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Journal of Chromatography B, 845 (2007) 210–217
JOURNAL OF CHROMATOGRAPHY B
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An improved method for haptoglobin 1-1, 2-1, and 2-2 purification using monoclonal antibody affinity chromatography in the presence of sodium dodecyl sulfate☆

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Received 30 May 2006; accepted 6 August 2006
Available online 7 September 2006

Abstract

Human haptoglobin (Hp) is classified as three phenotypes: Hp 1-1, 2-1, and 2-2. Previously, we had isolated this protein by affinity columns using either hemoglobin or monoclonal antibody (mAb) prepared against Hp β-chain (clone 8B1-3A). The isolated Hp from both methods, however, contaminates plasma apolipoprotein A-I (apoA-I). In the present report, we have developed a novel affinity column procedure using an mAb prepared against α-chain of Hp (clone 3H8) for Hp purification. Plasma was first chromatographed onto the column followed by a normal wash with a buffer containing 0.12 M NaCl and 0.02 M phosphate, pH 7.4 (PBS). The bound proteins were then prewashed with a 0.04% sodium dodecyl sulfate (SDS)–PBS, pH 7.4, to remove the low-affinity bound apoA-I from Hp. Finally, the bound Hp was eluted with a 0.1% SDS–PBS, pH 11, and collected in tubes containing 1 M Tris–HCl, pH 6.8. As a result, the isolated Hp was devoid of apoA-I and was able to retain the biological function by forming an Hp–hemoglobin complex. The homogeneity of each isolated Hp 1-1, 2-1, or 2-2 was greater than 95% with an yield greater than 50%. The procedure described here is significantly improved in time consumption, recovery, and purity. The rationale, design, and optimization for each step are described in detail.

Keywords: Human haptoglobin; Affinity column; Hemoglobin; Apolipoprotien A-I; Monoclonal antibody; Sodium dodecyl sulfate

1. Introduction

Hp is known as an acute phase protein; its plasma level elevates in response to infection and inflammation. The normal concentrations of Hp in human plasma are relatively high ranging from 1.0 to 1.5 mg/ml. Clinically, it is a useful marker for some inflammatory-related diseases [1–4].

Human Hp is tetrameric arrangement joined by disulfide linkages among two α and two β chains (Fig. 1) [5–7]. According to the length of α chain (α1 or α2), it results in three phenotypes of Hp in the population, Hp 1-1, 2-1, and 2-2. All the three phenotypes share the same two β chains (each with about M_r 40,000 kDa including 245 amino acids containing about 30% carbohydrate in moiety), with different compositions in α chains. Homozygous Hp 1-1 or 2-2 contains identical α1 (including 83 amino acids, ∼9 kDa) or α2 (including 142 amino acids, ∼16.5 kDa) chains, respectively, whereas heterozygous Hp 2-1 contains both α1 and α2. Owing to an extra thio-group on the α2 chain of Hp 2-1 and 2-2 (not 1-1), they form large polymers of monomeric, trimeric, tetrameric, pentameric, hexameric, and even larger arrangement through the disulfide linkages (Fig. 1).

The polymeric forms of Hp 2-1 and 2-2 are more prone to the development of inflammatory-related cardiovascular disease than that of Hp 1-1. Presumably, this is due to the complicated structure of Hp 2-1 and 2-2 as they form heterogeneous polymers, in which some of the biological entities are not fully expressed on the surface (Fig. 1). Recently, we have demonstrated that Hp is an extremely potent antioxidant that directly

☆ This paper was presented at the 10th International Symposium on Biochromatography, Lille, France, 26–28 April 2006.

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doi:10.1016/j.jchromb.2006.08.012

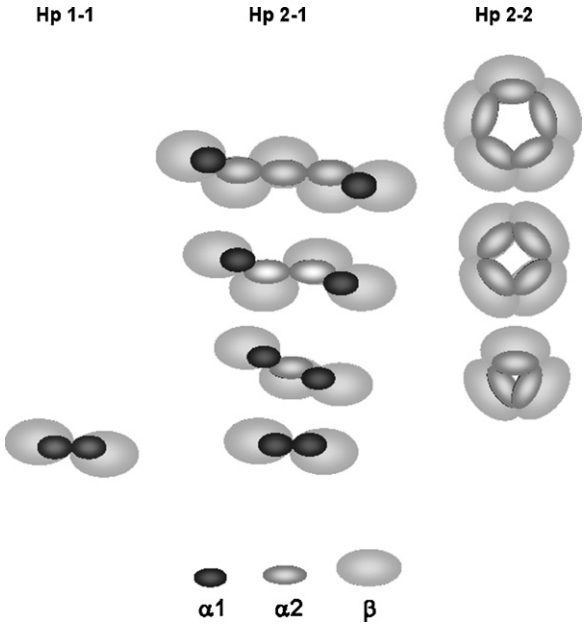

Fig. 1. Schematic drawing of molecular arrangement in human Hp phenotypes. Hp 1-1 possesses only the basic dimer $(\alpha1\text{-}\beta)_2$ whereas Hp 2-1 is comprised of many structures: a dimer $(\alpha1\text{-}\beta)_2$, a trimer $(\alpha\text{-}\beta)_3$, and other linear polymers. Here, α represents both α1 and α2 chains. Hp 2-2 is comprised of a trimer $(\alpha2\text{-}\beta)_3$ and other cyclic polymers. Each α1, α2, or β is 83, 142, or 245 amino acids in length, respectively. α2 is similar to α1, differing only by an additional insertion of a repeat identical to 3/4 of α1. Owing to the extra Cys-74 in α2, Hp 2-1 and 2-2 form complicated polymers.

inhibits low density lipoprotein (LDL) oxidation. The potency among the Hp phenotypes is ranked as follows: Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > Vitamin E [8]. Owing to the difficult procedures of Hp purification, the structural and functional relationship among the Hp phenotypes have not been yet well established.

