國　立　交　通　大　學

生物科技學院

生化工程研究所

碩士論文

單一型態 Haptoglobin 純化與分析

Analysis and isolation of single type haptoglobin

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指導教授：毛仁淡 教授

中 華 民 國 九十六 年 六 月

博碩士論文授權書

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論文名稱：__**Analysis and isolation of single type haptoglobinAbstract**__

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論文名稱：**Analysis and isolation of single type haptoglobinAbstract**

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致謝

兩年的碩士班生涯，轉眼間已經悄然走過。這段時間的酸甜苦辣，真的不是用簡單的幾句話就可以形容的，短短的兩年內，教導我最多東西的當然是我的指導教授毛仁淡博士，不僅在我實驗遇到瓶頸時，給予我關鍵性的方向，對於我待人處世的方法，更是加倍嚴格，兩年下來，除了從毛老師身上學習如何去做一個完整的研究外，也讓我學到了不少處世圓融的技巧，這是我在實驗外所得到最大的收穫，在此，對毛老師致以最大的謝意。

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單一型態 Haptoglobin 純化與分析

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中文摘要

人類 Haptoglobin (Hp)可區分為三種表現形：Hp 1-1, 2-1, 2-2。然而，Hp 2-1 和 2-2 (不包括 Hp 1-1) 本質上具有較複雜的多形性與異質性，使其具有不同的分子大小。人類 Hp 1-1、鹿 Hp 以及豬 Hp 在型態上個別為同質性二聚體 $(\alpha\beta)_2$、四聚體 $(\alpha\beta)_4$ 和二聚體 $(\alpha\beta)_2$。本篇報告中，我們建立了一個簡單純化具有同質性但不同物種 Hp 的純化方式。一開始先將血漿以 50%飽和硫酸銨沉澱，取其上層液通入高效液相層析儀搭配凝膠過濾管柱(gel-filtration column)並以 0.3 ml/min 磷酸生理食鹽水沖提。發現豬與鹿 Hp 的純度可達 90%以上，但是在純化的人類 Hp 1-1 有脂蛋白原 A-1(apolipoprotein A-1；apoA-1)及血清白蛋白(albumin)存在。因此針對去除脂蛋白原 A-1 與血清白蛋白提出了「策略性」的解決方法，以去除脂蛋白的血漿當純化起始物並搭配銅離子結合固定化金屬離子親和層析法{Cu (II)-immobilized metal ion affinity chromatography；Cu (II)-IMAC}搭配純化人類 Hp 1-1，其純度可達 95%以上。以不連續膠體電泳(SDS-PAGE)與西方點墨法 (Western blot)描繪純化後的蛋白質分子量形式。純化後的 Hp 保有原本可和血紅素(Hemoglobin)結合的能力，證實了純化過程中並沒有改變其蛋白質結構。最後在本篇報告中亦討論個別純化步驟中的純度(purity)與產率(yield)。

Student: Kuan-Yu Lin Advisor: Dr. Simon J.T. Mao
National Chiao Tung University Institute of Biochemical engineering

Analysis and isolation of single type haptoglobin

Abstract

Human haptoglobin (Hp) is classified as three phenotypes: Hp 1-1, 2-1, and 2-2, in which 2-1 and 2-2 (except 1-1) are polymeric and heterogeneous in nature with various molecular sizes. Thus far, the polymeric forms mimic to that of human Hp are found in some animals (such as deer). This report provides a simple protocol that can be used to isolate the Hp with homogeneous structure from different species. Plasma was first fractionated using a 50% saturated ammonium sulfate. The supernatant was then chromatographed on a HPLC gel-filtration column equilibrated with PBS (pH 7.4) at a flow rate of 0.3 ml/min. The homogeneity of isolated porcine and cervine Hp was greater than 90%, but the contamination of apolipoprotein A-1 (apoA-1) and albumin existed in that of human Hp 1-1. We proposed a "strategic solution" for the elimination of apoA-1 and albumin by using lipoprotein depleted plasma as a starting material, followed by a Cu (II)-immobilized metal ion affinity chromatography (IMAC). It was found 4 M urea being able to enhance the Hp binding affinity to IMAC for differential elution separated from the albumin. Finally, a purity with 95% homogeneity of human Hp 1-1 was achieved. The recovery of Hp in each isolation step is described and discussed.

Introduction

Hp is known as an acute phase protein. The normal human plasma Hp concentrations are relatively high ranging from 1.0 to 1.5 mg/ml [1-3]. Its level elevates in response to infection and inflammation [4-7]. Clinically, it is a useful marker for some inflammatory related diseases [4-7]. Therefore, use of purified Hp as a standard for the determination of Hp, such as immunoassay, becomes a subject of essence.

Human Hp consists of α and β chains joined by disulfide linkages. Depending on the structure of α chain (α1 or α2), it is classified as three phenotypes: Hp 1-1, 2-1, and 2-2 [8-10]. All the three phenotypes share the same β chains (each with ~40 kDa including 245 amino acids containing about 30% carbohydrate in moiety). Homozygous Hp 1-1 or 2-2 contains two identical α1 (each with ~9 kDa including 83 amino acids) or two α2 (each with ~16.5 kDa including 142 amino acids) [11], respectively, whereas heterozygous Hp 2-1 contains both α1 and α2 (Fig. 1). Owing to an extra thio-group on α2, Hp 2-1 and 2-2 form large polymers of trimeric, tetrameric, pentameric, hexameric, and even larger arrangement through the disulfide linkages or $(\alpha\beta)n$, where $n \geq 3$ (Fig. 1) [12-13].

In non-human mammalians, both dimeric and polymeric forms of Hp exist. The dimeric structure or $(\alpha\beta)_2$ is similar to human Hp 1-1 present in plasma of most

mammalians. Thus far, the polymeric forms mimic to that of human Hp are found only in ruminant families of *Artiodactyla* order [14-17].

There have been several methods for human Hp purification already established. However, the methods currently used for the purification of Hp frequently suffer some drawbacks. For example, Rademacher *et al*. utilized the chicken hemoglobin–Sepharose affinity column to isolate human Hp, while the use of 8 M urea to elute bound Hp often causes the dissociation hemoglobin from the Sepharose. The isolate also consistently contaminated apolipoprotein A-1 (apoA-1) [18]. Travis *et al.* employed Sephadex G-200 gel-filtration, but the purified Hp was accompanied with large amounts of IgM and α-2 macroglobulin [19]. Morimatsu *et al.* provided a modified method using HPLC with anion-exchange, Sephacryl S-300, TSK Phenyl-5PW, and TSK DEAE-5PW columns together; the procedures however were time-consuming and the yield was relatively low [20].

Tseng *et al*. have shown a convenient procedure using a monoclonal antibody-affinity column for human Hp 1-1, 2-1, and 2-2 isolation [21]. This is probably the only appropriate method for purifying Hp in three phenotypes in order to study their structural and functional relationship. One disadvantage is a requirement of preparation of Hp as an antigen prior to set up the monoclonal antibodies. Nevertheless, apoA-1 is still a major impurity using those methods mentioned above.

