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Haptoglobin 在生理與生化上之功能角色

Biochemical and physiologic role of haptoglobin

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摘要

Haptoglobin（Hp）是當動物受到感染或炎症反應時，血液中濃度會大量增加的一種急性反應期蛋白質。其最主要功能是可與血液中游離的血紅素形成複合物，而避免溶血時所造成的氧化壓力及組織傷害。

乳牛之乳房因細菌感染而產生炎症反應時，其牛奶中可發現大量體細胞數與 Hp 濃度上升。但是牛奶中 Hp 形成機制尚未明瞭。本研究發現牛奶中 Hp 濃度與體細胞數目呈現正相關。進一步經由反轉錄聚合酶連鎖反應，西方轉印法、免疫化學染色法、雙重免疫螢光染色法及 Hp release 實驗進行分析，證明牛奶中 Hp 可經由嗜中性球合成與分泌。本研究發現，當牛感染乳房炎時，牛奶中活化的嗜中性球會分泌大量 Hp，而利用 Hp 本身具有之抗氧化能力，可避免自由基形成所造成的細胞組織傷害。關於 Hp 之抗氧化能力，本研究進一步利用 Cu^{2+}所誘發的氧化試驗發現各個重組 Hp 次單位皆具有抗氧化能力，可保護低密度脂蛋白不被氧化，並且發現β次單位之抗氧化能力明顯高於 native Hp（3.5 X）、α次單位（10X）與臨床上使用藥物 Probucol（15 X）。此研究結果可提供未來設計 mini-Hp 作為強抗氧化藥物之參考，並且可進一步了解 Hp 其抗氧化能力之機制。在反芻動物 Hp 研究方面，本研究利用基因選殖分析發現，鹿 Hp 蛋白質之胺基酸序列與人類 *Hp 2* 類似，在α次單位內皆具有一段重複序列，並且-SH group 在胺基酸序

列上的位置與人類 *Hp 2* 相同，經蛋白質電泳分析顯示鹿 Hp 蛋白質為 tetramer $(\alpha\text{-}\beta)_4$ 之結構。為了進一步了解鹿 Hp tetramer 結構，利用已知可辨認人類與鹿 Hp α次單位之單株抗體（W1）進行分析，結果發現此單株抗體僅能辨認人類 *Hp 2* polymers，但卻無法辨認鹿之 Hp tetramer，顯示鹿 Hp tetramer 結構形成後此單株抗體位於α次單位上之抗原決定位（epitope）無法完全暴露在表面，而導致此抗體無法辨認鹿之 Hp tetramer，研究結果發現α次單位上胺基酸序列的差異所形成之空間障礙（steric hindrance）在人類與鹿形成 Hp polymer 上扮演重要的關鍵。此外，將鹿與其他哺乳類動物之 Hp 進行親緣關係與免疫化學之分析，發現鹿 *Hp 2* 基因可能在 2 千 5 百萬年前就存在。

綜合以上所述，本研究發現牛奶中嗜中性球可分泌大量 Hp，其抗氧化能力可保護細胞而避免遭受到氧化性傷害。而 Hp 之抗氧化功能區域主要位於β次單位上。Hp 胺基酸序列經由親緣關係分析，發現鹿 *Hp 2* 基因可能在 2 千 5 百萬年前就存在，其 Hp tetramer 之演化可能在生理方面佔有一定的優勢。

Biochemical and physiologic role of haptoglobin

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Abstract

Haptoglobin (Hp) is an acute phase protein responsive to inflammation and infection. One of the major functions of Hp is to capture released hemoglobin during excessive hemolysis and to scavenge the hemoglobin-induced free radicals during oxidative stress.

Hp and somatic cell counts (SCC) are sharply elevated in bovine milk following intramammary administration of endotoxin or bacteria. However, the sources of milk Hp responsible for such increase are not fully understood. In this study, our results reveal that milk Hp concentrations were correlated with SCC. Reverse transcriptase-polymerase chain reaction, Western blot, immunocytochemistry, double immunofluorescence, and Hp releasing experiment demonstrate that neutrophils were associated with the biosynthesis and release of Hp in milk. We propose neutrophils may play an essential role in elevating milk Hp in addition to previous suggestions that Hp may be derived from mammary tissues and circulation. During bovine mastitis, activated neutrophils produce significant amount of reactive oxygen species which may cause tissue damage. Hp is an extremely potent antioxidant that can directly scavenge the free radicals, it may there effectively utilize Hp to

attenuate such intracellular damage.

With respect to the antioxidant activity of Hp, we demonstrated that the antioxidant activity was found to be associated with both Hp α and β chains when assessed by Cu^{2+}-induced oxidation of low density lipoprotein. Interestingly, the antioxidant activity of β chain was extremely potent and markedly greater than that of native Hp (3.5 x), α chain (10 x) and probucol (15 x). The present study provides a potential utility for the future design of "mini-Hp" in developing a novel potent antioxidant. It may also provide a new insight in understanding the mechanism involved in the antioxidant nature of Hp.

Using ruminant Hp, we found that deer Hp mimics human *Hp 2* containing a tandem repeat over the α-chain based on our cloned cDNA sequence. Interestingly, the isolated deer Hp is homogeneous and tetrameric as a $(\alpha\text{-}\beta)_4$, although the location of –SH groups (responsible for the formation of polymers) is exactly identical to that of human. Most interestingly, an α-chain monoclonal antibody (W1) known to recognize both dissociated human and deer α-chains, only binds to the intact human Hp polymers, but not to deer Hp tetramers. It implicates that the epitope of deer α-chain is no longer exposed on the surface when forming tetramers. We propose that steric hindrance plays a major role in determining the polymeric formation in human and deer polymers. Phylogenetic and immunochemical analyses revealed that the *Hp 2* allele of deer might have arisen at least 25 million years ago. A mechanism involved in forming Hp tetramers is proposed and discussed, and the possibility

is raised that the evolved tetrameric structure of deer Hp might confer a physiological advantage.

Collectively, this thesis presents evidence that neutrophils were associated with the biosynthesis and release of Hp in milk. The major antioxidant domain of Hp was located in the β chain. Phylogenetic analysis revealed that the *Hp 2* allele of deer might have arisen at least 25 million years ago. The evolved tetrameric structure of deer Hp might be of a physiologic advantage.

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Dissertation index

Chapter 1

Overview

Haptoglobin (Hp) is an acute phase protein, responsive to infection and inflammation, ubiquitously present in the plasma of all mammals [1-4]. A recent study has found that Hp also exists in lower vertebrates, bony fish but not in frog and chicken [5]. The most frequently reported biological functions of the protein are to capture released hemoglobin (Hb) during excessive hemolysis and to transport Hb to the liver [6]. The captured hemoglobin is internalized by a macrophage/monocyte receptor, CD163, via endocytosis. Interestingly, the CD163 receptor only recognizes Hp and hemoglobin in complex, which indicates the exposure of a receptor-binding neoepitope [6]. Thus, CD163 is identified as a hemoglobin scavenger receptor. Besides the recycling of Hb-iron, the formation of the Hp-Hb complex possesses two additional benefits. First, Hp has a bacteriostatic effect by hampering the iron requiring process of bacterial replication as shown in rats inoculated with pathogenic *Escherichia coli* (*E. coli*) [7]. Second, Hp was assigned an antioxidant role by inhibiting Hb-driven free radical oxidative tissue damage [8-9]. Recently, we have shown that Hp is an extremely potent antioxidant, which directly protects low-density-lipoprotein (LDL) from Cu^{2+}-induced oxidation. The potency is markedly superior to probucol: one of the most potent antioxidants used in antioxidant therapy [10-12]. Transfection of Hp cDNA into Chinese hamster ovary cells protects them against oxidative stress [11].

Human Hp is originally synthesized as a single αβ polypeptide; following posttranslational cleavage by a protease, α and β chains are formed and then linked by disulfide bridges producing the mature Hp [13]. The gene is characterized by two common alleles *Hp 1* and *Hp 2* corresponding to α1β and α2β polypeptide chains, respectively, resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. DNA encoding for these two chains are linearly oriented in chromosome 16q22.1 [14] with 5 and 7 introns in *Hp 1* and *Hp 2*, respectively. These introns, however, are exclusively located at the region corresponding to the α chain [9]. The α2 contains a unique nonhomologous crossing-over in a part of the α1 [15]. Hp 2-1 and 2-2 form polymers due to an additional –SH group at the α2 chain, which determines its phenotype specificity [16]. All the phenotypes share the same β chains, each containing 245 amino-acid residues. The α1 chain (each with 83 amino acids) is "monovalent" forming one disulfide bridge with α and β subunit resulting in Hp dimer $(\alpha1\beta)_2$. In contrast, the α2 chain (each with 142 amino acids) is "divalent" containing one extra free –SH that is able to interact with an additional α2. As such, α2 chains can bind to either α1 or α2 chains to form large polymers [$(\alpha1\beta)_2$-$(\alpha2\beta)_n$ in Hp2-1 and $(\alpha2\beta)_n$ in Hp2-2].

Because of its weaker binding affinity to hemoglobin and retarded mobility (or penetration) between the cells, the polymeric structure of Hp 2-2 is dramatically more prevalent in some groups of patients with certain diseases, such as diabetes and inflammation-related diseases [17-19]. The human *Hp 2* allele has been proposed to be

originated from *Hp 1* about 2 million years ago and then gradually displaced *Hp 1* as a consequence of a nonhomologous crossing-over between the structural alleles (*Hp 1*) during meiosis [20-22], and is the first example of partial gene duplication of human plasma proteins [20,23-24]. Thus, only humans possess additional Hp 2-1 and 2-2 phenotypes.

All mammalian species studied to date have been shown to possess Hp. Analysis of the electrophoretic patterns of Hp-Hb complexes has suggested that most of mammals Hps are similar to human Hp 1-1 [25]. Only the Hp found in the plasma of ruminants resemble polymeric forms of human Hp 2-2 [26]. Structurally, bovine Hp is unique possessing only Hp 2-2 phenotype [27-28] with molecular weight ranged from 660 to 730 kDa [28]. 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. Recently, we have 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 [28].

1.1 Work objectives of present investigation

There are three objectives of this present dissertation:

1. Investigation of the relationship between milk Hp levels and somatic cell counts and

definition of the source of milk Hp from dairy cows with mastitis.

2. Production of the recombinant Hp subunits in *Escherichia coli* and determination of a major antioxidant domain of human Hp.

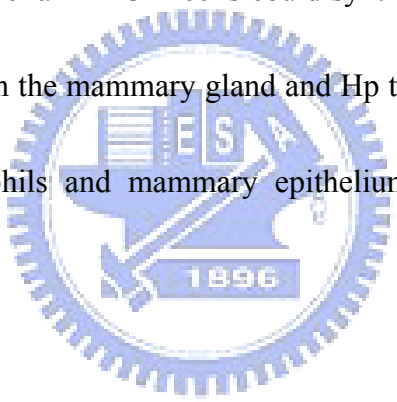
3. Definition of the molecular evolution of mammals Hp and study of the polymeric structure of deer Hp.

1.2 Neutrophils as one of the major haptoglobin sources in mastitis affected milk (Paper 1)

Bovine Hp, while not abundantly expressed in normal bovine plasma, is considered to be one of the sensitive acute phase proteins during bacterial infections [28-29]. Several studies indicate that its concentration increases dramatically in both plasma and milk during clinical mastitis of dairy cows [30-32]. In experimentally induced mastitis by intramammary bacterial-challenge with *Streptococcus uberis*, the observed increase in milk Hp has been suggested to originate from the circulation [33]. Hiss et al., 2004 also found elevated Hp in bovine milk after intramammary endotoxin challenge. It was further observed that Hp could be locally synthesized within the mammary gland [34]. However, the source of milk Hp found in naturally occurring mastitis is poorly understood

The purpose of the present study was to define the source of milk Hp from dairy cows with high somatic cell counts (SCC) (or mastitis). We investigated milk somatic cells for the

presence of Hp protein using Western blot, Hp mRNA expression using reverse transcriptase-polymerase chain reaction (RT-PCR), partial cell typing using double confocal immunofluorescence, and endogenously released Hp using ELISA. Finally, we localized Hp in normal and mastitic mammary gland tissue and in cultured mammary epithelial cell line (MAC-T). Our findings shows that the increased Hp was associated with neutrophils, in which Hp was released into the medium in an *ex vivo* experiment. In mastitis affected mammary glands, Hp was mainly localized within the epithelial cells. We also observed for the first time that cultured epithelial MAC-T cells could synthesize Hp in vitro. In addition to locally synthesized Hp within the mammary gland and Hp transferred from the circulation, we propose that milk neutrophils and mammary epithelium also contribute to milk Hp concentrations.



1.3 Cloning and expression of human haptoglobin subunits in *Escherichia coli*: delineation of a major antioxidant domain (Paper 2)

Since the purification for human native Hp and its corresponding α or β chain has been difficult, the purpose of the present study was to effectively express each recombinant Hp subunit and to localize its antioxidant domain(s). Using *E. coli*, we show that α1, α2, β, and α2β chain was abundantly expressed and primarily present in the inclusion bodies consisting of about 30% of the cell-lysate proteins. In human liver cells, however, the α2β is

postranscritionally cleaved into α and β chains between the residues Arg and Ile [35-36]. A more recent study suggests that a complement C1r-like protease is responsible for the cleavage [37]. Our data indicate that this protease is not present in the *E. coli.* Each cloned subunit retained its immunoreactivity as confirmed using antibodies specific to α or β chain. By circular dichroism, the structure of each expressed subunit was disordered as compared to the native Hp. The antioxidant activity was found to be associated with both α and β chains when assessed by Cu^{2+}-induced oxidation of low density lipoprotein (LDL). Of remarkable interest, the antioxidant activity of β chain was extremely potent and markedly greater than that of native Hp (3.5 x), α chain (10 x) and probucol (15 x). The present study is a clinically proved potent compound used for antioxidant therapy. The "unrestricted" structure of β subunit may therefore render its availability for free-radical scavenge, which provides a utility for the future design of a "mini-Hp" in antioxidant therapy. It may also provide a new insight in understanding the mechanism or specific amino acids involved in the antioxidant nature of Hp.

1.4 A unique tetrameric structure of deer plasma haptoglobin: an evolutionary advantage in the Hp 2-2 phenotype with homogeneous structure (Paper 3)

Similar to blood types, human Hp is classified as three phenotypes: Hp 1-1, 2-1, and 2-2. They are genetically inherited from two alleles *Hp 1* and *Hp 2*, but there is only Hp 1-1

phenotype or *Hp 1* in almost all the animal species [25]. The Hp 2-2 protein consists of complicated large polymers cross-linked by α2-β subunits or $(\alpha 2\text{-}\beta)_n$ (where $n \geq 3$, up to 12 or more) and is associated with the risk of the development of diabetic, cardiovascular, and inflammatory diseases [17-19]. In the present study, we found that deer plasma Hp mimics human *Hp 2* containing a tandem repeat over the α-chain based on our cloned cDNA sequence. Remarkably interesting, the isolated deer Hp is homogeneous and tetrameric as a $(\alpha\text{-}\beta)_4$, although the location of –SH groups (responsible for the formation of polymers) is exactly identical to that of human. Denaturation of deer Hp using 6M urea in reducing condition (143 mM β-mercaptoethanol) followed by renaturation still sustained the formation of $(\alpha\text{-}\beta)_4$ suggesting that the Hp tetramers are not randomly assembled. Interestingly, an α-chain monoclonal antibody (W1) known to recognize both human and deer α-chains, only binds to the intact human Hp polymers, but not to deer Hp tetramers. It implicates that the epitope of deer α-chain is no longer exposed on the surface when Hp tetramers are formed. We propose that steric hindrance plays a major role in determining the polymeric formation in human and deer polymers. Phylogenetic and immunochemical analyses revealed that the Hp 2 allele of deer might have arisen at least 25 million years ago. A mechanism involved in forming Hp tetramers is proposed and discussed, and the possibility is raised that the evolved tetrameric structure of deer Hp might confer a physiological advantage.

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Chapter 2

Neutrophils as one of the major haptoglobin sources in mastitis affected milk (Paper 1)

Abstract

The antioxidant haptoglobin (Hp) is an acute-phase protein responsive to infectious and inflammatory diseases. Hp and somatic cell counts (SCC) are sharply elevated in bovine milk following intramammary administration of endotoxin or bacteria. However, the sources of milk Hp responsible for such increase are not fully understood. The purpose of this study was to define the source of milk Hp from dairy cows with naturally occurring mastitis. Quarter milk samples selected from 50 dairy cows were separated into four groups according to SCC as group A: <100 (n=19); B: 100-200 (n=10); C: 201-500 (n=10); and D: >500 × 10^3 (n=11) cells/mL. Our results reveal that milk Hp concentrations were correlated with SCC (r=0.742; P<0.01), and concentrations in group D were ~10 fold higher than in group A. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis indicates that the milk somatic cell cells from group D were not only capable of synthesizing Hp but could also markedly increase Hp mRNA expression. Western blot, immunocytochemistry, double confocal immunofluorescence, and Hp releasing experiment demonstrate that neutrophils were associated with the biosynthesis and release of Hp in milk. It further shows that Hp

was significantly elevated in the epithelium of mammary gland tissue with mastitis and was also expressed in the cultured mammary epithelial cells. We propose neutrophils and epithelial cells may play an essential role in elevating milk Hp in addition to previous suggestions that Hp may be derived from mammary tissues and circulation.

Key words: haptoglobin / neutrophil / mastitis / MAC-T cells / immunocytochemistry

2.1 Introduction

Haptoglobin (Hp) is an acute phase protein responsive to inflammation and infection [1, 2, 3]. One of the major functions of Hp is to capture released hemoglobin during excessive hemolysis [4] and to scavenge the hemoglobin-induced free radicals during oxidative stress [5]. We have recently shown that Hp is an extremely potent antioxidant, which directly prevents low-density-lipoproteins (LDL) from Cu^{2+}- and radical compound-induced oxidation [6, 7]. Transfection of Hp cDNA into Chinese hamster ovary (CHO) cells protects them against oxidative stress [7]. Structurally, bovine Hp is unique possessing only Hp 2-2 phenotype [8, 9] with molecular weight ranged from 660 to 730 kDa [9] which is different from that of human expressed as Hp 1-1, 2-1, or 2-2.

Bovine Hp, while not abundantly expressed in normal bovine plasma, is considered to be one of the sensitive acute phase proteins during bacterial infections [3, 9]. Several studies indicate that its concentration increases dramatically in both plasma and milk during clinical

mastitis of dairy cows [1, 10, 11]. In experimentally induced mastitis by intramammary bacterial-challenge with *Streptococcus uberis*, the observed increase in milk Hp has been suggested to originate from the circulation [12]. Hiss et al., 2004 also found elevated Hp in bovine milk after intramammary endotoxin challenge [13]. It was further observed that Hp could be locally synthesized within the mammary gland [13]. The source of milk Hp found in naturally occurring mastitis is poorly understood.

The purpose of the present study was to define the source of milk Hp from dairy cows with high somatic cell counts (SCC) (or mastitis). We investigated milk somatic cells for the presence of Hp protein using Western blot, Hp mRNA expression using reverse transcriptase-polymerase chain reaction (RT-PCR), partial cell typing using double confocal immunofluorescence, and endogenously released Hp using ELISA. Finally, we localized Hp in normal and mastitic mammary gland tissue and in cultured mammary epithelial cell line (MAC-T). Our findings shows that the increased Hp was associated with neutrophils, in which Hp was released into the medium in an *ex vivo* experiment. In mastitis affected mammary glands, Hp was mainly localized within the epithelial cells. We also observed for the first time that cultured epithelial MAC-T cells could synthesize Hp in vitro. In addition to locally synthesized Hp within the mammary gland and Hp transferred from the circulation, we propose that milk neutrophils and mammary epithelium also contribute to milk Hp concentrations.

2.2 Materials and methods

2.2.1 Sample collection and SCC

Quarter milk samples from individual cows (*Bos taurus*) were collected at the Yong Rong Dairy Farm (Chyayi, Taiwan). SCC in each sample was measured using a Fossomatic 4000 cell counter (Foss Electric, Hillerød, Denmark) at the Livestock Research Institute, Council of Agriculture, Executive Yuan (Hsinchu, Taiwan). Quarter milk samples were then selected and assigned to four groups according to SCC (A: < 100; B: 100-200; C: 201-500; and D: > 500×10^3 cells/mL with n = 19, 10, 10, and 11, respectively).

2.2.2 Purification of bovine Hp and preparation of anti-Hp antibodies

Native bovine Hp was isolated from the plasma using an anti-bovine Hp immunoaffinity column, followed by gel-permeation chromatography (Superose-12) on a HPLC system as previously described [9]. Mouse and rabbit polyclonal antibodies against bovine recombinant Hp or mouse monoclonal antibody (mAb; 2H12) against bovine native Hp were prepared in our laboratory according to previously established procedures [9, 14].

2.2.3 Determination of milk Hp levels using ELISA

Bovine milk Hp concentrations were determined using a commercial bovine Hp ELISA kit

(Immunology Consultants Laboratory, Newberg, OR) according to the manufacture's instructions. One hundred μL of skimmed milk (1:10 dilution) were used for the determination of Hp, while skimmed milk was prepared as previously described [15].

2.2.4 Isolation of milk somatic cells and protein extraction

Milk somatic cells were isolated from 200 mL freshly collected milk. Briefly, milk samples maintained at 4 ºC were centrifuged at 1,000 g for 20 min at 4 ºC. After removing the fat layer and supernatant, the somatic cells were briefly washed twice with PBS at 4 °C and maintained on ice. Total cell proteins were extracted using a lysis buffer containing 0.3% Triton X-100 in PBS and sonicated at 4 ˚C for 5 min, followed by centrifugation at 10,000 g for 10 min at 4 ˚C. The remaining supernatant was stored at -20 ˚C until analyses by SDS-PAGE and Western blot.

2.2.5 Gel electrophoresis and Western blot

SDS-PAGE was carried out on 1.5-mm-thick slab gel, using a discontinuous system as previously described [8]. Unless otherwise specified, the gel containing 15% (for reducing) or 4% (for non-reducing samples) polyacrylamide was used with a top stacking gel of 5% polyacrylamide. Approximately 20 μg of the protein was loaded onto the gels and each tested sample was preheated at 100 ºC for 10 min in a loading buffer (12 mM Tris-HCl, pH

6.8, 0.4% SDS, 5% glycerol, 0.02% bromphenol blue) with or without 140 mM 2-mercaptoethanol. The samples were then run for about 1.5 h at 100 V and stained by Coomassie brilliant blue. Molecular-mass standard containing 12 prestained proteins (3.5-260 kDa) was purchased from Invitrogen (Carlsbad, CA). Western blot analysis was performed similar to that described previously [6].

2.2.6 Analysis of Hp mRNA expression of milk somatic cells

Total RNA was extracted from milk somatic cells using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacture's instructions. The first strand cDNA was synthesized using moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). Briefly, equal amount of total RNA (1 μg) was added to a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 0.5 μg oligo $(dT)_{18}$, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTP mix, 40 U of RNase inhibitor, and 200 U of MMLV reverse transcriptase, and proceeded at 37 °C for 50 min, followed by 70 °C for 15 min. Equal amount of total cDNA (100 ng) was amplified by PCR using Hp specific primers, while using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a house-keeping control. The primer design was based on the published nucleotide sequence of bovine Hp [15] with 5'-TGCTGCAGGGATCATCGGTGGCTCATTGGA-3' and 5'-CGGAAAACCATCGCTAA CAACTAAGCTTGGG-3' as a forward and reverse primer, respectively. The GAPDH

primers prepared were 5'-CCTGGAGAAACCTGCCAAGT-3' (forward) and 5'-GCCAA ATTCATTGTCGTACCA-3' (reverse). The PCR cycling profile was 95 ºC for 5 min followed by 30 cycles at 94 ºC for 30, 55 ºC for 30 s, 72 ºC for 50 s (or 30 s in GAPDH) with a final extension at 72 ºC for 10 min. The RT-PCR products (214 bp for GAPDH and 755 bp for Hp) were resolved on 1.5% agarose gel [14], followed by an ethidium bromide staining. The band intensity corresponding to Hp was determined using a Quantity One software of Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA).

2.2.7 Mammary epithelial cell culture (MAC-T) and Hp mRNA expression

MAC-T, an established and immortalized epithelial cell line isolated from bovine mammary tissue was cultured as previously described [16, 17]. In general, the cells (1×10^4 cells per well) were grown at 37 °C (5% CO_2) in a 24-well culture plate in complete Dulbecco's modified Eagle medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (containing no immunoreactive bovine Hp), 50 μg/mL of streptomycin, and 50 IU/mL of penicillin (Invitrogen). Hp mRNA expression were analyzed using RT-PCR similar to the procedures described above.

2.2.8 Immunocytochemical staining of somatic and MAC-T cells and mammary tissues

The labeled streptavidin biotin kit (LSAB) (Dakocytomation, Glostrup, Denmark) was used

for immunostaining according to the manufacturer's instructions. In brief, cytospins containing freshly isolated somatic or MAC-T cells were treated with ice-chilled methanol (100%) for 15 min and then rehydrated by PBS. Cells were permeabilized in PBS containing 0.3% Triton X-100 for 10 min, while the endogenous peroxidase was blocked by incubating with 3% H_2O_2. After blocking with 2% gelatin, cells were incubated with unlabeled mouse anti-CD5 mAb (lymphocyte marker), mouse anti-CD11b mAb (neutrophil marker) (Serotec, Oxford, UK), or mouse anti-Hp polyclonal antibody for 1 h. The slides were then incubated with biotinylated anti-mouse IgG for 30 min, followed by washes and incubation with HRP-conjugated streptavidin for 30 min. After washes, the slides were developed with 3-amino-9-ethylcarbazole (AEC) or 3,3'-diaminobenzidine (DAB) substrate and counterstained with hematoxylin. For normal and mastitic mammary tissues (n = 5 each), each section was deparaffinized in a 50 ºC oven for 10 min and then placed for two 5-min washes in xylene at 24 ºC. The sections were rehydrated through sequential soakings in 100, 95, 90, 80, and 50% alcohol for 2 min each, followed by final washes in deionized water. The sections were then stained using procedures described above.

2.2.9 Double immunofluorescence using confocal laser scanning microscopy

Cytospin prepared cells used for localization of CD11b and Hp were permeabilized as described above. After blocking, cells were co-incubated with unlabeled mouse anti-CD11b

mAb and rabbit anti-Hp polyclonal antibody for 1 h. The slides were then incubated with a mixture of fluorescent isothiocyanate (FITC) conjugated anti-mouse IgG and rhodamine conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) for 1 h in dark. Cells were washed with PBS before examination using a Fluoview FV500 confocal laser scanning microscopy (Olympus, Tokyo, Japan).

2.2.10 Time course of Hp protein released from somatic cells *in vitro*

Two ml of isolated somatic cells (adjusted to 1×10^4 cells/mL at 4 ºC) from Groups A and D (n = 3 for each) were suspended in Roswell Park Memorial Institute medium (RPMI) 1640 medium (JRH Biosciences, Lenexa, KS) containing 100 μg/mL ampicillin with or without protease inhibitor of 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St.Louis, MO) in a test tube and were incubated at 37 ºC for 0, 2, 4, 8 and 16 h, respectively, followed by centrifugation at 1,000 g at 4 ˚C for 5 min. The supernatant was then subjected to ELISA for the determination of Hp concentrations.

2.3 Results

2.3.1 Correlation between Hp levels and SCC in bovine milk

Selected bovine milk samples (n=50) were assigned into four groups according to their SCC (A: <100; B: 100-200; C: 201-500; and D: > 500 × 10^3 cells/mL with n= 9, 10, 10, and 11,

respectively). The mean ± SEM of milk Hp concentrations determined by an ELISA in groups A, B, C, and D were 0.23 ± 0.08, 0.74 ± 0.23, 1.11 ± 0.28, and 2.22 ± 0.53 μg/mL, respectively. The mean Hp concentration of group D was ~10-fold higher than that of group A ($P < 0.001$). The overall Hp concentrations were correlated with SCC (r = 0.742; $P < 0.01$).

2.3.2 Hp in milk somatic cells

Because Hp levels in the milk samples were positively correlated with SCC, we hypothesized that somatic cells might contribute to the presence of Hp in milk. We extracted protein from the somatic cells from the low (group A) and high SCC (group D) to examine the presence of Hp using a Western blot. Fig. 1 depicts the presence of Hp in group D, but not in group A. It implicates the endogenous synthesis and expression of Hp between the analyzed cells of groups A and D were different or/and altered. The electrophoretic pattern of bovine Hp (non-reduced) is similar to that previously reported by our laboratory [9].

2.3.3 Endogenous expression of Hp mRNA in milk somatic cells

To determine whether somatic cells could endogenously synthesize Hp, RT-PCR for the expression of Hp mRNA was conducted, while using GAPDH as a house-keeping control. Fig. 2 shows that the Hp mRNA expression was progressively increased with the SCC ($P <$

0.001). The mean Hp mRNA expression in groups C and D were evidently higher than those in groups A and B ($P < 0.001$).

2.3.4 Somatic cell typing

To identify the cell types in group D, we used anti-CD5 (lymphocyte marker) and anti-CD11b (neutrophil marker) mAb as a probe in immunostaining, while comparing group A as a non-mastitis control. We found lymphocytes to be a major cell type in group A and neutrophils the predominant cell type in group D (Fig. 3). The immunostaining was specific because the background control using non-immuned sera or non-related mAb was negative.

2.3.5 Colocalization of Hp and CD11b in neutrophils using confocal microscopy

In addition to RT-PCR, we tested whether neutrophils could express Hp *in situ.* Somatic cells isolated from group D enriched with neutrophils were examined for localization of Hp using a double confocal laser scanning microscopy. Fig. 4 shows that all the neutrophils identified by CD11b (in green FITC) exhibited expression of Hp (in red rhodamine), while no staining was observed for cells treated with non-immuned sera or unrelated mAb.

2.3.6 Release of Hp from the somatic cell *ex vivo*

To test the hypothesis that the neutrophil enriched population secreted Hp, we collected the

somatic cells from the groups A and D (n=3 quarters for each) and investigated their Hp secretion into the RPMI 1640 medium over time. Fig. 5 shows that both groups were able to secrete Hp into the medium with a markedly greater secretion in group D than in group A. Essentially, the released Hp reached maximal levels within the first 2 h tested. Thus, the milk neutrophils not only express Hp but also release it into the milk.

2.3.7 Localization of Hp in mammary tissue and MAC-T cell

We used mouse anti-bovine Hp polyclonal antibody to localize the bovine Hp near the area of alveoli in mammary tissues (n = 5). Results indicate that only mastitic mammary tissue expressed Hp relative to normal tissue. Figs. 6A and 6B illustrate a typical example showing that Hp was primarily localized at alveoli of mastitic mammary tissue, but not in the normal tissues (Figs 6C and 6D). To provide additional evidence that mammary alveolar epithelial cells can express Hp, we showed that cultured- MAC-T cells were able to express Hp mRNA (panel of Fig. 6E). We further demonstrated the presence of Hp protein in MAC-T cells using immunocytochemical staining (Figs. 6E and 6F).

2.4 Discussion

2.4.1 Correlation between Hp levels and SCC in bovine milk

The SCC of milk has been utilized as an indicator of mastitis because of its simple handling

procedures. In general, SCC of a quarter greater than 500×10^3 cells/mL is considered to be associated with mastitis [18, 19]. A limit of $\leq 100 \times 10^3$ cells/mL has been suggested for a healthy quarter [20]. If the SCC exceeds 200×10^3 cells/mL, the quarter is likely to be infected [21]. Results from the present study showed a 10-fold increase in Hp concentrations in milk containing SCC $> 500 \times 10^3$ (Group D) relative to SCC $< 100 \times 10^3$ (Group A). It suggests that the somatic cells were responsible for the elevation of Hp in milk, at least in part.

2.4.2 Bovine Hp in milk somatic cells

Previous studies have proposed that milk Hp can be derived from mammary tissues and circulating blood following intramammary infection [22, 13]. Although the factors attributed to the elevated milk Hp in naturally occurring mastitis are not fully understood, the present study suggests that neutrophils in milk are a major source of milk Hp of cow with mastitis. There are several lines of evidence to support this notion. First, Hp levels determined by ELISA were positively correlated with the SCC in our group analyses in which the increase in SCC was mostly attributed to neutrophils. Second, using Western blot analysis Hp was found abundantly in the cell lysate of group D, but not in that of the normal group A (Fig. 1). A recent study using matrix assisted laser desorption ionization-time of flight-mass (MALDI-TOF-MS) indicates that Hp is present and concentrated within the

granules of bovine granulocytes isolated from peripheral blood of healthy cattle, although the cell types (such as neutrophil) have not been specified [23]. Third, the Hp mRNA levels in cells of the high SCC groups B-D were significantly higher than in the cells of the normal group A, The underlying mechanism involved in high Hp expression in the higher SCC groups appears to be related to the different cell types. We also observed that neutrophils were the predominate cell type in group D, but not in group A when examined by immunocytochemistry (data not shown). This suggests that neutrophils in high SCC milk account for the major synthesis of Hp. Furthermore, using laser confocal scanning microscopy, we observed that all the neutrophils isolated from the somatic cells were capable of expressing Hp as determined by double immunofluorescence (Fig. 4). Fourth, these cells not only synthesized Hp but also released it into milk (Fig. 5).

This is the first study demonstrating that Hp is synthesized by cattle neutrophils using RT-PCR, inconsistent with an early report indicating that human neutrophils do not produce Hp [24]. In that study, the authors hypothesized that Hp of neutrophils was derived from the liver and stored in specific granules via endocytosis [24]. While conducting our investigation, a recent study reported that Hp can be released from human neutrophils [25] indicating that the presence of Hp mRNA in human neutrophils, although the level of expression was minimal. Taken together, it is conceivable that neutrophils may contribute to the increased level of Hp in milk of cow with mastitis.

2.4.3 Milk Hp from the other sources

It has been shown that Hp is expressed in the mammary tissue using ELISA and RT-PCR, although the cell types involved were not specified [13]. Using an epithelial cell line (MAC-T) and mammary gland tissue sections we further demonstrated that epithelial cells are able to endogenously synthesize and express Hp (Fig. 6), consistent with results by Thielen et al. [26] using an *in situ* hybridization on the epithelium of mastitic mammary tissue. Another study has also suspected the presence of Hp in epithelium of alveolus [6]. Nonetheless, the localization of Hp in bovine alveolar epithelium of mastitic tissue or cultured MAC-T cells is now demonstrated in the present study (Fig. 6).

2.4.4 Remarks on Hp secretion into the milk and its physiologic function

This study shows that neutrophils and mammary epithelial cells represent an additional extra-hepatic source of Hp in milk. The possible mechanism by which neutrophils migrate into the alveolus of mammary gland and how Hp is recruited in milk are explained and summarized in Fig. 7. The physiologic benefits of local Hp expression in mammary tissue during infection remain illusive. During mastitis, activated neutrophils produce significant amount of reactive oxygen species (in order to kill the bacteria) which may cause tissue damage [27, 28, 29]. Hp is an extremely potent antioxidant that can directly scavenge the

free radicals, it may there effectively utilize Hp to attenuate such intracellular damage [7]. Further understanding of the role played by neutrophils as a source of Hp may provide insight in to understanding an additional function of neutrophils in milk.

