CHAPTER III

RESULTS

3.1. Preparation of normal, cord blood and Hb Bart's hydrops fetalis hemolysates

In order to prepare hemolysates for using as immunogens for production of antibodies and using as antigens for examining the activity of antibodies, blood of normal adult, normal cord blood and Hb Bart's hydrops fetalis were collected using EDTA as anti-coagulant. Hemolysates were prepared from all blood samples by organic CCl_4 extraction as was described in the materials and methods. To verify that the obtained hemolysates contained corresponding Hbs, the hemolysates were analyzed for their hemoglobin contents by cellulose acetate electrophoresis. As shown in figure 3.1, normal adult hemolysate contained carbonic anhydrase enzyme (CA), Hb A_2, Hb A and a very small amout of Hb F. Normal cord blood hemolysate contained Hb F and trace amount of Hb A. Hemolysate of Hb Bart's hydrops fetalis contained Hb Bart's and Hb Portland which migrated faster than Hb A (Figure 3.1). These results demonstrated that all obtained hemolysates contained correspondence Hbs and could be used in the further experiments.

3.2. Purification of hemoglobin Bart's and Hb Portland

The main objective of this study is to produce monoclonal antibody to Hb Bart's and ζ globin chain. Purifed Hb Bart's and Hb Portland were therefore needed for using as immunogen. Hb Bart's hydrops fetalis hemolysate was separated

by cellulose acetate electrophoresis. After electrophoresis, the bands of Hb Bart's and Hb Portland were cut apart from each other. The the Hbs on the membrane were eluted as was described in the materials and methods. By this technique, approximately 853 μg of purified Hb Portland and approximately 2.826 mg of purified Hb Bart's were obtained from starting 19.2 mg of total Hbs in Hb Bart's hydrops fetalis hemolysate. The percent recovery of purified Hbs was calculated by the following equation.

$$\textbf{\% Recovery of Hb} = \frac{\text{purified Hb (mg)} \times 10^4}{\text{Total applied hemolysate (mg)} \times \text{\%Hb contained in hemolysate}}$$

Where

%Hb contained in hemolysates for Hb Bart's and Portland were 90% and 10% in Hb Bart's hydrops fetalis hemolysate (Rifkind *et al.*, 1984)

By the above equation, the percent recovery of Hb Portland and Hb Bart's were 44.42% and 16.35% respectively. To verify the purity of the obtained Hb Bart's and Hb Portland, the obtained purified Hbs were re-analyzed for their hemoglobin contents by cellulose acetate electrophoresis. As shown in figure 3.2, the purified Hb Portland contained a band of Hb Portland. The purified Hb Bart's contained a band of Hb Bart's. Bands of Hb Bart's and Hb Portland of the purified materials were at the same position of Hb Bart's and Hb Portland containing in the Hb Bart's hydrops fetalis hemolysate that were used as hemoglobin markers. The purified Hbs were used as immunogens and used for characterization of monoclonal antibodies in the further study.

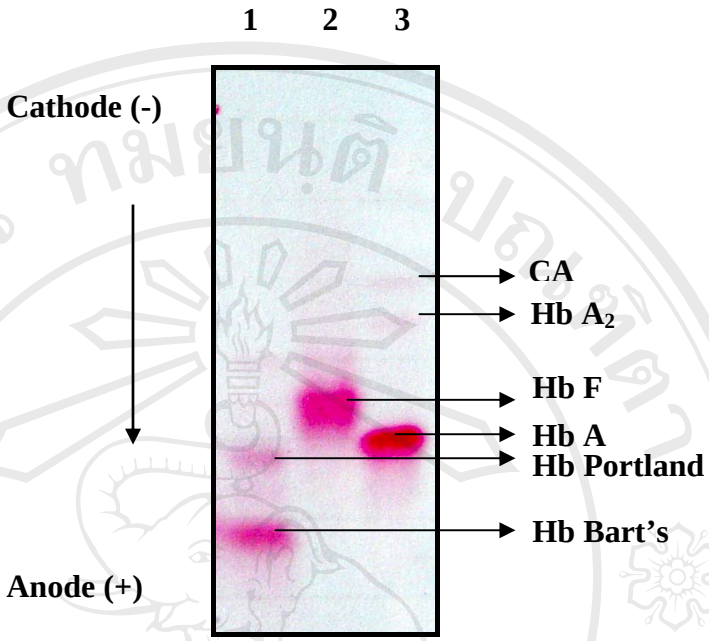


Figure 3.1. Analysis of normal adult, cord blood and Hb Bart's hydrops fetalis hemolysate by cellulose acetate electrophoresis. Hemolysates were prepared from EDTA bloodof normal adult, normal cord blood and Hb Bart's hydrops fetalis. The obtained hemolysates were analyzed for their hemoglobin by cellulose acetate electrophoresis. Two microlitters of the hemolysate were applied onto the cellulose acetate membrane and performed the electrophoresis at 160 V for 2 hr. The membrane were stained with Ponceau S. Lane 1, normal adult hemolysate; Lane 2, cord blood hemolysate; Lane 3, Hb Bart's hydrops fetalis hemolysate.

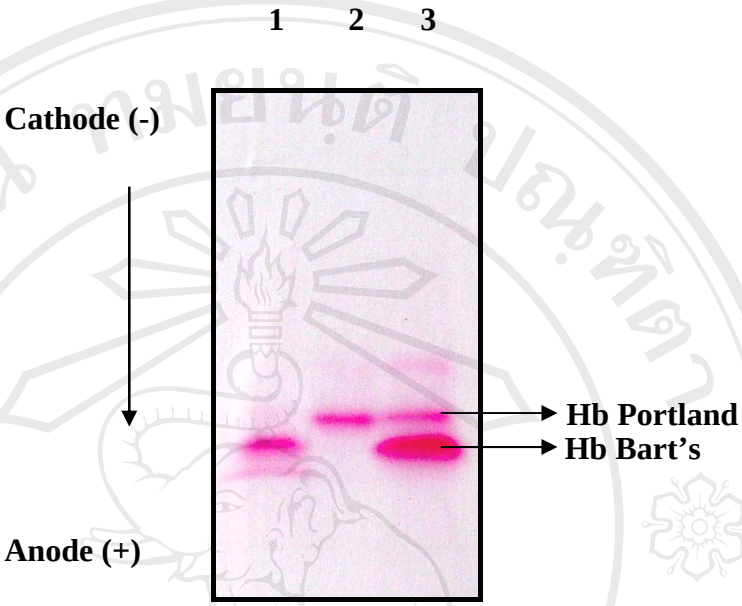


Figure 3.2. Cellulose acetate electrophoresis analysis of the purified Hb Bart's and Hb Portland. Hb Bart's and Hb Portland were purified from Hb Bart's hydrops fetalis hemolysates. The purified Hbs and Hb Bart's hydrops fetalis hemolysate, using as the hemoglobins marker, were analyzed by cellulose acetate electrophoresis. The membrane were stained with Ponceau S. Lane 1, purified Hb Bart's; Lane 2, purified Hb Portland; Lane 3, Hb Bart's hydrops fetalis hemolysate.

