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Short communication

Simple high-performance liquid chromatographic purification procedure for porcine plasma haptoglobin

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Abstract

Haptoglobin is an acute-phase protein and its plasma levels increase consistently in response to infection and inflammation. Some evidence has demonstrated that haptoglobin is involved in the immune responses. In this study, we established a novel high-performance liquid chromatographic purification procedure for porcine plasma haptoglobin. The procedure required an ammonium sulfate fractionation and a HPLC Superose 12 gel-permeation chromatography. Purified porcine haptoglobin possessed one heavy (β) and light chain (α) on sodium dodecyl sulfate–polyacrylamide gel electrophoresis, under reducing conditions, with a M_r (molecular mass) of about 42 000 and 14 000 for heavy (β) and light chains (α), respectively. Although the N-terminal amino acid sequence of porcine heavy chain of haptoglobin has never been reported previously, the analyses of N-terminal amino acid sequence showed a great sequence similarity to that of human and other animal species. In addition, Western blot using our specific antibody prepared against porcine M_r 42 000 chain did react with human haptoglobin and likewise, the antibody against human haptoglobin also cross-reacted with purified porcine M_r 42 000 chain. Thus, it confirmed that the identity of the porcine protein purified from our procedures was as haptoglobin.

Keywords: Porcine haptoglobin; Haptoglobin

1. Introduction

Haptoglobin (Hp) is a hemoglobin-binding protein present in the plasma of all vertebrates, and is believed to participate in hemoglobin transport. Haptoglobin, an α-2 glycoprotein, is also known as an acute phase protein in response to a variety of injuries or inflammatory disease states [1–4]. Its plasma concentrations are useful as diagnostic analyses and clinical evolution of many inflammatory diseases. Human haptoglobin is a glycoprotein with tetrameric structure, the typical structure haptoglobin 1-1 is composed of an α (light) chain of molecular mass (M_r) 9000 including 83 amino acids and a β (heavy) chain of M_r 40 000 including 245 amino acids and an approximate 30% (w/w) carbohydrate moiety. The α and β chains are connected each other by a single disulfide bond, forming an αβ unit. One

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αβ unit is further linked to another αβ unit by a disulfide bond between α chains [5]. Likewise, the tetrameric arrangement is also found in rat, rabbit and pig haptoglobins [2,6–8]. However, the two αβ units joined by a non-covalent interaction rather than a disulfide bridge are found in dog, cat and bear haptoglobins [9,10].

The haptoglobin β chain is a glycoprotein that was first detected in the extracts of rabbit uteri on six to seven days of pregnancy [11]. Several functional differences between haptoglobin phenotypes have been demonstrated that appear to have important biological and clinical consequences. Previous studies on the molecular structures of haptoglobin have focused on human haptoglobin, but the exact physiological function has not been fully understood.

The most common procedures for purification of haptoglobin from serum are electrophoresis, affinity chromatography on insolubilized hemoglobin, or multiple high-performance liquid chromatography (HPLC) steps [4,12,13]. These methods are useful but are troublesome and time-consuming. In the present report, we established a simple purification method with high yield for porcine haptoglobin from serum. The N-terminal amino acid sequence of porcine β chain and its immunochemical homogeneity in plasma are also reported for the first time.

2. Experimental

2.1. Animals and chemicals

Pigs were raised in our experimental farm of Pig Research Institute Taiwan (PRIT, Miaoli, Taiwan) with routine animal care. Goat anti-human haptoglobin antibody was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). Neuraminidase was from Sigma (St. Louis, MO, USA) with a specific activity of 1 unit/mg. All other chemicals were purchased from Sigma or Merck (Darmstadt, Germany) without any further purification.