The purpose of the present study was to establish a “universal” purification method that could be employed for most of the mammalian species possessing a homogeneous Hp such as pig, deer, and human Hp 1-1. However, in many cases containing of apoA-1 and albumin is unavoidable in final Hp preparation using a gel-filtration HPLC procedure [21-22]. Thus, we propose a “strategic solution” for the elimination of apoA-1 by using lipoprotein depleted plasma as a starting material [23-24]. Removal of albumin was achieved using a Cu (II)-immobolized metal ion affinity chromatography (IMAC) equilibrated with 4 M urea [25-26]. In addition, we described a simple and rapid hemoglobin binding assay to identify the homogeneity of a given Hp in the plasma of different species. Their purification procedures and recovery of Hp in each step are also described and discussed.

Materials and methods

2.1. Materials

Human plasma samples free of AIDS and hepatitis were a gift from the Hsinchu Blood Center of Chinese Blood Services Foundation. Cervine blood was obtained from Pingtung County Livestock Disease Control Center. Human hemoglobin, mouse polyclonal antibodies against bovine Hp, and mouse polyclonal antibody against porcine Hp β chain were prepared as previously established [27-28]. Goat anti-mouse IgG was purchased from Chemicon (Temecula, CA). Superose-12 10/300 GL column was obtained from Amersham (Uppsala, Sweden). His.Bind® resin (metal ion chelating beads) was purchased from Novagen (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) without any further purification. The buffers used in this report were all filtered through a 0.45-μm filter before use (Millipore, MA, USA). Unstain and prestain molecular weight markers were purchase from Fermentas (Burlington, Canada) and Invitrogen (Carlsbad, CA). The dialysis kit was purchased form PIERCE (Rockford, Il).

2.2. Analysis of Hp phenotype by native-PAGE

Hp phenotyping was conducted via native polyacrylamide gel electrophrosis (native-PAGE) using hemoglobin-supplemented serum or plasma [21]. Briefly, 6 μl

of plasma and 3 μl of hemoglobin (6 mg/ml) were pre-mixed for 30 min at room temperature and equilibrated with 3 μl of a sample buffer (containing 0.625 M Tris base at pH 6.8, 50% glycerol, and 1% bromophenol blue) followed by a 7% native-PAGE, for that a 5.5% polyacrylamide (26.5: 1 acrylamide: bis-acrylamide) was employed as a top stacking gel (pH 6.8). Electrophoresis was conducted at 20 mA until the dye front reached the separating gel. After electrophoresis, the Hp-hemoglobin complexes were visualized by gently shaking the gel in freshly prepared chromogenic solution {25 mg of 3,3'-diaminobenzidine (DAB) dissolved in 0.5 ml of dimethyl sulfoxide (DMSO), 30 ml of phosphate-buffered saline (PBS), and 50 μl of 30% H_2O_2} [13, 21].

2.3. SDS-PAGE and Western Blot Analysis

For 15% SDS-PAGE, isocratic gel containing 15% polyacrylamide (w/v) was used with a stacking gel of 5% polyacrylamide. Typically, 5 μg or less of the Hp samples were mixed with 5x concentrated loading buffer (12 mM Tris-HCl, pH 6.8, 0.4% SDS, 5% glycerol, 0.02% bromophenol blue) with or without 2.88 mM 2-mercaptoethanol [29]. The electrophoresis was performed at 20 mA for about 1 h, after which time the gel was soaked in a transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS, and 20% methanol at pH 8.3 for 30 s. In addition,

SDS-PAGE in 4% gel was performed according to the method of Weber and Osborn [30] in order to determine the high molecular weight proteins.

Electrotransfer onto a nitrocellulose paper was performed at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). The transferred membrane was immersed in blocking solution (1% Gelatin in PBS) for 1 h. After washing with PBS, the membrane was incubated with the diluted primary antibody in wash buffer (0.1% gelatin and 0.05% Tween20 in PBS) for 1 h, followed by a 3x washes. Subsequently, a 5,000-fold diluted secondary antibody was incubated with the membrane for 45 min. After additional washes, the membrane was developed in the chromogenic solution as described [31].

2.4. Preparation of apoA-1 depleted plasma

Human lipoproteins were depleted from plasma by first adjusting the density to 1.21 g/ml with solid KBr and then ultracentrifuged at 45,000 rpm for 40 h at 4℃ in a fixed angle SW7O Ti rotor (Beckman Optima LE-80 ultracentrifuge) [23-24]. Lipoprotein depleted plasma (LPPP) was them extensively dialyzed against PBS with 3 changes.

2.5. Fractionation of plasma from each species using 50% saturated ammonium

sulfate

Plasma collected in 0.1% (w/v) ethylenediaminetetraacetic acid (EDTA) was centrifugated at 1,200 g for 30 min at 4℃ to remove blood cells. Solid ammonium sulfate was added to the plasma (included LPDP) to give a final saturated concentration of 50%. After a gentle stirring for 1 h at 4℃, the reaction mixture was centrifuged at 4,000 g for 30 min at 4℃. The supernatant was then dialyzed at 4℃ overnight against PBS with 3 changes [28].

2.6. Purification of Hp by Superose-12 on high performance liquid chromatography (HPLC)

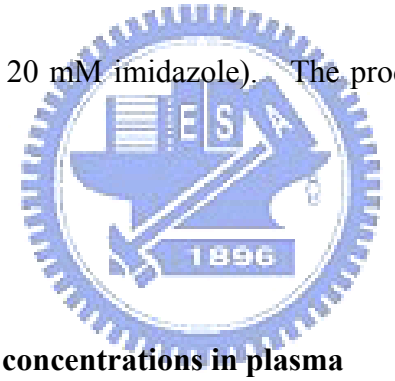
The dialyzed supernatant (described above) was concentrated by a 50-kDa cut-off Amicon Ultra Centrifugal Filter Device (Millipore). The concentrated sample was then chromatographied by Superose-12 column on HPLC according to the method previously described [26]. PBS containing 0.01% NaN_3 was used as the mobile phase with a flow rate of 0.3 ml/min for 60 min at room temperature [21, 28].

2.7. Immobilized metal ion affinity chromatography (IMAC)

Exclusively, human Hp product after Superose-12 chromatography has to be purified by an additional IMAC. Prior to IMAC, the sample was dialyzed in binding

buffer (4 M urea, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8).

The column packed by His.Bind® resin was pre-washed with 5 resin volume of distilled water to remove the storage solution (20% ethanol) and coupled by adding 2 resin volume of 100 mM $CuSO_4$ solution. Subsequently, the column was equilibrated in binding buffer and the samples were conducted. Unbound protein was exhausted by a 10x resin volume of binding buffer. Additional 10x resin volume of washing buffer (binding buffer containing 5 mM imidazole) was then utilized to remove weakly-binding impurity followed by 10x resin volume elution (binding buffer containing 20 mM imidazole). The product was further dialyzed in PBS [32-34].



2.8. Determination of Hp concentrations in plasma

For human Hp determination**,** a GenWay human Hp ELISA quantitation kit (40-288-20080F) was purchased from GenWay Biotech (San Diego, CA), in which one human Hp calibrator, chicken anti-human Hp (capture antibody), and HRP-conjugated chicken anti-human Hp antibody (HRP-antibody) were provided by the manufacturer. Initially, 0.5 μl of the capture antibody diluted in 50 μl of PBS was immobilized onto a 96-well ELISA plate for 1 h at room temperature and followed by an addition of 300 μl blocking buffer (1% of bovine serum albumin in

PBS). After washes, 100 μl of human Hp standard (ranging from 1 to 90 ng/ml) and tested plasma (1:20000 dilution) in washing buffer (0.1% BSA, 0.05% Tween-20 in PBS containing) was then added to each well and incubated for 90 min at 24°C. Following 3 times washes, 100 μl of diluted HRP-antibody was added and incubated for 1 h at 24°C. The plate was then developed by the addition of 150 μl of tetramethylbenzidine (TMB) solution and stopped by adding 100 μl of 1N HCl. The absorbance was read at 450 nm.

