CHAPTER I

INTRODUCTION

I. INTRODUCTION

Alkaline phosphatase (ALP, orthophosphoric monoester phosphohydrolase (alkaline optimum; EC 3.1.3.1)) is a widely distributed and well known enzyme displaying a pH optimum in the alkaline range. It cleaves phosphate (orthophosphate (Pi)) groups from monophosphate ester substrates and exhibits phosphotransferase and protein phosphatase activity (McCarthy *et al.*, 1998). Under physiological conditions ALP can hydrolyze a large number of natural substrates including pyridoxal-5-phosphate, β-glycerophosphate, phosphoethanolamine and pyrophosphate (PPi) (Stinson *et al.*, 1986).

In mammals, four different isoenzymes of ALP are known: intestinal (IAP), placental (PLAP), placental-like (GCAP) and tissue non-specific (TNAP) (Trowsdale *et al.*, 1990). The latter ALP isoform, present in bone, liver and kidney, differs from the other isoforms only by posttranslational modifications (Haillot, 1992; Miura *et al.*, 1994). The placental-type ALP presumably occurred late in evolution; it has been found only in Hominidae (Doellgast & Benirschke, 1979).

Molecular Genetic and Cellular Expression of Alkaline Phosphatase Isoenzymes

The alkaline phosphatases comprise a multigene enzyme family that hydrolyze phosphate esters and are widely distributed in nature. Three main classes have been isolated from humans, the placental, intestinal, and liver/bone/kidney forms. Adult IAP is encoded by a locus on chromosome 2, band q34-q37, where are also located loci that encode alkaline phosphatase of the mature placenta and closely similar (placenta - like) germ cell alkaline phosphatase (Griffin *et al.*, 1987). The human

liver/bone/kidney alkaline phosphatase (TNAP) locus maps to human chromosome bands 1p36.1-p34 (Smith *et al.*, 1988). The close association of these three loci presumably reflects their common, comparatively recent, evolutionary ancestry. These last three genes and their products have correspondingly similar sequences of bases and amino acids. These similarities are particularly marked in the case of placental and germ-cell alkaline phosphatase, which are different by only 7-10 amino acid residues (98% homology), depending on the source. The sequence homology between the placental and IAP isoenzymes is 86.5%, and between IAP isoenzymes and tissue non specific alkaline phosphatases is 56.6% (Fishman, 1987).

Expression of IAP is normally almost entirely confined to enterocytes. However, further report has confirmed the expression of small but significant amounts of alkaline phosphatase with the characteristics of the IAP isoenzyme in kidney and shown it to be localized to the distal (S3) segment of the proximal tubule (Verpooten *et al.*, 1989), with potential benefits in the investigation of renal pathology. Trace expression of this isoenzyme has also been detected in other, non-intestinal tissue from a case of hypophosphatasia (Mueller *et al.*, 1983).

Structures of the Alkaline Phosphatase Isoenzymes

Alkaline phosphatase is the ectoenzyme attached to outer surfaces of cells by a COOH-terminal glycan-phosphatidyl-inositol (GPI) anchor (Figure 1). The primary structure of human alkaline phosphatase contain a sequence of 36 amino acids which was conformationally close to the active center and absent from the enzyme of *E. coli* (Hoylaerts *et al.*, 1992). Placental, germ-cell, and IAP show stereospecific, uncompetitive inhibition by compounds like L-phenylalanine.

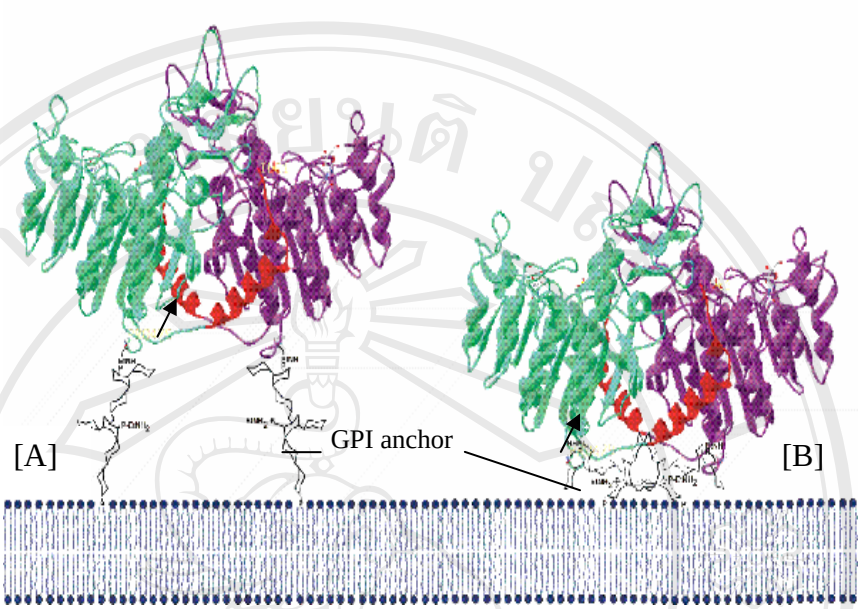


Figure 1. Models of association of dimeric GPI-anchored alkaline phosphatase with the bilayer surface. The structure of ALP (PLAP represented model) is shown in dimer. (A) The N-terminal α-helix and loop (residues 1-24) are pointed by arrows and the GPI anchor covalently linked to the C-terminus of each monomer is shown. The model of the crystal structure of the ALP dimer showing the two GPI anchors in a fully extended conformation ("lollipop model") with the protein moiety some distance from the membrane. (B) The protein portion may "flop down" onto the membrane causing it to be in direct contact with the bilayer surface, with the GPI anchor folded up underneath the protein. (Sharom Laboratory Department of Molecular and Cellular Biology Research GPI-anchored membrane proteins and lipid rafts.htm)

