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Evidence of Tandem Repeat and Extra Thiol-groups Resulted in the Polymeric Formation of Bovine Haptoglobin: A Unique Structure of Hp 2-2 Phenotype

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Human plasma Hp is classified as 1-1, 2-1, and 2-2. They are inherited from two alleles *Hp 1* and *Hp 2*, but there is only *Hp 1* in almost all the animal species. Hp 2-2 molecule is extremely large and heterogeneous associated with the development of inflammatory-related diseases. In this study, we expressed entire bovine Hp in *E. coli* as a αβ linear form. Interestingly, the antibodies prepared against this form could recognize the subunit of native Hp. In stead of a complicated column method, the antibody was able to isolate bovine Hp via immunoaffinity and gel-filtration columns. The isolated Hp is polymeric containing two major molecular forms (660 and 730 kDa). Their size and hemoglobin binding complex are significantly larger than that of human Hp 2-2. The amino-acid sequence deducted from the nucleotide sequence is similar to human *Hp 2* containing a tandem repeat over the α chain. Thus, the *Hp 2* allele is not unique in human. We also found that there is one additional -SH group (Cys-97) in bovine α chain with a total of 8 -SH groups, which may be responsible for the overall polymeric structure that is markedly different from human Hp 2-2. The significance of the finding and its relationship to structural evolution are also discussed.

Keywords: Amino-acid sequence, Bovine and human haptoglobin, DNA evolution, Phenotype, Purification

Introduction

Haptoglobin (Hp) is an acute phase protein, which appears in the plasma of all mammals (Wang *et al*., 2001; Faijmakers *et al*., 2003; Gervois *et al*., 2004) and the level rises in response to infection and inflammation. The most-noted biological functions of Hp are to capture released hemoglobin during excessive hemolysis (Kristiansen *et al*., 2001) and to scavenge the free radicals during oxidative stress (Tseng *et al*., 2004b).

Similar to blood types, Hp of human is classified as three phenotypes: Hp 1-1, Hp 2-1 and Hp 2-2 (Schultze and Heremans, 1966). They are genetically inherited from Hp 1 and Hp 2 alleles corresponding to the formation of two polypeptide chains, α1β and α2β, respectively. However, it has been thought that only one allele exists in other mammals (Hp 1-1). Following posttranslational cleavage on αβ polypeptide, α and β chains are formed and then linked by disulfide bridges producing the mature Hp (Fig. 1). Amino-acid sequence of the Hp 2 produced α2 chain (a large peptide containing 142 amino acids) is the same as the Hp 1 produced α1 (a small peptide containing 83 amino acids), but the α2 contains an additional repeated region (59 residues). This repeated region provides an extra -SH group which gives rise to the polymeric form 2-1 and 2-2 as depicted in Fig. 2 (Wejman *et al*., 1984). It has been reported that the polymeric forms are associated with the risk of diabetes, kidney failure, autoimmune, and cardiovascular diseases (Miyoshi *et al*., 1991; Lange, 1992; Levy *et al*., 2000; Hochberg *et al*., 2002).

The gene frequencies of human Hp present geographical differences. In Asia (Taiwan, China, Korea, and Japan), Hp 1 allele frequency of homozygotes (~8%) is lower than that in Africa, Europe, and North America (~20% or greater). It has been proposed that the human Hp 2 originated from Hp 1 about 2 million years ago in India and then gradually displaced Hp 1 (Maeda, 1985; McEvoy and Maeda, 1988; Langlois and Delanghe, 1996). Presumably, α2 is a consequence of a nonhomologous crossing-over within the structural alleles (Hp 1) during meiosis (Maeda, 1985; McEvoy and Maeda, 1988). Thus, only humans possess additional Hp 2-1 and 2-2 phenotypes.

Bovine Hp has been identified as a large molecule with

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polymeric forms (Morimatsu *et al*., 1991; Morimatsu *et al*., 1992), but the molecular mechanism involved in such arrangement remains unknown. In this study, we cloned and expressed recombinant bovine Hp in the *E. coli* expression system. Recombinant Hp was made as an uncleaved linear αβ chain and used for raising the mouse and rabbit polyclonal antibodies. These antibodies were able to recognize the native α or β unit of bovine Hp or both. The IgG of rabbit anti-recombinant Hp was then utilized for the preparation of an immunoaffinity column. The purified Hp shows a copy of α and β chain on reduced SDS-PAGE, in which the molecular weight of α chain mimics that of human α2. Amino-acid sequence alignment (deducted from the nucleotide sequence) of bovine Hp reveals that the α chain possesses a unique tandem repeat. We suggest that Hp 2 allele is not unique in human, but the bovine tandem repeat is markedly different from that of human. Besides the sequence heterogeneity within the tandem repeat, bovine α chain is consisted of an additional -SH group which is responsible for even larger Hp polymers as compared with human. The significance and detailed analysis of the nucleotide sequence of bovine Hp and its relationship to evolution are also discussed.

Materials and Methods

Materials. *Escherichia coli* JM109, M15 [pREP4], the pQE30 expression vector, and the RNeasy Mini kit were purchased from Qiagen. Plasmid preparation and gel-extraction kits were purchased from BD Biosciences. The proofreading DNA-polymerase and dNTP were purchased from Invitrogen. All restriction enzymes were purchased from New England Biolabs. T4-DNA-ligase and isopropyl β-D-thiogalactopyranoside (IPTG) were purchased from Fermentas. Rabbit anti-Goat IgG and Goat anti-mouse IgG were purchased from Chemicon. The HiTrap chelating column, CNBr activated Sepharose 4B and nitrocellulose membrane were purchased from Amersham Biosciences. The P-2 polyacrylamide gel was purchased from Bio-Rad. The ProSieve color proteins of low molecular weight marker (10-120 kDa) were purchased from Cambrex Bio Science Rockland, while the high molecular weight marker (97-584 kDa) were purchased from Sigma. All other chemicals were purchased from Sigma and Merck without any further purification. The buffers used in this report were all filtered through a 0.45 μm filter before using. Bovine plasma of normal healthy Holstein cows (*Bos taurus*) were obtained from the County Livestock Disease Control Center of Pingtung, Taiwan.

