CHAPTER 4

I. Discussion

Colon cancer is a very common cancer in USA. It is second only to lung cancer. The strongest risk factor for colon cancer is age. The incidence rates rise from 10 per 100,000 at age 40-45 to 300 per 100,000 at age 75-80. The cumulative life time risk for the disease is 1 in 20. Men are more likely to develop colon cancer than women. In Thailand, the incidence rates are arisen in female rather than male (National Cancer Institute, 2002). The factor which linked to an increase change of developing colon cancer appears to be associated with a diet that is low in fiber and high in calories, protein and fat, especially in red meat. In addition, the life styles of living and alcohol consumption have been implicated as potential risk factors (Deerasamee *et al* , 1999). People with a family history of colon cancer, familial polyposis and those bowel disease, particularly ulcerative colitis and to a lesser extent Crohn's disease are at the high risk. The symptoms are referred to the bowel, with anemia due to blood loss, or with evidence of spread and the most commonly metastasis to liver (Wang *et al* , 1994).

In the USA, colonoscopy technique has been used to screen for colon cancer disease but it is expensive as compared with other modality such as fecal occult blood test (Vijan *et al*, 2001). There are several diagnostic tests for colorectal or colon cancer screening which frequently used in clinical laboratory such as serum carcinoembryonic antigen (CEA) ,serum lipid-associated sialic acid (LASA), serum cancer antigen (CA) 19-9 DNA flow cytometric ploidy (DNA index), DNA flow cytometric proliferation index (% S phase), p53 Tumor suppressor gene *ras* oncogene (National guideline clearing house, 2000). Intestinal alkaline phosphatase (IAP) which was reported to be raised in colon cancer by some literatures (Herz *et al*, 1989 ; Matsumoto *et al* , 1990 ; Celano *et al*, 1993) seem to be of little or no use in diagnosing or monitoring intestinal diseases (Domar *et al*, 1988) because this isoenzyme can be found in normal serum of blood group B and O secretors (Beckman, 1964 ; Komoda *et al*, 1981 ; Domar *et al* , 1991 ; Matsushita *et al* , 1998). This study tried to re-evaluate the different forms of IAP isoenzyme at fasting state which was increased in serum of colon cancer and to compare with those found in normal serum with no regard to blood group secretor status.

Measurement of ALP activity is routinely used for laboratory diagnosis of liver and skeletal diseases. Liver and bone isoenzymes have been recognized as a suitable markers of hepato-biliary , and of skeletal disorders among clinicians(Fishman, 1990 ; Van Hoof *et al* , 1990). ALP originating from intestine mucosa can be released into duodenal fluid as a mixture of three isoforms, free hydrophilic dimers hydrophobic dimers and more complex hydrophobic structure of larger size entitled " the intestinal variant"(Deng *et al*, 1992). It is interesting that in colon carcer what form of this isoenzyme was predominated in serum and how it was correlated with that found in tumor tissue.

In this study, total ALP activity was determined by using PNPP substrate in diethanolamine(DEA) buffer at pH 9.2. The buffer gave a lower result difference between the reference range of ALP activities for blood groups B and O secretors and other blood groups (Matsushita *et al*, 2002). The precision of measurements of enzyme activity in this study were accepted by the criteria of WHO (Whitehead, 1976). Since normal serum contained at least 3 different ALP isoenzymes(LAP, BLP and IAP), partial purification of the isoenzymes in serum was needed in order to get rid of the non interested ALP isoforms , such as liver ALP, high molecular mass ALP (fast liver ALP) and other ALP isoforms before observing the enzyme properties.

Fractionation of IAP on DEAE –Sephacel column yielded 3 protein peaks containing 4 or 5 peak activities of ALP. As identified by agarose gel electrophoresis, it was found that small amount of LAP(and BAP) co-eluted with IAP in the first protein peak. The IAP isoenzyme, eluted in the first protein peak of DEAE-Sephacel, was reported to be the normal molecular mass ALP (NIAP) and that eluted in the third protein peak was the high molecular mass IAP (HIAP) (Kaeoyot, 2003,see Appendix II). In this study the NIAP isoform was used to evaluate the enzyme properties.

