

## TABLE OF CONTENTS

| CONTENT  | PAGE |
|--|------|
| <b>ACKNOWLEDGEMENT</b>                         | iii  |
| <b>ABSTRACT (ENGLISH)</b>                      | v    |
| <b>ABSTRACT (THAI)</b>                         | viii |
| <b>LIST OF TABLES</b>                          | xv   |
| <b>LIST OF FIGURES</b>                         | xvi  |
| <b>ABBREVIATIONS</b>                           | xix  |
| <b>CHAPTER I INTRODUCTION</b>                  |      |
| 1.1. Statement and significance of the problem | 1    |
| 1.2. Literature reviews                        | 5    |
| 1.2.1. Structure and function of hemoglobin    | 5    |
| 1.2.2. Hemoglobin synthesis and oxygen binding | 8    |
| 1.2.3. Developmental change in hemoglobin      | 12   |
| 1.2.4. Thalassemia                             | 15   |
| 1.2.4.1. Introduction to thalassemia syndromes | 15   |
| 1.2.4.2. Alpha thalassemia                     | 17   |
| 1.2.4.3. Hemoglobin Bart's hydrops fetalis     | 22   |

|   |    |
|---|----|
| 1.2.4.4. Laboratory diagnosis of thalassemia                                | 25 |
| 1.2.4.5. Laboratory investigation for $\alpha$ -thalassemia                 | 26 |
| 1.2.5. Monoclonal antibody  | 28 |
| 1.2.5.1. Hybridoma technique  | 28 |
| 1.2.5.2. Thalassemia diagnosis by immunological method                      | 33 |
| 1.3. Objectives   | 34 |
| <br><b>CHAPTER II MATERIALS AND METHODS</b>                                 |    |
| 2.1. Materials  | 35 |
| 2.1.1. Human blood samples  | 35 |
| 2.1.2. Animal blood samples   | 35 |
| 2.1.3. Mouse  | 35 |
| 2.1.4. Cell fusions   | 36 |
| 2.1.5. Chemicals  | 36 |
| 2.2. Methods  | 36 |
| 2.2.1. Preparation of hemolysates for monoclonal antibody<br>production     | 36 |
| 2.2.2. Purification of hemoglobins  | 37 |
| 2.2.2.1. Purification of hemoglobin Bart's and Hb Portland                  | 37 |
| 2.2.2.2. Purification of hemoglobin A, A <sub>2</sub> , E and F             | 38 |
| 2.2.3. Measurement of isolated hemoglobin concentration                     | 39 |
| 2.2.4. Identification of hemoglobin by cellulose acetate<br>electrophoresis | 40 |

|  |    |
|--|----|
| 2.2.5. Production of monoclonal antibodies   | 40 |
| 2.2.5.1. Mouse immunization with Hb Bart's hydrops<br>fetalis hemolysate   | 40 |
| 2.2.5.2. Mouse immunization with purified hemoglobin<br>Portland   | 41 |
| 2.2.5.3. Indirect ELISA for detection of polyclonal<br>antibodies against hemoglobins                            | 41 |
| 2.2.5.4. Hybridoma technique   | 42 |
| 2.2.5.5. Single cell cloning by limiting dilution  | 43 |
| 2.2.6. Isotyping of monoclonal antibodies  | 44 |
| 2.2.7. Study of the specificity of monoclonal antibodies<br>with different hemolysates                           | 44 |
| 2.2.8. Study of the specificity of monoclonal antibodies<br>with purified hemoglobins                            | 45 |
| 2.2.9. Study of the cross reactivity of monoclonal antibodies<br>between human and other animal hemoglobins      | 45 |
| 2.2.10. Study of the specificity of monoclonal antibodies<br>with various globin chains by Western blot analysis | 46 |
| 2.2.11. Large scale production of monoclonal antibodies and<br>purification of monoclonal antibodies             | 47 |

**CHAPTER III RESULTS**

|  |    |
|--|----|
| 3.1. Preparation of normal, cord blood and Bart's hydrops fetalis hemolysates                          | 49 |
| 3.2. Purification of hemoglobin Bart's and Hb Portland   | 49 |
| 3.3. Purification of hemoglobin A, A <sub>2</sub> , F and Hb E by DEAE Sepharose column chromatography | 53 |
| 3.4. Production of monoclonal antibodies against hemoglobin Bart's and Hb Portland                     | 55 |
| 3.4.1. Antibody responses in BALB/c mice after immunizations with Bart's hydrops fetalis hemolysate    | 55 |
| 3.4.2. Antibody responses in BALB/c mouse after immunizations with purified hemoglobin Portland        | 55 |
| 3.4.3. Production of monoclonal antibodies   | 58 |
| 3.5. Isotyping of monoclonal antibodies  | 62 |
| 3.6. Study of the specificity of monoclonal antibodies by using different hemolysates                  | 62 |
| 3.7. Study of the specificity of monoclonal antibodies using purified hemoglobins                      | 65 |
| 3.8. Study of the cross reactivity of monoclonal antibodies between human and animal hemoglobins       | 65 |

|   |     |
|---|-----|
| 3.9. Large scale production of monoclonal antibodies<br>and purification of mAbs                                    | 68  |
| 3.10. Study of the specificity of monoclonal antibodies using<br>various globin chains by Western blotting analysis | 70  |
| <b>CHAPTER IV DISCUSSIONS</b>   | 76  |
| <b>CHAPTER V CONCLUSION</b>   | 86  |
| <b>REFERENCES</b>   | 88  |
| <b>APPENDIX A</b>   | 101 |
| <b>APPENDIX B</b>   | 108 |
| <b>CURRICULUM VITAE</b>   | 125 |

