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Analysis by Enzyme-Linked Immunosorbent Assay and 2-Dimensional Electrophoresis of Haptoglobin in the High-Density Lipoprotein Fraction in Cows

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ABSTRACT. Haptoglobin (Hp) is a hemoglobin (Hb)-binding acute-phase protein. Besides its relevance in inflammation, Hp is involved in the regulation of lipid metabolism. In cattle, in addition to the lipoprotein-deficient fraction, Hp is distributed in high-density lipoprotein (HDL) and very high-density lipoprotein (VHDL) fractions. The purpose of this study was to determine Hp concentrations in the lipoprotein fractions using an enzyme-linked immunosorbent assay (ELISA) based on the affinity with Hb, and also to detect structural differences of HDL Hp from that in the lipoprotein-deficient fraction using 2-dimensional electrophoresis. When purified Hp was used as the antigen for the ELISA, the detection limit was 7.4 *n*g/m*l* and linearity was obtained from 14.8 to 475 *n*g/m*l*. The correlation coefficient between the ELISA and single radial immunodiffusion was 0.884. The ELISA was shown to be applicable to evaluate Hp concentrations in the lipoprotein fractions. Hp concentrations in the lipoprotein fractions were in the range of 0.94 to 8.77 μg of Hp/m*l* (n=4), and concentration ratios were 0.2 to 0.3% of whole serum Hp. Of the lipoprotein fractions, Hp was most abundant in HDL, moderate in VHDL and faint in chylomicrons, the very low-density lipoprotein fraction and low-density lipoprotein fraction. By 2-dimensional electrophoresis, α- and β-chains of serum Hp were each separated into 5 spots, and their isoelectric point (pI) values were from 5.05 to 6.28 in the α-chain and from 5.92 to 6.95 in the β-chain. The pI values of HDL Hp were indistinguishable from those of serum Hp. These results indicate that the ELISA based on the affinity with Hb is useful for evaluating Hp concentrations in lipoprotein fractions, and also suggest that HDL Hp is structurally similar to that in the lipoprotein-deficient fraction.

KEY WORDS: bovine, enzyme-linked immunosorbent assay, haptoglobin, high-density lipoprotein, 2-dimensional electrophoresis.

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Haptoglobin (Hp) is an acute-phase protein that has an ability to bind with hemoglobin (Hb). Besides its relevance in inflammation, Hp is involved in the regulation of lipid metabolism. Hp is synthesized by adipocytes as well as by the liver [4]. In addition to the presence in the lipoprotein-deficient fraction, Hp is distributed in the lipoprotein fractions, and has an affinity to apolipoprotein (apo) A-I, a major apoprotein in high-density lipoprotein (HDL) [9, 15]. Hp acts as an anti-oxidant for low-density lipoprotein (LDL) [11]. Moreover, human Hp subtype patterns significantly correlate with serum lipid concentrations [2]. In cattle, Hp is detected in serum from cows with fatty liver [14, 23], and is induced by fatty liver-producing agents, ethionine [18] and glucocorticoids [5, 24]. We have recently shown that Hp [8] and Hb [8, 21] are distributed in the lipoprotein fractions, particularly in HDL and very high-density lipoprotein (VHDL) fractions, and concentration ratios of Hp in the lipoprotein fractions are 0.2 to 0.3% of total serum Hp, as indicated by densitometric scanning of immunoblotting.

The serum Hp concentration has been determined by single radial immunodiffusion (SRID) [12, 24] and enzyme-linked immunosorbent assay (ELISA) [14, 17]. SRID is too insensitive to determine the Hp concentrations in the lipoprotein fractions. The ELISA, in which test serum is directly coated on microplates, is sensitive, but its reliability is influenced by the serum albumin concentration [14]. An ELISA based on affinity with Hb has been developed [25]. In this method, Hb is initially coated on microplates, and Hp in serum specifically binds to Hb attached to the surface of microplate. This method seems to be superior to the previous ELISA in reducing the interference by other serum proteins such as albumin. The purpose of the present study was to examine whether the ELISA based on the affinity with Hb could be applicable to evaluate lipoprotein Hp concentrations. We next compared the isoelectric point (pI) values of α- and β-chains of HDL Hp with those of serum Hp, to assess structural differences between Hp species in HDL and the lipoprotein-deficient fraction.

