CHAPTER IV

DISCUSSION AND CONCLUSION

Thalassemia result from mutations of globin genes that cause reduced or absent hemoglogin production and thus interfere with the critical function of oxygen delivery. (Chong *et al*., 2000; Krishnsmurti *et al*., 1998; Weatherall and Clegg, 2001). Theoretically, there are as many types of thalassemia as there are types of globin chains. The most clinically relevant are the α and β-thalassemia (Sarnaik, 2005).

In the case of thalassemia, β-thalassemia results from reduced synthesis of the β-globin chains with normal synthesis of α-globin that shows an imbalance in globin chain synthesis. Therefore free α-globin chains are highly unstable and readily precipitate, damaging membrane structures and triggering the apoptotic cell death of erythroid precursors. From these, hemolysis and ineffective erythropoiesis together cause the anemia that occurs in β-thalassemia. The relative contributions of these two pathologic processes differ in various forms of β-thalassemia (Pootrakul *et al*, 2000). Hemolysis causes higher than normal levels of bilirubin, a yellow chemical substance that is a product of the metabolism of hemoglobin, and which is released by red blood cells damaged in the process of hemolysis. The release of higher levels of bilirubin gives the eyes and skin of patients with thalassemia major a yellowish color. Patients have slower rates of physical growth and it becomes difficult to carry out normal physical activities. The severe anemia also triggers several defense mechanisms in the body, the most significant of which is the hyper-activity of the bone marrow. In an

effort to counter the low level of red blood cells in the system, the bone marrow expands to about 30 times its normal size to produce more cells. This expansion of the bone marrow in turn forces the bones to expand, producing deformities of the skull, protrusions of the upper teeth, and distortions of the ribs and vertebrae. Bones become osteopenia and more fragile, often leading to fractures. However, in patients with thalassemia the spleen must cope with a huge number of red blood cells, produced by a hyperactive bone marrow (Rund and Rachmilewitz, 2005; Thein, 2005; Sarnaik, 2005).

It have been reported many factors that contribute to this clinical severity of β-thalassemia phenotype including type of β-globins gene mutation, variation in the amount of α-globin production or variation in fetal hemoglobin response (Thein, 2005). For instance, individuals who are heterozygous for β-thalassemia with just one copy of the β-globin gene have essentially no symptoms and no significant anemia (Weatherall & Clegg, 2001). However, many will develop a moderately severe anemia and a relatively serious condition, thalassemia intermediate, if they also have extra α-globin genes producing more than the usual quota of α-globin (Galanello *et al*, 1983; Thein *et al*, 1984; Kulozik *et al*, 1987). In this situation, a certain threshold of excess α-globin appears to have been exceeded, overwhelming the buffering capacity of AHSP. A recent study reported that AHSP could also contribute to the severity of drug/infection-induced hemolytic anemia (Kanno et al, 2005).

Recently, it has been shown that the AHSP, is an erythroid-specific protein, can act as a chaperone for free α-hemoglobin and prevent their precipitation (Gell *et al.*,2002). These findings suggest that AHSP may have an important role in normal erythropoiesis by stabilizing α-globin. The ratio of *AHSP*/*α-globin* gene expression

probably is conserved during all stages of normal erythropoiesis. This data presented herein strongly indicate that *AHSP* gene expression in human erythroid cells is related to α-*globin* gene expression (Oresco dos Santos *et al*, 2004). Furthermore, several studies have demonstrated the relationship between AHSP and the severity of β-thalassaemia. Galanello *et al* (2003) reported that reduced expression of AHSP was associated with a more severe phenotype among individuals with identical β-thalassemia and α-globin genotypes although no mutations or polymorphisms in the gene could be implicated. Kong *et al* (2004) demonstrated AHSP knock-out mice show ineffective erythropoiesis and pathological features similar to those of β-thalassemia and loss of AHSP exacerbates the hematological abnormalities in β-thalassemic mice. In addition, Kihm *et al.* (2002) showed that the AHSP molecule could modify the clinical picture of β-thalassemia. It has been suggested that altered levels or function of alpha hemoglobin stabilising protein (AHSP) might account for some of the clinical variability observed in patients with β-thalassemia (Luzzatto & Notaro, 2002). For this correlation indicated that the detection of AHSP level is important role to evaluate about the severity of β-thalassemia patients.

Currently, the molecular techniques were used for the detection of AHSP mRNA level but that tools have some limitation such as the problem in RNA extraction step, the complication of technique and expensive cost. The detection of AHSP level by using monoclonal antibody against AHSP and flow cytometry, that is the simple and convenience method, will be an interesting choice. In addition, monoclonal antibody specific to AHSP molecule have not been reported so far. Thus,

in this study, production and characterization of monoclonal antibody against AHSP were purposed.

