Chapter 2. EXPERIMENTAL PROCEDURES

2.1 Phenotyping

Hp phenotyping was conducted on a native polyacrylamide gel electrophoresis (PAGE) using hemoglobin-supplemented serum or plasma. Hemoglobin was purified from lysed red blood cells as previously described (60). Briefly, 6 μl plasma and 3 μl of 40 mg/ml hemoglobin were premixed and equilibrated with 3 μl of a sample buffer (containing 0.625 M Tris base (pH 6.8), 50% glycerol (v/v), and 0.125 mg/l bromophenol blue) followed by electrophoresis on a 7% native polyacrylamide gel (pH 8), with a 5.5% polyacrylamide (26.5:1 acrylamide: bis-acrylamide) employed as a top stacking gel (pH 6.8). Electrophoresis was conducted at an initial voltage of 120 V and increased to 150 V when the dye front reached the separating gel. After electrophoresis, the Hp-hemoglobin complexes were visualized by shaking the gel in freshly prepared peroxidase substrate (0.5 ml of dimethyl sulfoxide, containing 25 mg of 3,3'-diaminobenzidine, 49 ml of phosphate-buffered saline, and 100 μl of 35% hydrogen peroxide). The method was confirmed by Western blot using an α-chain specific monoclonal antibody prior to the entire phenotyping (59).

