CHAPTER III

RESULTS

3.1 Construction of plasmid expression vector encoding hAHSP-BCCP fusion protein

3.1.1 Amplification of hAHSP coding region

The hAHSP domain was amplified from pGEX2T-hAHSP plasmid vector using a pair of primer that was designed to have the short restriction sequences for *NdeI* and *EcoRI* at the upstream and downstream of hAHSP gene, respectively. The amplified product of 308 bp corresponding to cDNA encoding hAHSP protein was demonstrated by agarose gel electrophoresis (Figure 3.1, lane 1). The digested PCR product was ligated into the appropriately cut pAK400CB plasmid vector (Figure 3.2, lane 3). The resulting vector named pAK400CB-hAHSP (Figure 3.3) was transformed into the *E. coli* Nova Blue and the chloramphenicol-resistant colonies were selected for purification of the plasmid vector. The purified pAK400CB-hAHSP with molecular size approximately 4.3 kb was verified again by restriction fragment analysis with *Nde* I and *EcoR* I (Figure 3.4, lane 2), then the product from restriction enzyme treatment was reamplified by PCR (Figure 3.4, lane 5) and subjected for DNA sequencing. The nucleotide of the inserted sequence in pAK400CB-hAHSP was completely identical to the nucleotide sequence of the known hAHSP gene. Therefore, the pAK400CB-hAHSP was used to produce biotinylated hAHSP. Then,

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produced hAHSP recombinant protein was used as immunogen for monoclonal antibody production.

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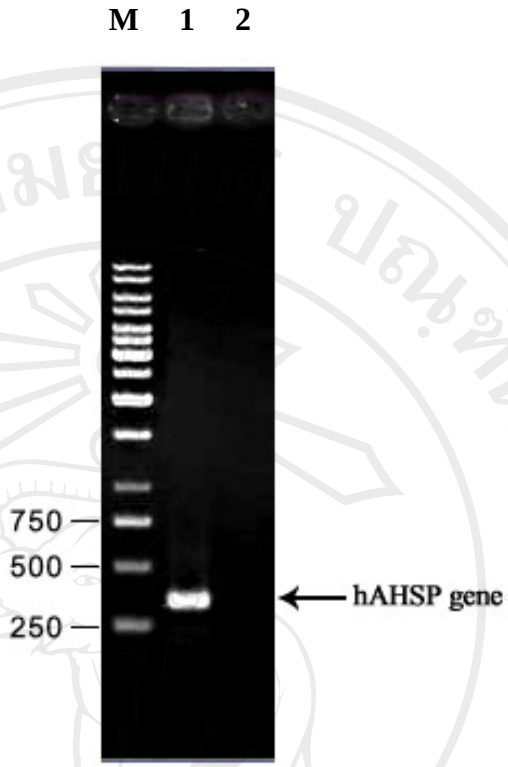

Figure 3.1 Agarose gel electrophoresis of amplified PCR product. The hAHSP gene was amplified from the pGEX2T-hAHSP plasmid vector by PCR. Lane 1: amplified PCR products of hAHSP gene with 308 bp, lane 2: product from non added template. DNA marker (1 kb ladder; Fermantas, MA, USA) are indicated in lane M.

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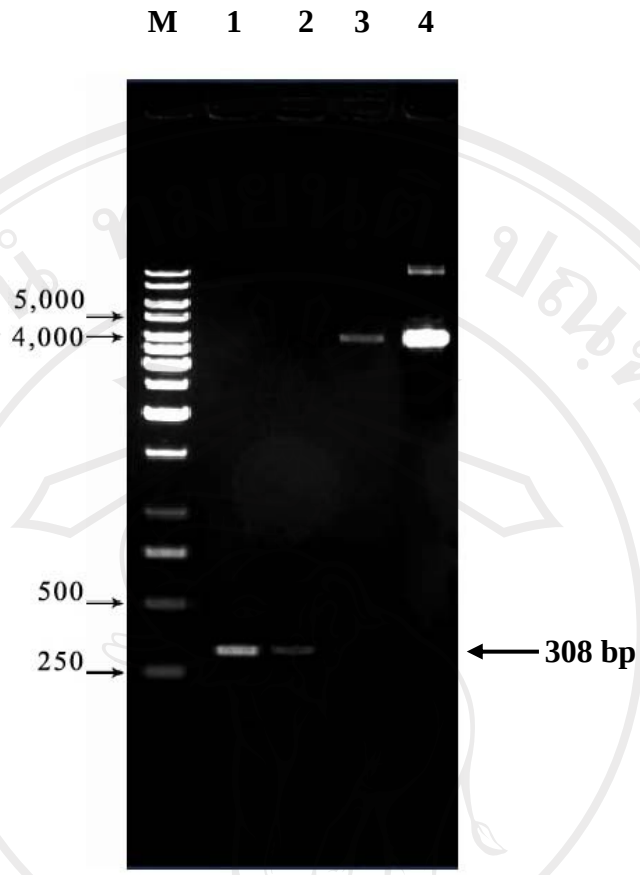

Figure 3.2 Agarose gel electrophoresis revealed PCR product of hAHSP (308bp) which was amplified from pGEX2T-hAHSP (land 1), the purified hAHSP fragment after *NdeI* and *EcoRI* treatment (land 2), the purified product from *NdeI* and *EcoRI*-treated pAK400CB plasmid vector (land 3) and undigested pAK400CB plasmid vector (land 4). DNA marker (1 kb ladder; Fermantas, MA, USA) are indicated in lane M.

