CHAPTER III

RESULTS

I. Blood Group Typing and ABH Secretor Status of Subjects

Blood group and secretor status of subjects collected from healthy volunteers were summarized in Table 1. In this study, 89 normal subjects, which were randomly selected from those who come for health checking up, were identified as 52 secretors and 37 non-secretors. The secretors consisted of 5 of blood group A, none of blood group AB, 16 of blood group B and 31 of blood group O. The non-secretors consisted of 6 of blood group A, 2 of blood group AB, 16 of blood group B and 13 of blood group O. It was obvious that the number of group O and B subjects were greater than the others. All serum specimens were aliquoted and stored at -20°C and used further for determination of liver function test, total ALP activity, IAP activity and IAP isoforms, respectively.

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Table1. The ABO blood group and ABH secretion status of the subjects

Blood group typing of specimens	Total (N)	Secretor status	
		Secretors (N = 52)	Non-secretors (N = 37)
Blood group A	11	5	6
Blood group AB	2	0	2
Blood group B	32	16	16
Blood group O	44	31	13
Total	89	52	37

II. Screening of Liver Disease on Subjects by Determination of the Liver Enzymes in Serum Specimens

1. Analytical precision of method for liver enzyme determination

The analytical precision of AST and ALT activity determination performed in pooled control serum in a Shimadzu UV 160 A were shown in Table 2. In the determination of optimal condition variation (intra-assay), the precision of analytical method was varied with activities of AST and ALT in control serum. The %CVs obtained by analyzing both enzymes in the serum control were 5.44 and 4.66%, respectively. The %CVs of routine condition variance (inter-assay) of AST and ALT in serum control that had been used previously for evaluation of the OCV were accepted by the criteria of WHO (WHO Document 1976; Lab/76.1:1-49). It was found that the RCV mean values of both enzyme determinations were closely to their corresponding OCV.

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Table 2. Analytical precision of AST and ALT determination

Enzyme	N	OCV (intra-assay)			N	RCV (inter-assay)		
		Mean	SD	%CV		Mean	SD	%CV
AST (U/L)	10	45.6	2.48	5.44	5	43.6	2.86	6.56
ALT (U/L)	10	81.6	3.8	4.66	5	82.1	4.46	5.43

2. AST and ALT activities in serum specimens

The means of AST and ALT activities in sera of volunteers (N = 89) were 20.2 ± 7.07 U/L (Mean ± SD) and 17.96 ± 10.62 U/L (Mean ± SD), respectively. The AST and ALT activities in all sera of volunteers were within the range of reference ranges shown in Table 3. Therefore all volunteers were free from liver disease shown by the screening of liver enzyme tests.

Table 3. AST and ALT activities in normal serum of all blood groups with no regard to ABH secretion status.

Screening test for liver enzymes	N	Detected ranges (U/L)	Mean ± SD (U/L)	Reference normal range of method* (U/L)	Reference normal range of the hospital** (U/L)
AST (U/L)	89	12.0-32.7	20.2 ± 7.07	5-34	7-40
ALT (U/L)	89	8.5-28.4	17.96 ± 10.62	10-35	3-37

***** Trace, Australia

****** Reference ranges shown on the requested form, established by Maharaj Nakorn Chiang Mai Hospital, Faculty of Medicine, Chiang Mai University

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III. Total ALP Activity in Serum of Subjects in Relation to Blood Group and Secretor Status

1. Analytical precision of total ALP activity determination

Optimal condition variation (OCV) and Routine condition variance (RCV) of total ALP activity determination in control sera determined in a Shimadzu UV 2450 double-beam UV- Visible Spectrophotometer was shown in Table 4. Two levels of control serum were used for evaluation of precision on total ALP activity analysis. Results were plotted on Levey-Jennings control charts for interpretation of precision. An increase in the percentage of coefficient of variation (%CV) of OCV was observed when the mean activities of the enzyme were decreased. The %CV of OCV determined in both control serums were ranged from 2.96-6.12%, respectively. In routine analyses of both control sera which were used previously for evaluation of OCV, it was found that the RCV mean values were very close to that of OCV. The %CV of both control serums in RCV were ranged from 5.17-8.62%. All %CV values were under most of the routine conditions and the ratio of RCV to OCV was less than two for both levels of determinations.

Figure 3 and 4 shows very good distributions of analytical ALP activity values performing in a Shimadzu UV 2450 (as report in Table 4). In conclusion, the precisions of quality control tests for determination of total ALP activity in both control sera were accepted by the criteria of WHO (WHO Document 1976; Lab/76.1:1-49).

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Table 4. Analytical precision of total ALP activity (U/L) determination in Shimadzu UV 2450 Spectrophotometer

Control serum	OCV(N=20)			RCV (N=10)		
	Mean	SD	%CV	Mean	SD	%CV
Level I	141.27	4.18	2.96	140.08	7.25	5.17
Level II	34.13	2.09	6.12	34.35	2.96	8.62

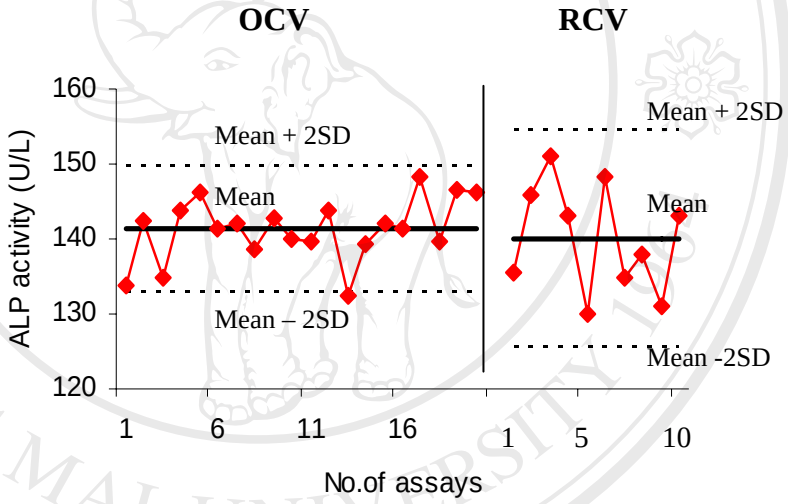

Figure 3. The distribution of ALP activity values in Level I control serum determined in Shimadzu UV 2450 Spectrophotometer.

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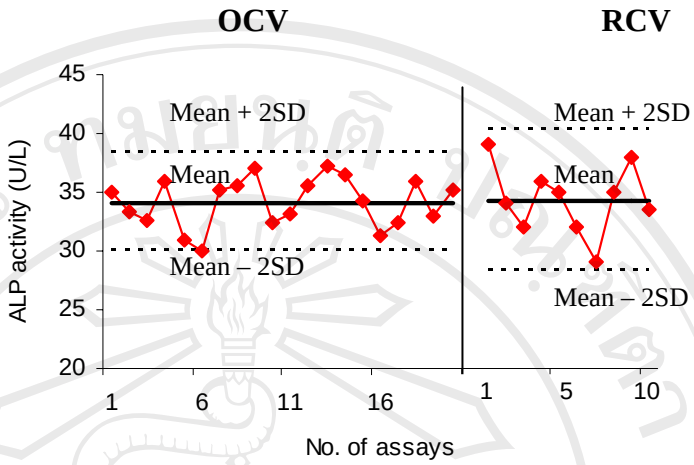

Figure 4. The distribution of ALP activity values in Level II control serum determined in Shimadzu UV 2450 Spectrophotometer.

2. Total ALP activity measured in normal serum

ALP activity in normal serum was measured in a double beam UV-2450 Spectrophotometer. The mean ± SD of total ALP activity in normal serum of total subjected (N= 89) was 65 ± 25.08 U/L.

Table 5 shows the mean ± SD of total ALP activity at fasting stage in serum of different blood group subjects with secretor status. There was no significant difference between the mean of total ALP activity determined in AMP buffer of blood group B secretors as compared with the O secretors, as well as the blood group B non-secretors with the O non-secretors. It was also no different between mean of group B secretors and non-secretors, but there was significant difference between mean of O secretors and non-secretors ($p < 0.001$). In further study, the B and O specimens of

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secretor and non-secretor subjects were regrouped in B or O group of each type of secretor status. It is obvious that the difference between the mean of total ALP activity in blood group B or O secretors and blood group B or O non-secretors was statistically significant at $p < 0.001$, however all values were within the reference normal range (40-125 U/L, (Bower, 1975)). Figure 5 shows the distributions of total ALP activity values in B or O serum of secretors and non-secretors (upper). The lower Figure 5 is the histogram of mean $\pm$ SD of both groups. As described above the means of two groups were significantly different. Figure 6 is the comparison of the distributions of total ALP activity values in B or O serum of secretors and non-secretors and total ALP activity of other blood groups. Mean of other blood groups was statistically lower than of B or O secretors and non-secretors ($p < 0.001$).

