CHAPTER IV

I. DISCUSSION

IAP is present in the apical microvilli of brush border of the enterocytes (Hanna *et al.*, 1979). IAP involved in the regulation of lipid transport. The increase in IAP activity in human serum induced by a single fatty meal was reported since 1966 (Langman *et al.*, 1966). Accumulated evidence studied in rat has shown that IAP in the intestinal mucosa plays a key role in the transport of fatty acids and triglyceride from the intestinal tract to the circulation (Mahmood *et al.*, 1994). Mechanism was postulated that after oral fat intake, IAP stimulates production of unilamellar membrane that moves through the Golgi apparatus along with dietary lipid (Zhang *et al*., 1996). This membrane, called as surfactant-like particle, surrounds the TG-rich fat droplets that contain apo-B-48 and accompanies them as they transverse the epithelial cells (Mahmood *et al.*, 2003).

Result of blood group typing and ABH secretion status obtained in this study was not coincided with the criteria ratio of secretors to non-secretors (approximately 80:20) distributed in population (Domar *et al.*, 1991; Ognibene *et al.*, 1997; Van Hoof *et al.*, 1989). In general, most of research studies recommend the use of sampling method to obtained specimens (samples) which represented their population. In this study, firstly, the saliva specimens were randomly collected before the corresponding blood specimens of the same samples were obtained. It was then difficult to get such of those specimens in the suggested ratio. Since the non-secretor subjects were hardily found among the population, therefore this thesis study was designed to

concentrate on the number of samples in this group of study rather than the percentage of secretors and non-secretors.

Isoenzyme analysis has shown that a small amount of IAP is a component in normal sera of about 25% at non fasting stage. The presence of these isoenzymes are more probable in individuals of B or O blood groups who are secretors positive and the concentration of IAP in their serum increases in those individuals in whom this occurs (Moss, 1973; Matsushita *et al.*, 1998). The concentration of IAP in serum is increased in a variety of disease. Sometimes, it appears as the predominant isoenzymes. An increased incidence of isoenzymes has been reported in various diseases of the digestive tract (Dent *et al.*, 1968), and in other diseases, such as cirrhosis of the liver (Stolbach *et al.*, 1967; Domar *et al.*, 1988), chronic hemodialysis (Fishman *et al.*, 1965; Walker, 1974). However, the isoenzymes are not invariable present in serum in some particular conditions presumably partly because of the association with blood group status which has already been demonstrated and then preventing from diagnostic interpretation. Many researchs studied in cell line (Celano *et al.*, 1993) or in serum and tissues of colon cancer (Lemtragool, 2004) showed the increase in the activity of this isoenzyme. As described, in serum, there was an involvement of blood group and secretor status in interpretation of the increase of this isoenzyme in diseases therefore, in this study the normal level of this isoenzyme was investigated. The level observed showing the relationship with ABO blood group and secretor status at fasting stage.

In determination of serum ALP isoenzymes by electrophoretic method, total ALP activity value of each determination affects the relative calculation values of the isoenzyme or isoform migrated on electrophoretic field. There was a report suggested

that the best buffer used to determine total ALP activity in serum with different blood groups was diethanolamine (DEA) buffer. DEA buffer showed the lowest change of total ALP activities in normal serum before and after fatty meal ingestion in blood group B or O secretors (Matsushita *et al.*, 2002). Other buffers such as N-methyl-D-glucamine (MEG) and 2-ethylaminothanol (EAE) caused the increased change of total ALP activity in serum of B or O secretor after fatty meal of about 10 % of the reference ranges of the corresponding buffer. The change caused by using AMP buffer was slightly higher than that of DEA buffer (6.7% vs 4.8%) (Matsushita *et al.*, 2002). The change of total ALP activity after food intake (essentially for fatty meal) was closely related to the change of NIAP isoform fraction in serum in group B or O secretors and non-secretor (Matsushita *et al.*, 1998). In this study, although the AMP buffer was used for measuring total ALP activity in serum samples but it had no effect on the activity values because the specimens were all of fasting sera.

Results from this study indicated that total ALP activity (Mean $\pm$ SD) in serum of B or O secretor was significantly different from that of non-secretors but it was still within the reference range of the activity values analyzed by using AMP buffer at 37^{o}C (40-125 U/L, (Bower, 1975)). As shown in this study, results of PAGE and Western blotting concluded that the different of mean between both groups caused by low level of HIAP fraction in serum of B or O non-secretors.