MATERIALS AND METHODS

Purification of Hp: Sera from 27 Holstein cattle were used. All sera were examined by SRID, and sera from 10 cows with infectious bovine rhinotracheitis, 2 cows with mastitis and 9 calves with experimental pneumonia [8, 13] were Hp-positive, whereas sera from 3 cows during midlactation and 3 calves (1 to 3 months old) were Hp-negative. Sera from Hp-positive cows were mixed, and 100 m*l* of the mixture was used to purify Hp. The purification procedures included ammonium sulfate fractionation and sequential chromatographies with DE-52 (Whatman International Ltd., Maidstone, Kent, UK), Sephacryl S-200 (Amersham Phar-

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macia Biotech, Little Chalfont, Backinghamshire, UK) and CM-52 (Whatman International Ltd.) as described previously [23]. The purity of Hp was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 12% constant gel and following Coomassie Brilliant Blue staining. Anti-bovine Hp was raised in rabbits. Immunoblot analysis was done as described previously [20]. The 2nd antibodies used were goat anti-rabbit IgG serum conjugated to horseradish peroxidase (ICN Pharmaceutical Inc.-Cappel Products, Costa Mesa, Calif, U.S.A.). Bands for Hp were detected by a chemiluminescense reagent (ECL, Amersham Pharmacia Biotech). The detection limit was approximately 10 *n*g of Hp under the conditions used [8].

Enzyme-linked immunosorbent assay: This was performed essentially as reported [25]. Briefly, microplates were coated with 200 μl of 50 mM sodium carbonate buffer (pH 9.6) containing 20 μg sheep Hb (Sigma Chemical Co., St Louis, Mo, U.S.A.), and incubated overnight at 4°C. After washing 6 times with 100 μl of phosphate-buffered saline (PBS) containing 0.05% Tween 20, 100 μl of serially diluted (twofold) purified Hp, serum or HDL was added to each well and incubated for 90 min at 37°C. Wells were washed 6 times with PBS/Tween 20, then incubated with 100 μl of anti-Hp rabbit serum, which had been diluted 5,000-fold with PBS containing 0.3% gelatin, for 45 min at 37°C. After being washed 6 times with PBS/Tween 20, 100 μl of anti-rabbit IgG goat serum conjugated to alkaline phosphatase (ICN Pharmaceutical Inc.-Cappel Products; diluted 2,000-fold with PBS/gelatin) was added. The plates were incubated for 45 min at 37°C, washed 6 times with PBS/Tween 20, and developed for 45 min by use of an Alkaline Phosphatase Substrate Kit (BioRad Laboratories, Hercules, Calif, U.S.A.). Absorbance was measured at 405 nm, using a microplate reader.

Two-dimensional electrophoresis: Serum (diluted 100-fold with PBS) or undiluted HDL was mixed with an equal volume of 9.5 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, 2.5% Bio-Lyte 5/7 (BioRad Laboratories) and 2.5% Bio-Lyte 3/10 (BioRad Laboratories), and incubated for 15 min at room temperature. The treated sample (100 μl) was applied to an Immobiline Dry Strip (pH 3-10L; Amersham Pharmacia Biotech), and electrophoresed for the 1st dimension, as directed in the manufacturer's manual. The 2nd dimension was SDS-PAGE using a 12% gel. Spots were detected by use of silver staining (BioRad Laboratories) or immunoblot analysis. The pI values were determined by simultaneous running of pI markers (BioRad Laboratories, pI 4.5–8.5).

Other methods: Chylomicron (CM)-very low-density lipoprotein (VLDL, d<1.006), LDL (d<1.063), HDL (d<1.21), VHDL (d<1.25) and the lipoprotein-deficient fraction (d>1.25) were prepared from 4 m*l* each of cow and calf sera, as described previously [8]. The lipoprotein fractions were dialyzed against PBS. The SRID was carried out as described [24]. Protein concentration was determined by the method of Lowry [10].