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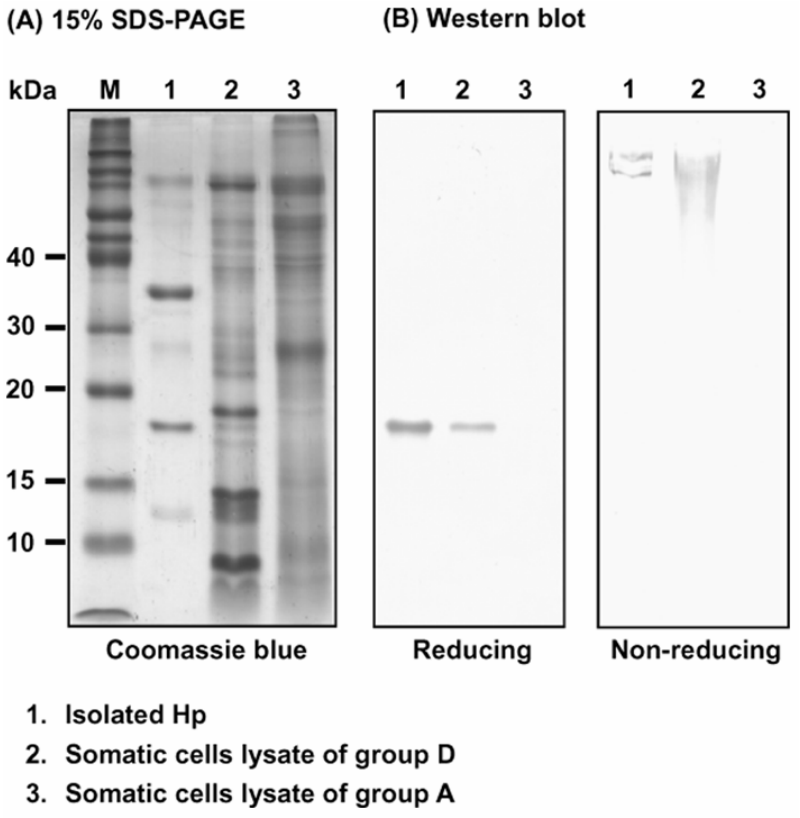

Figure 1. Typical SDS-PAGE pattern and Western blot analyses of milk somatic cell lysate from groups A and D. (A) Coomassie blue staining of somatic cell lysate run on a 15% SDS-PAGE under reducing condition. (B) Western blot analysis of the isolated Hp and somatic cell lysate under reducing (left panel with 15% SDS-PAGE) and non-reducing conditions (right panel with 4% SDS-PAGE) using a mouse polyclonal antibody prepared against bovine recombinant Hp. Lane M, molecular markers in kDa.

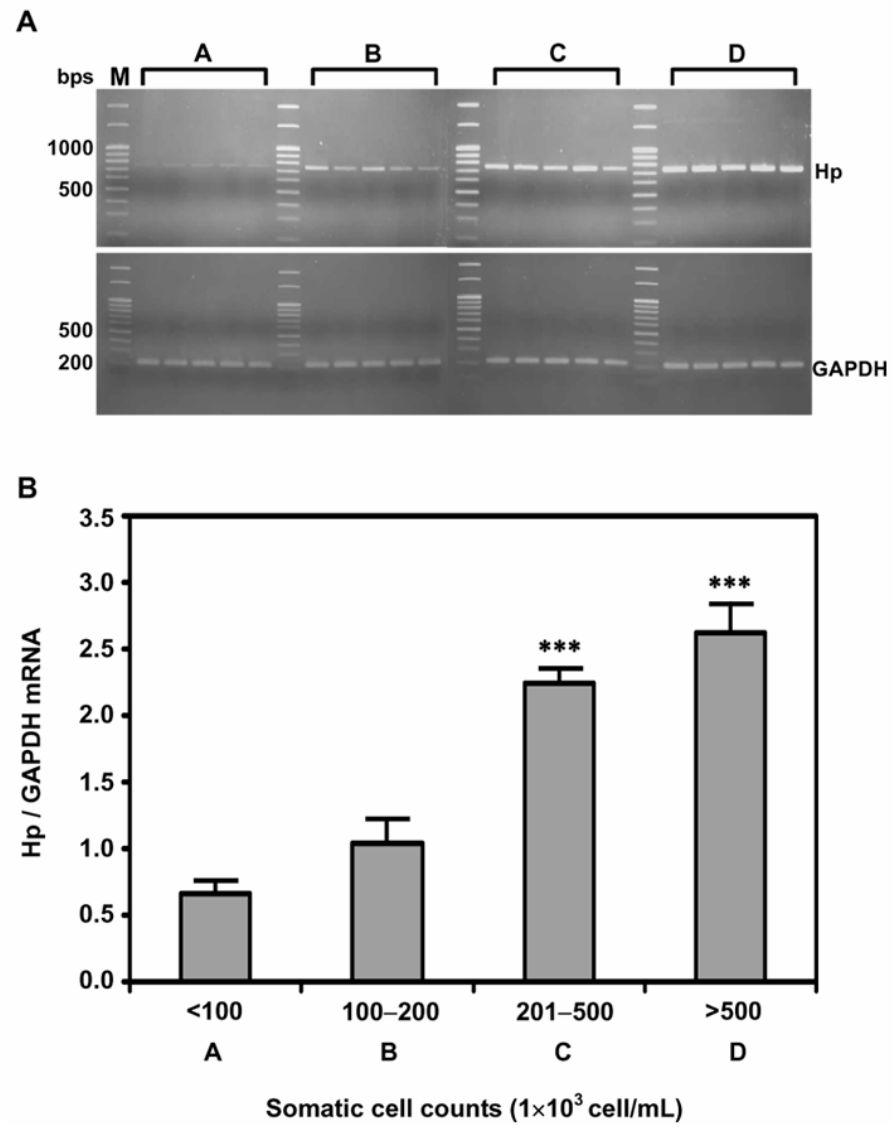

Figure 2. Expression levels of Hp mRNA in somatic cells. (A) Expression of Hp mRNA in milk somatic cells according to SCC (A: < 100; B: 100-200; C: 201-500; and D: > 500 × 10^3 cells/mL with n = 5 in each group). Equal amount of total cDNA (100 ng) was amplified by PCR, using GAPDH as a house-keeping control. (B) Each bar represents the mean ± SEM. *** $P < 0.001$ as compared to group A.

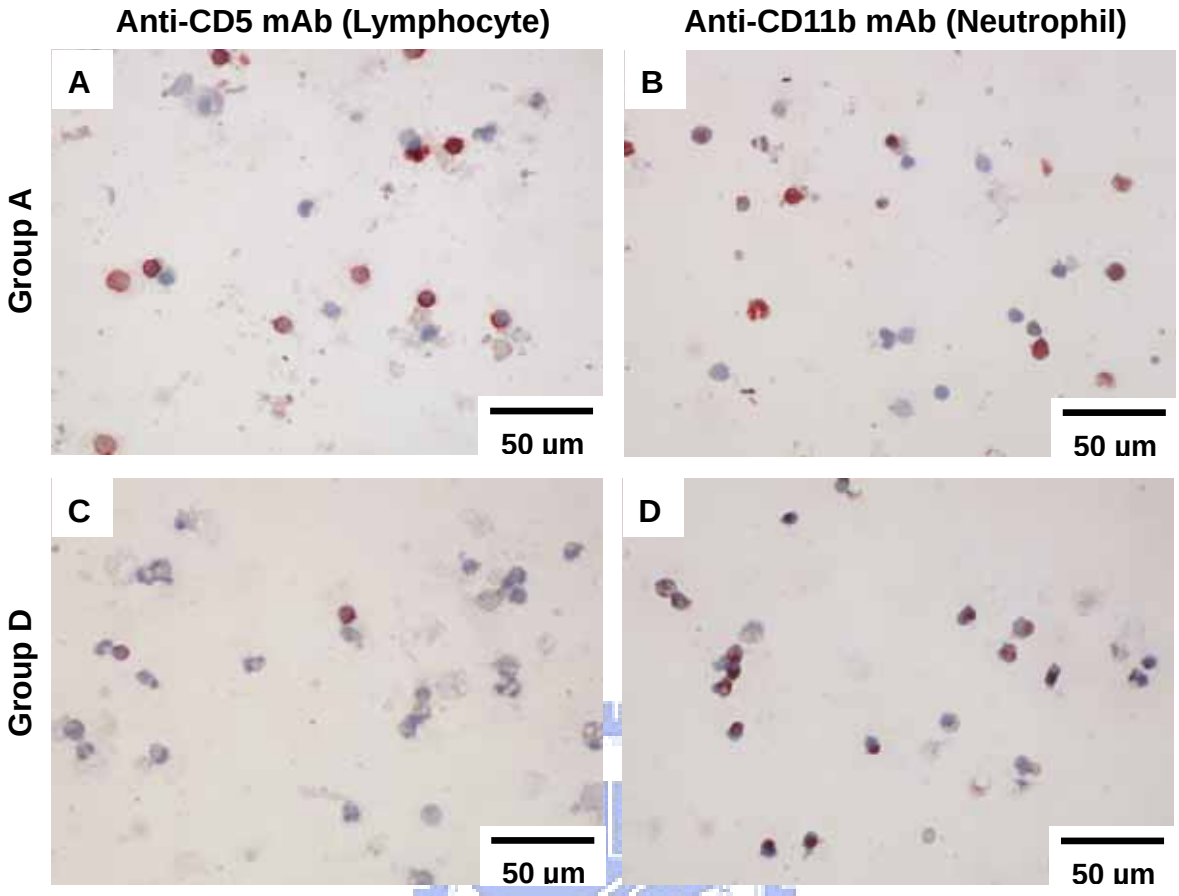

Figure 3. A typical example of immunocytochemical staining between somatic cells isolated from groups A and D. Cytospin prepared cells were incubated with mouse anti-CD5 (lymphocyte marker) (A and C) and anti-CD11b mAb (neutrophil marker) (B and D), followed by standard procedures using a LSAB method. AEC was used as the chromogenic substrate for CD5 or CD11b staining (in red), while hematoxylin was employed for nuclear staining

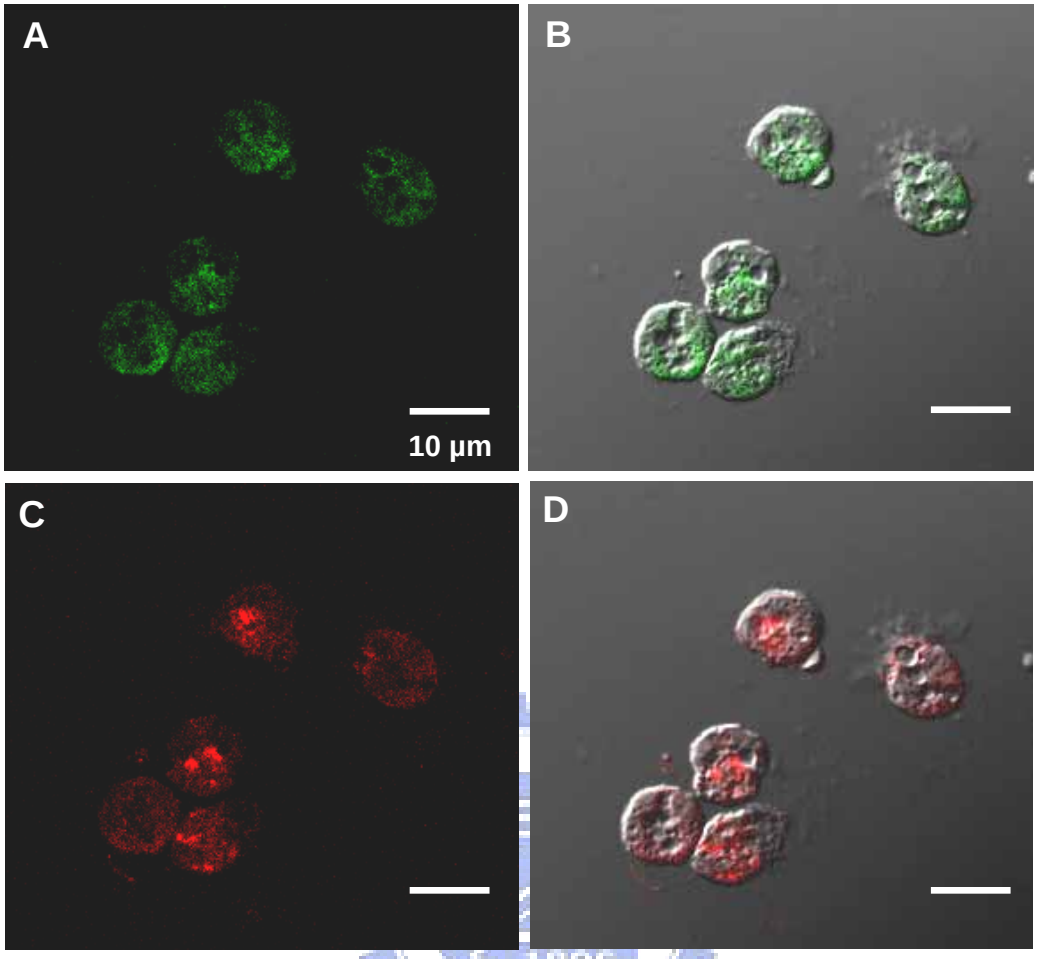

Figure 4. Colocalization of CD11b and Hp in milk neutrophils using confocal laser scanning microscopy. CD11b as a marker for neutrophils was visualized with a FITC-conjugated second antibody (A and B). Likewise, Hp was visualized with a rhodamine-conjugated second antibody (C and D).

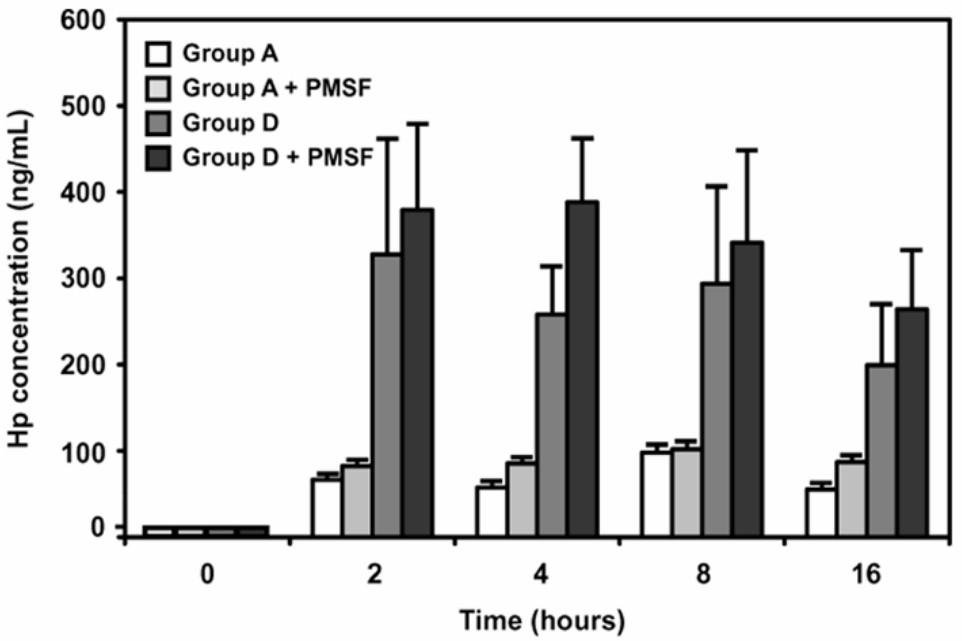

Figure 5. Time course of Hp protein released into RPMI 1640 medium by somatic cells from group A and D *ex vivo*. Freshly isolated somatic cells from milk samples of group A and D (n = 3 of each group) at 4 ºC were immediately suspended in RPMI 1640 and kept at 37 ºC over time. Supernatant containing Hp was determined using an ELISA. Protease inhibitor (PMSF) was also added in parallel to each group in an attempt to minimize the Hp degradation. Each bar represents the mean ± SEM.

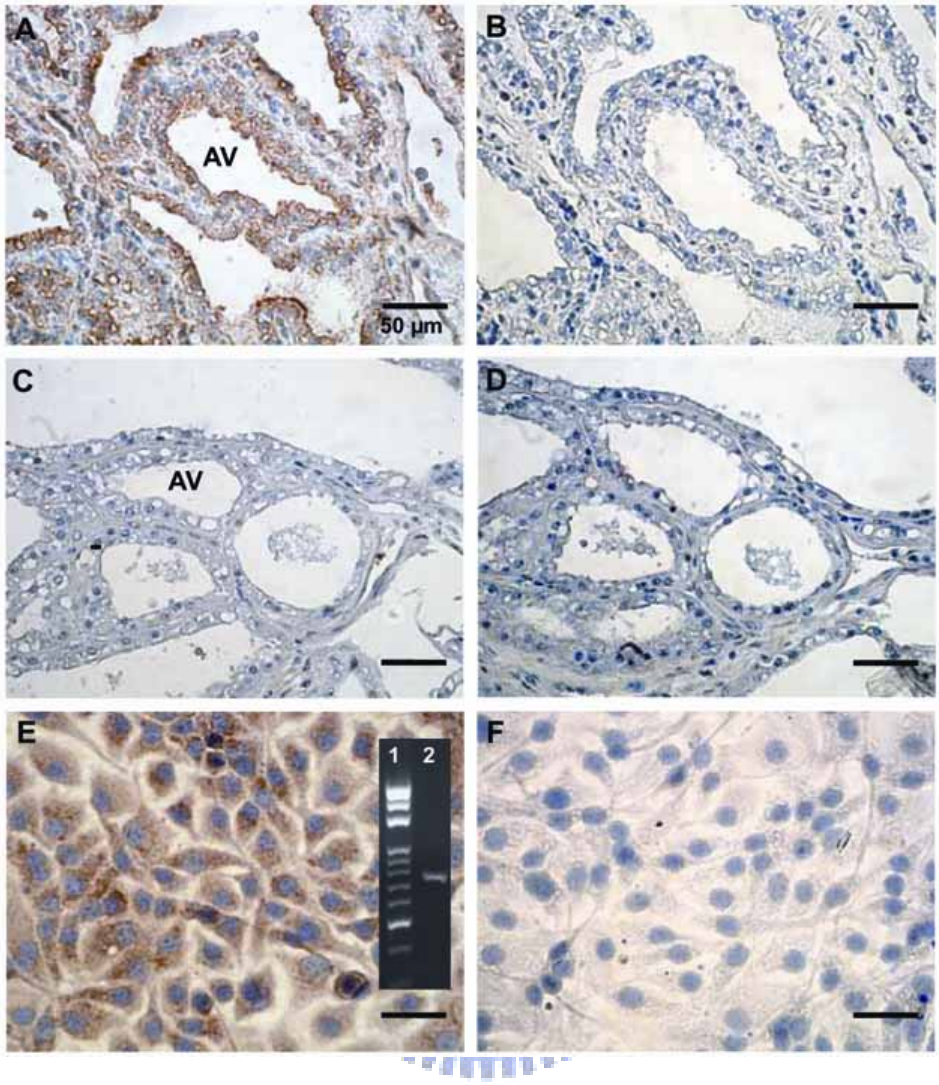

Figure 6. Immunocytochemical localization of Hp in mammary gland and MAC-T cell. Mammary tissues with (A and B) and without mastitis (C and D) were incubated with a mouse anti-Hp polyclonal antibodies (A and C), while using normal non-immuned mouse serum as a negative control (B and D). MAC-T cells were incubated with mouse anti-Hp polyclonal antibodies (E), while using normal non-immuned mouse serum as a negative control (F). DAB was used as a chromogenic substrate for Hp staining (in brown) and hematoxylin was employed as a nuclear stain (in blue). Panel in E represents the Hp mRNA expression (Lane 2) and DNA molecular weight markers (Lane 1) using RT-PCR. AV = alveolar.

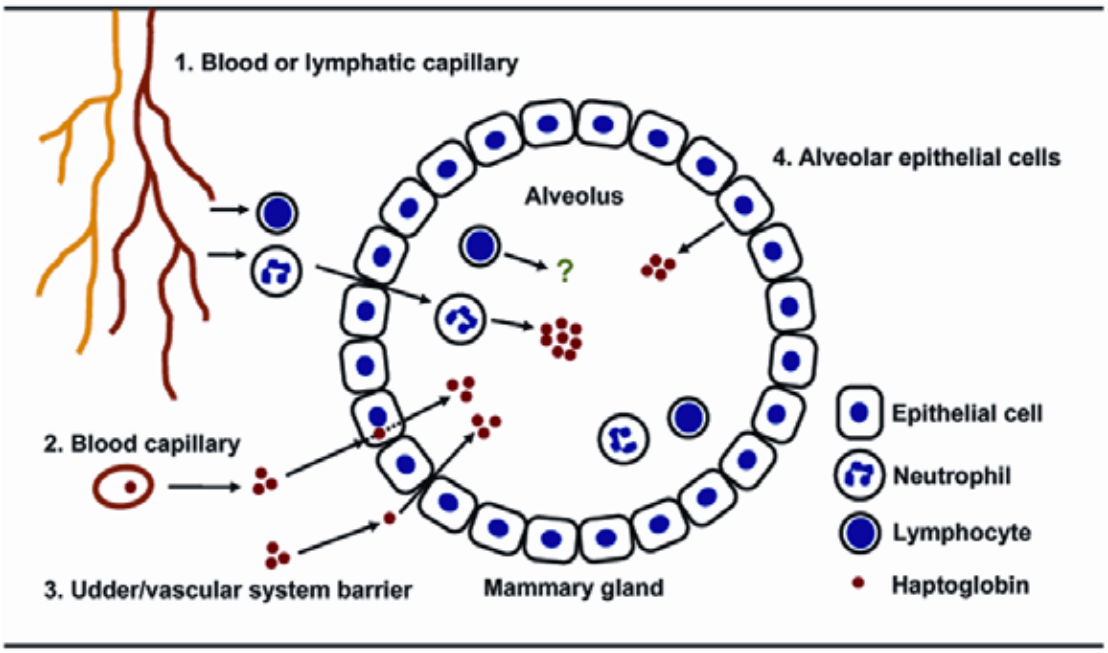

Figure 7. Schematic diagram of hypothetical pathways for the presence of bovine Hp in milk. There are at least four combined possible pathways for the presence of Hp in milk during mastitis. First, inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) may activate neutrophils and promote them to migrate through the endothelium, subepithelial matrix, and basement membrane of blood and lymph vessels into the infected mammary gland. Neutrophils then pass through the tight junction between the alveolar epithelial cells and enter into the alveolus to secrete cellular Hp (please see review article for more detail [30]) (pathway 1). Second, plasma Hp of hepatic origin mimicking albumin and immunoglobulin [31, 32] is able to directly enter into the epithelial cells via a receptor-mediated process and secrete into alveolus (including endocytosis and exocytosis) (pathway 2). Third, plasma Hp is able to spill into the udder by passive diffusion through a compromised udder/vascular system barrier (pathway 3). Fourth, the mammary epithelial cells are able to endogenously synthesize and express Hp in the alveolus (pathway 4).

Chapter 3

Cloning and expression of human haptoglobin subunits in *Escherichia coli*: delineation of a major antioxidant domain (Paper 2)

Abstract

Human plasma haptoglobin (Hp) comprises α and β subunits. The α subunit is heterogeneous in size, therefore isolation of Hp and its subunits is particularly difficult. Using *Escherichia coli*, we show that α1, α2, β, and α2β chain was abundantly expressed and primarily present in the inclusion bodies consisting of about 30% of the cell-lysate proteins. Each cloned subunit retained its immunoreactivity as confirmed using antibodies specific to α or β chain. By circular dichroism, the structure of each expressed subunit was disordered as compared to the native Hp. The antioxidant activity was found to be associated with both α and β chains when assessed by Cu^{2+}-induced oxidation of low density lipoprotein (LDL). Of remarkable interest, the antioxidant activity of β chain was extremely potent and markedly greater than that of native Hp (3.5 x), α chain (10 x) and probucol (15 x). The latter is a clinically proved potent compound used for antioxidant therapy. The "unrestricted" structure of β subunit may therefore render its availability for free-radical scavenge, which provides a utility for the future design of a "mini-Hp" in antioxidant therapy. It may also provide a new insight in understanding the

mechanism involved in the antioxidant nature of Hp.

Keywords: Human haptoglobin; α and β chains; cloning; mini-Hp fragment; antioxidant domain; monoclonal antibody; structure; circular dichroism

3.1 Introduction

Haptoglobin (Hp) is an acute-phase protein present in all mammals [1-3]. One of the major functions of Hp is to bind hemoglobin [4] and thereby prevent the oxidative tissue damage mediated by free hemoglobin [5]. We have recently demonstrated that chemically modified Hp losing its binding ability to hemoglobin, but is able to directly inhibit Cu^{2+} induced LDL oxidation [6]. In humans, Hp is originally synthesized as a single polypeptide containing both α and β chains (Fig. 1A) [7]. Posttranslational cleavage between the Arg of α chain and Ile of β chain (followed by removal of Arg) results in the formation of a single α and β chain that is subsequently linked by disulfide bridges to form mature Hp [8].

The Hp gene is characterized by two common alleles *Hp 1* and *Hp 2* corresponding to α1β and α2β polypeptide chains, resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. DNA encoding for these two chains are linearly oriented in chromosome 16q22.1 [9] with 5 and 7 introns in *Hp 1* and *Hp 2*, respectively. These introns, however, are exclusively located at the region corresponding to the α chain [5]. The α2 contains a unique nonhomologous

crossing-over in a part of the α1 [10]. Hp 2-1 and 2-2 form polymers due to an additional –SH group at the α2 chain, which determines its phenotype specificity [11]. All the phenotypes share the same β chains (each with 245 amino acids). The α1 chain (each with 83 amino acids) is "monovalent" forming one disulfide bridge with α and β subunit resulting in Hp dimer $(\alpha 1\beta)_2$. In contrast, the α2 chain (each with 142 amino acids) is "divalent" containing one extra free –SH that is able to interact with an additional α2. As such, α2 chains can bind to either α1 or α2 chains to form large polymers $[(\alpha 1\beta)_2\text{-}(\alpha 2\beta)_n$ in Hp2-1 and $(\alpha 2\beta)_n$ in Hp2-2]. The polymeric phenotypes have been reported to be more prevalent in some groups of patients with certain diseases, such as diabetic and autoimmune diseases [5,12-14].

The purposes of this study were to produce recombinant Hp (rHp) α1, α2, β and α2β subunits in *Escherichia coli* expression system and to test whether *E. coli* contains a protease that may specifically cleave the linear αβ chain as that in mammalian cells [6,15], and to determine which subunit of Hp possesses antioxidant activity. We demonstrated that *E. coli* was devoid of a specific protease responsible for the cleavage of α and β chains. Each expressed α or β subunit not only retained the immunoreactivity, but also possessed the antioxidant activity. However, a major antioxidant domain was located in the β chain with a superior potency to probucol. Remarkably interesting, the unfolded structure of Hp, based on circular dichroic spectra, dramatically increased in antioxidant activity. The present study provides a potential

utility for the future design of "mini-Hp" in developing a novel potent antioxidant. It may also provide a new insight in understanding the mechanism or specific amino-acids involved in the antioxidant nature of Hp.

3.2 Materials and methods

3.2.1 Materials

Escherichia coli JM109, M15 [pREP4], and the pQE30 expression vector were obtained from Qiagen (Hilden, Germany). Plasmid preparation and gel-extraction kits were purchased from BD Biosciences (Palo Alto, CA). The proofreading DNA-polymerase and dNTP were purchased from Invitrogen (Carlsbad, CA). All restriction enzymes were purchased from New England Biolabs (Beverly, MA). T4-DNA-ligase and HiTrap chelating column were purchased from Fermentas (Burlington, Canada) and Amersham Biosciences (Uppsala, Sweden), respectively.

3.2.2 Preparation of mouse mAb and human Hp

Mouse monoclonal antibodies (mAb) 3H8 and G2D specific to human Hp α or β subunit were produced in our laboratory according to the standard procedures previously established [16]. Native Hp was isolated from human plasma by an immunoaffinity column using procedures

previously established by us [17].

3.2.3 Plasmid construction of pQE30-Hp (α1, α2, β, α2β)

Gene fragments coding for human Hp α1, α2, β and α2β subunits were amplified by PCR using proofreading DNA-polymerase and oligonucleotide primers (Fig. 1A). The primer design was based on the published cDNA sequence of human Hp [18]. The primers were prepared for Hp α1 and α2 (forward 5'-GGGGTACCATGGTGGACTCAGGCAATGATGT-3' and reverse 5'-AACTGCAGTTACTGCACTGGGTTTGCCGGA-3'), Hp β (forward 5'-GGGGTACCATGATCCTGGGTGGACACCTGG-3' and reverse 5'-AACTGCAGTTAGTTCTCAGCTATGGTCTTCT-3'), and Hp α2β (forward 5'-GGGGTACCATGGTGGACTCAGGCAATGATGT-3' and reverse 5'-AACTGCAGTTAGTTCTCAGCTATGGTCTTCT-3'). Both of *Kpn* I and *Pst* I restriction sites were incorporated into the 5' end of the forward sequence and reverse sequence primers, respectively. The cDNA of Hp α1, α2, β and α2β were ligated into the *Kpn* I / *Pst* I sites of an *E. coli* expression vector, pQE30. The plasmids were screened in JM109 and then expressed in M15 [pREP4]. Finally, the sequence of pQE30-Hp (α1, α2, β and α2β) was confirmed by DNA sequencing.

3.2.4 Expression of recombinant Hp subunits

E. coli [M15 (pREP4)] was transformed with the recombinant plasmid and cultured in l L of Luria-Bertani (LB) medium containing ampicillin (100 μg/ml) at 37˚C on a rotary shaker. When the optical density reached 0.6 at 600 nm, the protein expression was induced by 1 mM IPTG at 37˚C for 2-4 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.

3.2.5 Disruption, wash, and isolation of inclusion bodies

The induced cells were then suspended in 40 ml of wash buffer and sonicated for 5 min at 4˚C, followed by centrifugation at 20,000 ×g for 20 min at 4˚C. The pellet containing the inclusion body was resuspended in 30 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 4˚C. Finally, the pellet was washed twice in wash buffer and stored frozen for late processing.

3.2.6 Solubilization of rHp subunits 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 4˚C for 12 h and the insoluble material was removed by centrifugation at 20,000 ×g for 20 min at 4˚C. Finally, the remaining soluble supernatant was then passed through a

syringe filter (0.45 μm) and proceeded directly for purification and refolding.

3.2.7 Purification and refolding of rHp subunits

Initially, a 1 ml-HiTrap chelating column was washed with 5 ml distilled water using a 10 ml-syringe. After loading 1 ml of 0.1 M $NiSO_4$, the column was extensively equilibrated with the binding buffer. The recombinant fusion protein was subsequently loaded onto the column and washed with the binding buffer. The bound protein was then treated with 6 M urea, starting with the binding buffer mentioned above and finished at one without urea. Finally, the recombinant proteins were eluted using a 20 ml linear gradient starting with an elution buffer containing 20 mM Tris-HCl, 0.5 M NaCl, and 20 mM imidazole without 2-mercaptoethanol, pH 8.0 and ending with the same buffer containing 500 mM imidazole. Protein fractions were pooled and then desalted on a P-2 column using 0.05 M ammonium bicarbonate, followed by lyophilization. Protein concentration was determined by the Lowry method [19], while using bovine serum albumin as a standard.

3.2.8 SDS-PAGE and Western blot analyses

Recombinant Hp subunits were characterized using SDS-PAGE containing 15% polyacrylamide as described previously [20]. In general, the tested sample was preheated at 100˚C 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. Western blot analysis was performed similar to that described previously [20]. In brief, the electrotransferred and blocked nitrocellulose was incubated with anti-Hp polyclonal or monoclonal antibodies (3H8 and G2D), followed by washes and incubation of peroxidase-conjugated anti-IgG. The membrane was developed using 3,3'-di-aminobenzidine (DAB) containing 0.01% H_2O_2 [20].

3.2.9 Determination of Immunoreactivity of rHp subunits by ELISA

ELISA was conducted according to the procedure previously established [20,21]. Half μg of each Hp subunit in 50 μl PBS (pH 7.4) was coated onto each well of an ELISA plate. After blocking and washes, 50 μl of diluted mouse mAb prepared against Hp α (3H8) or β chain (G2D) were added and incubated at room temperature for 2 h. Following washes and incubation of 50 μl of peroxidase-conjugated goat anti-IgG (1:3000 dilutions), the plate was developed using 0.04% 2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) containing 0.01% H_2O_2. The assay was conducted in triplicates. In general, the standard error of the mean (SEM) was less than 2%.

3.2.10 Preparation of human low density lipoprotein

Human low density lipoprotein (LDL; d. 1.012-1.063 g/ml) was prepared from human plasma by sequential ultracentrifugations according to the method previously established [22,23].

3.2.11 Antioxidant activity using Cu^{2+}-induced LDL oxidation

Thiobarbituric acid-reactive substances (TBARS) were used as an index for the measurement of LDL oxidation [23,24]. In a typical assay, 5 μM $CuSO_4$ and 20 μg of LDL (protein) were incubated with tested samples in a final volume of 100 μl. After 2h incubation at 37˚C, 250 μl of 20% tricholroacetic acid were added to precipitate proteins. Subsequently, 250 μl of 0.67% 2-thiobarbituric acid were added into the reaction mixtures and incubated at 80˚C for 30 min. The reaction mixtures were centrifuged at 3,000 ×g for 5 min. Supernatant (300 μl) in a 96-well plate was read at 540 nm [23]. The assay was conducted in triplicates. In general, the standard error of the mean was less than 2.5%.

3.2.12 Reduction and carboxymethylation of Hp

Tris-HCl buffer (0.01 M) containing 5.4 M urea and 1% (v/v) β-mercaptoethanol, pH 8.6. was added to 1 mg of Hp to make a final volume of 3.3 ml. The reaction mixture was flushed with nitrogen and incubated at room temperature. After 2 h, 20 mg of iodoacetic acid was slowly added and maintained the pH at 8.6 by the addition of 1 M NaOH for 30 min. Finally,

carboxymethylated (CM) Hp was desalted on a P2 column equilibrated with 0.1 M ammoniumbicarbonate and lyophilized [22].

3.2.13 Circular dichroic analysis

Lyophilized rHp subunits and CM-Hp were dissolved in 10 mM phosphate buffer (pH 7.4) with a final concentration of 0.2 mg/ml. About 300 μl aliquot was used for the analysis in a cuvette of 1 mm path length. Circular dichroic (CD) spectrum recorded between 190 and 300 nm (Jasco J-715 spectropolarimetry) was accumulated 20 times at a scanning rate of 50 nm/min [17,20].

3.3 Results and discussion

3.3.1 Plasmid construction and expression of rHp subunits

DNA sequencing analysis demonstrated that the cDNA of each Hp α1, α2, β or α2β (Fig. 1A) was inserted into the *E. coli* expression vector pQE30 (data not shown). The expression of $(His)_6$-tagged fusion proteins was constructed based on the T5 promoter transcription-translation system [25]. One advantage of this system is its ability to maintain target genes silently transcribed in the uninduced state, since the extremely high transcription rate initiated at the T5 promoter is efficiently regulated and repressed by the presence of high levels of the *lac* repressor. In addition, the pQE30 vector also permits the fusion of a 6× His-tag at the N-terminus of a given

recombinant protein, which is useful for rapid purification and permits the purification under denaturing conditions. Fig. 1B shows that clones containing cDNA of Hp or Hp subunit (α1, α2, β, or α2β) were abundantly and effectively induced by IPTG. Determined by densitometry on the SDS-PAGE gel, the average expressed level of each subunit was account for about 30% of the total lysate proteins (data not shown). Thus, this expression system is considerably effective in yield. The recombinant proteins were almost exclusively expressed in the inclusion bodies. Only trace amount (< 5%) of α subunits was found as a soluble form (data not shown). A full and intact length of α2β chain of Hp was expressed (Fig. 1B, lane 7). In human liver cells, however, the α2β is postranscritionally cleaved into α and β chains between the residues Arg and Ile [26,27]. A more recent study suggests that a complement C1r-like protease is responsible for the cleavage [15]. Our data indicate that this protease is not present in the *E. coli.* The recombinant α2β expressed may, therefore, provide a unique substrate for the identification of the specific protease involved in mammalian cells. This experiment is now in progress in our laboratory.