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Table 5. Total ALP activity (U/L) in different blood groups with secretor status

Blood group	Total ALP activity (U/L) Secretors			Total ALP activity (U/L) Non -secretors		
	N(men)	Detected range	Mean ± SD	N(men)	Detected range	Mean ± SD
A	5 (2)	30 – 45	43.3 ± 14.81	6 (5)	35 – 73	33.7 ± 7.85
AB	–	–	–	2 (0)	39 – 41	39.7 ± 0.85
B	16 (5)	40 – 105	61.0 ± 24.28	16 (4)	40 – 75	53.3 ± 15.75
O	31 (15)	43 – 115	72.6 ± 23.50*	13 (4)	42 – 76	54.4 ± 11.59*
B or O	47 (20)	40 – 115	65.1 ± 24.06**	29 (8)	40 – 76	53.8 ± 14.05**

*$p < 0.001$
**$p < 0.001$

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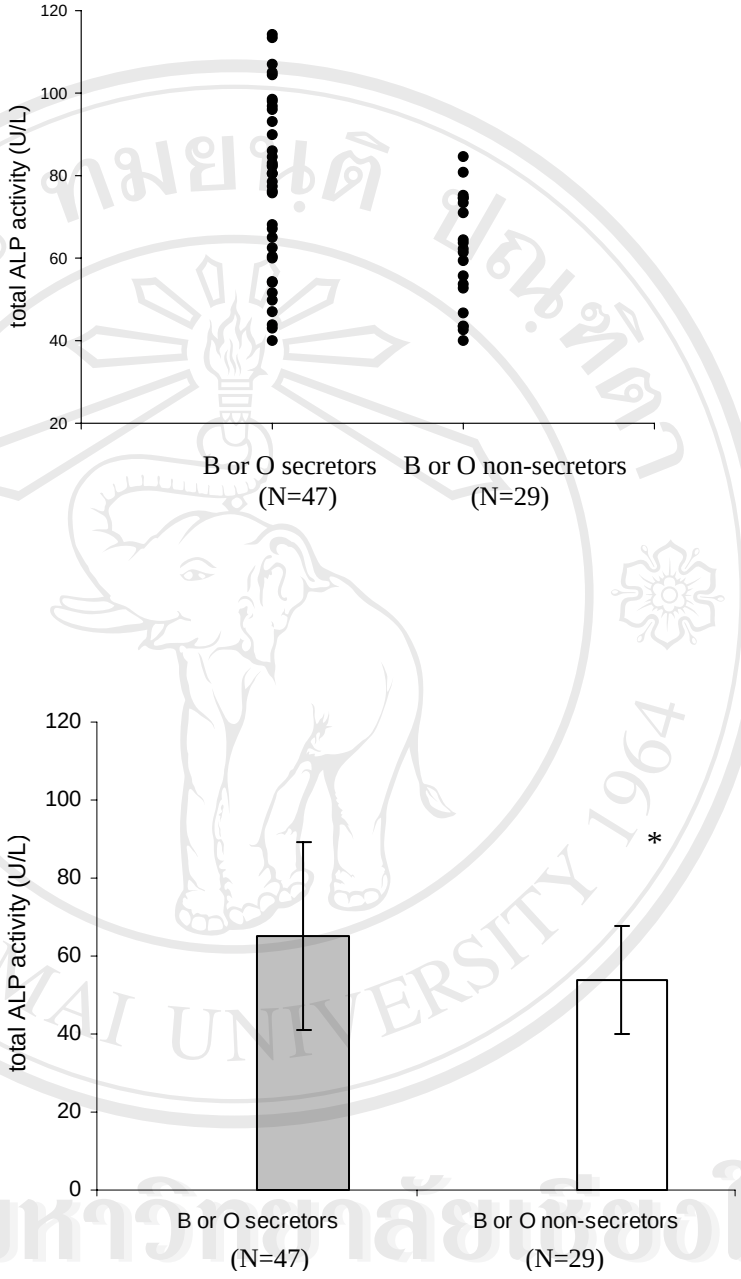

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Figure 5. The total ALP activity (U/L) in serum of B or O secretors and non-secretors. Upper: the distribution of total ALP activity in serum of B or O secretors and non-secretors. Lower: The comparison of the mean and standard deviation of total ALP activity in both group, * the different of mean was significant at $p < 0.001$.

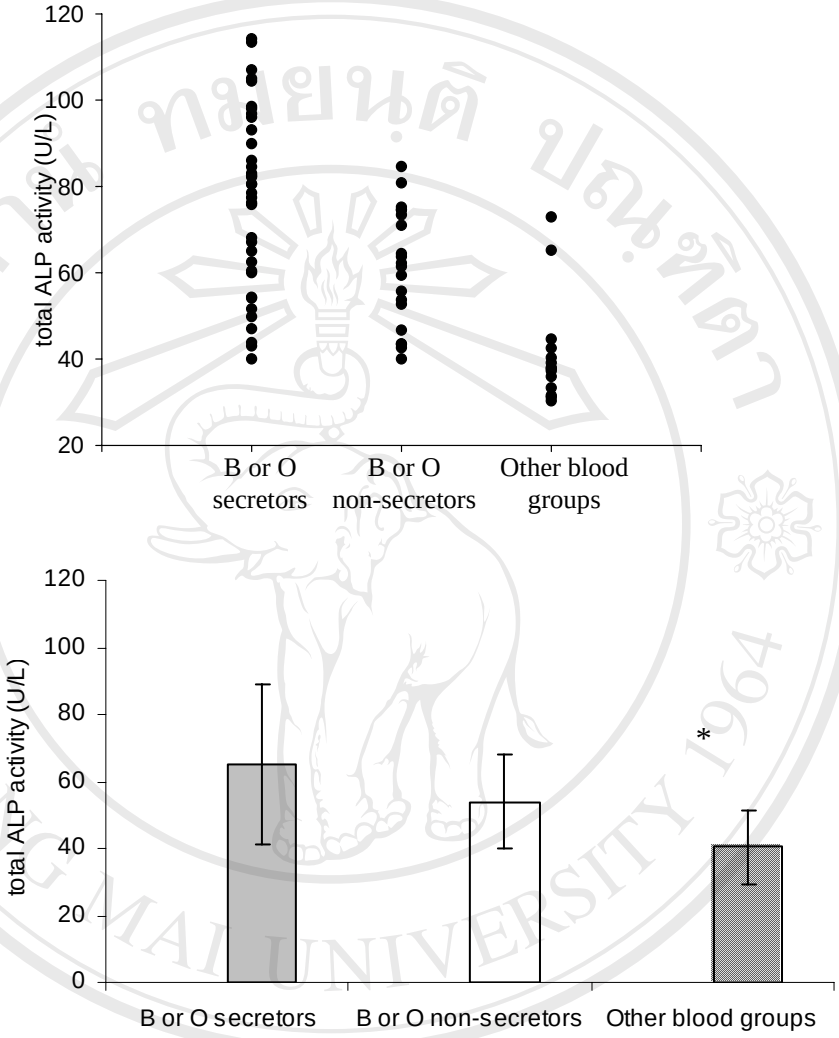

Figure 6. Total ALP activity (U/L) in serum of B or O secretors and non-secretors compared with the other blood group. Upper : the distribution of total ALP activity in serum of B or O secretors and non-secretors compared with the other blood groups and lower: the comparison of the mean and standard deviation of total ALP activity of both group with the other blood group , * the different of mean was significant at $p < 0.001$.

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Since the total ALP activity in B or O secretor was significantly different from that of non-secretor, therefore to observe whether the ages of subjects with regard to secretor status affected on total ALP activity or not, all subjects with regard to secretor status were regrouped according to their ranges of age. Table 6 demonstrated the relation of ages on the levels of total ALP activity in serum of B or O blood groups at fasting stage. Results show that means of total ALP activity in secretors of different age groups were closely with no statistical difference. The means of total ALP activity of B or O non-secretors of each age ranges were slightly increased as the ages increased and was significantly different from that of paired-range of age in B or O secretors ($p < 0.05$).

Table 6. The relation of total ALP activity (U/L) in serum of B or O secretors and B or O non- secretors with the ages of subjects.

Secretor status	Range of age (years)	N	Total ALP activity(U/L)	
			Detected range	Mean ± SD
B or O secretors	18-27	15	40 – 115	64.1 ± 35.50
	28-37	7	43 – 99	65.4 ± 19.09
	38-47	9	40 – 105	67.5 ± 19.70
	48-57	10	41 – 108	65.3 ± 3.34
	58-67	6	47 – 105	62.8 ± 15.56
B or O	18-27	9	42 – 100	56.8 ± 25.82
non- secretors	28-37	5	43 – 95	50.1 ± 16.46
	38-47	7	42 – 101	50.6 ± 15.40
	48-57	5	43 – 81	58.3 ± 23.12
	58-67	3	48 – 99	52.9 ± 22.62

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IV. Separation and Identification of ALP Isoenzymes in Tissue Extract and Serum Specimens by 6% Polyacryramide Gel Electrophoresis (PAGE) Containing Triton X-100

1. Identification of types of ALP isoenzymes by neuraminidase treatment on PAGE

Figure 7 shows the migration of ALP isoenzyme bands of each tissue extract separated by PAGE. It was demonstrated that the mobility of IAP isoforms extracted from bovine colon before (Lane 1) and after treating (Lane 2) with neuraminidase were unchanged whereas the isoforms of the other ALP isoenzymes were affected by treatment with neuraminidase. It was shown that the mobility of PLAP(Lane 4), LAP and BAP (mixed) (Lane 6) and LAP (Lane 8) isoforms, extracted from their corresponding tissues and treated with C-neu were retarded as compared with its own untreated control (paired-test). The mobility of ALP isoenzymes in liver disease serum of the untreated control (Lane 9) compared with that of treated isoenzyme (Lane 10) resemble the separation patterns of tissue- nonspecific ALP isoenzymes in Lane 5 vs Lane 6 and Lane 7 vs Lane 8, respectively. Therefore, IAP isoforms in serum separated by PAGE was identified as those bands which resisted to neuraminidase treatment.

Typical scanning patterns (by Scion image program) of ALP isoenzymes in tissue extracts are shown in Figure 8. It was confirmed and identified by the scanning that there are 3 isoforms of IAP separated by PAGE because of those bands were unaffected by neuraminidase treatment (panel A). The scanning of other ALP isoenzymes in panel B, C or D showed different patterns of peaks of control (C) as compared with peaks of its corresponding treated sample (T). The mobilities of

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treated isoenzymes were slower than that of untreated control by the concept of neuraminidase sensitive property.

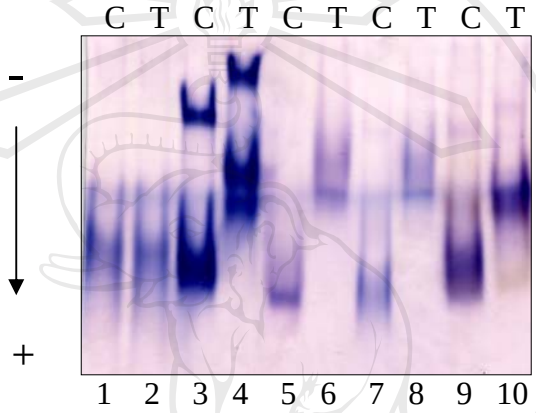

Figure 7. Polyacrylamide gel electrophoresis demonstrated ALP isoenzymes migration with and without neuraminidase treatment.
Lane 1, 2: IAP isoenzyme, Lane 3, 4: PLAP isoenzyme, Lane 5, 6: Liver& bone isoenzymes, Lane 7, 8: LAP isoenzyme and Lane 9, 10: liver disease serum. C and T represent control and neuraminidase treatment respectively.