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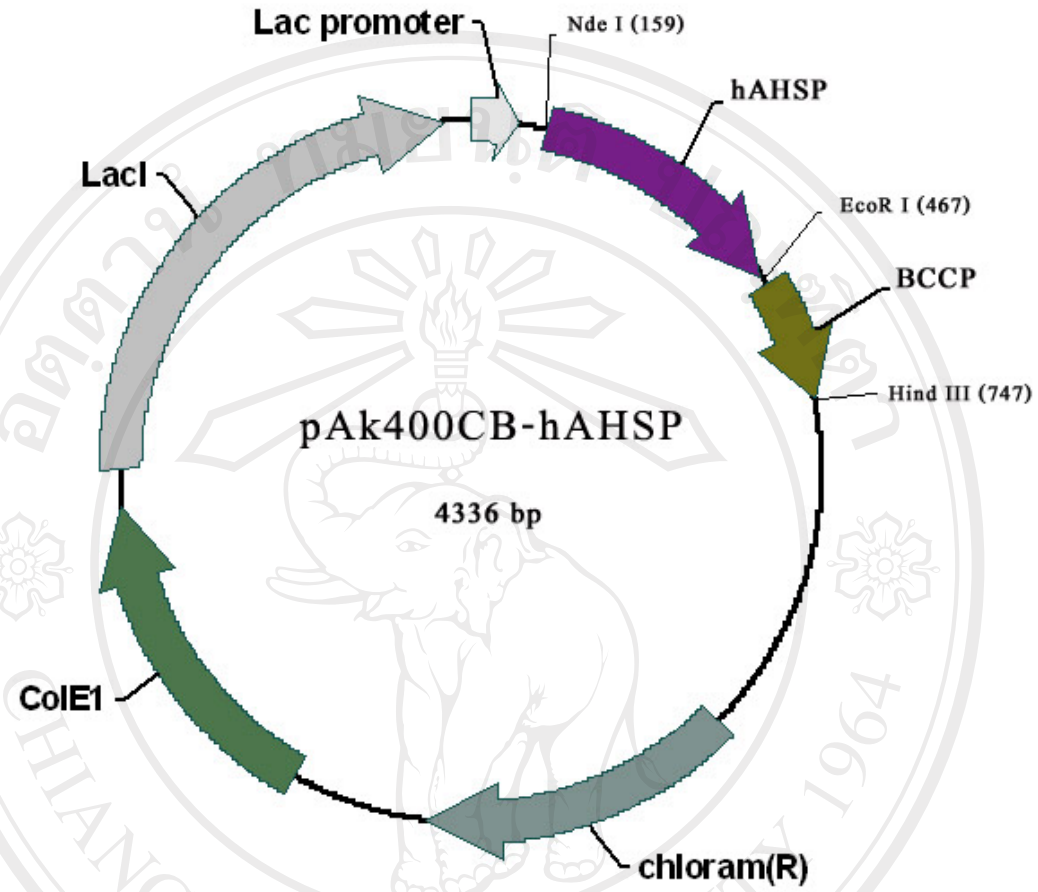

Figure 3.3 Schematic illustration represents the pAK400CB-hAHSP vector. The hAHSP was inserted between the *Nde* I and *EcoR* I restriction sites; the origin of replication (ColE1), lac promoter, lac repressor (lacI), chloramphenicol resistant gene (chloram(R)) and BCCP are shown.

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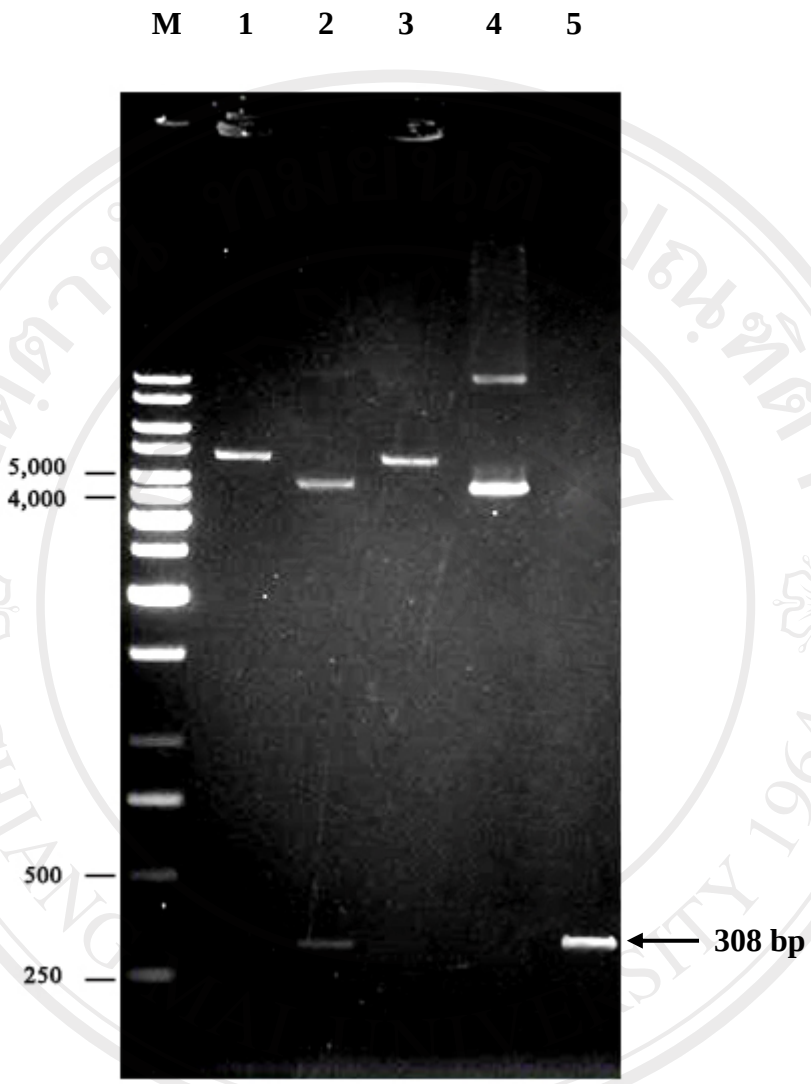

Figure 3.4 Analysis of plasmid vector purified from *E.coli* Nova Blue strain harboring pAK400CB-hAHSP. The plasmid vector was digested with either *NdeI* (land 1), or *EcoRI* (land 3). Inserted hAHSP gene was retrieved by double-digestion with *NdeI* and *EcoRI* (land 2). Land 4 demonstrates the uncut vector and lane 5 show re-amplification of hAHSP gene. DNA marker (1 kb ladder; Fermantas, MA, USA) are indicated in lane M.