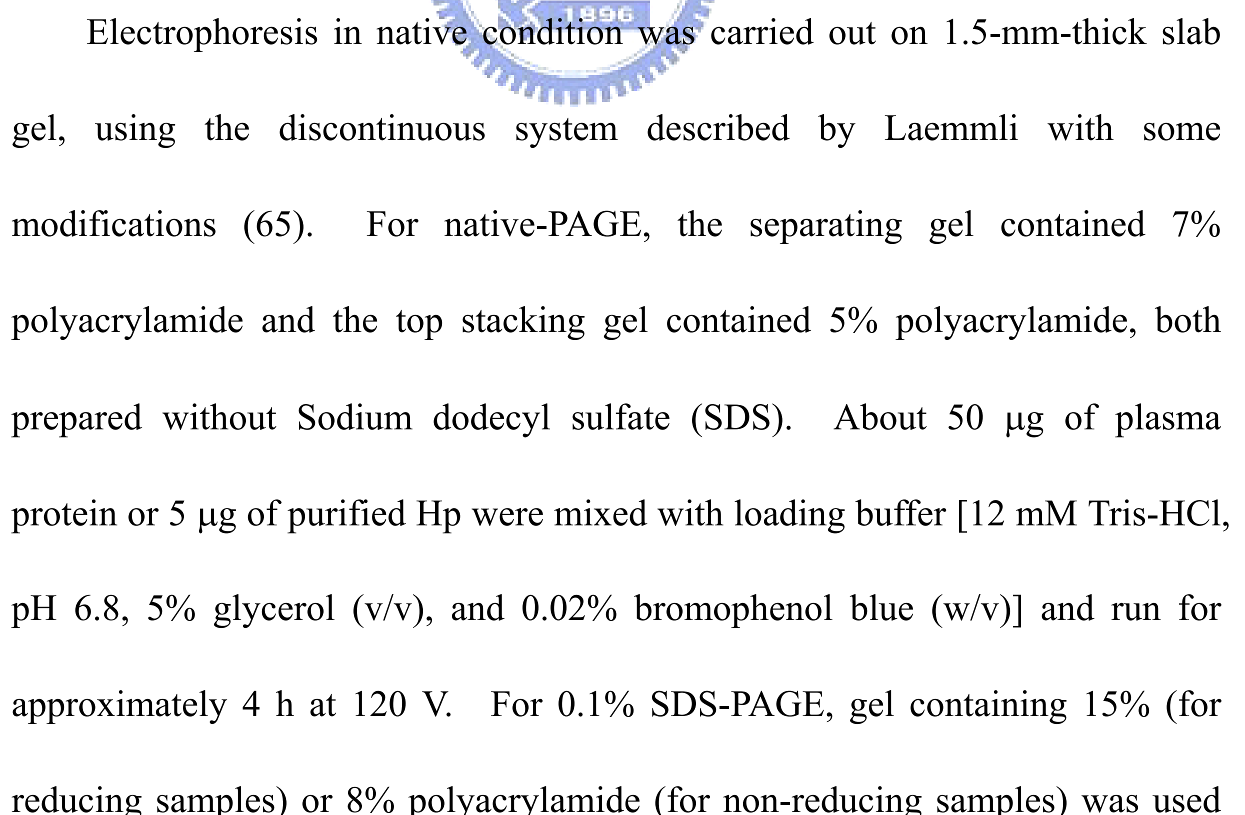
2.2 Purification of Hp1-1, 2-1, and 2-2

The plasma of each Hp phenotype was chromatographed on a mAb based affinity column followed by a HPLC procedure as described previousl (59, 61). Initially, two ml of filtered human plasma of each Hp-phenotype was loaded onto the antibody affinity-column (10 ml in bed volume) at room temperature. The column was then washed further with 50 ml of 0.02 M phosphate buffer containing 0.2 M NaCl, pH 7.4, and then eluted with 50 ml of a freshly prepared 0.15 M NaCl solution at pH 11 adjusted by ammonia (62). Five ml of each fraction was collected in a tube containing 0.25 ml of 1M Tris-HCl buffer, pH 6.8, to immediately neutralize the base. Pooled fractions containing Hp were then concentrated to a final volume of 1 ml using an Amicon-filter (Millipore, Billerica, Massachusetts USA) and filtered through a 0.45 μm membrane. Finally, the protein was rechromatographed on a gel-filtration HPLC column (Superose 12 column, 1x 30 cm, Pharmacia). The homogeneity of each isolated Hp type was greater than 95%.

2.3 Preparation of monoclonal antibodies (mAb)

In this study, Hp 2-1 (containing 1-1 molecules) or 2-2 were used for immunizing mice (n = 3). MAb were produced according to the standard

procedures previously described by us (63). In brief, 100 μg Hp 2-1 or 2-2 were premixed with adjuvant (Sigma-Aldrich, St. Louis MO) and used for each injection (n = 3). Typically, the titers of mouse antisera reached greater than 1 to 10,000 dilutions. Myeloma cell line (FO) was fused with spleen cells from immunized Balb/c mice at a ratio of 1:5. The culture medium (between days 14 and 21 after fusion) was assayed for the production of specific antibodies by a solid phase ELISA using Hp 1-1, 2-1, and 2-2 as a respective antigen. Each monoclonal was established by limiting dilutions at least two times (63, 64).



2.4 Native- and SDS-PAGE

Electrophoresis in native condition was carried out on 1.5-mm-thick slab gel, using the discontinuous system described by Laemmli with some modifications (65). For native-PAGE, the separating gel contained 7% polyacrylamide and the top stacking gel contained 5% polyacrylamide, both prepared without Sodium dodecyl sulfate (SDS). About 50 μg of plasma protein or 5 μg of purified Hp were mixed with loading buffer [12 mM Tris-HCl, pH 6.8, 5% glycerol (v/v), and 0.02% bromophenol blue (w/v)] and run for approximately 4 h at 120 V. For 0.1% SDS-PAGE, gel containing 15% (for reducing samples) or 8% polyacrylamide (for non-reducing samples) was used

with a top stacking gel of 5% polyacrylamide. Typically, 5 μg of the Hp samples were preheated at 100℃ for 10 min in 5x concentrated loading buffer (12 mM Tris-HCl, pH 6.8, 0.4% w/v SDS, 5% v/v glycerol, 0.02% w/v bromphenol blue) with or without 2.88 mM 2-mercaptoethanol. The samples were run for about 1.5 h at 100 V.

2.5 Western blot analysis

Following protein separation by electrophoresis, gels were soaked in transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% w/v SDS, and 20% v/v methanol at pH 8.3. Electrotransfer onto nitrocellulose paper (Pharmacia) was performed at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). Immunoblotting and developing were conducted according to standard procedures previously described (59-61).

2.6 Enzyme-linked immunosorbent assay

Monoclonal antibodies were analyzed for their specificity with three Hp phenotypes using an ELISA binding assay. Briefly, each microtiter well was coated with 50 μl of 200 ng of Hp 1-1, 2-1, or 2-2 followed by incubation at room temperature for 1 h and blocked by 5% skim-milk in PBS. Following washes, 50 μl of cultured medium (1:4 to 1:2048 dilutions) were added and

incubated for 1h, the secondary antibody (goat anti-mouse IgG conjugated with HRP) was then added and developed with 2, 2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) as previously described (66). Non-immunized mouse serum or cultured medium with antibody activity against other unrelated antigens was used as a negative control.

2.7 Determination of plasma Hp levels using a competitive ELISA

In brief, Hp 1-1, 2-1, or 2-2 (200 ng in 50 μl PBS) was immobilized onto a microtiter plate followed by washes and blocking in 5% skim-milk in PBS. After washing, 25 μl of competing antigen (1: 500 diluted human plasma or various amount Hp standard) in PBS containing 1% skim-milk were mixed with 25 μl of 3H8 mAb and added to each well. The plate was incubated at room temperature for 3 h and then washed and developed using the procedure similar to the ELISA binding assay mentioned above. Following washes and secondary antibody incubation (with goat anti-mouse IgG conjugated with HRP), the microtiter plate was developed with 2, 2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) and read at 415 nm.