Previously, we established a simple gel-filtration purification method with high yield for porcine plasma Hp [9]. As pigs only express one single phenotype of Hp, namely Hp 1-1, the molecular form of their Hp is simple and homogeneous. The same purification procedures are not practical for isolating human samples because of the heterogeneous nature of Hp 2-1 and 2-2 (Fig. 1). The most common procedures involved in the human Hp purification frequently suffer from some drawbacks. Using hemoglobin-sepharose affinity column, the harsh-elution condition (8 M urea) often causes dissociation of a hemoglobin subunit from the sepharose. Human plasma apoA-I of high density lipoprotein (HDL) appears to be a major contaminant [10]. Using immunoaffinity column chromatography previously established in our laboratory, human apoA-I is still coeluted with Hp. Another gel-filtration of high-performance liquid chromatography (HPLC) step is needed to remove apoA-I [11]. The step is time-consuming and attenuates the yield of Hp [11]. The major focus of the present study was to eliminate the apoA-I contamination over the affinity column. We investigated the interaction between HDL and Hp and developed a new eluting buffer system to remove the apoA-I prior to a final elution of Hp. The overall recovery of purified Hp using this procedure is greater than 50% with about 97% homogeneity.

2. Experimental

2.1. Materials

Monoclonal antibodies (mAb) against human Hp were produced using procedures previously described in our laboratory [12,13]. Rabbit anti-Goat IgG was purchased from Chemicon (Temecula, CA). CNBr activated Sepharose 4B was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were purchased from Sigma (St. Louis, MO) without any further purification. The buffers used in this report were all filtered through a 0.45 mm filter before use.

2.2. Preparation of ascetic fluid

Adult mice were injected intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethyldecanoic acid). After 7 days, 10^6 hybridoma cells in 0.5 ml PBS were injected into intraperitoneal cavity of each mouse. Following another 7–14 days, the ascetic fluid was drawn by an 18-gauge needle attached to a 5 ml syringe. Each mouse produced about 5–10 ml of ascetic fluid in three batches. The collected fluid was incubated at 37 °C for 1 h and stored at 4 °C overnight followed by a centrifugation at 3000 g for 10 min to remove an oil layer and cell pellet.

2.3. Purification of Hp mAb

Monoclonal antibodies prepared against human Hp were characterized according to the standard procedures established in our laboratory [12,13]. MAb 3H8 prepared against α-chain of Hp, which possessed the highest binding affinity to Hp, was selected for preparation of the affinity column. Briefly, 20 ml of ascetic fluid from the 3H8 monoclone was first precipitated by a 50% saturated ammonium sulfate. The precipitate was dissolved in 5 ml PBS, followed by an extensive dialysis against 20 mM phosphate buffer (PB) at pH 7.2 to remove the remaining ammonium sulfate. The sample was then passed through a diethylaminoethanol (DEAE)-cellulose column equilibrated with 20 mM PB at pH 7.2; the pass-through fraction eluted with the equilibrium buffer yield about 100 mg of almost pure IgG fraction.

2.4. ELISA evaluation of dissociation of bound Hp from mAb

All the reactions including those for enzyme-linked immunoabsorbent assay (ELISA), apoA-I binding assay, preparation of affinity column, and column chromatography were conducted at room temperature, unless otherwise specified. In brief, mouse 3H8 mAb against human Hp (0.5 μg in 50 μl) was first immobilized onto a microtiter well followed by three washes to remove unbound 3H8. Each well was then blocked by 2% (w/v) dry milk in a buffer containing 0.02 M phosphate and 0.12 M NaCl, pH 7.4 (PBS). After three washes, 50 μl of Hp (5 ng/μl) in PBS was added and incubated for 1 h. Following washes to remove the excess of Hp, the bound Hp were treated three times with various eluting reagents including a PBS, pH 11;

4 M urea-PBS, pH 11; and 0.01–2.5% SDS–PBS, pH 11. Final binding of Hp was determined using Goat anti-Hp followed by an HRP conjugated anti-Goat IgG. The microtiter plate was then developed with 100 μl tetramethylbenzidine (TMB) per well and stopped by adding 100 μl 1N HCl prior to the reading at 450 nm.