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3.2 Production of biotinylated hAHSP-BCCP fusion protein

The plasmid pAK400CB-hAHSP from individual clones were purified and characterized by reamplification using PCR and restriction fragment analysis with *Nde*I and *EcoR*I. Then the pAK400CB-hAHSP was transformed into *E. coli* strain Origami B, Nova Blue, TG1 and XL-1 Blue. The selected clones of *E.coli* (Origami B, Nova blue, TG-1, XL1-blue) containing pAK400CB-hAHSP were grown in LB broth with IPTG to allow protein expression. The bacteria culture was centrifuged and pellet was solubilized in protein extraction reagent. Then supernatant that containing the recombinant protein was obtained. To verify the presence of biotinylated hAHSP-BCCP fusion protein in bacterial lysate, indirect ELISA and Western immunoblotting were carried out.

3.2.1 Detection of biotinylated hAHSP-BCCP fusion protein by indirect ELISA

Indirect ELISA was performed to determine whether the recombinant protein produced and biotinylated *in vivo*. The ELISA plate was coated with avidin for capturing the biotinylated hAHSP-BCCP, followed by addition of rabbit anti-hAHSP polyclonal antibody (pAb) and swine anti-rabbit immunoglobulins conjugated HRP to detect the bound fusion proteins. By this system, only hAHSP-BCCP that was biotinylated by the *E.coli* biotin ligase showed the positive signal. As show in Figure 3.5, rabbit anti-hAHSP polyclonal antibody strongly reacted to recombinant protein hAHSP and hAHSP-BCCP from *E. coli* (Origami B and TG-1) but did not react with hAHSP-BCCP from *E. coli* (Nova Blue and XL1 Blue) or CD147-BCCP control. As expected, CD147-BCCP indicated positive reactivity with CD147 mAb M6-1E9. In addition, both of captured antigens, hAHSP-BCCP and CD147-BCCP strongly

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reacted with BCCP mAb indicating that recombinant protein hAHSP-BCCP and CD147-BCCP were biotinylated.

3.2.2 Analysis of hAHSP-BCCP fusion protein by Western immunoblotting

The bacterial lysate containing hAHSP-BCCP fusion protein was separated in 12% polyacylamide gel electrophoresis under reducing condition. Then the proteins were transferred to nitrocellulose membrane by electroblotting, then, probed with rabbit anti-hAHSP polyclonal antibody (pAb). Following, incubation with HRP-conjugate swine anti-rabbit immunoglobulins, an immuno-reactive band with molecular weight of approximately 30 kDa was visualized (Figure 3.6). The result showed that rabbit anti-hAHSP polyclonal antibody (pAb) reacted with hAHSP-BCCP protein from Origami B and TG1 strain but did not react with hAHSP-BCCP from Nova Blue and XL-1 Blue strain (data not shown). Moreover, rabbit anti-hAHSP pAb did not detect any cross-reactive protein in pAK400CB-CD147 from Origami B (Figure 3.7) or Origami B cell lysate (Figure 3.6).

As demonstrated by both ELISA and Western immunoblotting, the hAHSP-BCCP fusion protein was efficiently expressed and biotinylated in *E.coli* Origami B. Then, biotinylated hAHSP-BCCP fusion protein was further purified and immobilized on streptavidin magnetic beads prior for immunization.

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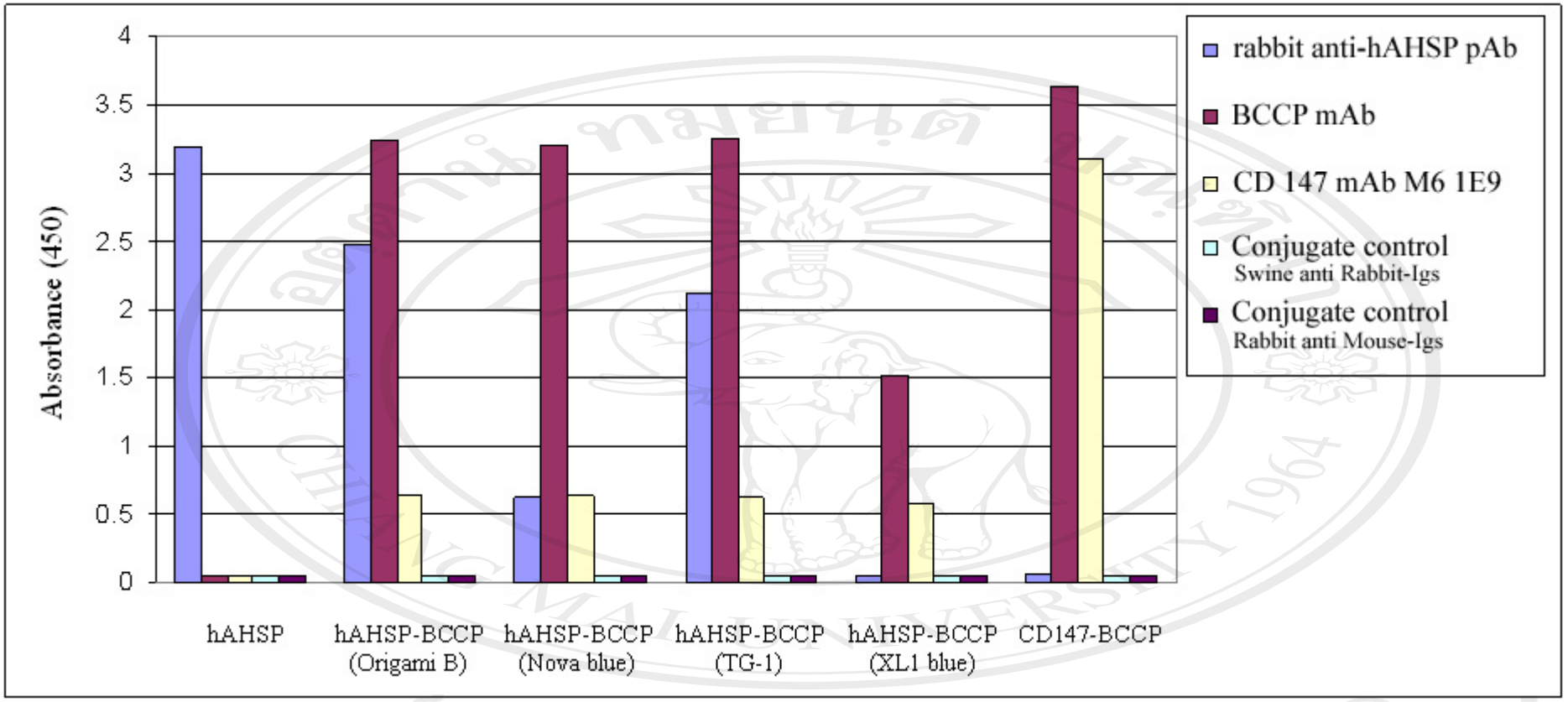