Gene cloning and Plasmid construction. Total RNA was isolated and purified from the bovine (*Bos taurus*) liver using the RNeasy Mini kit with the procedures recommended by the manufacturer. An oligo(dT) 18 mer was used for the reverse transcription step. The first strand cDNAs were synthesized with M-MLV reverse transcriptase at 37ºC for 50 min. Gene fragments coding for bovine Hp was amplified by PCR using proofreading DNA-polymerase and oligonucleotide primers (Lai *et al.,* 2007; Emamzadeh *et al*., 2006). The oligonucleotide primers prepared were 5'-TTCCTGCA GTGGAAACCGGCAGTGAGGCCA-3' (forward) and 5'-CGGAA AACCATCGCTAACAACTAAGCTTGGG-3' (reverse). Both *Pst*I and *Hind*III restriction sites were incorporated into the 5' end of the forward and reverse sequence primers. The cDNA of Hp was ligated into the *Pst*I/*Hind*III sites of an *E. coli* expression vector, pQE30, similar to that described previously (Wang *et al*., 2002; Lai *et al*., 2007). The plasmids were screened in JM109 and then expressed in M15 (pREP4).

Expression of Bovine recombinant Hp and isolation of inclusion bodies. *Escherichia coli* [M15 (pREP4)] was transformed with the recombinant plasmid and cultured in 1.2 L of Luria-Bertani (LB) medium containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) at 37ºC on a rotary shaker (Wang *et al*., 2002; Lai *et al*., 2007). When the optical density reached 0.6 at wavelength 600 nm, the expression of bovine recombinant Hp was then induced by 1 mM IPTG at 37ºC for 6 h. The medium was centrifuged at 8,000 *g* for 5 min and washed three times in a wash buffer containing 20 mM Tris-HCl, pH 8.0. The induced cells were then suspended in 40 mL of wash buffer and sonicated for 5 min at 4ºC, followed by centrifugation at 20,000 *g* for 20 min at 4ºC. The pellet containing the inclusion body was resuspended in 40 mL of 2 M urea containing 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton X-100, pH 8.0 and sonicated as above, followed by centrifugation at 20,000 *g* for 20 min at 4ºC. Finally, the pellet was washed twice in wash buffer and stored at −20ºC.

Solubilization of expressed Hp from inclusion bodies. The inclusion bodies were dissolved in a binding buffer containing 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine-HCl, and 1 mM 2-mercaptoethanol, pH 8.0. The mixture was gently stirred at 4ºC for 12 h and the insoluble material was removed by centrifugation at 20,000 *g* for 20 min at 4ºC. Finally, the remaining soluble supernatant was passed through a syringe filter (0.45 μm) and proceeded directly for purification and refolding.

Purification and refolding of expressed Hp. Purification of bovine expressed Hp was conducted using a nickel affinity column (Lai *et al*., 2007). Briefly, a 3 mL-HiTrap chelating column was washed with 15 mL distilled water. After loading 3 mL of 0.1 M $NiSO_4$, the column was extensively equilibrated with a binding buffer described above. The recombinant fusion protein was subsequently loaded onto the column and washed with the same buffer. The bound protein was then treated with a gradient, starting with 6 M urea and finishing at a buffer without urea. Finally, the Hp were eluted using a 30 mL linear gradient starting with an elution buffer containing 20 mM Tris-HCl, 0.5 mM NaCl, 20 mM imidazole, and 1 mM 2-mercaptoethanol, pH 8.0 and ending with the same buffer containing 300 mM imidazole. Protein fractions were pooled and then desalted on a P-2 column equilibrated with 0.05 M ammonium bicarbonate, followed by lyophilization. Protein concentration was determined using a Bio-Rad kit based on the Lowry method (Lowry *et al*., 1951), while using bovine serum albumin as a standard.

SDS-PAGE and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli's method (Laemmli, 1970) with some modifications using 5% polyacrylamide (w/v) on the stacking gel

as previously described (Yang and Mao, 1999). In general, the tested sample was preheated at 100ºC for 10 min in a buffer containing 12 mM Tris-HCl, 0.4% SDS, 5% glycerol, 2.9 mM 2-mercaptoethanol, and 0.02% bromphenol blue, pH 6.8 before loading to the gel. The samples were run for about 1.5 h at 100 V and stained using Coomassie brilliant blue R-250. Western blot analysis was performed similar to that described previously (Song *et al*., 2005; Xu *et al*., 2006b). In brief, the gel was electrotransferred to a nitrocellulose membrane at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a transfer buffer. The transferred membrane was then immersed in 2% gelatin (w/v) in PBS for 1 h at room temperature while shaking gently. After washing with PBS three times, 5 min each, the membrane was incubated with a primary rabbit or mouse polyclonal antibody against bovine Hp (1 : 5000 dilution in PBS washing buffer containing 0.2% (w/v) gelatin and 0.05% (v/v) Tween 20 for 1 h) at room temperature and washed three times for 5 min each. The membrane was then incubated with 1 : 5,000 diluted peroxidase-conjugated anti-IgG in washing buffer for 1 h. In addition, the membrane was washed twice with washing buffer and further washed once with PBS. Finally, the membrane was developed using 3,3-diaminobenzidine (DAB) as a substrate for horseradish peroxidase.

Preparation of polyclonal antibodies against bovine recombinant Hp. Female Balb/c mice (5 to 7 weeks of age) and rabbits (8-10 months of age) were used for raising the antibody against recombinant Hp. In brief, each animal was immunized by subcutaneous and intraperitoneal (only for mouse) injections of purified bovine recombinant Hp. About 1-2 mg of protein in sterile PBS were mixed and homogenized with an equal volume of Freund's complete adjuvant using a 3-way stopcock. A total emulsion of 500 μL containing 200 μg antigen was injected subcutaneously onto the six sites of the animal back with or without one additional intraperitoneal injection for mice or rabbits, respectively. After 21 d, each animal was boosted by 2 intramuscular injections with a total of 500 μL (50% sterile PBS solution and 50% incomplete adjuvant) containing 200 μg of bovine recombinant Hp. Seven to ten days following the final booster, blood was collected in 0.1% EDTA and plasma was obtained. All the procedures were conducted according to the regulation of National Science Council (NSC) and were approved by the Animal Study Group of the University. Titers and binding specificity were tested by ELISA and Western blotting, respectively. In general, the titers achieved were greater than 1 : 10,000 dilutions by an enzyme labeled immunosorbent assay (ELISA) (Chen *et al*., 2004; Xu *et al*., 2006). Additional booster injections were given when necessary.

Preparation of antibody affinity column. Dialyzed IgG fraction of rabbit polyclonal antibody prepared against recombinant Hp in a coupling buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3) was first coupled to CNBr-activated Sepharose-4B according to the method recommended by manufacturer as previously described (Tseng *et al*., 2004a; Yueh *et al*., 2007). Briefly, 5 g of freeze-dried Sepharose (1 g of freeze-dried powder gave about 3.5 mL final volume of gel) was swollen and suspended in 1 mM HCl and immediately washed with 20 × volume of the same solution within 15 min on a sintered glass filter. The gel was then washed with a coupling buffer and degassed. About 15 mL (6 mg/mL) of dialyzed rabbit anti-recombinant Hp IgG in coupling buffer was slowly added into the gel (CNBr-activated Sepharose-4B), while gently rotating for 1 h at room temperature. After which time, the gel was washed with 5 × volume of coupling buffer to remove unbound materials via a sintered glass filter. The gel was then treated with a blocking solution containing 0.1 M Tris-HCl buffer, pH 8.0, for 2 h at room temperature to saturate the remaining reactive-sites. The degassed gel was then washed with 3 cycles of 0.1 M acetate buffer containing 0.5 M NaCl , pH 4 and 0.1 M Tris-HCl containing 0.5 M NaCl, pH 8. Finally, the gel was equilibrated in PBS and packed onto a 1.5 × 25 cm column with a bed volume of approximately 17.5 mL of coupled Sephrose.