2.2. Purification of haptoglobin

Plasma samples were prepared from pig blood containing 0.1% (w/v) ethylenediaminetetraacetic acid (EDTA) followed by a centrifugation at 1200 *g* for 15 min at 4°C to remove the pellets. Saturated ammonium sulfate solution was added to the plasma to a final saturated concentration of 50%. After a gentle stirring for 30 min at room temperature, the precipitate was discarded by a centrifugation at 4000 *g* for 30 min at 4°C. The supernatant was then dialyzed at 4°C overnight against phosphate-buffered saline (PBS) containing 10 m*M* phosphate (pH 7.4) and 0.12 *M* NaCl. After dialysis, the solution was concentrated by Centricon (Amicon, Danvers, MA, USA) (centrifugation at 2000 *g* for 30 min) followed by an addition of ultrapure urea to 6 *M*. The final solution (100 μl) was applied to a Superose 12 HR column (30×1 cm I.D.) (Pharmacia Biotech, Sweden) pre-equilibrated with PBS using a Waters 600 HPLC controller system (Millipore, Milford, MA, USA). The flow-rate was 0.5 ml/min at room temperature and run for 40 min using PBS as a mobile phase. Fractions (0.5 ml) were collected using a Waters fraction collector (Millipore).

2.3. Gel electrophoresis and densitometry

One-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 12% polyacrylamide gel was performed according to Laemmli's method [14,15]. The samples (5–10 μg) for SDS–PAGE were preheated at 90°C for 8–10 min in a denaturation buffer [10 m*M* Tris–HCl, 5% (w/v) SDS and 5% (w/v) 2-mercaptoethanol, pH 7.6] before loading to the gel. For molecular mass calibration, a subset of the following standards (Bio-Rad Labs., Hercules, CA, USA) were included in each gel: myosin (M_r 200 000), β-galactosidase (116 000), phosphorylase B (97 000), bovine serum albumin (66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500) and lysozyme (14 400). After electrophoresis, the gels were removed and stained using Coomassie blue G-250.

The methodology used for two-dimensional (2D) PAGE analysis was performed according to the method of O'Farrel [16]. One-dimensional isoelectric focusing (IEF) gels were prepared using Bio-Rad ampholytes. Proteins were loaded onto pre-run IEF gels and run for 16 h at 400 V with an additional 1 h at 800 V. Subsequently, the IEF gels were loaded onto 9% SDS–polyacrylamide slab gels with a

4.75% stacking gel as the second dimension. Following SDS electrophoresis, the slab gels were processed as previously described [17]. Densitometric analysis of PAGE was performed using a Molecular Dynamics densitometer (Molecular Dynamics, Sunnyvale, CA, USA) for data acquisition and Image Quant software (Molecular Dynamics) for peak integration and analysis.

2.4. Preparation of antiserum

The polyclonal antibody against heavy chain (β) of porcine haptoglobin was raised in rats by subcutaneous and intramuscular injections of purified M_r 42 000 protein. To ensure the specificity of the antibody, the purified porcine haptoglobin was subjected to SDS–PAGE first. The gel containing the M_r 42 000 protein was sliced and homogenized in an equal volume of complete Freund's adjuvant by a tissue homogenizer. The emulsion was then injected into three female rats. After three weeks, a similar preparation was used for the booster injections with the addition of equal volumes of incomplete Freund's adjuvant. Ten days following the boost, blood was collected in 0.1% EDTA and antiserum were prepared.

2.5. 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 electrotransferred to a nitrocellulose membrane (Hybond-C extra, Amersham, Buckingham, UK) at 100 mA for 1 h in a semi-dry transfer cell (Pharmacia) containing a transfer buffer. The membrane was immersed in Tween-containing Tris-buffered saline (TTBS) [20 m*M* Tris–HCl, 0.5 m*M* NaCl, 0.05% (w/v) Tween 20 and 3% (w/v) gelatin, 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 [1:1000 dilution in TTBS containing 1% (w/v) gelatin] for 1 h at room temperature and washed three times with TTBS. The paper was then incubated with 1:2000 diluted antiserum against goat or rat immunoglobulin G (IgG) conjugated with horseradish peroxidase for 1 h in TTBS. Finally, the membrane was washed three times with TTBS and developed into a color immunoblot with TMB-stabilized substrate for horseradish peroxidase (Promega, Madison, WI, USA).

