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# Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography

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**Abstract**

Haptoglobin (Hp) is an acute-phase protein; its plasma levels increase consistently in response to infection and inflammation. The concentration of human plasma Hp is ranged between 1 and 1.5 mg/ml. Similar to blood type, individual human Hp is classified as Hp 1-1, 2-1, or 2-2. The structural and functional analysis of the Hp, however, has not been studied in detail due to its difficult isolation procedure. Previously, we reported a single step for the purification of porcine Hp. In this study, we established a purification method using a high capacity hemoglobin-affinity column. Briefly, DEAE-purified human hemoglobin was first coupled to Sepharose 4B to prepare an affinity column in a 15-ml bed volume. Following a flow through of human plasma and an extensive wash, the bound material was eluted with a solution of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), to remove low-affinity bound proteins. The high-affinity bound Hp was then eluted with 0.15 *M* NaCl containing 5 *M* urea, pH 11, and collected in tubes containing 100 μl of 1 *M* Tris buffer, pH 7.0. The biological activity of dialyzed Hp was retained as it formed a complex with hemoglobin on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Using this procedure, approximately 10 mg of Hp 1-1, with homogeneity greater than 96%, was obtained from 15 ml of human plasma. Affinity purified Hp 2-1 or 2-2, however, contained trace amounts of apoA-I with the similar approach. The Hp could be further purified by HPLC using a Superose 12 gel-permeation chromatography, if desired, to achieve 100% purity. All the phenotypes of purified Hp consisted of α and β chains on SDS–PAGE in the presence of a reducing reagent, further confirmed by a Western blot analysis. We conclude that human hemoglobin-affinity column was most suitable for the isolation of Hp 1-1 in large quantities. Whereas, one additional step using a gel-permeation was necessary for that of Hp 2-1 and 2-2.


*Keywords:* Affinity adsorbents; Haptoglobin; Proteins; Glycoproteins

## 1. Introduction

Haptoglobin (Hp), also known as an α-2 glycoprotein, is a hemoglobin-binding protein present in plasma of all vertebrates and is believed to participate in hemoglobin transport. The concentration of Hp in human plasma is relatively high ranging from 1.0 to 1.5 mg/ml [1,2], which may increase as an acute-phase protein in response to a variety of injuries and inflammatory disease states [3,4]. For this reason, Hp is useful as a diagnostic marker and as a clinical evaluation of many inflammatory diseases. Human Hp is a tetrameric structure linked by disulfide linkages among the two α and two β chains

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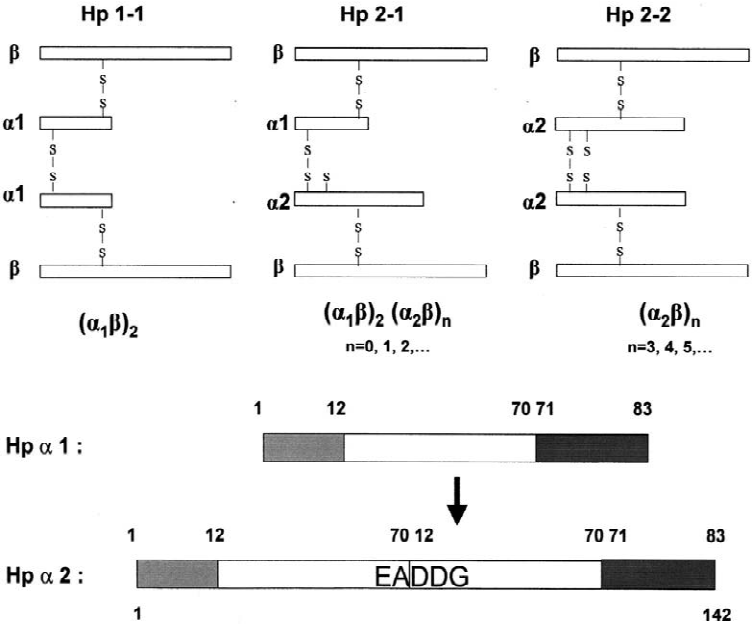


Fig. 1. Schematic drawing of the phenotype structure of human Hp 1-1, 2-1, and 2-2 (top). All three phenotypes share a common structure of β chains. The degree of polymerization within the inter-molecular arrangement is shown. The difference between α1 and α2 chains and their combinations determine the Hp phenotype (bottom). α2 is a duplicate of α1 with a repeat insert of residues 12–70. Making it simple, α2 (142 amino acids) contains two repeated domains showing a unique sequence of EADDG (residues 69–73) at the splicing site.