## LIST OF TABLES

| TABLE  | PAGE |
|--|------|
| 1.1. Examples of non-deletion mutants that cause $\alpha$ -thalassemia                               | 21   |
| 2.1. Eluting buffer gradients for each hemolysate  | 38   |
| 3.1. The reactivity of hybridoma clones obtained from the 1 <sup>st</sup> and 2 <sup>nd</sup> fusion | 60   |
| 3.2. The reactivity of hybridoma clones from the 3 <sup>rd</sup> fusion                              | 61   |
| A-1 Lists of chemicals used in this study  | 101  |

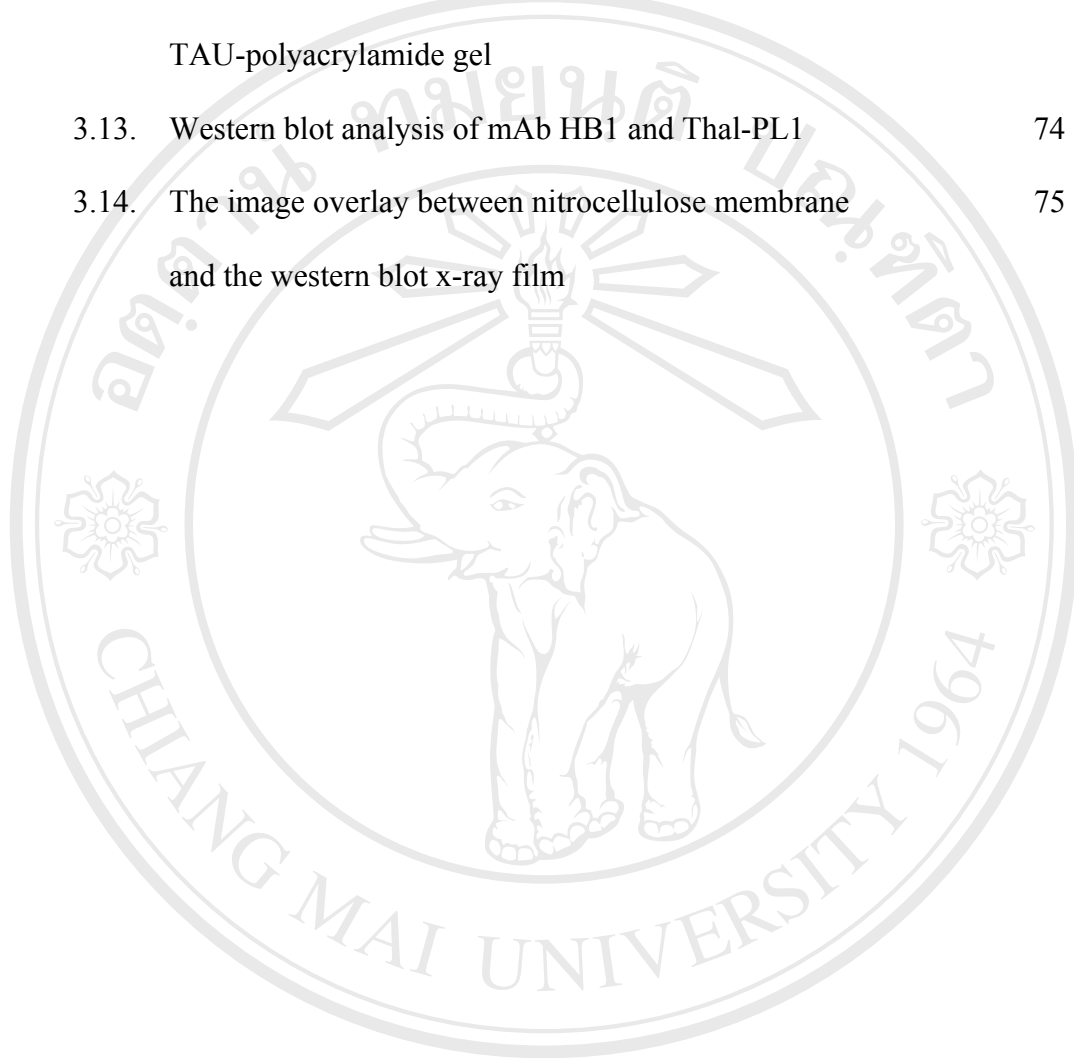
## LIST OF FIGURES

| <b>FIGURE</b> |   | <b>PAGE</b> |
|---------------|---|-------------|
| 1.1.          | Function of hemoglobin  | 5           |
| 1.2.          | Molecular structure of hemoglobin   | 7           |
| 1.3.          | The chromosome of $\alpha$ and $\beta$ globin genes family  | 7           |
| 1.4.          | $\alpha$ and $\beta$ globin genes in diploid cell   | 8           |
| 1.5.          | Hemoglobin synthesis in the developing red cell   | 10          |
| 1.6.          | Molecular structure of heme   | 11          |
| 1.7.          | Hemoglobin oxygen dissociation curve  | 12          |
| 1.8.          | Developmental change in globin chains and<br>production organs from fetus to adult  | 14          |
| 1.9.          | Incidence of thalassemia syndrome worldwide   | 16          |
| 1.10.         | A summary of major deletion in the $\alpha$ globin gene cluster   | 19          |
| 1.11.         | Hb Bart's hydrops fetalis   | 24          |
| 1.12.         | Blood smear of Hb Bart's hydrops fetalis  | 24          |
| 1.13.         | Simplified flow chart base on the MCH, Hb A <sub>2</sub> , Hb F value<br>and DNA analysis for screening of thalassemia carriers | 26          |

|       |   |    |
|-------|---|----|
| 1.14. | Metabolic pathway relevant to hybridoma selection in medium containing hypoxanthine, aminopterin and thymidine (HAT medium) | 30 |
| 1.15. | Production of hybridomas  | 32 |
| 3.1.  | Analysis of normal adult, cord blood and Bart's hydrops fetalis hemolysate by cellulose acetate electrophoresis             | 51 |
| 3.2.  | Cellulose acetate electrophoresis analysis of the purified Hb Bart's and Hb Portland  | 52 |
| 3.3.  | Cellulose acetate electrophoresis analysis of purified hemoglobin A, A <sub>2</sub> , E and F                               | 54 |
| 3.4.  | Antibody responses of mice immunized with Bart's hydrops fetalis hemolysate   | 56 |
| 3.5.  | Antibody responses of mouse immunized with purified hemoglobin Portland   | 57 |