For porcine Hp determination, a ICL Pig Haptoglobin ELISA Kit (E-5HPT) was purchased from Immunology Consultants Laboratory (Newberg, OR), in which one porcine Hp calibrator, rabbit anti-pig haptoglobin antibody precoated micro plate, and HRP-conjugated antibody against Hp (HRP-antibody) were provided by the manufacturer. The noncompetitive ELISA was conducted similar to that human ELISA described above.

For determination of cervine Hp, the method was similar human Hp quantitation by using GenWay human Hp ELISA quantitation kit. However, the HRP-antibody was changed to the rabbit anti-bovine Hp antibody conjugated HRP from an ICL Bovine Haptoglobin ELISA Kit (E-10HPT).

Results and Discussion

3.1. Phenotyping of porcine, cervine, and human Hp

Fig. 2 shows the phenotype of porcine, cervine, and human plasma using a hemoglobin-Hp binding assay on a native-PAGE. It demonstrates that porcine or cervine Hp is a homogeneous dimer $(\alpha 1\beta)_2$ or tetramer $(\alpha 2\beta)_4$, respectively. However, human 2-1 and 2-2 (except 1-1) are polymeric and heterogeneous in nature with various molecular sizes [13, 21].

3.2. Purification of porcine and cervine Hp using HPLC gel-filtration method

The purpose of this study was to test whether we were able to isolate plasma Hp from different mammalian species using a simple procedure (HPLC gel-filtration) previously established for that of pigs [28]. However, the same procedure failed to isolate human Hp 2-1 and 2-2 phenotypes (data not shown). Presumably, this was primarily due to the heterogeneity of the polymeric form of human Hp 2-1 and 2-2 that could not be eluted in a single peak. We, therefore, hypothesized that the established simple procedure might be available for those Hp with a homogeneous nature.

Fig. 3A shows that we are able to reproduce the isolation procedure for porcine Hp (pHp) from the plasma using a conventional HPLC gel-filtration method [28]. In

this experiment, plasma was firstly fractionated via a 50% saturated ammonium sulfate. The supernatant containing Hp was confirmed by a Western blot (data not shown). Following a dialysis against PBS, the supernatant fraction concentrated by a 50-kDa Amicon filter was applied onto a Superose-12 column HPLC and then eluted at a flow rate of 0.3 ml/min using PBS containing 0.01% NaN_3 as a mobile phase. Characteristics of each isolated fraction containing Hp is shown in Fig. 4A.

Next, we tested the hypothesis that a homogeneous Hp could be accomplished employing the same procedure described above. As demonstrated in Fig. 2, cervine Hp (cHp) is structurally homogeneous although its molecular form appears to be a tetramer. Interestingly, we were able to isolate it in a single peak (Fig. 3B). cHp was eluted approximately 5 min earlier than that of pHp consistent with the large tetrameric size of cHp. The purity of isolated protein is shown in Fig. 4B, there was somewhat a 5% contamination of hemoglobin (estimated by a scanning image), which was mainly due to trace amount of the hemolysis of erythrocytes during the blood collection. Since the hemoglobin concentration in erythrocytes is as high as about 300 mg/ml with superior binding affinity to Hp ($K_d \approx 1$ pmol/L) [35-36], the initial ammonium-sulfate fractionation was not able to dissociate hemoglobin from the hemoglobin-Hp complex. Hence, caution is need during the blood collection as well as preparation of plasma to avoid trace amount of hemolysis. The containing of

hemoglobin cannot be removed from the isolated Hp even using an antibody-affinity per our experience [13, 21]. Table 1 shows the overall theoretical yield of purified porcine and cervine Hp calculated from the gel-scanning densitometry. Greater than 90% purity of the isolated Hp was achieved. The final analytical recovery was about 30% and 20% for pig and deer Hp, respectively.

3.3. Purification of human Hp 1-1

As demonstrated in Fig. 2, human Hp (hHp) 1-1 is also structurally homogenous as a homodimer or $(\alpha\beta)_2$, we anticipated that it could be isolated using the same procedure described above. Previously we have shown that apoA-1 is a major contaminant while isolating hHp [13, 21]. It has been reported that there is a binding domain of apoA-1 located between Leu^{141} and Ala^{164} interacting with Hp [37]. Thus, the interaction between apoA-1 and Hp appears to be specific. To eliminate the presence of apoA-1, we conducted a conventional ultracentrifugation of plasma at a KBr density 1.21 g/ml to deplete HDL, a major apoA-1 containing lipoprotein [23] before the HPLC procedure. The chromatographic profile of using apoA-1 depleted plasma for Hp 1-1 isolation is shown in Fig. 3C. The elution of hHp peak was delayed about 1.5 min, but not significantly as compared to pHp. Fig. 4C reveals that these hHp fractions are nearly free of apoA-1. Instead, they contained abundant

serum albumin. The homogeneity of isolated Hp was about 60% and the yield was about 18% without further purification (Table 1 C). The strategy for hHp isolation was consequently focused to eliminate albumin and other non-Hp related proteins as described below.

3.4. Further purification of human Hp 1-1 by immobilized metal affinity chromatography (IMAC)

Immobilized metal ion affinity chromatography (IMAC) is now a wildly accepted technique for the purification of proteins [32-34]. Sharma and Agarwal [34] have shown that bovine serum albumin possesses a high binding affinity to Cu (II)-iminodiacetate derivetized agarose (IDA-agrose). We attempted to use this Cu (II)-IMAC method to "absorb" the albumin from our Hp preparation. First, we coupled Cu (II) to IDA-agrose using a standard procedure recommended by manufacturer, followed by extensive washes to remove the excess of Cu (II). After loading Hp preparation (Fig. 3C. peak 1), we used imidazole step gradient (0.5-10 mM) to differentiately elute the bound proteins from the IMAC column. However, Hp, albumin and other proteins were all retained on the column and coeluted with 5 mM imidazole (Fig. 5A). Thus, it suggests these proteins possessing almost the same affinity to Cu (II)-IMAC column. The interaction between proteins and

immobilized metal ions are extremely complex in nature, the protein retained on the gel is due to the combined effects of ionic, hydrophobic, and donor-acceptor interactions [34]. Certain side-chain groups of a given protein such as imidazole, thiol, and indole of histidine, cysteine, and tryptophane, respectively, are involved in the interaction with Cu (II) [34]. Next, we denatured the proteins of Hp preparation by treating with different concentrations of urea in order to differentiate their binding affinity to IMAC, if possible. Remarkable interestingly, sample pre-treated with 4 M urea only facilitated the binding of Hp onto the Cu (II)-IDA-gel. Fig. 5B shows a typical example that proteins other than Hp could be firstly eluted at 5 mM imidazole prior to that Hp at 7.5 mM imidazole. Presumably, 4 M urea enhances the Hp binding affinity by "exposing" more histidine residues of Hp (n = 22). Alternatively, 20 mM imidazole could be employed directly following a pre-elution with 5 mM imidazole (Fig. 5C). Under the latter condition, Hp was completely eluted as no apparent protein seen following an additional 100 mM EDTA "strip off". The final purity of human Hp 1-1 was approximately 95% with the yield 10% (Table 1).