3.3.2 Purification and refolding of rHp subunits

The present study shows the rHp subunits to be primarily present in the inclusion bodies of *E. coli*. Since binding of a protein containing 6 × His-tag to nickel-immobilized column is not

interfered by the chaotropic agent (such as urea or guanidine hydrochloride) at high concentration, 6 × His-tagged recombinant protein can be solubilized by the chaotropic extraction before loading to the column. Removal of contaminating materials followed by refolding the recombinant protein using non-denaturing buffer can then be performed before the elution from the column by 20 mM imidazole [28]. Using this strategy, we show that the purification for rHp subunits efficiently achieved with about 95% homogeneity as determined by a 15% SDS-PAGE (Fig. 1B). The final average yield of each recombinant purified was about 20% as calculated from the total *E. coli* proteins (Table 1). The average recovery from each step as judged by the SDS-PAGE, however, was greater than 70%.

With respect to the solubility of rHp subunits, we found that the solubility could be maintained when lyophilized protein was reconstituted into a final concentration less than 2 mg/ml by a PBS. It is of interest to point out that the eluted subunits were soluble if they were concentrated (up to 5 mg/ml) by Centricon tubes immediately following the desalting on a P-2 column, while using 0.05 M ammonium bicarbonate as a mobile phase.

3.3.3 Immunoreactivity of mAb for rHp subunits

To address whether the expressed proteins were immunoreactive, each subunit was examined by a Western blot analysis using Hp mAb specific to human α (3H8) and β chain (G2D) and a Hp

polyclonal antibody specific to α/β chains. Fig. 1C shows that each subunit was specifically recognized by each regionally specific mAb. Using a quantitative ELISA, the immunoreactivity of native Hp was higher in some extent than that of expressed subunit (Fig. 2). Although the reason is not readily clear, it is possible that those mAb were originally prepared against the native state of the Hp structure and the antibodies preferably recognized the native conformation of Hp. Another potential explanation is that recombinant subunits might not be completely refolded as same as that in its native state (discussed below in CD spectral data). Nevertheless, the immunoreactivity of each expressed subunit was retained.

3.3.4 Antioxidant domain of rHp subunits

To determine the antioxidant activity of each Hp subunit, a TBARS method using Cu^{2+}-induced LDL oxidation was conducted [23]. Recombinant β subunit was extremely potent to protect LDL from oxidation and was in a dose-dependent manner. A typical example of the inhibition in TBARS is shown in Fig. 3. Ranking of the 50% of inhibitory concentration (IC_{50}) in antioxidant activity was as follow: α2β> β> α1≥ α2≥ probucol (Table 2). Most importantly, the antioxidant activity of α2β and β was markedly higher than that of α1 and α2 subunits. Thus, the major antioxidant domain of Hp was located in the β subunit. It is of remarkable interest that the activity of β subunit was greater than that of native Hp molecule (Table 2). One

of the possible explanations is that the random structure of the subunit may expose a neo-epitope that renders its availability for further scavenging the free radicals (discussed below). In the next experiment, we chemically modified Hp by carboxymethylation (CM) (in the presence of 5.4 M urea) to dissociate the disulfide linkages between α and β chains. The antioxidant activity of CM-Hp was substantially increased by about 3.75 folds when compared to native Hp (Table 2). The activity of CM-Hp was almost identical to β subunit. Because the carboxymethylation was conducted in the presence of 5.4 M urea, it is worth mentioning that native Hp exposed to the denaturing agent (5.4 M urea or guanidine hydrochloride) did not alter the antioxidant potency after renaturation by desalting (data not shown). The data suggest that the antioxidant domain of native Hp was exposed further, while reducing irreversibly. Another essential feature was that the antioxidant activity of β subunit was superior to probucol (15X): one of the most established potent antioxidants. Since probucol has been used for the treatment of patients with xanthoma and atherosclerosis for decades [29-33] and its analogs have been designed for antioxidant therapy [34-35], the recombinant β subunit plus the success of large expression in *E. coli* may pave the way for the future design of "mini-Hp".

3.3.5 Circular dichroic analysis

To address the moderately decreased immunoreactivity and high antioxidant activity in

recombinant subunit were probably due to the conformational difference from the ordered structure of whole Hp molecule, the secondary structure of recombinant Hp subunits was studied by CD spectra. Fig. 4 depicts that all the α1, α2, β and α2β were typically disordered in structure (a sharp drop of ellipticity between 190-210 nm) as compared to native Hp. The result suggests that the conformational changes in each subunit may be responsible for the low binding to each chain specific mAb, as each mAb was originally prepared against the native Hp. Whereas, the random structure of β chain may facilitate its availability for scavenging free radicals. Furthermore, the carboxymethylation Hp also exhibited a disordered structure.

In conclusion, we demonstrate that Hp β chain is an extremely potent antioxidant directly preventing LDL against oxidation in the present study. Using a recombinant Hp cDNA without the signal sequence, the yield of each subunit was relatively high containing approximately 30% of total cell lysate proteins. Each expressed subunit retained the immunoreactivity as confirmed by α and β chain specific mAb (3H8 and G2D). It is conceivable that expressed β subunit may provide as an initial utility for the future design of "mini-Hp" for potent antioxidant. It may also provide a new insight in understanding the mechanism or specific amino-acids involved in free radical scavenge when site-directed mutagenesis is carried out for further studies. The latter experiment is now in progress in our laboratory.

Acknowledgments

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Table 1. Purification of recombinant Hp subunits from *E. coli*

Purification step	Yield (mg)				Purity (%) [a]			
	α1	α2	β	α2β	α1	α2	β	α2β
Wet cells in 100 ml culture	410	450	420	510	37.3	32.8	38.5	23.3
Washed inclusion bodies	146	118	135	166	42.6	42.4	44.6	30.4
Ni^{2+}-column purified protein	14 [b]	11 [b]	5 [b]	7 [b]	95	95	95	95

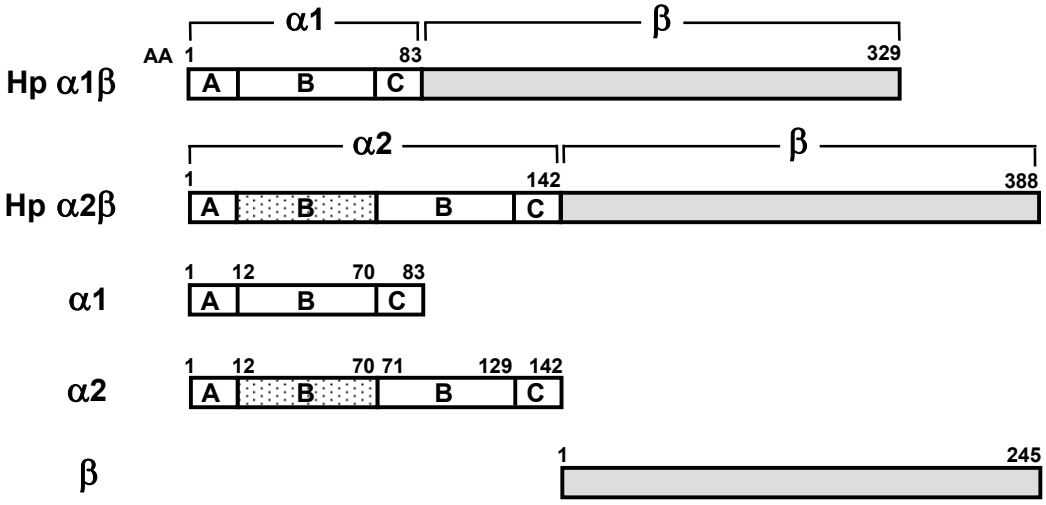
[a] Determined by densitometry of 15% SDS-PAGE.
[b] Determined using a Lowry method.

Table 2. Antioxidant potency in Cu^{2+}-induced LDL oxidation.

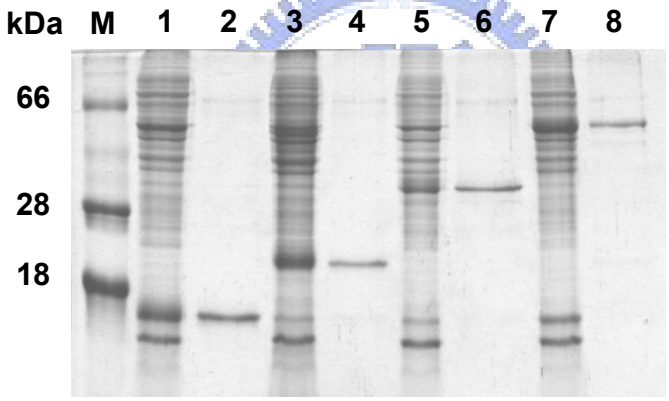
	Type	Cu^{2+}-induced IC_{50} (μM) [a]
α1	recombinant	7.50
α2	recombinant	8.72
β	recombinant	0.87
α2β	recombinant	0.65
Probucol	compound	13.70
Hp 1-1	native	3.00
CM Hp 1-1[b]	native	0.80

[a] IC_{50}: the concentration that inhibited 50% of LDL oxidation.
[b] CM Hp 1-1 was obtained by carboxymethylation to break up all the disulfide linkages.

A. Schematic drawing of cloned human Hp



B. SDS-PAGE



C. Western blot

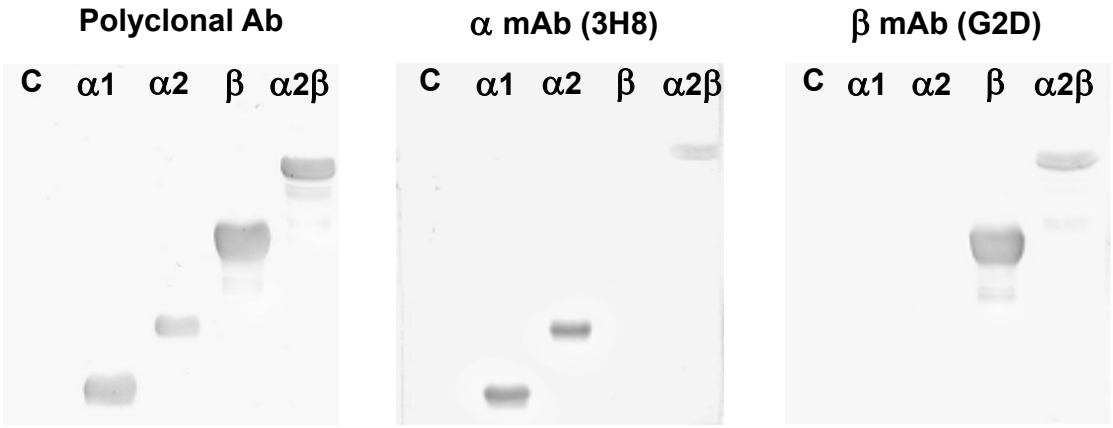


Figure 1. Gene construction and Hp expression. (A) Schematic drawing of cloned human Hp α1, α2, β and α2β subunits in *E. coli*. Human matured mRNA corresponding to Hp 1-1 is linear oriented (α1β). The linear amino-acid sequence (without signal peptide) containing residues 1-329 is shown on the top. Following a protease cleavage and a loss of the C-terminal residue Arg-84, it forms one α (residues 1-83) and one β chain (residues 1-245). Similarly, α2β forms one α2 (residues 1-142) and one β (1-245) chain. The amino acid sequence of α2 is identical to that of α1 with an insertion of repeated sequence B (dotted area or residues 12-70). It should be noted here, there are two amino acids in the inserted sequence B (dotted area) that are replaced by Asp-52 and Lys-53 in both native and our cloned sequence. (B) Expression and purification of recombinant Hp subunit in *E. coli*. Lane M, molecular markers; Lanes 1, 3, 5 and 7, whole cell lysate containing expressed α1, α2, β and α2β, respectively; Lanes 2, 4, 6 and 8, purified recombinant α1, α2, β and α2β, respectively. Notably, the α2β was not cleaved due to the lack of specific protease in *E. coli*. (C) Western blot of cell lysate containing α1, α2, β or α2β using Hp polyclonal antibody and α and β chain specific mAb. Lane C, whole cell lysate control. Other lanes represent the cell lysate containing expressed α1, α2, β or α2β subunit, respectively.

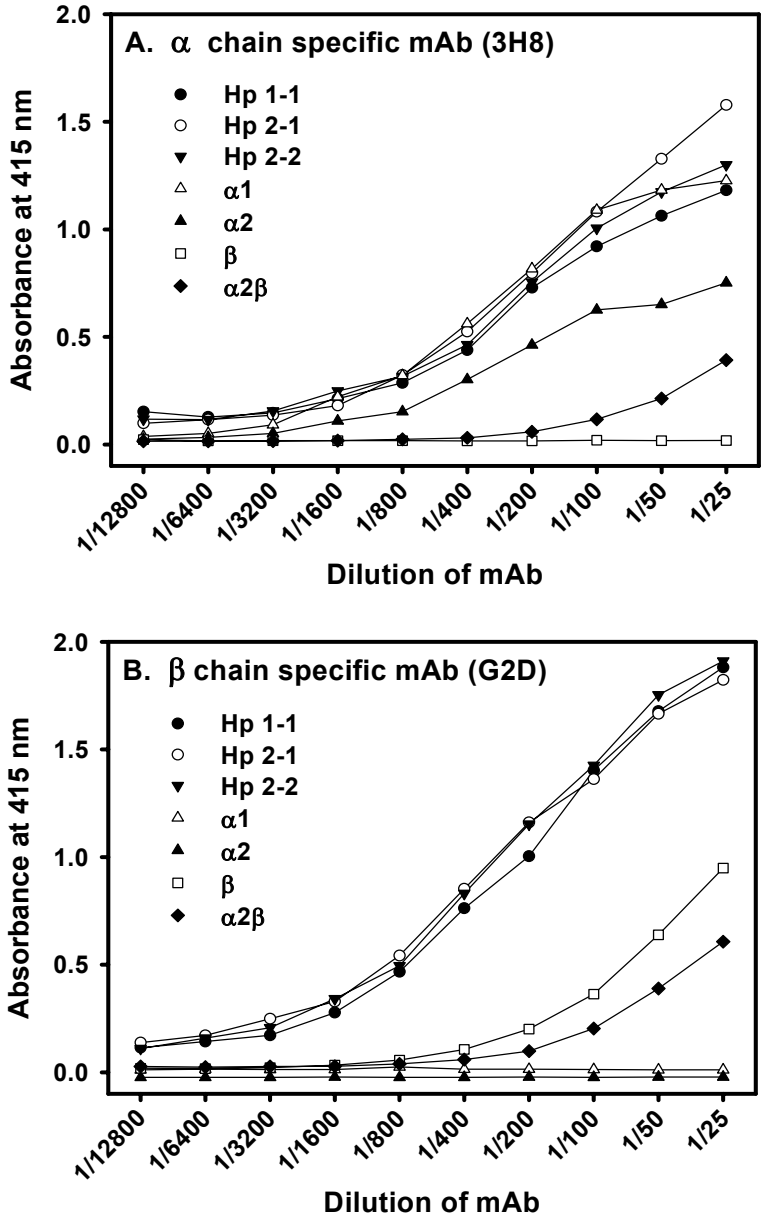


Figure 2. Immunoreactivity of native Hp 1-1, 2-1, and 2-2 and recombinant Hp measured by ELISA using Hp α and β specific mAb. The expressed α2β possesses full length of Hp, but is not cleaved as α and β subunits. (A) The immunoreactivity of native Hp is moderately higher than that of expressed α2β subunit using α chain mAb. (B) The immunoreactivity of native Hp is greater than β and α2β subunits using β chain mAb. This could be due to the structural difference found in Fig. 4.

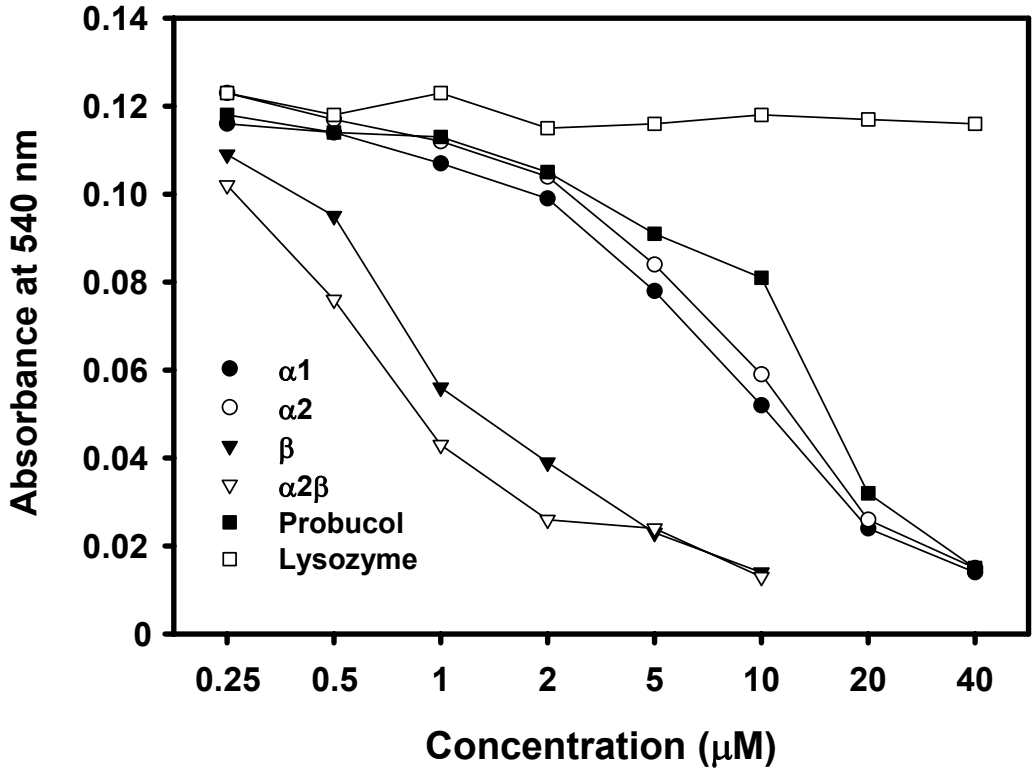


Figure 3. Antioxidant activity of each recombinant subunit. The assay was evaluated using the degree of inhibition of Cu^{2+}-induced formation of TBARS from LDL. LDL (20 μg) was incubated with 5 μM Cu^{2+} in the presence of recombinant α1, α2, β, α2β or probucol at 37˚C for 2 h in a final 100 μl of PBS. Lysozyme served as a control did not reveal any antioxidant activity.

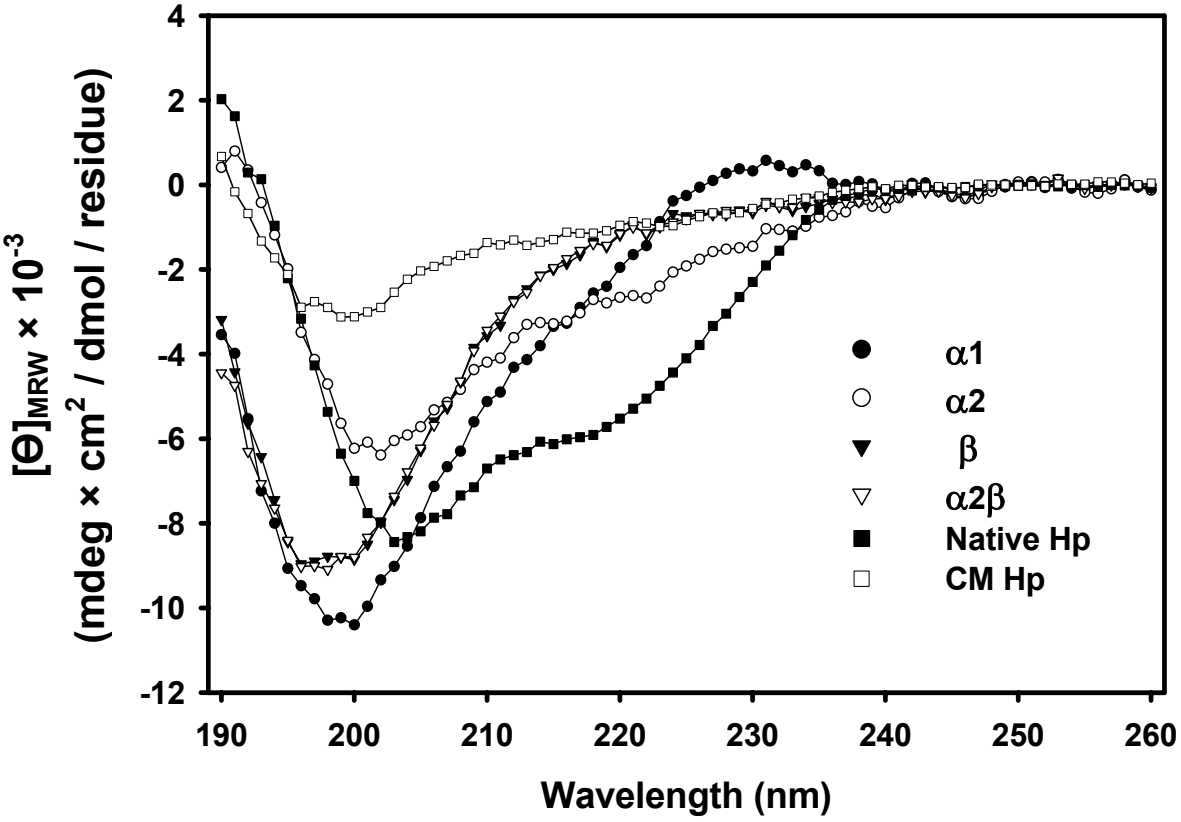


Figure 4. Circular dichroic spectra of recombinant α1, α2, β and α2β subunits. Recombinant Hp subunit or Hp (0.2 mg/ml) in 10 mM phosphate buffer, pH 7.4, was monitored by a circular dichroic spectrophotometer. Each spectrum represents a mean of 20 determinations. Essentially, recombinant α1, α2, β and α2β subunits and CM-Hp are typically random or disordered in structure (a sharp drop of ellipticity between 190~210 nm.

Chapter 4

A unique tetrameric structure of deer plasma haptoglobin: an evolutionary advantage in the Hp 2-2 phenotype with homogeneous structure (Paper 3)

Abstract

Similar to blood types, human plasma haptoglobin (Hp) is classified as three phenotypes: Hp 1-1, 2-1, and 2-2. They are genetically inherited from two alleles *Hp 1* and *Hp 2*, but there is only Hp 1-1 phenotype or *Hp 1* in almost all the animal species. The Hp 2-2 protein consists of complicated large polymers cross-linked by α2-β subunits or $(\alpha 2\text{-}\beta)_n$ (where $n \geq 3$, up to 12 or more) and is associated with the risk of the development of diabetic, cardiovascular, and inflammatory diseases. In the present study, we found that deer plasma Hp mimics human *Hp 2* containing a tandem repeat over the α-chain based on our cloned cDNA sequence. Remarkably interesting, the isolated deer Hp is homogeneous and tetrameric as a $(\alpha\text{-}\beta)_4$, although the location of –SH groups (responsible for the formation of polymers) is exactly identical to that of human. Denaturation of deer Hp using 6M urea in reducing condition (143 mM β-mercaptoethanol) followed by renaturation still sustained the formation of $(\alpha\text{-}\beta)_4$ suggesting that the Hp tetramers are not randomly assembled. Interestingly, an α-chain monoclonal antibody (W1) known to recognize both human and deer α-chains, only binds to the intact human Hp polymers, but not to deer Hp tetramers. It implicates that the epitope of

deer α-chain is no longer exposed on the surface when Hp tetramers are formed. We propose that steric hindrance plays a major role in determining the polymeric formation in human and deer polymers. Phylogenetic and immunochemical analyses revealed that the Hp 2 allele of deer might have arisen at least 25 million years ago. A mechanism involved in forming Hp tetramers is proposed and discussed, and the possibility is raised that the evolved tetrameric structure of deer Hp might confer a physiological advantage.

Keywords: deer and human haptoglobin; amino-acid sequence; purification; phenotype; monoclonal antibody

4.1 Introduction

Haptoglobin (Hp) is an acute phase protein (responsive to infection and inflammation) that is present in the plasma of all mammals [1-4]. A recent study has found that Hp also exists in lower vertebrates, bony fish but not in frog and chicken [5]. The most frequently reported biological functions of the protein are to capture released hemoglobin during excessive hemolysis [6] and to scavenge the free radicals during oxidative stress [7]. The captured hemoglobin is internalized by a macrophage/monocyte receptor, CD163, via endocytosis. Interestingly, the CD163 receptor only recognizes Hp and hemoglobin in complex, which indicates the exposure of a receptor-binding neoepitope [6]. Thus, CD163 is identified as a hemoglobin scavenger receptor. Recently, we have shown that Hp is an extremely potent

antioxidant, which directly protects low-density-lipoprotein (LDL) from Cu^{2+}-induced oxidation. The potency is markedly superior to probucol: one of the most potent antioxidants used in antioxidant therapy [8-10]. Transfection of Hp cDNA into Chinese hamster ovary (CHO) cells protects them against oxidative stress [9].

Human Hp is one of the largest proteins in the plasma originally synthesized as a single αβ polypeptide. Following posttranslational cleavage by a protease, α- and β-chains are formed and then linked by disulfide bridges producing the mature Hp [11]. The gene is characterized by two common alleles *Hp 1* and *Hp 2* corresponding to α1-β and α2-β polypeptide chains, respectively, resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. All the phenotypes share the same β-chain containing 245 amino-acid residues. As shown in Fig. 1A, the α1-chain containing 83 amino-acid residues possesses two "free" –SH groups. The one at the –COOH terminus always cross-links with a β-chain to form a basic α-β unit, and the other at the NH_2-terminus links with another $(\alpha\text{-}\beta)_1$ resulting in a Hp dimer $(\alpha1\text{-}\beta)_2$ or a Hp 1-1 molecule. In contrast, the α2-chain containing a tandem repeat of residues 12-70 of α1 with 142 amino-acid residues is "trivalent" providing an additional free –SH (Cys-15) that is able to interact with another α-β unit. As such, α2-chains can bind to either α1-β or α2-β units to form large polymers $[(\alpha1\text{-}\beta)_2\text{-}(\alpha2\text{-}\beta)_n$ in Hp2-1 and $(\alpha2\text{-}\beta)_n$ in Hp2-2] as shown in Fig. 1B.

Because of its weaker binding affinity to hemoglobin and retarded mobility (or

penetration) between the cells, the polymeric structure of Hp 2-2 is dramatically more prevalent in some groups of patients with certain diseases, such as diabetes and inflammation-related diseases [7,12-14]. The human *Hp 2* allele has been proposed to be originated from *Hp 1* about 2 million years ago and then gradually displaced *Hp 1* as a consequence of a nonhomologous crossing-over between the structural alleles (*Hp 1*) during meiosis [15-17], and is the first example of partial gene duplication of human plasma proteins [15,18,19]. Thus, only humans possess additional Hp 2-1 and 2-2 phenotypes.

In the present study, deer Hp protein was initially shown to be a homogeneous polymer using an electrophoretic hemoglobin typing gel. Following isolation and identification of the protein, the α-chain was found to be similar to human α2-chain based on its apparent molecular mass. We than cloned the cDNA of deer Hp showing that the putative amino-acid sequence mimics that of human Hp 2-2 (81.7% and 67.9% sequence homology in β- and α-chain, respectively), and that the α-chain of deer also possess a unique tandem repeat. Interestingly, deer Hp α-chain comprises seven –SH groups, that are oriented exactly the same as human Hp 2-2, but the molecular arrangement of deer Hp is strictly tetrameric or $(\alpha\text{-}\beta)_4$. It is thus totally different from human Hp 2-2 with $(\alpha\text{-}\beta)_n$ polymers, where $n \geq 3$. Using an α-chain monoclonal antibody (mAb) as a probe and denaturing/renaturing experiments, we further demonstrated that steric hindrance of the Hp α-chain plays a major role in determining the polymeric formation of human $(\alpha\text{-}\beta)_n$ and the deer $(\alpha\text{-}\beta)_4$ tetramer.

Amino-acid sequence alignment demonstrated that the evolved amino-acid sequences of ruminant β-chain are the most divergent among all mammals. By phylogenetic tree analysis, we identified the α-chain of dolphin and whale (a branch before the deer) belonging to the α1 type. This suggests that the deer tandem repeat sequence occurred between 25 and 45 million years ago, which is much earlier than the two million years proposed for humans. The evolved tetrameric structure of deer Hp might be of a physiologic advantage. We further proposed that a steric hindrance mechanism is involved in forming Hp tetramers.

4.2 Materials and methods

4.2.1 Animal plasma

Animal plasma of deer (*Cervus unicolor swinhoei*), goat (*Capra hircus*), cattle (*Bos taurus*), pig (*Sus scrofa domestica*), dolphin (*Steno bredanensis)*, and whale (*Delphinapterus leucas*) were obtained from the Pingtung County Livestock Disease Control Center and the Veterinary Medicine Teaching Hospital, National Pingtung University of Science and Technology.

4.2.2 Phenotyping

Hp phenotyping was performed by native PAGE using hemoglobin-supplemented serum or plasma [20]. Briefly, 6 μL plasma were premixed with 3 μL of 40 mg/mL hemoglobin for 15 min at room temperature. The reaction mixture was then equilibrated with 3 μL of a

sample buffer containing 0.625 M Tris (pH 6.8), 25% glycerol, and 0.05% bromophenol blue, followed by electrophoresis on a 7% native polyacrylamide gel (pH 8). Electrophoresis was performed at 20 mA for 2 h, after which time the Hp-hemoglobin complexes were visualized by shaking the gel in a freshly prepared peroxidase substrate (30 mL of phosphate-buffered saline containing 25 mg of 3,3'-diaminobenzidine in 0.5 mL of dimethyl sulfoxide and 0.01% H_2O_2). The results was confirmed by Western blot using an α-chain specific mAb prior to phenotyping.

4.2.3 Preparation of mouse mAb and human Hp

Mouse mAb W1 specific to human Hp α-chain was produced in our laboratory according to the standard procedures previously established [21]. Native human Hp was isolated from the plasma using an immunoaffinity column followed by a size-exclusion chromatography on an HPLC system using previously described procedures [20].

4.2.4 Purification of deer haptoglobin

Plasma samples enriched with Hp were prepared from deer blood containing 0.1% EDTA followed by centrifugation at 1,200 g for 15 min at 4ºC to remove the cells. Isolation was conducted performed according to the method previously established for porcine Hp [22]. Saturated ammonium sulfate solution was added to the plasma to a final saturated

concentration of 50%. After gentle stirring for 30 min at room temperature, the precipitate was discarded by centrifugation at 4,000 g for 30 min at 4ºC. The supernatant was then dialyzed at 4ºC for 16 h against PBS containing 10 mM phosphate (pH 7.4) and 0.12 M NaCl with three changes. After dialysis, the sample was concentrated and filtered through a 0.45 μm nylon fiber prior to size-exclusion chromatography. An HPLC system (Waters, Milford, MA) consisting of two pumps, an automatic sample injector, and a photodiode array detector, with a Superose-12 column (1x30 cm) (GE Healthcare, Uppsala, Sweden) pre-equilibrated with PBS, was used for further purification. The column was run for 60-80 min at room temperature with a flow-rate of 0.3 mL/min using PBS as a mobile phase. Fractions containing Hp were pooled and concentrated to a final volume of 1 mL using an Amicon centrifugal filter (Millipore, Billerica, MA) and stored at -20ºC until use.

4.2.5 Gel electrophoresis

SDS-PAGE was performed according to the method described by Laemmli [23] with some modifications using 5% polyacrylamide as a stacking gel [24]. In general, samples containing 143 mM β-ME was preheated at 100˚C for 10 min in a buffer containing 12 mM Tris-HCl (pH 6.8), 0.4% SDS, 5% glycerol, and 0.02% bromophenol blue before loading to the gel. The samples were run on a step gradient of polyacrylamide gel (10 and 15%) for about 1.5 h at 100V and stained using Coomassie brilliant blue. For determination of

molecular mass of Hp, the tested samples were prepared under the non-reducing condition using SDS-PAGE. Alternatively, the SDS gel was prepared in a 0.04 M phosphate buffer (pH 7.0) containing 4% polyacrylamide, and the samples were run for about 6 h at 30V. The molecular mass standards for SDS-PAGE, containing three prestained proteins (260, 160. and 110 kDa), was purchased from Invitrogen (Carlsbad, CA)

4.2.6 Immunoblot analysis

Western blot analysis was performed using a method similar to that described previously [24]. In brief, the electrotransferred and blocked nitrocellulose was incubated with anti-Hp mAb W1, followed by washes and incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA). The membrane was developed using 3,3'-diaminobenzidine containing 0.01% H_2O_2. Dot blots were performed by applying the samples (reduced or non-reduce) onto a nitrocellulose membrane using anti-Hp mAb W1 as a primary antibody.

4.2.7 Cloning and sequencing analysis of deer Hp

The entire procedure was similar to that described previously [9,10]. Briefly, total RNA was extracted from the deer whole blood using RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The gene for deer Hp from total RNA was

reverse transcribed and PCR amplified using proofreading DNA-polymerase (Invitrogen), forward primer 5'-TTCCTGCAGTGGAAACCGGCAGTGAGGCCA-3', and reverse primer 5'-CGGAAAACCATCGCTAACAACTAAGCTTGGG-3'. The PCR cycling profile was as follows: denaturation at 94ºC for 5 min, then 35 cycles of denaturation at 94ºC for 30 s, annealing at 55ºC for 30 s, and extension at 72ºC for 90 s; and final extension at 72ºC for 10 min. The PCR product was analyzed by electrophoresis through a 1% agarose gel and purified using a gel-extraction kit (BD Biosciences, Palo Alto, CA). The purified PCR product was cloned into a pGEM-T Easy vector (Promega, Madison, WI) and then the ligated plasmid was transformed into *Escherichia coli* JM109 (Qiagen). Finally, the sequence of deer Hp was confirmed by DNA sequencing.

4.2.8 Sequence alignment and phylogenetic analysis

The cDNA and amino acid sequence alignment, sequence pair distances, and phylogenetic tree construction were performed using DNASTAR software (Lasergene, Madison, WI).

4.2.9 Denaturation and renaturation of deer and human Hp 2-2

Purified deer Hp (0.1 mg/mL) or human Hp 2-2 (2 mg/mL) were mixed with PBS containing 6 M urea and 143 mM β-ME and incubated at room temperature for 30 min. The reaction mixture was first dialyzed in 200 mL of PBS at 4 ºC for 6 h, and this was repeated three times

(total 24 h) to allow renaturation. The mixture was finally dialyzed against 2 L PBS overnight. The concentrated Hp samples with or without reduction were incubated with hemoglobin for use on a typing gel as that for plasma phenotyping, and then stained by Coomassie brilliant blue.

4.3 Results

4.3.1 Identification of Hp phenotype

It has been claimed that Hp of ruminants (cattle, sheep, and goat) cannot enter polyacrylamide gels due to the large polymeric nature of the protein [25,26]. We tested whether this was also the case for the Hp of deer (another ruminant). Using a hemoglobin typing gel, we unexpectedly found deer plasma Hp to be a simple homogeneous molecule which is small enough to enter a 7% electrophoretic gel. An example of its phenotype and the electrophoretic property of deer Hp, compared to human Hp 1-1, 2-1, and 2-2, is shown in Fig. 2. This shows that deer Hp mimics one of the polymeric forms of human Hp 2-1 or 2-2: either a linear or cyclic tetramer.