3.3. Purification of hemoglobin A, A_2, F and Hb E by DEAE Sepharose column chromatography

For characterization of mAbs, various hemoglobins including Hb A, Hb A_2, Hb F and Hb E were needed. In this study, the mention Hbs were then purified from normal adult (A_2A), normal cord blood (AF) and adult Hb E heterozygote hemolysate (AE) by using DEAE Sepharose column as was described in material and methods. About 20 mg of purified Hb A and 2 mg of purified Hb A_2 were obtained from 360 mg of total Hbs of normal adult hemolysate. 25.4 mg of purified Hb F were obtained from approximately 100 mg of total Hbs of normal cord blood hemolysate. 3.4 mg of purified Hb E containing Hb A_2 (E/A_2), because of their similarity in the molecular net charge, were obtained from approximately 100 mg of total Hbs of adult with Hb E heterozygote. The percent recovery of Hb A, A_2, F and E/A_2 were 5.6%, 27.7%, 37.3% and 11.3% respectively.

The Hbs obtained from DEAE Sepharose column were analyzed for their purity by cellulose acetate electrophoresis. All purified Hbs showed a sing band of the correspondence Hb without contamination by the other type of Hb (Figure 3.3). These results indicated that the obtained purified Hbs were pure and could be used for characterization of the mAbs in further experiments.

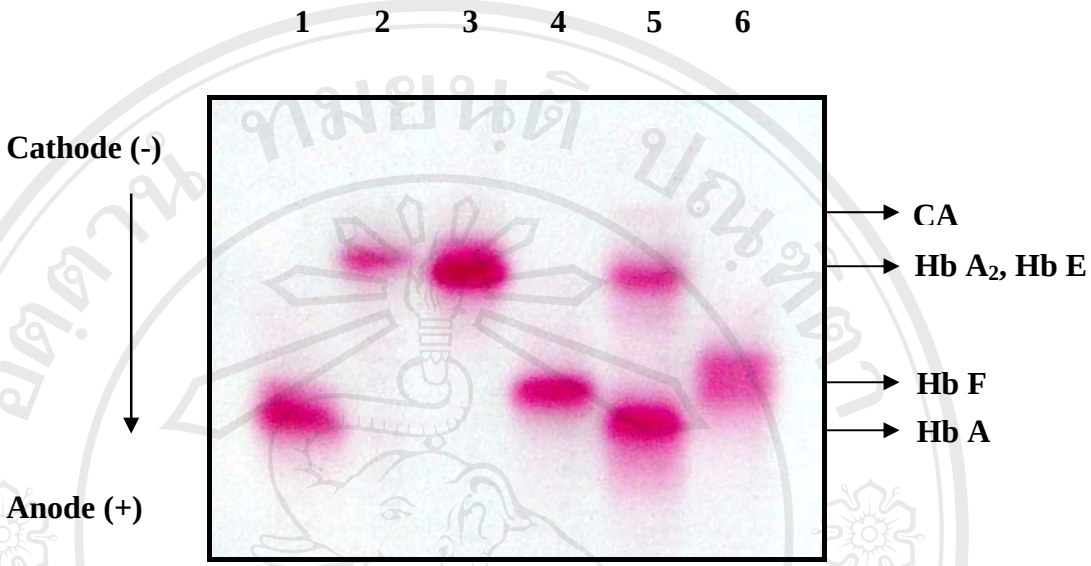


Figure 3.3. Cellulose acetate electrophoresis analysis of purified hemoglobin A, A_2, E and F. Hb A, A_2, E and F were purified from normal adult, cord blood and heterozygous β-thalassemia with Hb E hemolysate by DEAE Sepharose column chromatography. The obtained Hbs were analyzed by cellulose acetate electrophoresis. Lane 1, purified Hb A; Lane 2, purified Hb A_2; Lane 3, purified Hb E containing Hb A_2; Lane 4, purified Hb F; Lane 5, Hb E heterozygote hemolysate and lane 6, cord blood hemolysate.

3.4. Production of monoclonal antibodies against hemoglobin Bart's and Hb Portland

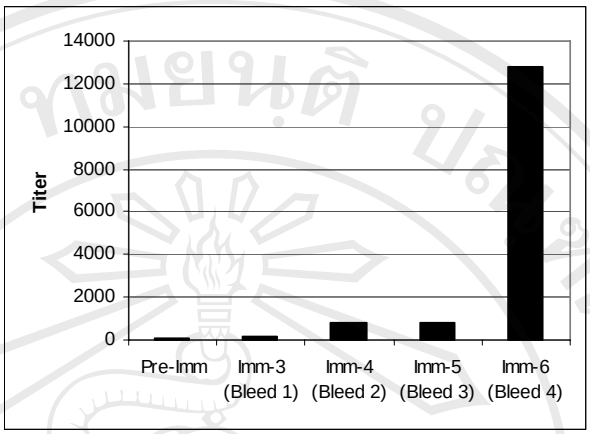
3.4.1. Antibody responses in BALB/c mice after immunizations with Hb Bart's hydrops fetalis hemolysate

Two female BALB/c mice, A and B, were immunized with 100 μg of Hb Bart's hydrops fetalis hemolysate at two weeks intervals. The antibody responses were determined by indirect ELISA technique. As was shown in figure 3.4, antibodies responses in mouse "A" were quite low in the first period of immunization (bleed 1-3), however, after the 6^{th} immunization (bleed 4) the antibody titer increased to more than 1: 12,000. The antibodies responses in mouse "B" were gradually rising from approximately titer of 1:100 at the first bleeding and stable at 1: 3,000 at the third and fourth immunization (bleed 3-4) (Figure 3.4). Both mice were used for further monoclonal antibody production.