Purification of bovine native Hp using antibody affinity-column chromatography. Hp positive bovine plasma was first confirmed by a Western blot. Typically, 1.5 mL of filtered bovine plasma was loaded onto the antibody affinity-column and allowed to enter into the top of the Sepharose at room temperature. After 1 h incubation, the unbound plasma materials were eluted and washed with 45 mL PBS, pH 7.4, and then eluted with 45 mL of a freshly prepared PBS with pH 11 adjusted by ammonia. Three milliliters of each fraction was collected in a tube containing 0.1 mL of 1 M Tris-HCl buffer, pH 6.8, to immediately neutralize the pH value. Pooled fractions containing Hp were then concentrated to a final volume of 0.2 mL using an Amicon centrifugal filter (Millipore).

Further purification of bovine native Hp by gel filtration column. Concentrated solution of affinity-isolated bovine Hp was filtered through a 0.45 μm nylon fiber prior to high performance liquid chromatography (HPLC). The HPLC system (Waters) consisted of two pumps, an automatic sample injector, and a photodiode array detector. Superose-12 column (1 × 30 cm) (Pharmacia) was used for further purification of affinity-isolated Hp. The column was pre-equilibrated with 50 mM ammonium bicarbonate. Affinity-isolated Hp (0.8 mL) was chromatographed on the column at a flow rate of 0.4 mL/min. Fractions containing bovine native Hp, identified by an ELISA and a Western blot, were pooled and concentrated to a final volume of 1 mL using an Amicon centrifugal filter and then lyophilized. The lyophilized bovine native Hp was stored at −80ºC until analyzing.

Phenotyping. Hp phenotype was determined by a native-PAGE using hemoglobin-supplemented serum or plasma (Liau *et al*., 2003). Briefly, 6 μL of plasma were first incubated with 3 μL of 5 mg/mL hemoglobin at room temperature for 10 min and then equilibrated with 3 μL of sample buffer (containing 0.625 M Tris-HCl, pH 6.8, 50% (v/v) glycerol, and 0.125 mg/L bromphenol blue). The reaction mixture was run on a step-gradient polyacrylamide gel (pH 8.8). The bottom gel layer containing a 7% polyacrylamide, while a 5% polyacrylamide (26.5 : 1 acrylamide: bis-acrylamide) was 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 the 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 30% hydrogen peroxide).

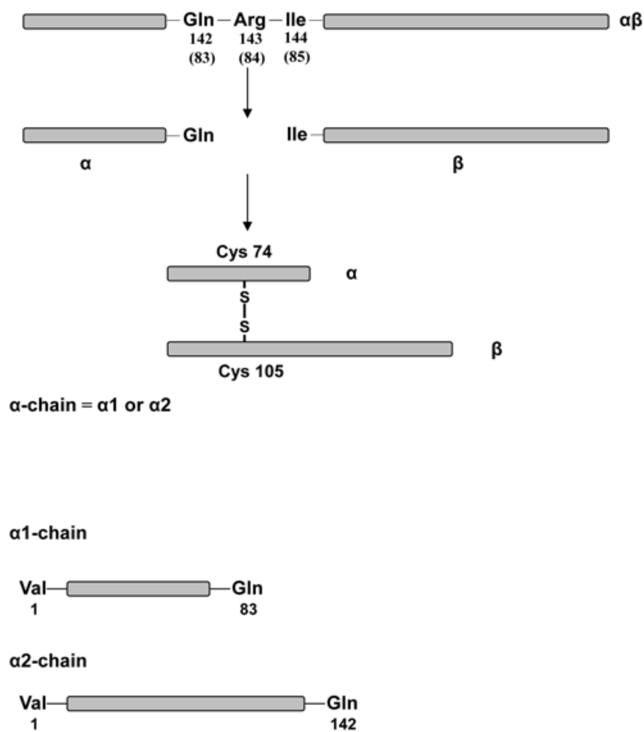

Fig. 1. Posttranslational cleavage between the Arg of α chain and Ile of β chain of human Hp. Following the posttranslational cleavage, the Arg residue is removed forming one single α and β chain. Eventually, the mature Hp is formed by the disulfide linkages between α and β chain with a basic α-β subunit. Depending on the Hp allele, the α chain can be classified as α1 and α2 containing 83 and 142 amino-acid residues, respectively.

Others. Human Hp 1-1, 2-1, and 2-2 were obtained using the procedures previously described (Tseng *et al.*, 2004a).

Results

Cloning and expression of recombined bovine Hp in *E. coli.* We constructed the plasmid using pQE30 as a vector and transformed it into *E. coli.* Protein extract of transformed cells induced by 1 mM IPTG revealed that bovine Hp was efficiently and highly expressed in *E. coli*, in which recombinant Hp accounted for more than 40% of the total cellular proteins as analyzed by SDS-PAGE in a reduced condition (Fig. 3, Lanes 1 and 2). Because substantial amount of recombinant Hp was found in the inclusion bodies, they were solubilized in 8 M urea prior to the isolation. Following renaturation using a urea-gradient from 6 to 0 M directly on a nickel affinity column, the Hp was then eluted using 300 mM imidazole. The isolated Hp shows a single band on SDS-PAGE with an apparent molecular weight of approximately 50 kDa (Fig. 3, Lane 3). This result displays that expressed bovine recombinant Hp is not cleaved into α and β subunits indicating that *E. coli* contains no C1r-like protease (Wicher and Fries, 2004) that is responsible for the cleavage of α and β chains in mammalian cells (Fig. 1).