[5–7]. Based on the length of α chains, there are three phenotypes of Hp in the population, Hp 1-1, 2-1, and 2-2 (Fig. 1), which are similar to that of blood types. All the phenotypes share the same two β chains [each with about molecular mass ($M_r$) 40 000 including 243 amino acids and approximate 30% (w/w) carbohydrate moiety] [5–7]. A typical structure of homozygous Hp 1-1 is composed of two identical α 1 chains (each with about $M_r$ 9000 including 83 amino acids). Whereas, Hp 2-2 is composed of two identical α 2 chains (142 amino acids) as compared to that of heterozygous Hp 2-1 containing each α 2 and α 1 (Fig. 1). Likewise, the tetrameric arrangement is also found in other animal species such as rat, rabbit, and pig [3,8–10]. However, the two αβ units joined by a non-covalent interaction, rather than a disulfide bridge, are found in dog, cat, and bear [11,12].

Several functional differences between Hp phenotypes have been demonstrated, appearing to have important biological and clinical consequences [1,2,13,14]. For example, patients with phenotype Hp 1-1 are less prone to the development of inflammatory-related cardiovascular diseases and diabetics than that with Hp 2-1 and 2-2 [13–15]. Although the protein has been well characterized genetically, the exact physiological role and the biochemical mechanism by which Hp 1-1 is more resistance to those inflammatory-related diseases are not well understood. The later has been hampered by the availability of Hp, which is mainly due to the considerably difficult procedures for Hp purification. Commercially prepared Hp is not only expensive lacking the biological activity, but also heterogeneous containing the mixture of three phenotypes isolated from the plasma pools.

Currently, the most common procedures involved for the purification of human Hp are associated with electrophoresis, affinity chromatography using a monoclonal antibody, and multiple high-performance liquid chromatography (HPLC) steps [16–20]. These methods are useful but are troublesome and time-consuming, and the quantity of Hp obtained is relatively small. Affinity column purification using chicken hemoglobin has been reported [19,21]. The binding affinity of chicken hemoglobin to human Hp,

however, is less than that of human hemoglobin [1,2]. The method [21] was satisfactory for Hp purification in species other than humans, but required a few column-steps for human Hp [19]. Previously, we established a simple purification method with high yield for porcine plasma Hp. The method, however, was not practical in the isolation of human samples due to the heterogeneity of human Hp [22]. In the present report, we describe a purification procedure for human Hp 1-1, 2-1, and 2-2 using an affinity column that was immobilized with highly purified human hemoglobin. Approximately 8–10 mg of human Hp can be obtained from 15 ml of plasma. The procedure can be easily scaled up for Hp 1-1 purification. A simple hemoglobin isolation procedure using an isocratic DEAE HPLC system is also described.

## 2. Experimental

### 2.1. Materials

Goat anti-human haptoglobin was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) without any further purification.

### 2.2. Preparation and purification of human hemoglobin

Fresh human blood collected in 0.1% EDTA was immediately centrifuged at 3000 *g* for 25 min, after which time plasma was removed by aspiration. The remaining red blood cells (RBCs) were washed five times with three volumes of phosphate-buffered saline (PBS) containing 0.12 *M* NaCl and 12 m*M* phosphate, pH 7.2, and then lysed with two volumes of deionized water at 4 °C. Cell debris was removed by centrifugation at 3500 *g* for 30 min. The supernatant containing mostly hemoglobin was fractionated by 50% saturated ammonium sulfate at 4 °C for 30 min followed by a centrifugation at 4500 *g* for 40 min at 4 °C. The supernatant was dialyzed against 0.02 *M* sodium phosphate, pH 8.0, at 4 °C overnight followed by a filtration through a 0.45 μm nylon fiber prior to HPLC.

The HPLC system (Waters) consisted of two pumps, an automatic sample injector, a photodiode array detector, and an interface module [23,24]. A Bio-Scale DEAE column (10×1.5 cm) packed with an anion-exchange Macro-Prep and equilibrated with 20 m*M* phosphate buffer, pH 8.0, at a flow-rate of 1 ml/min, was used for hemoglobin purification. Partially purified ammonium sulfate fraction of hemoglobin (total 50 mg in 2 ml) was applied to the column followed by an elution with the initial buffer at a flow-rate of 1 ml/min. The chromatographic profile was monitored by a photodiode array detector and read at 280 nm.