2.5. Effect of SDS on the dissociation of HDL from Hp

Human Hp was first immobilized (0.5 μg/50 μl) onto each microtiter well followed by three washes to remove unbound Hp similar to the process described above. The wells were then blocked by 2% dry milk in PBS. After three washes, 50 μl of HDL (5 μg/ml) in PBS were added to each well and incubated for 1 h. After the washes, the bound HDL was treated three times with SDS at various concentrations (0–0.1%) in PBS at pH 7.4. Determination of final bound HDL was conducted using Goat anti-apoA-I followed by an HRP-conjugated anti-Goat IgG. The microtiter plate was developed with 100 μl TMB and stopped by adding 100 μl 1 N HCl prior to the reading at 450 nm.

2.6. Preparation of antibody affinity column

Briefly, 1 g of freeze-dried sepharose (1 g of freeze-dried powder gave about 3.5 ml final volume of gel) was swollen and suspended in 1 mM HCl and immediately washed with 400 ml of 1 mM HCl solution within 15 min on a sintered glass filter. The gel was then washed with a coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3, and degassed. About 15 ml (2 mg/ml) of dialyzed anti-Hp IgG in coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3 was slowly added into the gel (CNBr-activated Sepharose 4B) while gently rotating for 1 h at room temperature. After coupling, the gel was washed with 500 ml of coupling buffer to remove unbound materials via a sintered glass filter. The gel was then blocked with a buffer containing 0.1 M Tris–HCl, pH 8.0, for 2 h to saturate the remaining reactive sites. The degassed gel was then washed with 3 cycles of 0.1 M acetate, pH 4 and 0.1 M Tris–HCl, pH 8, both containing 0.5 M NaCl. Finally, the gel was equilibrated in PBS and packed onto a 1.0 cm × 10 cm column with a bed volume of approximately 3.5 ml.

2.7. Initial purification of three Hp phenotypes on immunoaffinity chromatography

Initially, 1 ml of filtered human plasma of each Hp phenotype batch was loaded onto the affinity column (3 ml in bed volume). After which, the column was washed with 40 ml PBS and eluted with 20–60 ml of a freshly prepared eluting buffer, either a PBS (pH 11), a 0.1% SDS–PBS (pH 11), or a 0.1% SDS–PBS (pH 7.4). Two millilitre of each fraction was collected in a tube containing 0.1 ml of 1 M Tris–HCl buffer, pH 6.8, to immediately neutralize the pH before a final dialysis in PBS. The pooled fraction of desired was extensively dialyzed against 4 l of PBS (with three changes) to remove SDS.

2.8. Final affinity purification of bound Hp via prewash with 0.04% SDS–PBS, pH 7.4

The isolation procedures were similar to that described above, except two steps involved. First, following 1 ml plasma flown through the column and washed with 40 ml of PBS, the bound Hp was washed again by 20 ml of 0.04% SDS–PBS, pH 7.4, to remove apoA-I that associated with Hp. Next, the bound Hp was eluted with 0.1% SDS–PBS, pH 11, as described. The fractions containing Hp were pooled and extensively dialyzed against 4 l of PBS (with three changes) to remove the SDS. Homogeneity of each Hp phenotype was then analyzed on SDS–PAGE containing 1% 2-mercaptoethanol.

2.9. Gel electrophoresis and densitometry

SDS–PAGE was performed according to Laemmli's method with modifications by using 5% polyacrylamide (w/v) on the stacking gel as previously described [14]. Samples (typically 5 μg) for 15% SDS–PAGE were preheated at 100 °C for 10 min in a loading buffer (12 mM Tris–HCl, pH 6.8, 0.4% SDS (w/v), 5% glycerol (v/v), 2.88 mM of 2-mercaptoethanol, and 0.02% bromphenol blue (w/v)). The samples were run for about 1.5 h at 100 V and stained, using Coomassie brilliant blue R-250. Densitometric analysis of SDS–PAGE was performed using a Molecular Dynamics densitometer (Hercules, CA) for data acquisition and Image Quant software for integration and analysis.

2.10. Western blot analysis

Following the separation of proteins by SDS–PAGE, the gel was electrically transfered to a nitrocellulose-membrane attached with a 3 MM filter paper presoaked in a transfer buffer containing 48 mM Tris–HCl, 39 mM glycine, 0.037% SDS (w/v) and 20% methanol (v/v), pH 8.3. The rest of the procedures for immunoblot was similar to that described previously [9,10].

3. Results

In our previous studies, we have described mAb and hemoglobin affinity-column methods for the purification of human Hp [10,11]. Although these two methods, using PBS (pH11) as an eluent, were simple, the average Hp recovery was lower than 50% and an additional chromatographic step was needed to remove the contaminated apoA-I. To improve the recovery and the purity of isolated Hp in the present report, the steps described below were conducted.