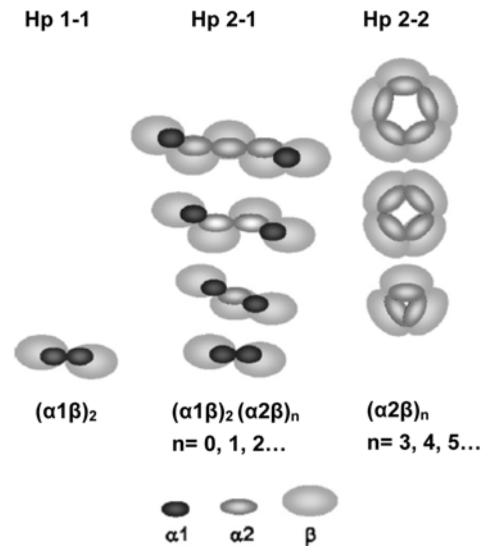

Fig. 2. Schematic drawing of molecular arrangement in human Hp phenotypes. Hp 1-1 possesses the simplest homodimer $(\alpha1\beta)_2$, whereas Hp 2-1 is comprised of polymeric structures in a linear form: a homodimer $(\alpha1\beta)_2$, a trimer $(\alpha\beta)_3$, and other polymers. Here, α represents both α1 and α2 chains. Hp 2-2 is comprised of cyclic structures: a trimer $(\alpha2\beta)_3$ and other cyclic polymers. Each α1, α2, or β contains 83, 142 or, or 243 amino-acid residues, respectively.

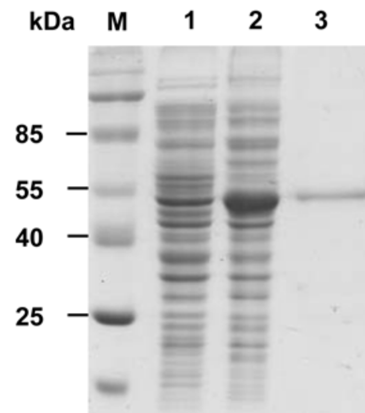

Fig. 3. Expression of recombinant bovine Hp in *E. coli*. Lane M, molecular markers; Lane 1, whole normal cell lysate; Lane 2, whole cell lysate containing expressed bovine Hp; Lane 3, purified recombinant bovine Hp. Cells were incubated at 37ºC for 6 h in the presence of the induction agent IPTG. SDS-PAGE gel (12%) was loaded with samples containing 1% mercaptoethanol.

Characterization of mouse and rabbit polyclonal antibodies prepared against bovine recombinant Hp. We prepared polyclonal antibodies against recombinant Hp (a single αβ polypeptide) in mice and rabbits to test whether the antibodies could recognize plasma native Hp (a cross-linked α-β subunit). Interestingly, we found mouse recombinant Hp antibody to be able to react with both native α and β chains on a Western blot, whereas rabbit antibody only reacted with the α chain (Fig. 4).

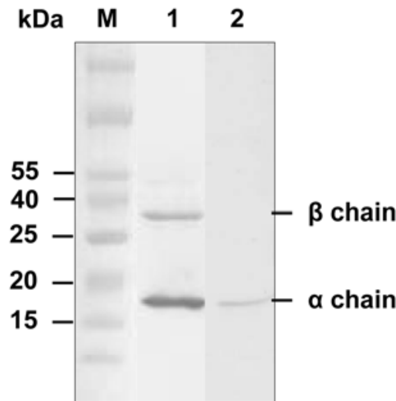

Fig. 4. Western blot of bovine plasma using mouse or rabbit polyclonal antibodies prepared against bovine recombinant Hp. Bovine plasma (1 μL) containing 1% mercaptoethanol was loaded on a 10-15% SDS-PAGE gradient gel. Lane M, molecular markers; Lane 1, developed by mouse anti-bovine recombinant Hp; Lane 2, developed by rabbit anti-bovine recombinant Hp. Notably, mouse antibody recognizes both α and β chains, while rabbit antibody only recognizes the α chain of bovine Hp.

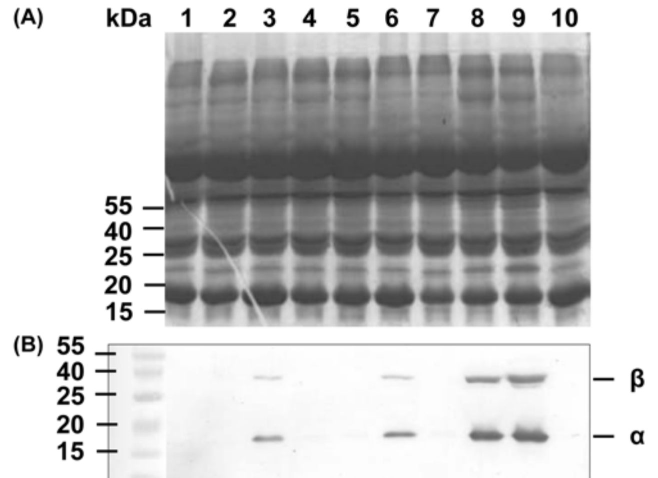

Fig. 5. Typical example of a Western blot of bovine plasma in reducing condition. Normal healthy cow plasma (1 μL) was loaded on a 10-15% SDS-PAGE gradient gel. A: Coomassie blue staining; B: Western blot using mouse anti-recombinant Hp. The data indicate approximately 40% of the normal cows (n = 50) expressed Hp in this study.

Determination of the presence of Hp in bovine plasma. In contrast to humans, it has been reported that bovine Hp is usually not expressed in the plasma (Conner *et al*., 1988; Morimatsu *et al*., 1992; Yoshino *et al*., 1992; Petersen *et al*., 2004). We randomly collected plasma from 50 normal healthy cows and determined whether Hp was present in their plasma using a Western blot. Notably, approximate 40% of the cows evaluated possessed plasma Hp (Fig. 5). Among those who possessed Hp, there was only one single phenotype as judged by the molecular pattern of α chain (Fig. 5).

Purification of bovine native Hp. With respect to the isolation of bovine Hp, a method of using HPLC system combined with anion-exchange, Sephacryl S-300, TSK Phenyl-5PW, and TSK DEAE-5PW columns together has been reported (Morimatsu *et al*., 1991). The procedures however are somewhat complicated and difficult to perform and time-consuming. The yield of final isolated protein is also relatively low. To test whether we could take the advantage of the rabbit polyclonal antibody prepared against linear form of recombinant Hp (a single αβ polypeptide), we attempted to isolate Hp from bovine plasma using an immunoaffinity column. Plasma of cows showing high levels of Hp (Fig. 5) were pooled and applied onto the column using a procedure recommended by the manufacturer. A typical affinity column chromatograph is shown in Fig. 6A, in which a major bound protein was eluted at pH 11. This fraction contained approximately 60% of bovine Hp, as analyzed on a SDS-PAGE in reducing condition. Following another gel-filtration using an HPLC column (Superpose-12) (Fig. 6B), the purification of bovine Hp was achieved with homogeneity almost greater than 90% (Fig. 6C). To identify its phenotype, the isolated bovine Hp was compared with human Hp 1-1, 2-1, and 2-2 standards. Clearly, bovine Hp was comprised of one β and one α chain with a molecular weight similar to that of human α2 (Fig. 6C). We, therefore, tentatively classified bovine phenotype as Hp 2-2.