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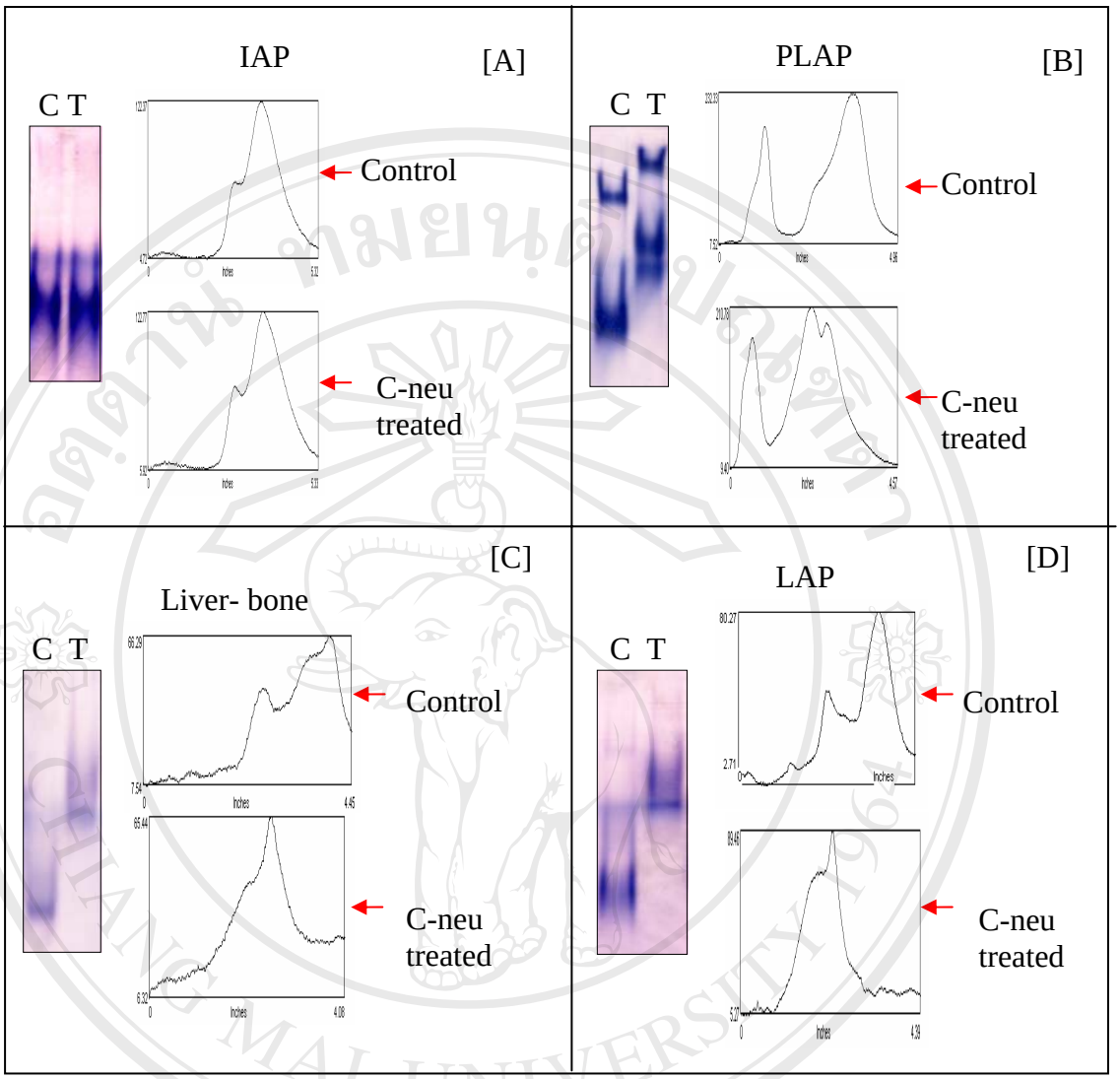

Figure 8. ALP isoenzyme scanning patterns before and after treating with neuraminidase. (Band scanning using Scion image program)

[A], [B], [C] and [D] were calf colon tissue extract (500 U/L), human placental extract (1,200 U/L), liver-bone control [Helena Laboratories, USA.] and bovine liver extract (250 U/L), respectively.

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2. Analytical precision of IAP isoforms separated on PAGE

Analytical precision of IAP isoforms separated by PAGE is shown in Figure 9. As shown, the control IAP from calf colon extract contains two high molecular mass (HIAP) and one normal molecular mass (NIAP) of IAP isoforms. After scanning, the activities of IAP isoforms were calculated. Table 7 shows the precision as %CV of separation of ALP isoenzymes by PAGE. The mean and SD of each IAP isoform was calculated as IAP activity (U/L). From Table 5, %CV of OCV for NIAP and two HIAP isoforms were 8.23, 8.68 and 9.46, respectively. The %CV of RCV was within the acceptable range but all the means of RCV were higher than those means of OCV. These results indicated changing in accuracy as compared with the mean of OCV. The distributions of three separated IAP isoforms activities were fairly good and shown in Figure 10.

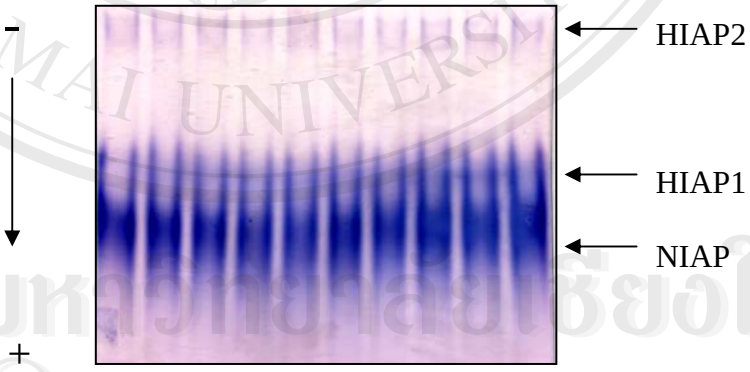

Figure 9. Separation of IAP isoforms on 6% PAGE containing Triton X-100. (IAP tissues extract from calf colon tissue, activity of 500 U/L)

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Table 7. The precision of IAP isoforms separated by PAGE

IAP isoforms	OCV(U/L) (N=10)			RCV(U/L) (N=5)		
	Mean	SD	%CV	Mean	SD	%CV
NIAP	10.00	0.82	8.20	12.93	1.23	9.51
HIAP1	12.11	1.05	8.67	15.79	1.36	8.61
HIAP2	11.61	1.10	9.47	12.43	1.16	9.33

3. Separation and identification of IAP isoforms in serum of secretors & non-secretors by PAGE

Figure 11 demonstrates the mobility patterns of ALP isoenzymes in normal serum separated by PAGE. Each serum specimen was untreated and pre-treated with neuraminidase before electrophoresis. It was shown by neuraminidase treatment that the B or O secretor serum demonstrated three bands of IAP isoforms separated on PAGE (Lane 3 ,C compared with Lane 4, T or Lane 5, C compared with Lane 6,T). The HIAP band was found in serum of secretors of B or O blood groups (Lane 3-6), and was disappeared in serum of non-secretors (Lane 7-10). The typical bands of IAP isoforms in serum of secretors consisted of two HIAP and one NIAP bands while in non-secretors serum, only NIAP band was detected. All TNAP bands (LAP and BAP) in each specimen in control lane (C) and migrated fastest to the anode. After treating with neuraminidase (lane T), the TNALP bands in sera of both secretors and non-secretors show the same retard mobilities which agreed with that observed in liver bone control (Panel C) separation in Figure 8.

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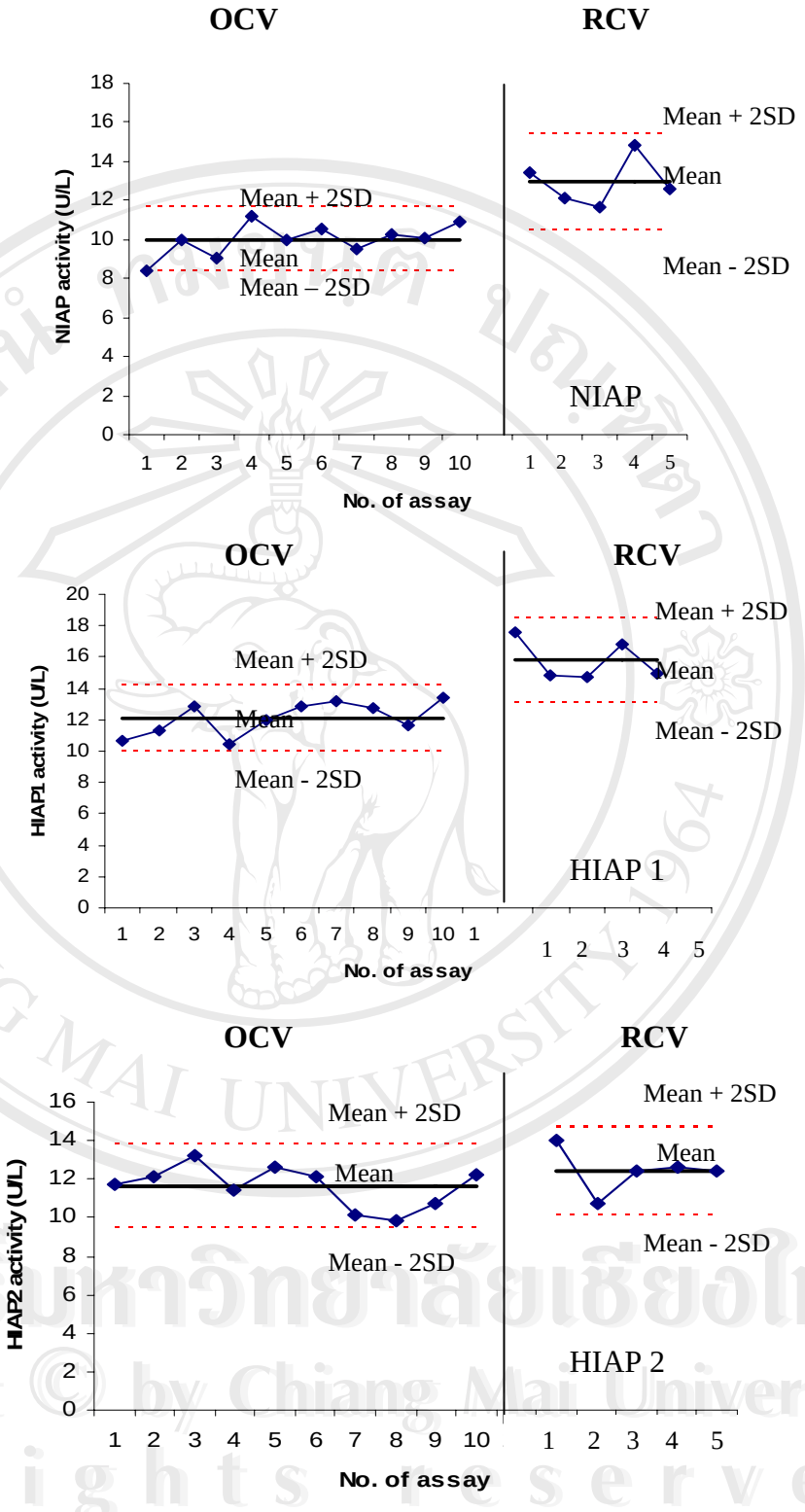