The action of these inhibitions depends on the residues located within this region (Hummer & Millan, 1991; Watanabe *et al.*, 1991). The particular, key role is played by the residue at portion 429 in determining patterns of inhibition (Hummer and Millan, 1991; Watanabe *et al.*, 1991). This position is occupied by glutamine in placental phosphatase, by serine in IAP, and by histidine in the less inhibited tissue-nonspecific alkaline phosphatase. While in germ-cell alkaline phosphatase, residue 429 is glycine (Hoylaerts & Millan, 1991). Since tissue-nonspecific alkaline phosphate was the product of a single structural gene, single-residue variations in primary structure cannot account for the small, tissue-specific differences in stability to heat that were among the first differential properties of these isoforms to be identified (Moss & King, 1962). The evidence from selective modification by glycosidase suggests that the differences in properties originate in differences in carbohydrate side chains (Moss & Whitaker, 1985). It is interesting to speculate whether these apparently tissue-specific differences in glycosylation are targeted specifically toward the enzyme and, therefore, by implication, have functional significance in particular cells, or whether they reflect differences in glycosylation patterns in those cells.

The structural differences between alkaline phosphatase isoenzymes were the built models of the tissue non-specific, intestinal and germ cell alkaline phosphatase molecules based on the 1.8A^{o} structure of placental alkaline phosphatse (McComb & Bowers, 1985) and performed a comparative structural analysis. The examination of monomer-monomer interface at this area was crucial for protein stability and enzymatic activity. The interface allows the formation of heterodimers among IAP, GCAP and PLAP but not between TNAP with any of three tissue-specific isoenzymes.

The active site cleft was mapped into three regions, i.e. the active site itself, the roof of the cleft, and the floor of the cleft. This analysis led to a structural fingerprint to the active site of each ALP isoenzymes that suggested a diversification in substrate specificity for this isoenzyme family (Le Du & Millan, 2002).

Post-translational Modification of Alkaline Phosphatase Isoenzymes

Oligosaccharides are the integral part of these ALP isoenzymes and greatly affect their physical properties and biological functions. The glycosylation pathway occurs in the cytosol, endoplasmic reticulum (ER) (Montreuil, 1980), and the Golgi complex (Figure 2). It also involves transport steps including processing glycosidase, and glycosyltransferase. Protein-bound oligosaccharides are classified according to the covalent linkage between amino acid and carbohydrate. The major linkage in avian and mammalian glycoproteins are the N-glycosidic linkage between Asn and GlcNAc and three types of O-glycosidic linkage, Ser (Thr)-GalNAc, Ser-xylose, and hydroxylysine-Gal (Kornfeld & Kornfeld, 1985). Most ALPs are well known as glycoprotein which glycosylation can alter their characteristics such as electrophoretic mobility and affinity precipitation with lectin (Lehmann, 1980).

Liver, bone and kidney isoforms have distinct oligosaccharide side chains. The liver and bone ALPs show both biantennary complex-type and hybrid-type which also show asparagine linked sugar chains with and without the internal fucose residue. The bone ALP posses many more hybride-type sugar chains than the liver ALP does. Both isoforms shows different sialylation at the glycosylation end (Koyama *et al.*, 1987).

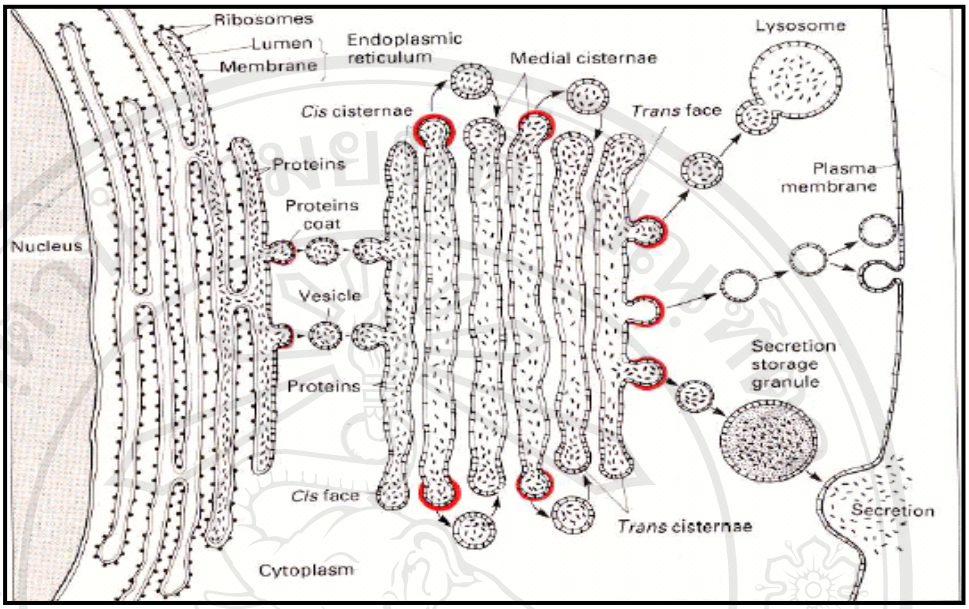


Figure 2. Protein secretory pathway as a model of targeting of ALP isoenzymes anchoring to the lipid bilayer membrane (or secreting the other proteins out of the cells as vaccuol).

The Gogi apparatus is highly specialized for a variety of functions, including protein glycosylation and subsequent transport of glycoprotein (e.g. ALP to their destination). (Campbell PN, Smit AD. Biochemistry Illustrated, 3rd Ed., Churchill Livingstone, NY., 1994, p. 253.)

The oligosaccharides of IAP is mainly a multi- and biantennary complex type (Tsumura *et al.*, 2001). The kidney also contains small amounts of IAP which exhibit a multiantennary complex type (unbound fraction) of carbohydrate side chain. Furthermore, it was reported that the oligosaccharide chains of the renal intestinal-type alkaline phosphatase were differrent from those of meconial and adult IAP as revealed by lectin affinity chromatography (Nishihara *et al.*, 1992). Therefore, the intestinal-type alkaline phosphatase can be heterogeneous. As comparison with calf IAP, the glycans linked to Asn249 are shown to have at least eight different, mainly non-fucosylated, biantennary or triantennary structures with a bisecting N-acetyl-glucosamine. The glycans linked to Asn 410 are a mixture of at least nine different, mainly tetraantennary, fucosylated structures with a bisecting N-acetylglucosamine. The majority of glycans are capped by alpha-galactose residues at their non-reducing terminal. In contrast to the glycans linked to other ALP isoenzymes, no sialylation was observed (Bublitz *et al.*, 2001).