The early study reported that age and sex affected the distribution of ALP isoenzymes including the IAP isoforms in serum. The method of fractionation of ALP isoenzymes in serum was the agarose gel electrophoresis (Van Hoof, 1989). The different of IAP isoforms with age and sex in normal sera of subjected with no information of blood group and secretor status was shown in both forms, the intestinal

IAP and the hydrophobic VAR IAP, however the data showed no significant difference. Results shown in this thesis was agreed with that work. This thesis study demonstrated that age (and sex) had no effect on total ALP, TIAP and NIAP activity at fasting and that the different lower in HIAP fractions of non-secretors than that of the secretors resulted in lower of total ALP activity (but within the reference range) in non-secretors serum. Moreover, by the PAGE method, the isoform which was normally detected in serum of B or O secretors and non-secretors was the NIAP which was not the VAR IAP as stated by Van Hoof *et al*. (1989). A report supported the prevalent distribution of IAP indicated that there were no components of intestinal variant ALP found in normal plasma (Griffiths *et al*., 1992). Since the 80% of population are B or O secretors, and in addition, the Mean ± SD of TIAP isoenzymes in B or O secretors and non-secretors observed in this study were more or less the same as determined by Matsushita (Matsushita *et al*., 2002), thus the Mean ± 2SD of total ALP and IAP activity determined in this study may be used as reference normal range of this isoenzymes in serum of Thai individuals.

In identification of the IAP isoenzyme and isoforms in electrophoretic field, the method utilized the resistant property of the IAP isoenzyme to digesting with neuraminidase enzyme (Jung *et al*., 1989) was used. In molecular level, the resistance or sensitive to neuraminidase digestion is concerned with that there are sialic acid sialylated on the carbohydrate side chains of the isoenzymes or not. Because of all IAP isoform molecules contain no sialic acid at the glycosylated ends; the resistance of this isoenzymes to neuraminidase treatment means no sialic acid to be cleaved by the enzyme. Most isoenzymes except for IAP are sensitive to neuraminidase, means that there are sialic acid presented on their molecules, therefore if the neuraminidase

enzyme presented, it would cleaved the sialic acid away which resulted in lossing of positive charge from the isoenzyme molecules and thus the migration of the enzyme on electrophoretic field will be retarded. Therefore, the isoenzyme which mobilities were not change after treating with neuraminidase was identified as the IAP isoenzyme (Bublitz *et al*., 2001).

This study used the PAGE method for distinguishing the IAP activity in serum from other source of activity by neuraminidase treatment. There are other methods such as L-phenylalanine inhibition of the intestinal enzyme activity (Fishman & Ghosh, 1967), levamisole inhibition of the other ALP sources (Van, 1976), cellulose acetate electrophoresis and other forms of zone electrophoresis (Fritache and Adams-Park), and isoelectric focusing (Griffiths, 1987). The method of PAGE is more sensitive than others because of the separated isoenzymes are activity staining (Chapman *et al.*, 1987). The technique were included the detergent Triton X-100 in the 6% gel in order to help dissociating the membrane bound tetramer from the soluble form which resulting in separation of NIAP from those of HIAP isoforms. The detergent micelles interact with the GPI anchor during electrophoresis, the inclusion of a charge detergent during electrophoresis will modify the electrophoretic mobility of anchor-bearing (hydrophobic) protein isoforms.

As in the results, the precision of IAP isoform by the PAGE method was very good but the mean of RCV is higher than that of the OCV which showed the change in accuracy by theory (Whitehead, 1976). The cause of change in accuracy was due to the staining color which was to be prepared freshly before used. The intensity of OCV isoenzyme bands were less stronger than those found in routine preparations which were precise as compared with the %CV of OCV.

In this study, although total ALP activity in blood group B and O secretors, and B and O non-secretors was not significant different, all data of IAP fractionations were shown in both separated results as B and O blood group and in combination into a group (as B or O blood group), respectively.

By PAGE method, NIAP and HIAP isoforms were detected in all serum of B or O and A secretors (no specimens for AB secretors), but in serum of B or O non-secretors and other blood groups only NIAP was present. These results were agreed with those observed by Matsushita (Matsushita *et al.*, 2002; Matsushita *et al.*, 1998). The mean of TALP, TIAP and HIAP isoform in serum of B or O secretors was higher than that found in other blood group. These results suggested that the different in TALP, TIAP in serum of secretors and non-secretors were closely related to the appearance of HIAP in circulation. Moreover the blood group type, B or O with secretor status affected the activities of both NIAP and HIAP in serum. This result was agreed with that found by Moss and Matsushita (Matsushita *et al.*, 1998; Moss, 1973). It was suggested by some worker that the HIAP forms only appeared in sera of Lewis blood group secretors [Le (a-b+)], and HIAP levels were dependent on ABO blood groups (D'Adamo & Kelly, 2001).