Figure 3.5 Detection of biotinylated hAHSP-BCCP fusion protein from various strains of *E. coli* by indirect ELISA. The biotinylated fusion proteins were captured by avidin which was coated into ELISA plate. The rabbit anti-hAHSP polyclonal antibody was added followed by HRP-conjugated swine anti-rabbit immunoglobulins or BCCP mAb was added followed by HRP-conjugated rabbit anti-mouse immunoglobulins. The bacterial extract containing CD147-BCCP fusion protein used as a control.

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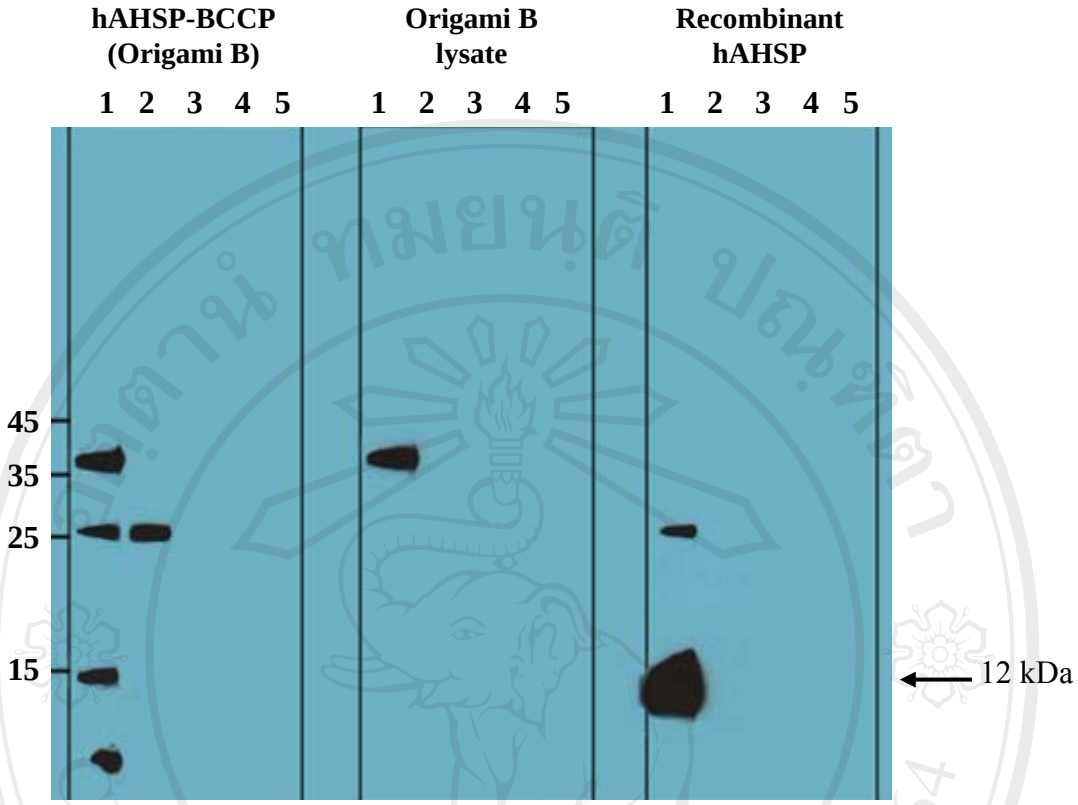

Figure 3.6 Analysis of biotinylated hAHSP-BCCP fusion protein by Western immunoblotting. The protein extract from Origami B harboring pAK400CB-hAHSP, Origami B lysate and recombinant hAHSP were separated by reducing SDS-PAGE, subsequently electroblotted onto nitrocellulose membrane. The proteins were probed with rabbit anti-hAHSP pAb (lane 1), BCCP mAb (lane 2), pooled CD147 mAb (M6-1B9, M6-1E9 anti-CD147 mAb (lane 3) followed by HRP-conjugated swine anti-rabbit immunoglobulins or HRP-conjugated rabbit anti-mouse immunoglobulins antibody. The immuno-reactive bands were visualized by chemiluminescent detection system. The conjugated controls were shown in lane 4 and 5. Molecular weight markers (kDa) are indicated in the left.