RESULTS

Purified Hp was free from apparent contaminants as indicated by SDS-PAGE and immunoblot analysis (Fig. 1). In the sheep Hb-coated ELISA, absorbances were increased in a dose-dependent manner when purified Hp or Hp-positive serum was used as the antigen, but not when Hp-negative serum was added (Fig. 2A). Bovine or human Hb was also used under the same condition (20 μg of Hb/200 μl). In these conditions, absorbance obtained at 1.9 μg/m*l* Hp and 250-fold-dilution of Hp-positive serum was approximately half that of the sheep Hb. Although the mechanistic basis for the preference of sheep Hb in the bovine Hp ELISA is not known, sheep Hb was used for the coating material because of its higher sensitivity. The standard curve for purified Hp is shown in Fig. 3. A significant increase of the absorbance compared with the blank value (0.118 ± 0.01) was observed at 7.4 *n*g/m*l* (0.170 ± 0.01). The curve was practically linear from 14.8 to 475 *n*g/m*l*. The accuracy of the standard curve was assessed by measuring the coefficient of variance (CV) at 9 Hp concentrations ranging from 7.4 to 1,900 *n*g/m*l*. The intra- and inter-assay CV values (means of 5 to 10 estimations at each concentration) were in the ranges of 2.3 to 7.6% and 3.6 to 13.0%, respectively. Addition of bovine serum albumin (up to 60 μg/m*l*) had no effect on the standard curve. In the standard curve for serum Hp (not shown), the intra- and inter-assay CV values were 5.8 to 9.1% and 8.9 to 15.0%, respectively. Three concentrations (68.8, 91.7 and 226 *n*g/m*l*) of purified Hp were added to 2,000-fold-diluted serum containing 105 *n*g/m*l* of Hp. The recoveries of the added Hp were from 110 to 128%. Using 21 Hp-positive sera, the correlation coefficient (r) between the ELISA and SRID methods was calculated to be 0.884.

When HDL Hp was used as the antigen, absorbance was increased with increased concentrations of HDL from Hp-positive cow serum, but not with HDL from Hp-negative serum (Fig. 2B). The intra- (n=9) and inter-assays (n=5) at 9 concentrations of HDL Hp showed that the CV values were 4.9 to 8.0% and 9.0 to 14.9%, respectively. To 8-fold-diluted HDL containing 110 *n*g/m*l* of Hp, 67.1 or 147 *n*g/m*l* of purified Hp was added, and their recoveries were 110 and 129%, respectively. By use of the ELISA, 0.94 to 8.77 μg of Hp (0.2 to 0.3 % of whole serum Hp) were found to be distributed in CM-VLDL, LDL, HDL and VHDL fractions (Table 1), and these values were comparable to those obtained by immunoblot analysis in the present and previous studies [8]. Other than the lipoprotein-deficient fraction, Hp was most abundant in HDL, moderate in VHDL, and faint in CM-VLDL and LDL fractions. The mode of distribution in the lipoprotein fractions was not largely different between different diseases and Hp concentrations.

Two-dimensional electrophoresis and subsequent immunoblot analysis revealed that the 23 kDa α-chain and the 35 kDa β-chain of purified Hp were each separated into 5 spots (Fig. 4A). Their pI values and density ratios (%; n=4) were: α-1, 5.05 (6.9%); α-2, 5.30 (19.8%); α-3, 5.60 (36.6%); α-4, 5.95 (30.6%); and α-5, 6.28 (6.1%); β-1, 5.92 (7.8%); β-2,