Figure 10. The distributions of activities of different isoforms of IAP isoenzyme extracted from calf colon tissue and separated by PAGE. Upper,middle and lower figures were NIAP, HIAP1 and HIAP2 isoforms, respectively.

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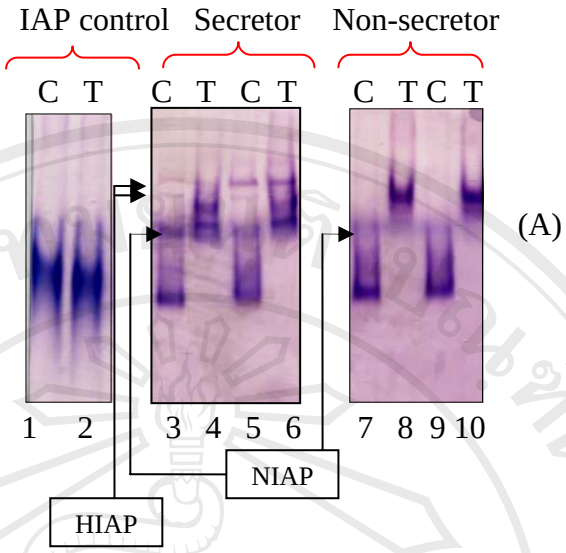
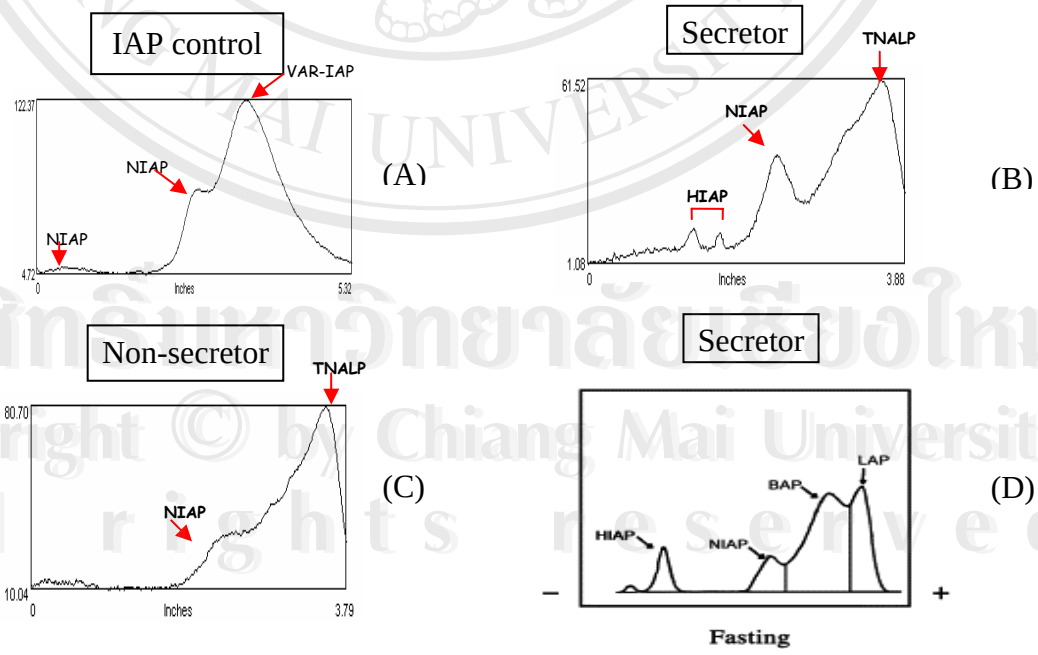


Figure 11. The migration of ALP isoenzymes in serum of secretor & non-secretor on 6% PAGE containing Triton X-100, before & after neuraminidase treatment.
Lane 1, 2: IAP isoenzyme control (Calf colon tissue), Lane 3, 4 and Lane 5, 6: sera of B or O secretors. Lane 7, 8 and Lane 9, 10: sera of B or O non-secretors. C and T represent control and neuraminidase treatment.



A, B and C are the Scion image scanning of ALP isoenzymes in lane 1 (C), 3 (C) and 7 (C). D is the densitrometric scanning of fasting serum B or O secretor performed by Matsushita, *et al.* (2002)

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4. IAP & TNAP activities in sera of secretors & non-secretors calculated from separated bands on PAGE.

ALP isoenzyme activities of the separated bands on PAGE were calculated after scanning the electrophorogram by Scion Image program (See Appendix C). As shown in the summary of ALP isoenzyme, activities separated by PAGE method were shown in Table 8. The mean of HIAP activity in B or O secretors was 7.3 ± 4.17 U/L (Mean ± SD), but in the non-secretors, this isoform could not be detected.

Table 9 is the effect of ages on the activities of ALP isoenzymes in sera of B or O secretors and non-secretors separated by PAGE. From statistical analysis, it could be concluded that the mean values of total ALP, IAP and TNAP activities in sera of B or O secretors and non-secretors with different age groups were not statistically significant from that of the group of ages between 18-27 years except for the IAP activities in sera of non-secretors whose ages were greater than 57 years.

Figure 12, the upper figure shows the distribution of total IAP (TIAP) activity in sera of secretors and non-secretors. The mean of TIAP activity in B or O secretors was higher than that found in non-secretors (Figure 12, Lower). TIAP activity in B or O secretors and non-secretors were 9.7± 4.64 U/L (Mean ± SD) and 0.9 ± 0.42 U/L (Mean ± SD), respectively. The difference between the means of TIAP activity in B or O secretors and non-secretors was statistically significant at $p < 0.0001$.

Figure 13 shows the distribution of NIAP activity values (upper figure) and the comparison of means of NIAP activity in sera of B or O secretors and non-secretors (lower figure). The means of NIAP activities in sera of secretors and non-secretors were 2.4 ± 0.92 U/L (Mean ± SD) and 0.9 ± 0.42 U/L (Mean ± SD),

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respectively. The difference between the means of NIAP activities in sera of B or O secretors and non-secretors was statistically significant at $p < 0.0001$.

The distribution of TNAP activities in sera of B or O secretors and non-secretors were shown in Figure 14 (upper figure). In the lower Figure 14, the comparison of TNAP activities of both secretor status were compared. The means of TNAP activity in sera of B or O secretors and non-secretors were 55.4 ± 24.60 U/L (Mean ± SD), and 52.9 ± 16.16 U/L, respectively. There was no statistically different between the means of TNAP activities in sera of B or O secretors and non-secretors.

Table 8. The summary of IAP isoenzyme activities in serum of B or O secretors and B or O non-secretors as separated by PAGE.

IAP isoenzyme activity (U/L)	B or O secretors Mean ± SD (N = 47)	B or O non-secretors Mean ± SD (N = 29)	P
TIAP	9.7 ± 4.64	0.9 ± 0.42	< 0.0001
NIAP	2.4 ± 0.92	0.9 ± 0.42	< 0.0001
HIAP	7.3 ± 4.17	0	NT
TNAP	55.4 ± 24.60	52.9 ± 16.16	NS

NT = no test for statistical difference.
NS = no significant difference

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Table 9. The ALP isoenzyme activities in serum of B or O secretors and non-secretors at various ranges of ages separated by PAGE.

	range of age	N	total ALP activity (U/L) by AMP method (Mean ± SD)	TIAP activity (U/L) (Mean ± SD)	NIAP activity (U/L) (Mean ± SD)	HIAP activity (U/L) (Mean ± SD)	TNALP activity (U/L) (Mean ± SD)
Secretors (N = 47)	18-27	15	64.1 ± 35.50	9.2±3.34	2.1±1.08	7.2±4.61	54.9±26.87
	28-37	7	65.4 ± 19.09	10.8±4.58	2.4±0.36	8.2±4.50	54.6±24.63
	38-47	9	67.5 ± 19.70	9.7±4.61	2.8±0.99	6.3±3.41	57.8±24.20
	48-57	10	65.3 ± 3.34	9.5±6.52	2.6±0.85	7.1±3.30	55.8±24.01
	58-67	6	62.8 ± 15.56	8.0±6.15	2.0±1.22	6.0±2.24	54.8±12.72
non-secretors (N = 29)	18-27	9	56.8 ± 25.82	0.7±0.15	0.7±0.15	0	56.1±25.57
	28-37	5	50.1 ± 16.46	0.8±0.22	0.8±0.22	0	49.3±18.5
	38-47	7	50.6 ± 15.40	0.9±0.16	0.9±0.16	0	49.7±23.02
	48-57	5	58.3 ± 23.12	0.8±0.25	0.8±0.25	0	57.5±12.34
	58-67	3	52.9 ± 22.62	1.1±0.12*	1.1±0.12*	0	51.8±16.24

*significant difference from the age range 18-27 years of non-secretor at $p < 0.05$

The levels of TIAP and NIAP activities in B or O non-secretors in all ranges of ages were significantly different from those of B or O secretors ($p < 0.0001$).