In separation of IAP isoforms in serum samples on DEAE- Sephacel column, the elution of IAP could be found in all fractions of separated peaks (identified by agarose gel electrophoresis) excepted for the isoenzyme which was eluted in the first protein peak (pooled activity fractions of 1-4). Apart from agarose gel electrophoresis, L-phenylalanine and levamisole inhibition tests were also used to identify the type of isoenzyme isolated in the first

protein peak. The IAP isoform, which was more inhibited by L-phenylalanine, will have to be further examined for its physico-chemical properties.

Heat inactivation of the ALP isoenzymes performed in this study was carried out at 1 hr inactivation time, although it was normally performed at 56°C for 10 min in clinical chemistry laboratory. Farly et al. (Farley *et al*, 1993) assessed the heat inhibition of ALP isoforms at 52°C for 30 min. Generally this technique is used to differentiate bone and liver isoform in serum samples. However, in this study it was used to distinguish the IAP isoform from other ALP isoenzymes in serum sample and LAP standard. The purpose of heat inactivation study was to show the more resistant property of the IAP isoform to heat than other ALP isoform (LAP) in serum samples. Since the isolated enzyme had low activity after separating on DEAE-Sepharose column, therefore the reduction of activity caused by heat inactivation must be measured carefully (although the quality control was shown acceptable). The decision of heat stability of the IAP isoenzyme was made at two different heat inactivation temperatures, 52 °C and 65°C for 1 hr respectively. The % remaining activity of IAP isoenzyme in blood group B normal serum, patient sera, normal and tumor tissue enzyme extracts, after heat treatment at two temperatures, were in agreement with that observed in the IAP standard.

In the study of enzyme properties, serum sample was allowed to react with MoAb specific to IAP, the reactivity of the enzyme to an anti-IAP Ab was recognized for all IAP isoenzymes containing in serum and tumor tissue extracts with positively inhibited by L-phenylalanine. There were two bands precipitated with the MoAb, the band migrated slower than the liver ALP to the anode and the band with high molecular mass migrated at the application point on agarose gel electrophoresis. The detection of high molecular mass isoform of IAP was confirmed by the separation on 7.5 % PAGE containing Triton X-100. The high molecular isoform reacted specifically with anti - IAP Ab and the complex of enzyme and MoAb could not enter the gel. These results demonstrated that there was at least two IAP isoforms presented in serum of normal, serum and tissue extracts of patients with colon cancer. These finding is consistent with one previous report that there were two IAP isoforms presented in normal serum bearing blood group secretor and non- secretor (Matsushita *et al* , 1998).

Glycosylation patterns of the IAP isoforms in normal, patient sera and colon cancer tissues were examined. The internal sugars of the carbohydrate side chains of the IAP molecule