It was concluded by PAGE method that among the secretors, the highest activities of HIAP in fasting sera were observed in subjects with blood group O, and the lowest activities were associated with blood group A (N = 11), while NIAP was present in serum of secretors and non-secretors regardless of ABO blood group. Western blot analysis confirmed this observation. However, more results were obtained from Western blot analysis. The new observation of the presence of HIAP

isoforms in sera of B or O non-secretors but in a lower quantity than those observed in B or O secretors affected some data calculated from PAGE technique.

In blood group A secretor, although there is small sample when compared with group B or O but the result obtained was correlated with those found by Matsushita *et al*. and Nakano *et al.* (Matsushita *et al.*, 2002; Matsushita *et al.*, 1998; Nakano *et al.*, 2006). The lower IAP activity of group A secretors than that observed in sera of blood group B or O secretor was explained. In blood group A, the binding of IAP to erythrocytes would results in rapid elimination of circulating IAP from the plasma. Erythrocytes produce soluble ABO antigens, and it seems possible that these complexes may further enhance the glycosylation-mediated removal of IAP. Some study revealed the possibility of the process which might be regulated either by the type of blood group present on the erythrocytes or other plasma membrane surface, e.g. in the liver, or by the secretor status that regulates the appearance of soluble blood groups in body fluids (Bayer *et al*., 1980). Considering that ABO blood antigens on erythrocytes bind to IAP (Bayer *et al*., 1980; Komoda and Sakagishi, 1978), it seems likely that IAP is rapidly eliminated by erythrocytes from the plasma in non-secretors, whereas the abundant soluble ABO antigens preferentially bind to IAP and thereby prevents the elimination in secretors (Domar *et al*., 1991).

Scion Image (Scion Corporation, Maryland USA.) is a useful program and easy to use when compared with densitometer scanning. The reliability of using the program is depended upon individual or user who selected and cropped on the band area. In using the acquired images can be shading corrected and frame averaged.

By using Western blot analysis to confirm the results obtained from PAGE, it was found that there are three molecular sizes of IAP isoforms at 75, 135 and 250 kDa,

respectively. These results were different from that reported by Matsushita *et al.* (1998) who found two molecular mass of IAP isoforms (at 68 and 140 kDa).The molecular mass of adult intestinal reported by another worker was at 77 and 170 kDa (Vockley & Harris, 1984). Result from the latter observation obtained from the purified adult intestinal IAP isoenzyme run on a native SDS-PAGE. The 77 kDa IAP was proved to be a subunit of IAP and that of the 170 kDa was the dimeric isoform. The different in molecular mass of IAP isoforms in this thesis study from other reports may be caused by different in % gel and treating sample protocol before the samples were subjected to electrophoretic separation.

It can be concluded from this study that serum levels of IAP isoenzyme, an isoenzyme fraction of ALP which resistant to neuraminidase, were vary among blood group and secretor status. The appearance of IAP isoforms in serum was related to blood group and ABH secretion status. By PAGE method, the HIAP activity determined in serum of blood group B or O secretors at fasting was higher than that in blood group A secretor, while it could not be detected in blood group B or O non-secretors. Total IAP activity in serum of blood group B or O secretors at fasting was about 13.8 % of total ALP activity which was lower than that detected by Matsushita (19.4%) (Matsushita *et al.*, 2002). The Western blot technique was more sensitive than PAGE for it can detect the HIAP isoforms in serum of both B or O secretors and non-secretors. The ratios of HIAP/NIAP in serum of B or O secretors, determined by Western blot technique were greater than that observed in B or O non-secretor. Results of this study are useful for help diagnosis and interpretation of disease associated with blood group and secretor status (Blackwell, 1989), essentially for

colon cancer in the previous study (Lemtragool, 2004, and in Appendix D) performed in this laboratory.

II. CONCLUSION

1. TALP activity in serum of B or O secretors and non-secretors were within the reference range. The appearance of IAP isoforms in serum was related to blood group and ABH secretion. By PAGE method, the mean of TIAP activity in sera of B or O secretors was higher than that of non-secretors and other blood groups.
2. In determination of IAP isoenzyme by PAGE method, all components of IAP isoenzyme were affected by neuraminidase treatment. This characteristic, with no relation to blood group and secretor status, was used to identified IAP isoforms in serum specimens.
3. The mean reference value determined by PAGE of TIAP, NIAP and HIAP in sera of B or O secretors were 9.7 ± 4.64, 2.4 ± 0.92 and 6.8 ± 4.17 (Mean ± SD), respectively. The Western blot technique was more sensitive than PAGE method for it can detect the HIAP isoforms in sera of B or O non-secretors. The ratios of HIAP/NIAP in B or O secretors as compared with non-secretors were 1.3 ± 0.22 and 0.8 ± 0.12, respectively.
4. The mean reference values of TIAP, NIAP and HIAP determined by PAGE, and HIAP/NIAP ratio determined by Western blot analysis can be applied to use in diagnosing of colon cancer disease (Appendix D).