CHAPTER III

RESULTS

I. Method for determination of total alkaline phosphatase activity

1. Quality control of total ALP activity determination.

Optimal condition variation (OCV) & Routine condition variance (RCV) of total ALP activity determination determined in Double-beam UV- visible Spectrophotometer (Shimudzu UV 160 A) and microplate reader were shown in Table 1. Two levels of control serum and 2 levels of eluated fraction from anion exchange chromatography (DEAE -Sephacel) were used for the evaluation of precision on the analyses. The results were plotted on Levey-Jennings control charts for interpretation of precision. The coefficient of variation (%CV) obtained by the microplate reader was lower than that determined by Shimudzu UV 160 A spectrophotometer. In the determination of OCV (intra-assay), the precision of analytical method determined by Shimudzu UV 160 A spectrophotometer was varied with the ALP activities in control serum. There were an increase in the percentage of coefficient of variation (%CV) when the mean activities of the enzyme were decreased. The eluated fraction level I showed the highest %CV in both instruments. The % CV of OCV determined in Shimudzu UV 160 A spectrophotometer were ranged from 3.6 –5.5%, respectively and in a microplate reader were varied from 2.95 - 7.54%, respectively. In routine analyses (inter-assay) of controls that had been used previously for evaluation of OCVs in both instruments, it was found that the RCV mean values were nearly the same as OCV. The % CV of controls in RCV by using Shimadzu UV 160 A spectrophotometer were from 4.81 - 9.53 %, respectively, and by a microplate reader were 3.61 - 8.23%, respectively. All % CV values were under most of the routine conditions and the ratio of RCV to OCV was less than two for both ALP analytical methods in both instruments.. Figure 4 & 5 shows examples of distribution of analytical ALP values performing in both instruments

Table 1. Quality control of ALP activity determination in serum and partial purified ALP fractions

Instrument / Method	OCV			RCV		
	$\bar{X}$	SD	%CV	$\bar{X}$	SD	%CV
1. UV-Visible Spectrophotometer						
Serum : Level 1	212.17	7.56	3.60	211.0	10.15	4.81
Level II	45.87	1.81	3.95	46.29	2.10	4.54
Fraction: Level I	7.20	0.40	5.55	6.95	0.60	8.63
Level II	3.73	0.18	4.94	3.67	0.35	9.53
2. Microplate reader*						
Serum :Level I	54.84	1.62	2.95	55.11	1.99	3.61
Level II	19.78	0.76	3.84	19.67	0.81	4.12
Fraction : Level I	6.10	0.46	7.54	6.07	0.49	8.07
Level II	4.00	0.16	4.00	4.01	0.33	8.23

* The microplate reader method was used in case of there was limited sample.

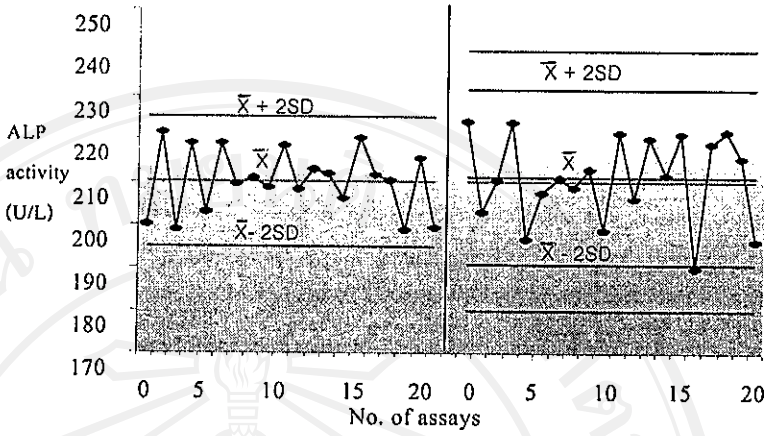


Figure 4. The distribution of ALP activity in Level I control serum in shimudzu UV- Spectrophotometer.

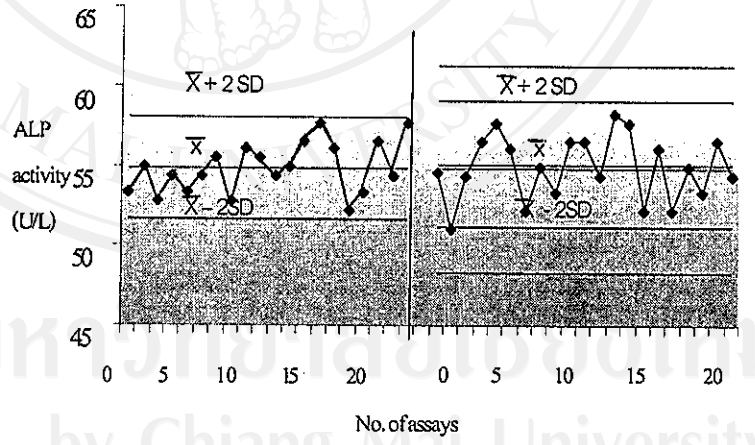


Figure 5. The distribution of ALP activity in Level I control serum in a microplate reader.

From results, the precisions of quality control tests for determination of ALP activity in control serum and fractions in both instruments were accepted by the criteria of WHO (WHO Document 1976; Lab/76.1: 1-49).

2. Total ALP activity measured in serum of normal and colon cancer patients

ALP activity measured in sera of normal and colon cancer patients were determined by a double beam UV-visible spectrophotometer. The mean $\pm$ SD of activity in normal serum (N=32) and colon cancer serum (N=5) were 183.87 $\pm$ 50.33 U/L and 270 $\pm$ 165.69 U/L, respectively. The mean ALP activity in patient serum was statistically different from that of normal serum at $p<0.025$ (Unpair t-test).

II. Liver function test for screening patient conditions

ALT activity in all sera of patients with colon cancer (N=5) were within the range of reference value. Three of five serum samples were hemolysed and therefore demonstrated higher values of AST activity than normal (Table2). All patients were diagnosed as colon cancer by colonoscopy and free from liver disease.

Table 2. The LFT determined in serum of colon cancer patients

Screening Test for LFT	Patient No 1	2	3	4	5	Average ($\bar{X} \pm$ SD)
ALT (U/L) (7-42 U/L)*	13.	12	23	33	34	23.00 $\pm$10.51 (N=5)
AST (U/L) (3-37 U/L)*	61*[1]	47*[1]	31.94	23.34	68*[1]	27.64$\pm$6.08 (N=2)

* Reference range, *[1]Not included in calculation

III. Preparation of ALP isoenzyme from tissues

1. Preparation of Liver ALP from bovine liver tissue

Liver ALP prepared from 20 g bovine liver had specific activity of 45.5 U/g. The enzyme precipitate dissolved in 0.1 mol/L Tris buffer, pH 7.0 was diluted to the final activity of 250 U/L before using as a control of tissue-non specific isoenzymes for IAP, tissue specific ALP in serum or those isolated from colon tissues.

2. Preparation of ALP isoenzyme from patient specimens

The protein concentrations, ALP activities and specific activity in normal and tumor tissues of patients with colon cancer were shown in Table 3 and Figure 6 & 7, respectively. The mean ALP and specific activity of ALP isoenzyme in tumor tissues was significantly different from that of normal colon tissues (paired t-test, $p < 0.05$). This result demonstrated that there was an increase in ALP activity in tumor tissue comparison with pair normal tissue of the same patient. The type of ALP isoenzyme was also identified and the result was shown. The mean of ALP activity with very high standard deviation observed in patient sera was higher than the reference range of ALP activity assayed by DEA kinetic method (< 270 U/L).

Table 3. ALP activities, protein concentrations and specific activities in tumor and corresponding normal tissues of patients with colon cancer.

Specimen No.	Serum ALP activity (U/L)	status	Weight of Tissue (g)	ALP activity (X ± SD), U/L	U/g tissue	Protein (g/L)	Specific activity (U/g)
1	260	Normal	0.036	106.80*	0.296667	33.00	3.236364
		Tumor	0.036	269.50*	0.748611	33.00	8.166667
2	370	Normal	0.036	142.8 ± 13.69	0.000397	23.33	6.120874
		Tumor	0.046	177.3 ± 10.78	0.385435	21.66	8.185596
3	154	Normal	0.045	93.22 ± 11.71	0.207156	50.83	1.833956
		Tumor	0.046	136.9 ± 8.51	0.297717	25.83	5.301974
4	494	Normal	0.100	82.7 ± 12.73	0.082725	96.50	0.857254
		Tumor	0.100	222.3± 5.78	0.222255	100.16	2.219000
5	80	Normal	0.083	173.0 ± 6.96	0.208464	58.33	2.966312
		Tumor	0.083	211.2 ± 6.28	0.254470	60.00	3.520167

* limited specimens

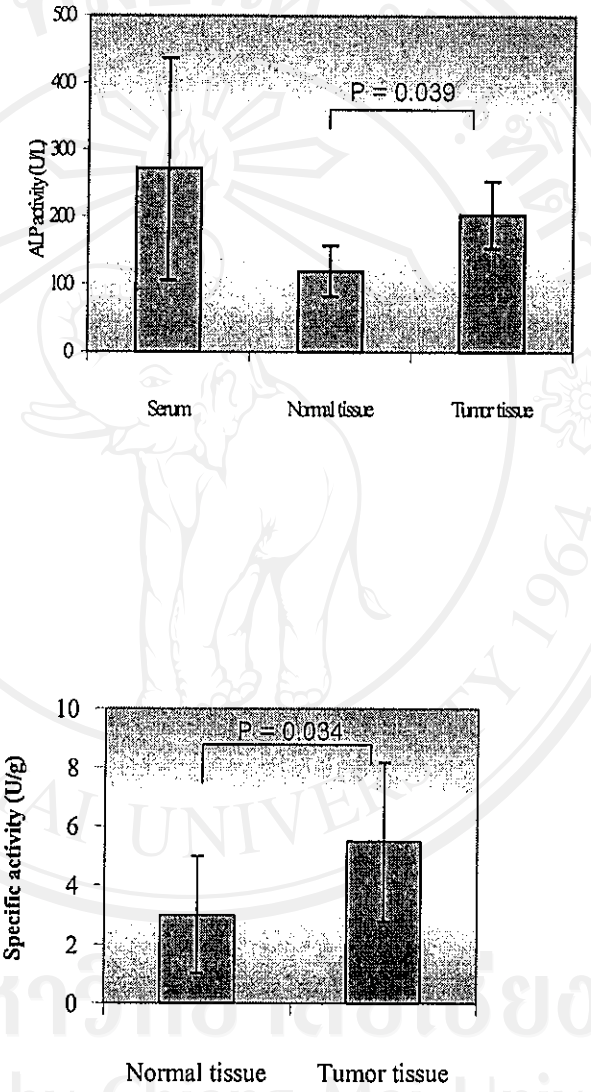


Figure 6. Comparison of ALP activity (upper figure) and specific activity (lower figure) in normal and tumor tissues of patients with colon cancer

IV. Partial purification of ALP isoenzymes from sera of normals and patients with colon cancer by anion exchange chromatography

Figure 7 shows the precision of five replicated protein eluted from DEAE-Sepacel column chromatography. The % CV of OCV and RCV were shown in table 4. The precision accepted by the criteria of WHO (WHO document 1976; Lab/76.1: 1-49).