3.4.2. Antibody responses in BALB/c mouse after immunizations with purified hemoglobin Portland

A female BALB/c mouse was immunized with 100 μg purified Hb Portland using Freund's adjuvant at two week intervals. The antibody responses were determined by indirect ELISA technique. The antibodies titer was 1: 10,000 after the third immunization (Figure 3.5). After the third immunization, this mouse was subjected for monoclonal antibody production.

(A)



(B)

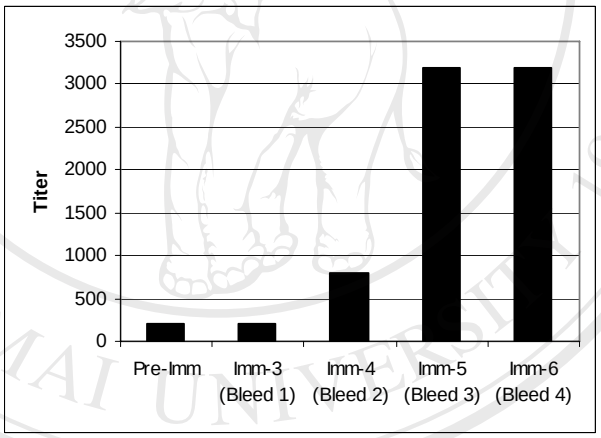
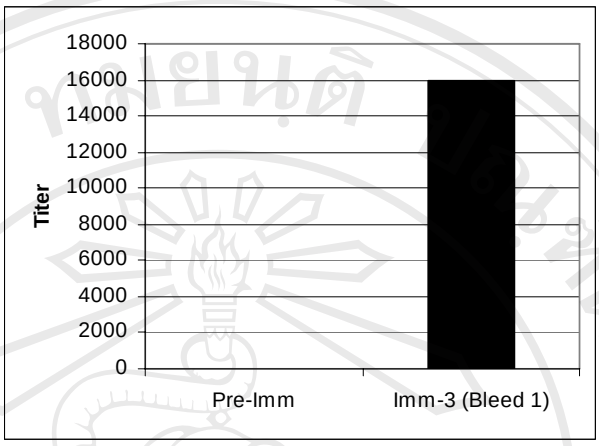


Figure 3.4. Antibody responses of mice immunized with Hb Bart's hydrops fetalis hemolysate. Two BALB/c mice were immunized with 100 μg Hb Bart's hydrops fetalis hemolysate diluted in PBS. Sera were collected at pre-immunization and after the first three immunizations (Bleed 1) and after the forth, the fifth and the sixth immunization (bleed 2-4). The antibody titers were examined by indirect ELISA. (A) serum titer of mouse "A", (B) serum titer of mouse "B".



(Portland)

Figure 3.5. Antibody responses of mouse immunized with purified hemoglobin Portland. A female BALB/c mouse was immunized with 100 μg purified Hb Portland diluted in Freund's adjuvant. Sera were collected at pre-immunization and after the third immunizations (Bleed 1) and determined by ELISA.

3.4.3 Production of monoclonal antibodies

In order to produce monoclonal antibodies against the hemoglobins containing in Hb Bart's hydrops fetalis, BALB/c mice with the highest titer of antibodies were used for hybridoma technique. The spleen cells were carefully homogenized and fused with myeloma cells using 50% polyethelene glycol (PEG). The culture supernatants from the wells containing the hybridoma cells were collected and screened for anti-hemoglobins antibodies by indirect ELISA.

In the first fusion experiment (using of mouse A: immunized with Hb Bart's hydrops fetalis hemolysate), fifty-three hybridoma clone were obtained. Nine clones were positive with Hb Bart's hydrops fetalis hemolysate. Three clones out of 9 hybridomas, named 3B11, 2F12 and 4H3, were selected for single cell cloning by limiting dilution.

In the second fusion experiment (mouse B: immunized with Hb Bart's hydrops fetalis hemolysate), 517 hybridoma clones were obtained and 140 clones were positive with Hb Bart's hydrops fetalis hemolysate. Ten clones, named 7E11, 10D2, 4D3, 3C11, 7G7, 6F3, 8F10, 9D9, 2A9 and 3D2 were selected for single cell cloning.

All hybridomas obtained from the first and the second fusions were then characterized for their specificity using normal adult and normal cord blood hemolysates. Unfortunately, all tested hybridoma clones were not specific for Hb Bart's hydrops fetalis hemolysate. All of them also strongly reacted with normal adults and normal cord blood hemolysate. Four hybridoma clones that strongly

reacted with all types of hemolysates were selected and renamed as HB1, HB2, HB3 and HB4. The ELISA results of mAbs HB1, HB2, HB3 and HB4 are shown in Table 3.1.

The third fusion experiment was performed using mouse immunized with Hb Portland. 309 hybridoma clones were obtained from this fusion. Antibody containing in the culture supernatants were screened by indirect ELISA using purified Hb Portland ($\zeta_2\gamma_2$) and purified Hb F ($\alpha_2\gamma_2$) as antigens. By this screening, 18 hybridoma clone were specifically positive with purified Hb Portland without cross reacted to purified Hb F. Five hybridomas (clone No. 38, 23, 149, 194 and 262) were selected for single cell cloning. The ELISA results of the five selected clone are shown in Table 3.2. Unfortunately, clone No.23 was reacted to both purified Hb Portland and purified Hb F when repeated the ELISA after limiting dilution. Clone No. 38 lost their activity during cultivation. Clones No. 194 and 262 died due to yeast contamination. Finally, clone No. 149, renamed as Thal-PL1, was the only clone remained in this study.

Table 3.1. Reactivity of hybridoma clones obtained from the 1st and 2nd fusion

Mouse	Clones	Optical density at 450 nm.			Rename
		Hb Bart's hydrops fetalis hemolysate	Cord blood hemolysate	Normal adult hemolysate	
A	4H3	2.892	2.826	2.951	HB3
B	7E11	3.416	3.177	3.227	HB1
	10D2	3.466	3.458	3.334	HB2
	4D3	2.556	2.56	2.488	HB4

For this valid ELISA

OD. value of less than 0.2 considered as negative.

OD. value of 0.4 - 0.2 considered as weak positive.

OD. value of 0.5 - 1.5 considered as positive.