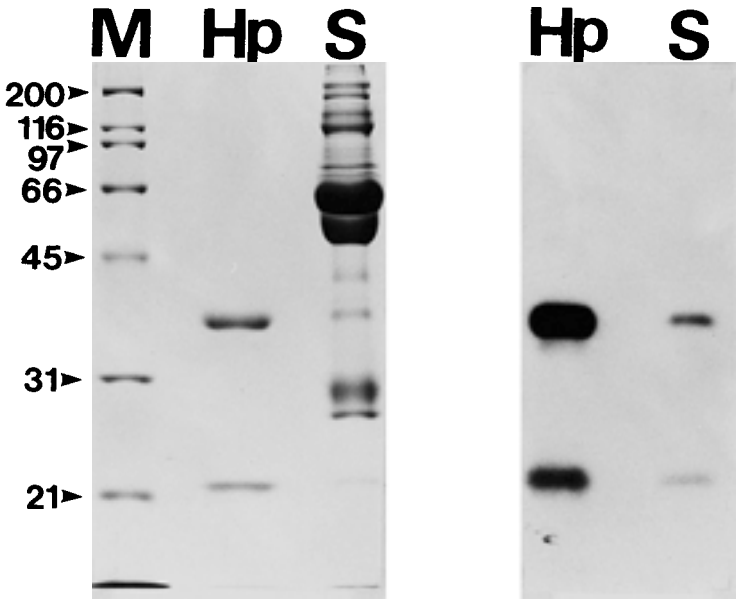

Fig. 1. Purity of Hp as indicated by Coomassie Brilliant Blue staining (left) and immunoblot analysis (right). M, molecular mass markers; Hp, purified Hp; and S, Hp-positive cow serum. Molecular weights are shown in kDa. The amounts of Hp used were 551 *n*g for the staining and 110 *n*g for the blotting. Serum was diluted 20-fold with PBS, and a 10 μl aliquot was applied.

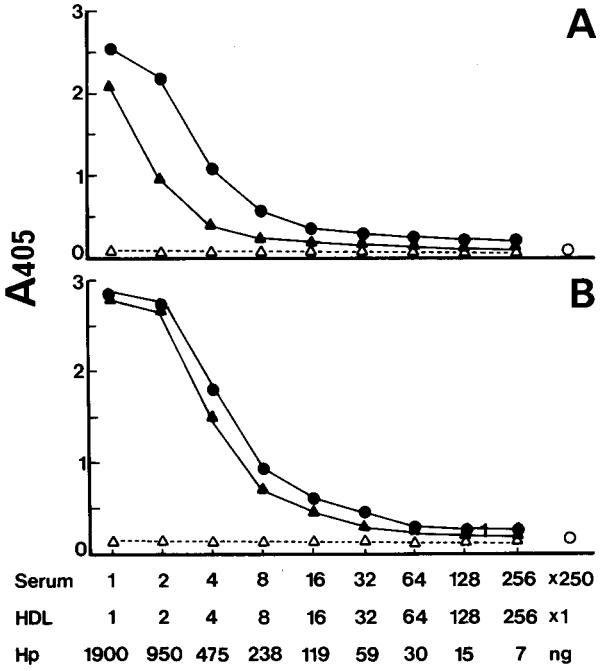

Fig. 2. ELISA of purified Hp, Hp-negative and Hp-positive cow sera (A), and HDL fractions from Hp-negative and Hp-positive cow sera (B). To the sheep Hb-coated wells, 0.1 m*l* of serially diluted purified Hp (●), Hp-negative serum (△), serum containing 285 μg of Hp/m*l* (▲), Hp-negative (△) and Hp-positive (▲) HDL fractions were separately added. To wells for blanks (○), 50 mM sodium carbonate buffer (pH 9.6) was added.

6.14 (15.8%); β-3, 6.39 (22.5%); β-4, 6.65 (30.4%); and β-5, 6.95 (23.6%). The separation into 5 spots of both α- and β-chains was reproduced when Hp-positive cow serum was analyzed by 2-dimensional electrophoresis and subsequent immunoblotting or silver staining (Fig. 4B, C). Their pI values and density ratios were essentially the same as those in purified Hp. The analysis of Hp-positive calf sera gave comparable results (figure not shown).

The spots of α- and β-chains in HDL from Hp-positive cow sera were shown by silver staining (Fig. 5A and B), but not by immunoblotting (figure not shown), probably because of the low Hp contents in this fraction. Spots for α-3, α-4 and α-5 were detected and showed pI values indistinguishable from those of purified Hp, whereas spots corresponding to α-1 and α-2 were masked by interference by a large spot of apoA-I (identified by immunoblot analysis using anti-apoA-I). All 5 spots for the β-chain of HDL were clearly detected. The 5 spots had nearly identical pI values and, moreover, except for β-1 (it was only faintly detected), their density ratios were similar to those in the purified Hp and serum Hp. The Hp-positive HDL was electrophoresed together with purified Hp (Fig. 5C). Although the presence of α-1 and α-2 was still obscure, the other HDL Hp spots increased in density, showing that their pI values were essentially identical to those of purified Hp. The 2-dimensional electrophoretic patterns of calf HDL were similar to those of cow HDL (not shown).