2.8 Antigenic mapping using synthetic peptide array

Twelve synthetic peptides in one nitrocellulose-array, each containing 15 amino acid residues, were designed corresponding to the residues 1-83 of Hp α1 chain. The specificity of this method was established as previously described (67). The synthetic peptides were prepared under a contract with a local biotechnology company (Genesis Biotech Inc., ROC). Briefly, the peptides were directly synthesized in situ on a nitrocellulose (NC) paper according to the method described (68). The NC membrane in 0.01M Tris buffered saline containing 0.05% (v/v) Tween-20 (TBST), was blocked with 5% skim-milk (w/v) in TBST for 2 h at room temperature followed by washing three times. After incubation with mAb for 2 h and three washes, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) in 0.5% milk/TBST was added and incubated. Finally, following the washes, chemiluminescent substrate (ECL^{TM} Western Blotting System, Amersham) was added, washed, and immediately developed by exposing onto a film.

2.9 Expression of the recombinant Hp α1 and α2 subunits

Total cellular RNA extracted from HepG2 cells (phenotype 2-1) was used for $\alpha1\beta$ and $\alpha2\beta$ cloning (39). The cDNA fragments encoding for Hp α1 and α2 (GenBank Accession Number K01763) subunits were amplified by

proofreading DNA-polymerase (Invitrogen, Carlsbad, CA, USA) and cloned into the *Kpn*I/*Pst*I sites of an *Escherichia coli* expression vector, pQE30 (Qiagen, Hilden, Germany). The plasmids were screened in JM109 and then expressed in M15 [pREP4]. Finally, the sequence of the resulting plasmid pQE30-Hp (α1 or α2) was confirmed by DNA sequencing. For protein expression, cells with pQE-Hp were cultured in Luria-Bertani medium containing ampicillin (100 μg/ml) and 1 mM IPTG at 37˚C on a rotary shaker (220 rpm) for 6 h. After growth, the induced cells were harvested at 8,000 rpm for 20 min and then suspended in 40 ml 20 mM Tris-HCl (pH 8.0). The cell suspension was subjected to sonication on ice for disruption and centrifuged at 13,000 rpm for 20 min at 4˚C. The pellets containing the inclusion body were resuspended in 30 ml 2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100 (pH 8.0) and sonicated as above, followed by centrifugation at 13,000 rpm for 20 min at 4˚C. The pellets were then dissolved in binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM β-mercaptoethanol, pH 8.0) and passed through the syringe filter (0.45 μm). Before loading with cell lysate, the column (Amersham, Uppsala, Sweden) was washed with 0.1 M $NiSO_4$ and equilibrated with binding buffer. The bound protein was subsequently flushed with washing buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM

imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol, pH 8.0) and a 6~0 M urea gradient to allow for protein refolding. Finally, the recombinant protein was eluted by a buffered solution (20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, pH 8.0) with linear gradient to 500 mM imidazole. Fractions containing the recombinant protein were pooled and desalted using a P-2 column (Bio-Rad).

2.10 Site directed mutagenesis

Hp α1 mutants were constructed by using PCR amplification. Plasmid pQE30-Hp α1 was used as a template for mutagenesis. Tyr-32 residue of Hp α1 gene was replaced by a phenylalanine residue using a pair of primers: A152T-F (5'-GCACTCGGTTCGCTTCCAGTGTAAGAACTAC-3') and A152T-R (5'-TCTTACACTGGAAGCGAACCGAGTGCTCCAC-3'). The underlined nucleotides represent the codon targeted for mutagenesis. The PCR was performed using 30 cycles under the following condition: 30 sec at 95°C, 30 sec at 57°C, and 3.5 min at 72°C. All DNA fragments carrying the mutation were sequenced to rule out secondary mutations.

2.11 CD Spectrum

The secondary structure of recombinant α subunits was determined using a computerized Jasco J-715 circular dichroic (CD) spectropolarimeter. Each protein sample was dissolved in 10 mM phosphate buffer at pH 7 with a final concentration of 0.2 mg/ml. About 300 μl of the protein solution were used in a cuvette of 1-mm path length. The obtained spectra were accumulated 25 times at a scanning rate of 50 nm/min. All the data were shown as the mean residue molar ellipticity $[\theta]_{MRW}$ (69).

2.12 Hemoglobin inhibition assay

Each microtiter well was immobilized with 200 ng of Hp 2-1 (in 50 μl PBS), followed by washes and blocking using 5% skim-milk. Each mAb was diluted to give about 50% of the maximal Hp binding. Excess hemoglobin (5 μg) dissolved in ELISA buffer was then premixed with titrated Hp mAb in a final volume of 100 μl. 50 μl of the mixture was then added to each well. The plate was incubated at room temperature for 1h, followed by washes, addition of secondary antibody and development using the procedures similar to the ELISA binding assay described above. Reaction mixtures without the Hp mAb or wells without Hp coating were used as a negative control for non-specific binding (NSB). Binding in the absence of hemoglobin was

expressed as maximal binding. % inhibition was evaluated as: 100[(A − B)/A], where A = Absorbance at 415 nm of maximal binding − NSB; B = Absorbance at 415 nm in the presence of hemoglobin − NSB.