OD. value of higher than 1.5 considered as strong positive.

Table 3.2. The reactivity of hybridoma clones from the 3rd fusion

Mouse	Clones No.	Optical density at 450 nm.		Rename
		Purified hemoglobin F	Purified hemoglobin Portland	
Mouse Portland	23*	0.100	1.582	-
	38**	0.060	0.955	-
	149	0.047	2.049	Thal-PL1
	194***	0.133	1.018	-
	262***	0.177	1.074	-

* Cross reaction with purified Hb F after repeated the ELISA

** Lost activity during cultivation

*** Death due to yeast contamination

For this valid ELISA

OD. value of less than 0.2 considered as negative.

OD. value of 0.4 - 0.2 considered weak as positive.

OD. value of 0.5 - 1.5 considered as positive.

OD. value of higher than 1.5 considered as strong positive.

3.5. Isotyping of monoclonal antibodies

After fusion experiments, five hybridoma clones were obtained. The generated mAbs were characterized for their immunoglobulin isotypes by using immunoglobulin isotyping ELISA kit. The monoclonal antibodys of clone HB1, HB2, HB3 and HB4 were IgM iostype, and that of clone Thal-PL1 was IgG isotype (Figure 3.6).

3.6. Study of the specificity of monoclonal antibodies by using different hemolysates

All five mAbs (HB1, HB2, HB3, HB4 and Thal-PL1) were first characterized for their specificity by three different types of hemolysates: normal adult, normal cord blood and Hb Bart's hydrops fetalis hemolysate by indirect ELISA. As predicted, mAbs clones HB1, HB2, HB3 and HB4 were strong positive with all types of hemolysate (Figure 3.7). Thal-PL1 was specifically reacted with only Hb Bart's hydrops fetalis hemolysate. Neither normal adults nor normal cord blood hemolysate were recognized by mAb Thal-PL1 (Figure. 3.7). All mAbs were further characterized for their specificity using purified Hb in the next experiment.

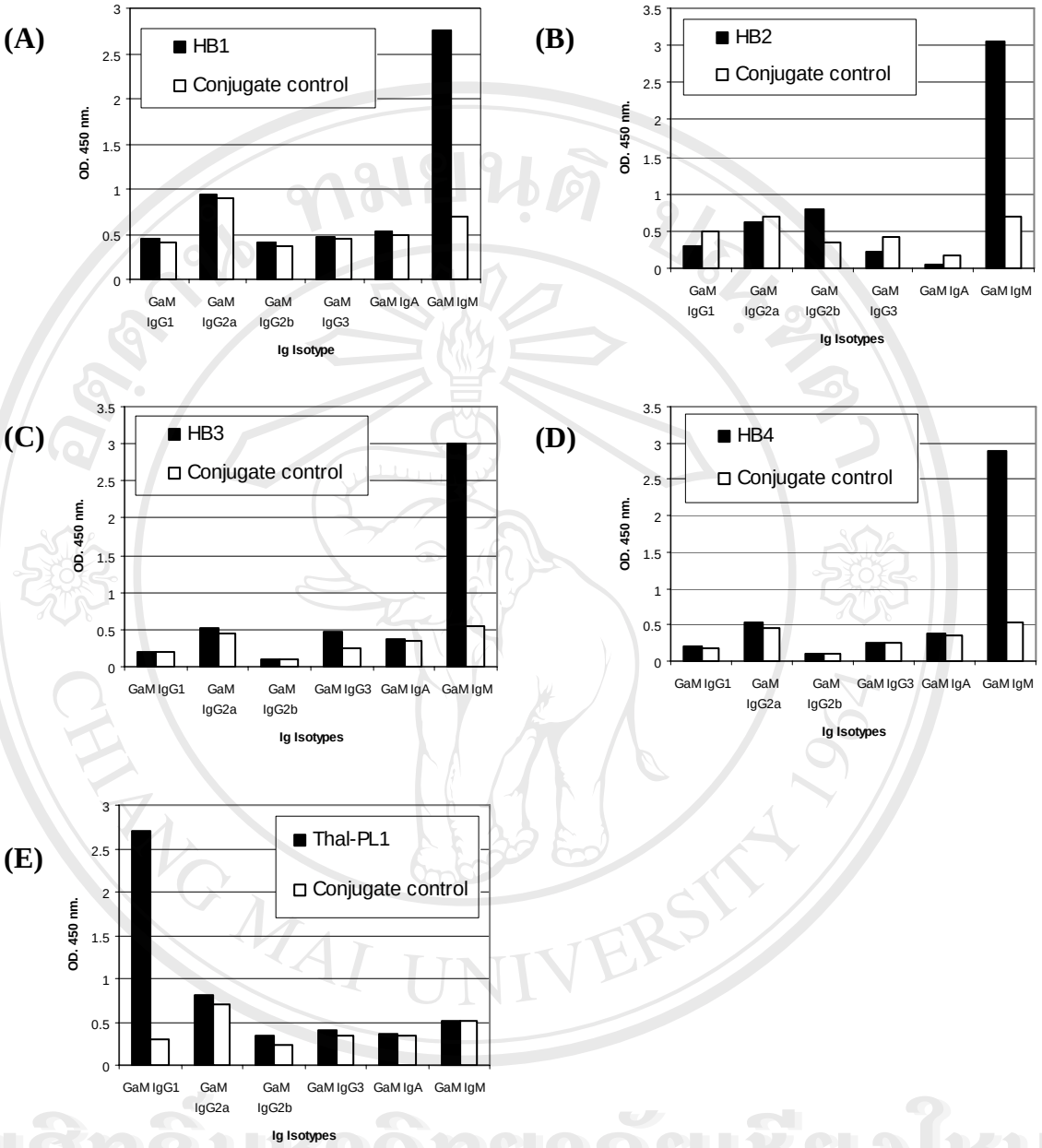


Figure 3.6. Immunoglobulin isotype of the generated monoclonal antibodies. Immunoglobulin isotypes of mAbs were determined by an ELISA kit. The antibody isotype is representing by the highest optical density at 450 nm. Conjugate control was obtained from wells without adding culture supernatant. (A), mAb HB1; (B), mAb HB2; (C), mAb HB3; (D), mAb HB4 and (E), mAb Thal-PL1. GaM was abbreviated of goat anti-mouse.