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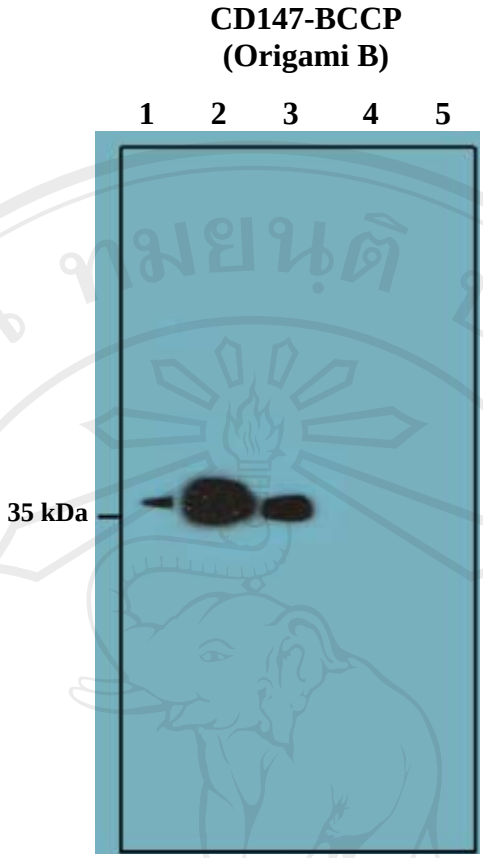

Figure 3.7 Analysis of biotinylated CD147-BCCP fusion protein by Western immunoblotting. CD147-BCCP fusion protein was run onto SDS-PAGE gel under reducing condition and blotted onto nitrocellulose membrane. The membrane were probed with rabbit anti-hAHSP polyclonal antibody (lane 1), BCCP mAb (lane 2), pooled CD147 mAb (M6-1B9, M6-1E9) (lane 3) followed by HRP-conjugated swine anti-rabbit immunoglobulins or HRP-conjugated rabbit anti-mouse immunoglobulins antibody. The immuno-reactive bands were visualized by chemiluminescent detection system. The conjugate control for HRP-conjugated swine anti-rabbit immunoglobulins and HRP-conjugated rabbit anti-mouse immunoglobulins were shown in lane 4 and 5, respectively. Molecular weight markers (kDa) are indicated in the left.

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3.3 Purification of biotinylated hAHSP-BCCP fusion protein by streptavidin-coated magnetic beads

Biotinylated hAHSP-BCCP fusion protein was concentrated and separated from the bacterial proteins by trap on the streptavidin-coated magnetic beads. The hAHSP-BCCP-immobilized magnetic beads were further used as immunogen to produce antibody against hAHSP. To verify the binding of hAHSP-BCCP on the magnetic beads, indirect immunofluorescence staining was performed using hAHSP polyclonal antibody. As shown in Figure 3.8, staining hAHSP-BCCP beads with rabbit anti-hAHSP polyclonal antibody showed high fluorescence intensity when compared with control indicating the immobilization of the hAHSP-BCCP fusion protein on the beads. Whereas, rabbit anti-hAHSP polyclonal antibody did not react with CD147-BCCP beads. Moreover, uncaptured streptavidin beads did not react with the rabbit anti-hAHSP polyclonal antibody ensuring the specificity of the test. Thus, the hAHSP-BCCP beads were used for mouse immunization.

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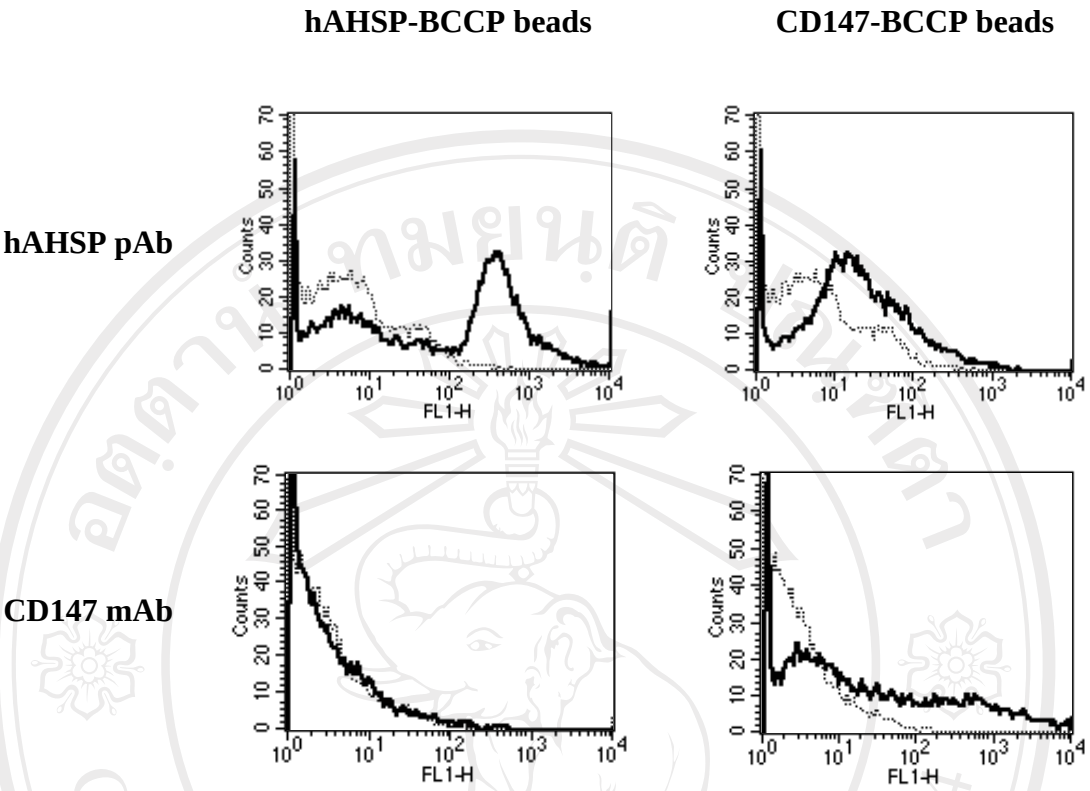

Figure 3.8 Flow cytometric analysis of captured streptavidin magnetic beads. The hAHSP-BCCP or CD147-BCCP was captured on streptavidin magnetic beads. The hAHSP-BCCP-beads and CD147-BCCP-beads were stained with hAHSP polyclonal antibody or CD147 mAb and detected by flow cytometry. Solid lines represent the immunofluorescence profiles of beads stained with indicated antibodies and dashed lines represent background fluorescence of conjugate control.

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3.4 Mouse immunization

In an attempt to produce hAHSP mAbs, the standard hybridoma technique was employed. Two groups of BALB/c mice were immunized three times at twice a week with two immunogens, hAHSP-BCCP or hAHSP.