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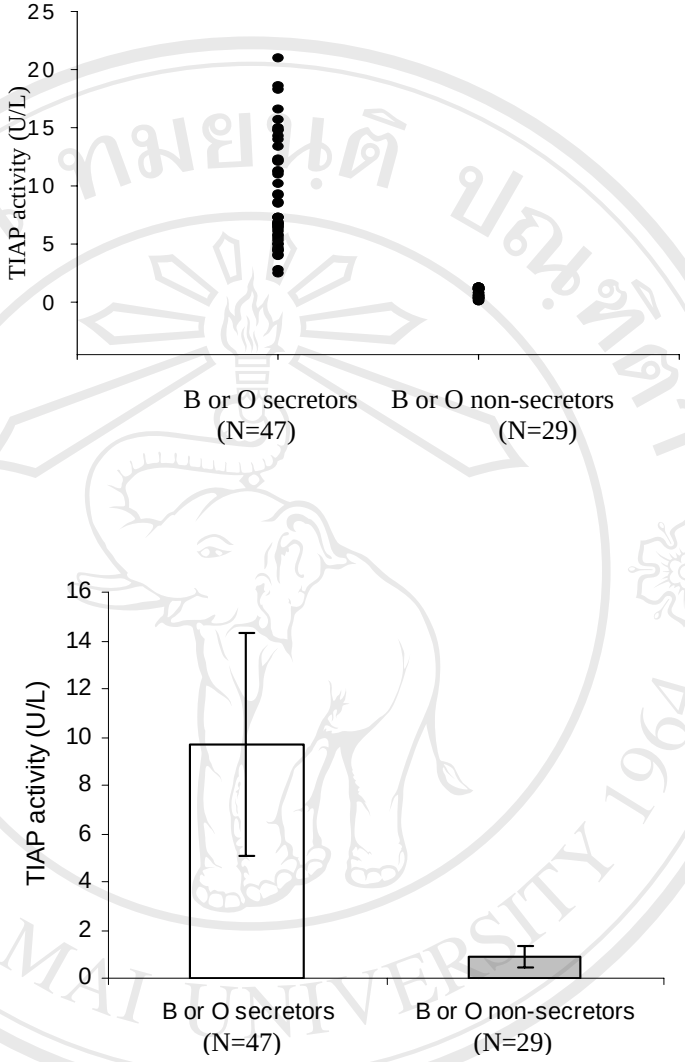

Figure 12. Comparison of TIAP activity (U/L) in sera of B or O secretors and non-secretors ($p < 0.0001$). Upper: The distribution of the analytical values and lower: the mean and standard deviation of TIAP activities

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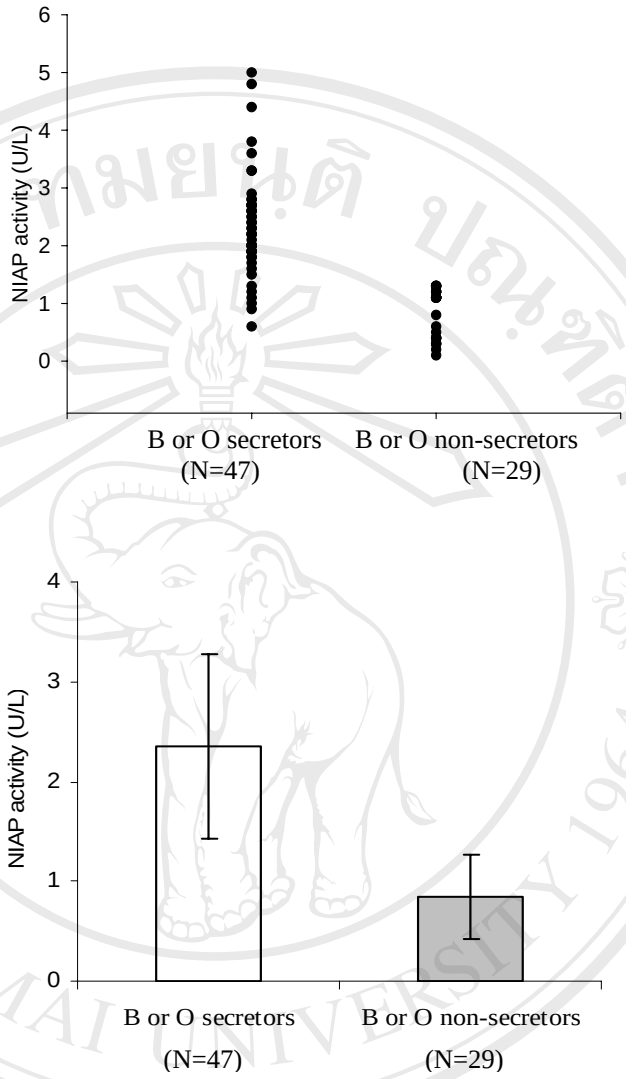

Figure 13. Comparison of NIAP activity (U/L) in sera of B or O secretors and non-secretors, $p<0.0001$. Upper: The distribution of the analytical values and lower: the mean and standard deviation of NIAP activities.

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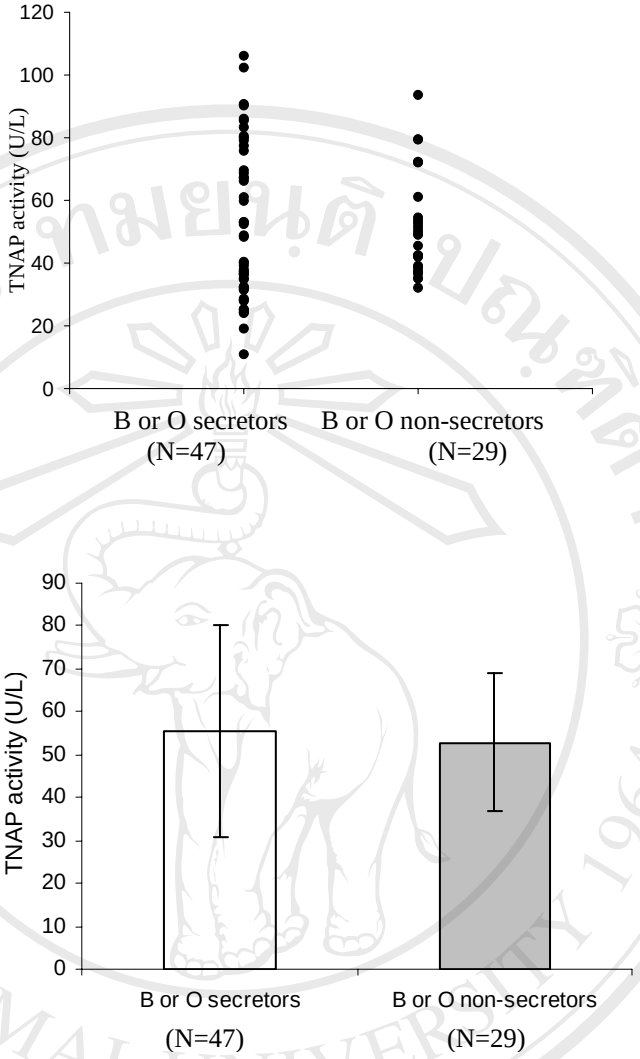

Figure 14. Comparison of TNAP activity (U/L) in sera of B or O secretors and non-secretors (NS). Upper: The distribution of the analytical values and lower: the mean and standard deviation of TNAP activities.

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The ALP isoenzymes in normal serum of other blood group (A, AB) were compared with B or O secretors and non-secretors. Figure 15 shows the patterns of ALP isoenzymes in blood group A and AB which were apparently identical to that of B non-secretors. The pattern of ALP isoenzymes in blood group O secretors was used as a control of secretors' serum separation.

The activities fractions of ALP isoenzymes in sera of B or O secretors and non-secretors are shown in Table 10. The mean activities of TNAP between two groups were not significantly different while consideration on the IAP fractions, they were significantly different between two groups of B or O secretor status ($p<0.001$). The IAP isoenzyme in sera of blood group B or O secretors had about 10 times higher than blood group A secretors. Serum specimens of group AB secretors could not be obtained during specimen collection. However, the group A secretor and A or AB non-secretors demonstrated the patterns of isoenzyme migration resembled group B or O non-secretor. The means of total ALP, TIAP, and TNAP activities of group A secretor and group A and AB non- secretors were significantly lower than that of group B or O secretor and non-secretor at $p< 0.001$.

Table 10 is the summary of ALP isoenzyme activities in normal serum in relation to ABH secretor status. The serum total ALP activities were strongly associated with ABO blood groups and secretor status. Serum TIAP activities were greater in the subjects with B or O secretors than in the others. The HIAP activities were higher than the NIAP activities in the B or O secretors while it was not present in the non-secretors sera. The means of TNAP activities in B or O secretors and non-secretors were more or less the same (NS). The difference between the means of TIAP activity in two groups of secretor status was not statistically significant.