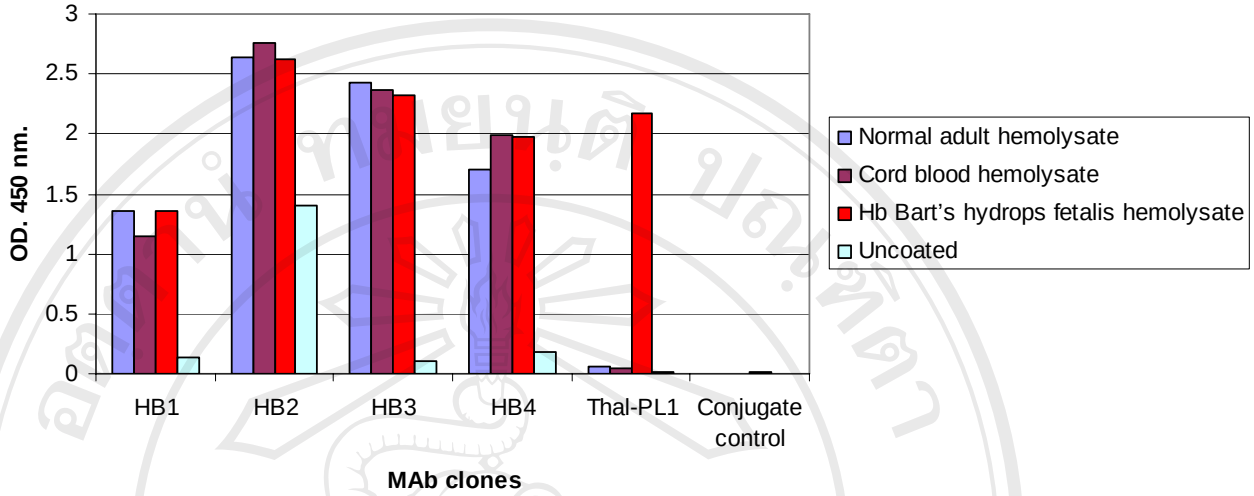


Figure 3.7. Characterization of the specificity of mAbs using three difference hemolysates. Normal adult, normal cord blood and Hb Bart's hydrops fetalis hemolysate were coated into 96 well plates and performed the indirect ELISA by using the culture supernatant of the indicated mAbs. Conjugate control was obtained from wells without adding culture supernatant.

3.7. Study of the specificity of monoclonal antibodies using purified hemoglobins

The mAbs were further characterized using purified Hb A, Hb A_2, Hb E/A_2, Hb F, Hb Portland and Hb Bart's. Similar to the previous study, mAbs HB1, HB2, HB3 and HB4 were strong positive with all purified hemoglobins. In addition, the HB2 mAb was also recognized the antigenic determinant of bovine serum albumin (BSA) using as blocking solution. In contrast, mAb Thal-PL1 was strong positive with purified Hb Portland and weak positive with purified Hb Bart's without cross reaction to other purified Hbs (Figure 3.8).

3.8. Study of the cross reactivity of monoclonal antibodies between human and animal hemoglobins

In order to study the cross reaction of the generated mAbs between human and animal hemoglobins, hemolysate from various animals including dog, cat, pig, cow, buffalo, chicken were prepared. Indirect ELISA was performed for this objective. As shown in Figure 3.9, mAb HB1 recognized an antigenic determinant presented in various animals hemolysate. Strong positive reactivity were observed in pig and chicken hemolysates while the lower O.D. were presented in dog, cat and cow hemolysates. MAbs HB2, HB3 and HB4 were strong positive with all animals and human hemolysate. In contrast mAb Thal-PL1 did not reacted to any animal hemolysates (Figure 3.9). From these results, mAbs HB1, HB2, HB3 and HB4 reacted to animal hemoglobin beside of human hemoglobin. For mAb Thal-PL1, these results confirmed the specificity of the antibody by without cross reaction to the other types of animal hemoglobins.

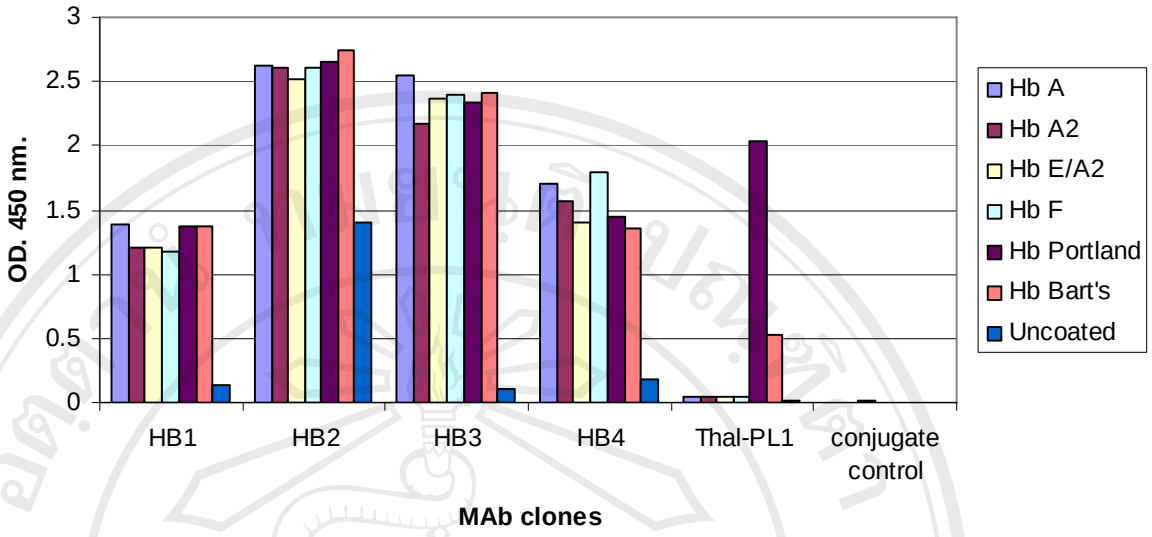


Figure 3.8. Characterization of the specificity of mAbs using various purified hemoglobins. Purified Hb A, Hb A_2, Hb E/A_2, Hb F, Hb Portland and Hb Bart's were coated on the 96 well plate. The indirect ELISA was performed by using the culture supernatant of the indicated mAbs. Conjugate control was obtained from wells without adding culture supernatant.