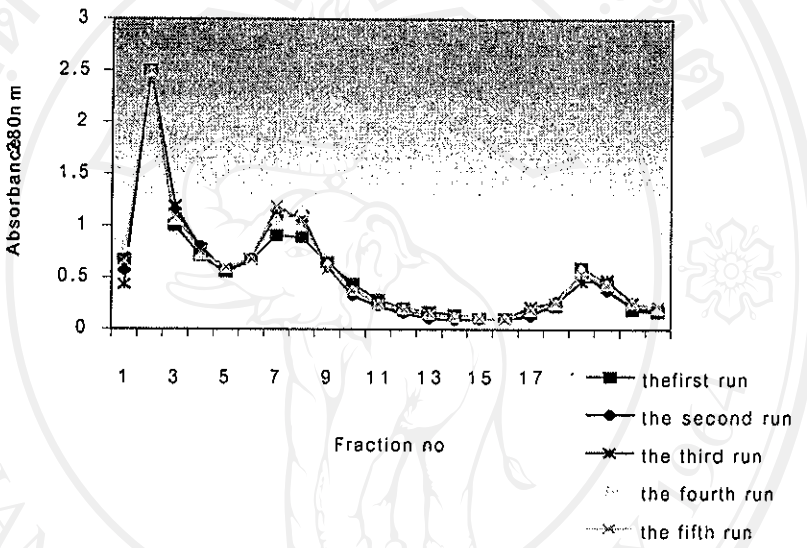


Figure 7. Optimal condition variance of partial purification of protein on DEAE-Sephacel column chromatography

Table 4. The precision of protein partial purification on DEAE -Sephacel column chromatography

Fraction No	N	OCV			N	RCV		
		$\bar{X}$*	SD	% CV		$\bar{X}$*	SD	% CV
3	5	1.099	0.062	5.63	2	1.0975	0.0955	8.701595
7	5	1.07	0.055	5.207	2	1.05	0.141	13.42857
19	5	0.5715	0.025	4.54	2	0.502	0.038	7.569721

* Absorbances at 280 nm.

Figure 8 and 9 are patterns of partial purification of IAP in normal and patient serum on DEAE -Sephacel column chromatography. All separations yielded 3 protein peaks. Protein eluates containing ALP activity were identified for IAP by L-phenylalanine and levamisole inhibition tests and agarose gel electrophoresis. The mean of total ALP activity in normal sera determined in DEA buffer , pH 9.2 using PNPP substrate (n = 32) was 184 $\pm$ 51.9 U/L ($\bar{X} \pm$ SD, with regardless to blood group secreting condition) and in colon cancer was 271.6 $\pm$ 165.69 U/L (n = 5). There was significant difference between mean of two groups of samples ($p < 0.025$). The eluates of fraction 1-4 in the first protein peak were pooled and assayed for the ALP activity and then lyophilized to concentrate the enzyme samples for used in the further study. The ALP activity of normal sera and colon cancer sera found in the first protein peak of eluates were 11.74 $\pm$ 4.07 U/L (n=32) and 17.60 $\pm$ 6.5 U/L (n=3), respectively. The mean of two groups of samples was different statistically at $p < 0.05$. The isolated fractions of the first protein peak of normal and patient sera were used in the identification and the study of IAP properties.

Table 5. Total activity of ALP in normal and colon cancer sera and pooled fraction (1-4), in the first protein peak.

Type of specimen	N	Mean	SD	Significant level
Serum				
Normal	32	184*	51.90	* $p < 0.025$
Colon cancer	5	271.60*	165.69	(Unpaired t-test)
Fraction (1-4) protein				
Normal	32	11.74**	4.07	** $p < 0.05$
Colon cancer	3	17.60**	6.50	(Unpaired t-test)

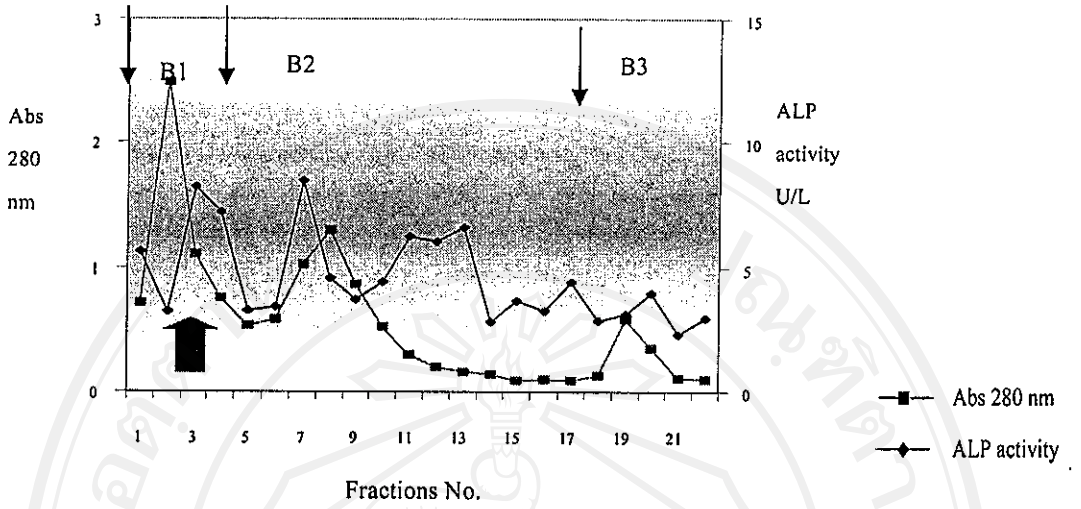


Figure 8. Pattern of partial purification of ALP in normal serum by DEAE-Sephacel column chromatography. B1,B2 and B3 were buffers used to elute ALP isoenzymes from DEAE-Sephacel column (see text).

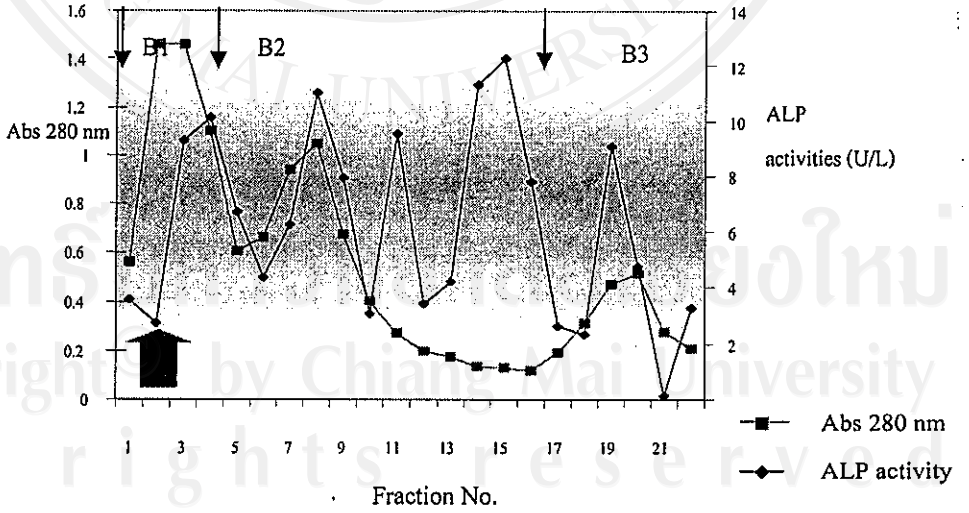


Figure 9. Pattern of partial purification of ALP in patient serum by DEAE-Sephacel column chromatography. B1,B2 and B3 were buffers used to elute ALP isoenzymes from DEAE-Sephacel column (see text).

V. Identification of Chromatographic Fractions of IAP isoenzymes.

1. Chemical inhibition tests

L- phenylalanine and levamisole have been used to differentiate the IAP and LAP isoforms in serum. IAP isoenzyme is sensitively inhibited by L-phenylalanine whereas it has less effect on LAP, BAP and ALP activity from kidney tissues. In contrast, levamisole has strongly effect on inhibiting the LAP, BAP and kidney ALP but not on IAP activity. Since IAP isoenzyme is not consistently found in all normal serum , therefore L- phenylalanine and levamisole were used to screen, identified and proved if the separated fraction obtained from anion exchange column chromatography was the IAP isoenzyme.

Results of testing showed that in normal serum samples, after fractionation on DEAE- Sephacel column The inhibition of L-phenylalanine data of greater than the mean of standard IAP (40.52%) were pooled and calculated as the more L-phenylalanine inhibition group. Those which were less than IAP standard was L-phenylalanine less inhibition group. Figure 10 shows chemical inhibitions on ALP activity in the first protein peak. The ALP in normal group with more positively inhibited by phenylalanine was used as the normal IAP control for those in samples of colon cancer and liver diseases. Table 6 is the comparison of chemical inhibition tests on ALP fractions of normal and patients with colon cancer and liver diseases. The ALP fraction of colon cancer was markedly inhibited by L-phenylalanine, and shows with low inhibition by levemisole. A pattern of inhibition was similar to the fraction of IAP standard and normal serum with more L-phenylalanine inhibition.

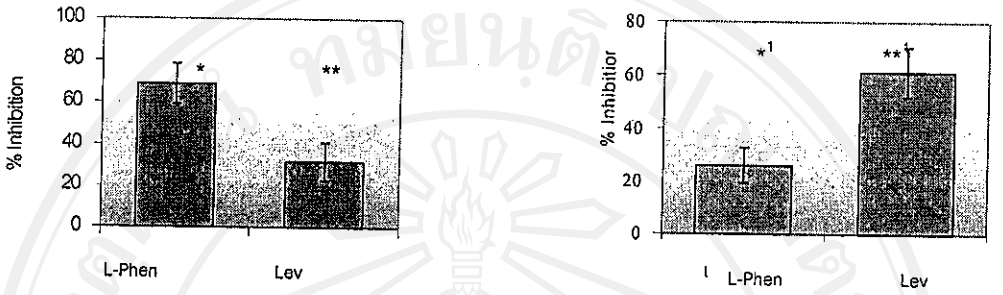


Figure 10. The inhibition effect of L-phenylalanine and levamisole on the fractionated normal serum ALP in the first protein peak. Left = L-phenylalanine more positive and right = L-phenylalanine less positive.