The Isoforms of Intestinal Alkaline phosphatase Isoenzyme

Human intestinal alkaline phosphatase (IAP) can be released by the enterocyte into duodenal fluid as a mixture of three isoforms. A proportion of the enzyme is associated with triple-layered membrane vesicles (vesicular IAP). Although, occasionally, free hydrophilic IAP dimers are present, the remaining enzyme usually consists of a mixture of hydrophobic IAP dimers and more complex hydrophobic IAP structures of larger size, both entities being identified as "intestinal variant" alkaline phosphatase (VAR IAP). The hydrophobicity of VAR IAP stems exclusively from its attached glycosyl-phosphatidylinositol (GPI) anchor. Both vesicular IAP and VAR IAP are converted to hydrophilic enzyme upon removal of the GPI tail by

phospholipase D (PLD) present in duodenal fluid. The IAP released into the vascular bed consists mainly of VAR IAP; vesicular IAP is absent. The enzyme characteristics of VAR IAP partially purified from duodenal fluid and from serum are identical. In plasma, VAR IAP appears to associate with (lipo) protein complexes and is thus protected from further degradation by plasma PLD. Such complex formation may explain why, in the serum of a healthy reference population, VAR IAP was more abundant than hydrophilic dimeric IAP (Jing *et al.*, 1992). Biochemical analysis of human IAP revealed multiple forms, differing carbohydrate content, electrophoretic mobility, and kinetic parameters. In addition, it has been shown that in human serum, IAP can circulate as hydrophilic or hydrophobic isoforms, or as a mixture of both. Aside from their different electrophoretic mobilities and hydrophobicities, both of these IAP isoforms have identical enzymatic characteristics and comparable affinities for monoclonal antibodies to IAP, which then the hydrophobic IAP isoform was designed as "intestinal variant" ALP (VAR IAP) (Van Hoof *et al*., 1988; Van Hoof *et al*., 1989; Van Hoof *et al*., 1990). VAR IAP is shown more abundant in serum samples from a healthy reference population than hydrophilic IAP. More important, serum samples that were positive for IAP contained VAR IAP exclusively (with no hydrophilic IAP), whereas in small sample population was hydrophilic IAP not accompanied by VAR IAP (Van Hoof *et al*., 1990). Because of the relative abundance of VAR IAP in human serum, a biochemical and morphological analysis both of hydrophilic and hydrophobic IAP isoforms were investigated. In an attempt to increase of the mechanism of release by the enterocyte and of vascular uptake, hydrophilic IAP and VAR IAP were isolated from serum and from duodenal fluid and their biochemical properties compared. The results are compatible with a mechanism

by which human IAP is apically released as vesicular IAP and VAR IAP, after which it can be slowly degraded further by phospholipase D (PLD). No evidence was found for the basolateral release of vesicular IAP, despite the abundance in serum of VAR IAP, which after gaining access to the circulation, is protected from further degradation by PLD (Jing *et al.*, 1992).

Physiological Rule of Intestinal Alkaline Phosphatase

IAP involved in the regulation of lipid transport. The increase in IAP activity in human serum was induced by a single fatty meal (Langman *et al.*, 1966). The magnitude of this response is dependent on fatty acid chain length (Glickman *et al.*, 1970). The postprandial rise in serum IAP activity was significantly greater after following a long-chain fatty acid meal than a medium-chain fatty acid meal in healthy humans under the physiological conditions (Day *et al.*, 1992). The correlation was found between lipid concentration and IAP activity in human lymph, which supported the speculation that IAP might be involved in lipid transport (Keiding, 1964).

Factors Affecting the Appearance of Intestinal Alkaline Phosphatase Isoenzyme in Serum

Ages and sexes have no effect on the distribution of IAP in serum. It has been reported that small quantities of IAP were present in serum. At fasting state, normal activity of IAP in normal serum was approximately 25%. There are no significant differences for these isoenzymes, between age groups and between sexes. The IAP activity rises following the ingestion of a fatty meal. The presence of IAP is more frequent in sera of healthy individuals with blood group B and O who are secretors (Bamford *et al.*, 1965; Langman *et al.*, 1966; Langman *et al.*, 1968.)

Intestinal Alkaline Phosphatase in Serum of Normal and Diseases

IAP is present in the apical microvilli of brush border of the enterocytes (Hanna *et al.*, 1979). Isoenzyme analysis has shown that a small amount of IAP is a component in normal sera of about 25%. 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 (Matsushita *et al.*, 1998; Moss, 1973). The high molecular mass intestinal alkaline phosphatase (HIAP) and normal molecular mass intestinal alkaline phosphatase (NIAP) were found in sera of normal individuals at fasting and after fatty meals. HIAP only appeared in sera of Lewis blood group secretors [Le (a-b+)], and HIAP levels were dependent on ABO blood groups. Among the secretors, the highest activities of HIAP in fasting sera were observed in subjects with blood group O and B and the lowest activities were associated with blood group A, while the HIAP activities were not changed after fatty meals. In contrast, NIAP was present in the serum of both secretors and non-secretors regardless ABO blood group. Trace amounts of NIAP were the remaining ratios of NIAP activity in fasting serum; however serum NIAP activities rose sharply after fatty meal. The remaining ratios of NIAP activity at fasting and 9 hr after fatty meals of secretors were approximately the same as those of non-secretors (Matsushita *et al.*, 1998). Large amounts of IAP entered the blood circulation of all individuals by the thoracic lymph (Keiding, 1964). The persistence in small amounts in the plasma of some individuals and absence in the others suggests the difference in the rate at which the isoenzymes are removed from the blood circulation. IAP is greater bound by erythrocytes of group A while it is bound to a lesser degree by those of group B or O (Bayer *et al.*, 1980). Blood

group antigens are present in the IAP molecules, as shown by the ability of individual preparations of the isoenzymes to react with anti-blood group antisera (Komoda & Sakagishi, 1978; Komoda *et al.*, 1981).