3.5. Molecular characteristics of isolated porcine, cervine, and human Hp

Fig. 6A shows a typical α and β subunit of isolated pig, deer, and human Hp on 15% SDS-PAGE in reducing condition. It was further confirmed by Western blot

using a polyclonal prepared against bovine Hp (Fig. 6B). This polyclonal antibody was found to be able to crossreact with Hp of human, pig, and all the ruminants. Clearly, cervine Hp is comprised of β and α2 subunit, the latter is distinct from other non-ruminant animals possessing only α1. We have recently cloned the cDNA of cervine Hp and proved its putative amino acid sequence mimics that human Hp 2-2 with a tandem repeat (GenBank accession no. EF601928). Both human and cervine α2 contain equal number of cysteines which are responsible for the formation of Hp formation polymers. Further characterization of the molecular form of each isolated Hp species without reducing condition was carried out. Fig. 7A reveals that the molecular weight of isolated porcine, cervine, and human Hp is 120, 220, 120 kDa, respectively. Again, their molecular pattern was further confirmed by a Western blot (Fig. 7B). Interestingly, cervine Hp consisting of α2 chain is tetrameric or $(\alpha\beta)_4$. The mechanism involved in the formation of only tetrameric is not readily known, but it certainly worth while to study its biochemical function with respect to the hemoglobin binding [13,21] and antioxidant activity [12,38] when compared to human Hp 1-1 dimer or $(\alpha\beta)_2$.

3.6. Analysis of hemoglobin-binding ability of isolated porcine, cervine, and human Hp

Fig. 9 reveals that isolated Hp possessed the ability to form a complex with hemoglobin using a 7% native PAGE analysis. Each isolated Hp resulted in a hemoglobin complex identical to that native Hp present in the plasma. The results suggest that the isolation Hp retained the hemoglobin binding nature under our isolation procedures [13,21].

3.7. Summary

In summary, the HPLC gel-filtration used in this study is appropriate for the isolation of those Hp with a homogeneous structure such as $(\alpha\beta)_2$ and $(\alpha\beta)_4$. Contamination of apoA-1 can be eliminated using HDL-depleted plasma. Albumin and some other proteins coeluted by gel-filtration column can be further removed by a Cu (II)-IMAC, while the presence of 4 M urea is essential to enhance or differentiate the binding affinity of Hp from the other contaminants. The purity of each isolates species is greater than 90% and retains its hemoglobin binding ability. Per our experience, once it contaminates hemoglobin during the collection of blood, it is almost impossible to remove using a conventional gel-filtration HPLC. Therefore, hemolysis should be avoided while preparing the plasma.

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Fig. 1. Schematic drawing of molecular arrangement in human Hp phenotypes. Hp 1-1 possesses only the basic dimer $(\alpha 1\beta)_2$ whereas Hp 2-1 is comprised of polymeric structures: starting with a dimer $(\alpha 1\beta)_2$, trimer $(\alpha\beta)_3$, and other linear polymers. Here, α represents both $\alpha 1$ and $\alpha 2$ chains. Hp 2-2 is comprised of a trimer$(\alpha 2\beta)_3$ and other cyclic polymers. Each $\alpha 1$, $\alpha 2$, or β is 83, 142, or 245 amino acids in length, respectively. $\alpha 2$ is similar to $\alpha 1$, differing only by an additional insertion of a repeat identical to 3/4 of $\alpha 1$. Owing to the extra Cys-74 in $\alpha 2$, Hp 2-1 and 2-2 form complicated polymers.

Fig. 2. Identification of the homogeneity of Hp in porcine, cervine, and human plasma using hemoglobin binding assay. Briefly, plasma (6 μl) was pre-incubated with hemoglobin (18 μg) at room temperature for 30 min before conducting the native-PAGE. The gel (7%) was run at 20 mA for 1.5 h and further developed with DAB in PBS containing 0.05% H_2O_2 based on the peroxidase activity of hempglobin.

Fig. 3. Typical chromatographic profile of Hp isolation using HPLC Superose-12 column. Dialyzed supernatant of 50% saturated ammonium-sulfate fraction was applied onto a HPLC system (see Materials and Methods). The chromatography was conducted at a flow-rate of 0.3 ml/min with a pressure of 200 psi and run for 60 min

at room temperature using PBS containing 0.01% NaN_3 as a mobile phase. The filled bar represented the pooled fractions corresponding to isolated Hp of each species.

Fig. 4. Analysis of isolated Hp from HPLC using 15% SDS–PAGE in the presence of reducing reagent. Following HPLC chromatography, each fraction was subjected to SDS-PAGE and run at 20 mA for 1.5 h. Lane M: molecular weight markers. Lane W: Western blot using porcine β chain specific, bovine Hp, and human Hp polyclonal antibodies for (A), (B), and (C), respectively, were used for Western blot.

Fig. 5. SDS-PAGE of human Hp fractions elution from immobilized metal affinity chromatography (IMAC) coupled with Cu (II). Following the binding of human crude Hp preparation (Fig. 3) to IMAC, the bound materials were stepwisely eluted with imidazole at the concentration indicated on the top of each lane. (A) Cu (II)-coupled column equilibrated with binding buffer (300 mM NaCl, 50 mM NaH_2PO_4, pH 8) and eluted with binding buffer containing imidazole from 0.5 to 10 mM. (B) Cu (II)-coupled column equilibrated with binding buffer containing 4 M urea and eluted with the binding buffer containing imidazole from 2.5 to 20 mM. (C) Cu (II)-coupled column equilibrated with binding buffer containing 4 M ureaand

eluted with 4 M urea containing 5 mM imidazole followed by a d 20 mM imidazole. Finally, the column was "striped off" by EDTA to confirm that no protein was retained in column. Lane M: molecular weight markers.

Fig. 6. Analysis of purified human, cervine, and porcine Hp by SDS-PAGE and Western-Blot in reducing condition. The purified human (1-1), cervine, and porcine Hp was analyzed by a 15% SDS-PAGE (A) and Western blot (B). Lane M: molecular weight markers (in kDa)

Fig. 7. Characterization of molecular form of human, corvine, and porcine Hp by 4% SDS-PAGE in non-reducing condition. The purified human (1-1), cervine, and porcine Hp was analyzed by a 4% SDS-PAGE (A) and Western blot (B)

Lane M: molecular weight markers (in kDa). The molecular weight of human (1-1), cervine, and porcine Hp were 120, 220, and 120 kDa, respectively, as estimated by an image analysis system Quantity One (Bio-Rad).

Fig. 8. Hemoglobin binding ability on Hp. Human, cervine, and porcine plasma were incubated with hemoglobin at room temperature for 30 min before conducting a 7% native-PAGE with a procedure similar to that described in Fig. 2.