3.1. Evaluation of dissociation of bound Hp from immobilized mab

To optimize the elution conditions for the yield of Hp while isolating, we evaluated the Hp and mAb interaction on an ELISA plate to mimic the Hp binding on mAb affinity column. Various modified eluting buffers including PBS (pH 11), 4 M urea-PBS (pH 11), and 0.1% SDS–PBS (pH 11) were studied. Fig. 2 demonstrates that the dissociation of bound Hp from mAb was not completed using PBS, pH 11. However, the bound Hp was

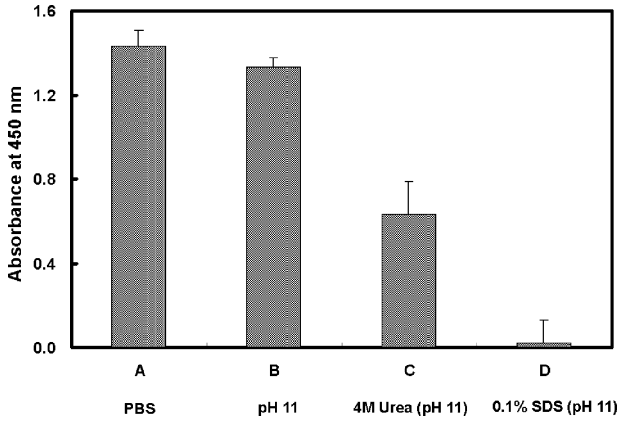

Fig. 2. Evaluation of dissociation of bound Hp from immobilized mAb at various elution conditions. To determine a simple and optional condition that is able to elute the Hp from immobilized mAb, Hp mAb was first immobilized on an ELISA plate followed by incubating 50 μg of purified Hp 2-1 in 50 μl PBS. After washing, bound Hp was then treated with various eluting buffers: (A) Total binding of Hp without eluting reagents; (B) PBS, pH 11; (C) 4 M urea-PBS, pH 11; (D) 0.1% SDS–PBS, pH 11. Binding of Hp was determined using Goat anti-Hp followed by HRP conjugated anti-Goat IgG for final development of chromogenicity. Each value represents a mean of triplicates ± SD.

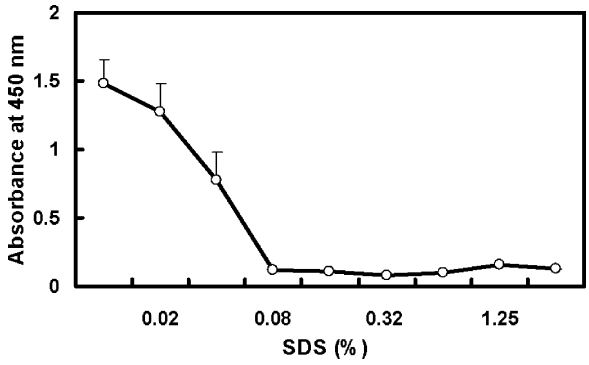

Fig. 3. Effect of SDS concentrations (pH 11) on the dissociation of bound Hp from immobilized mAb. The remaining Hp was determined in a manner similar to that described in Fig. 2. Each point represents a mean of triplicates ± SD. Typical deviation is shown in the first three doses.

completely dissociated when using the same PBS buffer containing 0.1% SDS. The buffer containing 4 M urea was only partially effective. The data suggest that SDS with pH 11 could be a useful reagent for eluting the bound Hp.

3.2. *Effect of the concentration of SDS–PBS, pH 11, on the dissociation of bound Hp from immobilized mAb*

To optimize the concentration of SDS capable of dissociating Hp from immobilized mAb, a series of diluted SDS was used for the assay. Fig. 3 shows that the SDS concentrations greater than 0.08% completely dissociated Hp from the mAb. As the mAb was immobilized on the plate as a capture, it is worth mentioning that SDS at 0–2.5% did not cause the dissociation of immobilized mAb from the plate (data not shown).

3.3. *Initial purification of Hp 1-1, 2-1, and 2-2 on immunoaffinity chromatography*

Fig. 4 shows the optimal conditions for the recovery of Hp phenotypes from the column. Overall, the Hp yield using SDS–PBS, pH 11, as an eluant was two times higher than the one using PBS, pH 11 (a conventional method) or SDS–PBS, pH 7.4. Interestingly, eluting buffer of SDS with pH 7.4 delayed the Hp retention time. One of the explanations is that antibody–antigen interaction is mostly ionic dependent; SDS at neutral pH

Table 1
Analytical recovery and yield of haptoglobin purified from 1 ml of human plasma

	Plasma	*Bound Hp eluted with		
		0.1% SDS–PBS	PBS	0.04% SDS–PBS prewash followed by 0.1% SDS–PBS
(a) Hp 1-1				
Protein (mg)	110	1.50	0.60	0.98
Moiety of Hp (mg)	1.69[a]	1.2[b]	0.5[b]	0.94[b]
Purity (%)[b]	1.54	81	83	96
Fold purification	1	53	54	62
Recovery (%)	100	71	30	56
(b) Hp 2-1				
Protein (mg)	102	1.15	0.79	0.69
Moiety of Hp (mg)	1.21[a]	0.82[b]	0.68[b]	0.67[b]
Purity (%)[b]	1.19	71	86	97
Fold purification	1	60	72	82
Recovery (%)	100	68	56	55
(c) Hp 2-2				
Protein (mg)	80	1.19	0.52	0.74
Moiety of Hp (mg)	1.01[a]	0.86[b]	0.37[b]	0.72[b]
Purity (%)[b]	1.26	72	71	97
Fold purification	1	57	56	77
Recovery (%)	100	85	37	71

[a] The concentration of each human Hp phenotype was determined using an ELISA currently used in our laboratory.
[b] Determined by densitometer using digital Image Quant software.
* Eluted at pH 11.