3. Results

Initially, an ammonium sulfate precipitation procedure was used to enrich the haptoglobin from porcine plasma. The SDS–PAGE profiles of total plasma, supernatant of 50% of saturated ammonium sulfate (SAS) fraction, and subsequent precipitate are

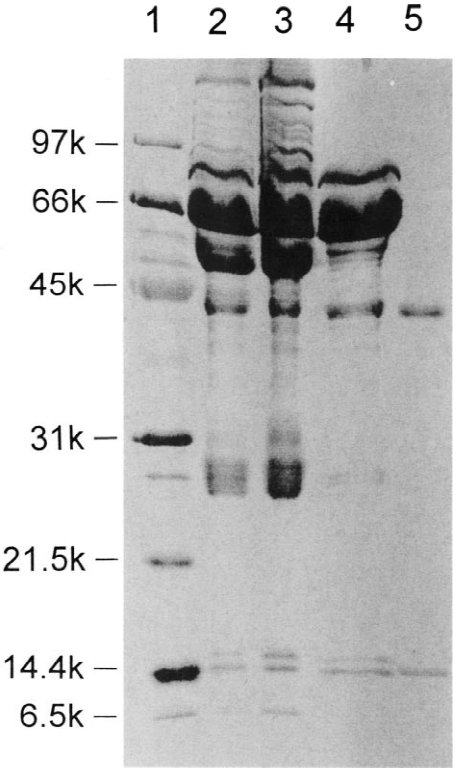

Fig. 1. SDS–PAGE profiles of purified porcine haptoglobin from each purification step. Lane 1: standard markers with molecular mass indicated on the left; lane 2: porcine total plasma; lane 3: precipitate of 50% saturated ammonium sulfate fraction; lane 4: supernatant of 50% saturated ammonium sulfate fraction; lane 5: purified haptoglobin from HPLC Superose 12 gel-permeation column containing β (M_r 42 000) and α (M_r 14 000) chains. SDS–PAGE containing 12% polyacrylamide was used in this preparation. For molecular mass standards see Experimental for more details. Each *K* value represents $M_r \cdot 10^3$.

shown in Fig. 1. Although both SAS fractions displayed the presence of the M_r 42 000 protein corresponding to the molecular mass of the β-subunit of haptoglobin, there were only five major proteins (with albumin as the most abundant one) presented in the supernatant fraction. The purification strategy was therefore focused on this supernatant fraction. The dialyzed supernatant was first concentrated using a Amicon filter followed by an addition of 6 *M* ultrapure urea and was then loaded on a Superose 12 gel-permeation HPLC column and eluted with PBS containing 0.01% NaN_3. A typical example of applying 2.5 mg of such a prepared supernatant onto the HPLC system is shown in Fig. 2. The protein eluted in the first peak (time 19–21 min) was collected and found to correspond, approximately, to M_r of 42 000 and 14 000 in SDS–PAGE (Fig. 1). These two protein moieties might represent porcine haptoglobin, since the SDS–PAGE employed here contained reducing reagents that can result in dissociation of the disulfide linkage between the β (M_r 42 000) and α (M_r 14 000) chains of haptoglobin.