Fig. 1

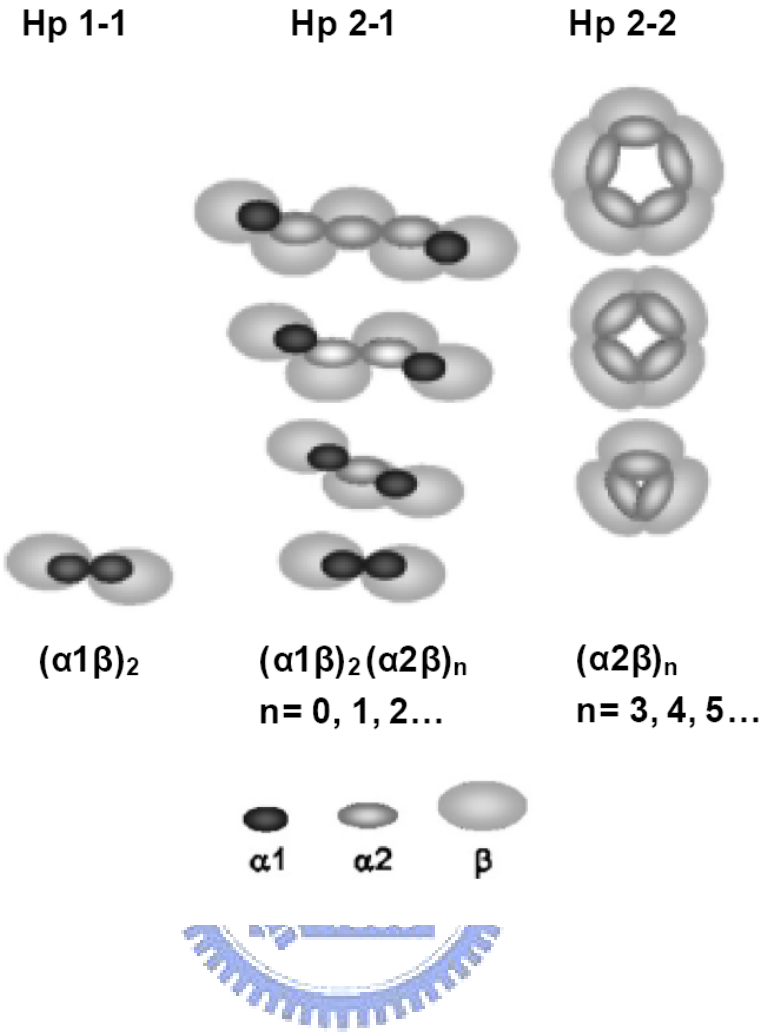


Fig. 2

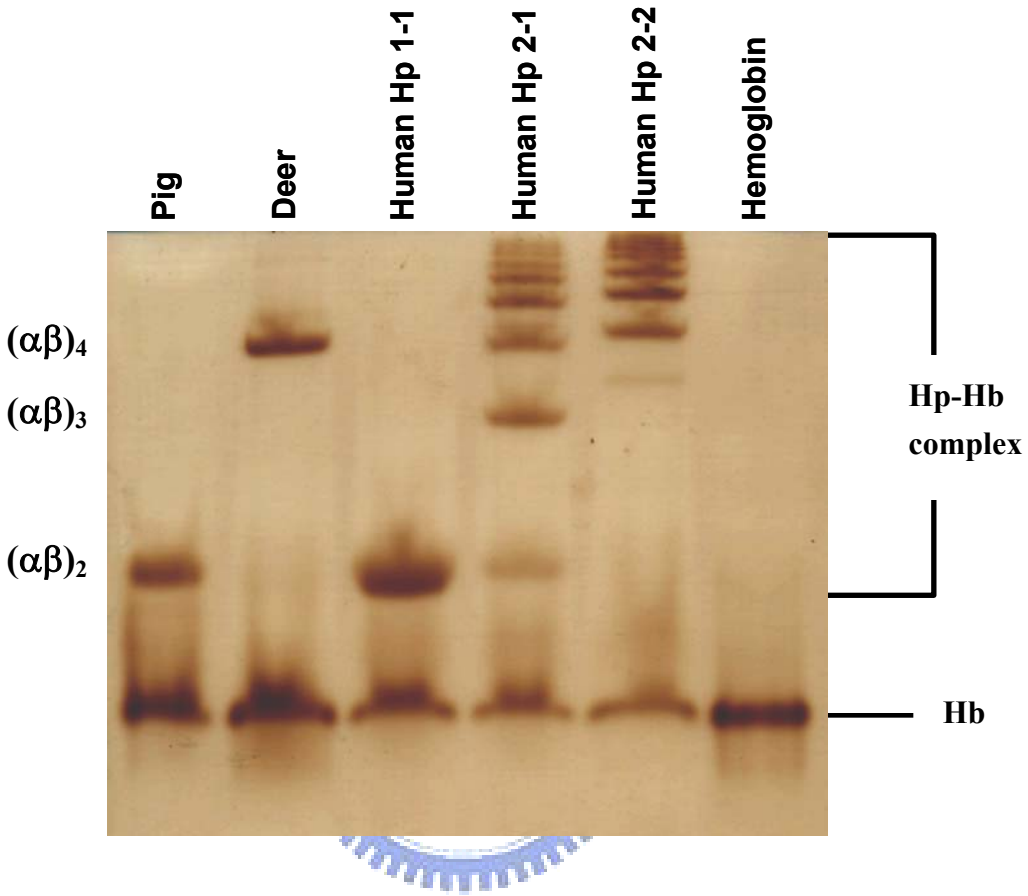

Pig
Deer
Human Hp 1-1
Human Hp 2-1
Human Hp 2-2
Hemoglobin
(αβ)4
(αβ)3
(αβ)2
Hp-Hb
complex
Hb