3.4.1 Antibody responses in BALB/c mice after immunization with hAHSP-BCCP beads

Two female BALB/c mice, mouse A and B, were intraperitoneally immunized three times with hAHSP-BCCP beads from *E.coli* Origami B strain at twice a week for 3 times. Sera were collected from the immunized mice 7 days after immunization. The obtained mouse sera were screened for anti-hAHSP antibodies by indirect ELISA. The antibody responses were gradually increased from titer 1:100 after first immunization to 1:12,800 at third immunization for mouse A and titer 1:100 to 1:6,400 for mouse B (Figure 3.9). Therefore, mouse A which high titer was selected for monoclonal antibody production.

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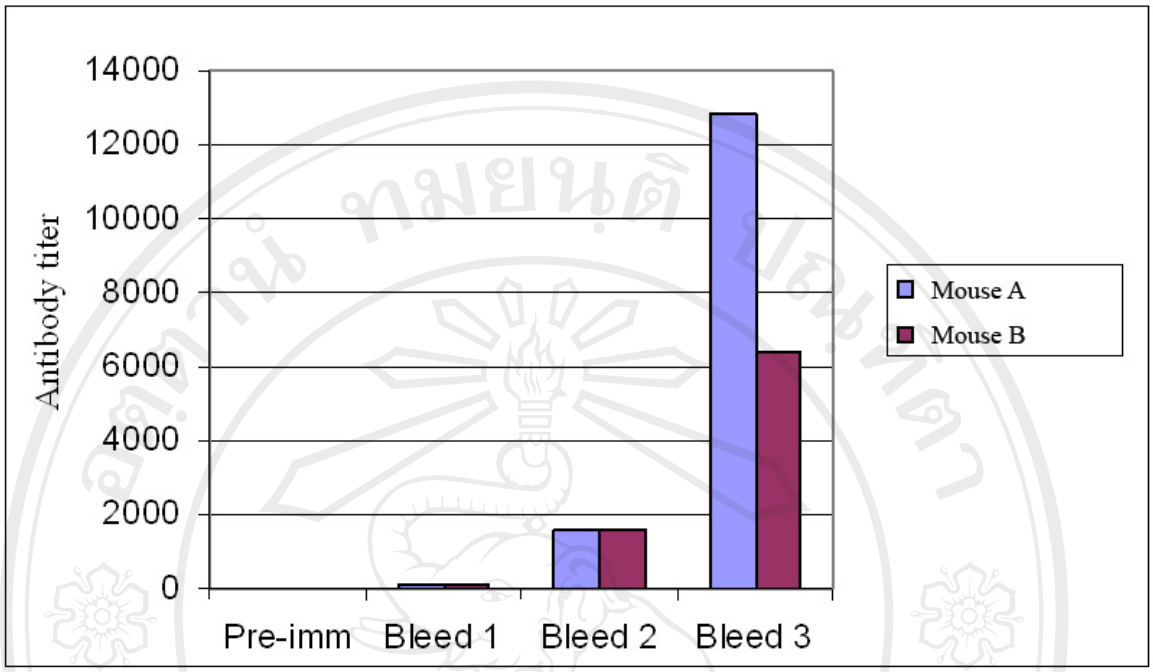

Figure 3.9 Antibody responses from two mice "A and B" immunized with hAHSP-BCCP beads. Sera were collected seven days after indicated immunization and determined for anti-hAHSP antibody by indirect ELISA.

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3.4.2 Antibody responses in BALB/c mouse after immunization with recombinant protein hAHSP

A female BALB/c mouse was immunized with 100 μg of hAHSP using Freund's adjuvant at two-week intervals. The antibody responses were determined by indirect ELISA. It was found that antibodies titer was 1:20,000 after the third immunization (Figure 3.10). After repeated immunization, this mouse was sacrificed for monoclonal antibody production.

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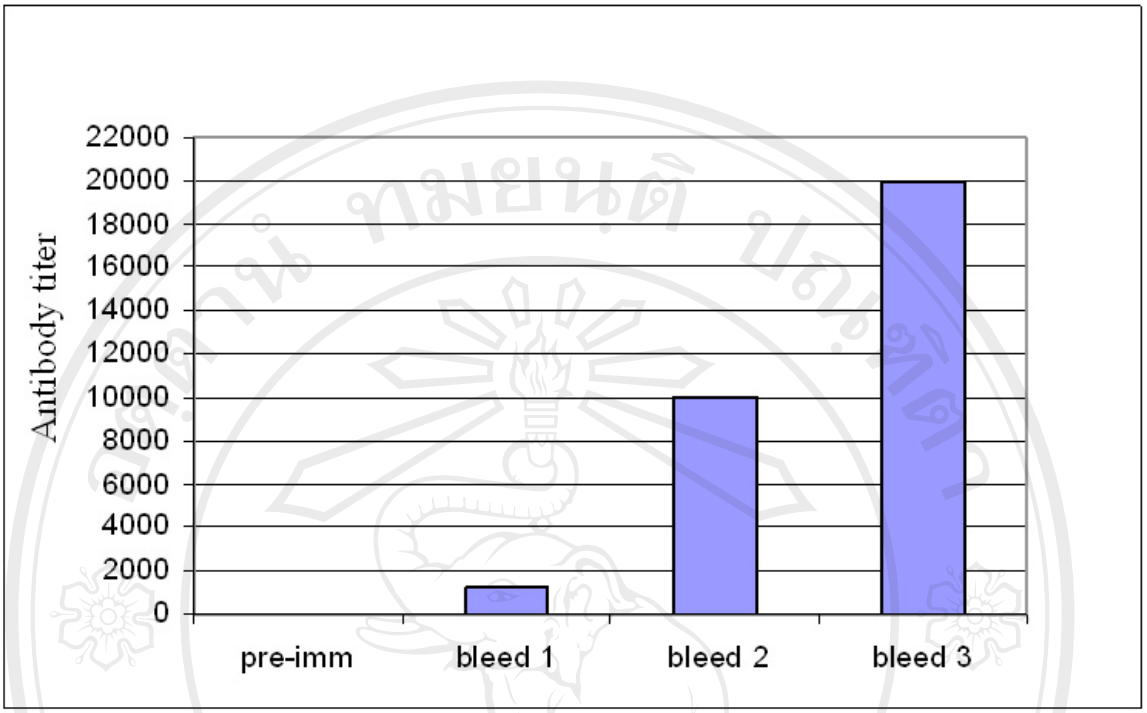

Figure 3.10 A female BALB/c mouse was immunized with 100 µg hAHSP in Freund's adjuvant. Sera were collected at pre-immunization and after 7 days of indicated immunization. Antibody response was determined in mouse sera by indirect ELISA.