Comparison of bovine Hp with human Hp 2-2 using SDS-PAGE. The putative bovine Hp 2-2 was further characterized on a 4% SDS-PAGE without a reducing reagent. Fig. 7A demonstrates that bovine Hp is consisted of at least two major molecular forms with molecular weight greater than 600 kDa. Using commercially available molecular markers and human Hp 2-2 as a calibrator (Fig. 7B), the estimated molecular weight of the two major forms of bovine Hp is about 660 and 730 kDa. These polymers totally differ from that of human Hp 2-2 which shows typical polymers following $(\alpha\beta)_n$; where n = 3, 4, 5, ---- up to 10 in this case. Of notice, $(\alpha\beta)_3$ was ran out of the gel (Fig. 7B). It remains ambiguous as to whether bovine Hp could be designated as a typical Hp 2-2.

Binding ability of isolated and native bovine Hp to hemoglobin. In the next experiment, we compared the hemoglobin binding ability of isolated bovine Hp with native Hp present in the plasma using a native-PAGE (5-7% step gradient). Fig. 8 reveals that the formation of hemoglobin complex of isolated bovine Hp consisted of two large molecular forms that were almost identical to that of native Hp in bovine plasma. This result suggests that the Hp isolated under our experimental condition did not alter its molecular form and essentially retained the hemoglobin binding ability. The “size” of the bovine Hp-hemoglobin complex was markedly greater than that of human Hp 2-1 and 2-2 (Fig. 8).

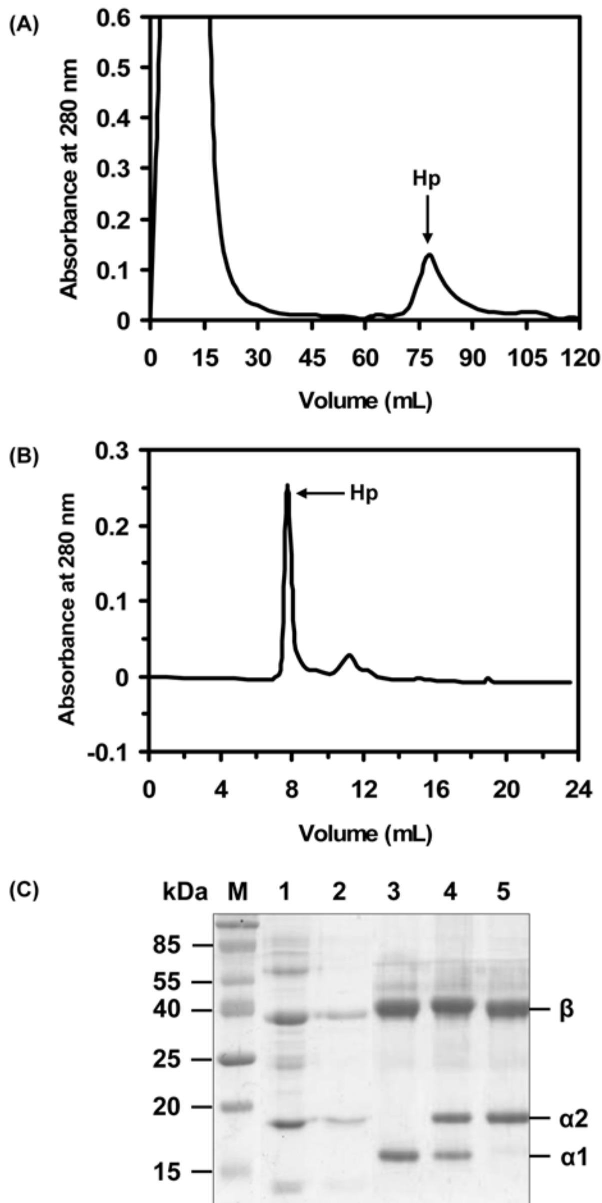

Fig. 6. Purification of bovine Hp using immunoaffinity column chromatography. A: For each chromatography, approximate 1.5 mL of bovine plasma (pooled from those with high expression of Hp) were applied onto an immunoaffinity column and extensively washed with PBS, pH 7.4. The bound Hp was then eluted by a buffer at pH 11. B: Pooled fraction A was re-chromatographed on a gel-filtration HPLC (Superose-12; 1 × 30 cm) for further purification. C: Homogeneity of final purified Hp on SDS-PAGE. Lane M, molecular markers; Lane 1, isolated bovine Hp from affinity column; Lane 2, final isolated bovine Hp from an additional separation on HPLC column; Lanes 3-5, purified human Hp 1-1, 2-1, and 2-2, respectively, represent as a reference for molecular weight evaluation.

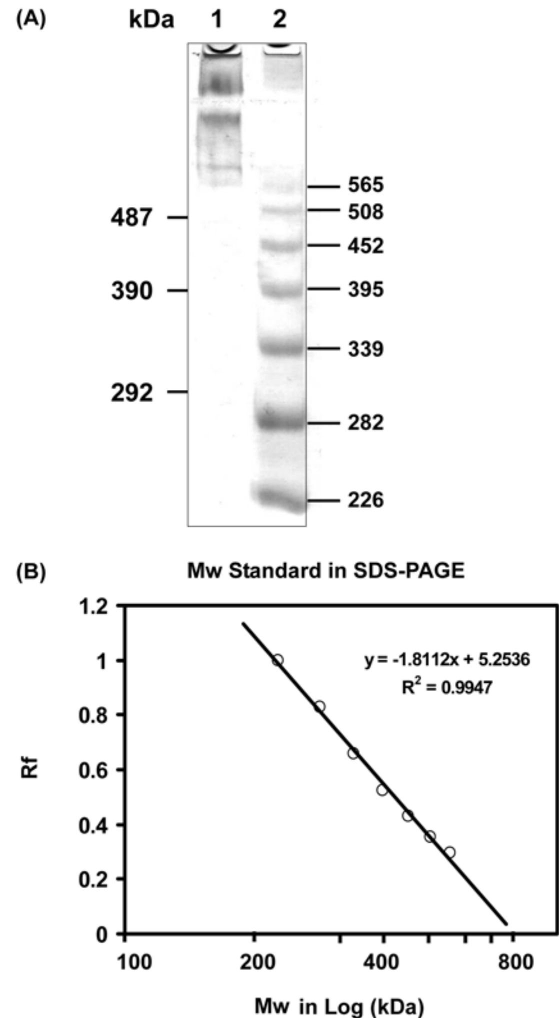

Fig. 7. Molecular weight characterization of purified bovine and human Hp 2-2 on 4% non-reducing SDS-PAGE. Electrophoresis was conducted for 3.5 h at 20 mA. A: Lane 1, isolated bovine Hp; Lane 2, purified human Hp 2-2. Molecular weight marked on the left margin (in kDa) represents those evaluated from a commercial source (gel not shown). Those marked on the right margin represent the true molecular weight calculated from the polymeric forms $(\alpha2\beta)_n$ (n = 4-10) of human Hp 2-2, in which the Hp trimer (n = 3) was running out of the gel. B: Linear plot of the molecular weight of human Hp 2-2 polymers. Extrapolation from the plot, the molecular weight of two major forms of bovine Hp is approximately of 660 and 730 kDa.