It was also noted that the first major peak (time 19–21 min) was not obtained without 6 *M* urea treatment. Because human haptoglobin is known as an α-2 glycoprotein, we determined whether or not the isolated porcine M_r 42 000 protein consisted of the carbohydrate moiety. This protein was treated with neuraminidase, an enzyme known to cleave terminal sialic acid from an oligosccharide chain, to partially remove the terminal sialic acid. Fig. 3 shows the decrease in molecular mass of the treated M_r 42 000 protein in SDS–PAGE suggesting the presence of carbohydrate moiety in the isolate. The commercially available source of neuraminidase might contain minor protease activity, if any, which

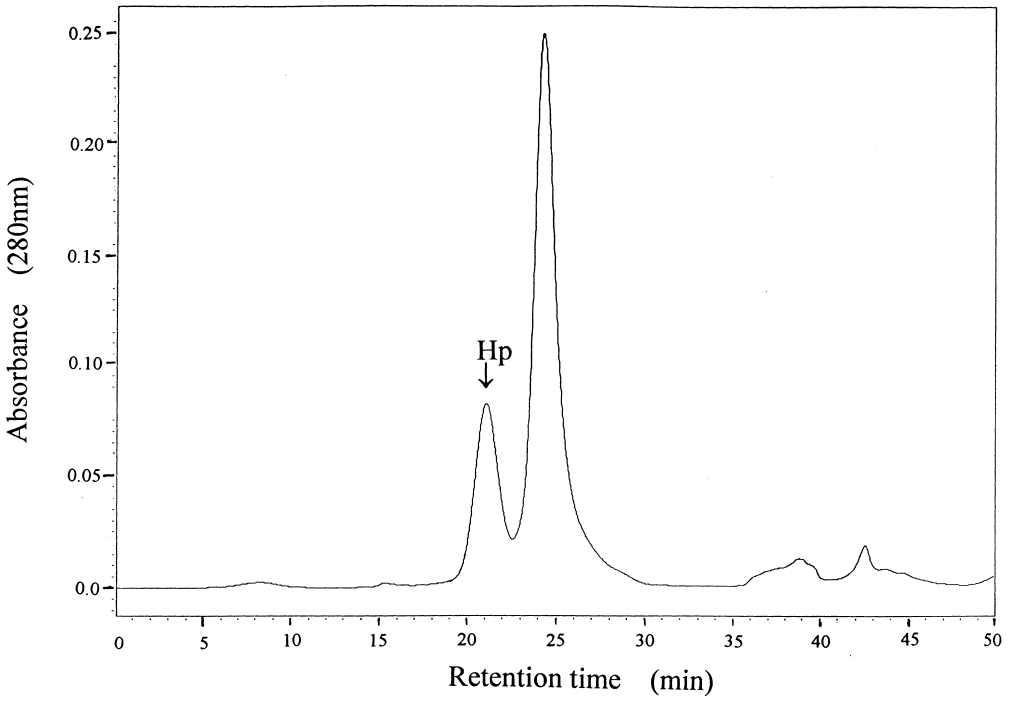

Fig. 2. Chromatographic profile of gel permeation using HPLC Superose 12 column (30×1 cm I.D.). About 2.5 mg protein (containing 6 *M* urea) of the dialyzed supernatant fraction of 50% saturated ammonium sulfate was applied onto the HPLC system. The chromatography was conducted at a flow-rate of 0.5 ml/min with a pressure of 1.8 MPa and run for 40 min at room temperature using PBS containing 0.01% NaN_3 as a mobile phase. Absorption was read at 280 nm simultaneously by a photodiode array detector. The minor peak at 15 min was an electronic signal, peak 20 (Hp) was the elution from the void volume as described in Results.