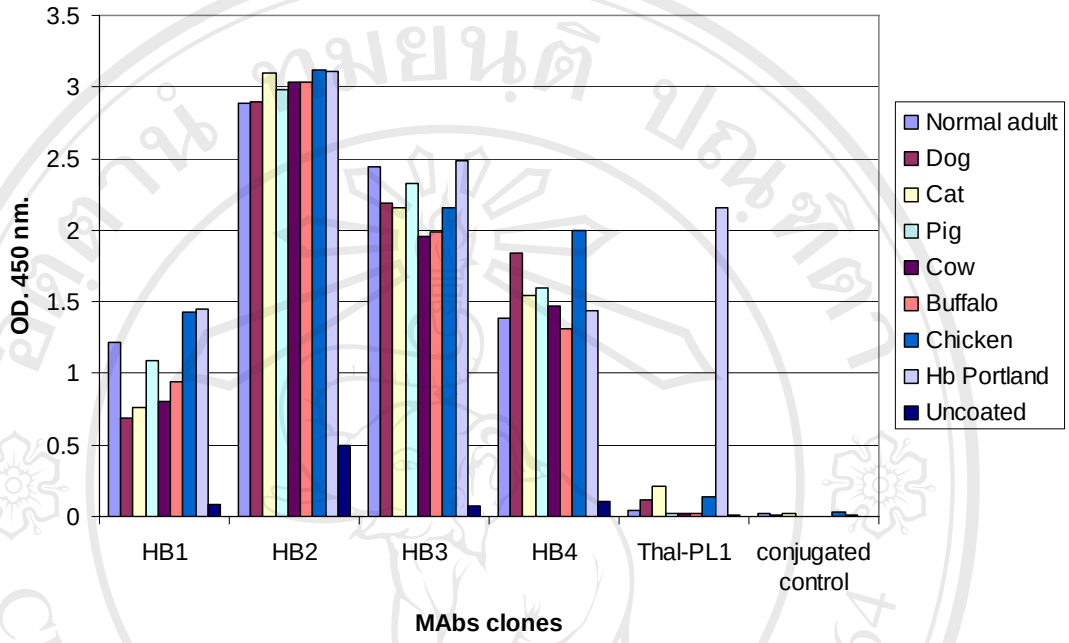


Figure 3.9. Cross reactivity of mAbs between human and animal hemolysates. The hemolysates of human and various animals were coated on 96 well plates and performed the indirect ELISA. Each bars represented the reaction of mAbs to the indicated hemolysates. The purified human Hb Portland was used as positive control for mAb Thal-PL1.

3.9. Large scale production of monoclonal antibodies and purification of mAbs

After examining the specificities of the generated mAbs, the mAbs HB1 and Thal-PL1 were subjected for large scale production. The HB1 and Thal-PL1 hybridomas were injected to BALB/c mice for induction of ascitic fluid as described in the Materials and Methods. Both hybridoma clones successfully induced ascitic fluids production. The ascitic fluids were purified using Hitrap^{tm} IgM column for mAb HB1 and Protein G column for mAb Thal-PL1. After the purification processes were completed, the purified mAbs were analyzed for their purity by SDS-PAGE (Figure 3.10).

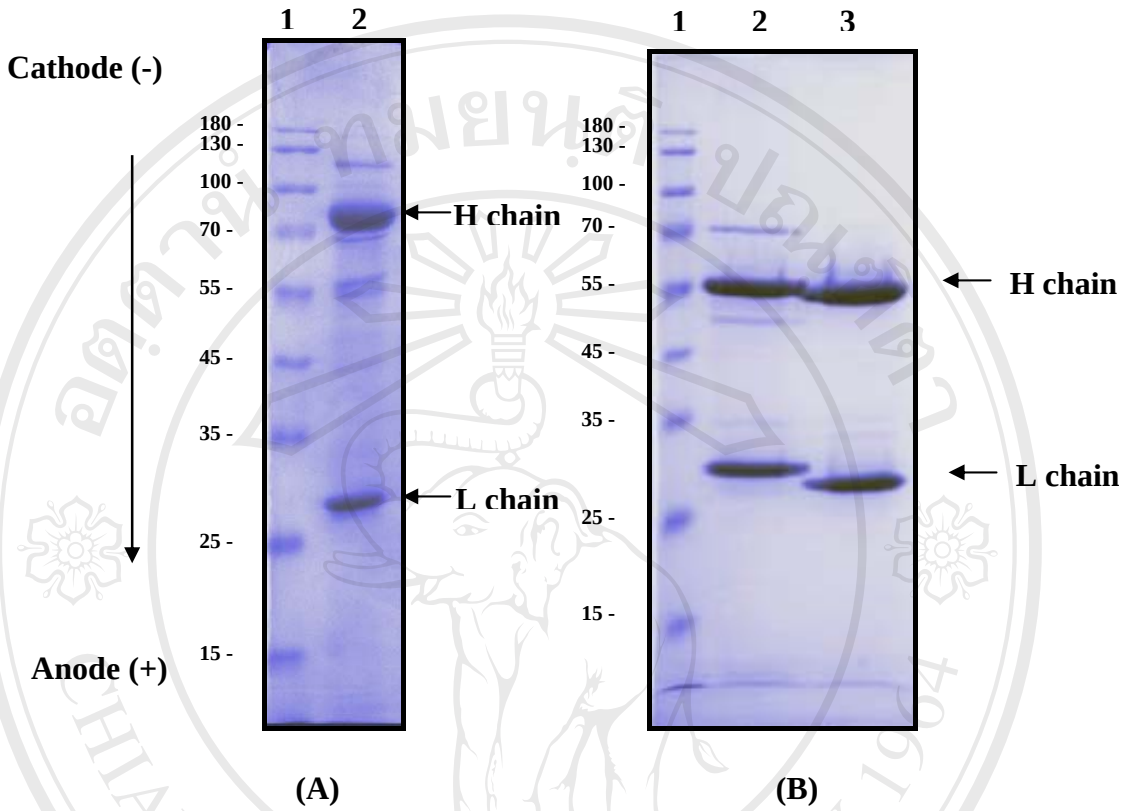


Figure 3.10. SDS-PAGE analysis of the purified mAb HB1 and Thal-PL1. 12.5 μg of purified mAbs HB1 and Thal-PL1 were loaded into each well of 12.5% SDS-polyacrylamide gel and performed the electrophoresis. Gels were srained with Coomassie brillant blue dye. (A): Lane 1, protein standard markers; Lane 2, purified HB1 mAb. (B): Lane 1, protein standard markers; Lane 2, purified MT45 mAb, using as the isotype matched control; Lane 3, purified Thal-PL1 mAb.