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 (Domar *et al*.,1988; Kreisher *et al*., 1965; Stolbach *et al*., 1967; 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 that the measurements of IAP isoenzyme in serum seem to be little or no use in monitoring intestinal disease. Nevertheless, the recognition of the possible presence of IAP is important for correct determination of the origin of hyperphosphatasemia (Moss, 1982).

In diseases, an increased prevalence of IAP isoenzyme was observed in fasting sera of diabetic patients (Giffiths & Black, 1987; Tibi et al., 1988) with no difference between types 1 and 2 diabetes mellitus (Tibi *et al.*, 1988). Within the diabetics and the non-diabetic control group, IAP activity was significantly higher in B, O secretors than A secretors or ABO non-secretors. There was no difference in IAP activity between type 1 and type 2 diabetics but the diabetics had a significantly higher activity of this isoenzyme than the corresponding blood group/secretor status category of the control group (Tibi *et al*., 1988).

Evidences of increase in IAP activity in colon cancer cells have been shown. The molecular events that regulate differentiation of human colon mucosal cells are

not known. Although a number of *in vitro* models to study this question exist, none have identified a gene product which could function as a mediator of cell differentiation. Although the Ki-ras gene is frequently mutated in human colon cancer, the Ha-ras protooncogene is maximally expressed in the most differentiated cells of intestinal mucosa. In order to study the effects of Ha-ras gene overexpression on the differentiation phenotype in human colon cancer cells, they have expressed the v-rasH oncogene in CaCO2 cells. This maneuver resulted in a marked induction of gene expression of multiple markers characteristic of intestinal brush border differentiation. These include a greater or an equal 30-fold induction of sucrase, a 10-fold increase in IAP, a 20-fold induction of transforming growth factor alpha, and a 5-fold increase in transforming growth factor beta 1 steady-state mRNA levels. Finally, the CaCO2-ras cells undergo a greater or an equal 95% reduction in DNA synthesis under serum-deficient conditions and cannot be restimulated after such treatment, suggesting terminal differentiation, whereas the same treatment has no effect on the proliferative capacity of the parent CaCO2 cell line. These studies with CaCO2 human colon cancer cells provide a model to study the role of v-rasH and related genes in colon epithelial differentiation (Celano *et al.*, 1993).

Another study which demonstrated the cellular expression of ALP isoenzymes was observed in LoVo, a continuous cell line derived from a human colon cancer cells. The LoVo cell produces two alkaline phosphatases; the heat-labile, L-homoarginine-insensitive, intestinal form, characteristic of its tissue of origin and the heat-stable, term-placental form, ectopically produced by a variety of tumors. Under basal conditions the activity levels of both enzymes are similar. Hyperosmolality and sodium butyrate induce increased levels of activity of the two alkaline phosphatases in

a disparate fashion; whereas hyperosmolality augments the activity of both to the same extent, the effect of butyrate is more pronounced on the activity of the IAP isoenzyme. When the two inducers are combined, induction of term-placental alkaline phosphatase is additive and that of the IAP isoenzyme is synergistic. The effect of hyperosmolality is blocked by cycloheximide, and induction by sodium butyrate is inhibited by thymidine, cordycepin and cycloheximide. The known alkaline phosphatase inducer, prednisolone, has no effect on the enzymes of LoVo cells. These results suggest that in these tumor cells the activity levels of the closely homologous term-placental and IAP appear to be independently controlled (Herz & Halwer, 1989).

Separation of Alkaline Phosphatase Isoenzyme from Tissues

Alkaline phosphatase is the ectoenzyme attached to outer surfaces of cells by a COOH-terminal glycan-phosphatidyl-inositol (GPI) anchor. The action of *n*-butanol in solubilizing the enzyme from cell membrane of all tissues, is at acid pH (e.g. from placenta and other different forms including intestine and colon tissues). The extraction which is effective at alkaline pH is now seen to be due to the cleavage of the GPI anchor with the activation of inositol-specific phospholipase in acid condition; the hydrophobic anchor is retained during extraction at alkaline pH (Roche & Sarles, 1954).

Determination of Intestinal Alkaline Phosphatase Isoenzymes by Electrophoretic Method

Human IAP isoenzyme is completely resistant to the digestion by neuraminidase (sialidase; EC 3.2.2.18) as detected by cellulose acetate electrophoresis whereas the other forms usually found in human sera are converted by this treatment

to variants that migrate more cathodically on cellulose acetate eletrophoresis (Komoda *et al.*, 1981). One component of enzyme extracted from intestinal mucosa was converted by neuraminidase (W. C. Griffiths *et al.*, 1985), although this variant was a minor fraction of the small intestinal mixture, it was predominant in the colon (W.C. Griffiths *et al.*, 1992). The ALPs extracted from ilium was different from those of duodenum due to N-terminal amino acid sequence but they are similar in anti-genically and nearly identical electrophoretic mobility (Bowers & McComb, 1975).