* vs $*^{I}$ $p < 0.005$

** vs $**^{I}$ $p < 0.005$

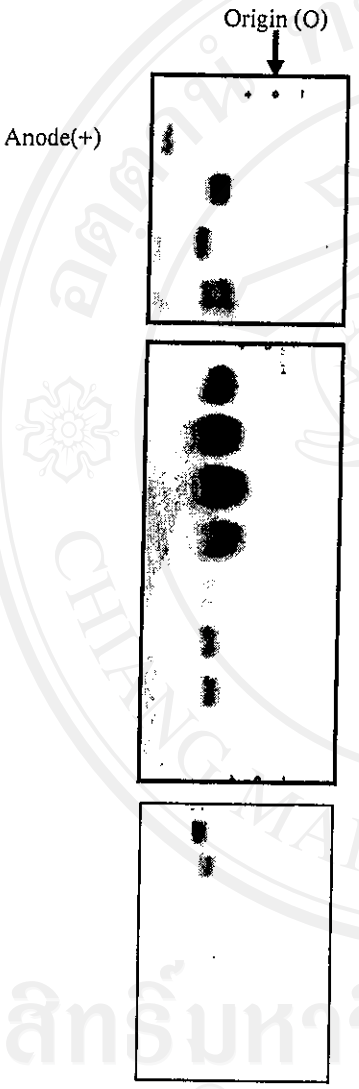
Table 6. Chemical inhibition tests on ALP fractions of normal and patients with colon cancer and liver disease.

Type of sample	N	L-phenylalanine inhibition (%)		Levamisole inhibition (%)		Significant level	
		Mean	SD	Mean	SD	Test	Result
Normal fractionated Enzyme							
L-Phe less positive	6	26.07*	6.67	61.21**	9.21	* vs $*^{1}$	P <0.005
L-Phe more positive	6	$68.75*^{1}$	10.36	$31.77**^{1}$	9.6	** vs $**^{1}$	P < 0.005
Colon cancer	3	$69.59*^{2}$	10.07	$46.63**^{2}$	11.20	$**^{1}$ vs $**^{2}$	NS
IAP standard	2	40.52	22.27	11.21	10.50		
Liver disease	2	25.69	7.26	$48.0**^{3}$	4.40	$**^{2}$ vs $**^{3}$	NS
LAP standard	2	20.28	6.47	65.76	13.45		

2. Identification of anion chromatographic fraction for IAP isoenzyme by electrophoretic method

On agarose gel electrophoresis, IAP and LAP standards were migrated with different rates. The isoenzyme migrated anodically was the liver ALP isoform and the other band migrated cathodic to the liver ALP band was the IAP isoenzyme (Figure 11, lane 1-4). All fractions isolated from DEAE -Sephacel column had been concentrated by lyophilization before subjecting to agarose gel electrophoresis. The dry fractions were reconstitued with distilled water 1/20 as compared with the original volume before lyophilization. The reconstituted fractions were used for identification, heat inactivation and amino acid inhibition experiments.

After partial purification by DEAE -Sephacel column chromatography, the IAP standard in 1-4 pooled ALP activity fraction of the first protein peak showed the most purified band compared with those found in other pooled protein fractions(5-6, 7-8, 13-14 pooled fractions). (Figure 11, lane 6-8). LAP standard eluted from DEAE sephacel column in pooled fraction 5-6 and 7-8 (Figure 11, lane 10, 11). Fractionated colon cancer serum (lane 13-16) demonstrated the same pattern of elution as the IAP standard. Figure 12 showed the identification and comparison of 1-4 pooled ALP fractions isolated from sera of normal and colon cancer by DEAE -Sephacel column chromatography. Agarose gel electrophoresis of partial purified patient serum revealed only one band (lane 6) migrated at the same position as IAP standard (lane 3) and in intact patient serum (lane 5). Figure 12 also demonstrated the separations of IAP isoenzyme in sera of normals and patients with colon cancer on agarose gel electrophoresis. The IAP bands separated from colon cancer sera stained with 5-Bromo-4-chloro-3-idolyl phosphate (lane 5) were more intended than those detected in normal (lane 7) and serum samples from liver disease patient (lane 8). The pattern of migration of IAP in serum and fractionated enzyme were compared with the fractionated IAP and LAP standards (lane 2,3 &4).



Lane 1. Albumin
Lane 2. IAP standard
Lane 3. LAP standard
Lane 4. IAP standard+LAP standard
Lane 5. IAP-DEAE-Sephacel Fraction 1-4
Lane 6. IAP-DEAE-Sephacel Fraction 5-6
Lane 7. IAP-DEAE-Sephacel Fraction 7,8
Lane 8. IAP-DEAE-Sephacel Fraction 13,14
Lane 9. LAP-DEAE-Sephacel Fraction 1-4
Lane 10. LAP-DEA-Sephacel fraction 5,6
Lane 11. LAP-DEAE-Sephacel Fraction 7,8
Lane 12 LAP-DEAE-Sephacel Fraction 13-14
Lane 13. Colon cancer serum
Lane 14. Colon cancer Sephacel fraction 1-4
Lane 15 Colon cancer Sephacel fraction 5,6
Lane 16. Colon cancer Sephacel fraction 7,8

Figure 11. Identification of ALP fractions by agarose gel electrophoresis

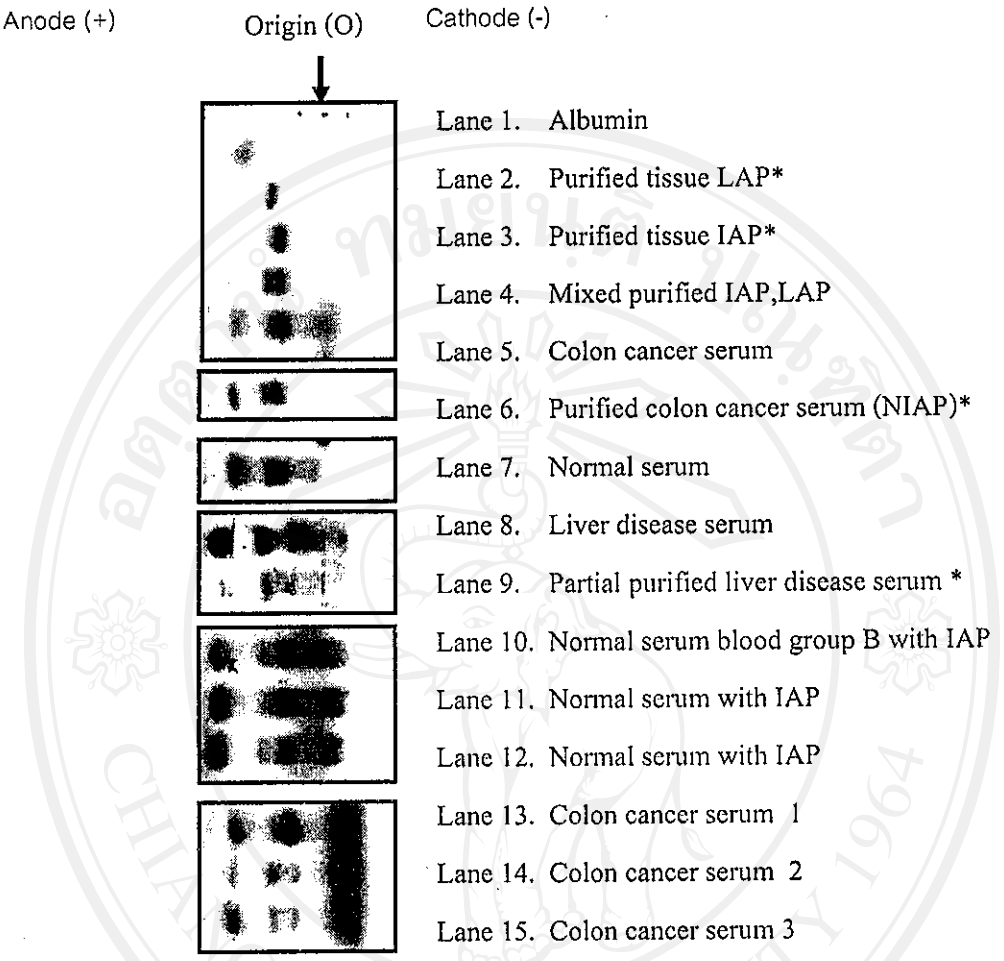


Figure 12. Agarose gel electrophoresis of ALP isoenzymes in normal and patient serum samples : comparison of IAP separation with tissue IAP and LAP standards.

* Pooled 1-4 fractions of the first protein peak

V. Study of IAP properties

1. Heat inactivation of the IAP isoenzyme

Heat inactivation performed in this study was carried out by heating two aliqouts IAP fractions, one was incubated at 52°C and the other was at 65 °C for 1 hr. The results of remaining activity were compared with those IAP and LAP standards which were inactvated by the same procedure. Figure 13 demonstrated the effect of heat treatment on IAP and LAP activities of standards performed as a function of time from zero (unheated) to 2 hrs. Results from the unknown expressed as percentage of remaining activity were compared with the standards. From Figure 13 [A] and [B] , % remaining activities of LAP and IAP standard after treating with heat for 60 and 120 min at 52 °C were 67.1 and 19 %, and 98.4 and 98.35 %, respectively. The % remaining activity at 65°C of LAP and IAP standard were 1.35 and 0.86, and 32.48 and 17.8 %, respectively. Results of the % remaining activity shown in Figure 14, the fractionated normal I, normal II, colon and liver disease sera were not different from IAP standard after the enzyme was inactivated at 52 °C. The percentage of ALP remaining activity of fractionated enzyme from normal, patient serum including tumor tissue were significantly different ($p < 0.01$) from the fractionated LAP standard except the normal II. Therefore, it could be interpreted that normal I and normal II were the representative samples of normal serum with and without IAP isoenzyme, respectively.

IAP fractionated from the tumor tissue was not different from the pair of- normal and tumor tissue (% remaining activity > 65 U/L) but slightly different from the fractionated normal tissue enzyme (%remaining activity = 58 U/L) when the enzyme was heated at 52 °C. The %remaining enzyme activity at 65°C in normal intact tissue(44.4%) was significantly higher than that of intact tumor enzyme, fractionated normal tissue enzyme and paired fractionated tumor tissue enzyme, respectively (% remaining activity > 33%)(Figure 15). The % remaining activity at 65°C of normal (1), colon cancer sera and in tissue (both intact and fractionated enzyme) were corresponded or higher than the IAP standard pretreated with heat by the same procedure as the unknown sera and tissue enzymes. From these results, it could be concluded that by comparing with the standard and the concept of heat treatment, the ALP detected in serum and tissue of patients with colon cancer were the IAP isoenzyme.