3.10. Study of the specificity of monoclonal antibodies using various globin chains by Western blotting analysis

Hemoglobin was composes of four globin chains to form the functional hemoglobin tetramer. To determine whether the generated mAbs react to the whole molecule of Hb or to the globin chains, mAbs HB1 and Thal-PL1 were then studied by Western blotting analysis. The globin chains were dissociated from hemoglobin by urea acid buffer and separated from each other according to their net charge on the TAU-PAGE. After electrophoresis, the ordering of globin chains migrate from anode to cathode were ζ, $^{A}\gamma$, δ, $^{G}\gamma$, β and α, respectively (Figure 3.11). Normal adult hemolysate contained a minute amount of δ chain and high amount of β and α chains. Hb Bart's hydrops fetalis hemolysate contained ζ, $^{A}\gamma$ and $^{G}\gamma$ chain. Purified Hb A_2 contained δ and α chains, respectively. The separated globin chains were transferred to a nitrocellulose membrane by semi-dry blotting. Then the membrane was cut into three strips and stained with Ponceus S dye (Figure 3.12). All strips were performed the antigen-antibody reactions with purified mAb Thal-PL1 or mAb HB1 or without primary antibody (or conjugated control) which were designed the strip No. 3, 2, 1 respectively (Figure 3.12). The specificity of mAbs was detected by chemiluminescence system. After detection, a single band with strong signal was observed on the strip No. 3 which was recognized only by Thal-PL1 mAb (Figure 3.13). The positive band appeared in the lane that Hb Bart's hydops fetalis hemolysate was loaded and must comprise three types of globin chains, ζ, $^{A}\gamma$ and $^{G}\gamma$. In contrast, no signal was observed on strips No. 1 and 2.

To clarify the specificity of mAbs Thal-PL1, the immuno-reactive band on x-ray film was overlaied onto the Ponceau S stained-nitrocellulose membrane to

indicate the position of Hb band. It was found that mAb Thal-PL1 was specifically bind to ζ globin chain. In contrast, mAb HB1 did not bind to any globin chain (Figure 3.14). MAb HB1 may recognize conformational epitope of the hemoglobin.

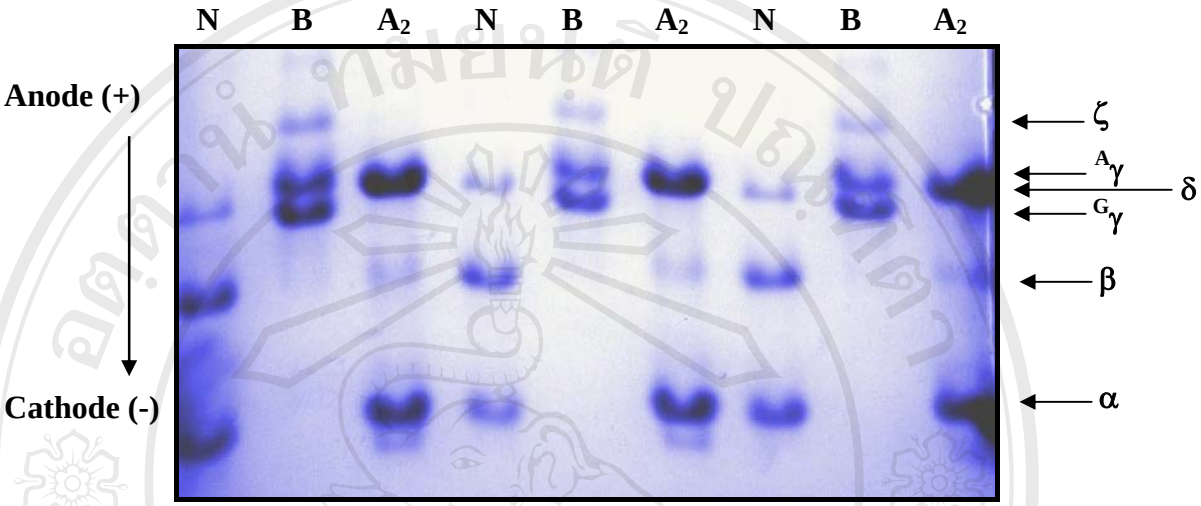
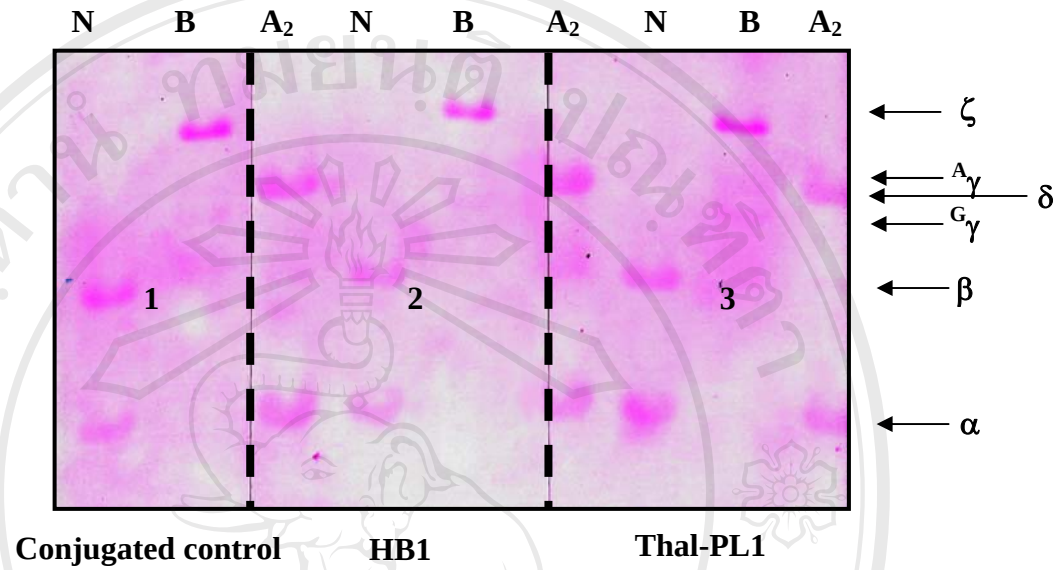