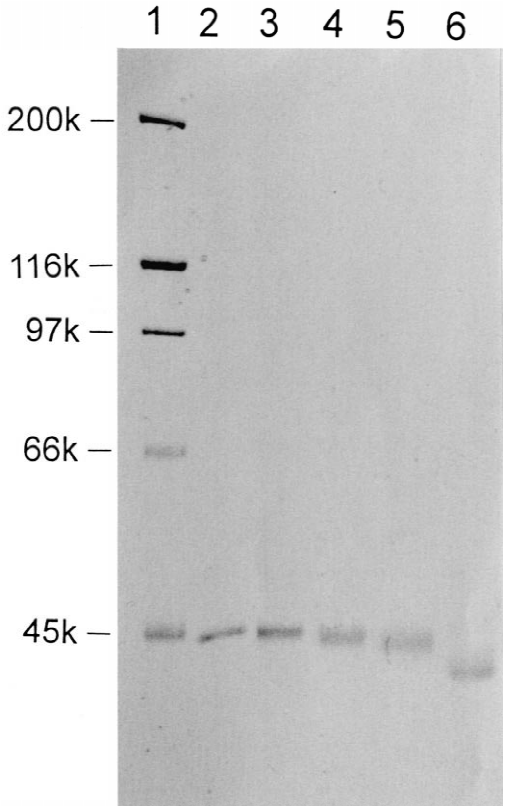

Fig. 3. Neuraminidase treatment of purified porcine M_r 42 000 protein. Lane 1: molecular markers; lane 2: purified porcine M_r 42 000; lanes 3, 4, 5 and 6: purified M_r 42 000 was treated with neuraminidase for 1, 2, 4 and 24 h, respectively. About 100 μg of isolated protein was treated with 1 μg of neuraminidase (specific activity 1 unit/mg) in 100 μl of PBS at room temperature. The tested sample was collected over time as indicated and the reaction for each sample was stopped immediately by adding SDS–PAGE loading buffer followed by heating at 90 for 30 min. Each *K* value in the molecular mass standards represents the $M_r \cdot 10^3$. SDS–PAGE containing 9% polyacrylamide was used.

might result in a non-specific cleavage of the M_r 42 000 protein. To rule out this possibility, we demonstrated that under the same experimental conditions, non-glycoproteins such as albumin did not show any decrease in molecular mass (data not shown). To identify the chemical nature of the porcine M_r 42 000 protein, the protein (Fig. 1) was eluted directly from the 1D and 2D gels and its amino acid sequence was determined. The first 20 amino acid sequence of N-terminus is shown in Table 1. Significant sequence homology was found among the porcine M_r 42 000 protein, the β-chain of human, bear, dog, cat, rabbit and bovine haptoglobins.

We further prepared rat antibody against this M_r 42 000 chain eluted from 2D SDS–PAGE as an antigen (Fig. 4A). The homogeneity of the M_r 42 000 chain was demonstrated in Fig. 4A as compared to that in whole porcine plasma in 2D gel (Fig. 4B). The antibody raised shows a single specificity against the M_r 42 000 protein on Western blot analysis (Fig. 5A) from whole porcine plasma and HPLC-purified protein. Using commercially available antibody against human haptoglobin, the immunoblot shows one heavy chain and light chain on whole porcine plasma and HPLC-purified protein (Fig. 5B). However, there was one extra-band with an apparent M_r of 55 000 when whole porcine plasma was blotted with anti-human haptoglobin antibody (Fig. 5B, lane 2), presumably it was due to the non-specificity from the commercially prepared antibody. Since immunochemically, antibody against human haptoglobin is known to cross react with porcine antigen [18] and vice versa our rat antibody prepared against the M_r 42 000 chain also reacts with human haptoglobin. Thus, the purified protein was

Table 1
N-Terminal amino acid sequence of isolated porcine β chain with M_r 42 000 (42 *K*) and its homology with haptoglobin β-chain from other species[a]

Porcine 42 K	I M G G S L D A K G X F P W Q A K M I S
Human	I L G G H L D A K G S F P W Q A K M V S
Bear	I M G G S V D A K G S F P X Q A K M V S
Dog	I M G G S V D A K G S F P W Q A K M V S
Cat	I M G G S V D A K G S F P W Q A K M V X
Rabbit	I I G G S L D A K G S F P W Q A K M V S
Bovine	I I G G S L D A K G S F P W Q

[a] Underline represents the identical sequence with isolated porcine M_r 42 000 protein (42 *K*).