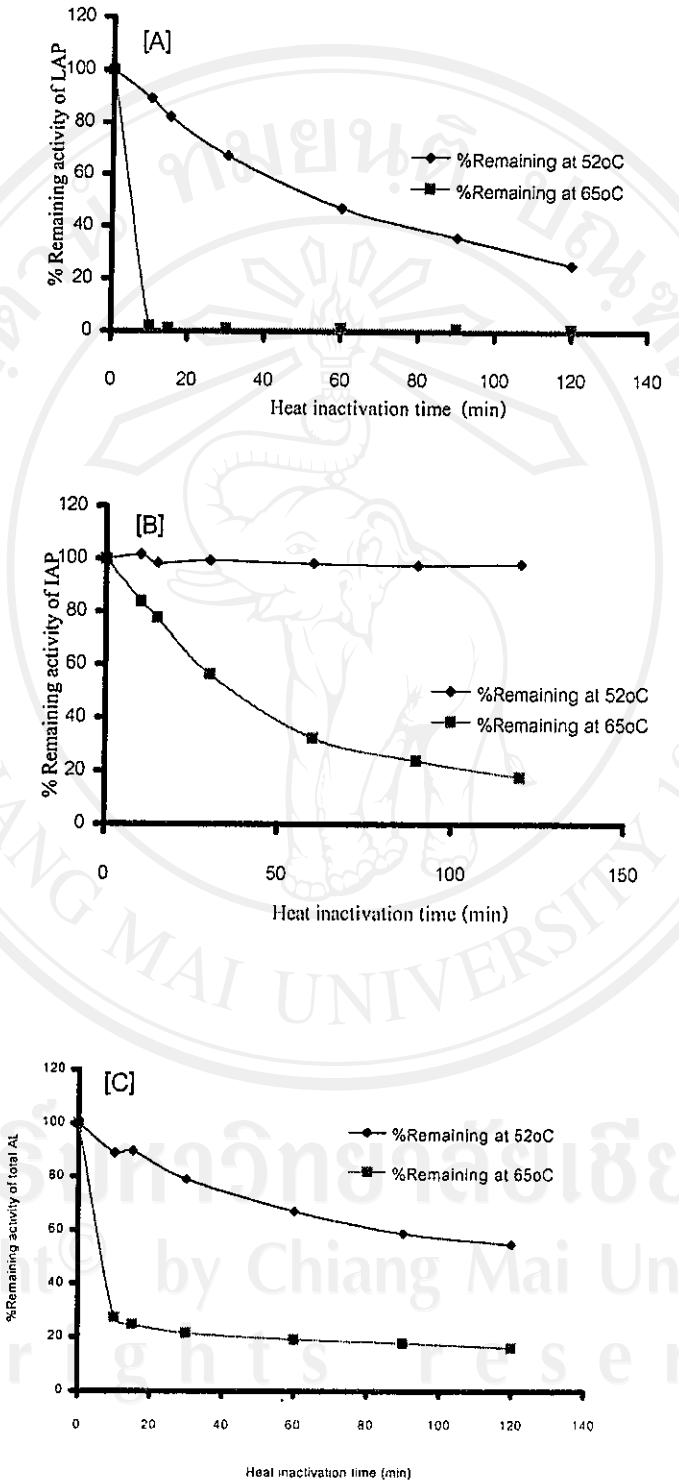


Figure 13. Heat inactivation of ALP isoenzymes activities. The percentage of remaining ALP activity were shown as a function of time (min) at 52 ^{o}C and 65 ^{o}C. [A] : LAP, [B] : IAP and [C] : LAP+IAP

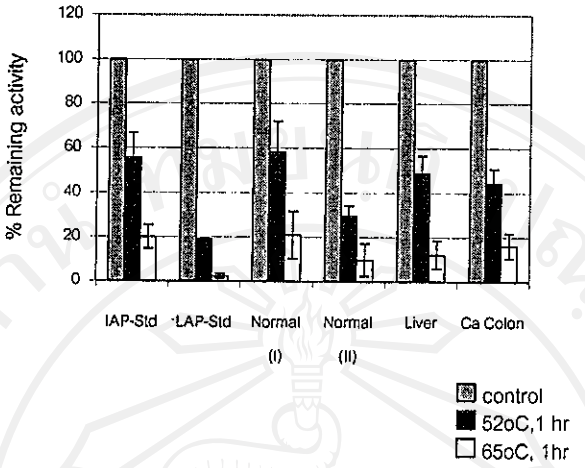


Figure 14. Heat inactivation of fractionated ALP in normal serum I (n=7), normal serum II (n=6), Liver disease serum (n=2), Ca colon serum (n=3) compared with IAP and LAP standards

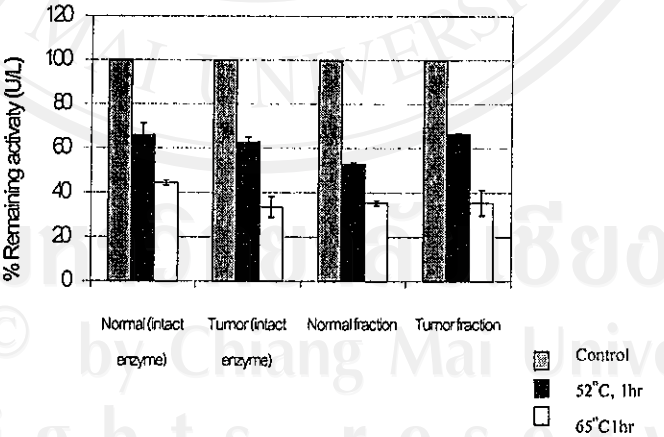


Figure 15. Heat inactivation of intact and fractionated ALP in normal and paired tumor tissue from the same patient. (n=2 for each)

2. Amino acid inhibition test

The selected amino acids , 0.5 mol/L of L -phenylalanine , 0.05 mol/L-arginine and 0.05 mol/L-alanine were used to inhibit IAP activity in fractionated samples of normal and patient sera and for those tumor tissue and their paired normal controls. The results in Table 7 shows that L-phenylalanine inhibited the enzyme activity in normal and tumor tissue of patient with colon cancer at the same rate as the IAP standard did and affected greatly on ALP activity in colon cancer serum. The % inhibition of ALP activity by L-alanine and L-arginine in normal serum was similar to the LAP standard. It was shown that L-alanine and L-arginine has more inhibited effect on ALP activity in fractionated colon cancer sera than those in normal and tumor tissues. These amino acid inhibition test showed strongly evidence of which the isoenzyme separated from serum and tissues of colon cancer patients were the IAP isoenzyme.

Table 7. Effect of Amino acid on ALP activity in fractionated sera and tissues

Sample	N	Amino acid		
		0.5 M L-phenylalanine	0.05 M L alanine	0.05 M L arginine
IAP standard	2	40.52±22.27	1.93±2.63	8.18±5.97
LAP standard	2	20.28±6.47	7.33±6.32	3.96±2.34
Normal serum	6	68.475±10.36	22.20±14.76	18.93±12.64
Colon cancer serum	3	69.59±10.07	75.58±10.38	51.26±23.04
Normal tissue	3	43.92±3.54	39.1±13.13	32.14±19.49
Tumor tissue	3	41.84±13.53	44.3±18.45	24.07±1.04

3. Reactivity with IAP monoclonal antibody

In this experiment, serum specimens were incubated with monoclonal antibody specific to IAP isoenzyme before subjecting to agarose or polycrylamide gel electrophoresis. Figure 16 reveals the reactivity of IAP in sera of colon cancer with MoAb on agarose gel electrophoresis. The 1:2,000 dilution of MoAb was optimal for reacting with IAP isoenzyme in serum sample. The reactive band was presented as a diffuse band between the application point and the IAP (NIAP) band (lane 7; 1:2,000 dilution)(Figure 16). The test performed on polyacrylamide gel showed reactivity of MoAb with 2 IAP isoforms (Figure 17). One isoform migrated as diffuse band; the other enzyme-MoAb complex was located at the stacking gel, suspected of its high molecular size. It was found again that the enzyme preferred to react with 1:2,000 dilution of MoAb.

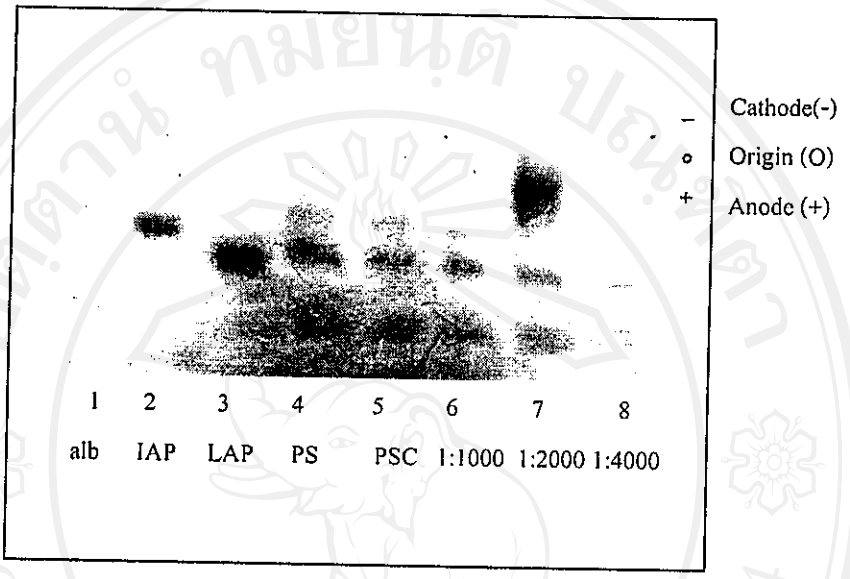


Figure 16. Agarore gel electrophoresis demonstrated colon cancer serum treated with various concentration of monoclonal anti IAP . Lane 1. albumin, lane 2. IAP standard, lane 3. LAP standard, lane 4. colon cancer serum (PS) , lane 5 colon cancer serum control (PSC), lane 6. colon cancer serum treated with anti IAP (1:1000), lane 7. colon cancer serum treated with anti IAP (1:2,000), lane8. colon cancer serum treated with anti IAP (1:4000)

