

TABLE OF CONTENTS

| | PAGE |
|--|--------|
| ACKNOWLEDGEMENT | I |
| ABSTRACT | II |
| TABLE OF CONTENTS | VII |
| LIST OF TABLES | XI |
| LIST OF FIGURES | XII |
| ABBREVIATIONS | XIV |
| I. INTRODUCTION | 1 |
| A. Statement of problem | 1 |
| B. Objectives of this study | 4 |
| II. LITERATURE REVIEWS | 5 |
| A. Basic knowledge on Alkaline phosphatase | 5 |
| B. Physiological role of ALP | 10 |
| C. Clinical significance | 11 |
| D. Clinical useful of serum ALP measurement | 12 |
| E. Multiple forms of ALP | 13 |
| F. Measurement of ALP activity and ALP isoenzymes | 15 |
| III. MATERIALS AND METHODS | 19 |
| A. Materials | 19 |
| 1. Specimens | 19 |
| 2. Instruments | 19 |
| 3. Chemical and Reagents | 20 |
| B. Methods | 22 |
| Part I. Methods for evaluation of serum specimens for pathological conditions | 22 |
| [1.] Determination of liver function tests | 22 |
| [2.] Methods for alkaline phosphatase activity determination | 22 |
| 2.1 Standard method measured with Shimudzu UV 160 A | 22 |
| 2.2 Total ALP activity measured with the microplate reader | 23 |

VIII

| | |
|---|----|
| 2.3 Quality control of total ALP activity determination | 24 |
| [3.] Methods for determinations of physicochemical properties of ALP isoenzymes in serum | 24 |
| 3.1 Studies of the chemical inhibitors of the ALP isoenzymes in serum | 24 |
| 3.2 Heat inactivation of ALP isoenzymes in serum | 25 |
| Part II. Methods for preliminary screening of types of ALP isoenzymes composed in serum samples | 25 |
| [1.] WGA precipitation of bone ALP isoenzyme | 25 |
| [2.] Screening of ALP isoenzymes in serum by cellulose acetate (CA) electrophoresis | 26 |
| Part III. Methods for characterization of sugar chains on the fractionated ALP isoenzymes molecules | 26 |
| [1.] Fractionation of ALP isoenzymes by anion exchange chromatography | 27 |
| [2.] Determination of protein in ALP isoenzyme fractions | 28 |
| [3.] Identification of fractionated ALP isoenzyme | 29 |
| 3.1 Identification of protein peaks of the separated ALP isoenzyme fractions | 29 |
| 3.2 Electrophoresis on agarose gel ; proof for purity | 29 |
| 3.3 Identification of fraction 1 and Fraction 2 ALP isoenzymes by heat inactivation | 30 |
| [4.] Lectin precipitation of sugar moieties of fractionated ALP isoenzymes | 30 |
| [5.] Study of sialic acid linked to the sugar chain moieties | 31 |
| 5.1 Preliminary studies of asialo-ALP isoenzyme preparation in serum | 31 |
| 5.1.1 Preparation of asialo-ALP isoenzymes in serum | 31 |
| 5.1.2 Heat inactivation of neuraminidase activity in the asialo-ALP isoenzymes | 31 |
| 5.1.3 Measurement of total sialic acid (TSA) in serum | 31 |
| 5.2 Sialylation of ALP isoenzyme fractions | 32 |
| 5.2.1 Optimization of neuraminidase (sialidase) on ALP isoenzyme fractions | 32 |
| 5.2.2 Preparation of asialo-ALP fraction | 33 |

| | |
|---|----|
| 5.2.3 Sialylation of asialo-ALP isoenzymes by α 2,6- sialyltransferase | 33 |
| Part IV. Application of carbohydrate heterogeneity of ALP molecules for separation of serum ALP isoenzymes in serum by CA electrophoresis | 34 |
| Part V. Statistical analysis | 34 |
| IV. RESULTS | 35 |
| Part I. Evaluation of serum specimens for pathological conditions | 35 |
| [1.] Routine laboratory data in healthy subjects and patients with liver disease | 35 |
| [2.] Analytical precision of ALP activity determination | 36 |
| [3.] Physicochemical properties of ALP isoenzymes in serum | 36 |
| Part II. Preliminary screening of types of ALP isoenzymes composed in serum samples | 37 |
| [1.] Quantification of bone ALP in healthy and patient sera by WGA precipitation technique | 37 |
| [2.] Patterns of ALP isoenzymes in serum using cellulose acetate electrophoresis | 37 |
| Part III. Characterization of sugar chain on the fractionated ALP isoenzyme molecules | 45 |
| [1.] Fractionation of ALP isoenzymes by anion exchange chromatography | 45 |
| [2.] Identification of fractionated ALP isoenzymes | 45 |
| [3.] Lectin precipitation of sugar moieties of the fractionated ALP isoenzymes | 52 |
| [4.] Study of sialic acid linked to the sugar chain moieties | 57 |
| 4.1 Evaluation of preliminary studies | 57 |
| 4.2 Sialylation of ALP isoenzyme fraction | 60 |
| 4.2.1 Optimization of neuraminidase concentration on the digestion of ALP isoenzyme fractions | 60 |