4.3.2 Isolation of deer Hp

The molecular size of the Hp α-chain has been conventionally used for identifying the phenotype of a given Hp protein. To further characterize the molecular form of deer plasma

Hp, we attempted to isolate the protein using a Sepharose-based immunoaffinity column [20,27]. A mouse mAb prepared against human α-chain (W1) was utilized for coupling to the Sepharose because this mAb was able to react with both human and deer α-chain on a Western blot (described below). First, plasma samples enriched with Hp were pooled and applied onto the affinity column. This procedure, however, failed to isolated deer Hp from the plasma due to the lack of the binding of deer proteins to the column. Next, we used combined ammonium-sulfate fractionation and size-exclusion chromatography procedures [22] for the isolation. A size-exclusion chromatographic profile for the fractions containing Hp is shown in Fig. 3A (second peak). The homogeneity of isolated Hp was approximately 90% as identified by SDS-PAGE (Fig. 3B). The presence of α-chains were confirmed by a Western blot using mAb W1 (Fig. 3B; right panel).

4.3.3 Hemoglobin binding of isolated Hp

In the next experiment, we tested the hemoglobin binding ability of isolated deer Hp. Fig. 3C shows that the isolated Hp was able to form an Hp-hemoglobin complex under 7% native-PAGE. Furthermore, it demonstrates that the deer protein is consisted of one major molecular form that is identical to its native form in the plasma based on electrophoretic mobility. It appears that the Hp isolated under our experimental conditions was not significantly altered with regard to its molecular and biochemical properties.

4.3.4 Molecular mass estimation of deer and human Hp 2-2 using SDS-PAGE and Western blot

Western blot analysis using the α chain-specific mAb W1 indicates that the mAb recognizes both human and deer α-chains (Fig. 4A). It also reveals that the deer α-chain belongs to the α2 group, with a molecular mass of approximately 18 kDa on both SDS-PAGE and Western blot. We therefore tentatively classified the deer Hp as phenotype 2-2. In isolated deer Hp, there was a trace amount of hemoglobin (approximately 14 kDa), with a molecular mass comparable to the human Hp α1-chain. The estimated molecular mass of the deer β-chain was about 36 kDa, slightly lower than that of human. The isolated deer Hp was further characterized using 4% non-reduced SDS-PAGE under non-reducing conditions. Consistent with our hemoglobin binding assay, Fig. 4B (Left) demonstrates that isolated deer Hp consists of only one specific tetrameric form, i.e. $(\alpha\text{-}\beta)_4$, with a molecular mass about 216 kDa, which is close to that of the human Hp 2-2 tetramer (230 kDa) based on the gel profile.

4.3.5 Unique immunoreactivity of deer Hp defined by mAb W1

We then attempted to ensure that the polymeric forms of human and deer protein were an Hp by a Western blot analysis using W1 mAb. Figs. 3B and 4A clearly showed that this antibody was capable of binding both human and deer α-chains in its reduced form.

Interestingly, Fig. 4B (right panel) shows that this mAb recognized all the human Hp 2-2 polymers, but not intact deer Hp 2-2. However, after adding a reducing reagent (β-mercaptoethanol; β-ME) directly to intact deer Hp, the immunoreactivity was recovered on a dot-blot assay (Fig. 4C). It appears that the antigenic epitope of deer α-chain is masked in the tetrameric form. This also explains why the W1 mAb coupled affinity-column failed to bind deer plasma Hp in the purification procedure desceibed above.

4.3.6 Cloning of deer Hp cDNA

Evidently, the molecular form of deer "Hp 2-2" totally differs from that of human Hp 2-2, with the latter found as typical polymers or the form $(\alpha\text{-}\beta)_n$, where n = 3-12 (Fig. 4B). It remains ambiguous as to whether deer Hp should be designated as a typical Hp 2-2. The most significant feature of the molecular structure of human Hp 2-2 is that it includes a tandem repeat in the α2-chain. To determine whether this is also true in deer Hp, we cloned the deer Hp cDNA. The complete linear nucleotide sequence corresponding to the α-β chain determined by our laboratory has recently been submitted to GenBank (accession number EF601928). Based on the cDNA sequence, the deer α- and β-chains comprise 136 and 245 amino-acid residues, respectively, which is similar to that of human with 142 (α2) and 245 (β) residues (Figs. 5A,B). A tandem repeat of the deer α-chain was observed (discussed below).

4.3.7 Amino-acid sequence alignment of deer and human Hp 2-2

The putative amino-acid sequence alignment reveals that deer Hp is somewhat homologous to human Hp 2-2 (80% and 68% for β- and α-chain, respectively). The divergence and identity of the β-chain with the other mammals are shown in Fig. 5C. The sequence of deer is relatively similar to that of cattle [28], another ruminant. We also created a brief phylogenetic tree for possible molecular evolution of the Hp β-chain using the CLUSTAL method in DNASTAR MEGALIGN software. The result shows that evolved amino-acid sequences of ruminant Hp β-chains are the most divergent among all mammals (Fig 5D).

4.3.8 Analysis of –SH groups of deer Hp α-chain and its implication for the formation of tetramer

As shown in Fig. 6 in the form of simplified ABC domains, the human α2-chain contains identical ABC domains to α1 with insertion of a tandem repeat region (B1). The latter contains amino-acid residues between Asp12 and Ala70 (a total of 59 residues). The sequence homology between the repeat regions of human α2-chain is 96% with only two amino acids mutated (replacement of Asn52 and Glu53 in the B region by Asp-52 and Lys-53 in the B1 region). This tandem repeat is responsible for the formation of Hp polymers due to an extra –SH group (Fig. 1A). Such repeats also exist within the deer α-chain (B1 and B repeat), where the B1 region is residues 9-65. Thus, at the molecular level, the deer α-chain

belongs to the α2 group, and is identical to the human α2 chain in possessing a tandem repeat. Interestingly, the sequence homology between the two repeat units (B1 and B) of deer is only 68% (Fig. 6).

As shown in a schematically in Fig. 1A, the human α2-chain consists of seven –SH groups (Cys15, 34, 68, 74, 93, 127 and 131) in 142 residues. Among these, there are two disulfide linkages within the α-chain (Cys34 and 68 and Cys93 and 127), and the one at the C terminal region (Cys131) cross-links with the β-chain (Cys105) to form a basic α-β unit. Under such an arrangement, Cys15 and Cys74 are available to link with other α-β units. As a result, human α2 forms $(\alpha\text{-}\beta)_n$ polymers (where $n \geq 3$) as shown in Fig. 4B. Interestingly, the number and location of –SH groups in deer α2-chain are identical to those in human (Fig. 6), but the deer Hp only yields a tetrameric $(\alpha\text{-}\beta)_4$ form. As the identity between the tandem repeats of deer is only 68% (compared with 96% in human), we hypothesized that these amino-acid difference eventually determines the conformation between Cys15 and 74 and drive the construction of the $(\alpha\text{-}\beta)_4$ structure of deer Hp (see Discussion).

To test whether the deer Hp can also form multiple polymers *in vitro*, we denatured the protein by 6 M urea with addition of 143 mM β-ME. Under these conditions, the deer protein was completely dissociated, similar to the profile shown in Fig. 4A for SDS-PAGE analysis (data not shown). We then slowly renatured the deer Hp by stepwise dialysis in order to determine possible formation of other large polymers (greater than tetramer).

Figure 7 show that the renatured protein retained the tetramer form, and no other polymers larger than tetramers were observed on SDS-PAGE, although some trimers were produced. Under the same condition, human Hp 2-2 was renatured to $(\alpha\text{-}\beta)_n$. The data suggest that the formation of deer Hp tetramer is specific, not randomly assembled. This assembly seems to be dependent on the unique orientation of the –SH groups within the Hp. In addition, each renatured protein retained its hemoglobin-binding ability (Fig. 7). A hypothetical model explaining the formation of Hp tetramers is detailed below.

4.4 Discussion

4.4.1 Isolation of deer native Hp

We have recently developed several lines of human Hp mAb and routinely utilized these antibodies for the isolation of human Hp 1-1, 2-1, and 2-2 phenotypes [20,29]. As only W1 (specific to α-chain) is able to cross-react with the deer α-chain on a Western blot, we attempted to utilize this mAb for the affinity isolation of deer Hp in this study. Interestingly, the W1 mAb only recognize the human Hp but not deer Hp in their intact form (Figs. 4B and 4C). We therefore used a previously described HPLC-based size-exclusion chromatography procedure [22] for the isolation of deer Hp. However, this procedure is only suitable for isolating the Hp with a homogeneous structure and not possible for human Hp 2-2 or 2-1 [20]. One minor disadvantage of the method was the contamination of the isolated Hp by a trace

amount of hemoglobin (Fig. 4A). This is observed mainly because Hp-hemoglobin complexes are formed prior to the purification; as such, hemolysis should be kept to a minimum in order to reduce the hemoglobin level while collecting the blood.

4.4.2 Presence of Hp in deer plasma

Not all the deer possess a high level of plasma Hp. About 30% of the plasma samples that we screened (total n = 15) exhibited low Hp levels on hemoglobin binding assay (Fig. 2). Based on chromogeneity, the concentrations of deer plasma were approximately 1 mg/mL of those used for purification when compared with human Hp 1-1 standard. In reindeer (n = 6), a mean plasma value of 0.6 mg/mL has been reported [30].

4.4.3 Primary structure of deer α-chain and its relationship to Hp polymers

There are several lines of evidence to support the conclusion that the genotype of deer Hp belongs to *Hp 2* with phenotype as 2-2. First, analysis of mercaptoethanol-reduced plasma indicates a molecular mass of 18 kDa for the α-chain, which is similar to that of human α2 based on a Western blot (Fig. 4A). Second, the molecular mass of α-chain from a purified sample is also similar to that of human α2 (Fig. 4A). Third, by putative amino-acid sequence alignment, deer α-chain contains a unique tandem repeat that is consistent with that found in human. Forth, the total number of –SH groups and their location resulting from the

tandem repeat are completely identical to that of human, although the sequence homology between the repeat was 68% in deer, compared to 96% in human (Fig. 6).

It remains unclear why the apparent molecular mass of the deer α-chain on PAGE is somewhat higher than that of human. We then therefore attempted to determine whether it was due to additional carbohydrates moieties on the deer α-chain. Using the Pro-Q Emerald Glycoprotein Gel Stains (Molecular probes, Eugene, OR), we did not identify any carbohydrates associated with the α-chain of either species (data not shown).

4.4.4 Hypothetical model for the formation of the deer Hp tetramer

The ability of the deer Hp to refold and reassemble into its tetrameric forms *in vitro* indicates that the assembly of α- and β-chains into predetermined polymers is dependent on their biochemical nature (Fig. 7). As shown in Fig. 8A, we proposed a model to explain the formation of tetramers. This suggests that the two –SH groups of deer α-chain are located on two flat surfaces at different angle to each other. Under this condition, a homodimer cannot form due to the availability of another free –SH group of a α-β unit for cross-linking with another α-β unit. Figure 8B illustrates that there is no steric hindrance for tetramer formation, although there are two possible configurations for the tetramer. Some trimers may form, but there is some hindrance preventing the subunits from coming close together in the cyclic center (Fig. 8C). Therefore, the formation of trimers takes place at a much lower

extent than tetramers. No higher order polymers are formed, because the distance between the –SH groups is too great to allow cross-linking for $(\alpha\text{-}\beta)_5$ pentamers or other larger polymers (Fig. 8D).

For a higher order polymer (n>5) the angle (θ) between the sides containing the –SH groups of two polymers would be 90-360/n degrees. If the distance between the –SH sites is approximately form a 90°, and the side of the Hp subunit contributes the base of the triangle, the distance is proportional to sin θ. As θ approaches 90° as n approaches infinity, the distance between the –SH sites also comes close to maximum as n increases. In fact, few trimers are seen in our renaturing experiment (Fig. 7) and no polymers of an order of five or higher are observed.

For human Hp 2-2 on the other hand, the formation of higher order polymers is possible (Fig. 9). The assumed positions of the –SH groups differs from those in deer Hp. They are located at the edges of the same plane, so formation of an identical "stacking" dimer or $(\alpha\text{-}\beta)_2$ is not possible due to the steric hindrance between the two –SH groups (Fig. 9A). However, formation of some trimers by linking together via the two –SH groups at the edge is possible, but not to a great extent due to the limited space in the cyclic center (Fig. 9B). This explains why there are only trace amount of trimers in all the human Hp 2-2 samples (Fig. 2). The cyclic center provides sufficient room to facilitate the formation of polymers of an order greater than four α-β units. Such configuration also renders the binding of the W1 mAb.

In contrast, the cyclic center of deer Hp teramers is totally blocked and is not accessible for mAb binding (Figs. 4B,C).

4.4.5 Evolution

In vertebrates, a recent study has suggested that the Hp gene appeared early in vertebrate evolution, between the emergence of urochordates and bony fish [5]. All mammalian species studied to date have been shown to possess Hp. Analysis of the electrophoretic patterns of Hp-Hemoglobin complexes has suggested that most of these Hps are similar to human Hp 1-1 [31]. Only the protein found in ruminants (cattle, sheep, and goat) resemble polymeric forms of human Hp 2-2 [25], but whether they also possessed a tandem repeat remained unexplored [28].

It is thought that humans originally had a single Hp 1-1 phenotype [32]. Maeda et al. [15] proposed that the tandem repeat sequence of human α2 has evolved two million years ago from a nonhomologous unequal crossover between two *Hp 1* alleles [*Hp 1S and Hp 1F*] during meiosis. The unique feature of the *Hp 2* allele is that it is present only in humans and not found in any primates, including New and Old world monkeys, chimpanzees, and gorillas [17]. We have recently found that cattle also possess *Hp 2* as the sole genotype [28]. It is likely that ruminants including deer, cattle, goat, and sheep may all possess a sole *Hp 2*-type allele. In the present study, we have shown that the inserted tandem repeat region in deer Hp

appears to have extensively evolved, as 32% of the repeated region has undergone mutation, compared to that of only 4% (two amino-acid residues) in human Hp (Fig. 6). Thus, we propose that the occurrence of the tandem repeat in deer was much earlier than in humans.

Figure 10 depicts the phylogenetic tree constructed by assuming all eutherian orders (mammals) radiated at about the same point in evolutionary time (approximately 75 million years ago) [33]. The phylogenetic analysis indicates that the crossing-over of deer α-chains occurred after divergence of the line leading to ruminants and pig, as pig possesses only Hp 1-1 phenotype [22]. As dolphins and whales are the closest divergence before the ruminants, we further examined the size of α-chain in whales and dolphins as well as other ruminants (cattle and goat) to determine the possible time of the tandem repeat evolution in deer Hp. Interestingly, the inserted panels of Fig. 10 shows that the α-chains of all the ruminants tested are of α2 type, except for dolphins (n = 5) and whales (n = 5).

These data suggests that the crossing-over of tandem repeat in ruminants occurred approximately at least 25 or between 25 and 45 million years ago (Fig. 10), which is much earlier than that two million years proposed in humans [15]. The molecular evolution of the ruminants, which are the latest mammals in the phylogenetic tree (divergence after dolphins), is remarkably rapid based on the molecular evolution model for growth hormone and prolactin, when compared with other mammals [34,35]. This model appears to be consistent with the overall amino-acid alterations (32%) within the tandem repeat of deer Hp α-chain.

A similar alteration in cattle has also been reported recently [28].

Whether this alteration is adaptive during evolution remains to be addressed. For example, in cattle, there is an extensive family of at least eight prolactin-like genes that are expressed in the placenta [36,37]. These genes appear to be arranged as a cluster on the same chromosome. Phylogenetic analysis suggests that all of these genes are the consequence of one or more duplications of the prolactin gene; detailed analysis suggests that a rapid adaptive change has played a role in molecular evolution [38].

4.4.6 Evolutionary advantage of deer Hp protein being a tetramer

In addition to the superior binding affinity to hemoglobin, Hp is an anti-inflammatory molecule and a potent antioxidant [9]. In humans, the large complicated polymers of Hp 2-2 are a risk in the association of diabetic nephropathy [39,40]. One explanation is that the large polymer dramatically retards penetration of the molecular into the extracellular space [39]. We have shown in the present study that deer Hp 2-2 was not able to form the complicated polymers, because the diversity in amino acid sequence between the tandem repeat of α-chain has produced steric hindrance (Fig. 8) that may be advantageous of deer.

In conclusion, we have shown that deer possess an *Hp 2* with a tandem repeat that could have occurred at least 25 or between 25 and 45 million years ago based on the phylogenetic analysis. The phenotypic and biochemical structure of their Hp is markedly homogeneous,

with a tetrameric arrangement due to the orientation of the two available –SH groups, preventing the formation of the complicated Hp polymers found for human Hp 2-2. In terms of molecular evolution, this steric hindrance may have conferred an advantage on deer Hp that compensate for the undesired tandem repeat in the α-chain.

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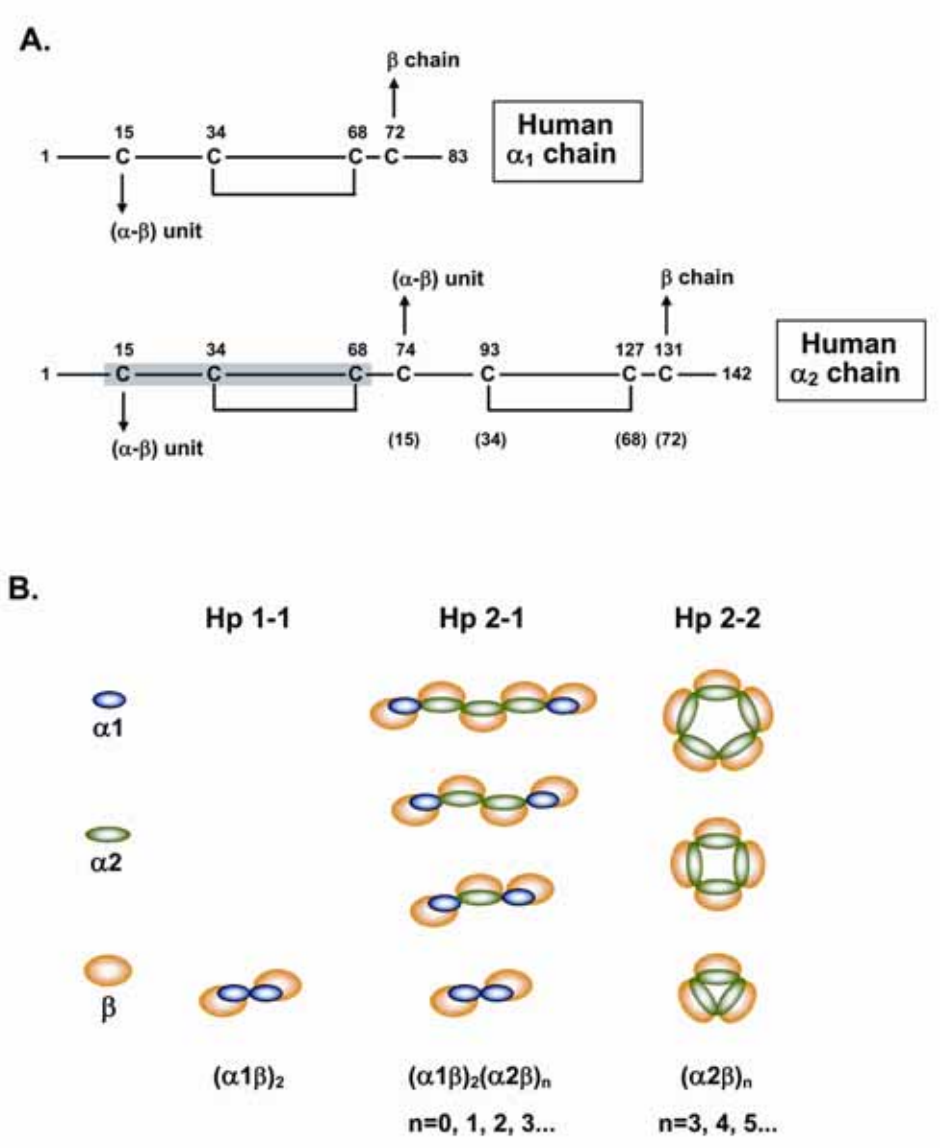

Figure 1. Schematic drawing of the human Hp α-chain and molecular arrangement of Hp phenotypes. (A) Human Hp α1-chain includes two avaiable –SH groups. That at the C-terminus always links to a β-chain to form a basic α1-β unit and the other at N-terminus links either α1-β unit or $(\alpha2\text{-}\beta)_n$ units. The sequence of α2 is identical to that of α1 except for a partial repeat insertion of residues 12-70. However, the extra Cys74 means that Hp 2-1 and 2-2 form complicated polymers. (B) Hp 1-1 forms the simplest homodimer $(\alpha1\text{-}\beta)_2$, whereas Hp 2-1 is polymeric in linear form, forming a homodimer $(\alpha1\text{-}\beta)_2$, a trimer $(\alpha\text{-}\beta)_3$, and other polymers. Here, α represents α1- or α2-chains. Hp 2-2 forms cyclic structures: a trimer $(\alpha2\text{-}\beta)_3$ and other cyclic polymers.

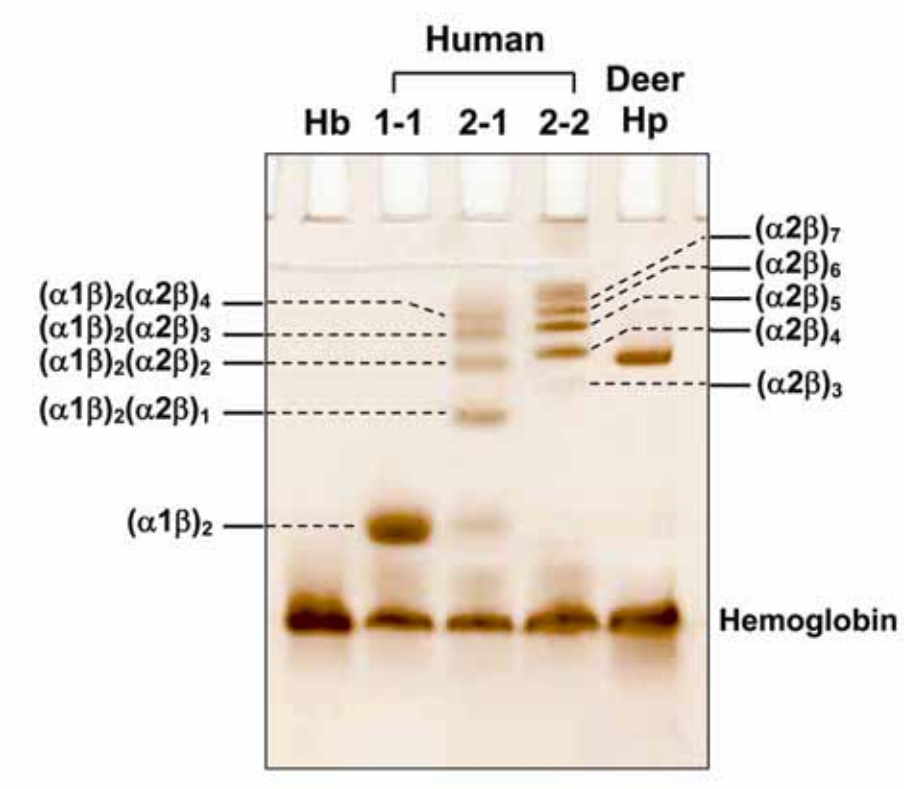

Figure 2. Hemoglobin-binding patterns of deer and human plasma Hp on 7% native-PAGE. Lane 1: hemoglobin only. Lanes 2, 3, and 4: human plasma of Hp 1-1, 2-1, and 2-2 with hemoglobin, respectively. Lane 5: deer plasma with hemoglobin.

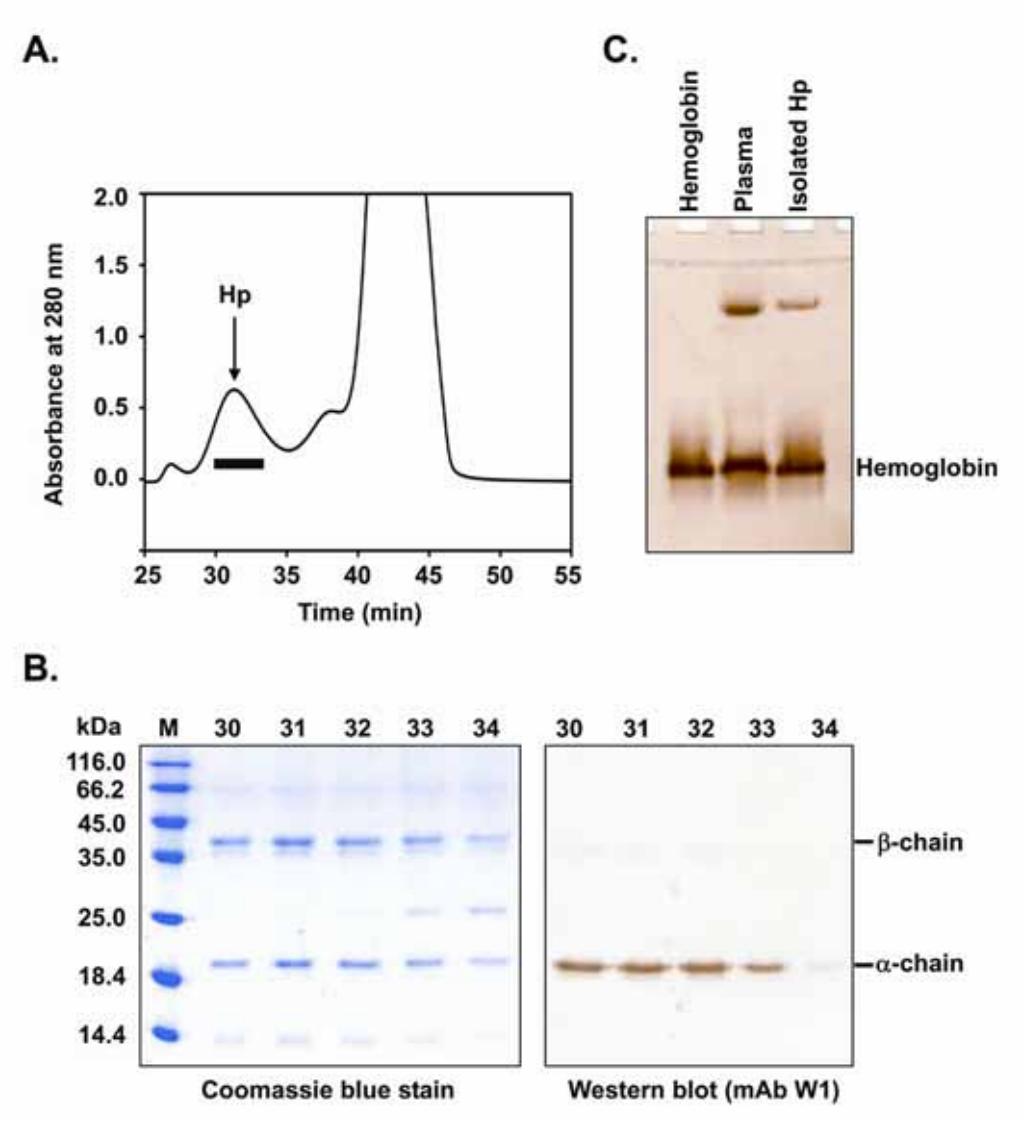

Figure 3. Isolation of deer Hp using size-exclusion Superose-12 column on an HPLC system. (A) A dialyzed supernatant of 50% saturated ammonium-sulfate fraction from plasma was applied on Superose-12 column (1x30 cm) at a flow-rate of 0.3 mL/min, while using PBS as the mobile phase. The bar represents the pooled fractions corresponding to Hp. (B) SDS-PAGE and Western blot analyses of eluted Hp fractions. (C) Hemoglobin-binding property of isolated Hp and plasma containing native Hp on 7% native-PAGE. Lane M, molecular markers in kDa (Invitrogen).

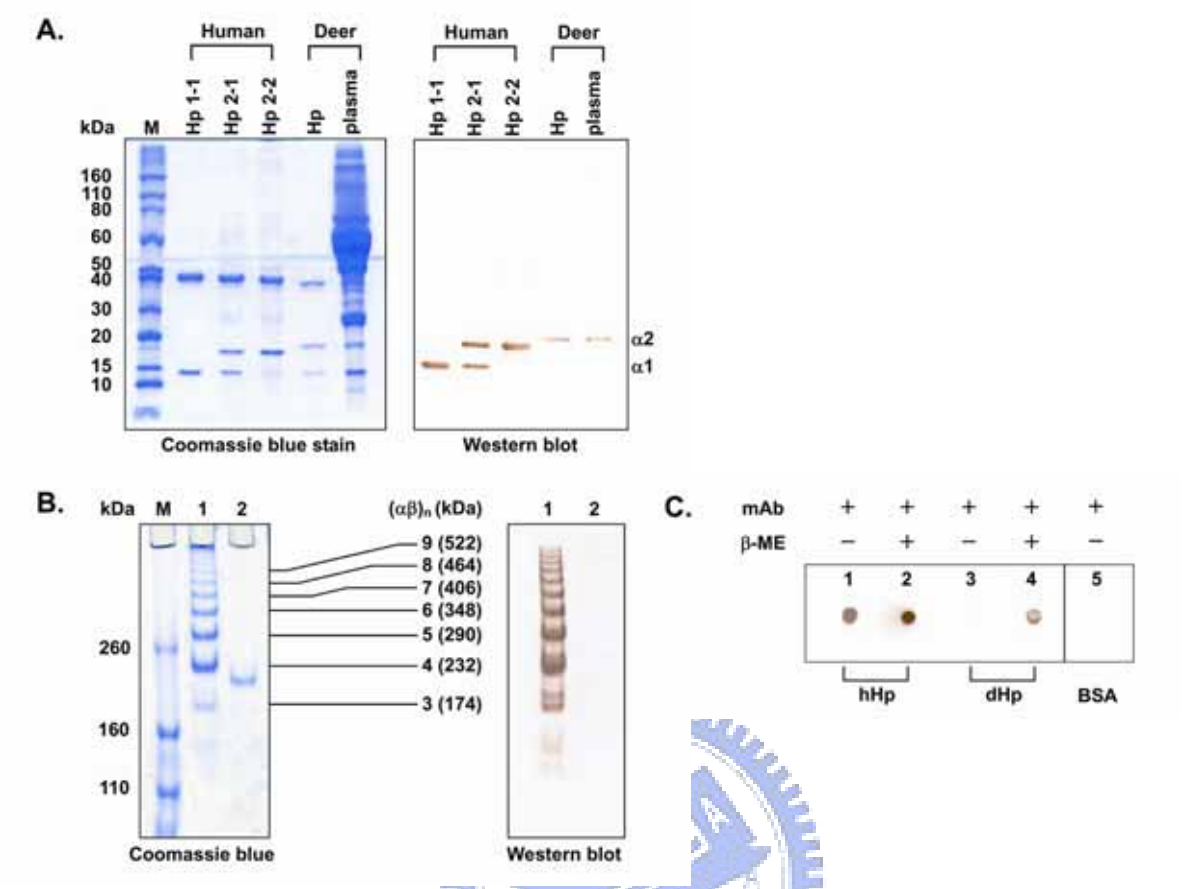

Figure 4. SDS-PAGE, Western blot, and molecular mass analyses of isolated deer and human Hp. (A) The isolated proteins were run on 10-15% PAGE under reducing condition. The Western blot was performed using a human α-chain specific mAb (W1) that cross-reacts with the deer α-chain. Lane M, molecular markers in kDa (Invitrogen). (B) Left panel**:** Western blot analysis of the polymeric structure of isolated human and deer Hp under 4% non-reducing SDS-PAGE using α-chain specific mAb W1. Lane M: molecular markers in kDa (Invitrogen). Lane 1, isolated human Hp 2-2. Lane 2, isolated deer Hp. Right panel: On the Western blot, mAb W1 only recognizes human polymeric Hp, but not deer tetrameric Hp. (C) Dot-blot analysis of isolated human Hp (hHp) and deer Hp (dHp) using α-chain specific mAb W1 in the presence or absence of the reducing reagent β-ME (143 mM). BSA was used as a negative control.

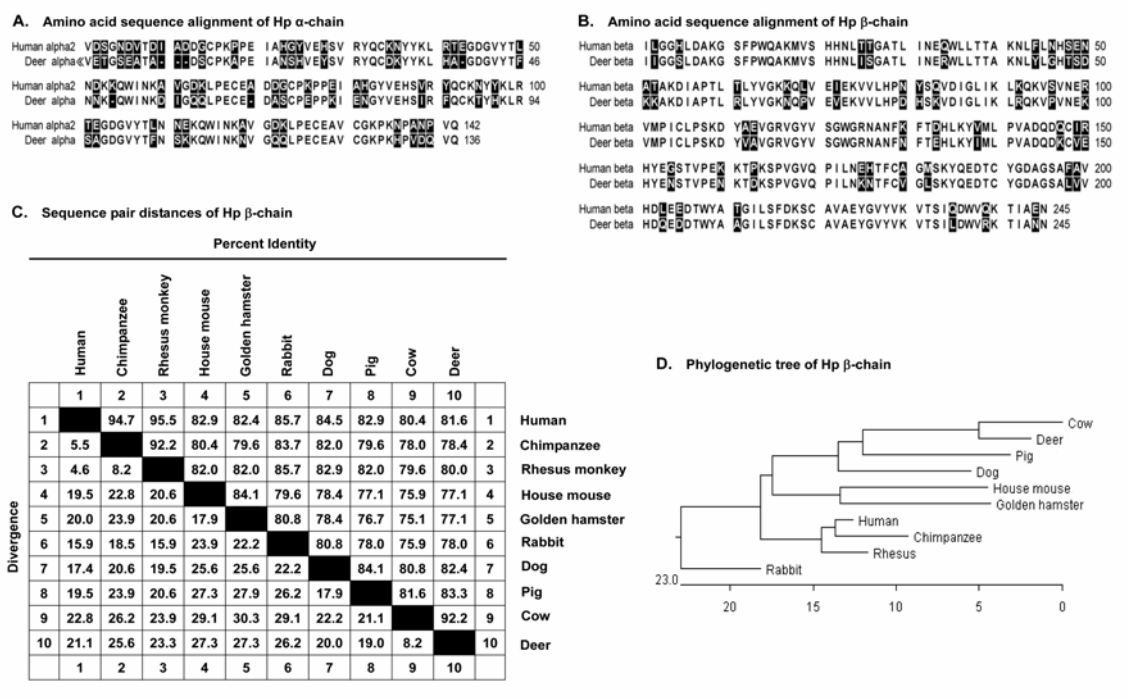

Figure 5. Putative amino-acid sequence analysis and divergence of mammal Hps. (A, B) Amino-acid sequence alignment of the α- or β-chains of human and deer. Variable regions are shaded in black. The cDNA nucleotide sequence corresponding to deer Hp in this study has been deposited in GenBank under the accession number of EF601928. (C) Divergence of the amino-acid sequence of Hp β-chains among ten mammals. (D) Phylogenetic tree constructed according to the amino-acid sequence of Hp β-chains for ten mammals. The tree was plotted using the MEGALIGN program in the DNASTAR package. Branch lengths (%) are proportional to the level of sequence divergence, while units at the bottom indicate the number of substitution events.