were characterized by using lectin precipitation technique and Con A affinity colum chromatography (Farley *et al*, 1993). WGA precipitation test gave similar pattern of precipitating the IAP isoenzyme in patient serum samples as compared with that in tumor tissue extract and the liver ALP isoform which predominated largely in serum of normal and liver disease. These result suggested that the common internal sugars of the carbohydrate side chains of ALP isoenzymes may be biantennary complex type with the N -linked asparagine, rich with N-actylglucosamine and low containing mannose and fucose sugar (Cummings *et al*, 1982). Varied amounts of ALP in normal and patient sera and enzyme in tumor tissue extracts of patients precipitated with Con A. The enzyme in normal tissue extract of colon cancer patient showed the same pattern of low precipitation with Con A as liver ALP standard and liver ALP in liver disease serum. Low precipitation with Con A may be caused by the large molecular size of ALP which was attached with a piece of plasma membrane and could not be precipitated with Con A or bound to the Con A column. In this *in vitro* study, the large molecular size of ALP molecule was reduced by digestion with PI-PLC and the forms of enzyme obtained were able to bound to the Con A column. It was reported by Moss (Moss, 1977) that , the way to reduce the molecular size of ALP could be accomplished by two enzymes which are abundant in serum, Phospholipase D (PL-D) and PI- PLC. Both enzymes function to digest the phospholipid plasma membrane from the enzyme molecules *in vivo*. From results, it was demonstrated that the % relative activity of normal serum of blood group A digested with PI-PLC was different from that of blood group B (containing IAP isoform) with known secretor status. The % relative activity observed in serum of patients with colon cancer digested with PI-PLC was agreed with that observed in blood group B normal serum. Surprisingly tumor tissue enzyme extract digested with PI-PLC showed different elution pattern (of % relative activity) from that observed in its corresponding serum elution pattern. The increase in % relative activity of IAP in all ConA elution fractions of tumor tissue extract demonstrated the hetergeneities of the internal sugar of the IAP isoforms. The type of glycosylation of the IAP in the tumor tissue enzyme extract was suggested to be the N-linked Asn which rich in mannose sugar. In this experiment, it could be suggested that the butanol used to extract the enzyme from membrane (see method of preparation ALP from tissue) may also involved in determining the forms of

enzyme detected in ConA column separation (Moss, 1997) and molecular weight determination by Western blot analysis (Matsushita *et al*, 1998).

Determination of sialylation of the carbohydrate side chian was also performed to characterize the glycosylated end of the ALP isoenzymes (Griffiths et al, 1992). Neuraminidase from different sources of microorganisms had no effect on mobility of IAP isoenzyme standard, IAP of blood group B normal and colon cancer serum (all were NIAP obtained from the first protein peak of DEAE-Sephacel column chromatography) as detected by agarose gel electrophoresis. These results suggested that no sialylation at the glycosylated end of these IAP isoenzyme molecules. The contrast result was observed in tissue IAP isoenzyme separated by PAGE using boric acid buffer containing Triton X-100 for separation. The presence of only HIAP isoform was detected in both normal and tumor tissue enzyme extracts. These HIAP isoform was affected with the digestion with neuraminidase. More over,by the PAGE technique, it could be seen that one component of the NIAP band of IAP standard and IAP in patient serum was affected by neuraminidasedigestion. Therefore, it could be concluded that in patient serum there must be more NIAP isoforms , one is IAP (NIAP) with no sialylation and the other one with sialylation. It was reported by Deng *et al.* (Deng *et al,* 1992) that the IAP isoform normally presented in serum of normal with or without secretor status was originated in small intestine which known as the " intestinal variant " or NIAP. This isoform was resistant to neuraminidase treatment. The IAP isoforms with high molecular mass or HIAP comes from the colon. This form was sialylation and sensitive to neuraminidase treatment (Griffiths *et al,* 1992).

Blood group and secretor status influenced the appearance of IAP isoforms in healthy serum (Komoda et al, 1981). In this study, although NIAP bands obtained from agarose gel had not been scanned to determined the relative IAP activity (because it contained more than one component at the position of separation, therefore it could not be scanned and calculated correctly), however it could be evaluated qualitatively by visualization. The presences of NIAP bands on agarose gel electrophoresis were observed in 13 out of 32 normal serum samples whereas both NIAP and HIAP were found only in one of normal serum sample. In the condition that blood group secretor status was unable to use in interpretation, it was observed that most of the NIAP bands were visualized in 7 blood group B and 4 blood group O with

only 2 for blood group A subjects. Matsushita *et al.*, (Matsushita et al, 1998) examined the IAP isoforms by Triton -PAGE technique (6.0% acrylamide gel concentration) following by calculation the percentages of their activities from the scanning compared with its total activity and found that at fasting condition, total ALP, HIAP, NIAP activities determined by DEA method were 133.9 ± 37.2, 7.0 ± 5.1 and 3.4 ± 1.8 U/L ($\bar{X}$ ± SD) respectively. In addition, it was demonstrated that the HIAP isoform was chiefly present in blood group B and O secretors. A large amount of NIAP is secreted into circulation in blood group B or O secretor (Matsushita *et al*, 2002) and was increased after fatty meal ingestion.