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3.5 Production of monoclonal antibody against hAHSP

To generate hybridomas produced monoclonal antibody against hAHSP, splenocytes from immunized mice with high titer of antibody response 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-hAHSP antibodies by indirect ELISA.

In the first fusion experiment, mouse A that immunized with hAHSP-BCCP beads was selected for monoclonal antibody production. After fusion, cultures supernatants from 310 hybridomas were screened for anti-hAHSP antibodies by indirect ELISA. The hAHSP-BCCP or CD147-BCCP was added into avidin-coated wells. Then, culture supernatants were added into each well followed by anti-mouse Igs-HRP and the color was developed by adding TMB substrate. For screening, hybridomas producing mAb which showed reactivity to hAHSP-BCCP but did not react to CD147-BCCP will be collected. From this experiment, culture supernatant from all hybridoma tested clones showed negative reactivity with both hAHSP-BCCP and CD147-BCCP.

In the second fusion experiment, BALB/c mouse immunized with recombinant protein hAHSP was used for fusion. After fusion, the results showed that 15% of hybridoma clones were grown. Then, culture supernatants from 163 hybridomas were screened by indirect ELISA using recombinant hAHSP protein as antigen. It was found that culture supernatant from two hybridomas showed strongly positive with recombinant hAHSP protein. The positive hybridoma clones were then re-cloned by limiting dilution. Afterward, culture supernatant containing mAb from single clone

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was confirmed against hAHSP by indirect ELISA. Then, positive clones was propagated and named MT-hAHSP1 and MT-hAHSP2.

3.6 Characterization of the generated mAbs MT-hAHSP1 and MT-hAHSP2

The reactivity of generated hAHSP mAbs named MT-hAHSP1 and MT-hAHSP2 were examined by indirect ELISA and Western immunoblotting

3.6.1 Characterization of the generated hAHSP mAbs by ELISA

All hAHSP mAbs (MT-hAHSP1 and MT-hAHSP2) were characterized by indirect ELISA using hAHSP recombinant protein and hAHSP-BCCP from various bacteria strains including Origami B, Nova Blue, TG1 and XL-1 Blue. As shown in Figure 3.11, MT-hAHSP1 and MT-hAHSP2 strongly reacted to hAHSP but did not react to hAHSP-BCCP., Whereas rabbit anti-hAHSP pAb, as positive control, strongly reacted to hAHSP, hAHSP-BCCP (Origami B and TG1) and showed weakly positive with hAHSP-BCCP (Nova Blue) but did not react to hAHSP-BCCP from XL-1 Blue. In addition, both of captured antigens, hAHSP-BCCP and CD147-BCCP, reacted with BCCP mAb. This result indicated that MT-hAHSP1 and MT-hAHSP2 recognized to recombinant protein hAHSP but negative to hAHSP-BCCP.

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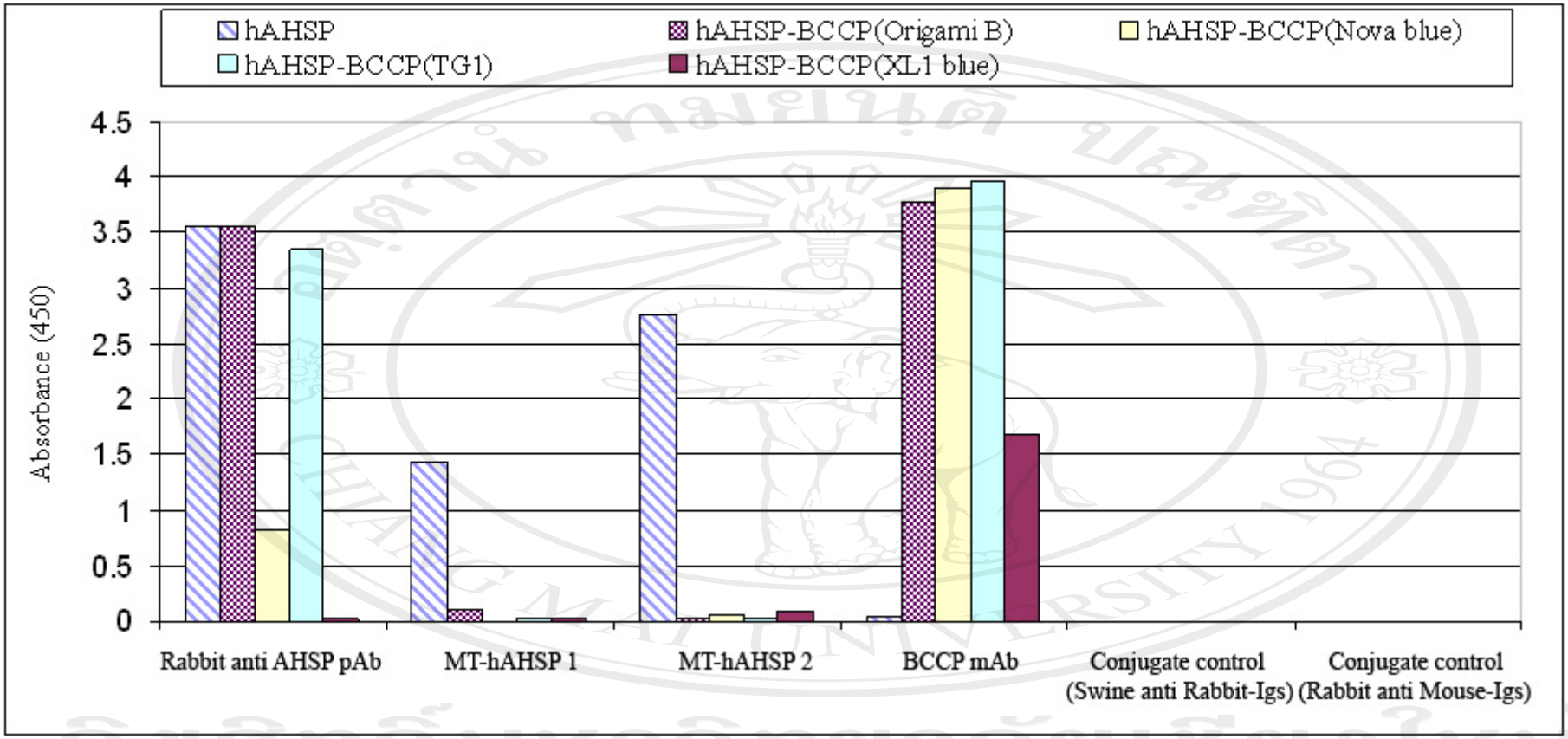