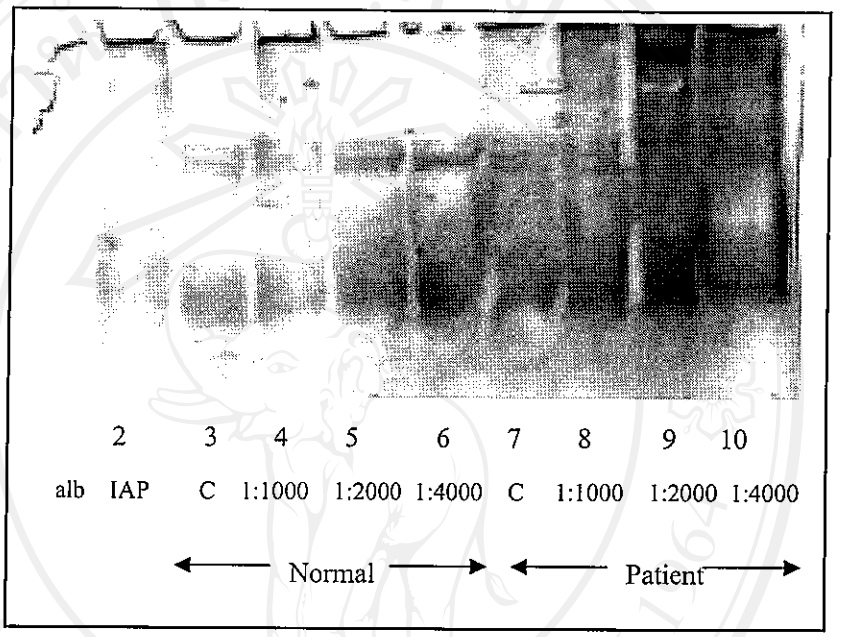


Figure 17. Polyacylamide gel electrophoresis demonstrated normal serum and colon cancer serum treated with various concentrations of monoclonal anti IAP. Lane 1 albumin, lane 2 IAP standard, lane 3 normal serum, lane 4 normal serum treated with antiIAP (1:1,000), lane 5 normal serum treated with anti IAP(1:2,000), lane 6 normal serum treated with anti IAP (1:4,000), lane 7 colon cancer serum, lane 8 colon cancer serum treated with anti IAP (1:1,000), lane 9 colon cancer serum treated with anti IAP (1:2,000), lane 10. colon cancer serum treated with anti IAP (1:4,000)

VI. Biochemical characterizations of The IAP isoforms

1. Sialylation (Neuraminidase treatment)

The treatment of ALP isoenzymes with neuraminidase reduced the anode electrophoretic mobility of liver and bone ALP so that they were able to separate from each other and would be able to be quantitated by densitometric scanning. There were many sources of neuraminidase produced by microorganism such as from *Clostridium perfringen* (Cn) and *Vibrio chlorera* (Vn). It was specific to α 2,6 and α 2,3 linked of sialic acid to the end of carbohydrate side chain of the glycosylated enzyme. In this experiment, the IAP in samples were separately digested by each type of the neuraminidase described above to observe the type and the sialylation of the separated IAP molecules. The results of digested enzyme fraction was compared with the corresponding undigested control and visualized on agarose gel electrophoresis.

Figure 18 showed the comparative effect of Cn and Vn on IAP and LAP isoforms. On agarose gel electrophoresis, it was demonstrated that both Cn and Vn had no effect on IAP standard mobilities (lane 3& 4 vs lane 2). In contrast, they had similar effect on retarding LAP activity so that they migrated slower than the untreated control (lane 6 & 7 vs lane 5). In normal serum, Cn and Vn showed different effect on mobility of ALP isoenzyme. The more retard effect was observed in lanc 9 by which the Vn could separate the ALP isoenzyme, which migrated as band to the anode, into two bands. The band migrated to the anode suspected to be the bone ALP and the band migrated to the cathode was the liver isoform band (lane10 vs lane 6 & 7).

Figure 19 demonstrated the mobility patterns of different ALPs in serum samples treated with Vn. Normal serum of blood group A contained only LAP and showed the rate of migration equaled to the standard LAP after treating with Vn (lane 5 vs lane 6). The blood group B normal sera with known secretor status (lane 8 vs 7) showed the migration pattern similarly to colon cancer serum (lane 10 vs lane 9). The IAP separation shown in lane 8 and lane 10 were unaffected by neuraminidase treatment as compared with the IAP standard (lane 4 & lane 3). Two liver disease sera, one that contained IAP showed two bands after separation (lane 12 vs lane 11). The IAP band was unaffected by Vn and the other one had the mobility pattern like LAP standard after treating with Vn (lane 13 vs lane 14).

Mobility of tissue IAP (limited sample) was demonstrated in Figure 20 using polyacrylamide gel electrophoresis. It was found that the IAP extract from tumor tissue was sensitive to neuraminidase and the mobility was retarded and located at the stacking gel region.

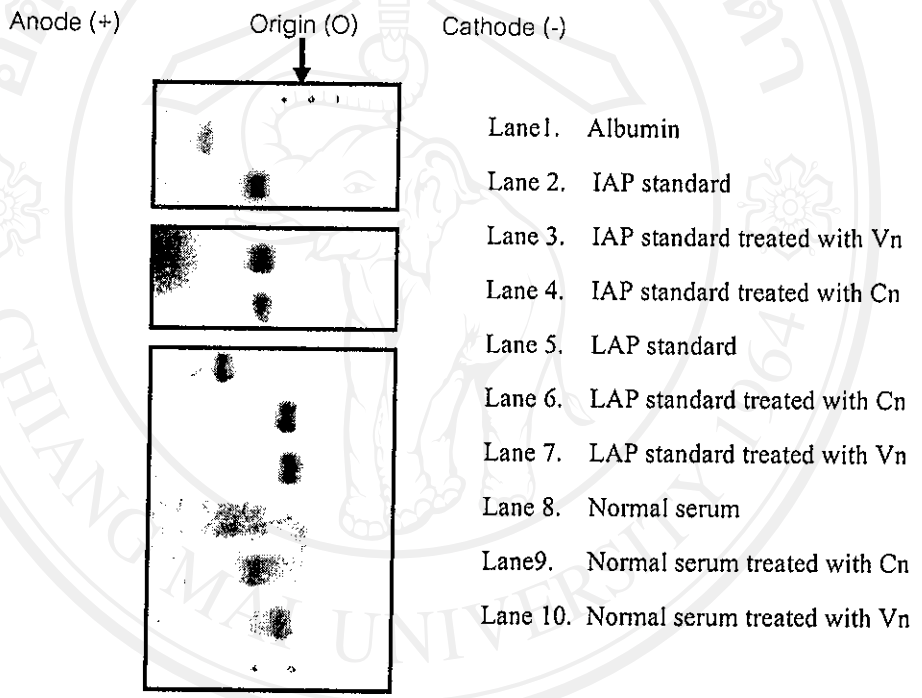


Figure 18. Agarose gel electrophoresis of ALP isoenzymes in normal sera untreated and treated with neuraminidase (Cn and Vn), demonstrated the patterns of migration of ALP isoenzymes in normal serum as compared with standard ALP isoenzymes.

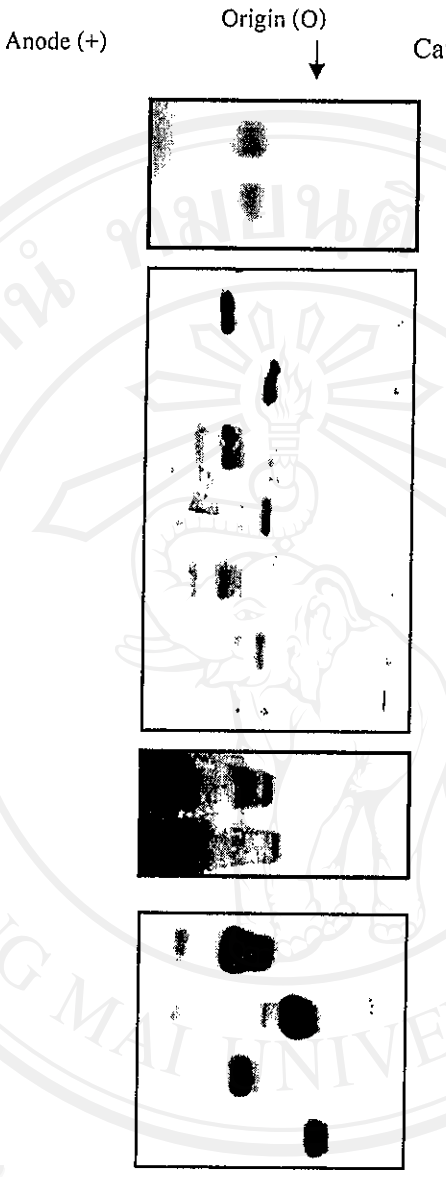


Figure 19. Agarose gel electrophoresis of ALP isoenzymes in sera of normal, colon cancer, and liver diseases untreated and treated with neuraminidase (Cn and Vn), demonstrated the patterns of migration of ALP isoenzymes in normal and patient sera comparison with standard ALP isoenzymes.

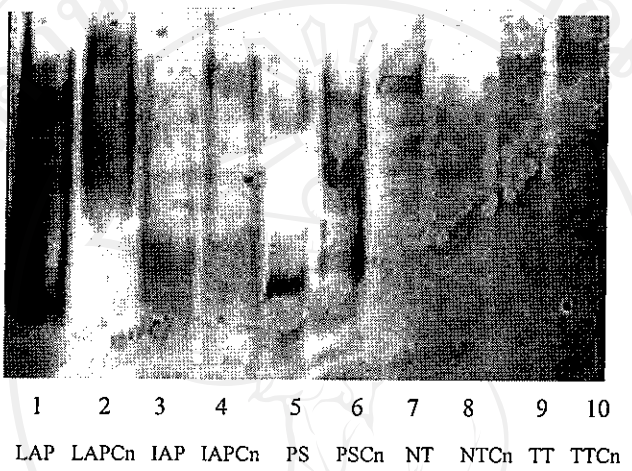


Figure 20. Effect of neuraminidase (Cn) on IAP isoforms detected by polyacrylamide gel electrophoresis. Lane 1 LAP standard, lane 2 LAP standard treated with Cn, lane 3.IAP standard, lane 4. IAP standard treated with Cn, lane 5. colon cancer serum (PS), lane 6. colon cancer serum treated with Cn (PSCn), lane 7. normal tissue (NT), lane 8. normal tissue treated with Cn (NTCn), lane 9. tumor tissue (TT), lane 10. tumor tissue treated with Cn (TTCn)

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 the ALP isoenzymes. This experiment was a screening test for the presence of high molecular forms of IAP which could not be precipitated with Con A in the reaction tube. The result of low precipitation of lectin with ALP isoenzyme will yield low-% precipitation of lectin glycoprotein complex.