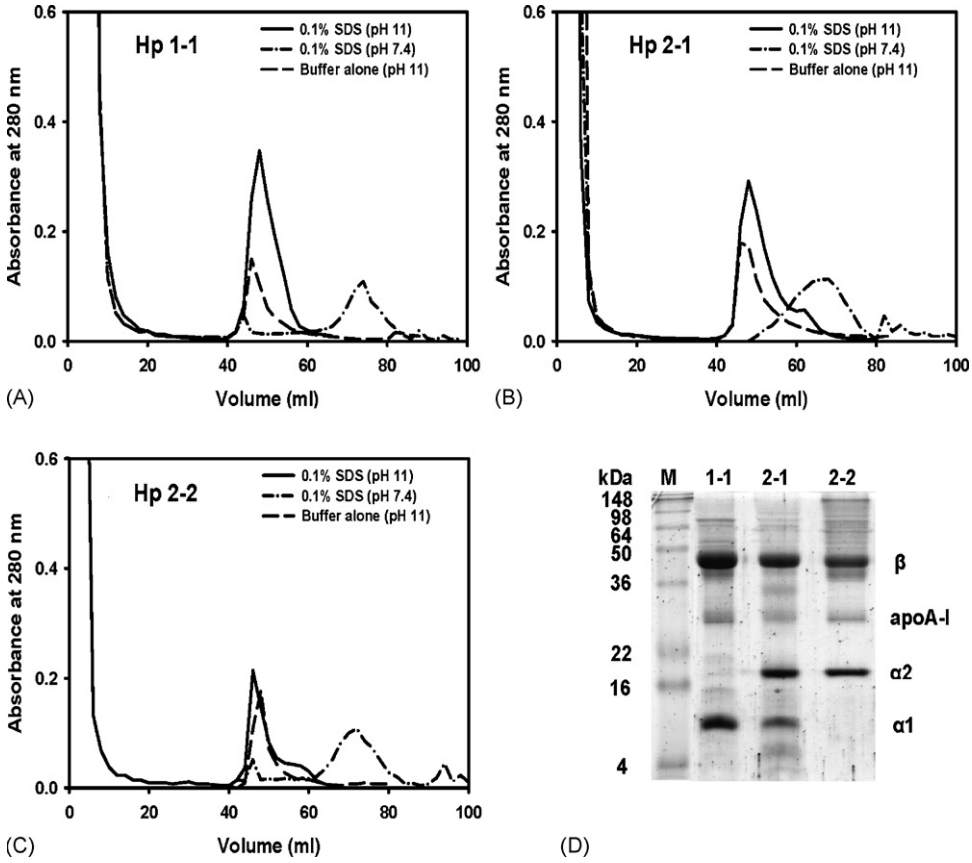

Fig. 4. Elution profile of bound Hp from immobilized mAb on immunoaffinity column (A–C) and contamination of apoA-I in isolated Hp (D). (A–C) One ml of human plasma was applied onto the immunoaffinity column and washed with a PBS, pH 7.4. The bound Hp was subsequently eluted with a 0.1% SDS–PBS, pH 11 and with 0.1% SDS–PBS, pH 7.4. The overall data suggest that the eluting buffer containing 0.1% SDS at pH 11 results in high yield for Hp purification. (D) Analysis of isolated Hp by a 15% SDS–PAGE containing 1% 2-mercaptoethanol. It reveals that isolated Hp contaminates apoA-I.

slowly alters the conformation of antigen or antibody via a protein–SDS micelle formation [15]. Further conformational change is induced at high pH resulting in the dissociation of antigen–antibody complex. Thereby, elution of Hp by SDS combined with pH 11 resulted in high recovery of Hp 1-1 (71%), 2-1 (68%), and 2-2 (85%) from the column (Table 1). Nonetheless, the isolated Hp appeared to contaminate some apoA-I (Fig. 4D).

3.4. Removal of apoA-I contamination

ApoA-I is a major apolipoprotein residued in HDL [16]. It has been known for some time that apoA-I is a major contaminant in isolated Hp using variety of the methods [9,10]. To minimize the apoA-I contamination, we evaluated the HDL and Hp interaction on an ELISA plate immobilized with Hp.