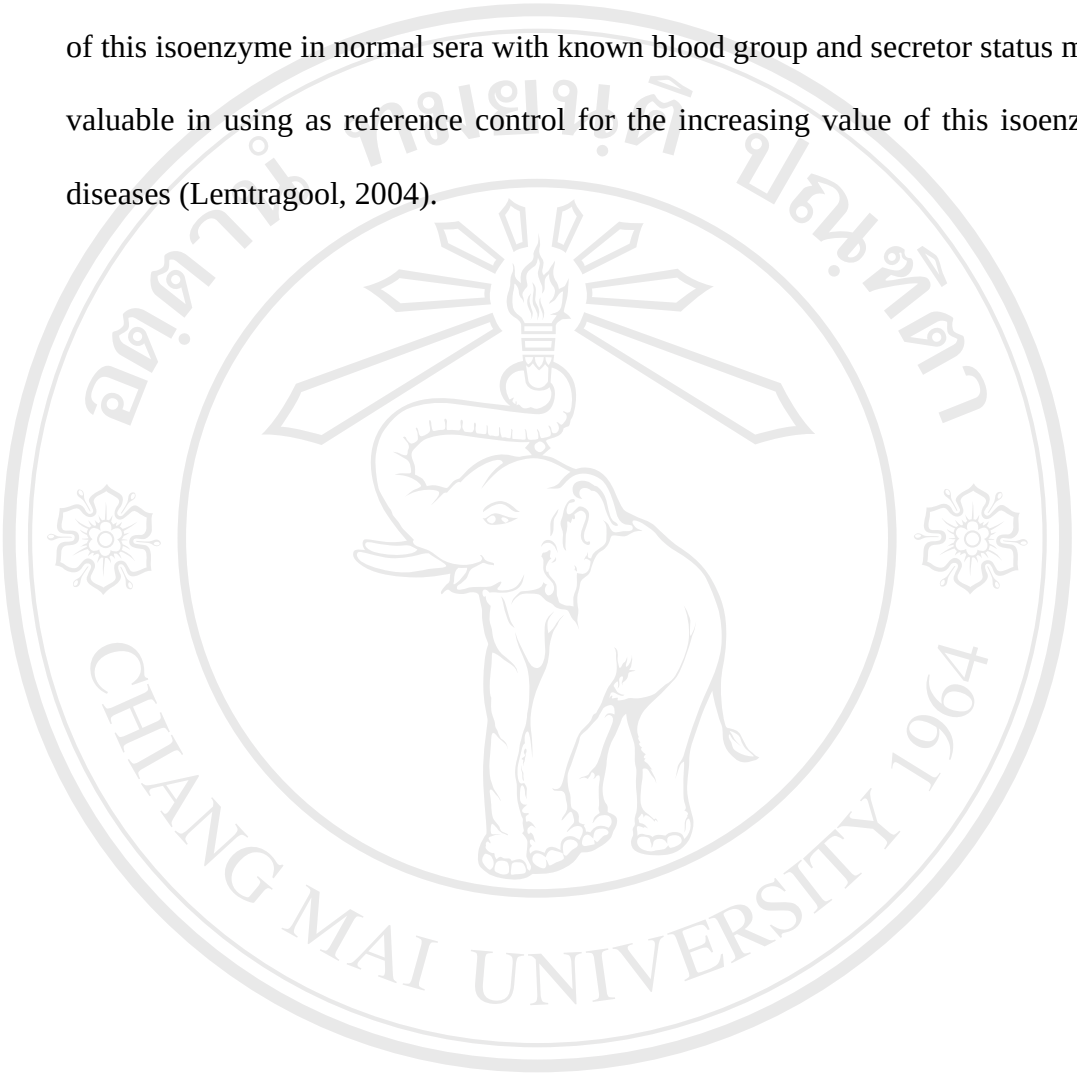
Method for Determination of Total ALP Activity in Serum Affected the Intestinal Alkaline Phosphatase Activity Fractions

Different buffers used in methods for determination of total ALP affected the activity of individaul ALP isoenzyme in serum. Tissue non-specific ALP and IAP are activated differently by respective buffers. It is shown that in comparison of four transphosphorylating accepter buffers (2-amino-2-methyl-1-propanol,AMP; *N*-methyl -D-glucamine, MEG; diethanolamine, DEA; and 2-ethylaminoethanol, EAE), which used to determine total ALP activity in serum, the DEA method showed a lower different result between the reference range of ALP activities for blood groups B or O. DEA method may be a better procedure as a routine assay of ALP activity than 2-Amino-2- methyl propanol (AMP), MEG and EAE methods (Matsushita *et al.*, 2002).

Advantageousness of Determination of Intestinal Alkaline Phosphatase Activity and Intestinal Alkaline Phosphatase Isoforms in Normal Serum

Total IAP activity and isoforms in serum can be determined by using a combination of two methods. Firstly total activity of ALP is measured and following by separation of the ALP isoenzymes by electrophoretic method. After activity staining, the isoforms of fractionated IAP are able to be calculated from their

percentages of total ALP activity. Although the activities and isoforms of IAP in serum are dependent on blood groups and fatty meal intake but the level and isoforms of this isoenzyme in normal sera with known blood group and secretor status might be valuable in using as reference control for the increasing value of this isoenzyme in diseases (Lemtragool, 2004).



II. LITERATURE REVIEW

ABH Secretor Status

The term "ABH secretor," as used in blood banking, refers to secretion of ABO blood group antigens in fluids such as saliva, sweat, tears, semen, and serum. A person who is an ABH secretor will secrete antigens according to their blood group; for example, a group O individual will secrete H antigen, a group A individual will secrete A and H antigens, etc. Soluble (secreted) antigens are called substances. One of the primary differences in physiology between secretors and non-secretors involves qualitative and quantitative differences in components of their saliva, mucus, and other bodily secretions. ABH secretion is controlled by two alleles, *Se* and *se*. *Se* is dominant and *se* is recessive (or amorphic). As a general rule, about 20% of the populations are non-secretors (with the remaining 80% being secretors) (D'Adamo & Kelly, 2001).

In genetics of the secretor system, a person can be classified as either a secretor (Se) or a non-secretor (se). This is completely independent of whether they are a blood type A, B, AB, or O. This means that someone can be an A secretor or an A non-secretor, a B secretor, or B non-secretor etc.

Metabolic Differences between Secretors and Non-secretors

Similar to the ABO blood types, it appears additional genetic information must be linked to the secretor gene, because predictable trends in non-blood type aspects of physiology have a close association with secretor/non-secretor status. Aspects of physiology such as the relative activity of an enzyme called IAP; propensities toward clotting, reliability of some tumor markers, and generalized performance of the immune system have predictable trends depending upon the secretor status

(http://www.right4cu.us/secretor.html).