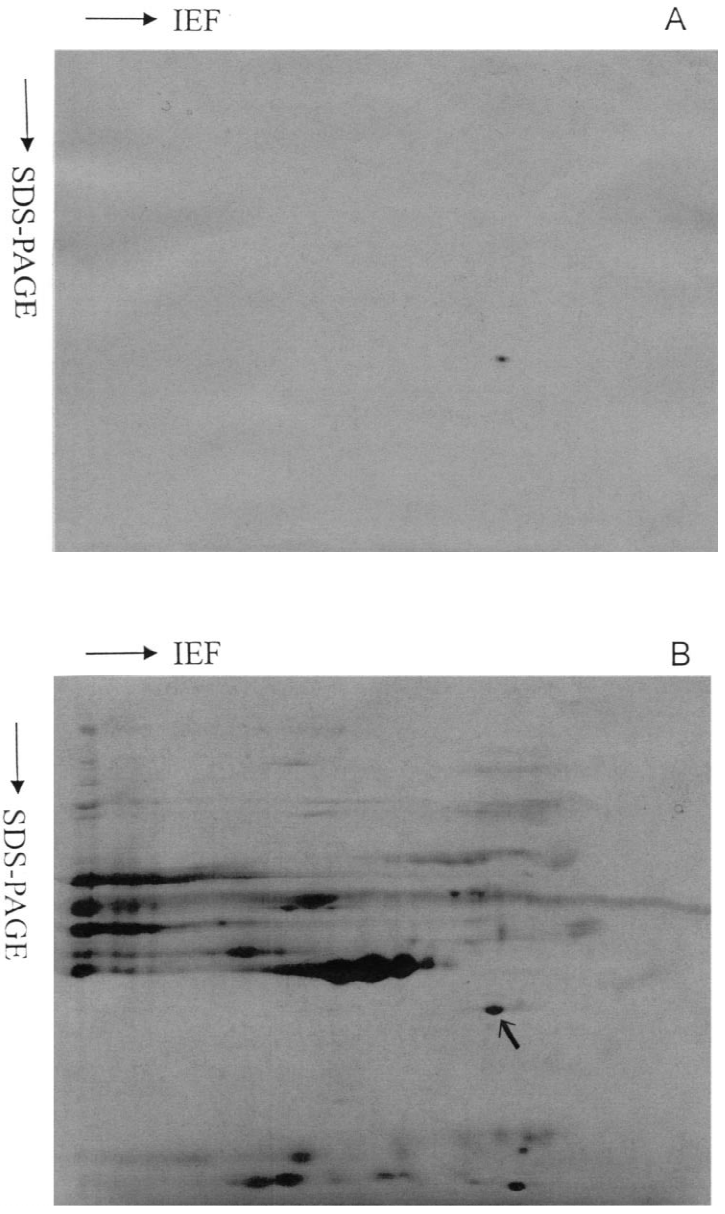

Fig. 4. Two-dimensional electrophoresis of purified porcine haptoglobin β chain (A) as compared to that of whole plasma (B). The location of isolated or plasma M_r 42 000 protein is indicated as an arrow which shows a p*I* value of approximately 4.8–5.2.

identified as porcine haptoglobin based on the similarities of its N-terminal sequence and its immunochemical properties to human haptoglobin.

Based on the β-subunit, Table 2 shows the theoretical yield of purified porcine haptoglobin calculated from the gel-scanning densitometry. Greater than 98% purity of the isolated protein was achieved. The final analytical recovery was about 45.0% with a 10-fold purification.