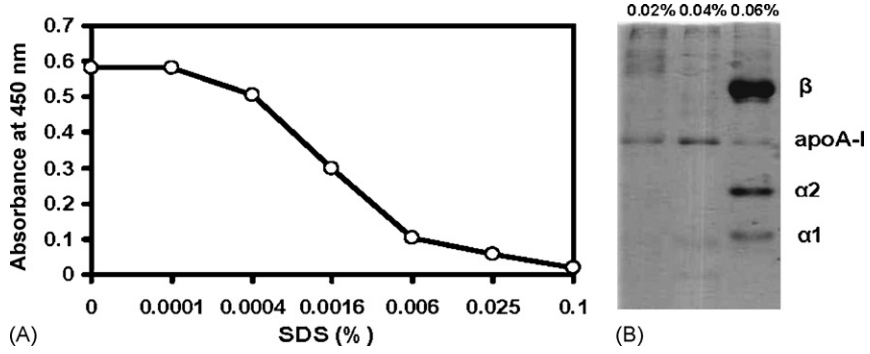

Fig. 5. Binding of HDL with Hp and its dissociation by SDS. (A) Hp was first immobilized on an ELISA plate followed by incubating 50 μg of purified HDL in 50 μl PBS. After washing with PBS, the bound HDL was dissociated from Hp by treating it with SDS in a dose-dependent fashion. Binding levels of HDL were determined using Goat anti-apoA-I followed by HRP conjugated anti-Goat IgG similar to that described in Fig. 2. The data suggests that Hp-associated apoA-I can be eliminated in the presence of SDS. (B) Eluant from affinity column, eluted with 0.02%, 0.04%, and 0.06% SDS–PBS, pH 7.4, was analyzed by SDS–PAGE in the presence of reducing reagent. It suggests to avoid using of 0.06% SDS for apoA-I removal.

Fig. 5A reveals that apoA-I was able to bind Hp, but bound apoA-I was dissociated from Hp at SDS (pH 7.4) concentrations greater than 0.025%. In the next experiment, we mimicked the above condition directly on the affinity column. By SDS–PAGE, concentrations between 0.02 and 0.04% of SDS could wash bound apoA-I away from the column, but higher concentrations of SDS (≧0.06%) removed both apoA-I and Hp from the immobilized mAb (Fig. 5B). Likewise, 0.04% SDS–PBS (pH 11) also removed apoA-I and Hp (data not shown). The data indicate that apoA-I can be selectively removed using 0.04% SDS–PBS, pH 7.4, prior to a final elution of Hp.

3.5. Final affinity purification of bound Hp via prewash with 0.04% SDS–PBS, pH 7.4

Finally, we used 0.04% SDS as a prewash for the removal of bound apoA-I from Hp over the affinity column. Fig. 6 shows a typical chromatographic profile for the plasma of Hp 1-1, 2-1, or 2-2. Following a flow through of plasma and washes with a PBS, pH 7.4, the column was prewashed with 0.04% SDS–PBS, pH 7.4, to remove bound apoA-I as well as other non specifically bound proteins as fraction 1 (F1). Next, 0.1% SDS–PBS, pH 11 was used to elute bound Hp from mAb as fraction 2 (F2). Fig. 6 exhibits that prewash fraction (F1) had most of apoA-I