Table 8 showed the comparison of different lectins which were used to isolate different ALP isoenzymes in samples by the affinity precipitation. Con A , Pea and WGA had different effects on ALP in normal sera. It was separated into two groups according to their heat sensitive property (the more resistance to heat, the more containing of IAP isoenzyme in the samples). Normal sera group which was resistant to heat treatment had more affinity precipitation with 3 lectins than the heat sensitive group. ALP in colon cancer sera revealed more affinity precipitation with WGA than those observed in two normal groups but there was slightly different between patient and from normal sera for the precipitation with Con A and Pea . It was demonstrated that ALP in all groups of patient sera, normal and tumor tissue and partial purified standards were preferentially precipitated with WGA. It could be suggested the glycosylation of ALP isoenzymes in all serum samples and standard might contain certain sugars which were common and similar to each other.

The ALP in liver disease sera showed more affinity precipitation with Con A than that found in normal (both groups) and colon cancer sera. There were an increase in affinity precipitations of ALPs with ConA, Pea and WGA observed in tumor tissues compared with those observed in normal tissues. The increase in affinity precipitation with lectins of the glycosylated ALP in tumor tissues might be due to the increase in IAP activity from the activity presented in normal tissue which had previously been demonstrated in Table 3 and Figure 6. Figure 21 showed the effect of lectin on precipitating of ALP isoenzymes in different samples shown in Table 8.

Table 8. Lectin precipitation of carbohydrate side chains of ALP isoenzymes in normal and colon cancer sera comparison with those of liver disease sera and standards

Test group	N	Condition	Con A (6g/L) treated (X ± SD)	Pea (1g/L) treated (X ± SD)	WGA (3g/L) treated (X ± SD)
1	3	Normal (sensitive to heat)	20.04± 5.23*	18.03 ± 7.37	19.58 ± 4.53*[8]
2	5	Normal (resistant to Heat)	37.4 ± 2.85*[1]	29.83 ± 4.33	46 ± 7.10*[9]
3	3	Colon cancer patients	20.39 ± 5.59*[2]	15.15 ± 2.09	53.42 ± 13.43*[10]
4.	3	Liver disease patients	49.89 ± 14.31*[3]	26.28 ± 5.10	53.27 ± 7.63*[11]
5	2	Partial purified Intestinal ALP standard	62.9 ± 4.62*[4]	35.73 ± 0.12	78.81 ± 1.57*[12]
6	2	Partial purified liver ALP standard	21.46 ± 5.73*[5]	5.27 ± 6.08	77.49 ± 1.33*[13]
7	3	Normal tissue ALP	30.23±03.18*[6]	43.63 ± 10.93	39.27±10.22*[14]
8	5	Tumor tissue ALP	52.75± 15.75*[7]	58.50±14.68	64.41± 20.33*[15]

Unpaired t-test was used for determining the significant difference:

ConA; $*^3$ vs $*^2$, $p< 0.005$; $*^3$ vs *, $p<0.005$; $*^3$ vs $*^1$, NS; $*^5$ vs $*^4$, $p<0.001$ and $*^7$ vs $*^6$, $p<0.05$

WGA; $*^{10}$ vs $*^9$, NS; $*^{10}$ vs $*^8$, $p<0.005$; $*^{13}$vs$*^{12}$, NS; $*^{15}$ vs $*^{14}$, NS

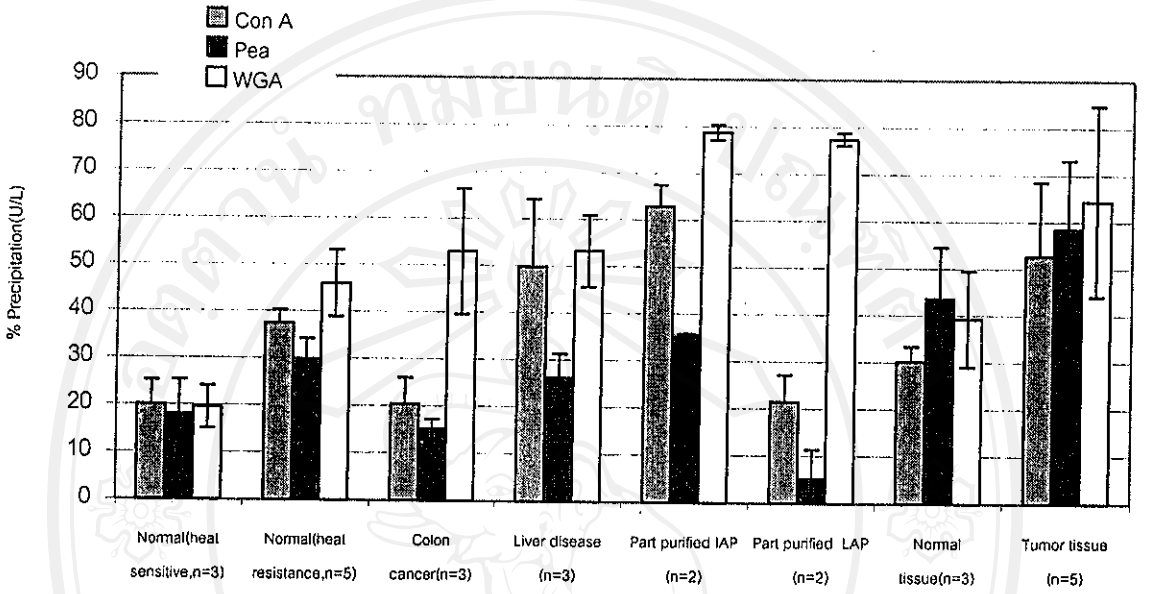


Figure 21. Lectin precipitation of ALP isoenzymes in normal, patient sera and tissues of patients with colon cancer comparison with the partial purified standards.

3 . Affinity chromatography: Concanavalin A chromatography

The separation patterns of the heterogeneous carbohydrate side chains of the ALP molecule on Con A - Separose column were shown in Figure 22 to 24 . The carbohydrate side chains of ALP molecule which occupied by α-D-manose and α-D-glucose would have more affinity binding to Concanavalin A -Sepharose column than those contained other sugars (fucose, galactosamine etc). Figure 22 showed a typical elution of enzyme from the Con A column. Three elution peaks normally obtained are the unbound, weakly bound and strongly bound (by manose) fractions. Figure 24 [A] and [B] were the compared results of the % relative activity of IAP and LAP standards, respectively before and after treating with phosphatidylinositol phospholipase C (PI-PLC). The relative IAP activity of the unbound IAP standard decreased after treating with PI-PLC and the form of digested molecule was shift to the strongly bound peak. As the PI-PLC removed the GPI and lipid residues from the unbound molecules then the enzyme which bared the carbohydrate side chain could have more affinity binding to the ConA column. The LAP standard also demonstrated the unbound fraction but after treating with the PI-PLC , the % relaive activity of ALP was slightly increased in weakly bound fraction.This result was concluded that the carbohydrate side chains of both IAP & LAP isoforms were different.

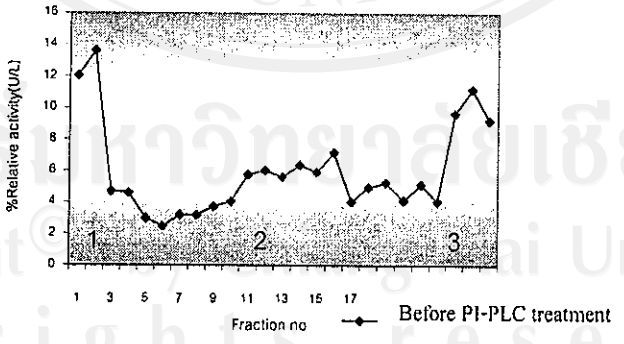


Figure 22. The elution profile of sugars containing in ALP protein ; Peak 1 : The unbound fraction (the molecule contained GPI or lipid residues), Peak 2 : Weakly bound fraction and Peak 3 : Strongly bound fraction.

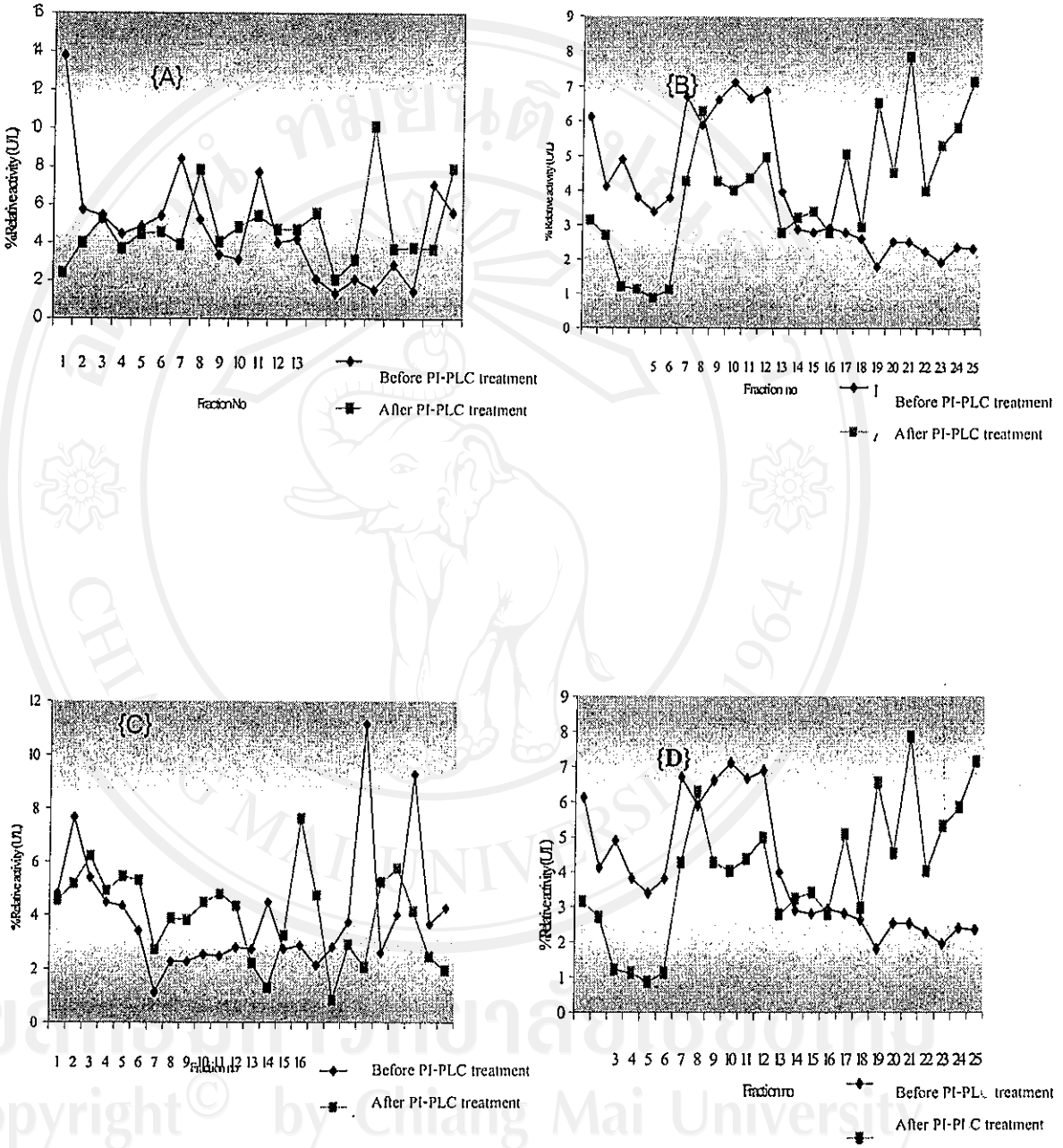


Figure 23 The % relative ALP activity of IAP (Figure23-A), LAP standard (Figure 23-B) and normal sera blood group A (Figure 23-C) and normal sera blood group B (Figure 23-D) respectively : comparison between before and after treating with PI-PLC before separation on ConA column.