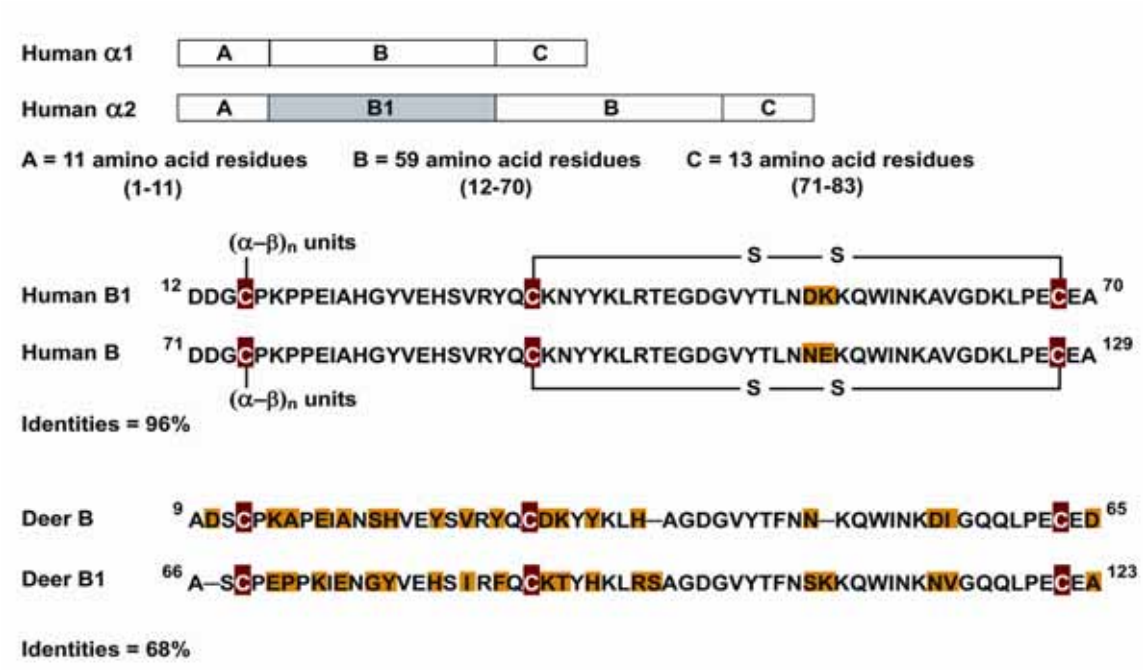

Figure 6. Schematic drawing of tandem repeat region (B and B1) of deer and human α-chain. The most significant feature of human α2 is that it matches the ABC domains of α1 but with an additional insertion of a redundant sequence (B1 region). The repeat unit contains 59 amino acid residues between Asp12 to Ala70. The sequence homology in the repeat region of human is 96% (two amino acids mutated). Deer also have a redundant sequence (B and B1), but the sequence homology between the two repeat units is approximately 68%. The full length of the α-chain contains 142 and 136 residues in human and deer, respectively. The position and number of Cys (total of seven) residues are completely identical between the two species (the one at C-terminal region is not shown). Divergence of the amino acids within the species is marked in yellow.

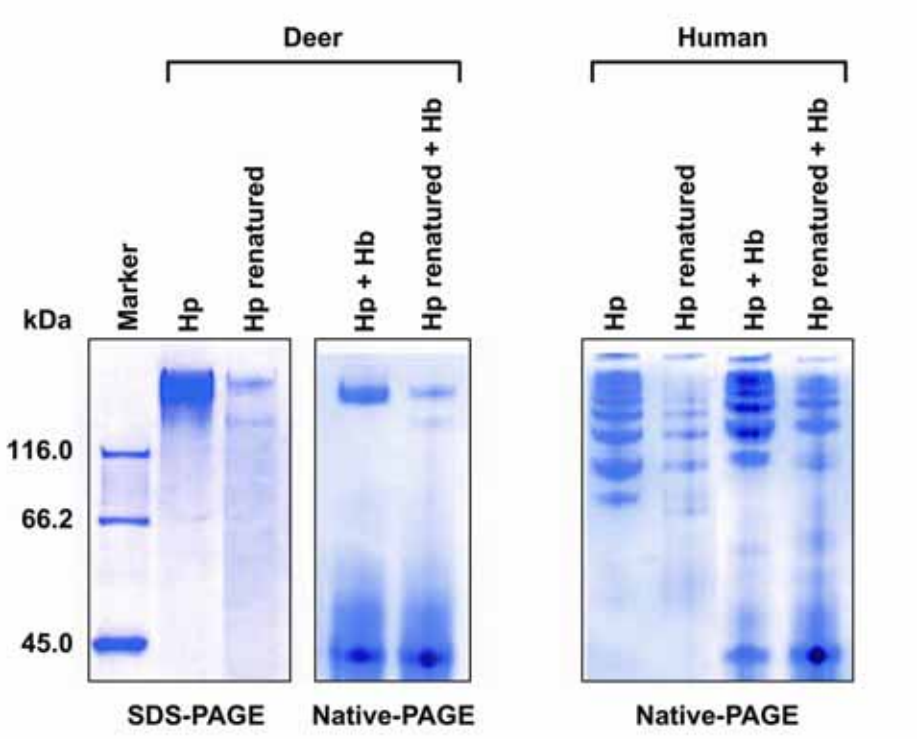

Figure 7. SDS-PAGE and native PAGE analyses of renaturation of deer and human Hp polymers. Denaturation of deer Hp using 6M urea under reducing conditions (143 mM β-ME) followed by renaturation resulted in the formation of $(\alpha\text{-}\beta)_4$ and some $(\alpha\text{-}\beta)_3$.

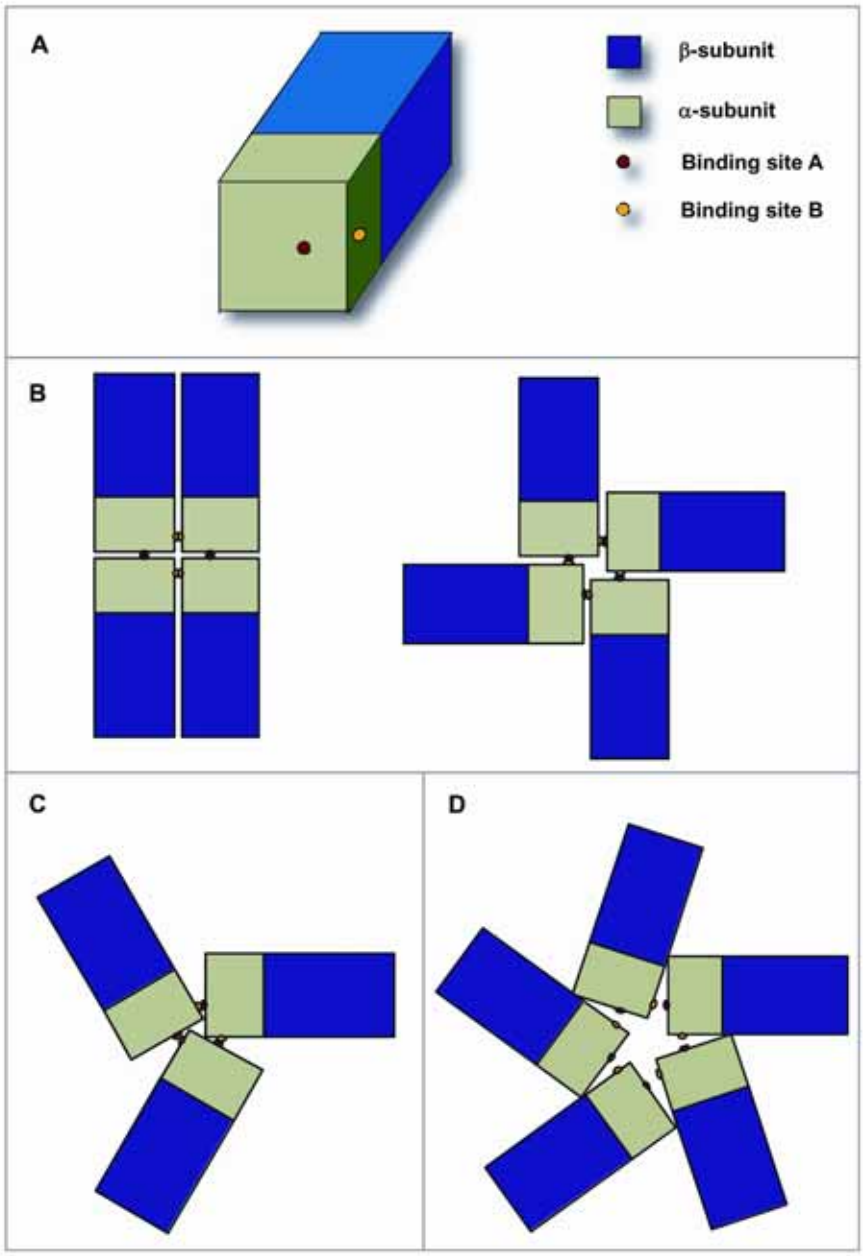

Figure 8. A hypothetical model illustrating the steric hindrance involved in the formation of a deer Hp tetramer. (A) A basic Hp subunit comprising one α- and one β-subunit. The –SH groups that connect the Hp subunits into polymers are assumed to be located with a steric hindrance between the SH binding sites A and B. (B) The two different possible forms of tetramers. (C) The trimeric form of deer Hp is possible to assemble according to this model, but steric hindrance is seen which prevents the –SH groups from linking to some extent. (D) Formation of a pentamer or higher-order polymer is not possible.

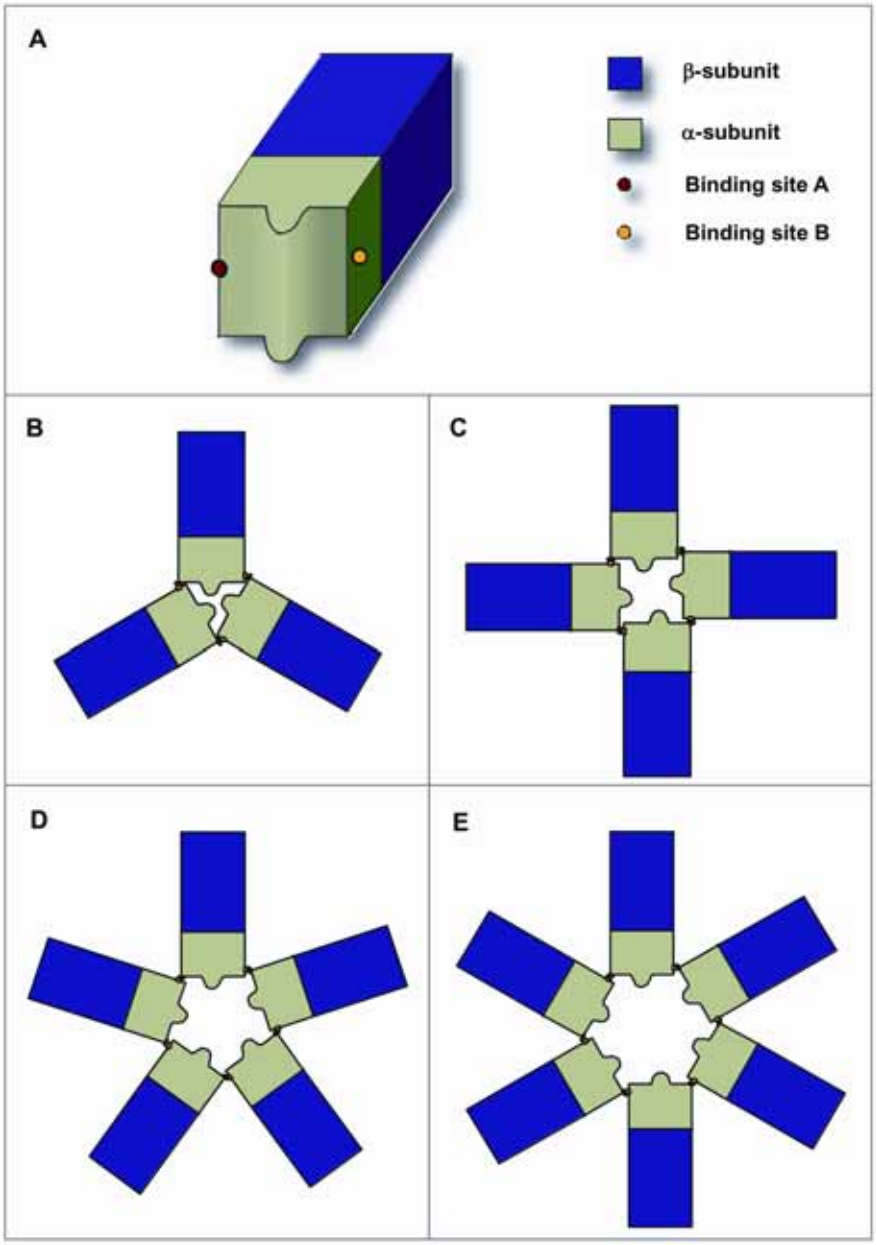

Figure 9. Model of formation of human Hp 2-2 polymers. The positioning of the -SH groups involved in polymer formation differs from those in deer Hp. (A) A basic human Hp 2-2 subunit comprised of one α- and one β-subunit. The –SH groups that connect the Hp subunits into polymers are located at the edge of the surface. The hindrance between the –SH binding sites A and B prevents formation of a dimer. (B) A trimer is able to form to some extent with some steric hindrance. (C-E) Polymers of a higher order than tetramers can form without any steric hindrance.

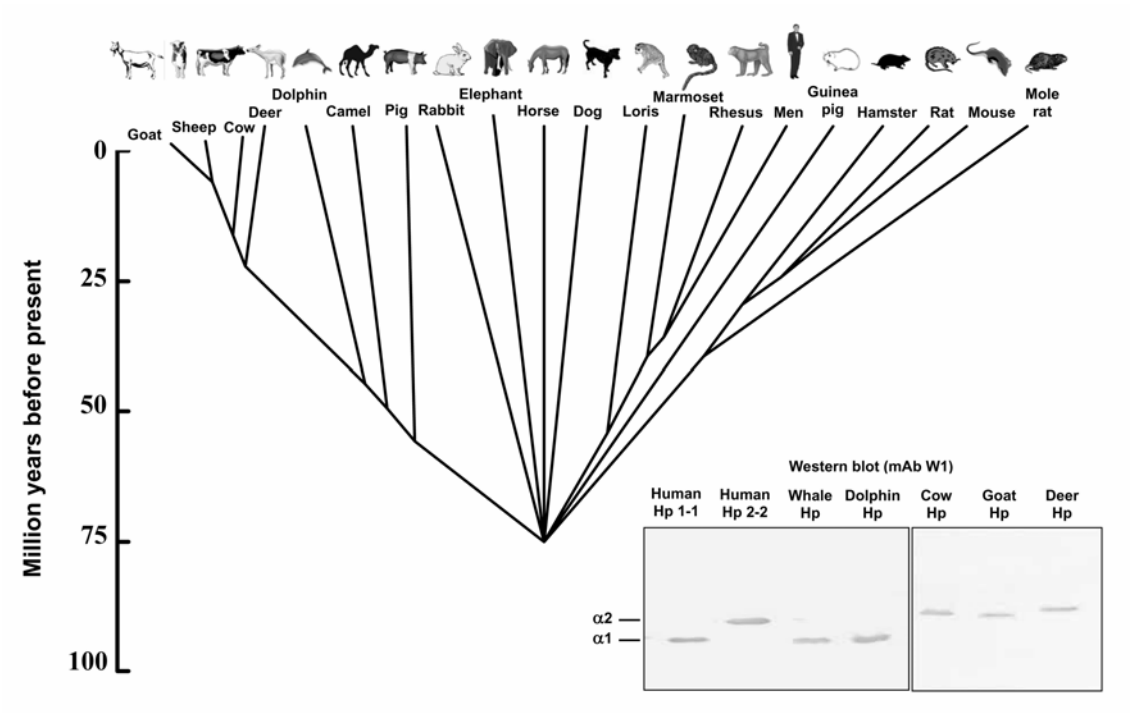

Figure 10. Phylogenetic tree illustrating the molecular evolution of mammals, and phenotyping of human, whale, dolphin and ruminant α-chains. The tree is constructed by assuming all eutherian orders radiated at about the same point in evolutionary time, approximately 75 million years ago. Alternative branching orders give essentially identical results. Within a eutherian order, branch points are assigned using evolutionary times based on fossil records [30]. Western blot analysis of Hp of six mammals (with a branching point before and after deer) was conducted using a 10-15% SDS-PAGE gradient gel under reducing conditions with an α-chain specific mAb (W1) prepared against human Hp.

Chapter 5

Discussion

This thesis presents evidence that neutrophils were associated with the biosynthesis and release of Hp in milk. It further shows that Hp was significantly elevated in the epithelium of mammary gland tissue with mastitis and was also expressed in the cultured mammary epithelial cells. We propose neutrophils and epithelial cells may play an essential role in elevating milk Hp in addition to previous suggestions that Hp may be derived from mammary tissues and circulation. During bovine mastitis, activated neutrophils produce significant amount of reactive oxygen species which may cause tissue damage. Hp is an extremely potent antioxidant that can directly scavenge the free radicals, it may there effectively utilize Hp to attenuate such intracellular damage. Using recombinant Hp, we further find the major antioxidant domain was located in the β chain. The present study provides a potential utility for the future design of "mini-Hp" in developing a novel potent antioxidant. On the other hand, we cloned the cDNA of deer Hp showing that the putative amino-acid sequence mimics that of human Hp 2-2, and that the α-chain of deer also possess a unique tandem repeat. By phylogenetic analysis, we have shown that deer possess an *Hp 2* with a tandem repeat that could have occurred at least 25 million years ago. The evolved tetrameric structure of deer Hp might be of a physiologic advantage. We further proposed that a steric hindrance mechanism is involved in forming Hp tetramers.

Appendix

Curriculum Vitae

Curriculum Vitae

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EDUCATION

2004-2009 Ph.D. Institute of Molecular Medicine and Bioengineering, National Chiao Tung University, Hsinchu, Taiwan (國立交通大學生化工程研究所)

2002-2004 M.S. Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung, Taiwan (國立屏東科技大學生物科技研究所)

1997-2002 B.S. Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan (國立屏東科技大學獸醫學系)

PROFESSIONAL CERTIFICATE

- 專門職業及技術人員高等考試 獸醫師類科及格證書（(91)專高字第 9021 號）
- 獸醫師證書（台獸師字第 4710 號）
- 行政院農業委員會實驗動物人道管理訓練合格證書（實動訓字(94)第 189 號）

DISSERTATION

Biochemical and Physiologic Role of Haptoglobin

Advisor: Chair Professor Simon J.T. Mao (毛仁淡 講座教授)

This work comprises antioxidant role of human haptoglobin, molecular evolution of mammals haptoglobin, and functional role of bovine milk haptoglobin. Most of the dissertation has been published as journal articles (see publication list).

RESEARCH EXPERIENCE

Doctoral Research:

Institute of Molecular Medicine and Bioengineering, National Chiao Tung University, Hsinchu, Taiwan

2004-2009 (research advisor: Dr. Simon J.T. Mao) (指導教授：毛仁淡 講座教授).

- Cloning and expression of the Human haptoglobin subunits.
- Delineation of a major antioxidant domain of Human haptoglobin.
- Determination of the polymeric structure of deer haptoglobin.
- Definition of the molecular evolution of mammals haptoglobin.
- Definition of the milk haptoglobin source from dairy cows with mastitis.

Graduate Research:

Institute of Biotechnology, National Pingtung University of Science and Technology, Pingtung, Taiwan

2002-2004 (research advisor: Dr. Ming-Huei Liao) (指導教授：廖明輝 教授).

- Cloning and expression of the major outer membrane protein gene from *Chlamydophila psittaci and Chlamydophila abortus*.
- Antigenic analysis of the major outer membrane protein from *Chlamydophila psittaci and Chlamydophila abortus*.
- Development of the diagnostic reagent of chlamydiosis.

Undergraduate Research:

Department of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan

1999-2002 (research advisor: Dr. Ming-Huei Liao) (指導教授：廖明輝 教授).

- Development and application of diagnostic technology of chlamydiosis.
- Antigenic detection of Avian chlamydiosis in the south of Taiwan.

TEACHING EXPERIENCE

Teaching Assistant. "Biotechnology & Biomedicine seminar in English"

Department of Biological science and Technology, National Chiao Tung University, September 2006-January 2007.

Teaching Assistant. "Biochemical Experiments"

Department of Biological Science and Technology, National Chiao Tung University, February 2006-June 2006.

Teaching Assistant. "Introduction of Biochemical Engineering"

Institute of Molecular Medicine and Bioengineering, National Chiao Tung University, September 2005-January 2006.

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6. Lai Y.A., **Lai I.H.**, Tseng C.F., Lee J., Mao S.J.T. Evidence of tandem repeat and extra thiol-groups resulted in the polymeric formation of bovine haptoglobin: a unique structure of Hp 2-2 phenotype. **J. Biochem. Mol. Biol. (2007)** 40:1028-1038. **(SCI IF: 2.141; Category of Biochemistry & Molecular Biology: 157/263)**
7. **Lai I.H.** Cloning and antigenic analysis of the major outer membrane protein gene from *Chlamydophila psittaci*. Master thesis, National Pingtung University of Science and Technology, Taiwan. (2004).

PRESENTATIONS

1. **Lai I.H.**, Lu Y.P., Liao M.H., Mao S.J.T. Relationship between milk haptoglobin levels and somatic cell counts in dairy cows with mastitis. The Symposium of the Taiwan Association of Veterinary Medicine and Animal Husbandry, Taipei, Taiwan, May, 2009. P65.
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7. **Lai I.H.**, Lu Y.P., Lee J.W., Wang S.H., Mao S.J.T. Determination of the source of milk haptoglobin from dairy cows with mastitis. The Symposium of the Taiwan Association of Veterinary Medicine and Animal Husbandry, Taipei, Taiwan, December, 2008. P63.
8. **Lai I.H.**, Lu Y.P., Lee J.W., Mao S.J.T. Neutrophils as one of the major haptoglobin sources in mastitis affected milk. The Symposium of the Taiwan Association of Veterinary Medicine and Animal Husbandry, Pingtung, Taiwan, May, 2008. P27.
9. **Lai I.H.**, Yang M.C., Shiau C.H., Liao M.H., Mao S.J.T. A unique tetrameric structure of deer plasma haptoglobin: an evolutionary advantage in hp 2-2 phenotype with homogeneous structure. The Symposium of the Taiwan Association of Veterinary Medicine and Animal Husbandry, Pingtung, Taiwan, May, 2008. P78.
10. **Lai I.H.**, Lin K.Y., Yang M.C. A unique tetrameric structure of deer plasma haptoglobin: an evolutionary advantage in hp 2-2 phenotype with homogeneous structure. NCTU Conference of Biological technology, Hsinchu, Taiwan, May, 2008. P29.
11. **Lai I.H.**, Lin K.Y., Larsson M., Yang M.C., Shiau C.H., Liao M.H., Mao S.J.T. A unique tetrameric structure of deer plasma haptoglobin: an evolutionary advantage in hp 2-2 phenotype with homogeneous structure. The Twenty-third Joint Annual Conference of Biomedical Sciences, Taipei, Taiwan. March, 2008. P102.
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human haptoglobin subunits in Escherichia coli: The potential use of mini-haptoglobin as an antioxidant. NCTU Conference of Biological technology, Hsinchu, Taiwan, November, 2006. P29.

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20. Lai W.Y., **Lai I.H.**, Mao S.J.T. Conversion of recombinant protein expression in Escherichia coli from inclusion body to soluble fraction by a single amino acid mutation. The Twenty first Joint Annual Conference of Biomedical Sciences, Taipei, Taiwan, March, 2006. P262.
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22. Lin H.H., Tseng C.F., **Lai I.H.**, Mao S.J.T. Antioxidant domain of human plasma haptoglobin and its mechanism involved, The Twentieth Joint Annual Conference of Biomedical Sciences, Taipei, Taiwan, March, 2005. P396.
23. **Lai I.H.**, Lu Y.P., Hung H.H., Liao Y.K., Liu H.J., Liao M.H. Cloning and antigenic analysis of the major outer membrane protein gene from chlamydophila psittaci. The Symposium of the Taiwan Association of Veterinary Medicine and Animal Husbandry, Taichung, Taiwan, March, 2004. P39.
24. Lu Y.P., **Lai I.H.**, Liao M.H., Huang H.T., Hung H.H. Development and application of diagnostic technology of Chlamydiosis. The Symposium of the Taiwan Association of Veterinary Medicine and Animal Husbandry, Taipei, Taiwan, 2002. P110.
25. **Lai I.H.**, Liao M.H., Lu Y.P., Huang H.T., Hung H.H. Antigenic detection of Avian Chlamydiosis in the south of Taiwan. The Symposium of the Taiwan Association of Veterinary Medicine and Animal Husbandry, Taipei, Taiwan, 2001. P90.

Publications

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Original article

Neutrophils as one of the major haptoglobin sources in mastitis affected milk

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Abstract – The antioxidant haptoglobin (Hp) is an acute-phase protein responsive to infectious and inflammatory diseases. Hp and somatic cell counts (SCC) are sharply elevated in bovine milk following intramammary administration of endotoxin or bacteria. However, the sources of milk Hp responsible for such increases are not fully understood. The purpose of this study was to define the source of milk Hp from dairy cows with naturally occurring mastitis. Quarter milk samples selected from 50 dairy cows were separated into four groups according to SCC as group A: < 100 ($n = 19$); B: 100–200 ($n = 10$); C: 201–500 ($n = 10$); and D: $> 500 \times 10^3$ ($n = 11$) cells/mL. Our results reveal that milk Hp concentrations were correlated with SCC ($r = 0.742$; $P < 0.01$), and concentrations in group D were ~10-fold higher than in group A. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis indicates that the milk somatic cells from group D were not only capable of synthesizing Hp but could also markedly increase Hp mRNA expression. Western blot, immunocytochemistry, double confocal immunofluorescence, and Hp releasing experiments demonstrate that neutrophils were associated with the biosynthesis and release of Hp in milk. It further shows that Hp was significantly elevated in the epithelium of mammary gland tissue with mastitis and was also expressed in the cultured mammary epithelial cells. We propose that neutrophils and epithelial cells may play an essential role in elevating milk Hp in addition to previous suggestions that Hp may be derived from mammary tissues and circulation.

haptoglobin / neutrophil / mastitis / MAC-T cells / immunocytochemistry

1. INTRODUCTION

Haptoglobin (Hp) is an acute phase protein responsive to inflammation and infection [6, 7, 24]. One of the major functions of Hp is to capture released hemoglobin during excessive hemolysis [12] and to scavenge the hemoglobin-induced free radicals during oxidative stress [16]. We have recently shown that Hp is an extremely potent antioxidant that directly prevents low-density-lipoproteins from Cu^{2+}- and radical compound-induced oxidation [13, 30].

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Transfection of Hp cDNA into Chinese hamster ovary cells protects them against oxidative stress [30]. Structurally, bovine Hp is unique possessing only the Hp 2-2 phenotype [14, 15] with a molecular weight ranged from 660 to 730 kDa [15] which is different from that of humans expressed as Hp 1-1, 2-1, or 2-2.

Bovine Hp, while not abundantly expressed in normal plasma, is considered to be one of the sensitive acute phase proteins during bacterial infections [15, 24]. Several studies indicate that its concentration increases dramatically in both plasma and milk during clinical mastitis of dairy cows [6, 8, 21]. In experimentally induced mastitis by intramammary bacterial-challenge with *Streptococcus uberis*, the observed increase in milk Hp has been suggested to originate from the circulation [23]. Hiss et al. [9] also found elevated Hp in milk after intramammary endotoxin challenge. It was further observed that Hp could be locally synthesized within the mammary gland [9]. The source of milk Hp found in naturally occurring mastitis is poorly understood.

The purpose of the present study was to define the source of milk Hp from dairy cows with high somatic cell counts (SCC) (or mastitis). We investigated milk somatic cells for the presence of Hp protein using Western blot, Hp mRNA expression using reverse transcriptase-polymerase chain reaction (RT-PCR), partial cell typing using double confocal immunofluorescence, and endogenously released Hp using ELISA. Finally, we localized Hp in normal and mastitic mammary gland tissue and in a cultured mammary epithelial cell line (MAC-T). Our findings show that the increased Hp was associated with neutrophils, in which Hp was released into the medium in an ex vivo experiment. In mastitis mammary glands, Hp was mainly localized within the epithelial cells. We also observed for the first time that cultured epithelial MAC-T cells could synthesize Hp in vitro. In addition to locally synthesized Hp within the mammary gland and Hp transferred from the circulation, we propose that milk neutrophils and mammary epithelium also contribute to milk Hp concentrations.

2. MATERIALS AND METHODS

2.1. Sample collection and SCC

Quarter milk samples from individual cows (*Bos taurus*) were collected at the Yong Rong Dairy Farm (Chyayi, Taiwan). SCC in each sample was measured using a Fossomatic 4000 cell counter (Foss Electric, Hillerød, Denmark) at the Livestock Research Institute, Council of Agriculture, Executive Yuan (Hsinchu, Taiwan). Quarter milk samples were then selected and assigned to four groups according to SCC (A: < 100; B: 100–200; C: 201–500; and D: $> 500 \times 10^3$ cells/mL with $n = 19$, 10, 10, and 11, respectively).

2.2. Purification of bovine Hp and preparation of anti-Hp antibodies

Native bovine Hp was isolated from the plasma using an anti-bovine Hp immunoaffinity column, followed by gel-permeation chromatography (Superose-12) on a HPLC system as previously described [15]. Mouse and rabbit polyclonal antibodies against bovine recombinant Hp or mouse monoclonal antibody (mAb; 2H12) against bovine native Hp were prepared in our laboratory according to previously established procedures [3, 15].

2.3. Determination of milk Hp levels using ELISA

Bovine milk Hp concentrations were determined using a commercial bovine Hp ELISA kit (Immunology Consultants Laboratory, Newberg, OR, USA) according to the manufacture's instructions. One hundred μL of skimmed milk (1:10 dilution) were used for the determination of Hp, while skimmed milk was prepared as previously described [2].

2.4. Isolation of milk somatic cells and protein extraction

Milk somatic cells were isolated from 200 mL freshly collected milk. Briefly, milk samples maintained at 4 °C were centrifuged at 1 000 *g* for 20 min at 4 °C. After removing the fat layer and supernatant, the somatic cells were briefly washed twice with PBS at 4 °C and maintained on ice. Total cell proteins were extracted using a lysis buffer containing 0.3% Triton X-100 in PBS and sonicated at 4 °C for 5 min, followed by centrifugation at 10 000 *g* for 10 min at 4 °C. The remaining

supernatant was stored at –20 °C until analysis by SDS-PAGE and Western blot.

2.5. Gel electrophoresis and Western blot

SDS-PAGE was carried out on 1.5-mm-thick slab gel, using a discontinuous system as previously described [14]. Unless otherwise specified, the gel containing 15% (for reducing) or 4% (for non-reducing samples) polyacrylamide was used with a top stacking gel of 5% polyacrylamide. Approximately 20 μg of the protein was loaded onto the gels and each tested sample was preheated at 100 °C for 10 min in a loading buffer (12 mM Tris-HCl, pH 6.8, 0.4% SDS, 5% glycerol, 0.02% bromphenol blue) with or without 140 mM 2-mercaptoethanol. The samples were then run for about 1.5 h at 100 V and stained by Coomassie brilliant blue. Molecular-mass standard containing 12 pre-stained proteins (3.5–260 kDa) was purchased from Invitrogen (Carlsbad, CA, USA). Western blot analysis was performed similar to that described previously [13].

2.6. Analysis of Hp mRNA expression of milk somatic cells

Total RNA was extracted from milk somatic cells using an RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacture's instructions. The first strand cDNA was synthesized using moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). Briefly, equal amounts of total RNA (1 μg) were added to a reaction mixture containing 50 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 0.5 μg oligo $(dT)_{18}$, 75 mM KCl, 3 mM $MgCl_2$, 0.5 mM dNTP mix, 40 U RNase inhibitor, and 200 U MMLV reverse transcriptase, and proceeded at 37 °C for 50 min, followed by 70 °C for 15 min. Equal amounts of total cDNA (100 ng) were amplified by PCR using Hp specific primers, while using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a house-keeping control. The primer design was based on the published nucleotide sequence of bovine Hp [15] with 5′-TGCTGCAGGGATCATC GGTGGCTCATTGGA-3′ and 5′-CGGAAAACCAT CGCTAACAACTAAGCTTGGG-3′ as the forward and reverse primer, respectively. The GAPDH primers prepared were 5′-CCTGGAGAAACCTGCCA AGT-3′ (forward) and 5′-GCCAAATTCATTGTC GTACCA-3′ (reverse). The PCR cycling profile was 95 °C for 5 min followed by 30 cycles at 94 °C for 30, 55 °C for 30 s, 72 °C for 50 s (or 30 s in GAPDH) with a final extension at 72 °C for 10 min. The RT-PCR products (214 bp for GAPDH and 755 bp for Hp) were resolved on 1.5% agarose gel [14], followed by an ethidium bromide staining. The band intensity corresponding to Hp was determined using a Quantity One software of Gel Doc 2000 Gel Documentation System (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. MAC-T and Hp mRNA expression

MAC-T, an established and immortalized epithelial cell line isolated from bovine mammary tissue was cultured as previously described [10, 18]. In general, the cells (1×10^4 cells per well) were grown at 37 °C (5% CO_2) in a 24-well culture plate in complete Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (containing no immunoreactive bovine Hp), 50 μg/mL of streptomycin, and 50 IU/mL of penicillin (Invitrogen). Hp mRNA expression was analyzed using RT-PCR similar to the procedures described above.

2.8. Immunocytochemical staining of somatic and MAC-T cells and mammary tissues

The labeled streptavidin biotin kit (LSAB) (Dakocytomation, Glostrup, Denmark) was used for immunostaining according to the manufacturer's instructions. In brief, cytospins containing freshly isolated somatic or MAC-T cells were treated with ice-chilled methanol (100%) for 15 min and then rehydrated by PBS. The cells were permeabilized in PBS containing 0.3% Triton X-100 for 10 min, while the endogenous peroxidase was blocked by incubation with 3% H_2O_2. After blocking with 2% gelatin, the cells were incubated with unlabeled mouse anti-CD5 mAb (lymphocyte marker), mouse anti-CD11b mAb (neutrophil marker) (Serotec, Oxford, UK), or mouse anti-Hp polyclonal antibody for 1 h. The slides were then incubated with biotinylated anti-mouse IgG for 30 min, followed by washes and incubation with HRP-conjugated streptavidin for 30 min. After washes, the slides were developed with 3-amino-9-ethylcarbazole or 3,3′-diaminobenzidine (DAB) substrate and counterstained with hematoxylin. For normal and mastitic mammary tissues (n = 5 each), each section was deparaffinized in a 50 °C oven for 10 min and then placed for two 5-min washes in xylene at 24 °C. The sections were rehydrated through sequential soakings in 100, 95, 90, 80, and 50% alcohol for 2 min each, followed by final washes in deionized water. The sections were then stained using procedures described above.

2.9. Double immunofluorescence using confocal laser scanning microscopy

Cytospin prepared cells used for localization of CD11b and Hp were permeabilized as described above. After blocking, the cells were co-incubated with unlabeled mouse anti-CD11b mAb and rabbit anti-Hp polyclonal antibody for 1 h. The slides were then incubated with a mixture of fluorescent isothiocyanate (FITC) conjugated anti-mouse IgG and rhodamine conjugated anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 h in the dark. The cells were washed with PBS before examination using a Fluoview FV500 confocal laser scanning microscopy (Olympus, Tokyo, Japan).

2.10. Time course of Hp protein released from somatic cells in vitro

Two mL of isolated somatic cells (adjusted to 1×10^4 cells/mL at 4 °C) from groups A and D ($n = 3$ for each) were suspended in Roswell Park Memorial Institute medium (RPMI) 1640 medium (JRH Biosciences, Lenexa, KS, USA) containing 100 μg/mL ampicillin with or without protease inhibitor of 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) in a test tube and were incubated at 37 °C for 0, 2, 4, 8 and 16 h, respectively, followed by centrifugation at 1 000 *g* at 4 °C for 5 min. The supernatant was then subjected to ELISA for the determination of Hp concentrations.