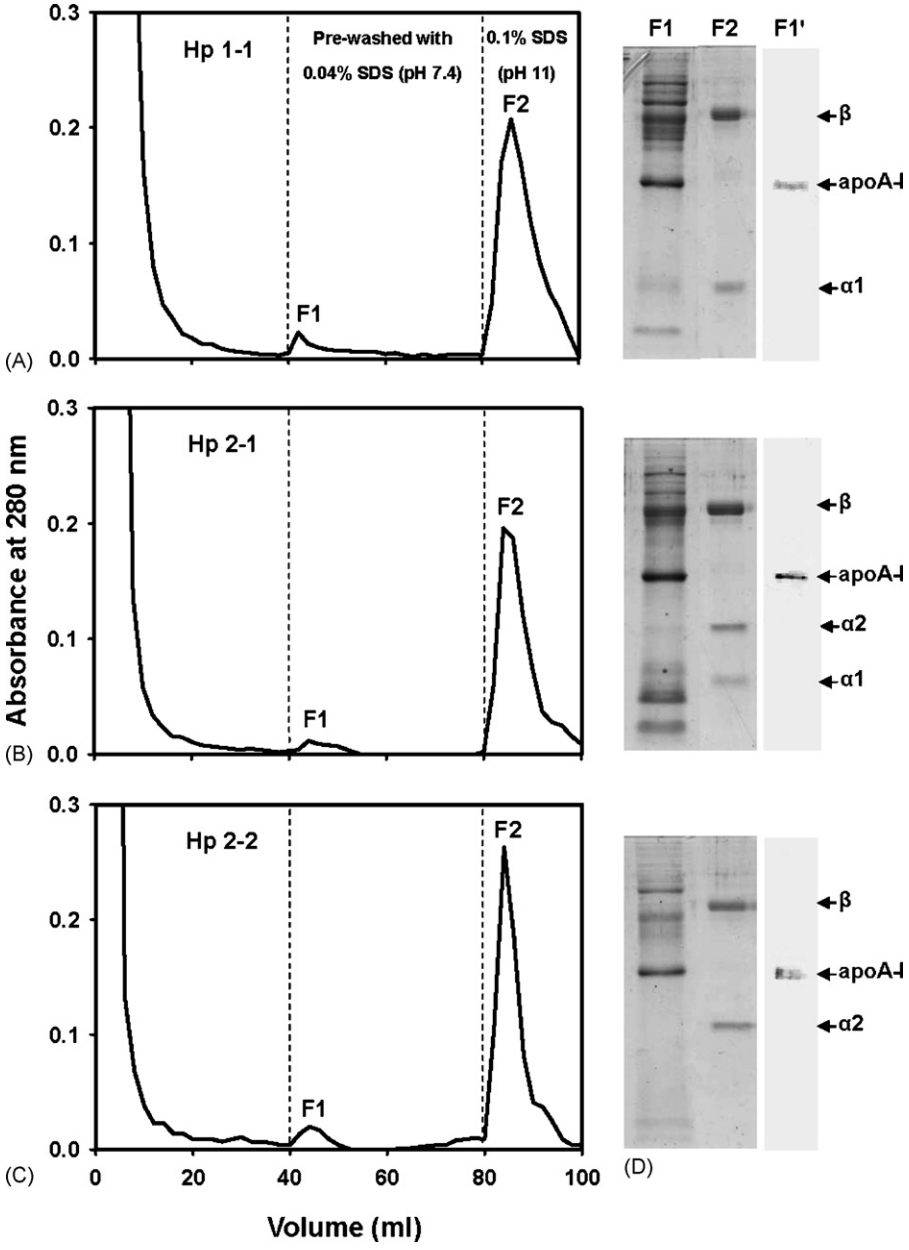

Fig. 6. Final purification of human Hp 1-1 (A), 2-1 (B), and 2-2 (C) from immunoaffinity column prewashed with 0.04% SDS–PBS, pH 7.4. (A–C) Initially, 1 ml of human plasma was passed through the column with PBS, pH 7.4 and washed with the same buffer. The bound Hp was then prewashed with 40 ml of 0.04% SDS–PBS, pH 7.4 to remove Hp associated apoA-I (F1). Finally, Hp was eluted from immobilized mAb by 0.1% SDS–PBS, pH 11 (F2). (D) Analysis of F1 and F2 on 15% SDS–PAGE containing 1% 2-mercaptoethanol and F1 on Western blot (F1'). The α and β subunits corresponding to each Hp phenotype are shown.

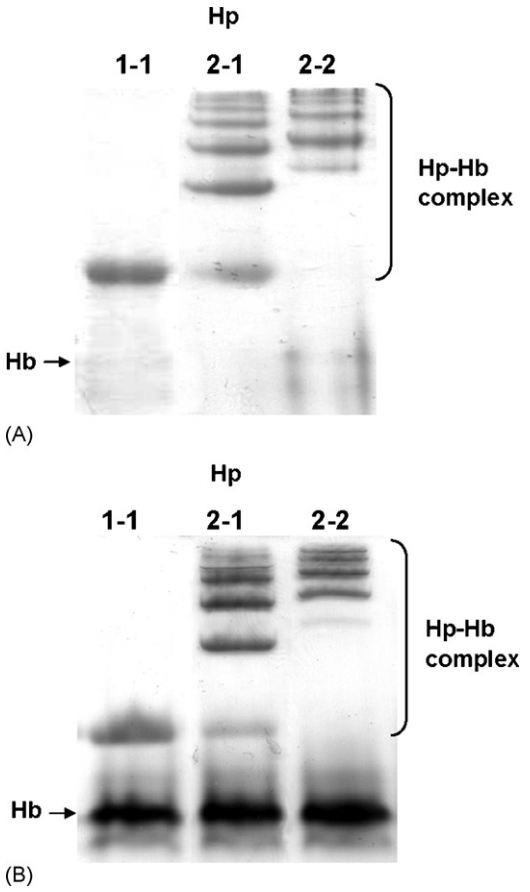

Fig. 7. Analysis of hemoglobin-binding property of isolated Hp 1-1, 2-1, and 2-2 (A) and Hp 1-1, 2-1, and 2-2 from human plasma (B) on 7% native-PAGE. Briefly, each isolated Hp (5 μg) or each plasma (6 μl) was incubated with hemoglobin (Hb) (5 μg) at room temperature for 30 min. Following electrophoresis, the gel was stained directly by a freshly prepared DAB solution containing 0.05% H_2O_2 as a developer for the endogenous peroxidase activity of hemoglobin.

contaminant as identified by a SDS–PAGE and confirmed by a Western blot analysis. The homogeneity of final isolated Hp was about 97% (Fig. 6, F2 fraction). The final recovery of isolated Hp 1-1, 2-1, and 2-2 was approximately of 56, 55, and 71%, respectively (Table 1).

3.6. Analysis of hemoglobin-binding property of isolated Hp 1-1, 2-1, and 2-2

Fig. 7 reveals that final isolated Hp possessed the ability to form a complex with Hb using a 7% native PAGE analysis. Each phenotype formed a complex with Hb as a monomer (Hp 1-1) and polymers (Hp 2-1 and 2-2), whereas Hp 2-1 shared one completely identical monomer to Hp 1-1.

4. Discussion

Clinically, Hp phenotypes are found to be related to several inflammatory diseases. For example, polymeric form of Hp 2-1 or 2-2 is associated with the complications of myocardial infarction [17], kidney failure [18], and diabetics [19]. In a prospective study, human subjects with Hp 2-2 are at a 5-fold increased risk for the development of CAD as compared with those with Hp 1-1 [20]. The risk in heterozygous Hp 2-1 participates is intermediate [20]. Low levels of Hp are also found among HIV-1 seropositive patients with Hp 2-2 [21]. Owing to the difficult purification procedures for each Hp phenotype, the relationship between the Hp levels and its affected diseases are rarely reported. One of the difficulties is to isolate Hp 2-1 and 2-2 because of their heterogeneity with polymeric molecular forms (Fig. 1). On the contrary, it is essential to study the structural and functional relationship among the Hp phenotypes. Previously, Rademacher and Steele [22] utilized the chicken hemoglobin-sepharose affinity column to isolate human Hp; the harsh-elution condition (8 M urea) causes the dissociation of a hemoglobin subunit from the sepharose matrix. Meanwhile, human apoA-I appears to be another major contaminant. Travis and Sanders [23] employ Sephadex G-200 gel-filtration, but the purified Hp is accompanied by large amounts of IgM and a-2 macroglobulin. Morimatsu et al. [24] provide a modified method using HPLC with anion-exchange, Sephacryl S-300, TSK Phenyl-5PW, and TSK DEAE-5PW columns together; the procedures, however, are time consuming and the yield is relatively low.