Fig. 3

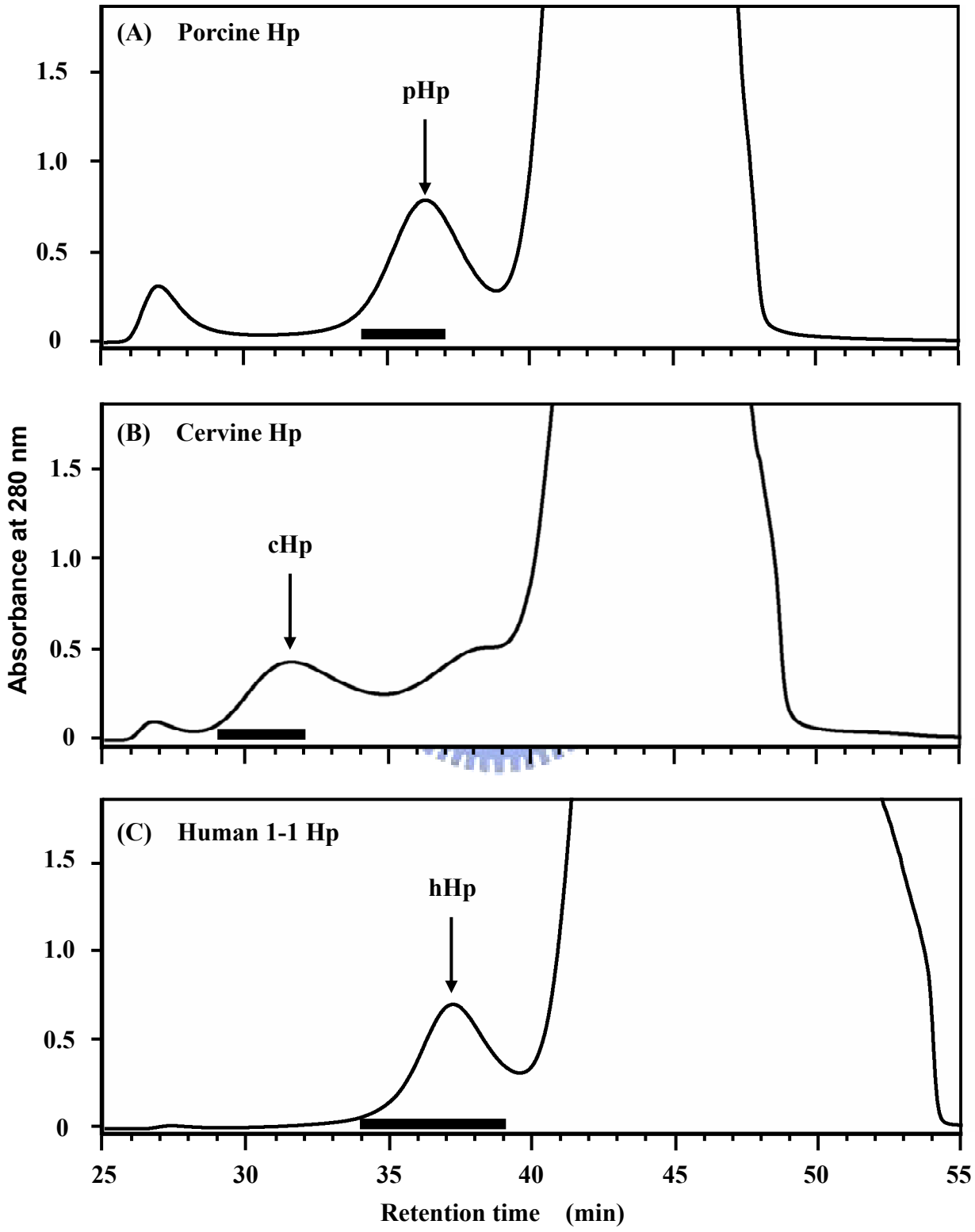

(A) Porcine Hp
pHp
(B) Cervine Hp
cHp
(C) Human 1-1 Hp
hHp
Absorbance at 280 nm
1.5
1.0
0.5
0
25
30
35
40
45
50
55
Retention time (min)

Fig. 4

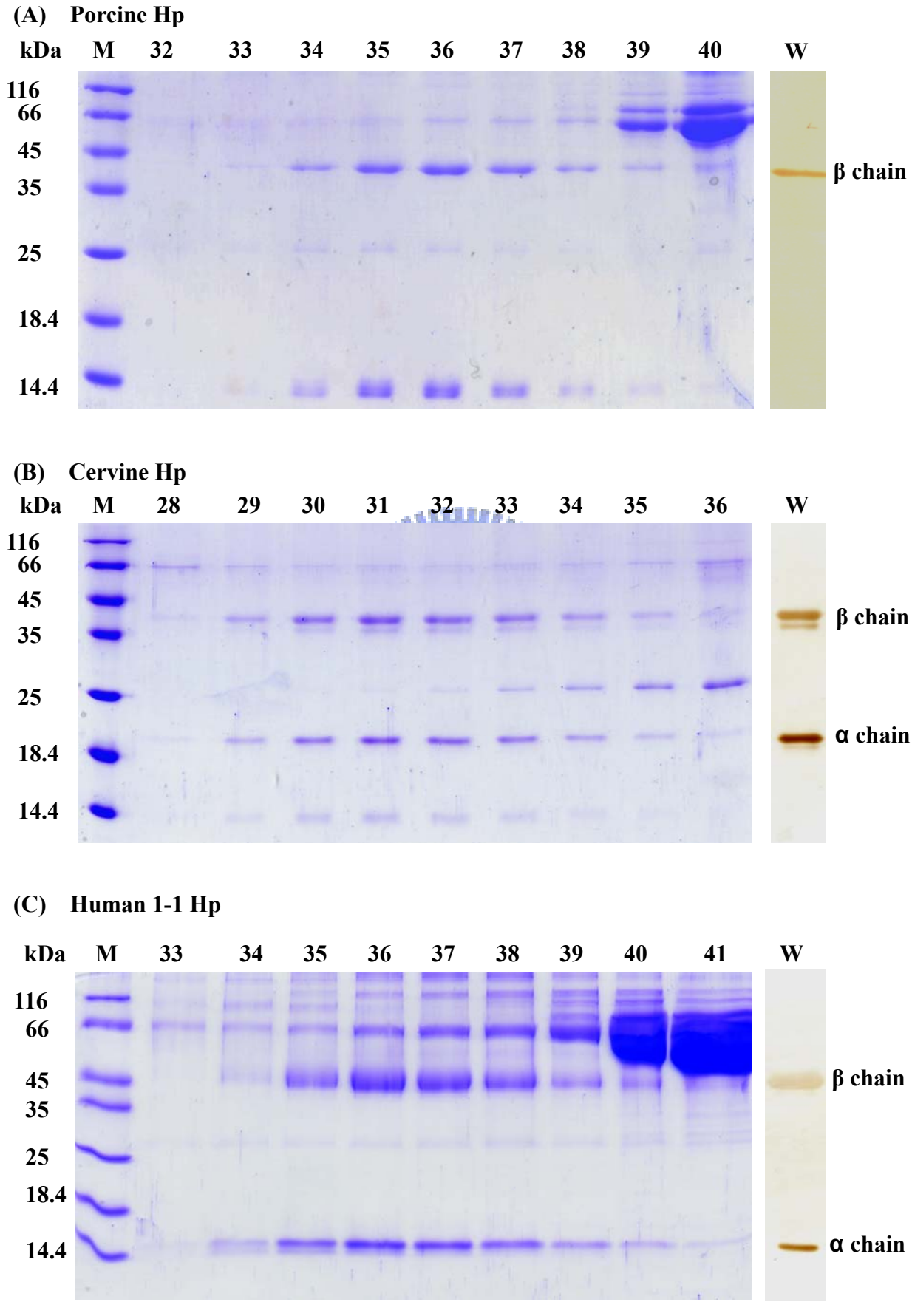
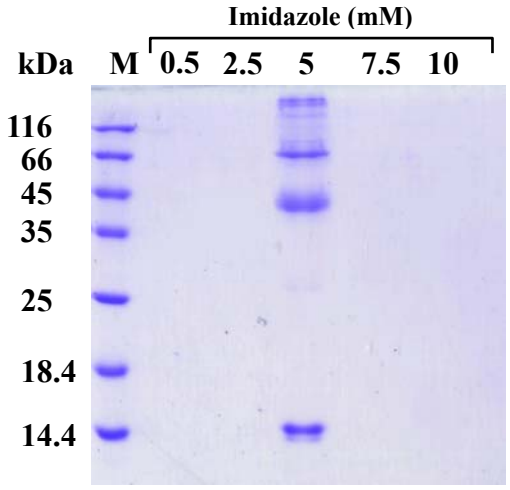
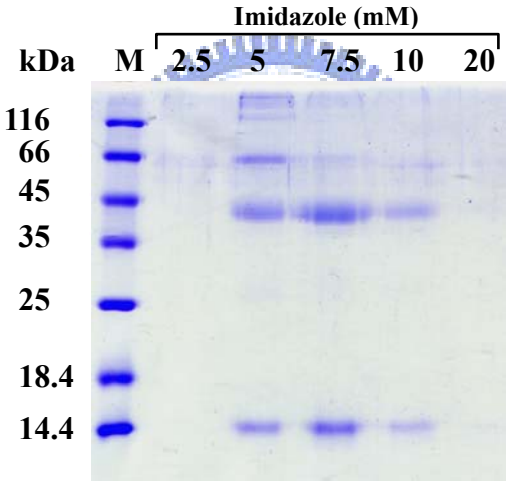