Table 1. Concentrations of Hp in the lipoprotein fractions from cows with mastitis and calves with pneumonia

HDL from	CM-VLDL	LDL	HDL	VHDL	LP-d
Hp-negative					
ELISA	<0.01	<0.01	<0.01	<0.01	<0.01
IB	ND	ND	ND	ND	ND
Hp-positive					
ELISA	<0.01	0.04 (0.011)	0.88 (0.252)	0.02 (0.007)	350(99.7)
IB	ND	ND	0.165	ND	99.8
Mastitis (*E. coli*)					
ELISA	0.02 (0.001)	0.02 (0.001)	1.90 (0.109)	1.13 (0.064)	1,750 (99.8)
IB	ND	ND	0.296	0.196	99.5
Mastitis (*Klebsiella* spp.)					
ELISA	0.06 (0.002)	0.07 (0.002)	5.33 (0.180)	3.32 (0.112)	2,950 (99.7)
IB	ND	ND	0.251	0.089	99.5
Pneumonia					
ELISA	<0.01	0.13 (0.012)	1.95 (0.184)	0.77 (0.072)	1,056 (99.7)
IB	ND	ND	0.196	0.121	99.7

The lipoprotein fractions were separately prepared from the 5 sera. The Hp-negative and Hp-positive sera were mixtures from 3 and 12 cows, respectively. Hp concentration is μg/m*l* and numbers in parenthesis are % distribution of Hp in the lipoprotein and lipoprotein-deficient fractions. Densitometric data of immunoblot analysis (IB) are expressed as %. LP-d, lipoprotein-deficient fraction; ND, not detected.

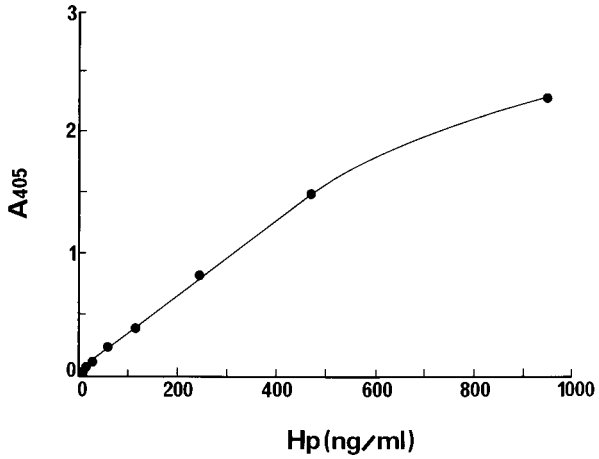

Fig. 3. Standard curve of Hp. Data for purified Hp in Fig. 2 were replotted.

DISCUSSION

The present study indicated that the ELISA method based on the affinity with Hb could be applicable to evaluate Hp concentrations in the lipoprotein fractions. Concentrations of Hp in the lipoprotein fractions determined by the ELISA were comparable to those obtained by immunoblot analysis. This study also indicated that α- and β-chains of Hp showed polymorphism with respect to pI values. Evaluation of pI values of HDL Hp (although data for α-1 and α-2 were lacking) suggested that HDL Hp is structurally similar to serum Hp.

We have previously determined the serum Hp concentration by an ELISA in which serum was directly coated on the microplate [17]. However, this method showed great variability [25] and, moreover, was influenced by the concentration of serum albumin [14]. The ELISA used in the present study was sensitive and was not influenced by the concentration of serum albumin. The VHDL fraction contains the bulk of serum albumin [8]. However, VHDL Hp concentrations determined by this ELISA were not largely different from those obtained by immunoblotting. The present results provided evidence that this ELISA was a useful tool to determine the lipoprotein Hp concentration, which is prerequisite for forthcoming studies on functional roles of lipoprotein Hp. This ELISA method appears to be applicable for determination of Hp concentrations in other biological fluids such as cell culture medium. In the culture of hepatocytes, serum albumin is secreted into the medium and, moreover, is used as a carrier of fatty acids.