### 2.3. Preparation of human hemoglobin-affinity column

DEAE-purified human hemoglobin was first coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's procedures. Briefly, 5 g of freeze–dried Sepharose was swollen and suspended in 1 m*M* HCl and immediately washed 3× within 15 min with the same solution on a glass filter [23,25]. The gel was then washed with a coupling buffer containing 0.1 *M* $NaHCO_3$, and 0.5 *M* NaCl, pH 8.0, and subsequently degassed. A 2-ml volume of hemoglobin (25 mg/ml), pre-dialyzed in the coupling buffer, was slowly added to the gel (in 25 ml), while gently stirring for 1 h at room temperature. After coupling, the gel was washed 3× with the coupling buffer (200 ml) to remove uncoupled hemoglobin via a glass filter. Finally, the gel was treated with 0.1 *M* Tris–HCl, pH 8.0, for 2 h at room temperature to saturate the remaining reactive sites of Sepharose. The coupling efficiency of hemoglobin to gel was approximately of 98%. The degassed gel was then packed onto a 20×1.5 cm column and extensively washed with two cycles of PBS, pH 7.2, and 0.15 *M* NaCl, pH 11, which was adjusted by ammonium as previously described [25].

### 2.4. Isolation of Hp by human hemoglobin-affinity column

Initially, 15 ml of human plasma was loaded onto the hemoglobin-affinity column (15 ml in bed volume) at room temperature without incubation, fol-

lowed by an extensive wash with 200 ml of PBS. The bound materials were first eluted with three volumes of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), as fraction 1 [25] and then eluted with three volumes of 5 *M* urea in 0.15 *M* NaCl, pH 11 (freshly prepared and filtered), as fraction 2. A 5-ml volume of each fraction was collected in a tube containing 0.1 ml of 1 *M* Tris–HCl, pH 7.0, to immediately neutralize the pH value. Pooled fractions containing Hp were then dialyzed at 4 °C overnight with three changes of PBS.

### *2.5. Gel electrophoresis and densitometry*

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and native PAGE were performed according to the Laemmli's method [26] with some modification as previously described [22]. Samples (typically 10 μg) for SDS–PAGE were preheated at 100 °C for 10–15 min in an SDS loading buffer [50 m*M* Tris–HCl, 2% (w/v) SDS, 100 m*M* 2-mercaptoethanol, pH 6.8]. For molecular mass calibration, a subset of the following standards was included in each gel: β-galactosidase (116 000), phosphorylase B (97 000), bovine serum albumin (BSA, 66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), lysozyme (14 400), and aprotinin (6500). The samples were run for 0.5 to 1 h at 120 V and stained by a Coomassie brilliant blue G-250. Densitomertic analysis of SDS–PAGE gel was performed using a Molecular Dynamics densitometer for data acquisition and Image Quant software for integration and analysis.

### *2.6. Immunoblot analysis*

Following the separation of proteins by SDS–PAGE, the gel was soaked in a transfer buffer containing 50 m*M* Tris–HCl, 50 m*M* boric acid, and 1 m*M* EDTA, pH 8.2, for 30 min. The gel was then transferred to a nitrocellulose membrane (Pharmacia) at 100 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a transfer buffer. The membrane was immersed in 1% BSA, Tween-containing Tris-buffered saline (TTBS) [20 m*M* Tris–HCl, 50 m*M* NaCl, 0.05% (w/v) Tween 20, pH 7.4] for 1 h with gentle shaking at room temperature. Following a wash with TTBS for 3 min, the membrane was incubated with a primary antibody [1:2500 dilution in TTBS containing 1% (w/v) BSA] for 1 h at room temperature and washed three times with TTBS. The membrane was then incubated with 1:5000 diluted antiserum against goat immunoglobulin G (IgG) conjugated with horseradish peroxidase for 1 h in TTBS containing 1% (w/v) BSA. Finally, the membrane was washed three times with TTBS and developed into a color immunoblot with 3,3′-diaminobenzidine (DAB)-stabilized substrate for horseradish peroxidase [22].