X

| | |
|--|----|
| 4.2.2 The digesting effect of different sources of neuraminidase on ALP isoenzyme fractions | 60 |
| 4.2.3 Electrophoretic patterns α 2,6- sialyltransferase treated asialo- liver ALPs | 60 |
| Part IV. Application of sialic acid linked- properties of the ALP molecules on the separation of ALP isoenzymes in serum by CA electrophoresis | 67 |
| V. DISCUSSION | 69 |
| VI. SUMMARY | 76 |
| VII. REFERENCES | 77 |
| VIII. APPENDIX | 86 |
| IX. CURRICULUM VITAE | 92 |

LIST OF TABLES

| TABLE | PAGE |
|---|------|
| 1. Liver function test finding in sera of healthy subjects and total patients with liver disease | 39 |
| 2. Comparison of liver function tests in sera of patients with differential diagnosis of liver disease | 40 |
| 3. Analytical performances of the measurements of ALP activities in two different instruments | 41 |
| 4. Quantitative measured of bone ALP isoenzyme in sera and patients and healthy subjects | 44 |
| 5. Effect of lectins on precipitation fraction 1, liver ALP isoenzyme, separated from sera of patients and healthy subjects | 54 |
| 6. Effect of lectins on precipitation of fraction 2, bone ALP isoenzyme, separated from sera of patients and healthy subjects | 55 |
| 7. Effect of lectins on precipitation of fraction 4, fast liver ALP isoenzyme, separated from sera of patients and healthy subjects | 56 |

LIST OF FIGURES

| FIGURE | PAGE |
|---|------|
| 1. Evolution of the ALP gene family | 6 |
| 2. Ribbon drawing of a monomer of ALP | 7 |
| 3. Schematic representation of the various forms of ALP | 9 |
| 4. Ion-exchange column chromatography separation of ALP-isoenzyme | 28 |
| 5. The quality control chart showing intra-and inter-assay precision of ALP values in a microplate EL 340 | 42 |
| 6. Sensitivities of ALP Activities to chemical inhibitors | 43 |
| 7. Elution profiles of ALP separated from sera of liver disease patients and healthy control by DEAE Sepharose anion exchange chromatography | 47 |
| 8. Elution profiles of ALP separated from control, normal and hepatoma sera by DEAE Sepharose anion exchange chromatography | 48 |
| 9. Agarose gel electrophoresis of ALP isoenzyme fractionated from hepatoma serum | 49 |
| 10. Agarose gel electrophoresis of column-fractionated ALP isoenzyme | 50 |
| 11. Heat inactivation of fraction 1 and 2 ALP isoenzymes activities | 51 |
| 12. Effect of heat on ALP isoenzymes incubated with neuraminidase at different lengths of time | 58 |
| 13. Heat inactivation of neuraminidase activity (measured by the releasing of TSA product) | 59 |
| 14. Effect of various concentrations of C-Neu and comparison effect of C-Neu and V-neu at the concentration 125 mU/L on the fractionated ALP isoenzymes | 62 |
| 15. Agarose gel electrophoretic patterns of C-Neu and V-Neu desialylation of fractionated liver ALP isoenzymes from healthy subjects and patients | 63 |

XIII

| | |
|---|----|
| 16 a. Agarose gel electrophoretic patterns of liver ALP isoenzyme treated with α 2,6- ST in healthy subject as compared with cirrhosis and CHCA patients | 64 |
| 16 b. Agarose gel electrophoretic patterns of ALP isoenzyme treated with α 2,6- ST in patients with hepatoma | 65 |
| 16 b. Agarose gel electrophoretic patterns of liver ALP isoenzymes treated with α 2,6- ST in patients with cholestasis, hepatitis and hepatoma | 66 |
| 17. Densitometric scanning of ALP isoenzymes in serum of liver disease patients by CA electrophoresis | 68 |

ABBREVIATIONS

| | |
|--------|---------------------------------|
| ABS | absorbance |
| ALP | alkaline phosphatase |
| ALT | alanine aminotransferase |
| AST | aspartate aminotransferase |
| CA | cellulose acetate |
| CMP | cytidine- 5'- monophosphate |
| Con A | Concanavalin A |
| CV | coefficient of variation |
| Da | dalton |
| DBIL | direct bilirubin |
| DEAE | diethylaminoethyl |
| Fig | Figure |
| g | gram |
| GlcNAc | N-acetylglucosamine |
| GPI | glycan-phosphatidyl-inositol |
| h | hour |
| HCL | hydrochloride |
| L | litre |
| M | Molar |
| min | minute |
| mL | millilitre |
| mM | millimolar |
| mmol | millimole |
| Mr | Molecular weight |
| mU | milliunit |
| N | normal |
| NANA | neuraminic acid |
| NaOH | sodium hydroxide |
| nm | nanometer |
| OD | optical density |
| PSA | <i>Pisum sativum</i> agglutinin |
| r | correlation |
| SD | standard deviation |

XV

| | |
|--------------------|-----------------------|
| ST | sialyltransferase |
| TBIL | total bilirubin |
| TSA | total sialic acid |
| U | unit |
| WGA | wheat germ agglutinin |
| % | percent |
| α | alpha |
| $^{\circ}\text{C}$ | degree Celsius |
| μL | microlitre |