Sequence alignment of bovine and human Hp 2-2. To further define that cow exhibits the “Hp 2-2” phenotype, the cloned bovine Hp cDNA was sequenced (Fig. 9). Amino-acid sequence alignment, deducted from the nucleotide sequence, reveals that the putative sequence of bovine Hp is somewhat similar to human Hp 2-2 with 80% and 68% sequence homology for β and α chain, respectively (Fig. 9).

In humans, the α chain is responsible for the determination of Hp phenotypes. The Hp 1-1 or 2-2 homozygote is consisted of α1 (83 amino acids) or α2 (142 amino acids) chains, while Hp 2-1 is a heterozygote consisting of one α1 and one α2. As shown in Fig. 10 (by simplified ABC domains), the uniqueness of human α2 is that it not only contains identical

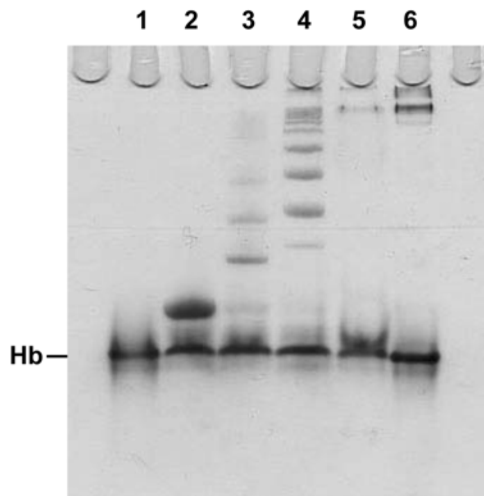

Fig. 8. Hemoglobin-binding property of isolated and native bovine plasma Hp. Bovine and human plasma were incubated with hemoglobin at room temperature for 30 min before conducting a 4% native-PAGE (see Materials and Methods). Lane 1, hemoglobin only; Lanes 2, 3, and 4, human plasma of Hp 1-1, 2-1, and 2-2 with hemoglobin, respectively; Lane 5, bovine plasma with hemoglobin; Lane 6, isolated bovine Hp with hemoglobin.

ABC domains of α1, but also an additional insertion of a redundant sequence (B1) between amino acid residues Asp-12 and Ala-70 (total 59 residues). The sequence homology between B1 and B in the repeat region is 96% with only two amino acids mutated. In cows, there also exists a repeat sequence within the α chain, but the sequence homology between the two repeat units (B1 and B) is 68% (Fig. 10), which significantly differs from that of human with 98% identity. Accordingly, we designated bovine Hp α genotype being unique as α2.

Discussion

Expression of bovine Hp. In the present study, we have shown bovine recombinant Hp expression in *E. coli* being abundant (>50% of the total endogenous proteins) and was mostly located in the inclusion bodies. The expressed bovine recombinant Hp being not cleaved into α and β subunits in *E. coli* was expected, since C1r-like protease, responsible for the cleavage of α and β chain in those mammalian cells (Wicher and Fries, 2004), is not present in *E. coli* (Lai *et al.*, 2007). However, it makes the isolation of recombinant Hp relatively simple when using a nickel-affinity column in the presence of urea (Fig. 3). One advantage in expressing the Hp in *E. coli* is that the resulting protein (in an intact αβ polypeptide chain) can be utilized for the preparation of polyclonal antibodies that in turn can be used as a probe for Western blot (Fig. 5) and for bovine Hp purification (Fig. 6).

Isolation of bovine native Hp. Rather than using combined anion-exchange, Sephacryl S-300, TSK Phenyl-5PW, and TSK DEAE-5PW columns for the isolation of bovine plasma Hp (Morimatsu *et al.*, 1991); we used a straightforward purification method achieved by an affinity column coupled with the polyclonal antibody prepared against bovine recombinant Hp, followed by HPLC gel-filtration (Fig. 6). The latter procedures are essentially simple; it can be routinely utilized for the purification of bovine Hp. Notably, the estimated molecular weight of the two major forms of bovine Hp we isolated is about 660 and 730 kDa (Fig. 7), which differs from previously reported values (1000-2000 kDa). The reason for this discrepancy is readily not known, but one of the explanations is that there were at least four chromatographic procedures involved in the former purification method (Morimatsu *et al.*, 1991). Bovine Hp might be reoxidized via the disulfide linkages (described below). It is of interest that the two large molecular forms we isolated could form hemoglobin complexes with the electrophoretic property almost identical to that of the native Hp that exists in the bovine plasma (Fig. 8). This result suggests that the Hp isolated under our experimental condition did not produce an observable alteration.

Presence of Hp in cow plasma. Hp level is abundant in human plasma with an approximate concentration of 100 mg/dL (Dobryszycka, 1997). It has been reported that Hp is not present in normal healthy cow plasma (Conner *et al.*, 1988; Morimatsu *et al.*, 1992; Yoshino *et al.*, 1992; Petersen *et al.*, 2004), but was inducible by giving inflammatory reagents (Petersen *et al.*, 2004). However, a recent report using immunoassay (Western blot and ELISA) indicated that Hp is present in cattle with a mean concentration of 8.4 ± 3.4 mg/dL (Chan *et al.*, 2004). The absence of plasma Hp in the latter investigation, however, is not particularly mentioned. In the present study, we show that about 40% of normal cows possess Hp (assessed by Western blot) in their plasma indicating that the presence of Hp in bovine plasma is dependent on the individuals (Fig. 5). Nevertheless, plasma of cows should be prescreened for Hp levels in order to isolate Hp. This can be simply done using a hemoglobin binding assay we described as the entire assay can be virtually finished within 60 min (Fig. 8). Undoubtedly, the factor(s) influencing the expression of plasma Hp in the normal cows remains unclear and deserves future investigations.

Primary structure of bovine α chain and its relationship to Hp polymers. It is worth mentioning that during the investigation of this work, a cDNA sequence of bovine Hp had also been filed in the NCBI database (GenBank accession no. NM_001040470), although a follow-up of structural analysis and its significance were not conducted.