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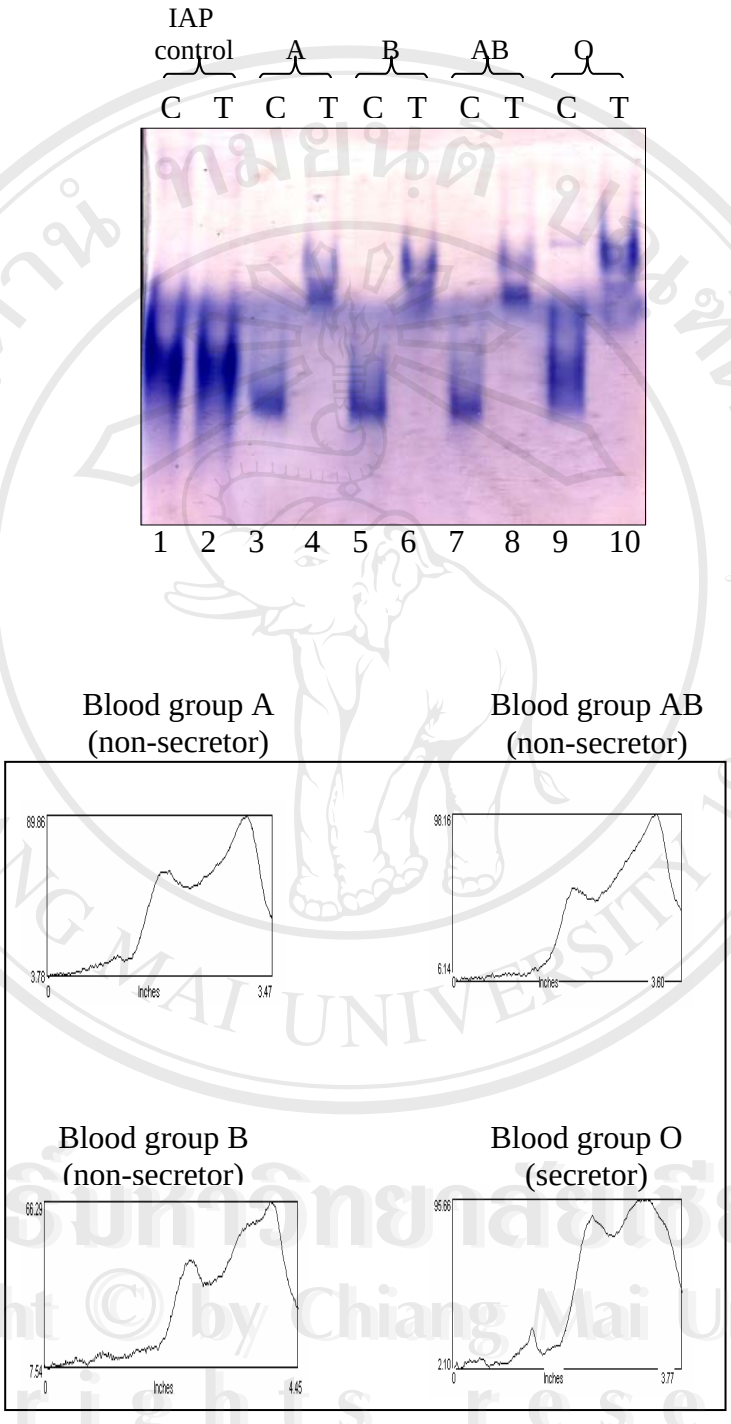

Figure 15. The ALP isoenzymes in normal serum of other blood group (A, AB) compared with B or O separated by PAGE method.

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Table 10. The summary of ALP isoenzyme activities in normal sera of different blood groups with regard to secretor status and separated by PAGE method.

Blood group	N (men)	total ALP activity (U/L) Mean ± SD	TIAP (U/L) Mean ± SD	NIAP (U/L) Mean ± SD	HIAP (U/L) Mean ± SD	TNAP (U/L) Mean ± SD
A secretors	5(2)	43.3±14.81	1.5±0.42	0.8±0.34	0.7±0.19	41.8±8.06
A non-secretors	6(5)	33.7±7.85	0.7±0.12	0.7±0.12	0	33.0±14.74
AB secretors	-	-	-	-	-	-
AB non-secretors	2(0)	39.7±0.85	0.8±0.07	0.8±0.07	0	38.9±0.78
B secretors	16(5)	61.0±24.28	9.5±4.55	2.5±1.14	7.0±3.64	51.5±27.12
B non-secretors	16(4)	53.3±15.75	0.9±0.43	0.9±0.43	0	52.4±17.97
O secretors	31(15)	72.6±23.50	9.8±4.60	2.2±0.80	7.6±4.09	62.8±20.94
O non-secretors	13(4)	54.4±11.50	0.9±0.40	0.9±0.40	0	53.5±19.50
B or O secretors	47(20)	65.1±24.06	9.7±4.64	2.4±0.92	7.3±4.17	55.4±24.60
B or O non-secretors	29(8)	53.8±14.05	0.9±0.42	0.9±0.42	0	52.9±16.16

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V. Molecular Mass Determination and Confirmation of the Presence of the IAP Isoforms in Normal Sera by Western Blot Analysis

In Figure16, the Western blot analysis of NIAP and HIAP in serum of B or O blood groups with different secretor status are shown. Both NIAP and HIAP were found in both sera of B or O secretors and non-secretors (Figure 16, Lane 4-6 and 7-9, respectively). It appeared that there were three bands of IAP isoforms detected by Western blot analysis. The NIAP band with the molecular size of 75 kDa and 2 bands of HIAP at the molecular sizes of 135 and 250 kDa, respectively. Both HIAP bands (135 & 250 kDa) in serum of B or O non-secretors (Figure 16, Lane 7-9) shows lower intensity (or density) of bands than that found in B or O secretors (Lane 4-6). LAP from liver extract (Lane 2) which used as negative control for those containing IAP isoforms could not react with mouse monoclonal antibody against bovine IAP.The IAP isoforms in the extract from bovine colon and used as a control migrated at 135 and 250 kDa. The molecular size of 75 kDa, however, could not be seen in this figure. Figure 17 demonstrated the SDS-PAGE of control and unknown separations shown in Figure 16, the proteins were stained with Coomassie Blue and the bands were compared with the same molecular weight marker used in Figure 16. The lower molecular size of IAP control separated on SDS- PAGE appeared at the same mobility as 75 kDa molecular weight marker.

Figure 18 is the confirmation of the molecular sizes of IAP isoforms in sera of B or O secretors and non-secretors determined by Western blot technique. From the Scion image scanning, the molecular sizes of the separated IAP isoforms in sera of B or O secretors and non-secretors were identical to those of the molecular weight marker.The IAP control showed two bands at 135 and 250 kDa.

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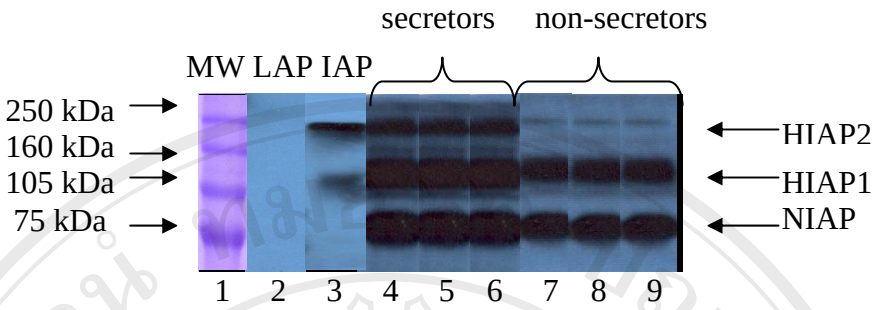

Figure 16. Molecular masses of three IAP isoforms in serum of B or O secretors and non-secretors on Western blot analysis.
Lane 1: Molecular mass markers, Lane 2: LAP control, Lane 3: IAP control, Lane 4-6 normal serum of B or O secretors and Lane 7-9 normal serum of B or O non-secretors.

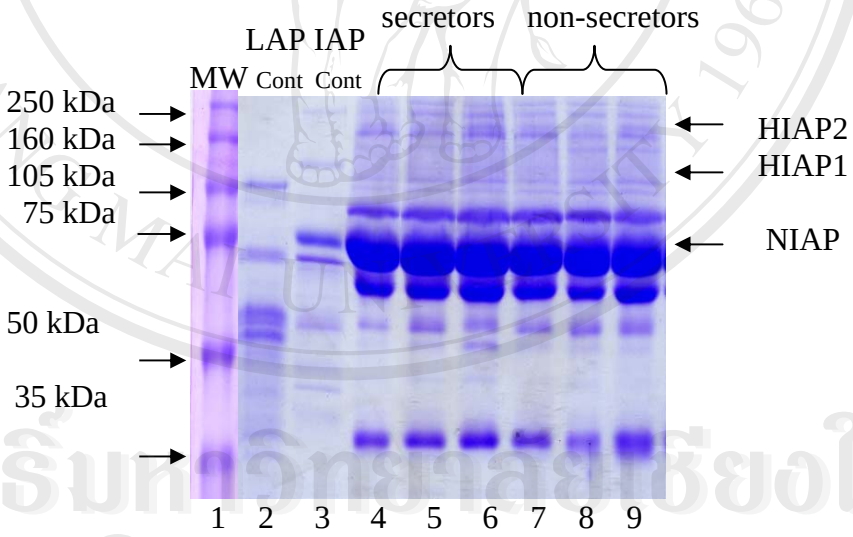

Figure 17. The ALP isoenzymes in serum of B or O secretors and non- secretors on SDS-PAGE.
Lane 1: Molecular mass markers, Lane 2: LAP control, Lane 3 : IAP control, Lane 4-6 serum of B or O secretors and Lane 7-9 serum of B or O non-secretors.