Relationship of Serum IAP Isoenzyme with ABO Blood Group and Secretor Status

By the use of sensitive immunocatalytic assays, based on isoenzyme specific monoclonal antibodies, the activities of the three main human alkaline phosphatases were determined in serum. The activity of IAP was found to be strongly correlated with ABO blood groups and secretor phenotypes, while neither the placenta alkaline phosphatase activity nor the tissue unspecific alkaline phosphatase activity demonstrated any dependence on blood groups or secretor phenotypes (Domar *et al.*, 1991). Non-secretors, independent of ABO blood groups, demonstrated low activities of IAP in serum, amounting to approximately 20% of the activities in the secretor groups (Domar *et al.*, 1991; Ognibene *et al.*, 1997; Van Hoof *et al.*, 1989). Within the secretor group, the lowest activities were observed for blood group A (2.8 ± 1.1 IU/l; mean ± SEM) and the highest for blood groups B and O (14.1 ± 1.1 IU/l and 19.0 ± 2.5 IU/l, respectively). These results confirm that the activities of IAP in serum have to be related to both ABO blood groups and secretor phenotypes in order to be informative in clinical contexts (Domar *et al.*, 1991).

In the determination of reference intervals for serum total ALP activity and isoenzymes, it was demonstrated that the reference intervals were dependent on the ABO blood groups. Serum total ALP activity and isoenzymes (ALP-IZ: high ALP-IZ: high molecular-, liver-, bone- and intestinal-types) in individuals were determined in 200 healthy subjects aged 20-39 years. ALP activity was determined according to the JSCC method. ALP-IZ was stained by the formazan method after isolation by TITAN III - Lipo plate electrophoresis. For the electrophoresis, treated serum with

neuraminidase and untreated one were concomitantly used for detecting liver ALP and bone AP respectively. As a result of comparison of mean ALP-IZ activity among the ABO blood groups, total ALP, IAP and liver ALP activities in the type B and O individuals were significantly higher than in the type A and AB individuals. It is well known that the activities of total ALP and IAP in type B and O individuals are higher than in type A and AB individuals, but there have been no reports showing that the activity of liver AP in type B and O individuals is higher than in type A and AB individuals. Furthermore, in the type B and O individuals there was a low correlation ($r = 0.195$, $p < 0.05$) between the activities of liver ALP and IAP (Nakata & Tozawa, 1995).

In the determination of IAP isoforms (HIAP & NIAP) in serum by using four different buffers (2-amino-2-methyl-1-propanol, AMP; N-methyl-D-glucamine, MEG; diethanolamine, DEA and 2-ethylaminoethanol, EAE) in measuring total ALP activity, the experiment classified 80 healthy subjects into two groups of blood group B or O secretors ($n = 36$) and other blood groups ($n = 44$). Results of the study showed that the mean ALP activities at fasting in blood group B or O secretors from AMP, MEG, DEA and EAE methods were 15.5%,24.0%, 11.0% and 22.1% higher than those in other blood groups, respectively. The reference ranges of ALP activity at fasting with the AMP method in blood group B or O secretors and other blood groups were 63.5 $\pm$ 17.4 U/l (mean $\pm$S.D.) and 55.0 $\pm$14.5 U/l (mean $\pm$S.D.), respectively. The difference between the reference ranges of ALP activity in blood group B or O secretors and other blood groups was statistically significant ($p < 0.01$). HIAP and NIAP in serum at fasting only appeared in blood group B or O secretors, and the activities of HIAP and NIAP were 4.7 ± 3.4 U/l (mean ± S.D.) and 2.2 ± 1.2

U/l (mean ± S.D.), respectively. The activity of ALP (HIAP ± NIAP) in blood group B or O secretors was 56.6 ± 15.1 U/l (mean ± S.D.), and this reference range was approximately the same as the ALP activity (55.0 ± 14.5 U/l) of other blood groups. The same results were observed with MEG, DEA and EAE methods. It was concluded from these results that the differences in ALP activity in blood group B or O secretors and other blood groups were closely related to the HIAP and NIAP levels (Matsushita *et al.*, 2002).

The study of IAP isoforms showed the dependent of the molecular size of the isoenzyme on the secretor status. The differences in IAP are almost exclusively related to one fraction of this enzyme. Normal molecular mass intestinal alkaline phosphatase (NIAP) is present in the serum of both secretors and non-secretors, regardless of ABO blood group. However, the high molecular mass intestinal alkaline phosphatase (HIAP) only appears in serum of Le (a-b+) blood group secretors. For Lewis blood-group system, a group of dominantly and independently (secretor) inherited antigens associated with the ABO blood factors. The antigens are glycolipids present in plasma and secretions that may adhere to the erythrocytes. The phenotype Le (b+) is the result of the interaction of the Le gene Le (a+) with the genes for the ABO blood groups. There are only three phenotypes: Le (a-b-); Le (a+b-); and Le (a-b+). Lewis phenotypes may change during pregnancy. Examples of Le (a+b+) are only transient. Lewis antibodies are only found in Le (a-b-) individuals, and are almost entirely IgM. They are the only blood group antibodies which have never been implicated in HDN (hemolytic disease of the newborn) (D'Adamo & Kelly, 2001).

Food Intake Affected the Increase of IAP Activity in Serum in Relation to Blood Group and Secretor Status

The separation of ALP isoenzymes by electrophoretic technique showed that after normal meals, the band of activity becomes much stronger in blood group O and B secretors; in blood group A secretors a weaker though distinct band appears; while in non-secretors little or none is detectable. Fat ingestion seems to stimulate the appearance of the IAP in the serum. In blood group O or B secretors a synthetic breakfast of protein and carbohydrate did not seem to alter the serum ALP characteristics, but the substitution isocalorically of fat for part of protein and carbohydrate resulted in a notable increase in serum ALP activity. These findings agree well with independent observations showing that the administration of fat, but not protein or carbohydrate, increases the ALP content of human thoracic-duct lymph. Further study of IAP should, however, increase them understanding of the relation between blood groups and alimentary function, and perhaps give us some idea of the physiological role of one form of ALP (Hodson, 1962).