(A) Porcine Hp
kDa
M
32
33
34
35
36
37
38
39
40
W
116
66
45
35
25
18.4
14.4
β chain
(B) Cervine Hp
kDa
M
28
29
30
31
32
33
34
35
36
W
116
66
45
35
25
18.4
14.4
β chain
α chain
(C) Human 1-1 Hp
kDa
M
33
34
35
36
37
38
39
40
41
W
116
66
45
35
25
18.4
14.4
β chain
α chain

Fig. 5

(A) Human Hp without urea



(B) Human Hp with 4M urea



(C) Human Hp with 4M urea

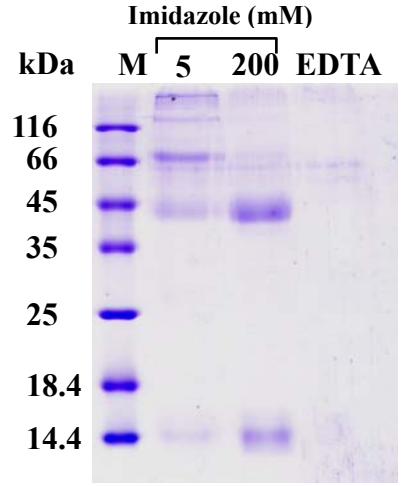
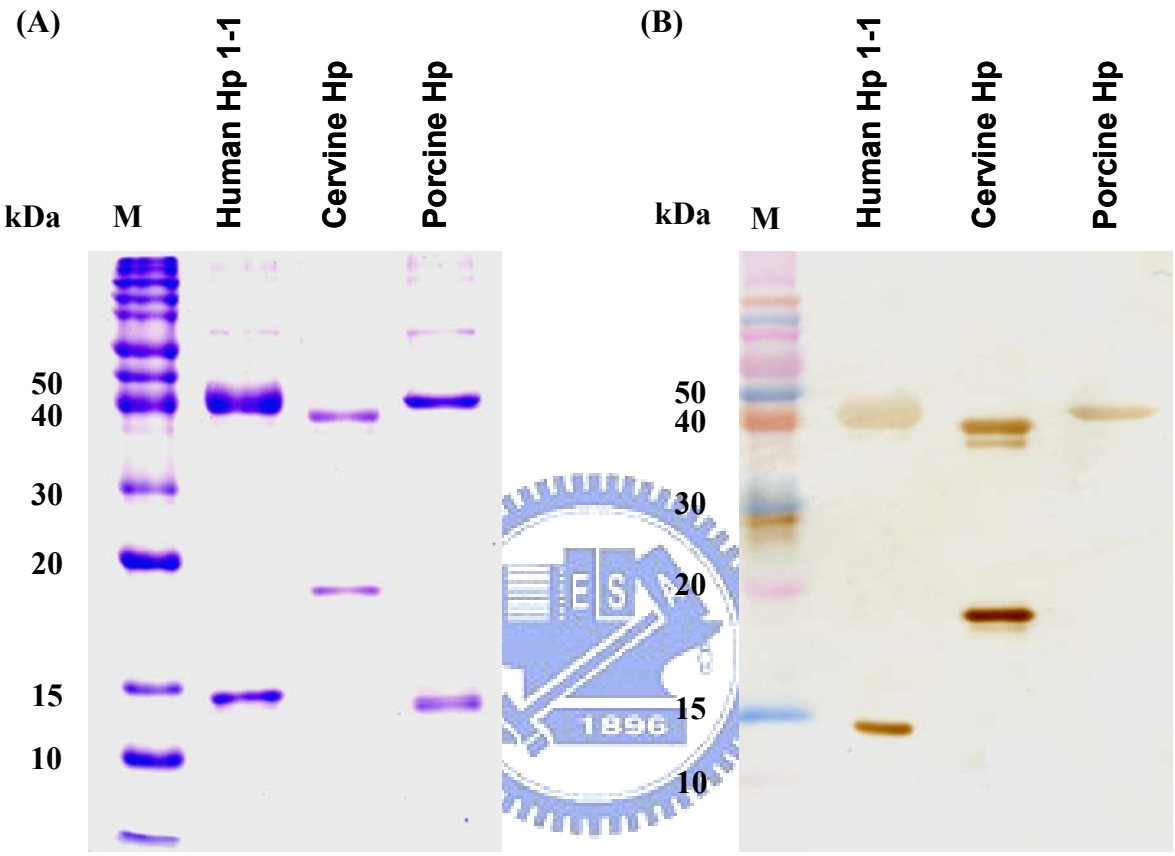