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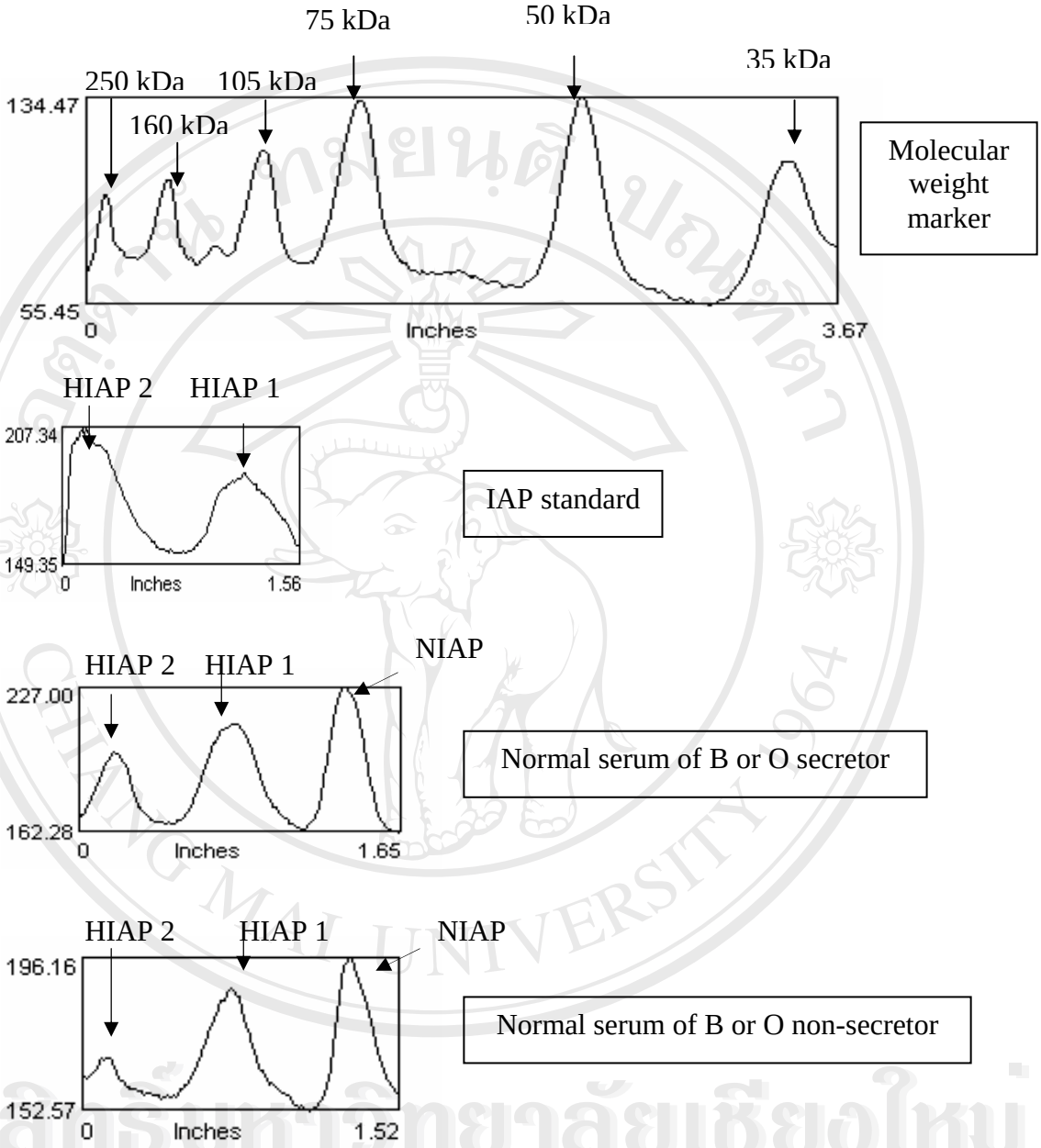

Figure 18. Molecular weight determination of IAP isoforms as compared with molecular weight marker (Full range Rainbow, Amersham, USA.)

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Four examples of IAP isoform detections were shown in Figure 19. The Western blot analysis confirmed the appearances of two isoforms of HIAP in sera of B or O non-secretors which had the same mobility as the B or O secretors. Both HIAP isoforms were migrated at 135 and 250 kDa.

Distribution of intensity of NIAP isoform values (upper) and the comparison of mean intensity of NIAP isoform (lower) in sera of secretors and non-secretors separated by SDS-PAGE and detected by Western blot analysis were shown in Figure 20. The mean intensity of NIAP isoform in sera of B or O secretors and non-secretors shown in Table 11 were 57.3 ± 9.60 (Mean ± SD) and 32.8 ± 5.42 (Mean ± SD), respectively. The difference between the mean intensity of NIAP isoforms in sera of B or O secretors and non-secretors was statistically significant ($p < 0.0001$).

The distribution of intensity of HIAP values (upper) and the comparison of mean intensity of HIAP isoform (lower) in sera of B or O secretors and non-secretors determined by Western blot analysis were demonstrated in Figure 21. The mean intensity of HIAP isoforms in sera of B or O secretors and non-secretors were 74.22 ± 12.57 (Mean ± SD), 25.1 ± 3.38 (Mean ± SD), respectively (Table 11). The difference between the mean intensity of HIAP isoforms in sera of B or O secretors and non-secretors was statistically significant at $p < 0.0001$.

The ratios of HIAP/NIAP of different secretor status were calculated and shown in Figure 22. The upper Figure 22 is the distribution of HIAP/NIAP values of B or O secretors as compared with those of non-secretors. Mean of HIAP/NIAP of B or O secretors was higher than that of non-secretors and they were statistically significant ($p < 0.001$).

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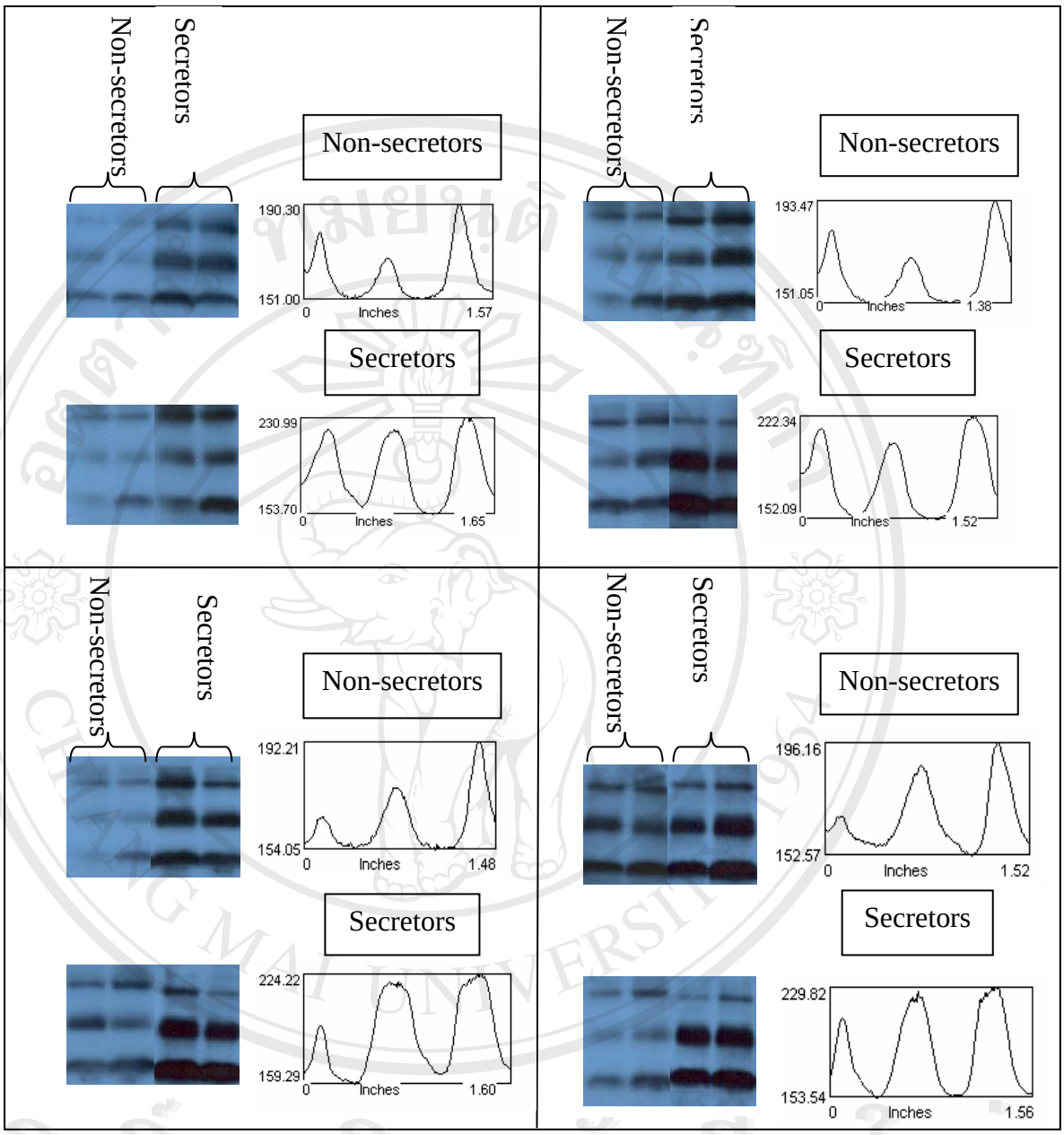

Figure 19. The comparison of IAP isoform separation detected in serum of B or O secretors and non- secretors by Western blot analysis.