3. RESULTS

3.1. Correlation between Hp levels and SCC in bovine milk

Selected bovine milk samples ($n = 50$) were assigned into four groups according to their SCC (A: < 100; B: 100–200; C: 201–500; and D: > 500 × 10^3 cells/mL with $n = 9$, 10, 10, and 11, respectively). The mean ± SEM of milk Hp concentrations determined by an ELISA in groups A, B, C, and D were 0.23 ± 0.08, 0.74 ± 0.23, 1.11 ± 0.28, and 2.22 ± 0.53 μg/mL, respectively. The mean Hp concentration of group D was ∼10-fold higher than that of group A ($P < 0.001$). The overall Hp concentrations were correlated with SCC ($r = 0.742$; $P < 0.01$).

3.2. Hp in milk somatic cells

Because Hp levels in the milk samples were positively correlated with SCC, we hypothesized that somatic cells might contribute to the presence of Hp in milk. We extracted protein from the somatic cells from the low (group A) and high SCC (group D) to examine the presence of Hp using a Western blot. Figure 1 depicts the presence of Hp in group D, but not in group A. It implicates the endogenous synthesis and expression of Hp between the analyzed cells of groups A and D was different or/and altered. The electrophoretic pattern of bovine Hp (non-reduced) was similar to that previously reported by our laboratory [15].

3.3. Endogenous expression of Hp mRNA in milk somatic cells

To determine whether somatic cells could endogenously synthesize Hp, RT-PCR for the expression of Hp mRNA was conducted, while using GAPDH as a house-keeping control. Figure 2 shows that the Hp mRNA expression was progressively increased with the SCC ($P < 0.001$). The mean Hp mRNA expression in groups C and D were evidently higher than those in groups A and B ($P < 0.001$).

3.4. Somatic cell typing

To identify the cell types in group D, we used anti-CD5 (lymphocyte marker) and anti-CD11b (neutrophil marker) mAb as a probe in immunostaining, while comparing group A as a non-mastitis control. We found lymphocytes to be a major cell type in group A and neutrophils the predominant cell type in group D (data not shown). The immunostaining was specific because the background control using non-immuned sera or non-related mAb was negative (data not shown).

3.5. Colocalization of Hp and CD11b in neutrophils using confocal microscopy

In addition to RT-PCR, we tested whether neutrophils could express Hp in situ. Somatic cells isolated from group D enriched with

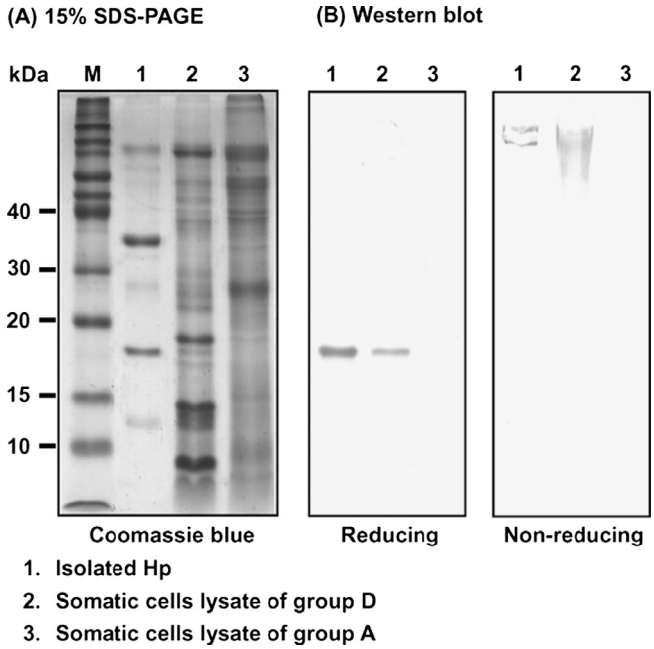

Figure 1. Typical SDS-PAGE pattern and Western blot analyses of milk somatic cell lysates from groups A and D. (A) Coomassie blue staining of somatic cell lysates run on a 15% SDS-PAGE under reducing conditions. (B) Western blot analysis of the isolated Hp and somatic cell lysates under reducing (left panel with 15% SDS-PAGE) and non-reducing conditions (right panel with 4% SDS-PAGE) using a mouse polyclonal antibody prepared against bovine recombinant Hp. Lane M, molecular markers in kDa.

neutrophils were examined for localization of Hp using a double confocal laser scanning microscopy. Figure 3 shows that all the neutrophils identified by CD11b (in green FITC) exhibited expression of Hp (in red rhodamine), while no staining was observed for cells treated with non-immuned sera nor unrelated mAb (data not shown).

3.6. Release of Hp from the somatic cell ex vivo

To test the hypothesis that the neutrophil enriched population secreted Hp, we collected the somatic cells from the groups A and D (n = 3 quarters for each) and investigated their Hp secretion into the RPMI 1640 medium over time. Figure 4 shows that both groups were able to secrete Hp into the medium with a markedly greater secretion in group D than in group A. Essentially, the released Hp reached maximal levels within the first 2 h tested. Thus, the milk neutrophils not only express Hp but also release it into the milk.

3.7. Localization of Hp in mammary tissue and MAC-T cell

We used mouse anti-bovine Hp polyclonal antibody to localize the bovine Hp near the area of alveoli in mammary tissues (n = 5). The results indicate that only mastitic mammary tissue expressed Hp relative to normal tissue. Figures 5A and 5B illustrate a typical example showing that Hp was primarily localized at alveoli of mastitic mammary tissue, but not in the normal tissues (Figs. 5C and 5D). To provide additional evidence that mammary alveolar epithelial cells can express Hp, we showed that cultured- MAC-T cells were able to express Hp mRNA (panel of Fig. 5E). We further demonstrated the presence of Hp protein in MAC-T cells using immunocytochemical staining (Figs. 5E and 5F).

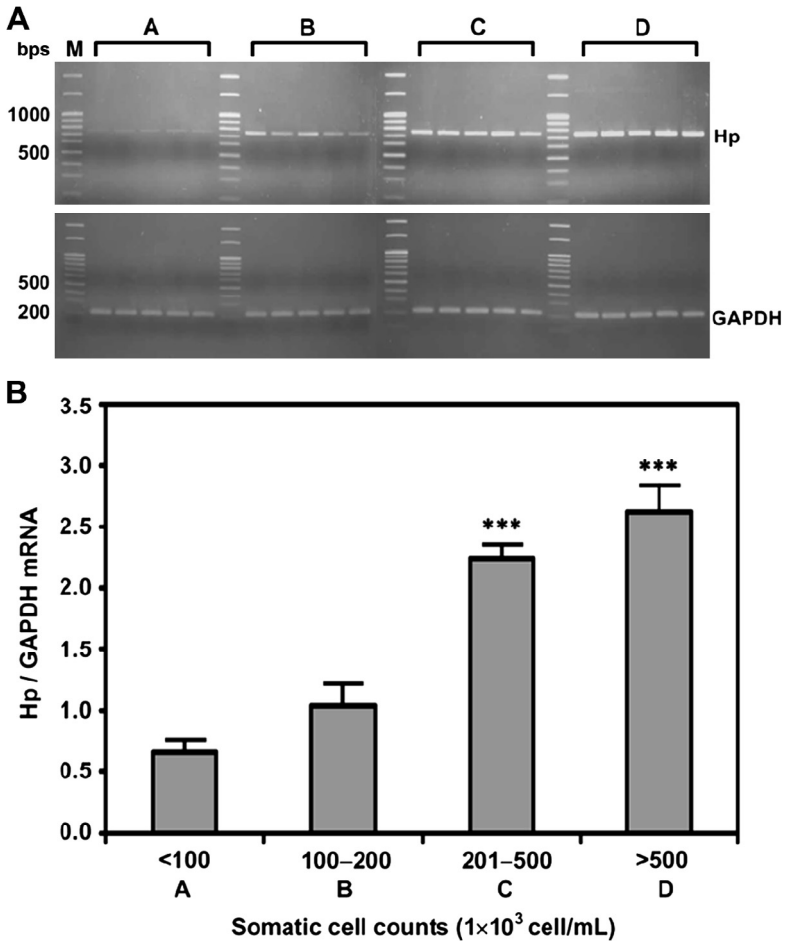

Figure 2. Expression levels of Hp mRNA in somatic cells. (A) Expression of Hp mRNA in milk somatic cells according to SCC (A: < 100; B: 100–200; C: 201–500; and D: > 500 × 10^3 cells/mL with $n = 5$ in each group). An equal amount of total cDNA (100 ng) was amplified by PCR, using GAPDH as a housekeeping control. (B) Each bar represents the mean ± SEM. *** $P < 0.001$ as compared to group A.

4. DISCUSSION

4.1. Correlation between Hp levels and SCC in bovine milk

The SCC of milk has been utilized as an indicator of mastitis because of its simple handling procedures. In general, SCC of a quarter greater than 500 × 10^3 cells/mL is considered to be associated with mastitis [22, 26]. A limit of ≤ 100 × 10^3 cells/mL has been suggested for a healthy quarter [19]. If the SCC exceeds 200 × 10^3 cells/mL, the quarter is likely to be infected [25]. The results from the present study showed a 10-fold increase in Hp concentrations in milk containing SCC > 500 × 10^3 (group D) relative to SCC < 100 × 10^3 (group A). This suggests that the somatic cells were responsible for the elevation of Hp in milk, at least in part.

4.2. Bovine Hp in milk somatic cells

Previous studies have proposed that milk Hp can be derived from mammary tissues and

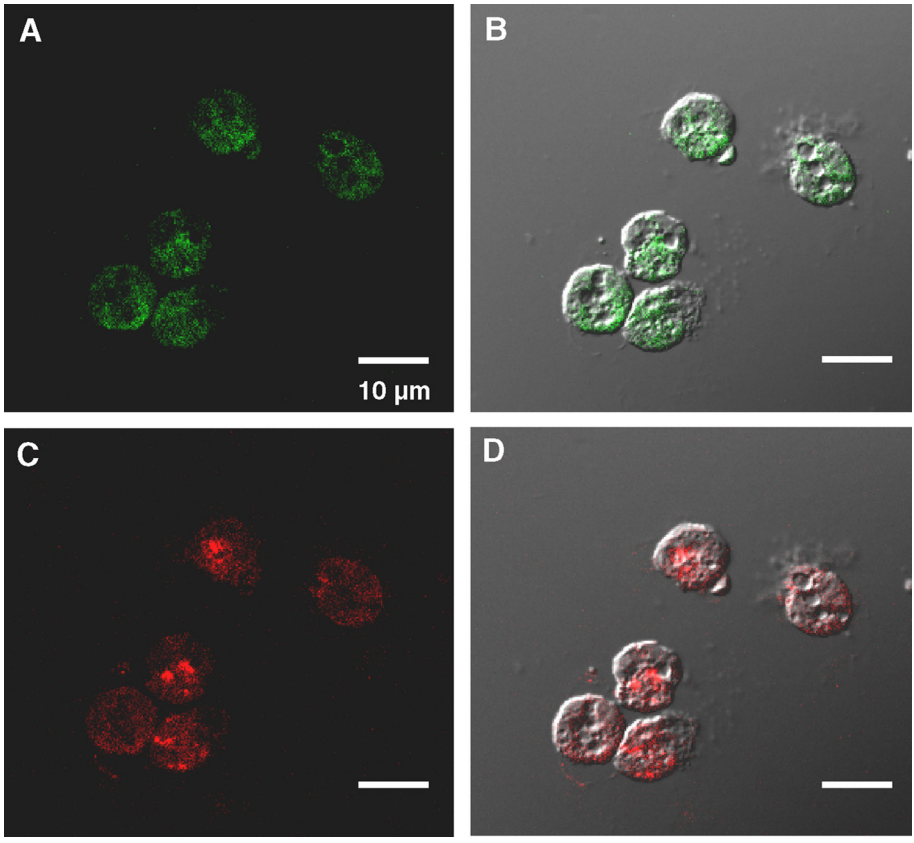

Figure 3. Colocalization of CD11b and Hp in milk neutrophils using confocal laser scanning microscopy. CD11b as a marker for neutrophils was visualized with a FITC-conjugated second antibody (A and B). Likewise, Hp was visualized with a rhodamine-conjugated second antibody (C and D).

circulating blood following intramammary infection [5, 9]. Although the factors attributed to the elevated milk Hp in naturally occurring mastitis are not fully understood, the present study suggests that neutrophils in milk are a major source of milk Hp in cows with mastitis. There are several lines of evidence to support this notion. First, Hp levels determined by ELISA were positively correlated with the SCC in our group analyses in which the increase in SCC was mostly attributed to neutrophils. Second, using Western blot analysis, Hp was found abundantly in the cell lysate of group D, but not in that of the normal group A (Fig. 1). A recent study using matrix assisted laser desorption ionization-time of flight-mass indicates that Hp is present and concentrated within the granules of bovine granulocytes isolated from peripheral blood of healthy cattle, although the cell types (such as neutrophils) have not been specified [4]. Third, the Hp mRNA levels in cells of the high SCC groups B-D were significantly higher than in the cells of the normal group A. The underlying mechanism involved in high Hp expression in the higher SCC groups appears to be related to the different cell types. We also observed that neutrophils were the predominate cell type in group D, but not in group A when examined by immunocytochemistry (data not shown). This suggests that neutrophils in high SCC milk account for the major synthesis of Hp. Furthermore, using laser confocal scanning

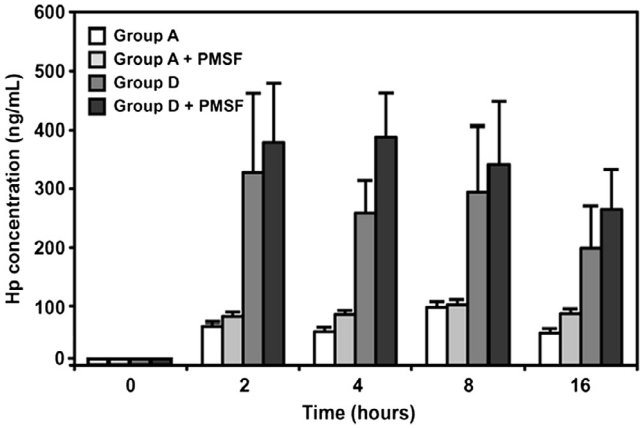

Figure 4. Time course of Hp protein released into RPMI 1640 medium by somatic cells from groups A and D ex vivo. Freshly isolated somatic cells from milk samples of groups A and D (n = 3 of each group) at 4 °C were immediately suspended in RPMI 1640 and kept at 37 °C over time. Supernatant containing Hp was determined using an ELISA. Protease inhibitor (PMSF) was also added in parallel to each group in an attempt to minimize the Hp degradation. Each bar represents the mean ± SEM.

microscopy, we observed that all the neutrophils isolated from the somatic cells were capable of expressing Hp as determined by double immunofluorescence (Fig. 3). Fourth, these cells not only synthesized Hp but also released it into milk (Fig. 4).

This is the first study demonstrating that Hp is synthesized by cattle neutrophils using RT-PCR, which is inconsistent with an early report indicating that human neutrophils do not produce Hp [32]. In that study, the authors hypothesized that Hp of neutrophils was derived from the liver and stored in specific granules via endocytosis [32]. While conducting our investigation, a recent study appeared, reporting that Hp can be released from human neutrophils [28] indicating the presence of Hp mRNA in human neutrophils, although the level of expression was minimal. Taken together, it is conceivable that neutrophils may contribute to the increased level of Hp in milk from cows with mastitis.

4.3. Milk Hp from the other sources

It has been shown that Hp is expressed in the mammary tissue using ELISA and RT-PCR, although the cell types involved were not specified [9]. Using an epithelial cell line (MAC-T) and mammary gland tissue sections we further demonstrate that epithelial cells were able to endogenously synthesize and express Hp (Fig. 5), which is consistent with the results by Thielen et al. [29] using an in situ hybridization on the epithelium of mastitic mammary tissue. Another study has also suspected the presence of Hp in the epithelium of alveolus [6]. Nonetheless, the localization of Hp in bovine alveolar epithelium of mastitic tissue or cultured MAC-T cells is now demonstrated in the present study (Fig. 5).

4.4. Remarks on Hp secretion in milk and its physiologic function

This study shows that neutrophils and mammary epithelial cells represent an additional extra-hepatic source of Hp in milk. The possible mechanism by which neutrophils migrate into the alveolus of mammary gland and how Hp is recruited in milk are explained and summarized in Figure 6.

The physiologic benefits of local Hp expression in mammary tissue during infection

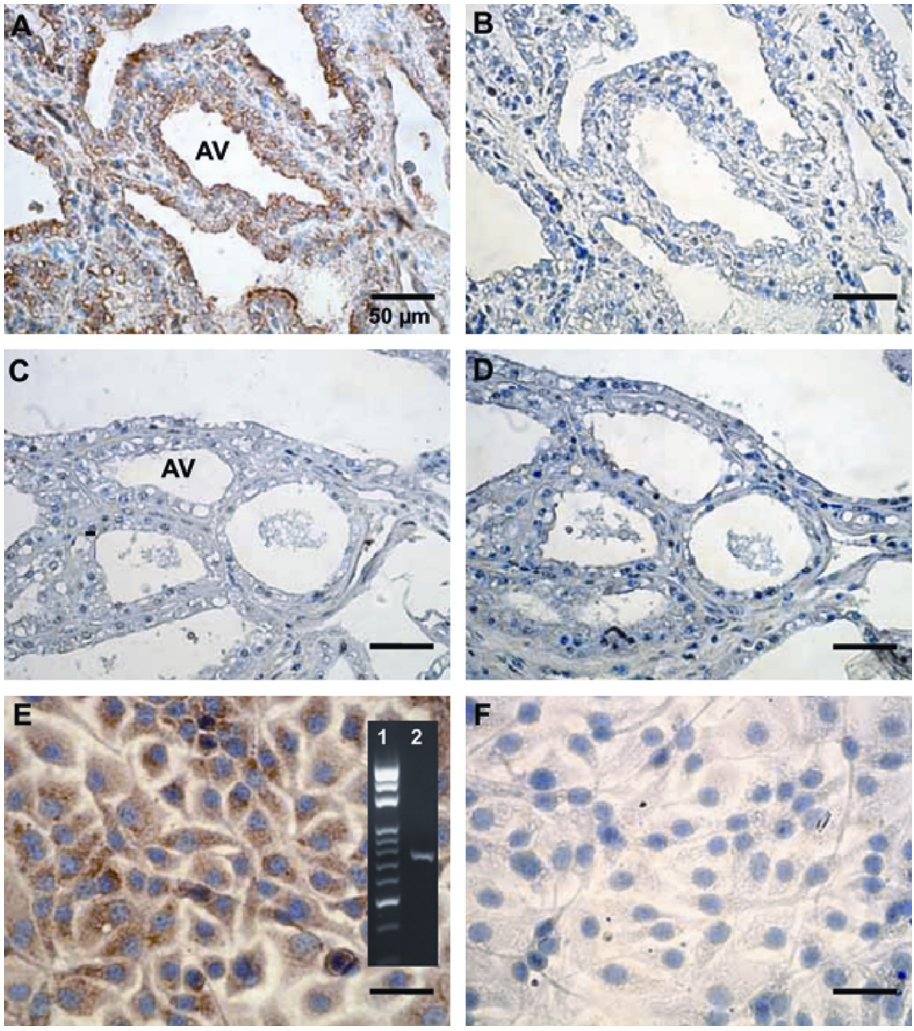

Figure 5. Immunocytochemical localization of Hp in mammary gland and MAC-T cells. Mammary tissues with (A and B) and without mastitis (C and D) were incubated with mouse anti-Hp polyclonal antibodies (A and C), while using normal non-immuned mouse serum as a negative control (B and D). MAC-T cells were incubated with mouse anti-Hp polyclonal antibodies (E), while using normal non-immuned mouse serum as a negative control (F). DAB was used as a chromogenic substrate for Hp staining (in brown) and hematoxylin was employed as a nuclear stain (in blue). Panel in E represents the Hp mRNA expression (Lane 2) and DNA molecular weight markers (Lane 1) using RT-PCR. AV = alveolar.

remain illusive. During mastitis, activated neutrophils produce significant amounts of reactive oxygen species (in order to kill the bacteria) which may cause tissue damage [1, 17, 31]. Hp is an extremely potent antioxidant that can directly scavenge free radicals, it may effectively utilize Hp to attenuate such intracellular damage [30]. A further understanding of the role played by neutrophils as a source of Hp may provide insight into the understanding of an additional function of neutrophils in milk.

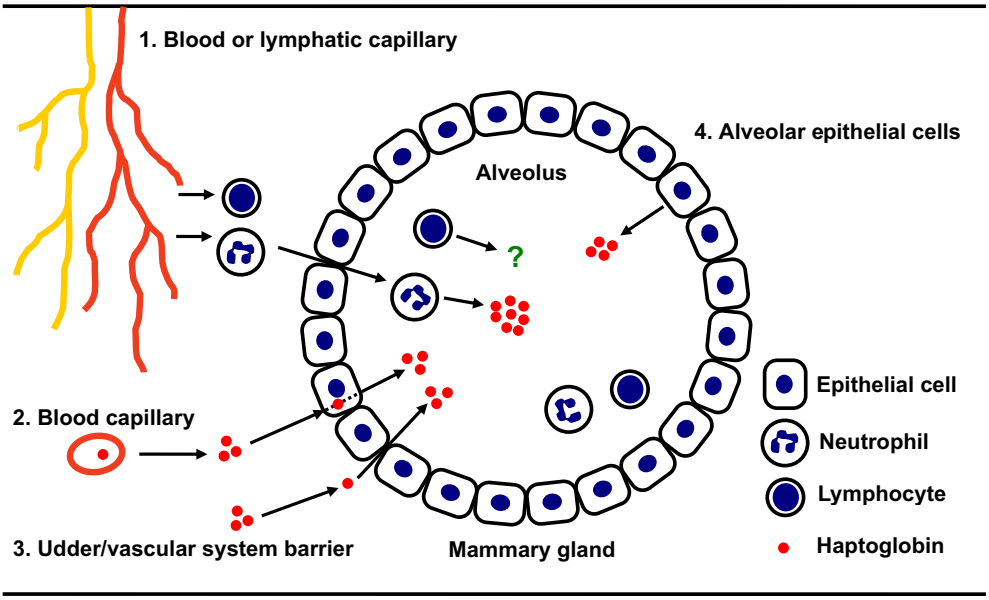

Figure 6. Schematic diagram of hypothetical pathways for the presence of bovine Hp in milk. There are at least four combined possible pathways for the presence of Hp in milk during mastitis. First, inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) may activate neutrophils and promote them to migrate through the endothelium, subepithelial matrix, and basement membrane of blood and lymph vessels into the infected mammary gland. Neutrophils then pass through the tight junction between the alveolar epithelial cells and enter into the alveolus to secrete cellular Hp (please see review article for more detail [27]) (pathway 1). Second, plasma Hp of hepatic origin mimicking albumin and immunoglobulin [11, 20] is able to directly enter into the epithelial cells via a receptor-mediated process and secrete into alveolus (including endocytosis and exocytosis) (pathway 2). Third, plasma Hp is able to spill into the udder by passive diffusion through a compromised udder/vascular system barrier (pathway 3). Fourth, the mammary epithelial cells are able to endogenously synthesize and express Hp in the alveolus (pathway 4). (A color version of this figure is available at www.vetres.org.)

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A unique tetrameric structure of deer plasma haptoglobin – an evolutionary advantage in the Hp 2-2 phenotype with homogeneous structure

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Keywords
amino acid sequence; deer and human haptoglobin; monoclonal antibody; phenotype; purification

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Database
The sequence corresponding to deer Hp is available in the DDBJ/EMBL/GenBank database under the accession number EF601928

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Similar to blood types, human plasma haptoglobin (Hp) is classified into three phenotypes: Hp 1-1, 2-1 and 2-2. They are genetically inherited from two alleles ***Hp 1*** and ***Hp 2*** (represented in bold), but only the Hp 1-1 phenotype is found in almost all animal species. The Hp 2-2 protein consists of complicated large polymers cross-linked by α2-β subunits or $(\alpha 2\text{-}\beta)_n$ (where $n \geq 3$, up to 12 or more), and is associated with the risk of the development of diabetic, cardiovascular and inflammatory diseases. In the present study, we found that deer plasma Hp mimics human ***Hp 2***, containing a tandem repeat over the α-chain based on our cloned cDNA sequence. Interestingly, the isolated deer Hp is homogeneous and tetrameric, i.e. $(\alpha\text{-}\beta)_4$, although the locations of −SH groups (responsible for the formation of polymers) are exactly identical to that of human. Denaturation of deer Hp using 6 M urea under reducing conditions (143 mM β-mercaptoethanol), followed by renaturation, sustained the formation of $(\alpha\text{-}\beta)_4$, suggesting that the Hp tetramers are not randomly assembled. Interestingly, an α-chain monoclonal antibody (W1), known to recognize both human and deer α-chains, only binds to intact human Hp polymers, but not to deer Hp tetramers. This implies that the epitope of the deer α-chain is no longer exposed on the surface when Hp tetramers are formed. We propose that steric hindrance plays a major role in determining the polymeric formation in human and deer polymers. Phylogenetic and immunochemical analyses revealed that the ***Hp 2*** allele of deer might have arisen at least 25 million years ago. A mechanism involved in forming Hp tetramers is proposed and discussed, and the possibility is raised that the evolved tetrameric structure of deer Hp might confer a physiological advantage.

Haptoglobin (Hp) is an acute-phase protein (responsive to infection and inflammation) that is present in the plasma of all mammals [1–4]. A recent study has found that Hp also exists in lower vertebrates (bony fish) but not in frog and chicken [5]. The most frequently reported biological functions of the protein are to capture released hemoglobin during excessive hemolysis [6] and to scavenge free radicals during oxidative

Abbreviations
Hp, haptoglobin; β-ME, β-mercaptoethanol.

stress [7]. The captured hemoglobin is internalized by a macrophage/monocyte receptor, CD163, via endocytosis. Interestingly, the CD163 receptor only recognizes Hp and hemoglobin in complex, which indicates exposure of a receptor-binding neo-epitope [6]. Thus, CD163 is identified as a hemoglobin scavenger receptor. Recently, we have shown that Hp is an extremely potent antioxidant that directly protects low-density lipoprotein (LDL) from Cu^{2+}-induced oxidation. The potency is markedly superior to that of probucol, one of the most potent antioxidants used in antioxidant therapy [8–10]. Transfection of Hp cDNA into Chinese hamster ovary (CHO) cells protects them against oxidative stress [9].

Human Hp is one of the largest proteins in the plasma, and is originally synthesized as a single αβ polypeptide. Following post-translational cleavage by a protease, α- and β-chains are formed and then linked by disulfide bridges producing mature Hp [11]. The gene is characterized by two common alleles, ***Hp 1*** and ***Hp 2b***, corresponding to α1-β and α2-β polypeptide chains, respectively, resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. All the phenotypes share the same β-chain containing 245 amino acid residues. As shown in Fig. 1A, the α1-chain containing 83 amino acid residues possesses two available −SH groups; that at the C-terminus always cross-links with a β-chain to form a basic α-β unit, and that at the N-terminus links with another $(\alpha\text{-}\beta)_1$, resulting in an Hp dimer $(\alpha1\text{-}\beta)_2$, i.e. a Hp 1-1 molecule. In contrast, the α2-chain, containing a tandem repeat of residues 12–70 of α1 with 142 amino acid residues, is 'trivalent' providing an additional available −SH group (Cys15) that is able to interact with another α-β unit. As such, α2-chains can bind to either α1-β or α2-β units to form large polymers $[(\alpha1\text{-}\beta)_2\text{-}(\alpha2\text{-}\beta)_n$ in Hp2-1 and $(\alpha2\text{-}\beta)_n$ in Hp2-2] as shown in Fig. 1B.

Because of its weaker binding affinity to hemoglobin and retarded mobility (or penetration) between the cells, the polymeric structure of Hp 2-2 is dramatically more prevalent in some groups of patients with certain diseases, such as diabetes and inflammation-related diseases [7,12–14]. The human ***Hp 2*** allele has been proposed to have originated from ***Hp 1*** about two million years ago and then gradually displaced ***Hp 1*** as a consequence of nonhomologous crossing-over between the structural alleles (***Hp 1***) during meiosis [15–17], and is the first example of partial gene duplication of human plasma proteins [15,18,19]. Thus, only humans possess additional Hp 2-1 and 2-2 phenotypes.

In the present study, deer Hp protein was initially shown to be a homogeneous polymer using an electrophoretic hemoglobin typing gel. Following isolation

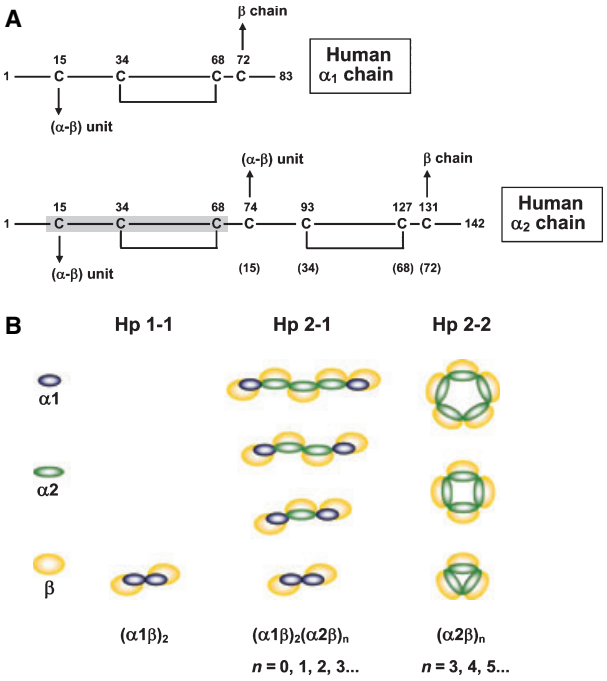

Fig. 1. Schematic drawing of the human Hp α-chain and the molecular arrangement of Hp phenotypes. (A) The human Hp α1-chain includes two avaiable −SH groups. That at the C-terminus always links to a β-chain to form a basic α1-β unit, and that at the N-terminus links either an α1-β unit or $(\alpha2\text{-}\beta)_n$ units. The sequence of α2 is identical to that of α1 except for a partial repeat insertion of residues 12–70. However, the extra Cys74 means that Hp 2-1 and 2-2 form complicated polymers. (B) Hp 1-1 forms the simplest homodimer $(\alpha1\text{-}\beta)_2$, whereas Hp 2-1 is polymeric in linear form, forming a homodimer $(\alpha1\text{-}\beta)_2$, trimer $(\alpha\text{-}\beta)_3$ and other polymers. Here, α represents α1- or α2-chains. Hp 2-2 forms cyclic structures: a trimer $(\alpha2\text{-}\beta)_3$ and other cyclic polymers.

and identification of the protein, the α-chain was found to be similar to the human α2-chain based on its apparent molecular mass. We then cloned the cDNA of deer Hp, showing that the putative amino acid sequence mimics that of human Hp 2-2 (81.7% and 67.9% sequence homology in the β- and α-chains, respectively), and that the α-chain of deer Hp also possesses a unique tandem repeat. Interestingly, deer Hp α-chain comprises seven −SH groups, that are oriented exactly the same as in human Hp 2-2, but the molecular arrangement of deer Hp is strictly tetrameric, i.e. $(\alpha\text{-}\beta)_4$. It is thus totally different from human Hp 2-2, which has $(\alpha\text{-}\beta)_n$ polymers, where $n \geq 3$. Using an α-chain mAb as a probe and denaturing/renaturing experiments, we further demonstrated that steric hindrance of the Hp α-chain plays a major role in determining the polymeric formation of human $(\alpha\text{-}\beta)_n$ and the deer $(\alpha\text{-}\beta)_4$ tetramer. Amino acid sequence alignment demonstrated that the evolved amino acid

sequences of the ruminant β-chain are the most divergent among all mammals. By phylogenetic tree analysis, we identified the α-chain of dolphin and whale (a branch before the deer) as belonging to the α1 type. This suggests that the deer tandem repeat sequence arose between 25 and 45 million years ago, which is much earlier than the two million years proposed for humans. It is possible that the evolved tetrameric structure of deer Hp might confer a physiological advantage. We further proposed that a steric hindrance mechanism is involved in forming Hp tetramers.

Results

Identification of Hp phenotype

It has been claimed that the Hp of ruminants (cattle, sheep and goat) cannot enter polyacrylamide gels due to the large polymeric nature of the protein [20,21]. We tested whether this was also the case for the Hp of deer (another ruminant). Using a hemoglobin typing gel, we unexpectedly found deer plasma Hp to be a simple homogeneous molecule that is small enough to enter a 7% electrophoretic gel. An example of its phenotype and the electrophoretic properties of deer Hp, compared to human Hp 1-1, 2-1 and 2-2, is shown in Fig. 2. This shows that deer Hp mimics one of the polymeric forms of human Hp 2-1 or 2-2: either a linear or cyclic tetramer.

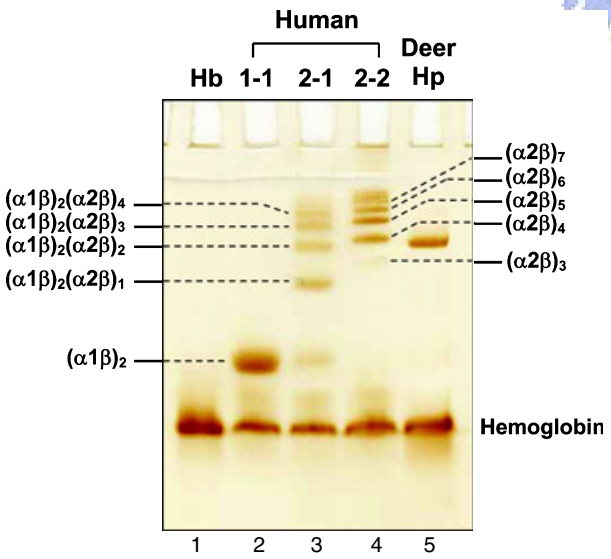

Fig. 2. Hemoglobin-binding patterns of deer and human plasma Hp on 7% native PAGE. Lane 1, hemoglobin only. Lanes 2, 3 and 4, human plasma of Hp 1-1, 2-1 and 2-2 phenotypes with hemoglobin, respectively. Lane 5, deer plasma with hemoglobin.