Figure [23-C] and [23-D] are the Con A elution patterns of ALP in blood group A and B normal sera , respectively. The blood group A normal serum showed the elution pattern resemble the LAP standard whereas the blood group B (with secretor status) elution pattern was more similar to the IAP than that of the LAP standard. It could be suggested that the blood group B normal serum contained more IAP than the LAP isoform and therefore it was eluted with a high buffer volume containing competitive sugar (mannose) after it was digested with PI-PLC.

Figure 24 demonstrated the elution patterns of ALP in serum of patient with colon cancer [24 – A], the extracted normal tissue [24 – B] and the corresponded tumor tissue extract [24 –C] from the same patient . ALP in extracted normal tissue of patient with colon cancer ,before and after treating with PI-PLC were eluted from Con A column with the same patterns as IAP standard and ALP in blood group B serum with secretor status. In contrast, the ALP in patient serum and ALP extracted from tumor tissue exhibited different pattern of elution from those observed in group B normal serum and IAP standard. In patients' sera, there was an increase in % relative activity at weakly bound fractions whereas in the ALP extracted from tumor tissue, the % relative activity after treating with PI-PLC were increased in all 3 peaks which eluted from the Con A column. These results suggested that there were heterogeneities of the carbohydrate side chains of IAP molecules extracted from tumor tissue. The IAP isoenzyme of tumor tissue after digesting with PI-PLC may yield more isoforms of IAP.

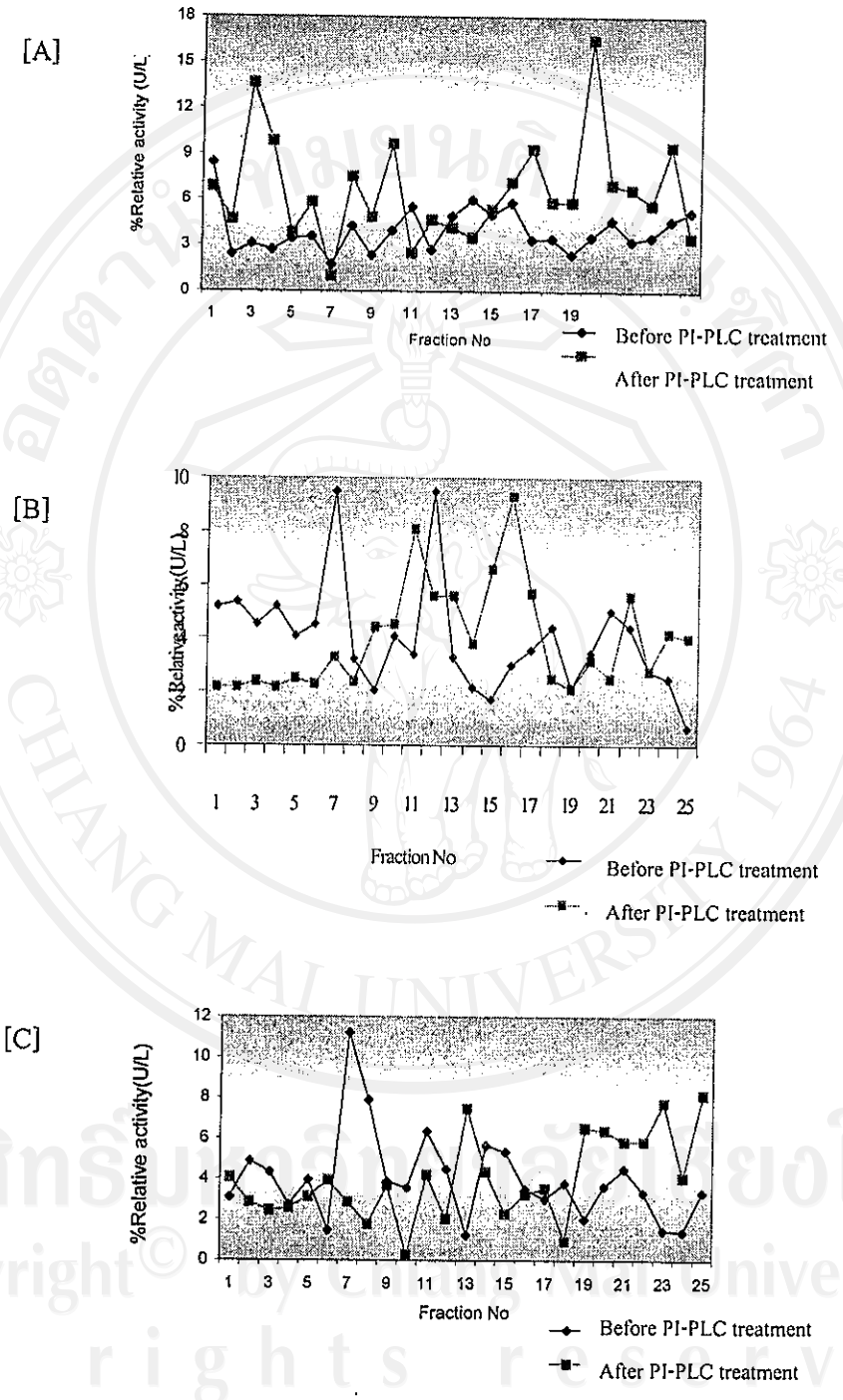


Figure 24 The % relative activity of ALP in serum, normal and corresponding tumor tissues extract of patient with colon cancer. The same samples, untreated and pre-treated with PI-PLC were separated on ConA column and the patterns of elution were compared. {A} ;serum {B} : normal tissue and {C}: tumor tissue of the same patient

4. Molecular weight determination : Western blot analysis

Figure 25 demonstrated the molecular mass of IAP isoforms observed in normal serum and three colon cancer sera. The molecular mass of IAP isoform in normal serum (lane 6) was larger than that of the IAP isoforms found in patient sera (lane 7,8 & 9). The molecular mass of IAP isoforms in patient sera were separated at 116, 205, 225 and greater than 225 kDa respectively. LAP standard (lane 3) was used as a negative control for those containing IAP isoforms including IAP standard (lane 4 & 5).

Normal serum samples may or may not contained the IAP isoforms. Figure 26 revealed the presence of IAP isoforms in blood group B normal serum (lane 5) whereas it could not be seen in blood group A normal serum (lane 6). The molecular sizes of IAP isoforms in blood group B normal serum were at 116 kDa and approxiamtly at 225 kDa (lane 5) which were the same as the IAP isoforms presented in colon cancer serum (lane 7). There were two IAP isoforms detected in one of liver disease serum (lane 8), showing the same molecular mass as the IAP isoforms separated from blood group B normal serum and colon cancer serum.

The comparison of IAP isoforms in serum , tumor and corresponding normal tissue extracts of the same patient was demonstrated in Figure 27. Blood group B normal serum (secretor) showed the same pattern of IAP separations and the molecular sizes as observed in patient sera (lane 2 v.s. lane 4 & lane 7). These observations were agreed with those found in Figure 25 (lane 6,7 & 8). IAP in tumor tissue extracts were varied in the number of isoforms and the molecular sizes(lane 6 & lane 9). One patient sample (P_1) demonstrated the molecular sizes of three IAP isoforms which were more or less the same as that found in the corresponding serum (lane 6 v.s lane 4). The IAP isoforms found in tumor tissue extract of the second patient (P2) were separated at different positions from that found in P1 tissue extract and its corresponded serum enzyme.

In Figure 28, the IAP isoforms in tumor tissue and normal tissue extract of the same patient were compared. The IAP in normal tissue extract migrated at the position which the molecular size was greater than 225 kDa. The IAP isoforms in all tumor tissue extracts(lane 5,7 & 9) were varied in molecular sizes, 205 and 225 kDa (also observed in Figure 27). IAP isoenzyme detected by Western Blot analysis were all high molecular mass isoforms.

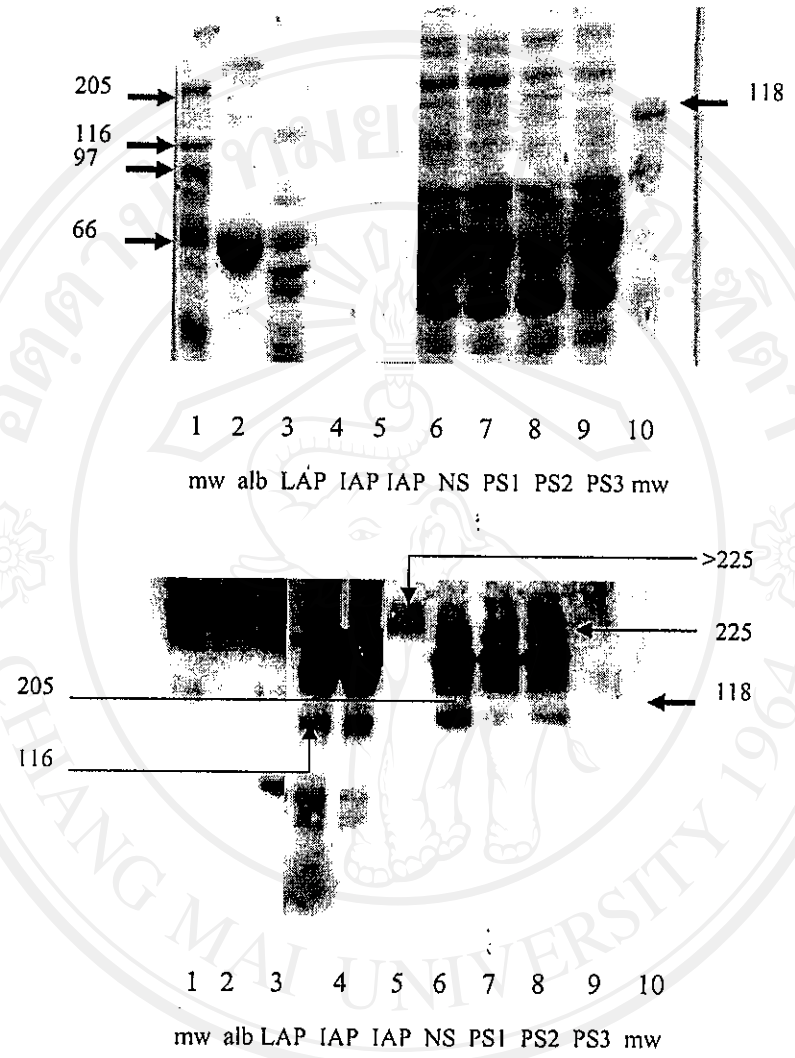


Figure 25. The comparison of molecular weight of IAP isoforms in 3 patient sera with that in normal serum. Lane 1 & 10,the molecular weight markers (mw); lane 2 , albumin ; lane 3, LAP standard ; lane 4 , IAP standard ; lane 5, IAP standard in lane 4 (diluted 1:2) ; lane 6 , normal serum (NS) ; lane 7,8 &9 (PS1, PS2 & PS3), colon cancer sera.