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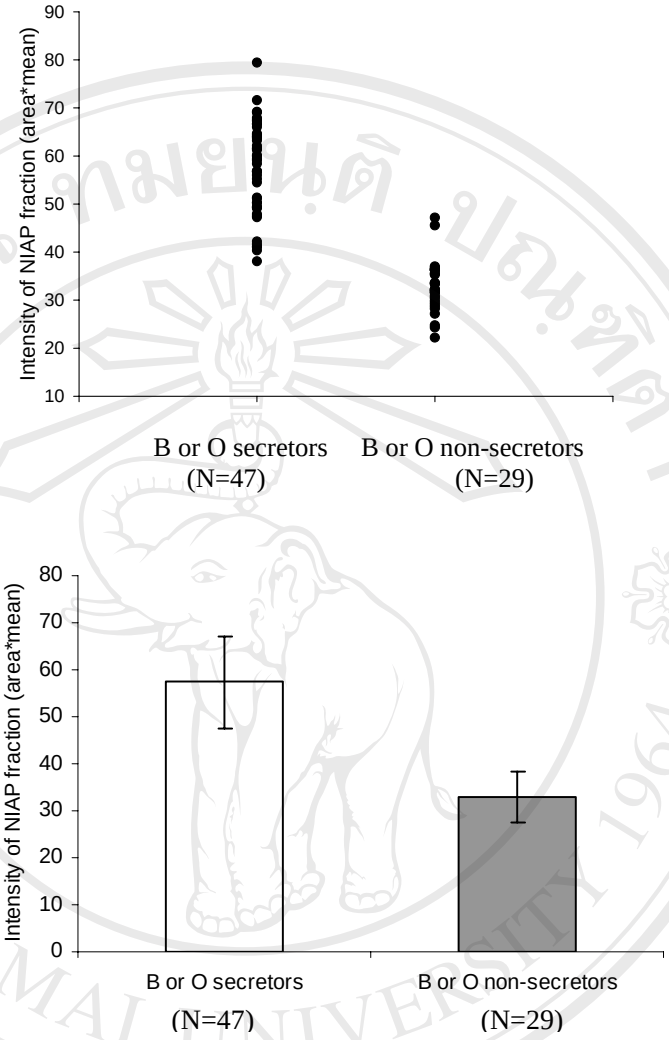

Figure 20. The intensity (density) of NIAP fraction in sera of B or O secretors and non-secretors.
Upper: The distribution in fractions of NIAP
Lower: The comparison of mean $\pm$ SD of NIAP fraction in sera of both groups of secretor status.

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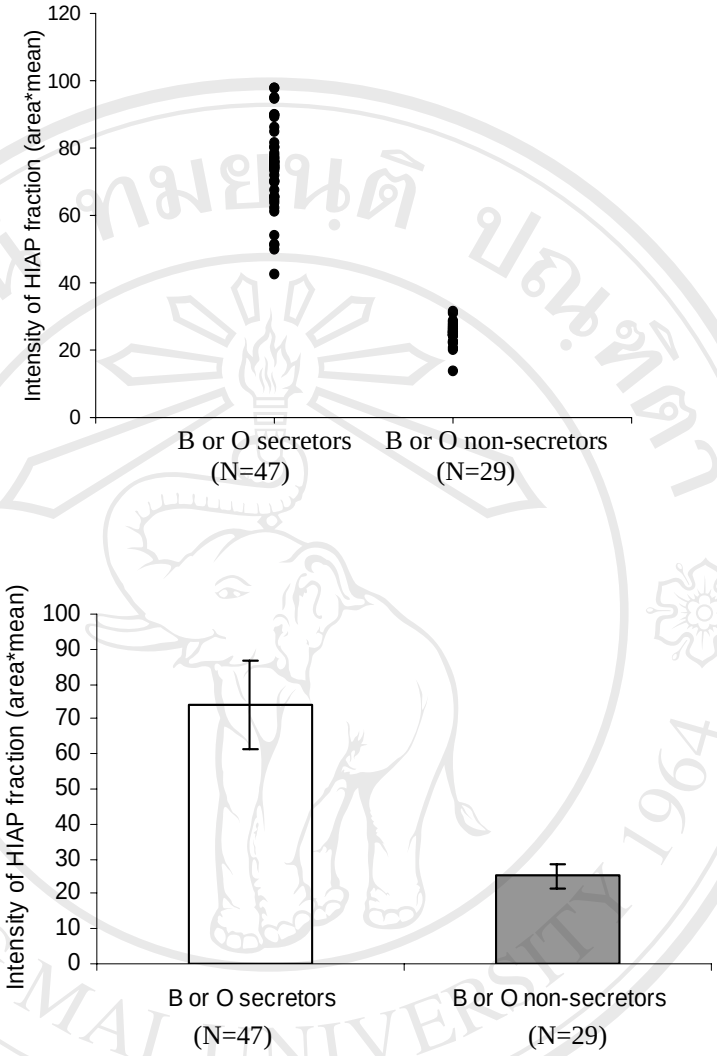

Figure 21. The intensity (density) of HIAP fractions in sera of B or O secretors and non-secretors.
Upper: The distribution in fractions of HIAP and Lower: The comparison of mean ± SD of HIAP fractions in sera of both groups of secretor status.

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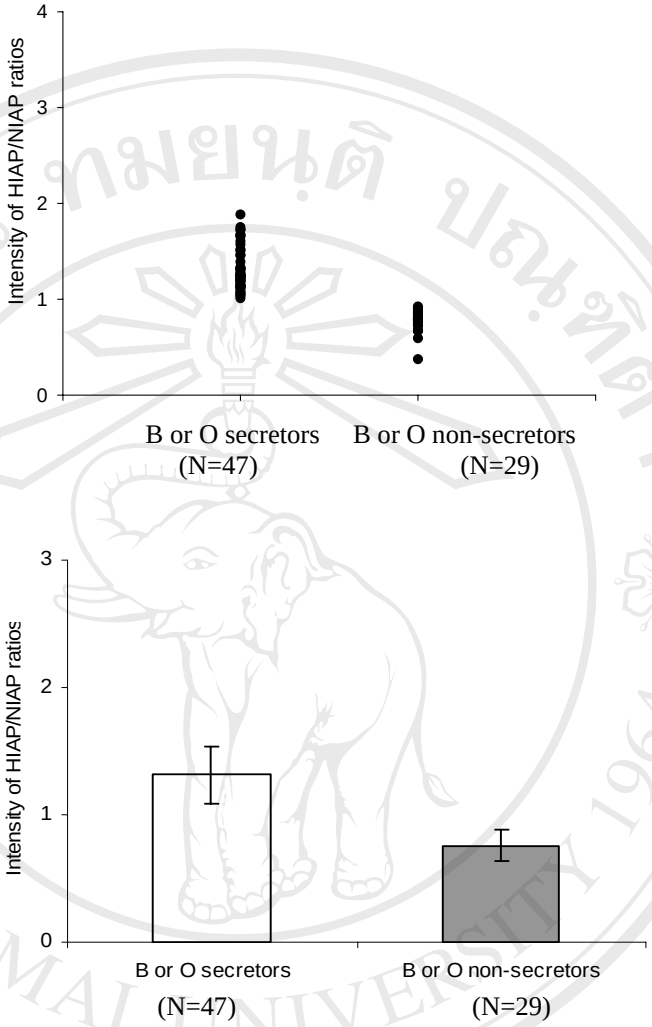

Figure 22. The intensity (density) of HIAP/NIAP ratios in sera of B or O secretors and non-secretors.
Upper: The distribution in fractions of HIAP/NIAP ratios
Lower: The comparison of mean $\pm$ SD of HIAP/NIAP ratios in sera of both groups of secretor status.

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Table 12 is the summary of the fractions of IAP isoenzyme detected in normal sera by Western blot analysis. The NIAP fractions in sera of B and O, or B or O secretors were significantly higher that of the non-secretors. The intensity of HIAP fractions in B and O, or B or O secretors was higher than their corresponding NIAP fractions. As similar as in NIAP fractions, the HIAP intensity fractions in B and O, or B or O secretors were higher and statistically different from those of non-secretors. These results confirmed that the activities of NIAP and HIAP in normal serum were dependent on ABO blood group and secretor status.

The ratios of HIAP/NIAP in different blood groups and secretor status were also shown in Table 11 and 12. The ratios were compared between those observed by the Western blot technique and by PAGE. The higher ratio could be seen in sera of B and O, B or O secretors by both techniques as compared with other blood group with no regard to secretor status. The ratios of HIAP/NIAP in B and O or B or O secretors determined by PAGE were greater than that found by using the Western blot technique.

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Table 11. The fractions of IAP isoenzyme in normal sera of B or O secretors and non-secretors.

Samples	NIAP fraction (area*mean) Mean ± SD	HIAP fraction (area*mean) Mean ± SD	HIAP/NIAP ratios Mean ± SD	HIAP/NIAP ratios by PAGE** Mean ± SD
B or O secretors (N=47)	57.3 ± 9.60	74.2 ± 12.57	1.3 ± 0.22*	3.0 ± 1.66
B or O non-secretors (N=29)	32.8 ± 5.42	25.1 ± 3.38	0.8 ± 0.12*	-

*$p < 0.0001$
**data calculated from values for preparing Table 10.

Table 12. Summary of the fractions of IAP isoenzyme detected in normal serum by the Western blot analysis.

Blood group	N (men)	total ALP activity (U/L) Mean ± SD	NIAP fraction (area*mean) Mean ± SD	HIAP fraction (area*mean) Mean ± SD	HIAP/NIAP ratios Mean ± SD	HIAP/NIAP ratios by PAGE* Mean ± SD
A secretors	5(2)	43.3 ± 14.81	35.5 ± 8.45	25.3 ± 8.49	0.8 ± 0.23	0.9 ± 0.96
A non-secretors	6(5)	33.7 ± 7.85	30.9 ± 5.16	24.1 ± 8.14	0.6 ± 0.19	-
AB secretors	-	-	-	-	-	-
AB non-secretors	2(0)	39.7 ± 0.85	31.2 ± 7.74	25.5 ± 7.73	0.6 ± 0.12	-
B secretors	16(5)	61.0 ± 24.28	53.5 ± 8.55	76.5 ± 8.58	1.4 ± 0.51	2.8 ± 1.53
B non-secretors	16(4)	53.3 ± 15.75	38.4 ± 8.78	24.5 ± 8.79	0.8 ± 0.13	-
O secretors	31(15)	72.6 ± 23.50	53.6 ± 10.15	76.4 ± 10.15	1.5 ± 0.87	3.5 ± 2.34
O non-secretors	13(4)	54.4 ± 11.50	36.8 ± 3.75	27.2 ± 3.75	0.8 ± 0.08	-
B or O secretors	47(20)	65.1 ± 24.06	57.3 ± 9.60	74.2 ± 12.57	1.3 ± 0.22	3.0 ± 1.66
B or O non-secretors	29(8)	53.8 ± 14.05	32.8 ± 5.42	25.1 ± 3.38	0.8 ± 0.12	-

*data calculated from values for preparing Table 10.

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