| 3.6.  | Immunoglobulin isotype of the generated monoclonal antibodies   | 63 |
| 3.7.  | Characterization of the specificity of mAbs using three difference hemolysates  | 64 |
| 3.8.  | Characterization of the specificity of mAbs using various purified hemoglobins  | 66 |
| 3.9.  | Cross reactivity of mAbs between human and animal hemolysates   | 67 |
| 3.10. | SDS-PAGE analysis of the purified mAb HB1 and Thal-PL1  | 69 |
| 3.11. | Globin chain analysis by Urea Acid Triton X-100 polyacrylamide gel (TAU-PAGE)   | 72 |



|  |    |
|--|----|
| 3.12. Nitrocellulose membrane after blotting with<br>TAU-polyacrylamide gel                | 73 |
| 3.13. Western blot analysis of mAb HB1 and Thal-PL1  | 74 |
| 3.14. The image overlay between nitrocellulose membrane<br>and the western blot x-ray film | 75 |



ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่  
Copyright © by Chiang Mai University  
All rights reserved

## ABBREVIATIONS

|       |   |
|-------|---|
| Ab    | antibody  |
| APS   | ammonium persulphate                              |
| BSA   | bovine serum albumin                              |
| °C    | degree Celcius                                    |
| EDTA  | ethylene diamine tetraacetic acid                 |
| ELISA | enzyme-linked immunosorbent assay                 |
| FCS   | fetal calf serum                                  |
| gm    | gram  |
| HAT   | hypoxanthine aminopterin and<br>thymidine         |
| HGPRT | hypoxanthine guanine<br>phosphoribosyltransferase |
| IgG   | immunoglobulin G                                  |
| IgG1  | immunoglobulin G1                                 |
| IgG2a | immunoglobulin G2a                                |
| IgG2b | immunoglobulin G2b                                |
| IgG3  | immunoglobulin G3                                 |
| IgA   | immunoglobulin A                                  |

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่  
Copyright © by Chiang Mai University  
All rights reserved

IgM immunoglobulin M

Igs immunoglobulins

mg milligram

mL milliliter

M molar

mM millimolar

mAb monoclonal antibody

mA milli ampere

OD. optical density

PBS phosphate buffered saline

rpm revolution per minute

TBE tris-borate-EDTA

$\mu\text{g}$  microgram

$\mu\text{L}$  microliter

V volt

$\alpha$  alpha

$\beta$  beta

$\delta$  delta

$\gamma$  gamma

$\varepsilon$  epsilon

$\zeta$  zeta

ลิขสิทธิ์มหาวิทยาลัยเชียงใหม่  
 Copyright © by Chiang Mai University  
 All rights reserved