To generate mAb against hAHSP, two immunogens, hAHSP-BCCP and hAHSP, were used to immunize BALB/c mice. The hAHSP recombinant protein was kindly gift from Dr. Mitchell J. Weiss. For hAHSP-BCCP fusion protein, it was constructed from plasmid expression vector named pAK400CB. To compare whether each strain of *E. coli* express different hAHSP structure, pAK400CB harboring hAHSP was transformed in various strains of *E.coli* include Origami B, Nova blue, TG-1 and XL1 blue. The description of bacterial Origami B strain which contains double mutation on both thioredoxin (*trxB*) and glutathione reductase (*gor*). The *trxB* and *gor* mutations were selectable on kanamycin and tetracycline, respectively. Protein expression from this strain could form disulfide bond in the cytoplasm., Nova blue and XL1 blue, non expression host strain that lacks a source of T7 RNA polymerase. Its have been used for purpose cloning or plasmid preparation. TG1, The amber codon suppressing *E.coli* strain that used as the bacterial hosts for phagemids (Rietsch and Beckwith, 1998; Bessette *et al*., 1999).

Currently, *E. coli*, the number one bacterium of recombinant DNA technology, has been extensively studied as production host for heterologous proteins (Baneyx, 1999; Swartz, 2001). Despite its extensive knowledge on genetics and molecular biology, there is no a prior guarantee that every gene can be expressed efficiently in this Gram-negative bacterium. Factors influencing the expression level include unique and subtle structural features of the gene sequence, the stability and efficiency of mRNA, correct and efficient protein folding, codon usage, and

degradation of the recombinant protein by ATP-dependent proteases and toxicity of the protein.

Recombinant fusion proteins have been successfully used in production of monoclonal antibody (Tayapiwatana et al., 2006). Gene fusion techniques permit the assembly of recombinant protein with a protein fusion partner that has been designed for purification or detection purpose (LaVallie and McCoy, 1995). The binding interaction of biotin to avidin/streptavidin had been used widely in biochemistry and molecular biology. Herein, described the intracellular expression and concomitant site-specific *in vivo* biotinylation of hAHSP. For this study, nucleotide sequence encoding hAHSP protein was cloned into pAK400CB vector (Santala and Lamminmaki, 2004). We described the intracellular expression and concomitant site-specific *in vivo* biotinylation of the hAHSP. This approach is based on the cytoplasmic expression of hAHSP fused to the biotin acceptor domain of *E.coli* BCCP which is a natural substrate of biotin ligase that catalyse the conjugation of the biotin moiety to a specific lysine residue in the BCCP. The hAHSP-BCCP fusion protein was expressed into *E. coli* Origami B, Nova Blue, and TG1 or XL-1 Blue strains after culturing at 25^{o}C with IPTG induction. The biotinylated of recombinant AHSP-BCCP molecule, biotinylated hAHSP-BCCP, were obtained in bacterial extract and verified by indirect ELISA. It was found that biotinylated hAHSP-BCCP from *E. coli* Origami B and TG1 were strongly recognized by rabbit anti-AHSP polyclonal antibody (pAb). The hAHSP-BCCP from Nova Blue strain weakly reacted with rabbit anti-AHSP pAb. Whereas no reactivity was observed with biotinylated hAHSP-BCCP from *E. coli* XL-1 Blue strain. However, BCCP mAb react to biotinylated hAHSP-BCCP fusion protein indicating the presence of hAHSP-BCCP

with biotin attached. In contrast, there did not show significant signal on the well to apply with crude extract from CD147-BCCP. In addition, the presence of biotinylated hAHSP-BCCP fusion protein was confirmed by Western blotting. The result showed that rabbit anti-hAHSP pAb reacted with biotinylated hAHSP-BCCP protein from Origami B and TG1 strain but did not react with hAHSP-BCCP from Nova Blue and XL-1 Blue strain. The expected protein band with approximately 30 kDa was also detected by rabbit anti-hAHSP pAb and BCCP mAb. This results suggested that, *E.coli* Origami B and TG1 strain could produce the correct conformation of recombinant protein hAHSP more than *E.coli* Nova Blue and XL1 Blue strain. In recombinant bacteria, the rapid production of plasmid- encoded gene products triggers the accumulation of high concentration of folding intermediates which further form insoluble aggregates designated as inclusion body (Kiefhaber *et al*., 1991). Such, the fusion protein from *E.coli* Nova Blue and XL1 Blue strain may misfolding. The result indicated that host strain of *E. coli* have affect to recombinant protein production. Then, biotinylated hAHSP-BCCP expressing in *E.coli* Origami B strain was selected and used as immunogen for monoclonal antibody production.