## 3. Results

### *3.1. Purification of human hemoglobin*

A typical HPLC profile for the purification of ammonium sulfate fractionated hemoglobin is shown in Fig. 2. Both SDS–PAGE and native-PAGE analyses show that the homogeneity of purified hemoglobin was greater than 96% (Fig. 3).

### *3.2. Preparation of hemoglobin-affinity column*

In theory, the purity of human hemoglobin obtained from ammonium sulfate fractionation was

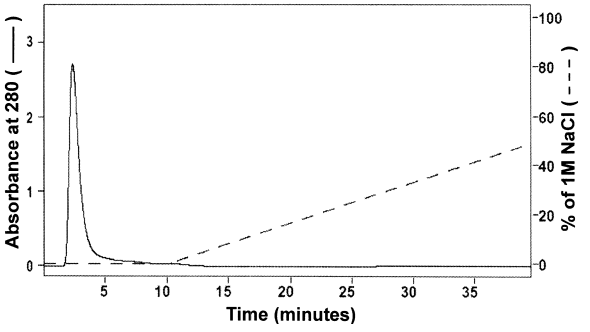


Fig. 2. Typical purification profile of human hemoglobin on DEAE HPLC. About 5 mg of 50% saturated ammonium sulfate top fraction was applied to a DEAE column (10×1.5 cm) pre-equilibrated with 20 m*M* phosphate, pH 8.0. A mobile phase containing the same buffer was run through for 10 min at a flow-rate of 1 ml/min. A linear gradient was produced from 0 to 0.3 *M* of NaCl to regenerate the DEAE resin. The same procedure was used for the purification of hemoglobin in large scale (total of 50 mg in 2 ml) as described in the Experimental section.

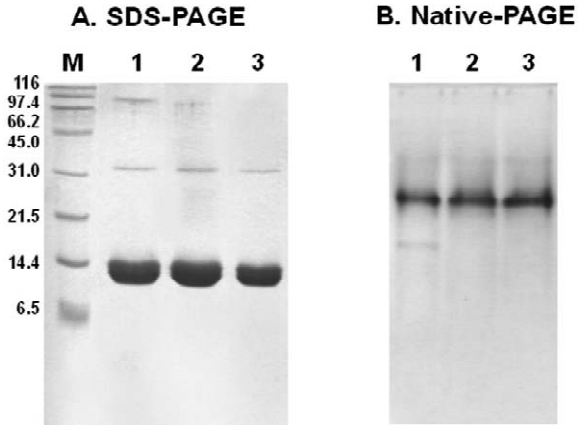


Fig. 3. Analyses of purified human hemoglobin using SDS–PAGE (A) and native-PAGE (B). (A) Lane M represents the molecular mass markers (molecular masses $\times 10^{-7}$). Lanes 1–3 represent hemoglobin obtained from the RBC lysate, top fraction of 50% saturated ammonium sulfate precipitation, and DEAE HPLC, respectively. Approximately 15 μg of each protein was loaded on an 18% SDS–PAGE in the presence of a reducing reagent. (B) Lanes 1–3 represent hemoglobin obtained from RBC lysate, top fraction of 50% saturated ammonium sulfate precipitation, and DEAE HPLC, respectively. Each protein was loaded on a 10% native-PAGE.

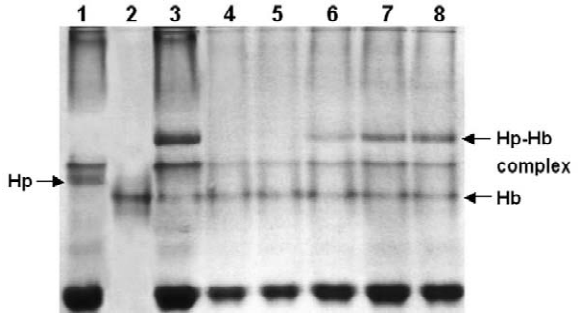


Fig. 4. Evaluation of binding capacity of hemoglobin-conjugated Sepharose to Hp in plasma. Briefly, plasma containing Hp was passed through 1 ml of conjugated Sepharose column. The pass-through fraction was collected and mixed with free hemoglobin. The unbound or remaining Hp, if any, was able to form a Hp–hemoglobin complex displaying an extra band in a 7% native-PAGE. Lanes: 1=human plasma prior to the affinity column; 2=purified hemoglobin; 3=plasma spiked with purified hemoglobin showing a Hp–hemoglobin complex; 4–8=samples of 0.25, 0.5, 0.75, 1.0, and 2.0 ml plasma passed through a hemoglobin-Sepharose containing 1 mg of hemoglobin, respectively. No Hp was detected in lanes 4 and 5 when 0.25–0.5 ml of plasma was applied onto the conjugated Sepharose. According to our calculation, 1 mg of hemoglobin coupled on Sepharose could bind about 0.75 to 1.13 mg Hp (lanes 5 and 6).