Figure 3.11 Characterization of generated mAbs against hAHSP. The indirect ELISA was performed by using various hAHSP from *E. coli* strains as antigen. The culture supernatant from indicated mAbs were screened. Conjugate control was obtained from well without adding culture supernatant.

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3.6.2 Characterization of the generated hAHSP mAbs by Western immunoblotting

The hAHSP or hAHSP-BCCP from various bacterial strains (Origami B, Nova Blue, TG1 and XL-1 Blue) and CD147-BCCP were separated by SDS-PAGE under reducing condition. After blotting on nitrocellulose membrane, the membrane was probed with the generated MT-hAHSP1, MT-hAHSP2 or rabbit anti-hAHSP pAb. In this study, rabbit anti-hAHSP pAb showed positive signal with hAHSP and hAHSP-BCCP at the molecular weight approximately of 12 kDa. Then, both hAHSP rabbit pAb and BCCP mAb reacted to hAHSP-BCCP from *E.coli* Origami B strain that had a band of biotinylated protein at the molecular weight approximately of 27 kDa. In contrast, Origami B control did not show biotinylated protein band. In addition, the results showed that both of MT-hAHSP1 and MT-hAHSP2 did not react to hAHSP, hAHSP-BCCP and CD147-BCCP (data not shown).

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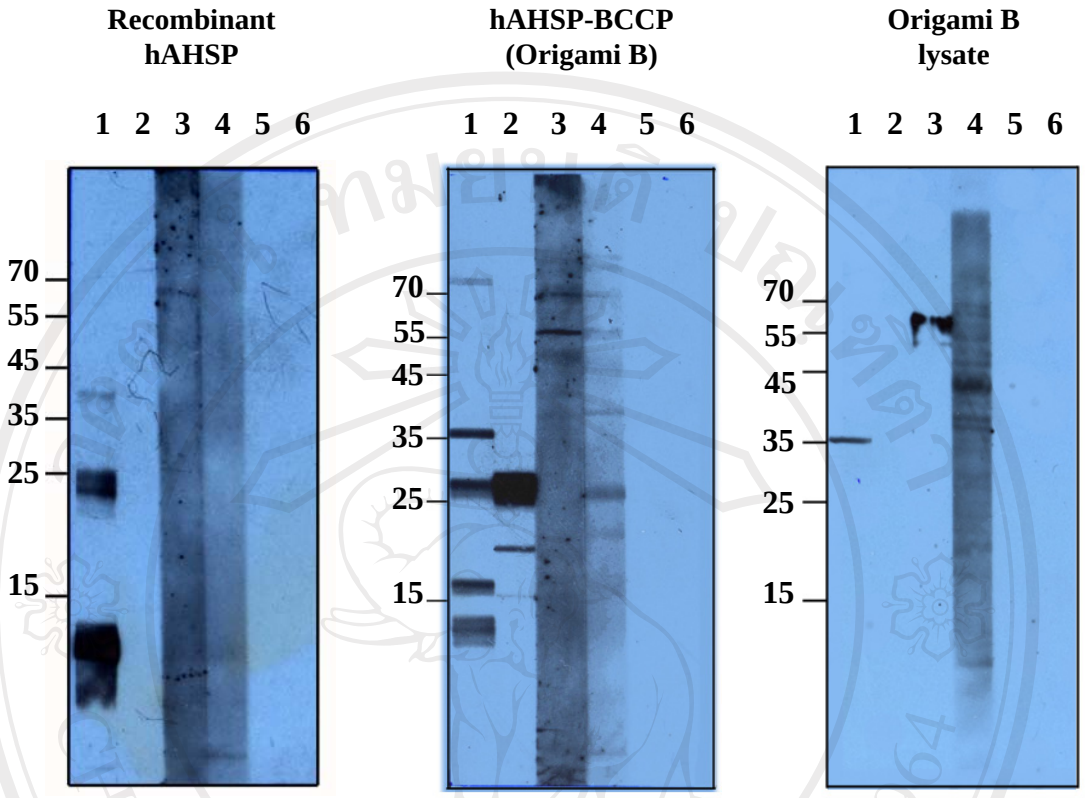

Figure 3.12 SDS-PAGE and Western blot analysis of produced mAb to hAHSP. The hAHSP, hAHSP-BCCP (Origami B) and Origami B bacterial lysate were run in 10% SDS-PAGE. After proteins were transferred on to nitrocellulose membrane, The produced hAHSP mAbs were added and the antigen-antibody reaction was developed by enhanced chemiluminescence detection system. Each membrane strip was reacted with the following antibodies: Lane 1, rabbit anti-hAHSP pAb; Lane 2, BCCP mAb; Lane 3, MT-hAHSP1; Lane 4, MT-hAHSP2; Lane 5, conjugate control of swine anti rabbit Igs-HRP; Lane 6, conjugate control of rabbit anti mouse Igs-HRP. Molecular weight markers (kDa) are indicated in the left.

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