Isolation of deer Hp

The molecular size of the Hp α-chain has been conventionally used for identifying the phenotype of a given Hp protein. To further characterize the molecular form of deer plasma Hp, we attempted to isolate the protein using a Sepharose-based immunoaffinity column [22,23]. A mouse mAb prepared against the human α-chain (W1) was utilized for coupling to the Sepharose because this mAb was able to react with both human and deer α-chains on a western blot (described below). First, plasma samples enriched with Hp were pooled and applied to the affinity column. This procedure, however, failed to isolated deer Hp from the plasma due to the lack of binding of deer proteins to the column. Next, we used combined ammonium-sulfate fractionation and size-exclusion chromatography procedures [24] for the isolation. A size-exclusion chromatographic profile for the fractions containing Hp is shown in Fig. 3A (second peak). The homogeneity of isolated Hp was approximately 90%, as determined by SDS–PAGE (Fig. 3B). The presence of α-chains was

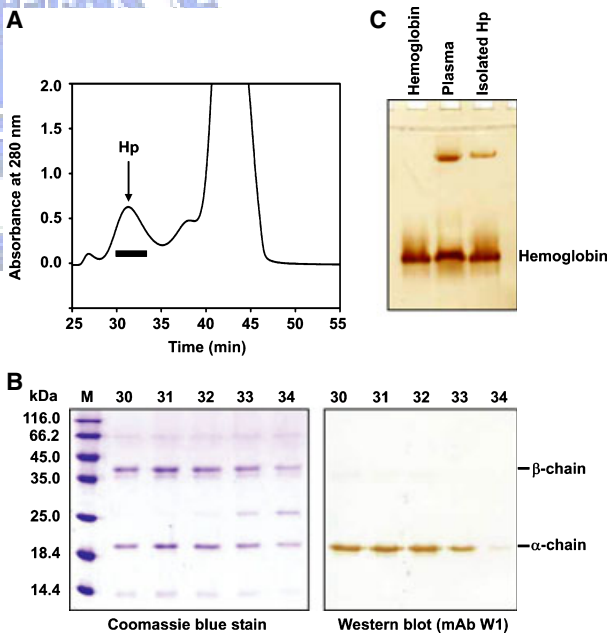

Fig. 3. Isolation of deer Hp using a size-exclusion Superose-12 column on an HPLC system. (A) A dialyzed supernatant of the 50% saturated ammonium sulfate fraction from plasma was applied to Superose-12 column (1 × 30 cm) at a flow rate of 0.3 mL·min^{-1}, using NaCl/Pi as the mobile phase. The bar represents the pooled fractions corresponding to Hp. (B) SDS–PAGE and western blot analyses of eluted Hp fractions. (C) Hemoglobin-binding properties of isolated Hp and plasma containing native Hp on 7% native PAGE. Lane M, molecular markers in kDa (Invitrogen).

confirmed by western blot using W1 mAb (Fig. 3B; right panel).

Hemoglobin binding of isolated Hp

In the next experiment, we tested the hemoglobin-binding ability of isolated deer Hp. Fig. 3C shows that the isolated Hp was able to form an Hp–hemoglobin complex under 7% native PAGE. Furthermore, it demonstrates that the deer protein consists of one major molecular form that is identical to its native form in the plasma based on electrophoretic mobility. It appears that the Hp isolated under our experimental conditions was not significantly altered with regard to its molecular and biochemical properties.

Molecular mass estimation of deer and human Hp 2-2 using SDS–PAGE and western blot

Western blot analysis using the α chain-specific mAb W1 indicated that the mAb recognizes both human and deer α chains (Fig. 4A). It also reveals that the deer α-chain belongs to the α2 group, with a molecular mass of approximately 18 kDa on both SDS–PAGE and western blot. We therefore tentatively classified the deer Hp as phenotype 2-2. In isolated deer Hp, there was a trace amount of hemoglobin (approximately 14 kDa), with a molecular mass comparable to that of the human Hp α1-chain. The estimated molecular mass of the deer β-chain was about 36 kDa, slightly lower than that of human. The isolated deer Hp was further characterized using 4% SDS–PAGE under non-reducing conditions. Consistent with our hemoglobin binding assay, Fig. 4B (left panel) demonstrates that isolated deer Hp consists of only one specific tetrameric form, i.e. $(\alpha\text{-}\beta)_4$, with a molecular mass about 216 kDa, which is close to that of the human Hp 2-2 tetramer (230 kDa) based on the gel profile.

Unique immunoreactivity of deer Hp defined by mAb W1

We then attempted to ensure that the polymeric forms of human and deer protein were an Hp by western blot analysis using W1 mAb. Figs 3B and 4A clearly showed that this antibody was capable of binding both human and deer α-chains in its reduced form. Interestingly, Fig. 4B (right panel) shows that this mAb recognized all the human Hp 2-2 polymers, but not intact deer Hp 2-2. However, after adding a reducing reagent (β-mercaptoethanol; β-ME) directly to intact deer Hp, the immunoreactivity was recovered on a dot-blot

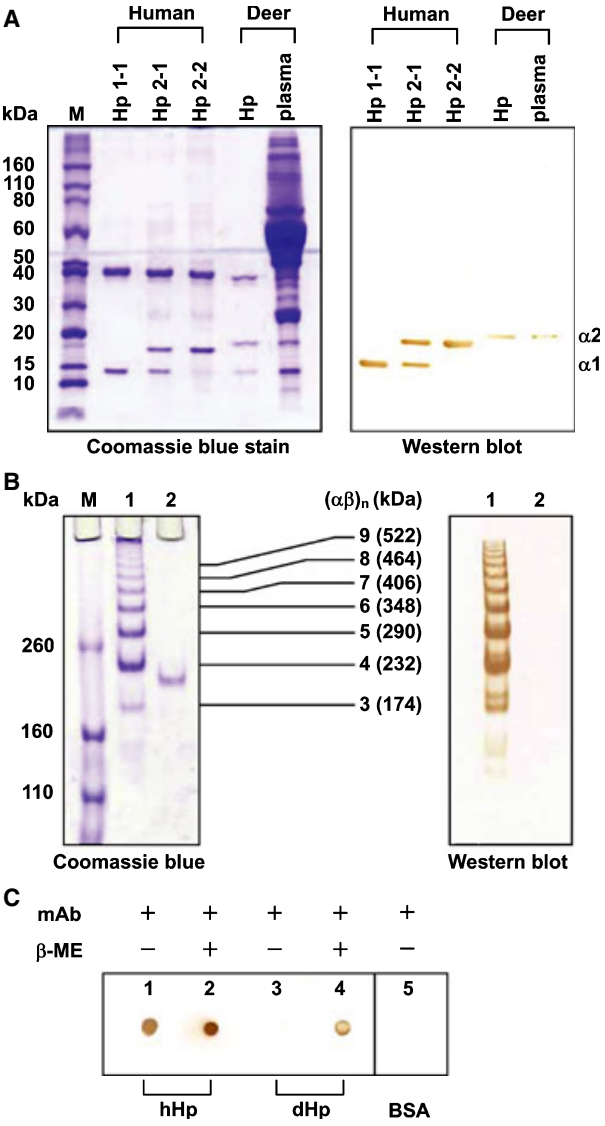

Fig. 4. SDS–PAGE, western blot and molecular mass analyses of isolated deer and human Hp. (A) The isolated proteins were run on 10–15% PAGE under reducing conditions. The western blot was performed using a human α-chain-specific mAb (W1) that cross-reacts with the deer α-chain. Lane M, molecular markers in kDa (Invitrogen). (B) Left panel: western blot analysis of the polymeric structure of isolated human and deer Hp under 4% non-reducing SDS–PAGE using α-chain-specific mAb W1. Lane M, molecular markers in kDa (Invitrogen). Lane 1, isolated human Hp 2-2. Lane 2, isolated deer Hp. Right panel: On the western blot, mAb W1 only recognizes human polymeric Hp, but not deer tetrameric Hp. (C) Dot-blot analysis of isolated human Hp (hHp) and deer Hp (dHp) using α-chain-specific mAb W1 in the presence or absence of the reducing reagent β-ME (143 mM). BSA was used as a negative control.

assay (Fig. 4C). It appears that the antigenic epitope of deer α-chain is masked in the tetrameric form. This also explains why the W1 mAb-coupled affinity

column failed to bind deer plasma Hp in the purification procedure described above.

Cloning of deer Hp cDNA

Evidently, the molecular form of deer 'Hp 2-2' totally differs from that of human Hp 2-2, with the latter found as typical polymers or the form $(\alpha\text{-}\beta)_n$, where n = 3–12 (Fig. 4B). It remains ambiguous as to whether deer Hp should be designated as a typical Hp 2-2. The most significant feature of the molecular structure of human Hp 2-2 is that it includes a tandem repeat in the α2-chain. To determine whether this is also true in deer Hp, we cloned the deer Hp cDNA. The complete linear nucleotide sequence corresponding to the α-β chain as determined by our laboratory has been submitted to GenBank (accession number EF601928). Based on the cDNA sequence, the deer α- and β-chains comprise 136 and 245 amino acid residues, respectively, which is similar to that of human, with 142 (α2) and 245 (β) residues (Fig. 5A,B). A tandem repeat of the deer α-chain was observed (discussed below).

Amino acid sequence alignment of deer and human Hp 2-2

The putative amino acid sequence alignment reveals that deer Hp is somewhat homologous to human Hp 2-2 (80% and 68% for β- and α-chains, respectively). The divergence and identity of the β-chain with that of other mammals are shown in Fig. 5C. The sequence for deer is relatively similar to that of cattle [25], another ruminant. We also created a brief phylogenetic tree for possible molecular evolution of the Hp β-chain using the CLUSTAL method in DNASTAR MEGALIGN software. The result shows that the evolved amino acid sequences of ruminant Hp β-chains are the most divergent among all mammals (Fig. 5D).

Analysis of –SH groups of the deer Hp α-chain and their implication for formation of the tetramer

As shown in Fig. 6 in the form of simplified ABC domains, the human α2-chain contains identical ABC

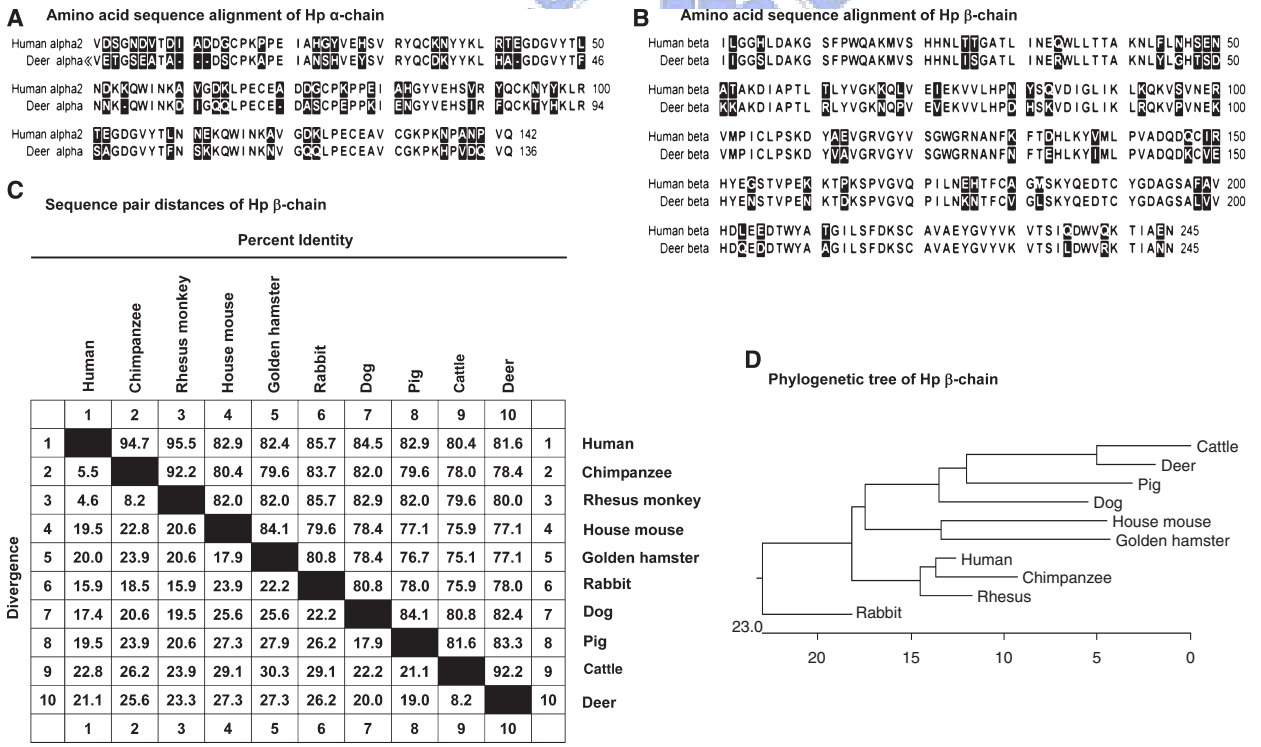

Fig. 5. Putative amino acid sequence analysis and divergence of mammal Hps. (A,B) Amino acid sequence alignment of the α- and β-chains of human and deer. Variable regions are shaded in black. The cDNA nucleotide sequence corresponding to deer Hp in this study has been deposited in GenBank under the accession number of EF601928. (C) Divergence of the amino acid sequences of Hp β-chains among ten mammals. (D) Phylogenetic tree constructed according to the amino acid sequences of Hp β-chains for ten mammals. The tree was plotted using the MEGALIGN program in the DNASTAR package. Branch lengths (%) are proportional to the level of sequence divergence, while units at the bottom indicate the number of substitution events.

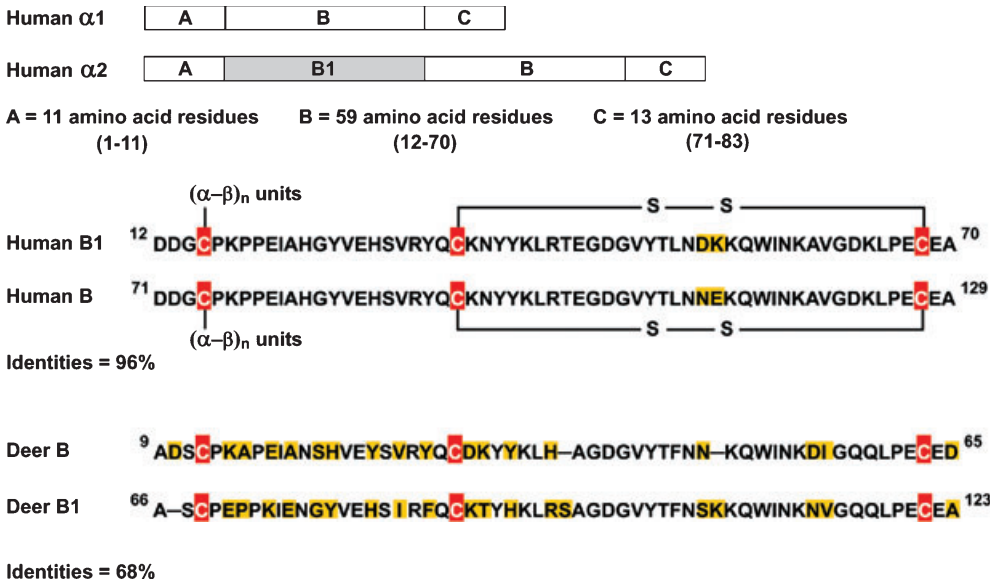

Fig. 6. Schematic drawing of tandem repeat region (B and B1) of deer and human α-chain. The most significant feature of human α2 is that it matches the ABC domains of α1 but with an additional insertion of a redundant sequence (B1 region). The repeat unit contains 59 amino acid residues between Asp12 and Ala70. The sequence homology in the repeat region of human is 96% (two amino acids mutated). Deer also have a redundant sequence (B and B1), but the sequence homology between the two repeat units is approximately 68%. The full length of the α-chain contains 142 and 136 residues in human and deer, respectively. The positions and number of Cys residues (total of seven) are completely identical between the two species (the one at the C-terminal region is not shown). Divergence of the amino acids within the species is marked in yellow.

domains to α1 with insertion of a tandem repeat region (B1). The latter contains amino acid residues between Asp12 and Ala70 (a total of 59 residues). The sequence homology between the repeat regions of the human α2-chain is 96%, with only two amino acids mutated (replacement of Asn52 and Glu53 in the B region by Asp52 and Lys53 in the B1 region). This tandem repeat is responsible for the formation of Hp polymers due to the extra −SH group (Fig. 1A). Such repeats also exist within the deer α-chain (B1 and B repeat), where the B1 region is residues 9–65. Thus, at the molecular level, the deer α-chain belongs to the α2 group, and is identical to the human α2-chain in possessing a tandem repeat. Interestingly, the sequence homology between the two repeat units (B1 and B) of deer is only 68% (Fig. 6).

As shown schematically in Fig. 1A, the human α2-chain consists of seven −SH groups (Cys15, 34, 68, 74, 93, 127 and 131) in 142 residues. Among these, there are two disulfide linkages within the α-chain (Cys34 and 68 and Cys93 and 127), and the one at the C-terminal region (Cys131) cross-links with the β-chain (Cys105) to form a basic α-β unit. Under such an arrangement, Cys15 and Cys74 are available to link with other α-β units. As a result, human α2 forms $(\alpha\text{-}\beta)_n$ polymers (where $n \geq 3$) as shown in Fig. 4B. Interestingly, the number and location of −SH groups in the deer α2-chain are identical to those in human (Fig. 6), but the deer Hp only yields a tetrameric $(\alpha\text{-}\beta)_4$ form. As the identity between the tandem repeats of deer is only 68% (compared with 96% in human), we hypothesized that these amino acid differences determine the conformation between Cys15 and 74 and drive the construction of the $(\alpha\text{-}\beta)_4$ structure of deer Hp (see Discussion).

To test whether the deer Hp can also form multiple polymers *in vitro*, we denatured the protein using 6 M urea with addition of 143 mM β-ME. Under these conditions, the deer protein was completely dissociated, similar to the profile shown in Fig. 4A for SDS–PAGE analysis (data not shown). We then slowly renatured the deer Hp by stepwise dialysis in order to determine possible formation of other large polymers (greater than tetramer). Figure 7 shows that the renatured protein retained the tetramer form, and no other polymers larger than tetramers were observed on SDS–PAGE, although some trimers were produced. Under the same conditions, human Hp 2-2 was renatured to $(\alpha\text{-}\beta)_n$. The data suggest that formation of deer Hp tetramer is specific, not randomly assembled. This assembly seems to be dependent on the unique orientation of the −SH groups within the Hp. In addition, each renatured protein retained its hemoglobin-binding ability (Fig. 7). A hypothetical model explaining the formation of Hp tetramers is described below.

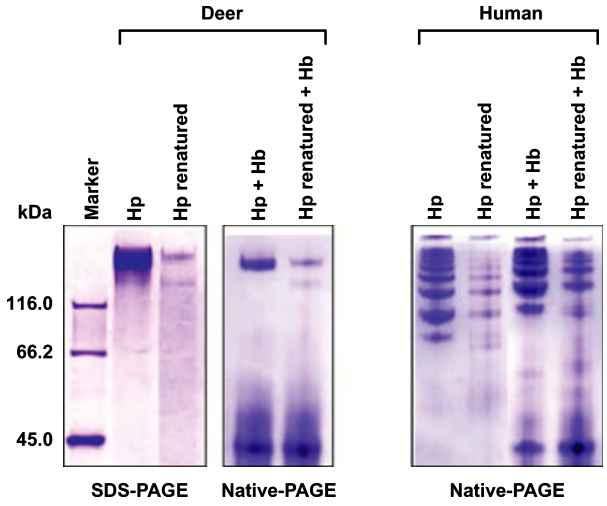

Fig. 7. SDS–PAGE and native PAGE analyses of renaturation of deer and human Hp polymers. Denaturation of deer Hp using 6 M urea under reducing conditions (143 mM β-ME) followed by renaturation resulted in the formation of $(\alpha\text{-}\beta)_4$ and some $(\alpha\text{-}\beta)_3$.

Discussion

Isolation of deer native Hp

We have recently developed several lines of human Hp mAb and routinely utilized these antibodies for the isolation of human Hp 1-1, 2-1 and 2-2 phenotypes [22,26]. As only W1 (specific to the α-chain) is able to cross-react with the deer α-chain on a western blot, we attempted to utilize this mAb for the affinity isolation of deer Hp in this study. Interestingly, the W1 mAb only recognizes the human Hp but not deer Hp in its intact form (Fig. 4B,C). We therefore used a previously described HPLC-based size-exclusion chromatography procedure [24] for the isolation of deer Hp. However, this procedure is only suitable for isolating the Hps with a homogeneous structure, and is not suitable for human Hp 2-2 or 2-1 [22]. One minor disadvantage of the method was the contamination of the isolated Hp by a trace amount of hemoglobin (Fig. 4A). This is observed mainly because Hp–hemoglobin complexes are formed prior to the purification; as such, hemolysis should be kept to a minimum in order to reduce the hemoglobin level while collecting the blood.

Presence of Hp in deer plasma

Not all deer possess a high level of plasma Hp. About 30% of the plasma samples that we screened (total $n = 15$) exhibited low Hp levels in the hemoglobin-binding assay (Fig. 2). Based on chromogeneity, the concentrations of deer plasma were approximately 1 $mg{\cdot}mL^{-1}$ of those used for purification when compared with human Hp 1-1 standard. In reindeer ($n = 6$), a mean plasma value of 0.6 $mg{\cdot}mL^{-1}$ has been reported [27].

Primary structure of the deer α-chain and its relationship to Hp polymers

There are several lines of evidence support the conclusion that the genotype of deer Hp is ***Hp 2***, with an Hp 2-2 phenotype. First, analysis of mercaptoethanol-reduced plasma indicates a molecular mass of 18 kDa for the α-chain, which is similar to that of human α2 based on a western blot (Fig. 4A). Second, the molecular mass of the α-chain from a purified sample was also similar to that of human α2 (Fig. 4A). Third, by putative amino acid sequence alignment, the deer α-chain contains a tandem repeat that is consistent with that found in human. Fourth, the total number of −SH groups and their location resulting from the tandem repeat are completely identical to that of human, although the sequence homology between the repeats was 68% in deer, compared to 96% in human (Fig. 6).

It remains unclear why the apparent molecular mass of the deer α-chain on PAGE is somewhat higher than that of human. We therefore attempted to determine whether it was due to additional carbohydrate moieties on the deer α-chain. However, using Pro-Q Emerald glycoprotein gel stains (Molecular Probes, Eugene, OR, USA), we did not identify any carbohydrates associated with the α-chain of either species (data not shown).

Hypothetical model for the formation of the deer Hp tetramer

The ability of the deer Hp to refold and reassemble into its tetrameric form *in vitro* indicates that the assembly of α- and β-chains into predetermined polymers is dependent on their biochemical nature (Fig. 7). As shown in Fig. 8A, we proposed a model to explain the formation of tetramers. This suggests that the two −SH groups of the deer α-chain are located on two flat surfaces at different angles to each other. Under these conditions, a homodimer cannot form due to the availability of another free −SH group of the α-β unit for cross-linking with another α-β unit. Figure 8B illustrates that there is no steric hindrance for tetramer formation, although there are two possible configurations for the tetramer. Some trimers may form, but there is some hindrance preventing the subunits from coming

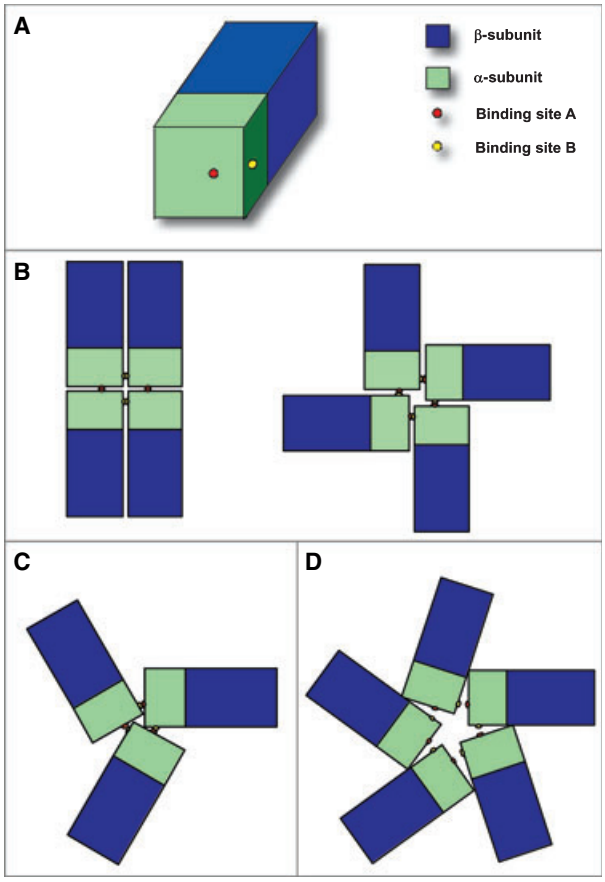

Fig. 8. A hypothetical model illustrating the steric hindrance involved in formation of a deer Hp tetramer. (A) A basic Hp subunit comprising one α- and one β-subunit. The –SH groups that connect the Hp subunits into polymers are assumed to be located with steric hindrance between the SH binding sites A and B. (B) The two different possible forms of tetramers. (C) A trimeric form of deer Hp is possible to assemble according to this model, but steric hindrance is seen which prevents the –SH groups from linking to some extent. (D) Formation of a pentamer or higher-order polymer is not possible.

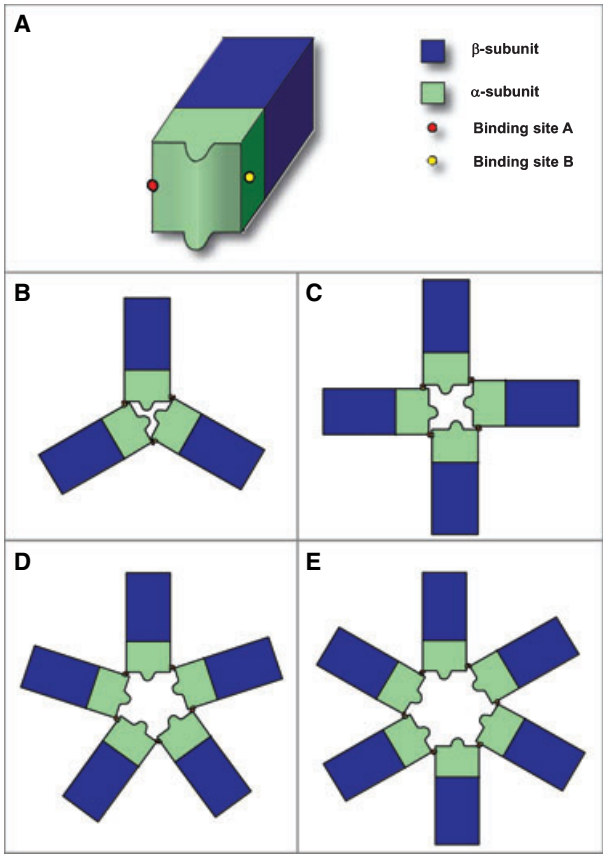

Fig. 9. Model of formation of human Hp 2-2 polymers. The positioning of the –SH groups involved in polymer formation differs from those in deer Hp. (A) A basic human Hp 2-2 subunit comprising one α- and one β-subunit. The –SH groups that connect the subunits into polymers are located at the edge of the surface. The hindrance between the –SH binding sites A and B prevents formation of a dimer. (B) A trimer is able to form to some extent with some steric hindrance. (C–E) Polymers of a higher order than tetramers can form without any steric hindrance.

close together in the cyclic center (Fig. 8C). Therefore, the formation of trimers takes place to a much lower extent than that of tetramers. No higher-order polymers are formed, because the distance between the –SH groups is too great to allow cross-linking for $(\alpha\text{-}\beta)_5$ pentamers or other larger polymers (Fig. 8D).

For a higher-order polymer ($n > 5$), the angle (θ) between the sides containing the –SH groups of two polymers would be $90–360/n$ degrees. If the distance between the –SH sites is approximately 90°, and the side of the Hp subunit contributes the base of the triangle, the distance is proportional to $\sin\theta$. As θ approaches 90° as n approaches infinity, the distance between the –SH sites also comes close to a maximum as n increases. In fact, few trimers are seen in our renaturing experiment (Fig. 7) and no polymers of an order of five or higher are observed.

For human Hp 2-2, on the other hand, the formation of higher-order polymers is possible (Fig. 9). The assumed positions of the –SH groups differ from those in deer Hp. They are located at the edges of the same plane, so formation of an identical 'stacking' dimer or $(\alpha\text{-}\beta)_2$ is not possible due to steric hindrance between the two –SH groups (Fig. 9A). However, formation of some trimers by linking together via the two –SH groups at the edge is possible, but not to a great extent due to the limited space in the cyclic center (Fig. 9B). This explains why there are only trace amount of trimers in all the human Hp 2-2 samples (Fig. 2). The cyclic center provides sufficient room to facilitate

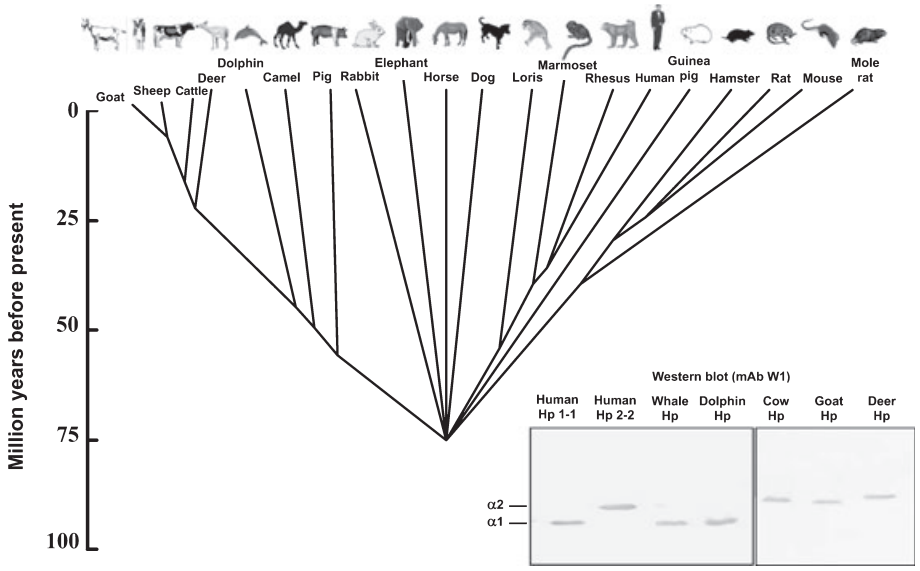

Fig. 10. Phylogenetic tree illustrating the molecular evolution of mammals, and phenotyping of human, whale, dolphin and ruminant α-chains. The tree is constructed by assuming that all eutherian orders radiated at about the same point in evolutionary time, approximately 75 million years ago. Alternative branching orders give essentially identical results. Within a eutherian order, branch points are assigned using evolutionary times based on fossil records [30]. Western blot analysis of Hp of six mammals (with a branching point before and after deer) was conducted using a 10–15% SDS–PAGE gradient gel under reducing conditions with an α-chain-specific mAb (W1) prepared against human Hp.

formation of polymers of an order greater than four α-β units. Such configuration also allows binding of the W1 mAb. In contrast, the cyclic center of deer Hp tetramers is totally blocked and is not accessible for mAb binding (Fig. 4B,C).

Evolution

In vertebrates, a recent study has suggested that the Hp gene appeared early in vertebrate evolution, between the emergence of urochordates and bony fish [5]. All mammalian species studied to date have been shown to possess Hp. Analysis of the electrophoretic patterns of Hp–hemoglobin complexes has suggested that most of these Hps are similar to human Hp 1-1 [28]. Only the protein found in ruminants (cattle, sheep and goat) resembled polymeric forms of human Hp 2-2 [20], but whether they also possess a tandem repeat remains unexplored [25].

It is thought that humans originally had a single Hp 1-1 phenotype [29]. Maeda *et al.* [15] proposed that the tandem repeat sequence of human α2 evolved two million years ago from a nonhomologous unequal crossover between two ***Hp 1*** alleles (***Hp 1S*** and ***Hp 1F***) during meiosis. A unique feature of the ***Hp 2*** allele is that it is present only in humans and is not found in any primates, including New and Old World monkeys, chimpanzees and gorillas [17]. We have recently found that cattle also possess ***Hp 2*** as the sole genotype [25]. It is likely that ruminants including deer, cattle, goat and sheep may all possess a sole ***Hp 2***-type allele. In the present study, we have shown that the inserted tandem repeat region in deer Hp appears to have extensively evolved, as 32% of the repeated region has undergone mutation, compared to that of only 4% (two amino acid residues) in human Hp (Fig. 6). Thus, we propose that the occurrence of the tandem repeat in deer was much earlier than in humans.

Figure 10 depicts a phylogenetic tree constructed by assuming that all eutherian orders (mammals) radiated at about the same point in evolutionary time (approximately 75 million years ago) [30]. The phylogenetic analysis indicates that crossing-over of deer α-chains occurred after divergence of the line leading to ruminants and pig, as pig possesses only the Hp 1-1 phenotype [24]. As dolphins and whales are the closest divergences before the ruminants, we further examined the size of the α-chain in whales and dolphins as well as other ruminants (cattle and goat) to determine the possible time of the tandem repeat evolution in deer Hp. Interestingly, the inserted panel of Fig. 10 shows that the α-chains of all the ruminants tested are the α2 type, except for dolphins ($n = 5$) and whales ($n = 5$).

These data suggests that the crossing-over resulting in the tandem repeat in ruminants occurred at least 25 million years ago or between 25 and 45 million years ago (Fig. 10), which is much earlier than the two million years proposed in humans [15]. The molecular evolution of the ruminants, which are the latest mammals in the phylogenetic tree (diverging after dolphins), is remarkably rapid, based on molecular evolution models for growth hormone and prolactin, when compared with other mammals [31,32]. This model appears to be consistent with the overall amino acid alterations (32%) within the tandem repeat of deer Hp α-chain. A similar alteration in cattle has also been reported recently [25].

Whether this alteration is adaptive during evolution remains to be addressed. For example, in cattle, there is an extensive family of at least eight prolactin-like genes that are expressed in the placenta [33,34]. These genes appear to be arranged as a cluster on the same chromosome. Phylogenetic analysis suggests that all of these genes are the consequence of one or more duplications of the prolactin gene; detailed analysis suggests that a rapid adaptive change has played a role in molecular evolution [35].

Evolutionary advantage of deer Hp protein being a tetramer

In addition to the superior binding affinity of Hp to hemoglobin, Hp is an anti-inflammatory molecule and a potent antioxidant [9]. In humans, the large complicated polymers of Hp 2-2 are a risk in the association of diabetic nephropathy [36,37]. One explanation is that the large polymer dramatically retards penetration of the molecule into the extracellular space [36]. We have shown in the present study that deer Hp 2-2 was not able to form complicated polymers, because the diversity in amino acid sequence between the tandem repeat of α-chain has produced steric hindrance (Fig. 8) that may be advantageous to deer.

In conclusion, we have shown that deer possess an ***Hp 2*** allele with a tandem repeat that could have occurred at least 25 or between 25 and 45 million years ago based on the phylogenetic analysis. The phenotypic and biochemical structure of their Hp is markedly homogeneous, with a tetrameric arrangement due to the orientation of the two available –SH groups, preventing the formation of the complicated Hp polymers found for human Hp 2-2. In terms of molecular evolution, this steric hindrance may have conferred an advantage on deer Hp that compensates for the undesired tandem repeat in the α-chain.

Experimental procedures

Animal plasma

Animal plasma of deer (*Cervus unicolor swinhoei*), goat (*Capra hircus*), cattle (*Bos taurus*), pig (*Sus scrofa domestica*), dolphin (*Steno bredanensis*) and whale (*Delphinapterus leucas*) were obtained from the Pingtung County Livestock Disease Control Center and the Veterinary Medicine Teaching Hospital, National Pingtung University of Science and Technology, Taiwan.

Phenotyping

Hp phenotyping was performed by native PAGE using hemoglobin-supplemented serum or plasma [22]. Briefly, 6 μL plasma were premixed with 3 μL of 40 $mg \cdot mL^{-1}$ hemoglobin for 15 min at room temperature. The reaction mixture was then equilibrated with 3 μL of a sample buffer containing 0.625 M Tris (pH 6.8), 25% glycerol and 0.05% bromophenol blue, followed by electrophoresis on a 7% native polyacrylamide gel (pH 8). Electrophoresis was performed at 20 mA for 2 h, after which time the Hp–hemoglobin complexes were visualized by shaking the gel in a freshly prepared peroxidase substrate (30 mL $NaCl/P_i$ containing 25 mg of 3,3′-diaminobenzidine in 0.5 mL dimethyl sulfoxide and 0.01% H_2O_2). The results were confirmed by western blot using an α-chain-specific mAb prior to phenotyping.