In our previous studies, we utilized hemoglobin or Hp mAb (prepared against Hp β-chain; clone 8B1-3A) affinity column to isolate three phenotypes of Hp [10,11]. Both methods showed that there was a contamination of apoA-I, when eluting Hp by PBS, pH 11. To remove contaminated apoA-I, a re-chromatography on an HPLC sephorose-12 gel-filtration was required [11]. For this reason, one major focus of this report was an attempt to minimize the contamination of apoA-I. To test the possibility that plasma HDL (apoA-I containing lipoprotein) may directly bind to Hp, we immobilized purified Hp on an ELISA plate followed by the addition of excess amount of HDL to saturate its interaction with Hp, if any. Most interestingly, we found that HDL could bind Hp (Fig. 5) although the binding affinity between HDL and Hp was not readily known. Such binding is not non specific, since Hp can inhibit the apoA-I-dependent lecithin:cholesterol acyl transferase (LCAT) activity in vitro [25], which plays a role in the reverse cholesterol transport [26]. The data suggest that HDL is associated with Hp in plasma, at least in part. Fortunately, the binding affinity seemed to be differentially lower than that of Hp–mAb. This is the reason why 0.04% SDS–PBS (pH 7.4) only removed apoA-I from Hp but not Hp from immobilized mAb (Fig. 5). But, SDS concentrations greater than or equal to 0.06% or 0.04% SDS–PBS at pH 11 should be avoided for prewash as Hp could come off the column. In an early study [9], we proposed to use HDL-depleted plasma (a bottom fraction obtained by ultracentrifugation at KBr d.1.21 g/ml) for Hp purification, assuming that the contamination of plasma apoA-I could be eliminated. This experiment was conducted in the present study. The isolated Hp still contained some apoA-I but not as much as that of whole plasma (data not shown). Therefore, the use of HDL-deficient plasma for Hp purification may not be considered in the future.

SDS is used more often than any other detergent as an excellent denaturing or unfolding reagent [15,27]. It breaks mostly the quaternary and tertiary protein–protein interaction [28,29].

As such, at low SDS concentration (0.04%), pH 7.4, it dissociates the apoA-I from Hp. While at 0.1% of SDS, pH 11, it elutes bound Hp from the immobilized mAb over the affinity column. However, one concern is that SDS may alter the conformation of isolated Hp. Biswas and Das reported that α-crystallin was able to refold to native structure after unfolding by SDS [29]. To address whether isolated Hp could refold closely to its native conformation, we monitored the structure of Hp 1-1 using a circular dichroic spectrophotometer. We found SDS-eluted Hp being slightly more disordered than that of Hp eluted without using SDS. Following extensive dialysis in 4 l PBS with three changes, the disordered structure, however, refolded to the original alpha-helical content (about 30% helix) as the values previously reported [11] (data not shown). Furthermore, we have shown that the formation of Hp–Hb complex is dependent on the overall conformation of Hp [8]. In the present study, the purified Hp following a dialysis could form a complex with Hb (Fig. 7), suggesting that Hp has refolded to native form. Nevertheless, the purified Hp also retained its immunochemical property as determined by Western blot and ELISA (data not shown).

Taken together, the present method using SDS as an eluant has certain advantages. First, Hp can be purified in a predictable way by passing it through the immobilized mAb. Secondly, the bound apoA-I (or other non specially bound proteins) is selectively removed prior to final elution of Hp. Third, the technique allows isolation of the polymeric form of Hp 2-1 and 2-2 (Fig. 7) unlike standard methods which may significantly lose a part of them. Some standard methods, which rely on different molecular masses or charges can distribute the component to different fractions. Fourthly, the simple technique can be used for analytical purpose, for example, for the determination of polymeric forms of Hp that may be of importance for the investigation of metabolism in pathological cases. Because SDS is used in the elution buffers, it is worth mentioning that a large volume of PBS (300 ml), pH 7.4, is required for the regeneration of the column, which is time consuming and is considered to be one disadvantage of the present method.

Acknowledgements

This work was supported by the Grant NHRI-EX92-9229SI (S.J.T.M.) from the National Health Research Institute; and NSC95-2313-B-009-003-MYZ (S.J.T.M.) and NSC93-2815-C-009-044-B from National Science Council, Taiwan, ROC. We also appreciate National Science Council for the special project award to Sunny C.H. Yueh for the years of 2005–2007. We specially thank Miss Wen-Chao Wang for performing the native gel electrophoresis.

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