Because the major isolation procedure was simple

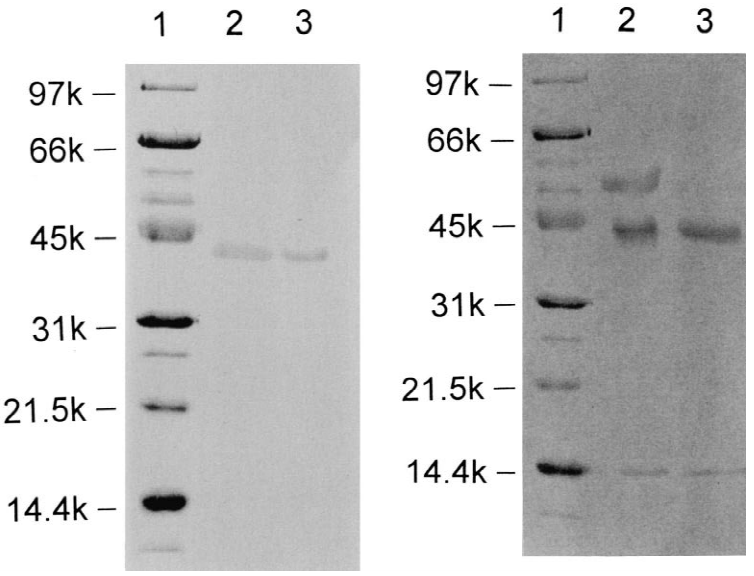

Fig. 5. Western blot analysis on porcine haptoglobin using a rat antibody prepared against porcine haptoglobin β chain and a commercially available antibody against human haptoglobin. (A) Lane 1: standard markers with molecular mass indicated on the left; lanes 2 and 3: immunoblot of rat anti-porcine haptoglobin β chain with porcine plasma and purified porcine haptoglobin, respectively. (B) Lane 1: standard markers with molecular mass indicated on the left; lanes 2 and 3: immunoblot of goat anti-human haptoglobin with porcine plasma and purified porcine haptoglobin, respectively.

and can be achieved within few hours, we recommend that this procedure should be widely used for the purification of porcine haptoglobin.

4. Discussion

The acute phase serum protein response to infection, inflammation or trauma has been identified in a number of species and consists of alterations to the serum concentrations of several proteins. It is known that the profile of acute phase protein response to stimulation differs between species. In pig, individual proteins have been identified as acute phase proteins in association with infection or pathological lesion [19]. Acute inflammation can be induced in pigs using a single subcutaneous turpentine injection. The concentration of haptoglobin increases five- to seven-times 48 h after a single injection [20]. Moreover, since pig model has been popularly and widely used in studying the pathogenesis of human disease, it is of worthy to isolate porcine haptoglobin for the studies related to its potential role in inflammatory diseases.

Methods designed for the isolation of human have been found insufficient when applied to pig plasma

Table 2
Analytical recovery of porcine plasma haptoglobin purified from each step

Fraction	Total protein yield (mg)	Purity (%)	Theoretical amount of haptoglobin (mg)	Final yield (%)	Fold purification
Plasma	24.5	9.8	2.40	100.0	–
50% SAS[a]	7.9	14.0	1.11	46.3	1.4
HPLC	1.1	98.5	1.08	45.0	10.1

[a] Supernatant fraction of 50% saturated ammonium sulfate.

due to the formation of a material identified as albumin dimer [21]. Small-scale separation is possible by preparative PAGE, but is time consuming [21]. Prior to the development of purification procedure, we have used DEAE-, CM- and other gel-permeation HPLC columns such as TSK G6000 PW for separation of molecular masses between 2000 and 100 000; and yet none of these procedures were able to successfully isolate porcine haptoglobin (data not shown). Presumably, this was due to the interaction (complex formation) of haptoglobin with other serum proteins [22]. It is not known, however, if silica-based (normal-phase) columns would result in a better separation. The Superose 12 column used in this report is, however, designed for the isolation of macromolecules with molecular mass greater than $1 \cdot 10^6$, such as serum lipoproteins [23–25] and other plasma proteins with large molecular mass.

The mechanism by which urea treatment succeeded the purification is not readily clear, but it would be of interest to be delineated. In summary, the purification approach reported in this paper represents a significant improvement for the isolation of porcine haptoglobin from the conventional methods.

Acknowledgements

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