As judged by our cDNA sequence of bovine Hp α chain in the present study, there are several lines of evidence to support

(A)

Nucleotide sequence of bovine Hp β-chain

```
ATCATCGGTGGCTCATTGGATGCCAAGGGCAGCTTTCCCTGGCAGGCCAAGATGGTCTCCCAGCATAACC   70
TCATCTCGGGAGCCACGCTCATCAATGAACGATGGCTCCTCACCACAGCTAAAAATCTCTACCTGGGTCA  140
CAGTAGTGACAAAAAAGCAAAGGACATCACTCCTACTTTAAGACTCTATGTGGGGAAGAACCAGCTTGTA  210
GAGGTGGAGAAGGTGGTTCTCCACCCTGACCACTCCAAGGTAGACATTGGGCTCATCAAACTCAGACAGA  280
AGGTACCTGTCAATGACAAAGTAATGCCCATCTGCCTACCTTCAAAAGATTATGTGAAGGTGGGTCGTGT  350
GGGTTATGTG TCTGGCTGGGGGCGAAATGAAAACTTCAACTTTACGGAGCATCTGAAGTATGTCATGCTA  420
CCTGTGGCTGACCAAGACAAGTGTGTGAAACACTATGAGGGCGTCGACGCACCTAAAAATAAGACAGCTA  490
AGAGCCCCGTAGGGGTGCAACCCATACTGAATGAGAACACCTTCTGCGTCGGCCTGTCCAAGTACCAGGA  560
CGACACCTGCTATGGCGACGCCGGCAGCGCCTTCGTCGTTCACGACAAGGAAGACGACACCTGGTATGCG  630
GCCGGGATCCTGAGCTTTGACAAGAGCTGTGCTGTGGCTGAGTATGGTGTGTACGTGAAGGTGACCTCCA  700
TTCTGGACTGGGTTCGGAAAACCATCGCTAACAAC                                      735
```

Putative amino-acid sequence alignment between bovine and human Hp β-chain

```
Bovine Hp      IIGGSLDAKGSFPWQAKMVSQHNLISGATLINERWLLTTAKNLYLGHSSDKKAKDITPTL    60
Human Hp 2-2   ILGGHLDAKGSFPWQAKMVSHHNLTTGATLINEQWLLTTAKNLFLNHSENATAKDIAPTL    60

Bovine Hp      RLYVGKNQLVEVEKVVLHPDHSKVDIGLIKLRQKVPVNDKVMPICLPSKDYVKVGRVGYV   120
Human Hp 2-2   TLYVGKKQLVEIEKVVLHPNYSQVDIGLIKLKQKVSVNERVMPICLPSKDYAEVGRVGYV   120

Bovine Hp      SGWGRNENFNFTEHLKYVMLPVADQDKCVKHYEGVDAPKNKTAKSPVGVQPILNENTFCV   180
Human Hp 2-2   SGWGRNANFKFTDHLKYVMLPVADQDQCIRHYEGSTVPEKKTPKSPVGVQPILNEHTFCA   180

Bovine Hp      GLSKYQDDTCYGDAGSAFVVHDKEDDTWYAAGILSFDKSCAVAEYGVYVKVTSILDWVRK   240
Human Hp 2-2   GMSKYQEDTCYGDAGSAFAVHDLEEDTWYATGILSFDKSCAVAEYGVYVKVTSIQDWVQK   240

Bovine Hp      TIANN                                                          245
Human Hp 2-2   TIAEN                                                          245
```

(B)

Nucleotide sequence of bovine Hp α-chain

```
GTGGAAACCGGCAGTGAGGCCACAGCCGACAGCTGCCCAAAGGCCCCCGAGATTGCTAATAGCCATGTGG   70
AGTACTCGGTTCGCTATCAGTGTGACAAATATTACAAACTGCATGCTGGAAATGGGGTGTATACTTTTAA  140
CAATAAGCAATGGATAAACAAGGACATTGGACAGCAACTTCCTGAATGTGAAGAAGATGACAGCTGCCCA  210
GAGCCCCCCAAGATTGAAAATGGCTACGTGGAGTACTTGGTTCGCTATCAGTGCAAACCCTATTACACAC  280
TGCGCACCTGTGGAGATGGAGTGTACACCTTTAACAGTAAGAAGCAGTGGATAAATAAGAACATTGGACA  350
GAAACTCCCTGAATGTGAGGCAGTGTGCGGGAAGCCCAAGCACCCCGTGGACCAGGTGCAG           411
```

Putative amino-acid sequence alignment between bovine and human Hp α-chain

```
                         B1
Bovine Hp      VETGSEAT- - -ADSCPKAPEIANSHVEYSVRYQCDKYYKLHA -GNGVYTFNNK -QWINKD    55
Human Hp 2-2   VDSGNDVTDIADDGCPKPPEIAHGYVEHSVRYQCKNYYKLRTEGDGVYTLNDKKQWINKA    60

                         B
Bovine Hp      IGQQLPECEE DDSCPEPPKIENGYVEYLVRYQCKPYYTLRTCGDGVYTFNSKKQWINKNI   115
Human Hp 2-2   VGDKLPECEADDGCPKPPEIAHGYVEHSVRYQCKNYYKLRTEGDGVYTLNNEKQWINKAV   120

Bovine Hp      GQKLPECEAVCGKPKHPVDQVQ                                         137
Human Hp 2-2   GDKLPECEAVCGKPKNPANPVQ                                         142
```

Fig. 9. Nucleotide sequence of bovine Hp and its putative amino-acid sequence alignment between human and bovine β (A) and α (B) chains. The alignment, deducted from the nucleotide sequence, was constructed using ClustalW (Labarga *et al*., 2005; Pillai *et al*., 2005). Repeated sequences within the α chain are shown in the brackets with two distinct regions. Locations of cysteine residues (in shaded brackets) are almost the same between the two species, except that bovine Hp α chain has an additional Cys-97 in a total of 8 -SH groups. This extra -SH group (Cys-97) might be responsible for the formation of a larger bovine Hp polymer than that of human Hp 2-2.

that the genotype of bovine α chain mimics that of human α2. First, plasma Hp α chain displays a molecular weight of 16.7 kDa, similar to that of human α2 on a Western blot (Fig. 5). Second, the isolated bovine Hp possesses a α chain that is almost identical to that of human in its molecular weight (18.2 kDa) (Fig. 6). Third, by putative amino-acid sequence alignment,