In this study, although the sample size of patient was very small (datails of patient shown in Appendix III) , the results obtained were promising. Total ALP activity in patient serum (N= 5, mean ± S.D = 270.1 ± 165.29 U/L) was significantly different from that of normal serum (N=32, mean ± S.D = 183.87 ± 50.33 U/L (mean ± S.D.). Only 3 serum samples from patients demonstrated increased in total ALP activity but as observed by agarose gel electrophoresis, the IAP isoforms could be seen in all patient samples. The separated IAP isoforms demonstrated diffused HIAP bands at the application area , with the varied amount of NIAP isoforms co-migrated with liver (or bone) ALP to the anode. It seems that the HIAP bands were the main IAP isoforms in colon cancer sera. The percentages of HIAP and NIAP were not calculated because the NIAP band was usually comigrated with liver-bone isoforms on the agarose gel. Moreover from result of this study , it was shown that there may be two component of NIAP isoforms migrated at the same mobility and one component was sialylated.

Molecular weight determination of IAPs, separated by SDS-PAGE and analysed by Western Blotanalysis found in this study, were different from that determined by Matsushita et al. (Matsushita *et al,* 1998). The molecular weight of NIAP which separated at 68 kDa could not be detected in all samples performed in this experiment. It may be due to the antigenicity of the anti-IAP Ab was nonspecific to rect with this isoform of IAP. The molecular mass of HIAPs found in normal and patient sera and in both normal and tumor tissue extracts of colon cancer patients were heterogeneous. They were still differed from that reported by Matsuchita *et al* (140 kDa) (Matsuchita *et al,* 1998). One that is close to that previous report was 116 kDa which could not detected in both normal and tumor tissue enzyme extracts of patients.

The activity of IAP in serum of BO secretors is also increased in cirrhosis (Komoda *et al,* 1981 ; Farley *et al,* 1993), chronic renal failure (Skillen *et al,* 1972 and Pfleiderer *et al,* 1980) and in fasting serum of diabetic patients (Tibi *et al,* 1988; Griffiths *et al,* 1985). Although the measurements of IAP in serum was suggested to be little or no use in diagnosing or monitoring intestinal diseases (Okochi *et al,* 1987), the properties and characteristics observed in this study showed correlation of the IAP isoforms detected in serum and patient tissues. The high molecular weight (HIAP) and the NIAP which sensitive to neuraminiadse treatment were the proposed isoforms which can be applied to use as a diagnostic tool for detecting the colon cancer in clinical laboratory. The technique useful for differentiating these isoforms is neuraminidase treated sample separated on 7.5 % PAGE in the presence of Triton X-100

II. Summary

1. There were two isoforms of IAP presented in normal and patient sera, NIAP and HIAP. The NIAP isoforms showed at least two components with reacted differentially with neuraminidase. One component was neuraminidase resistant (no sialylation) and the other component was neuramindase sensitive. Both isoforms were similar in biochemical characterization and properties.

2. The IAP in both normal and tumor tissue extracts characterized by having high molecular mass were sensitive to neuraminidase treatment. Only HIAP isoforms with molecular size approximately 205 & 225 kDa were found in normal and colon cancer tissues. In serum of colon cancer, 3 isoforms of HIAP with the same molecular sizes of 116, 205 and 225 kDa were elucidated by Western blot analysis.

3. Using the information described above, the technique for differentiating these IAP isoforms in serum of colon cancer disease could be proposed. By using 7.5% PAGE in the presence of Triton X-100, the neuraminidase sensitive NIAP and HIAP isoforms in patient serum could be visualized and that was useful for interpreting colon cancer condition.