasm and pleomorphic vesicular nuclei. The resected margins including vermiform appendix are all negative for malignancy but the apical node at the vesicular margin, L, show metastatic deposits with perinodal soft tissue invasion although no tumor thrombus is included. The colonic polyps are similar to each other. They are composed of proliferative colonic crypts with distorted lumina lined by absorptive columnar cells bearing stratified hyperchromatic nuclei and decreased amount of globet cells. The stalk is composed of intact muscularis mucosae and congested submucosa. The omentum is free without metastatic deposits.

P4 Male

DIAGNOSIS

Colon, ascending and transverse, 6x,biopsy.

ADENOCARCINOMA,

MODERATELY DIFFERENTIATED

Note: Tissue are submitted in the same containers without definitive labels.

Macroscopic Description:

The specimens labeled " ulcerated mass at asending and transverse colon" are six pieces of white tissue. They are submitted together as A:3, B3.

Microscopic Description:

Section show invasive growths of moderately differentiated adenocarcinoma with desmoplastic stroma. The cancer froms tubular and cribiform structures lined by tall columnar cells with large vesicular nuclei bearing prominent nucleoli and abundant mitotic figures. Cancer cell necrosis is minimal. There are inflamed and edematous colonic mucosa with regenerative colonic crypts and moderate infiltration of chronic inflammatory cells including many eosinophils.

P5 Female Age: 76 Years

DIAGNOSIS:

Colon, site 6x, biopsy T67800P107030M81413

ADENOCARCINOMA, 6/6

WELL DIFFERENTIATED

Macroscopic Description:

The specimens are six pieces of white tissue measuring 0.1-0.2 cm in diameters. They are submitted together as A: 3, B: 3.

Microscopic Description:

Section show invasive growths of welldifferentiated adenocarcinoma with inflamed desmoplastic stroma. The cancer forms tubular and tubulopapillary structures lined by tall columnar cell with bizarre vesicular nuclei and mitotic figures. Lymphatic invasions are observed.

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