adequate in preparing affinity column for Hp purification as that described using chicken hemoglobin [19]. However in a preliminary application, we found that column immobilized with ammonium sulfate fraction of hemoglobin could produce significant plasma clots and subsequently demolished the chromatography (data not shown). This clotting effect, however, was not observed when DEAE-purified hemoglobin was employed for affinity column. Using native-PAGE to evaluate the binding capacity of Sepharose 4B immobilized with human hemoglobin, the capacity we estimated was approximately between 0.75 and 1.13 mg of Hp 1-1 per mg of hemoglobin (Fig. 4). This binding capacity was 20-times greater than that reported using chicken hemoglobin [19].

### *3.3. Isolation of human Hp by hemoglobin-affinity column chromatography*

Fig. 5 shows a typical chromatography using an affinity column conjugated with highly purified human hemoglobin. Initially, 15 ml of plasma of Hp 1-1 was applied to the column followed by an extensive wash (Fig. 5); the bound protein was first eluted with 0.15 *M* NaCl, pH 11 (fraction 1) to remove the low-affinity binding proteins such and apoA-I. The column was then eluted with 0.15 *M* NaCl containing 5 *M* urea, pH 11 (fraction 2) for high-affinity binding Hp. Each eluent was immediately neutralized in the tube containing 100 μl of 1 *M* Tris–HCl, pH 7.0 (Fig. 5). SDS–PAGE analysis on fraction 1 revealed that it contained mostly high-molecular-mass proteins and apoA-I (Figs. 6 and 7), but not in fraction 2. The purity of Hp 1-1 in fraction 2 was approximately 96%. The recovery of Hp in fraction 2 was approximately 45.5% from the plasma with a final of 77-fold purification (Table 1). Under the same condition, however, some apoA-I was found to be co-eluted in the fraction 2 of Hp 2-1 and 2-2 (Figs. 6 and 7). The contaminated apoA-I could be further removed (data not shown) using a single step on HPLC Superose 12 as previously described by us [22]. A typical Western blot analysis showing three isolated phenotypes of Hp is depicted in Fig. 7. The presence of apoA-I in Hp 2-1 and 2-2 was