To generate a monoclonal antibodies (mAbs) specific for a desired antigen, an immune response of mouse was raised by immunizing the protein of interest. The harvested cells from spleen were fused with myeloma cells and HAT selection. The survival cells, called hybridoma, are combination advantage nature of myeloma which is immortal cell and HGPRT gene from immune B cell. This hybridoma can grow continuously *in vitro* and secrete a single monoclonal antibody. By limiting techniques, the interest clones could be selected and they can be grown in tissue culture dish or *in vitro* or producing ascitic fluid in mouse or *in vivo*. These mAbs has

been steady expansion into numerous ELISA developments. Its specificity and reproducibility can solve the problems from traditional antisera.

For mouse immunization with biotinylated hAHSP-BCCP, the biotinylated hAHSP-BCCP protein from Origami B expression was trapped on streptavidin-cated magnetic beads. Because the protein-coated beads serve as particulate antigens that are strongly immunogenic for induction of immune responses (Heinz et al., 1995; Telino et al., 2006). To determine the existence of biotinylated hAHSP-BCCP on streptavidin magnetic beads, immunofluorescence staining and flow cytometry were performed. The positive reactivity was observed when staining biotinylated hAHSP-BCCP with rabbit hAHSP pAb or BCCP mAb indicating the immobilization of hAHSP-BCCP fusion protein on the magnetic beads. Then, biotinylated hAHSP-BCCP coated beads were called hAHSP-BCCP bead and used for immunization. For immunization, two female BALB/c mice, A and B, were intraperitoneally immunized three times with hAHSP-BCCP beads and recombinant protein hAHSP at two-week intervals. To determine the antibody response in immunized BALB/c mice, indirect ELISA was performed by using recombinant hAHSP as coated antigen. The results showed that antibody titer from mouse "A" was increased to 1:20,000 which was higher than mouse "B" after third immunization. The different immune response between those two mice may be due to the individual genetics variation (Lavrovsky *et al.*, 1979). Therefore, mouse "A" was selected for fusion. To get monoclonal antibody specific to hAHSP, hybridomas secreting monoclonal antibody were screened against hAHSP by indirect ELISA using hAHSP-BCCP fusion protein as antigen. To eliminate any monoclonal antibody specific to BCCP part, the culture supernatant containing antibody were counter screened with irrelevant protein,

CD147-BCCP. By this screening, hybridomas that indicated the difference of absorbance greater than 0.3 when tested with hAHSP-BCCP and CD147-BCCP were further screened for the specific hAHSP antibody. From hAHSP-BCCP immunization, the results showed that antibodies from all hybridoma showed negative reactivity with both hAHSP-BCCP and CD147-BCCP. However, hAHSP-BCCP from Origami B showed the strongly positive result with rabbit anti-hAHSP pAb to confirm that hAHSP-BCCP could be used for positive control of the detection system.

The second immunization using hAHSP, various culture supernatant containing antibody were screened against hAHSP by indirect ELISA. It was found that two hybridoma clones producing antibody showed strongly positive with recombinant hAHSP protein. By limiting dilution technique, single clone from two interested hybridoma was obtained. The clone was named “MT-hAHSP1 and MT-hAHSP2 and propagated for further characterization.

In this study, hAHSP-BCCP fusion protein from each strain of *E.coli* (Origami B, Nova Blue, TG1 and XL-1 Blue) was used to characterize the generated hAHSP mAb. The MT-hAHSP1 and MT-hAHSP2 strongly reacted to hAHSP but did not react to hAHSP-BCCP assuming this expression system may alter the hAHSP structure. The another reason was two generated mAbs, MT-hAHSP1 and MT-hAHSP2, may recognized hAHSP domain where was linked to BCCP. By Western immunoblotting, the results showed that both of MT-hAHSP1 and MT-hAHSP2 did not react to hAHSP, hAHSP-BCCP from *E. coli* various strains and CD147-BCCP under reducing condition. The results indicated that MT-hAHSP1 and MT-hAHSP2 recognized to conformational epitope.

In conclusion, hAHSP mAb were produced and named MT-hAHSP1 and MT-hAHSP2. Both hAHSP mAbs react to conformational epitope of hAHSP recombinant protein. However, the reactivity of MT-hAHSP1 and MT-hAHSP2 to native AHSP will be further characterized. The development of immunological assays to detect hAHSP level by using MT-hAHSP1 and MT-hAHSP2 will be applied for evaluation of severity in patients with β-thalassemia.