Fig. 6



15% SDS-PAGE

Western-blot

Fig. 7

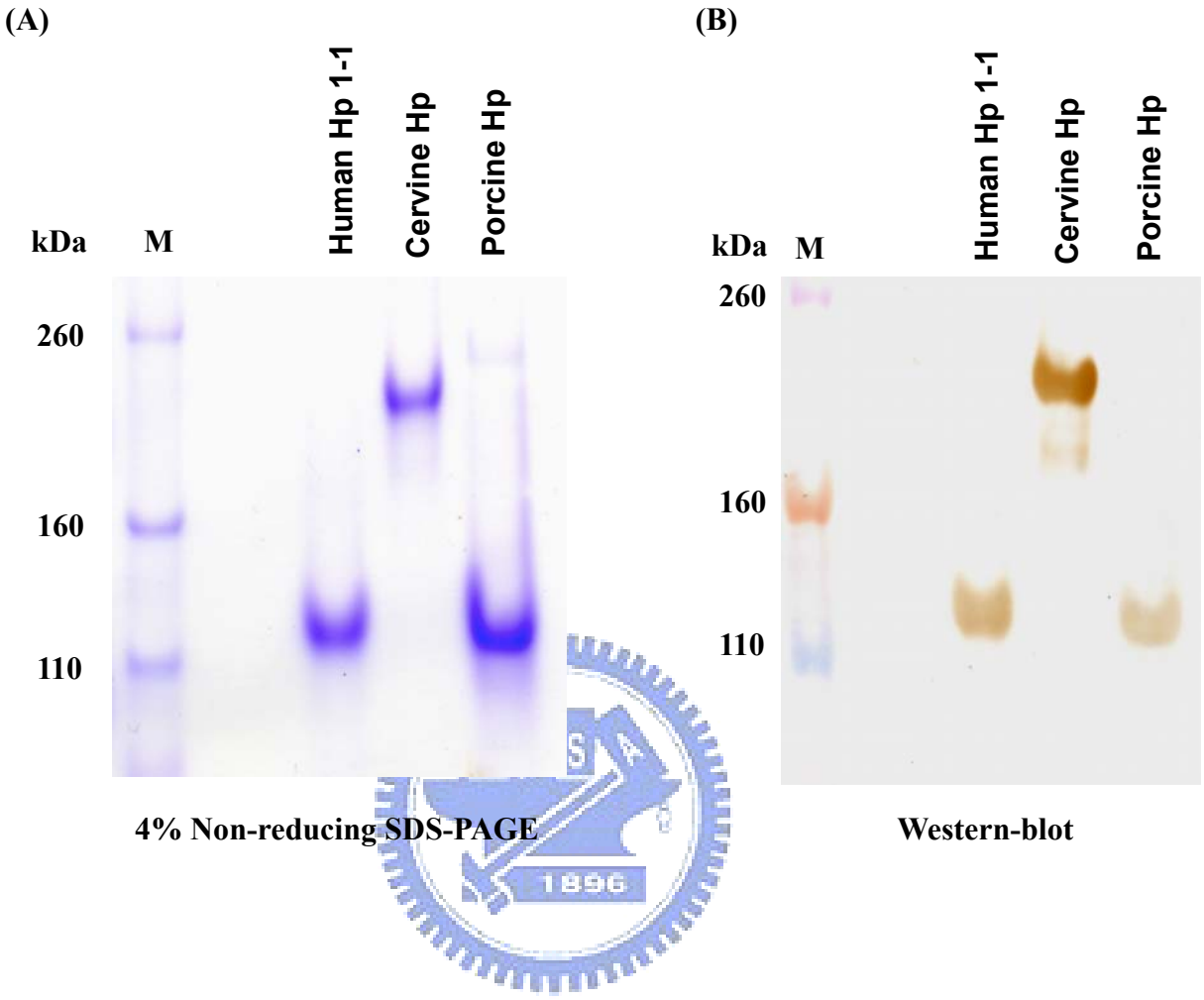

(A)
kDa
M
Human Hp 1-1
Cervine Hp
Porcine Hp
260
160
110
4% Non-reducing SDS-PAGE
(B)
kDa
M
Human Hp 1-1
Cervine Hp
Porcine Hp
260
160
110
Western-blot

Fig. 8

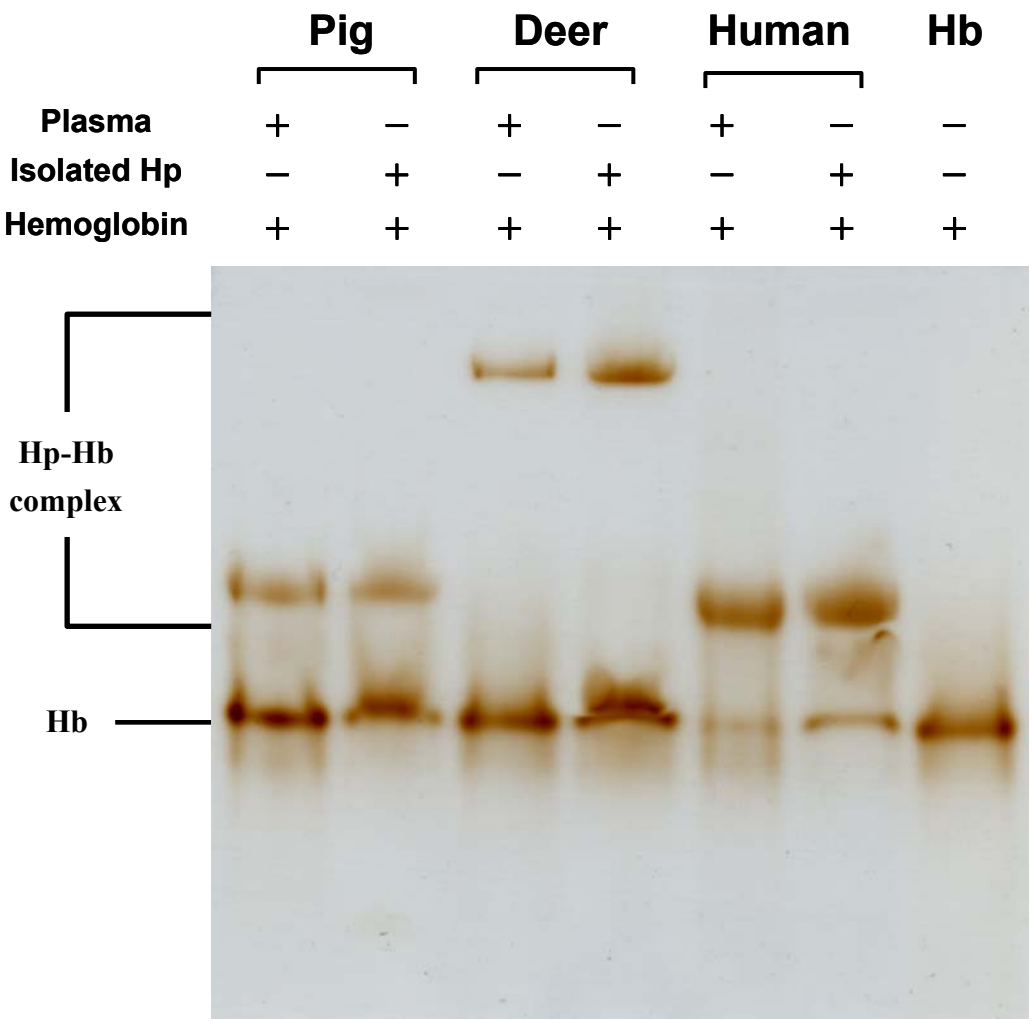

Pig
Deer
Human
Hb
Plasma + − + − + − −
Isolated Hp − + − + − + −
Hemoglobin + + + + + + +
Hp-Hb complex
Hb

Table 1. Analytical recovery of haptoblobin from each step

Steps	Total protein (mg)	Moiety of Hp (mg)	Hp purity (%)	Fold purification	Recovery (%)	Yield (mg)
(A) Porcine Hp						
Plasma	62.64	0.40[a]	0.64	1	100	---
$(NH_4)_2SO_4$ fractionation	16.98	0.26[a]	1.53	2.4	65	---
Superpose-12	0.144	0.129[d]	90.1	141	32.3	0.129
(B) Cervine Hp						
Plasma	62.6	0.44[b]	0.70	1	100	---
$(NH_4)_2SO_4$ fractionation	16.82	0.2[b]	1.18	1.7	45	---
Superpose-12	0.092	0.084[d]	91.4	131	19.1	0.084
(C) Human Hp 1-1						
Plasma	3398.5	34.4[c]	1.01	1	100	---
LPDP[e]	1163.5	27.3[c]	2.4	2.4	79.3	---
$(NH_4)_2SO_4$ fractionation	570.6	10.8[c]	1.9	1.9	31.3	---
Superpose-12	10.2	6.3[d]	62	61	18.1	---
IMAC	3.8	3.6[d]	96	95	10.5	3.6

[a] The moiety of porcine Hp was determined by an ELISA (Immunology Consultants Laboratory, USA).

[b] The moiety of cervine Hp was determined by a modified ELISA (GenWay, USA).

[c] The moiety of human Hp was determined by an ELISA (GenWay, USA).

[d] Determined by densitometer using Quantity One software (Bio-Rad).

[e] Lipoprotein-depleted plasma (LPDP) was used to remove HDL containing apoA-1.

IMAC: Immobilized metal affinity chromatography