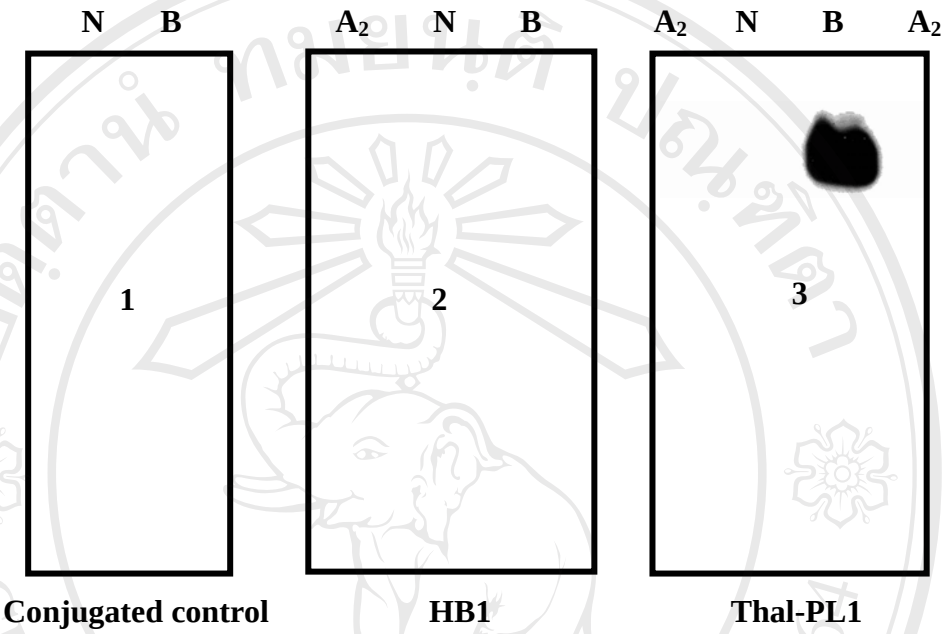
Figure 3.11. Globin chain analysis by Urea Acid Triton X-100 polyacrylamide gel (TAU-PAGE). 10 μg of normal adult hemolysate, Hb Bart's hydrops fetalis hemolysate and purified Hb A_2 were loaded into each well of 12% TAU-polyacrylamide gel and performed electrophoresis. Lane N, normal adult hemolysate; Lane B, Hb Bart's hydrops fetalis hemolysate; Lane A_2, purified Hb A_2. In this figure, the slab gel was stained with Coomassie brillant blue R250. The figure show 3 sets of loading samples.



N = Normal adult hemolysate, **B =** Hb Bart's hydrops fetalis hemolysate,

A_2 = Purified hemoglobin A_2**,** ▪▪▪ = Trace of cutting

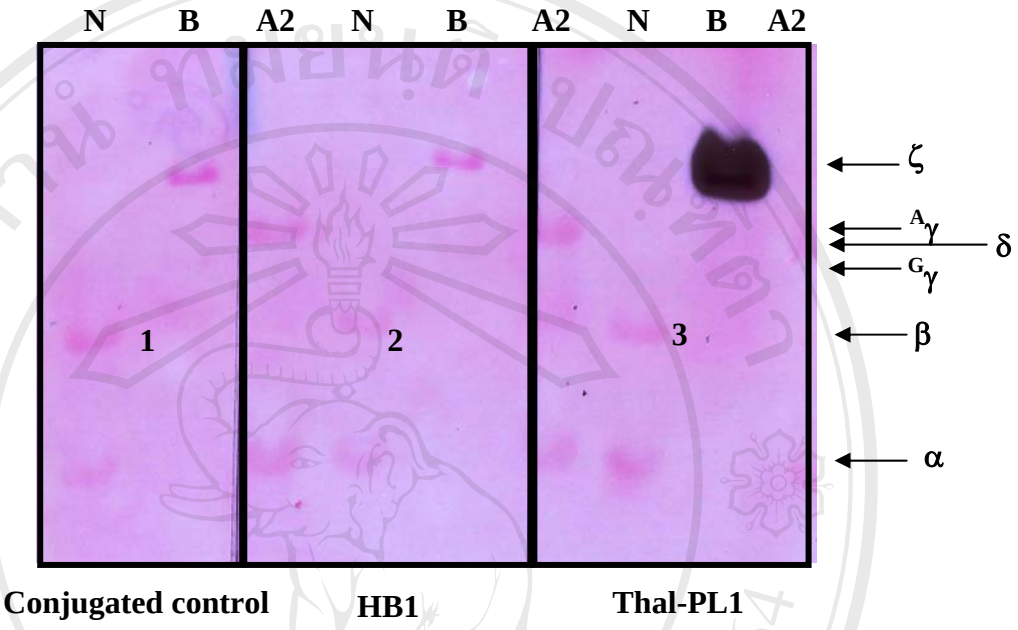
Figure 3.12. Nitrocellulose membrane after blotting with TAU-polyacrylamide gel. Globin chains on the TAU-polyacrylamide gel were transferred to nitrocellulose membrane by semi-dry blotting. The nitrocellulose membrane was stained with Ponceus S dye. The nitrocellulose membrane was cut into three strips, as indicated by dashed lines, for determination of the specificities of purified mAbs HB1, Thal-PL1, and conjugated control.



N = Normal adult hemolysate, **B =** Hb Bart's hydrops fetalis hemolysate,

A_2 = Purified hemoglobin A_2

Figure 3.13. Western blot analysis of mAb HB1 and Thal-PL1. Chemiluminescence system was used to detect the antigen-antibody reaction between the purified mAbs HB1 and Thal-PL1 and the globin chains on the nitrocellulose membranes. Each membranes strip was reacted with the following antibodies: No. 1, 0.1% Tween-PBS without primary antibodies (conjugated control); No. 2, 10 μg/ml purified mAb HB1; No. 3, 10 μg/ml purified mAb Thal-PL1.



N = Normal adult hemolysate, **B =** Hb Bart's hydrops fetalis hemolysate, **A_2** = Purified hemoglobin A_2

Figure 3.14. Image overlay between nitrocellulose membrane and the western blot x-ray film. The membrane was stained with Ponceus S dye before overlaid with the western blot x-ray film. The appeared band on the x-ray film was at the position of ζ globin chain.