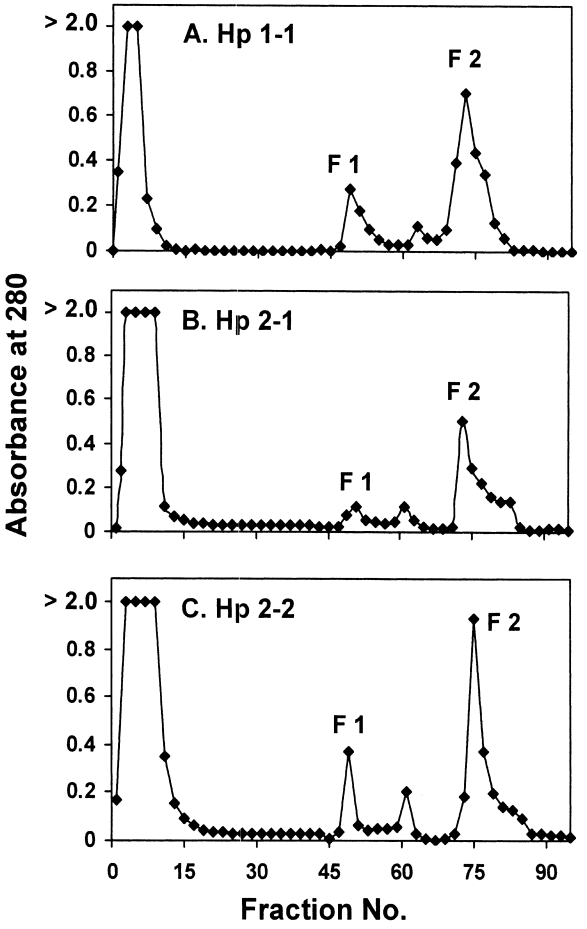


Fig. 5. Typical elution profile of hemoglobin-affinity column chromatography of plasma containing Hp 1-1 (A), Hp 2-1 (B), and Hp 2-2 (C). Initially, 15 ml of human plasma was applied to the hemoglobin-affinity column followed by an extensive wash with 200 ml of PBS. The bound materials were first eluted with three volumes of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), as fraction 1 and then eluted with three volumes of freshly prepared and filtered 5 *M* urea in 0.15 *M* NaCl, pH 11, as fraction 2. A 5-ml volume of each fraction was collected in a tube containing 0.1 ml of 1 *M* Tris–HCl, pH 7.0, to immediately neutralize the pH value.

unavoidable using hemoglobin-based affinity column and was confirmed by a monoclonal antibody prepared against apoA-I (Fig. 7). Nevertheless, the major isolation procedure was simple and can be achieved within a few hours. This procedure should be widely used for the purification of Hp and particularly for 1-1 phenotype.

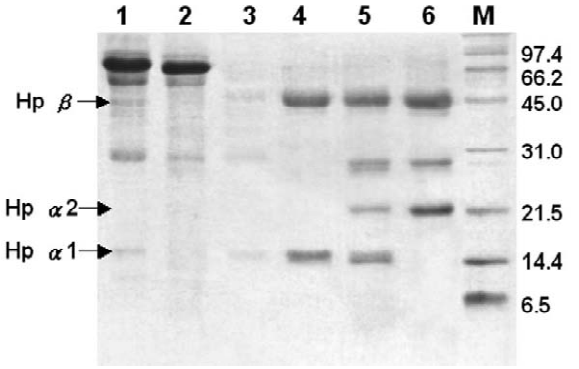


Fig. 6. Analyses of isolated Hp from affinity column on 15% SDS–PAGE. Lanes: M=molecular mass markers, 1=plasma of Hp 1-1, 2=a typical pass-through fraction (Hp 1-1 plasma) from hemoglobin-affinity chromatography, 3=a typical sample from fraction 1 (Hp 1-1) contaminated mostly apoA-I and proteins with large molecular mass. Lanes 4 and 6 represent fraction 2 of isolated Hp 1-1, 2-1, and 2-2, respectively. Notably, apoA-I is co-eluted in Hp 2-1 and 2-2.

## 4. Discussion

The acute phase serum protein, Hp, response to infection, inflammation, and trauma has been identified in a number of species. Methods designed for large isolation of human Hp have been complicated and time-consuming. We have recently described a single-step purification procedure for porcine Hp

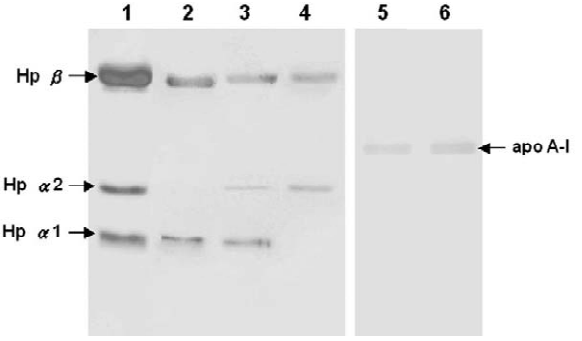


Fig. 7. Western blot analyses on affinity-purified human Hp 1-1, 2-1, and 2-2 by a goat antibody prepared against human haptoglobin. Lanes: 1=Hp standard purified from a human plasma pool, 2–4=affinity-purified Hp 1-1, 2-1, and 2-2, respectively, 5 and 6=the apoA-I co-eluted in affinity-purified Hp 2-1 and 2-2 fractions by a mouse monoclonal antibody prepared against human apoA-I. Purified Hp 1-1 did not reveal immunoreactive apoA-I (data not shown).