Concentration of the IAP is lowest in the serum during fasting and rises after ingestion of fat, reaching a peak at about seven to eight hours. This increase is most marked in group O and B secretors, but it is detectable in most people. The concentration of IAP in human thoracic-duct lymph rises after a fatty meal, and presumably most of the IAP enters the blood by way of the lymphatic system. In a measurement of the ALP concentration in the mucosa of the human small intestine, one experiment found no correlation between ALP levels and ABO groups or secretor type, but the other observed that group O and B secretors had the highest mean concentration of ALP, group A secretors had the next highest concentration, and non-

secretors had the lowest amount; in that study, however, there was a marked overlap between the three groups in the range of enzyme activity, and the differences observed were much smaller than those found in serum. In view of these data it seems likely that the ABO and secretor genes influence the rate at which the IAP enters the blood, or its catabolism, rather than its synthesis in the intestine. (http://www.dadamo.com/wiki/wiki.pl/Intestinal_Alkaline_Phosphatase_(IAP)

Disease Susceptibility among Secretors and Non-secretors

(http://www.right4eu.us/secretor.html)

In digestive system, as a general rule, a higher intensity of oral disease is found among non-secretors. This includes dysplasia (precancerous changes to the tissue) and an increase in cavities. Statistically speaking, blood type A secretors has the lowest number of cavities. Non-secretors also tend to have more digestive problems. Several studies have indicated that non-secretors have a significantly higher rate of duodenal and peptic ulcers. Non-secretors are also less resistant to infection by *Helicobacter pylori* (a microbe associated with ulcers). It appears that this organism can colonize more readily and generate more inflammation in individuals incapable of secreting their blood type into the digestive tract. Non-secretors are at an increased risk for development of celiac disease (up to 48% of patients with celiac disease have been reported to be non-secretors).

In the respiratory system, with regards to aspects of lung function, being a non-secretor takes its usual place as a health disadvantage. Several researchers have suggested that being a non-secretor might predispose an individual to damaging effects, while being a secretor might add a degree of protection against harmful environmental assaults to the lungs. Among coal miners, asthma was significantly

related to non-secretor phenotype. Secretors also appear to receive a degree of protection against some of the deleterious effects of cigarette smoking. Evidence suggests that the ability to secrete ABO blood type antigens might decrease the risk of chronic obstructive pulmonary disease (COPD). Being a non-secretor also offers a slight increase risk for having a problem with habitual snoring.

Non-secretors appear to have an increase in the prevalence of a variety of autoimmune diseases including ankylosing spondylitis, reactive arthritis, psoriatic arthropathy, Sjogren's syndrome, multiple sclerosis, and Grave's disease.

In diabetes, heart disease & metabolic syndrome X, the non-secretors are at a greater risk of developing diabetes (especially adult onset diabetes); and they might be at a greater risk of developing complications from diabetes. Data allows the conclusion that non-secretors are a risk factor for myocardial infarction and heart disease (note: this is particularly true for men).

Several different researchers have noted a connection between a metabolic syndrome called "Syndrome X" and non-secretor blood types. Syndrome X is a clustering of metabolic problems comprised of insulin resistance (your cells do not respond effectively to the insulin that you create), elevated plasma glucose (high blood sugar), lipid regulation problems (elevated triglycerides, increased small low-density lipoproteins, and decreased high-density lipoproteins), high blood pressure, a prothrombic state (tendency to clotting), and obesity (especially central obesity or a predisposition to gaining weight in the abdomen).

In metabolic disorders seem to interact to promote the development of diabetes (adult onset type II), atherosclerosis, and cardiovascular disease. And while insulin resistance might lie at the heart of the problem, all of these metabolic disorders

appear to contribute to health problems.

Alcoholism has been associated with the non-secretor blood type. On the positive side, alcohol consumption appears to exert a protective effect on lung function and to lower the risk of heart disease more in non-secretors than in secretors. The key principle with the use of alcohol is for non-secretors (and everybody actually) is moderation.

Antibody levels of the secretors are known to have higher levels of IgG and IgA antibodies. The lack of IgA antibodies perhaps explains the link between non-secretor status and an increased frequency of heart valve problems secondary to bacteria infection. Because IgA functions protect an organ from invasion, most if not all non-secretors have problems with gut permeability ("leakygut").

Serum Intestinal Alkaline Phosphatase and Blood Group Associated Diseases

A natural question is whether the blood group associations noted in disease could be more readily explained by relationships with the serum isoenzyme patterns of ALP. For instance, it was not clear that the presence of the slow-moving intestinal component in blood group A patients correlated with liability to gastric cancer. An association between peptic ulcer and the presence of the IAP isoenzyme seems unlikely, because the disease is common in individuals of blood group O but not in those of blood group B. But simple comparisons of serum isoenzyme patterns in healthy individuals and patients with gastric cancer are impossible, for the presence or absence of the IAP isoenzyme in the blood seems to depend greatly on diet. H. HARRIS and his colleagues have shown that in normal individuals after an overnight fast the slow-moving component is present in considerably reduced amounts and can be clearly detected only in blood group O and B secretors (Blornstrand & Werner,

1965; Keiding, 1964; Laemmeli, 1970).

IAP, an isoenzyme manufactured in the small intestine, has the primary function of splitting cholesterol and long chain fatty acids. IAP is seen normally in the serum of subjects who have B or O blood types, especially after a fatty meal. Pathologically the band obtained by electrophoretic method may be present in perforation of the bowel, ulcerative disease of the intestine and faintly in liver cirrhosis. Acute infarction of the intestine will cause a release of IAP from the mucosa.