Preparation of mouse mAb and human Hp

Mouse mAb W1 specific to the human Hp α-chain was produced in our laboratory according to standard procedures [38]. Native human Hp was isolated from plasma using an immunoaffinity column followed by size-exclusion chromatography on an HPLC system using previously described procedures [22].

Purification of deer haptoglobin

Plasma samples enriched with Hp were prepared from deer blood containing 0.1% EDTA, followed by centrifugation at 1200 ***g*** for 15 min at 4 °C to remove the cells. Isolation was performed according to the method previously established for porcine Hp [24]. Saturated ammonium sulfate solution was added to the plasma to a final saturated concentration of 50%. After gentle stirring for 30 min at room temperature, the precipitate was discarded by centrifugation at 4000 ***g*** for 30 min at 4 °C. The supernatant was then dialyzed at 4 °C for 16 h against $NaCl/P_i$ containing 10 mM phosphate (pH 7.4) and 0.12 M NaCl with three changes. After dialysis, the sample was concentrated and filtered through a 0.45 μm nylon fibre prior to size-exclusion chromatography. An HPLC system (Waters, Milford, MA, USA), consisting of two pumps, an automatic sample

injector and a photodiode array detector, with a Superose-12 column (1 × 30 cm) (GE Healthcare, Uppsala, Sweden) pre-equilibrated with NaCl/P_i, was used for further purification. The column was run for 60–80 min at room temperature with a flow rate of 0.3 mL·min^{-1} using NaCl/P_i as the mobile phase. Fractions containing Hp were pooled and concentrated to a final volume of 1 mL using an Amicon centrifugal filter (Millipore, Billerica, MA, USA), and stored at −20 °C until use.

Gel electrophoresis

SDS–PAGE was performed according to the method described by Laemmli [39] with some modifications, using 5% polyacrylamide as the stacking gel [40]. In general, samples containing 143 mM β-ME were preheated at 100 °C for 10 min in a buffer containing 12 mM Tris–HCl (pH 6.8), 0.4% SDS, 5% glycerol and 0.02% bromophenol blue before loading to the gel. The samples were run on a step gradient of polyacrylamide gel (10 and 15%) for about 1.5 h at 100 V and stained using Coomassie brilliant blue. For determination of the molecular mass of Hp, the tested samples were prepared under the non-reducing conditions using the SDS gel. Alternatively, the SDS gel was prepared in a 0.04 M phosphate buffer (pH 7.0) containing 4% polyacrylamide, and the samples were run for about 6 h at 30V. The molecular mass standard for SDS–PAGE, containing three prestained proteins (260, 160 and 110 kDa), was purchased from Invitrogen (Carlsbad, CA, USA).

Immunoblot analysis

Western blot analysis was performed using a method similar to that described previously [40]. In brief, the electrotransferred and blocked nitrocellulose was incubated with anti-Hp mAb W1, followed by washes and incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (Chemicon, Temecula, CA). The membrane was developed using 3,3′-diaminobenzidine containing 0.01% H_2O_2. Dot blots were performed by applying the samples (reduced or non-reduced) onto a nitrocellulose membrane using anti-Hp mAb W1 as the primary antibody.

Cloning and sequencing analysis of deer Hp

The entire procedure was similar to that described previously [9,10]. Briefly, total RNA was extracted from deer whole blood using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The gene for deer Hp from total RNA was reverse-transcribed and PCR-amplified using proofreading DNA polymerase (Invitrogen), forward primer 5′-TTCCTGC AGTGGAAACCGGCAGTGAGGCCA-3′ and reverse primer 5′-CGGAAAACCATCGCTAACAACTAAGCTT GGG-3′. The PCR cycling profile was as follows: denaturation at 94 °C for 5 min, then 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 90 s, then final extension at 72 °C for 10 min. The PCR product was analyzed by electrophoresis through a 1% agarose gel, and purified using a gel extraction kit (BD Biosciences, Palo Alto, CA). The purified PCR product was cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA), and then the ligated plasmid was transformed into *Escherichia coli* JM109 (Qiagen). Finally, the sequence of deer Hp was confirmed by DNA sequencing.

Sequence alignment and phylogenetic analysis

The cDNA and amino acid sequence alignment, sequence pair distances and phylogenetic tree construction were performed using DNASTAR software (Lasergene, Madison, WI, USA).

Denaturation and renaturation of deer and human Hp 2-2

Purified deer Hp (0.1 mg·mL^{-1}) or human Hp 2-2 (2 mg·mL^{-1}) were mixed with NaCl/P_i containing 6 M urea and 143 mM β-ME and incubated at room temperature for 30 min. The reaction mixture was first dialyzed in 200 mL NaCl/P_i at 4 °C for 6 h, and this was repeated three times (total 24 h) to allow renaturation. The mixture was finally dialyzed against 2 L NaCl/P_i overnight. The concentrated Hp samples with or without reduction were incubated with hemoglobin for use on a typing gel as that for plasma phenotyping, and then stained by Coomassie brilliant blue.

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Cloning and expression of human haptoglobin subunits in *Escherichia coli*: Delineation of a major antioxidant domain

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Abstract

Human plasma haptoglobin (Hp) comprises α and β subunits. The α subunit is heterogeneous in size, therefore isolation of Hp and its subunits is particularly difficult. Using *Escherichia coli*, we show that α1, α2, β, and α2β chain was abundantly expressed and primarily present in the inclusion bodies consisting of about 30% of the cell-lysate proteins. Each cloned subunit retained its immunoreactivity as confirmed using antibodies specific to α or β chain. By circular dichroism, the structure of each expressed subunit was disordered as compared to the native Hp. The antioxidant activity was found to be associated with both α and β chains when assessed by Cu^{2+}-induced oxidation of low density lipoprotein (LDL). Of remarkable interest, the antioxidant activity of β chain was extremely potent and markedly greater than that of native Hp (3.5×), α chain (10×) and probucol (15×). The latter is a clinically proved potent compound used for antioxidant therapy. The "unrestricted" structure of β subunit may therefore render its availability for free-radical scavenge, which provides a utility for the future design of a "mini-Hp" in antioxidant therapy. It may also provide a new insight in understanding the mechanism involved in the antioxidant nature of Hp.

Keywords: Human haptoglobin; α and β chains; Cloning; Mini-Hp fragment; Antioxidant domain; Monoclonal antibody; Structure; Circular dichroism

Haptoglobin (Hp)[1] is an acute-phase protein present in all mammals [1–3]. One of the major functions of Hp is to bind hemoglobin [4] and thereby prevent the oxidative tissue damage mediated by free hemoglobin [5]. We have recently demonstrated that chemically modified Hp losing its binding ability to hemoglobin, but is able to directly inhibit Cu^{2+} induced LDL oxidation [6]. In humans, Hp is originally synthesized as a single polypeptide containing both α and β chains (Fig. 1A) [7]. Posttranslational cleavage between the Arg of α chain and Ile of β chain (followed by removal of Arg) results in the formation of a single α and β chain that is subsequently linked by disulfide bridges to form mature Hp [8].

The Hp gene is characterized by two common alleles ***Hp 1*** and ***Hp 2*** corresponding to α1β and α2β polypeptide chains, resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. DNA encoding for these two chains are linearly oriented in chromosome 16q22.1 [9] with 5 and 7 introns in ***Hp 1*** and ***Hp 2***, respectively. These introns, however, are exclusively located at the region corresponding to the α chain [5]. The α2 contains a unique nonhomologous crossing-over in a part of the α1 [10]. Hp 2-1 and 2-2 form polymers due to an additional –SH group at the α2 chain, which determines its phenotype specificity [11]. All the phenotypes share the same β chains (each with 245 amino acids). The α1 chain (each with 83 amino acids) is "monovalent" forming one disulfide bridge with α and β subunit resulting in Hp dimer $(\alpha 1\beta)_2$. In contrast, the α2 chain (each with 142 amino

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[1] *Abbreviations used:* Hp, Haptoglobin; rHp, recombinant Hp; LDL, low density lipoprotein; DAB, 3,3′-di-aminobenzidine; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid); SEM, standard error of the mean; TBARS, thiobarbituric acid-reactive substances; CD, circular dichroic; CM, carboxymethylation.

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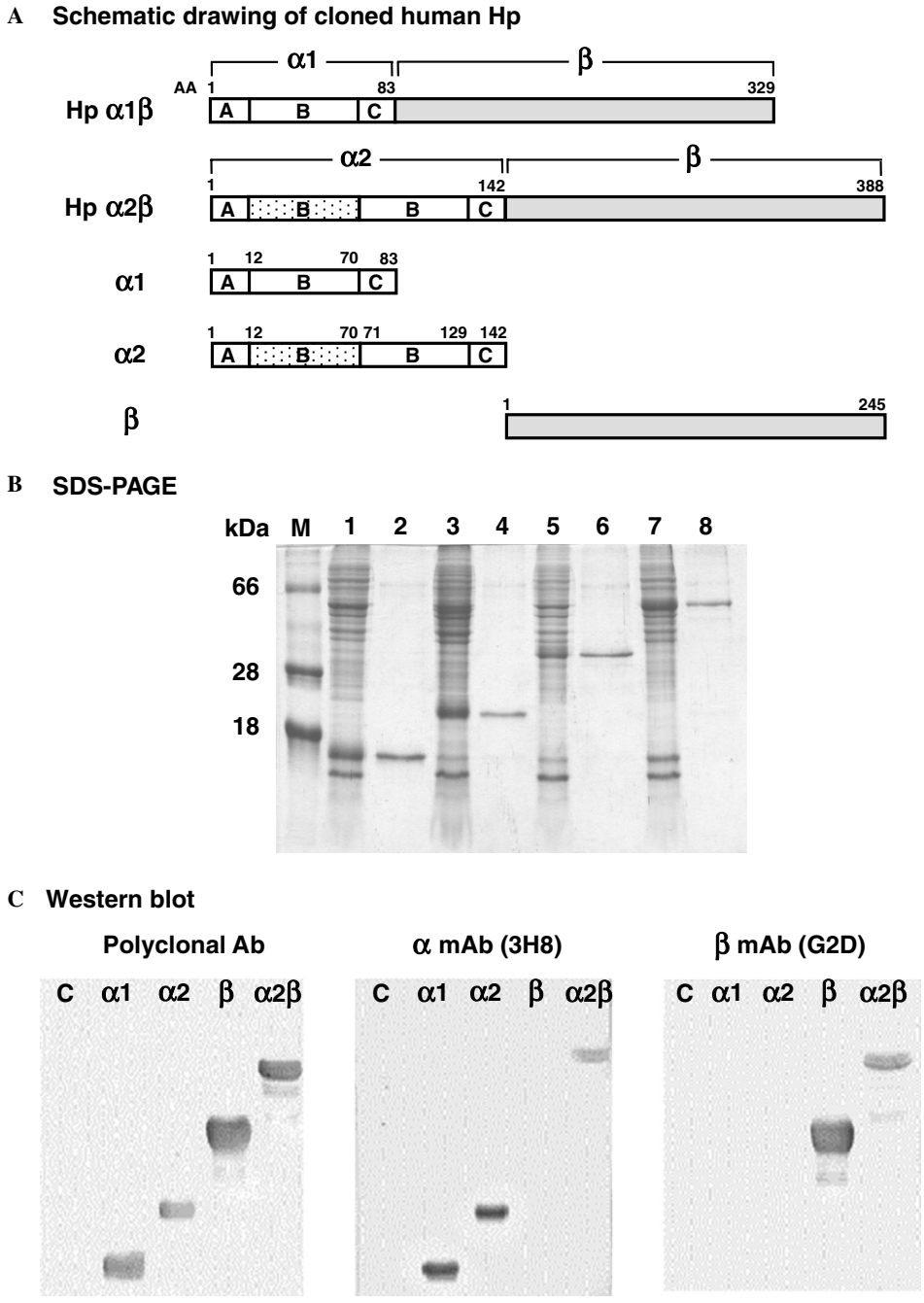

Fig. 1. Gene construction and Hp expression. (A) Schematic drawing of cloned human Hp α1, α2, β and α2β subunits in *E. coli*. Human matured mRNA corresponding to Hp 1-1 is linear oriented (α1β). The linear amino acid sequence (without signal peptide) containing residues 1–329 is shown on the top. Following a protease cleavage and a loss of the C-terminal residue Arg-84, it forms one α (residues 1–83) and one β chain (residues 1–245). Similarly, α2β forms one α2 (residues 1–142) and one β (1–245) chain. The amino acid sequence of α2 is identical to that of α1 with an insertion of repeated sequence B (dotted area or residues 12–70). It should be noted here, there are two amino acids in the inserted sequence B (dotted area) that are replaced by Asp-52 and Lys-53 in both native and our cloned sequence. (B) Expression and purification of recombinant Hp subunit in *E. coli*. Lane M, molecular markers; lanes 1, 3, 5 and 7, whole cell lysate containing expressed α1, α2, β and α2β, respectively; lanes 2, 4, 6 and 8, purified recombinant α1, α2, β and α2β, respectively. Notably, the α2β was not cleaved due to the lack of specific protease in *E. coli*. (C) Western blot of cell lysate containing α1, α2, β or α2β using Hp polyclonal antibody and α and β chain specific mAb. Lane C, whole cell lysate control. Other lanes represent the cell lysate containing expressed α1, α2, β or α2β subunit, respectively.

acids) is "divalent" containing one extra free –SH that is able to interact with an additional α2. As such, α2 chains can bind to either α1 or α2 chains to form large polymers [$(\alpha1\beta)_2$–$(\alpha2\beta)_n$ in Hp2-1 and $(\alpha2\beta)_n$ in Hp2-2]. The polymeric phenotypes have been reported to be more prevalent in some groups of patients with certain diseases, such as diabetic and autoimmune diseases [5,12–14].

The purposes of this study were to produce recombinant Hp (rHp) α1, α2, β and α2β subunits in *Escherichia coli* expression system and to test whether *E. coli* contains a

protease that may specifically cleave the linear αβ chain as that in mammalian cells [6,15], and to determine which subunit of Hp possesses antioxidant activity. We demonstrated that *E. coli* was devoid of a specific protease responsible for the cleavage of α and β chains. Each expressed α or β subunit not only retained the immunoreactivity, but also possessed the antioxidant activity. However, a major antioxidant domain was located in the β chain with a superior potency to probucol. Remarkably interesting, the unfolded structure of Hp, based on circular dichroic spectra, dramatically increased in antioxidant activity. The present study provides a potential utility for the future design of "mini-Hp" in developing a novel potent antioxidant. It may also provide a new insight in understanding the mechanism or specific amino acids involved in the antioxidant nature of Hp.

Materials and methods

Materials

Escherichia coli JM109, M15 [pREP4], and the pQE30 expression vector were obtained from Qiagen (Hilden, Germany). Plasmid preparation and gel-extraction kits were purchased from BD Biosciences (Palo Alto, CA). The proofreading DNA-polymerase and dNTP were purchased from Invitrogen (Carlsbad, CA). All restriction enzymes were purchased from New England Biolabs (Beverly, MA). T4-DNA-ligase and HiTrap chelating column were purchased from Fermentas (Burlington, Canada) and Amersham Biosciences (Uppsala, Sweden), respectively.

Preparation of mouse mAb and human Hp

Mouse monoclonal antibodies (mAb) 3H8 and G2D specific to human Hp α or β subunit were produced in our laboratory according to the standard procedures previously established [16]. Native Hp was isolated from human plasma by an immunoaffinity column using procedures previously established by us [17].

Plasmid construction of pQE30-Hp (α1, α2, β, α2β)

Gene fragments coding for human Hp α1, α2, β and α2β subunits were amplified by PCR using proofreading DNA-polymerase and oligonucleotide primers (Fig. 1A). The primer design was based on the published cDNA sequence of human Hp [18]. The primers were prepared for Hp α1 and α2 (forward 5′-GGGGTACCATGGTGGACTCAGG CAATGATGT-3′ and reverse 5′-AACTGCAGTTACTG CACTGGGTTTGCCGGA-3′), Hp β (forward 5′-GGGG TACCATGATCCTGGGTGGACACCTGG-3′ and reverse 5′-AACTGCAGTTAGTTCTCAGCTATGGTCTTCT-3′), and Hp α2β (forward 5′-GGGGTACCATGGTGGACTC AGGCAATGATGT-3′ and reverse 5′-AACTGCAGTT AGTTCTCAGCTATGGTCTTCT-3′). Both of *Kpn*I and *Pst*I restriction sites were incorporated into the 5′ end of the forward sequence and reverse sequence primers, respectively. The cDNA of Hp α1, α2, β and α2β were ligated into the *Kpn*I/*Pst*I sites of an *E. coli* expression vector, pQE30. The plasmids were screened in JM109 and then expressed in M15 [pREP4]. Finally, the sequence of pQE30-Hp (α1, α2, β and α2β) was confirmed by DNA sequencing.

Expression of recombinant Hp subunits

Escherichia coli [M15 (pREP4)] was transformed with the recombinant plasmid and cultured in 1 L of Luria–Bertani (LB) medium containing ampicillin (100 μg/ml) at 37 °C on a rotary shaker. When the optical density reached 0.6 at 600 nm, the protein expression was induced by 1 mM IPTG at 37 °C for 2–4 h. The medium was centrifuged at 8000*g* for 5 min, and washed three times in a wash buffer containing 20 mM Tris–HCl, pH 8.0.

Disruption, wash and isolation of inclusion bodies

The induced cells were then suspended in 40 ml of wash buffer and sonicated for 5 min at 4 °C, followed by centrifugation at 20,000*g* for 20 min at 4 °C. The pellet containing the inclusion body was resuspended in 30 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 4 °C. Finally, the pellet was washed twice in wash buffer and stored frozen for late processing.

Solubilization of rHp subunits 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 4 °C for 12 h and the insoluble material was removed by centrifugation at 20,000*g* for 20 min at 4 °C. Finally, the remaining soluble supernatant was then passed through a syringe filter (0.45 μm) and proceeded directly for purification and refolding.

Purification and refolding of rHp subunits

Initially, a 1 ml-HiTrap chelating column was washed with 5 ml distilled water using a 10 ml-syringe. After loading 1 ml of 0.1 M $NiSO_4$, the column was extensively equilibrated with the binding buffer. The recombinant fusion protein was subsequently loaded onto the column and washed with the binding buffer. The bound protein was then treated with 6 M urea, starting with the binding buffer mentioned above and finished at one without urea. Finally, the recombinant proteins were eluted using a 20 ml linear gradient starting with an elution buffer containing 20 mM Tris–HCl, 0.5 M NaCl, and 20 mM imidazole without 2-mercaptoethanol, pH 8.0 and ending with the same buffer containing 500 mM imidazole. Protein fractions were pooled and then desalted on a P-2 column using 0.05 M

ammonium bicarbonate, followed by lyophilization. Protein concentration was determined by the Lowry method [19], while using bovine serum albumin as a standard.

SDS–PAGE and Western blot analyses

Recombinant Hp subunits were characterized using SDS–PAGE containing 15% polyacrylamide as described previously [20]. In general, the tested sample was preheated at 100 °C 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. Western blot analysis was performed similar to that described previously [20]. In brief, the electrotransferred and blocked nitrocellulose was incubated with anti-Hp polyclonal or monoclonal antibodies (3H8 and G2D), followed by washes and incubation of peroxidase-conjugated anti-IgG. The membrane was developed using 3,3′-di-aminobenzidine (DAB) containing 0.01% H_2O_2 [20].

Determination of immunoreactivity of rHp subunits by enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted according to the procedure previously established [20,21]. Half micrograms of each Hp subunit in 50 μl PBS (pH 7.4) was coated onto each well of an ELISA plate. After blocking and washes, 50 μl of diluted mouse mAb prepared against Hp α (3H8) or β chain (G2D) were added and incubated at room temperature for 2 h. Following washes and incubation of 50 μl of peroxidase-conjugated goat anti-IgG (1:3000 dilutions), the plate was developed using 0.04% 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) containing 0.01% H_2O_2. The assay was conducted in triplicates. In general, the standard error of the mean (SEM) was less than 2%.

Preparation of human low density lipoprotein

Human low density lipoprotein (LDL; d. 1.012–1.063 g/ml) was prepared from human plasma by sequential ultracentrifugations according to the method previously established [22,23].

Antioxidant activity using Cu^{2+}-induced LDL oxidation

Thiobarbituric acid-reactive substances (TBARS) were used as an index for the measurement of LDL oxidation [23,24]. In a typical assay, 5 μM $CuSO_4$ and 20 μg of LDL (protein) were incubated with tested samples in a final volume of 100 μl. After 2 h incubation at 37 °C, 250 μl of 20% tricholroacetic acid were added to precipitate proteins. Subsequently, 250 μl of 0.67% 2-thiobarbituric acid were added into the reaction mixtures and incubated at 80 °C for 30 min. The reaction mixtures were centrifuged at 3000*g* for 5 min. Supernatant (300 μl) in a 96-well plate was read at 540 nm [23]. The assay was conducted in triplicates. In general, the standard error of the mean was less than 2.5%.

Reduction and carboxymethylation of Hp

Tris–HCl buffer (0.01 M) containing 5.4 M urea and 1% (v/v) β-mercaptoethanol, pH 8.6, was added to 1 mg of Hp to make a final volume of 3.3 ml. The reaction mixture was flushed with nitrogen and incubated at room temperature. After 2 h, 20 mg iodoacetic acid was slowly added and maintained the pH at 8.6 by the addition of 1 M NaOH for 30 min. Finally, carboxymethylated (CM) Hp was desalted on a P2 column equilibrated with 0.1 M ammoniumbicarbonate and lyophilized [22].

Circular dichroic analysis

Lyophilized rHp subunits and CM-Hp were dissolved in 10 mM phosphate buffer (pH 7.4) with a final concentration of 0.2 mg/ml. About 300 μl aliquot was used for the analysis in a cuvette of 1 mm path length. Circular dichroic (CD) spectrum recorded between 190 and 300 nm (Jasco J-715 spectropolarimetry) was accumulated 20 times at a scanning rate of 50 nm/min [17,20].

Results and discussion

Plasmid construction and expression of rHp subunits

DNA sequencing analysis demonstrated that the cDNA of each Hp α1, α2, β or α2β (Fig. 1A) was inserted into the *E. coli* expression vector pQE30 (data not shown). The expression of $(His)_6$-tagged fusion proteins was constructed based on the T5 promoter transcription–translation system [25]. One advantage of this system is its ability to maintain target genes silently transcribed in the uninduced state, since the extremely high transcription rate initiated at the T5 promoter is efficiently regulated and repressed by the presence of high levels of the *lac* repressor. In addition, the pQE30 vector also permits the fusion of a 6 × His-tag at the N-terminus of a given recombinant protein, which is useful for rapid purification and permits the purification under denaturing conditions. Fig. 1B shows that clones containing cDNA of Hp or Hp subunit (α1, α2, β, or α2β) were abundantly and effectively induced by IPTG. Determined by densitometry on the SDS–PAGE gel, the average expressed level of each subunit was account for about 30% of the total-lysate proteins (data not shown). Thus, this expression system is considerably effective in yield. The recombinant proteins were almost exclusively expressed in the inclusion bodies. Only trace amount (<5%) of α subunits was found as a soluble form (data not shown). A full and intact length of α2β chain of Hp was expressed (Fig. 1B, lane 7). In human liver cells, however, the α2β is postranscriptionally cleaved into α and β chains between the residues Arg and Ile [26,27]. A more recent study suggests that a complement C1r-like protease is responsible for the cleavage [15]. Our data indicate that this protease is not present in the *E. coli.* The recombinant α2β expressed may, therefore, provide a unique substrate for the identification

of the specific protease involved in mammalian cells. This experiment is now in progress in our laboratory.

Purification and refolding of rHp subunits

The present study shows the rHp subunits to be primarily present in the inclusion bodies of *E. coli*. Since binding of a protein containing 6× His-tag to nickel-immobilized column is not interfered by the chaotropic agent (such as urea or guanidine hydrochloride) at high concentration, 6× His-tagged recombinant protein can be solubilized by the chaotropic extraction before loading to the column. Removal of contaminating materials followed by refolding the recombinant protein using nondenaturing buffer can then be performed before the elution from the column by 20 mM imidazole [28]. Using this strategy, we show that the purification for rHp subunits efficiently achieved with about 95% homogeneity as determined by a 15% SDS–PAGE (Fig. 1B). The final average yield of each recombinant purified was about 20% as calculated from the total *E. coli* proteins (Table 1). The average recovery from each step as judged by the SDS–PAGE, however, was greater than 70%.

With respect to the solubility of rHp subunits, we found that the solubility could be maintained when lyophilized protein was reconstituted into a final concentration less than 2 mg/ml by a PBS. It is of interest to point out that the eluted subunits were soluble if they were concentrated (up to 5 mg/ml) by Centricon tubes immediately following the desalting on a P-2 column, while using 0.05 M ammonium bicarbonate as a mobile phase.

Immunoreactivity of mAb for rHp subunits

To address whether the expressed proteins were immunoreactive, each subunit was examined by a Western blot analysis using Hp mAb specific to human α (3H8) and β chain (G2D) and a Hp polyclonal antibody specific to α/β chains. Fig. 1C shows that each subunit was specifically recognized by each regionally specific mAb. Using a quantitative ELISA, the immunoreactivity of native Hp was higher in some extent than that of expressed subunit (Fig. 2). Although the reason is not readily clear, it is possible that those mAb were originally prepared against the native state of the Hp structure and the antibodies preferably recognized the native conformation of Hp. Another potential explanation is that recombinant subunits might not be completely refolded as same as that in its native state (discussed below in CD spectral data). Nevertheless, the immunoreactivity of each expressed subunit was retained.

Table 1
Purification of recombinant Hp subunits from *E. coli*

Purification step	Yield (mg)				Purity[a](%)			
	α1	α2	β	α2β	α1	α2	β	α2β
Wet cells in 100 ml culture	410	450	420	510	37.3	32.8	38.5	23.3
Washed inclusion bodies	146	118	135	166	42.6	42.4	44.6	30.4
Ni^{2+}-column purified protein	14[b]	11[b]	5[b]	7[b]	95	95	95	95

[a] Determined by densitometry of 15% SDS–PAGE.
[b] Determined using a Lowry method.

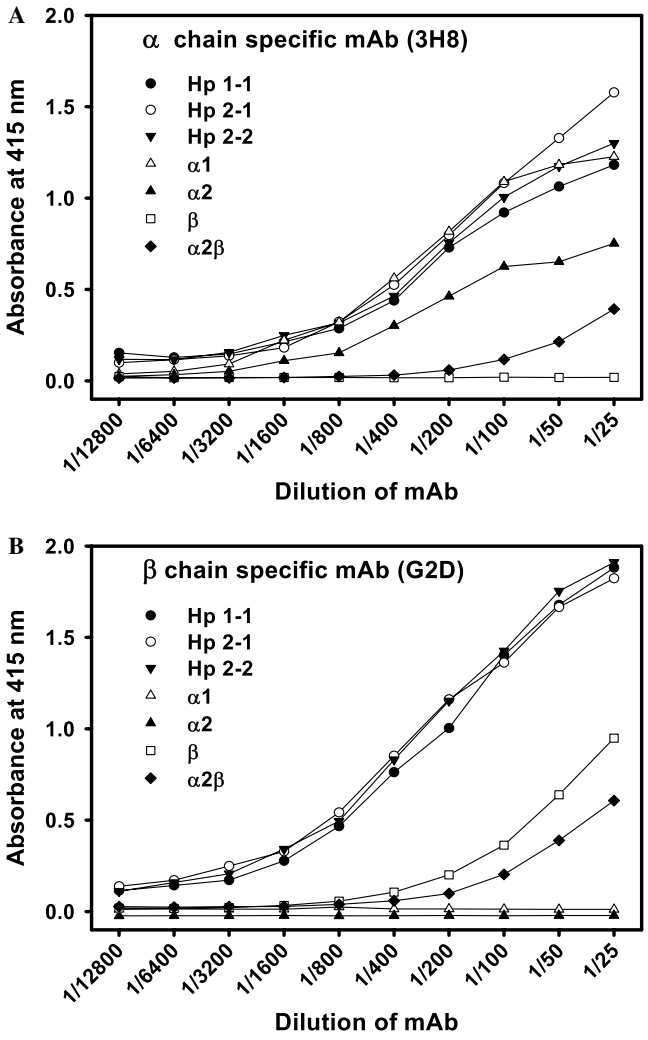

Fig. 2. Immunoreactivity of native Hp 1-1, 2-1, and 2-2 and recombinant Hp measured by ELISA using Hp α and β specific mAb. The expressed α2β possesses full length of Hp, but is not cleaved as α and β subunits. (A) The immunoreactivity of native Hp is moderately higher than that of expressed α2β subunit using α chain mAb. (B) The immunoreactivity of native Hp is greater than β and α2β subunits using β chain mAb. This could be due to the structural difference found in Fig. 4.

Antioxidant domain of rHp subunits

To determine the antioxidant activity of each Hp subunit, a TBARS method using Cu^{2+}-induced LDL oxidation was conducted [23]. Recombinant β subunit was extremely potent to protect LDL from oxidation and was in a dose-dependent manner. A typical example of the inhibition in TBARS is shown in Fig. 3. Ranking of the 50% of inhibitory concentration (IC_{50}) in antioxidant activity was as follow: $\alpha2\beta > \beta > \alpha1 \geqslant \alpha2 \geqslant$ probucol (Table 2). Most importantly, the antioxidant activity of α2β and β was markedly higher than that of α1 and α2 subunits. Thus, the major antioxidant domain of Hp was located in the β sub-

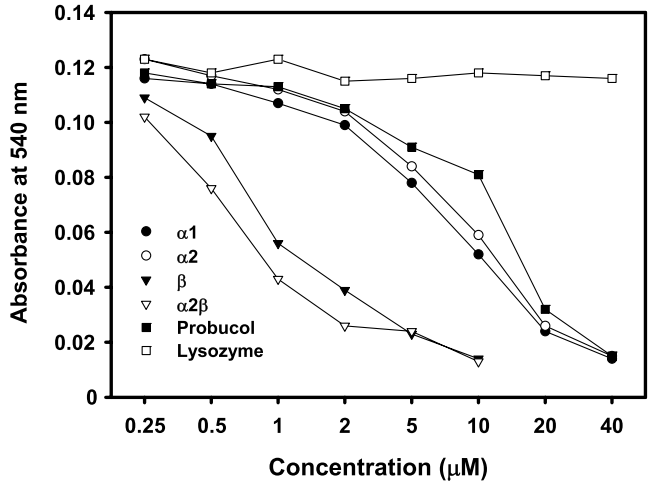

Fig. 3. Antioxidant activity of each recombinant subunit. The assay was evaluated using the degree of inhibition of Cu^{2+}-induced formation of TBARS from LDL. LDL (20 μg) was incubated with 5 μM Cu^{2+} in the presence of recombinant α1, α2, β, α2β or probucol at 37 °C for 2 h in a final 100 μl of PBS. Lysozyme served as a control did not reveal any antioxidant activity.

unit. It is of remarkable interest that the activity of β subunit was greater than that of native Hp molecule (Table 2). One of the possible explanations is that the random structure of the subunit may expose a neo-epitope that renders its availability for further scavenging the free radicals (discussed below). In the next experiment, we chemically modified Hp by carboxymethylation (CM) (in the presence of 5.4 M urea) to dissociate the disulfide linkages between α and β chains. The antioxidant activity of CM-Hp was substantially increased by about 3.75 folds when compared to native Hp (Table 2). The activity of CM-Hp was almost identical to β subunit. Because the carboxymethylation was conducted in the presence of 5.4 M urea, it is worth mentioning that native Hp exposed to the denaturing agent (5.4 M urea or guanidine hydrochloride) did not alter the antioxidant potency after renaturation by desalting (data not shown). The data suggest that the antioxidant domain of native Hp was exposed further, while reducing irreversibly. Another essential feature was that the antioxidant activity of β subunit was superior to probucol (15×): one of the most established potent antioxidants. Since probucol has been used for the treatment of patients with xanthoma and atherosclerosis for decades [29–33] and its analogs have been designed for antioxidant therapy [34,35], the recombinant β subunit plus the success of large expression in *E. coli* may pave the way for the future design of "mini-Hp".

Table 2
Antioxidant potency in Cu^{2+}-induced LDL oxidation

	Type	Cu^{2+}-induced IC_{50}[a](μM)
α1	Recombinant	7.50
α2	Recombinant	8.72
β	Recombinant	0.87
α2β	Recombinant	0.65
Probucol	Compound	13.70
Hp 1-1	Native	3.00
CM-Hp 1-1[b]	Native	0.80

[a] IC_{50}: the concentration that inhibited 50% of LDL oxidation.
[b] CM-Hp 1-1 was obtained by carboxymethylation to break up all the disulfide linkages.

Circular dichroic analysis

To address the moderately decreased immunoreactivity and high antioxidant activity in recombinant subunit were probably due to the conformational difference from the ordered structure of whole Hp molecule, the secondary structure of recombinant Hp subunits was studied by CD spectra. Fig. 4 depicts that all the α1, α2, β and α2β were typically disordered in structure (a sharp drop of ellipticity between 190 and 210 nm) as compared to native Hp. The result suggests that the conformational changes in each subunit may be responsible for the low binding to each chain specific mAb, as each mAb was originally prepared against the native Hp. Whereas, the random structure of β chain may facilitate its availability for scavenging free radicals. Furthermore, the carboxymethylation Hp also exhibited a disordered structure.

In conclusion, we demonstrate that Hp β chain is an extremely potent antioxidant directly preventing LDL against oxidation in the present study. Using a recombinant Hp cDNA without the signal sequence, the yield of each subunit was relatively high containing approximately 30% of total cell-lysate proteins. Each expressed subunit retained the immunoreactivity as confirmed by α and β chain specific mAb (3H8 and G2D). It is conceivable that expressed β subunit may provide as an initial utility for the future design of "mini-Hp" for potent antioxidant. It may also provide a new insight in understanding the mechanism or specific amino acids involved in free-radical scavenge when site-directed mutagenesis is carried out for further studies. The latter experiment is now in progress in our laboratory.

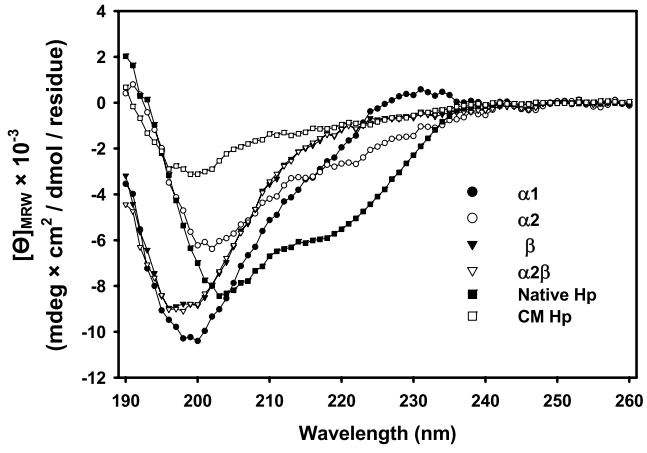

Fig. 4. Circular dichroic spectra of recombinant α1, α2, β and α2β subunits. Recombinant Hp subunit or Hp (final protein concentration 0.2 mg/ml) in 10 mM phosphate buffer, pH 7.4, was monitored by a circular dichroic spectrophotometer. Each spectrum represents a mean of 20 determinations. Essentially, recombinant α1, α2, β and α2β subunits and CM-Hp are typically random or disordered in structure (a sharp drop of ellipticity between 190 and 210 nm).

Acknowledgments

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