Table 1
Analytical recovery of haptoglobin 1-1 purified from 15 ml human plasma

| Total protein from plasma (mg) | Theoretical amount of Hp (mg) | Total Hp yield (mg) | Final yield (%) | Purity (%) | Fold purification |
|---|---|---|---|---|---|
| 1755 | 22 | 10 | 45.5 | >96 | 77 |

using HPLC gel-permeation chromatography in the presence of 5 *M* urea [22]. The procedure, however, could not be reproduced in human Hp isolation. Presumably, the human Hp structure is more complicated in its polymerization nature (Fig. 1) than that of pig. Thus, the purification for human Hp has been hampered by its structural diversity as each Hp 1-1, 2-1, and 2-2 has average molecular masses of 100 000, 220 000, and 400 000, respectively (Fig. 1). Although the procedure using a salting-out of plasma proteins followed by anion-exchange chromatography has been recommended, the reproducibility (including the yield) is rather poor due to the heterogeneity of its polymerization form of Hp 2-1 and 2-2 [20,27,28]. It is almost not feasible to isolate Hp 2-2 as a pool and to study its biochemical properties.

An immunoaffinity chromatography method to purify human Hp had been developed using a two-monoclonal antibody system [20], in which the phenotypes and the final purity of Hp were not specified. The yield, on the other hand, is limited and utilized only for the preparation of antigen and polyclonal antibodies [20].

With respect to hemoglobin-affinity column, Rademacher and Steele [19] have reported use of Sepharose immobilized with chicken hemoglobin. However, an attempt using human hemoglobin for the purification of human Hp was unsuccessful [19]. The method we employed was different from that of chicken hemoglobin-Sepharose chromatography. First, our human hemoglobin-Sepharose had a binding capacity 0.75–1.00 mg Hp/mg hemoglobin that was about 20 times greater than that of using chicken hemoglobin (Fig. 4). Second, our results demonstrated that highly purified hemoglobin via DEAE chromatography should be used for the affinity column rather than a crude extract of hemoglobin from ammonium sulfate fraction described previously [19]. Under this condition, the formation of plasma clots in the column could be eliminated. Third, the pH of each eluted fraction was immediately neutralized by a 1 *M* Tris buffer, pH 7.0, to restore the biological activity (complex formation between Hp and hemoglobin). It is worth mentioning that ammonium, rather than a high-capacity buffer solution, was used for adjusting the final pH of the saline solution (pH 11) in eluting the Hp; this was because the eluent could be easily neutralized by a Tris buffer. A similar procedure was employed previously in our laboratory [25]. Fourth, the most contaminants of proteins that bound weakly or non-specifically to the affinity column were differentially removed using pH 11 saline solution (Fig. 5, fraction 1). Fifth, unlike phenotypes Hp 2-1 and 2-2, Hp 1-1 could be isolated without apoA-I contaminant, as confirmed by a Western blot analysis (Fig. 7).

In the present study, about 8–10 mg of Hp 1-1 could be isolated from 15 ml of human plasma in one isolation. A similar yield of Hp 2-1 and 2-2 was obtained, but it required a further gel-filtration to remove apoA-I. The mechanism by which the affinity column favored the Hp 1-1 purification is not readily clear. Since Hp 2-1 and 2-2 molecules are largely polymerized by disulfide linkages with molecular weights ranging from 153 000 to 1 200 000 [6], these polymers may more accessibly ''trap'' the apoA-I than that of monomeric Hp 1-1. To address this assumption, we applied purified-apoA-I [25] directly to the affinity column. There was no apoA-I binding to the column suggesting that apoA-I did not interact with hemoglobin in the absence of Hp (data not shown). On the other hand, apoA-I may weakly bind to Hp and therefore was co-eluted with Hp during the purification. Regardless, the apoA-I deficient plasma, which can be easily obtained by a simple ultra-centrifugation for the removal of high-density lipoproteins [25], may be ultimately considered for the purification of all Hp phenotypes. This experimental procedure is currently in progress in our laboratory.

In conclusion, human hemoglobin could be con-

veniently isolated in large quantities by ammonium sulfate fractionation followed by a HPLC DEAE column. Immobilized human hemoglobin had a binding capacity about 20-times greater than that of chicken hemoglobin and could be more suitable for the purification of phenotype Hp 1-1. Accordingly, the procedure described in this report can be simply scaled up using a 100-ml bed affinity column for even larger Hp purification. This Hp purification procedure is currently used in our laboratory; the resulting Hp has been utilized in studying the structural and functional relationship and preparing polyclonal and monoclonal antibodies.

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