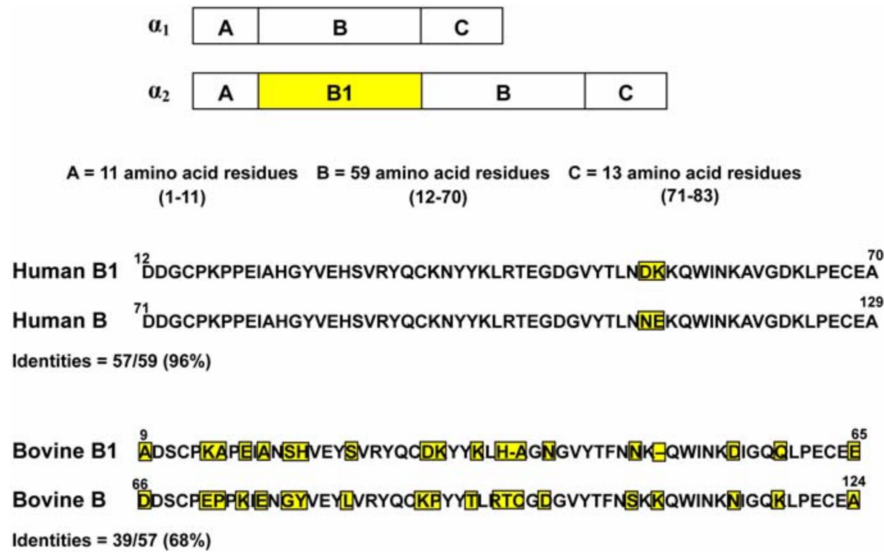

Fig. 10. Schematic drawing of the repeated region (B1 and B) of bovine and human α chain. The uniqueness of human α2 is that it not only matches the ABC domains of α1, but also with an additional insertion of a redundant sequence (B1 and B repeats). Each repeat unit contains 59 amino-acid residues between Asp-12 to Ala-70. The sequence homology in the repeat region of human is 96% with two amino acids mutated (in shaded bracket). In cows, there is also a redundant sequence (B1 and B) existing within the α chain, but the sequence homology between the two repeat units (B1 and B) is approximately 68%.

bovine α chain contains a unique redundant sequence, although the sequence homology between the repeated sequences (B1 and B in Fig. 10) was only 68% as compared to 96% in human.

It has been previously postulated that the molecular form of bovine Hp is extremely large, which cannot enter into the gel for SDS-PAGE (Eckersall and Conner, 1990). However, the molecular mechanism involved in the formation of the heterogeneous sizes of bovine Hp has not been elucidated (Morimatsu *et al*., 1991). In human, the α2 chain consists of 7 total -SH groups, in which four of them form intra-molecular linkages. Among the other 3 -SH, one (Cys-131) at the -COOH terminus always internally crosslinks the β chain forming a basic (α2-β) subunit, while the other 2 "free" -SH groups are responsible for crosslinking the other two (α2-β) subunits on the α2 chain. As a result, human Hp 2-2 forms cyclic polymers starting with a minimal trimer $(\alpha2\text{-}\beta)_3$ or $(\alpha2\text{-}\beta)_n$ (where $n \geq 3$) as shown in Fig. 1. Remarkably interesting, from the cDNA sequence we show that the α2 chain of bovine is comprised of an additional -SH group (Cys-97) forming a total of 8 -SH (Fig. 9). The role of this extra -SH is currently unknown, and we hypothesize that this extra group may form up to 3 "free" -SH in each α chain which is responsible for further polymerization with the other (α2-β) subunits resulting in an even larger molecular form of Hp as compared with human Hp 2-2 (Fig. 7). This notion is consistent to our hemoglobin typing gel depicted in Fig. 8. Thus, it could explain why the reported values of molecular weight of intact bovine Hp are extraordinarily large and could not enter the gel when routine SDS-PAGE was conducted (Eckersall and Conner, 1990).

Evolution. It has been well documented that almost all the animals possess Hp 1-1 as the only phenotype (Bowman, 1993). Based on the molecular weight of bovine Hp, some reports have pointed out that bovine Hp is an exception with a phenotype mimicking human Hp 2-2 (Eckersall and Conner, 1990; Morimatsu *et al*., 1991; Morimatsu *et al*., 1992; Yoshino *et al*., 1992; Katnik *et al*., 1998). Other ruminant Hp also putatively belongs to this category (Eckersall and Conner, 1990), but the protein has not yet been shown to contain the tandem repeat of α chain on the molecular level. Regardless, it has been thought that humans originally had a single Hp 1-1 phenotype (Smithies *et al*., 1962). Maeda *et al*. proposed that the repeated sequence of human α2 had evolved from a nonhomologous unequal crossover between two ***Hp 1*** genes [***Hp 1S and Hp 1F***] (Maeda *et al*., 1984). The uniqueness of ***Hp 2*** allele is that it is present only in humans and not found in all the primates including the new and old world monkeys, chimpanzees, and gorillas (McEvoy and Maeda, 1988). Because the bovine α chain also consists of a 2/3 α chain repeat similar to humans, our data suggest that the unequal crossover in human is no longer unique. It is of interest to point out that the unique bovine α2β gene (***Hp 2***) might exist in cattle as a sole genotype during evolution, since there is no Hp 1-1 or Hp 2-1 genotype identified thus far. We show that approximately 30% of the repeated region of bovine α2 chain underwent mutation. Thus, the evolved crossover leading to tandem repeat in cow could occur much earlier than that in humans. It seems that this "inserted region" in cows is not well reserved via evolution and its physiologic advantage remains elusive. Alternatively, the bovine Hp 2-2 gene may have pre-existed and not have been evolved from the crossover of Hp 1-1, which is a subject

of challenge. Nonetheless, the resulting point mutation produced an extra -SH group (Cys-97) that makes bovine Hp even a larger polymeric form than human Hp 2-2 (Fig. 7). Hp has been considered as an anti-inflammatory molecule. In humans, the large polymers of Hp 2-2 are a risk in the development of diabetic nephropathy (Nakhoul *et al*., 2001). One explanation is that the large polymer dramatically retards the penetration into the extracellular space (Nakhoul *et al*., 2001). We have although recently reported that Hp is an extremely potent antioxidant, the potency of Hp 2-2 is differentially less than that of 2-1 and 1-1 (Tseng *et al*., 2004b).

In conclusion, we have shown that although the bovine recombinant Hp is in a single polypeptide form from the *E. coli* expression system, the antibodies prepared against the $\alpha\beta$ chain can be utilized for the characterization of the molecular forms and isolation of native Hp. The purified bovine Hp classified, via an immunoaffinity column chromatography and HPLC gel-filtration, is polymeric and heterogeneous in size with molecular weights markedly larger than that of human Hp 2-2. One additional -SH group found in the repeat region of bovine α chain is probably responsible for further cross-linking among the α-β subunits in forming complicated Hp polymers. Due to a repeat sequence on bovine α chain that mimics the human $\alpha 2$, we defined bovine Hp genotype to be Hp 2-2, and its presence is not unique in humans.

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