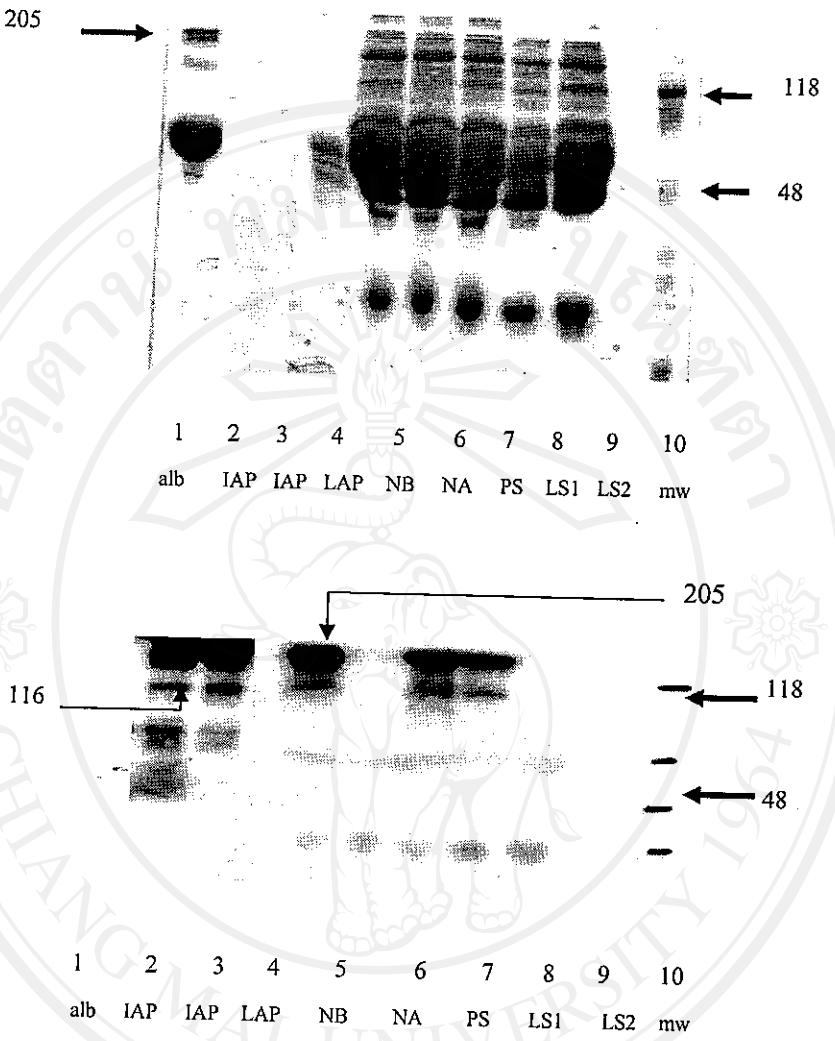


Figure 26. The comparison of IAP isoforms in normal , colon cancer and liver disease serum. Lane 1, albumin ; lane 2 &3, IAP standard ;lane 4, LAP standard ; lane5, blood group B normal serum (NB); lane 6, blood group A normal serum (NA) ; lane 7 colon cancer serum (PS); lane 8 & 9 ,liver disease serum (LS1 &LS2) ; lane 10, molecular weight marker (mw).

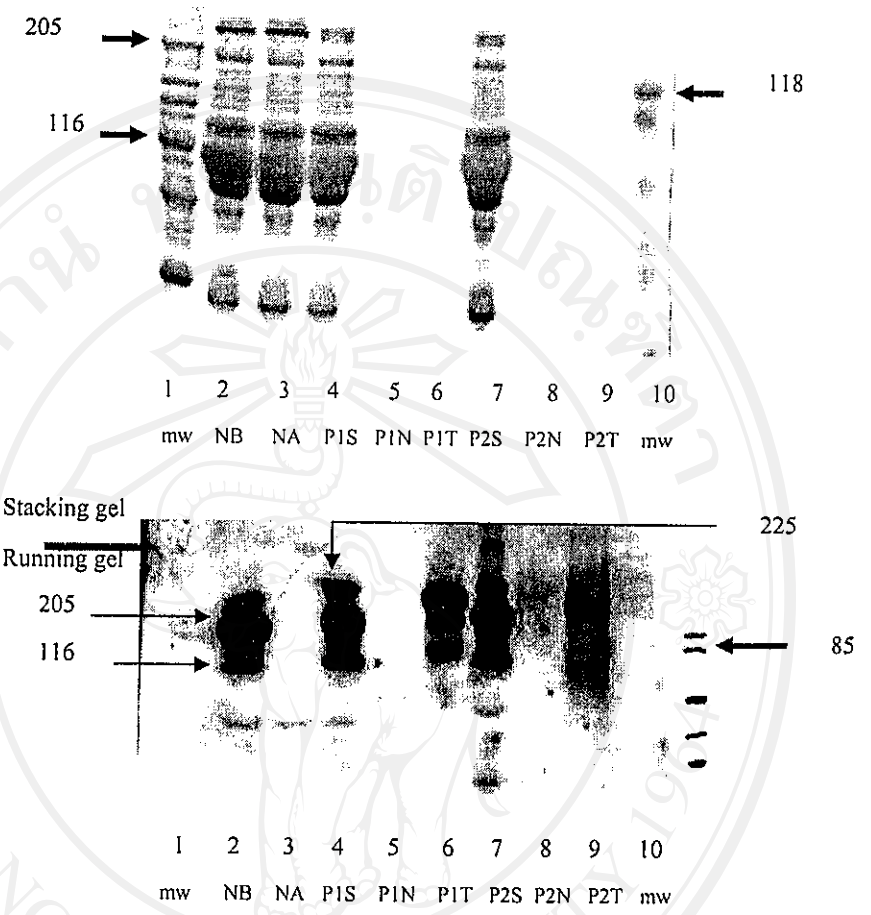


Figure 27. The comparison of IAP isoforms in serum , normal tissue and tumor tissue obtained from the same patient. Lane 1 &10, molecular weight markers ; lane 2 , blood group B normal serum ; lane 3, blood group A normal serum ; lane 4 ,5 & 6 and lane 7, 8 & 9, serum, paired normal and tumor tissue extracts of patient 1& 2, respectively.

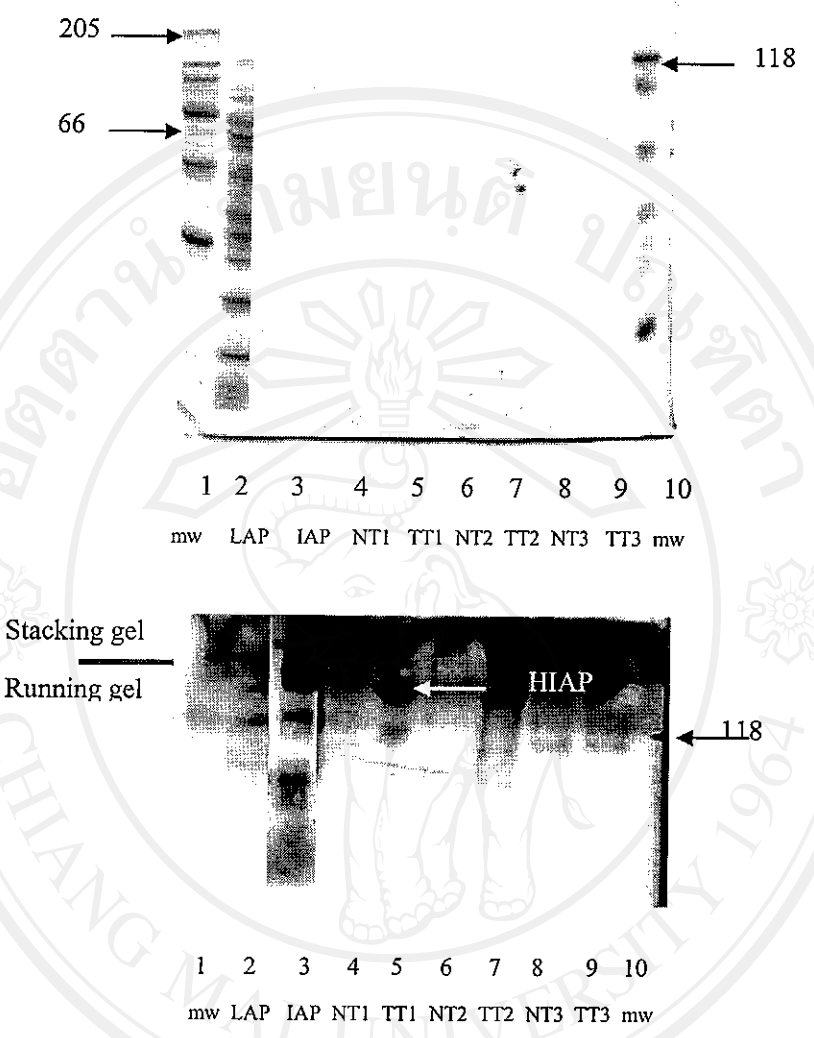


Figure 28. The comparison of IAP isoforms in tumor tissue extract with that in normal tissue extract of the same patient. Lane 1&10,the molecular weight markers (mw); lane 2, LAP standard ; lane 3, IAP standard ; lane 4 & 5; lane 6 &7 and lane 8 & 9 were paired normal (NT1,NT2,NT3) and tumor tissue extracts (TT1, TT2,TT3) of patient 1, 2 and 3, respectively.