Large erosive or ulcerative lesions of the stomach, duodenum or other small intestinal areas, or colon may result in an elevation of the serum ALP level. The small intestinal lesions associated with malabsorption are associated with an elevation of the serum IAP level only if there is an erosive or ulcerative mucosal lesion.

The prevalence and characteristics of IAP were identified in human serum. Separation of IAP by cellulose acetate electrophoresis in fasting serum samples of 8% of hospital patients (n = 500) and in 35% of fasting serum samples from patients with diabetes mellitus (n = 106) elucidated no different between the hospital and the DM patients. Furthermore, there is no different observed between types 1 and 2 DM patients (W.C. Griffiths *et al.*, 1992).

The characteristics of the IAP electrophoretic band were found heterogeneous and contained two major subtypes of ALP. Isoelectric focusing of IAP positive serum treated with levamisole and neuraminidase (EC 3.2.1.18) revealed two distinct regions of enzymatic activity that co-migrated with ALP extracted from small intestinal and colonic mucosa. Anodic IAP was resistant to treatment with levamisole and neuraminidase and co-migrated with ALP from small intestinal mucosa. The more-

cathodic IAP, which co-migrated with ALP from colonic mucosa, was completely inhibited by levamisole and converted by neuraminidase to a species with a more basic pI than that of neuraminidase-digested tissue-nonspecific form. This component of IAP was proposed to be the isoform of the vascular origin (W.C. Griffiths *et al.*, 1992). The descriptions by Aird and colleagues (1954) of the associations of blood group O with peptic ulcer and of blood group A with gastric cancer have been confirmed by others (Clarke *et al.*, 1955; Roberts, 1959); and the observations have been extended to cover associations of blood group A with pernicious anemia, and with salivary tumors (Cameron, 1958). Moreover, inability to secrete ABH blood group substances in the gastrointestinal mucus (a genetically determined characteristic) has been shown to be associated with peptic ulcer (Doll *et al.*, 1961; Glynn *et al.*, 1957) and possibly with gastric cancer and pernicious anemia (Callender *et al.*, 1957; Doll *et al.*, 1961). The causes of these associations are not known. But a simple protective action of the ABO (H) blood group substances in the mucus seems an unlikely explanation, because the total titer of blood group substance is the same for ABH secretors as for non-secretors (in non-secretors Lewis substance is substituted for the A, B, and H mucopolysaccharides). Furthermore a blood group effect on peptic ulcer can be shown in non-secretors alone. But the large quantities of blood group mucopolysaccharides found in the gastrointestinal mucosa suggest that they have some function (Glynn *et al.*, 1957; Szulman, 1960; Szulman, 1962). Since the prevalence of both pernicious anemia and gastric cancer is higher in individuals of blood group A, and duodenal ulcer in those of blood group O, a hypothesis relating blood group effects to acid secretion naturally followed. There was a report confirmed that acid output tended to be greater in blood group O than in blood group A subjects

(Sievers, 1959), but the observed differences, which were in elderly subjects, could well have been due to gastric atrophy (which would, by analogy with pernicious anemia, probably be more frequent and severe in blood group A than in blood group O individuals). Studies in younger healthy subjects have given conflicting results. In one series (Niederman *et al.*, 1962) gastric secretory potential, as measured by serum-pepsinogen levels, differed little between blood groups O and A; acid output was higher in blood group O subjects. In another study (Hanley, 1964) however, gastric acid output was found to be higher in individuals of blood group O than in those of blood group A and slightly but not significantly greater in non-secretors than secretors. Another approach has been to study possible associations between ulcer symptoms and prognosis and the ABO blood group and secretor characteristics. (Aird *et al.*, 1954)

Although earlier work was inconclusive, Langman and colleagues (1967) found a higher frequency of blood group O among patients with bleeding gastric or duodenal ulcers than among those with non-bleeding ulcers. Non-secretors seemed more liable to need operation for ulcer, though blood group did not seem to have any striking effect on likelihood of operation, nor did secretor status seem to influence the likelihood of hemorrhage. The association of blood group O with hemorrhage has been confirmed by others (Merikas *et al.*, 1966) and the strength of the association of blood group O with liability to ulcer rather than with bleeding clearly must be reassessed. All surveys of blood groups in ulcer patients so far reported have been largely retrospective, and since the individuals for whom blood group data are readily available are those with bleeding ulcer, a strong bias in favor of blood group O is introduced. The same kind of error may well be present in data collected on secretor

status, and an unselected consecutive ulcer series will be required to solve these problems.

In the previous study of the biochemical characterization of IAP in serum and tissues of normal and colon carcinoma, it was shown that there were two isoforms of IAP presented in serum of normal and patient sera, the NIAP and HIAP (Lemtragool, 2004). Both isoforms were similar in biochemical characterization (no sialylation) and properties (phenylalanine resistance, levamisol sensitive). As shown by the Western blot analysis, the IAP isoforms in both normal and tumor tissue extracts were high molecular mass HIAP isoforms with molecular size approximately 205 & 225 kDa. In serum of colon cancer, 3 isoforms of HIAP were elucidated , one at 116 kDa and the other two were with nearly the same molecular sizes as found in paired normal and tumor tissues i.e. at 205 and 225 kDa, respectively. Because of blood group and secretor status of serum specimens of normal and patients were unfortunately unidentified, therefore the increase of IAP isoforms in serum was in doubt of the influence of blood group and secretor status (ALP in most tissues is independent on blood group). This thesis try to clarify that problem by determining the level of total IAP isoenzyme and isoforms in normal serum with known blood group and secretor status. The advantage of this study will be valuable in diagnosis of colon carcinoma.

III. OBJECTIVES

1. To investigate the total ALP and IAP isoform activity in normal serum in relation to ABO blood group and secretor status
2. To differentiate the carbohydrate side chain of IAP isoforms in normal serum which may be influenced by ABO blood group and secretor status
3. To estimate the average values (Mean ± SD) of IAP isoforms in normal serum of B and O blood groups in relation with ABH secretion status