The pI values of α- and β-chains in cattle Hp appeared to be similar to those in mice [1] and humans [2], although their pI values were not precisely described. Our purpose for determination of pI values and concentration ratios of the separated spots was to examine whether HDL Hp was structurally different from that in the lipoprotein-deficient fraction. The obtained results appear to indicate that HDL Hp is similar, at least with respect to molecular sizes and charges to that in the lipoprotein-deficient fraction. In calves with experimental pneumonia, the HDL Hp concentration concomitantly increases as the serum Hp concentration increases [8]. Previous results, coupled with the present findings, suggest that Hp is associated with lipoprotein particles after it is induced and released into plasma. In addition to the liver, Hp is synthesized by the lung [22], uterus [7] and adipocytes [4]. An analysis of charge differences of Hp species in different tissues and cells may be helpful to identify the origin of lipoprotein Hp. In humans, there are several subtypes related to the polymorphism in the α-chain, and these subtypes show different pI values [2]. The presence in cattle of the subtypes has not yet been demonstrated.

The functional relevance of lipoprotein Hp remains unan-

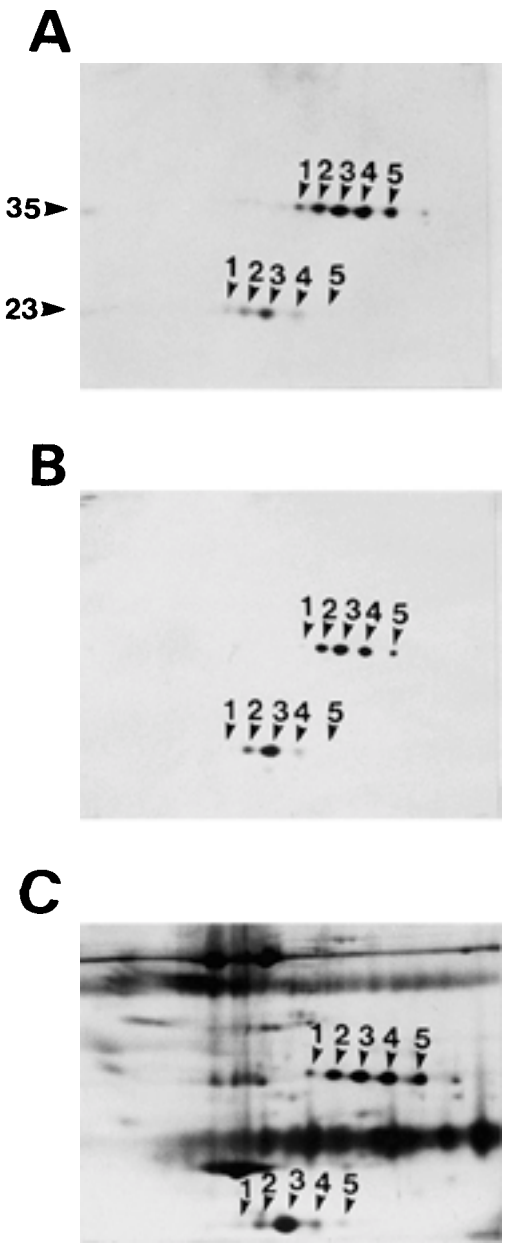

Fig. 4. Two-dimensional electrophoresis of purified Hp (A) and Hp-positive cow serum (B and C). Purified Hp (2.38 μg/50 μl) and 50 μl of 100-fold-diluted serum were treated and applied to a strip as described in MATERIALS AND METHODS. Hp was detected by immunoblot analysis (A and B) and by silver staining (C). In the left side of A, Hp was run by 1-dimensional SDS-PAGE and the numbers show molecular masses of the α-chain (23 kDa) and β-chain (35 kDa) of Hp.

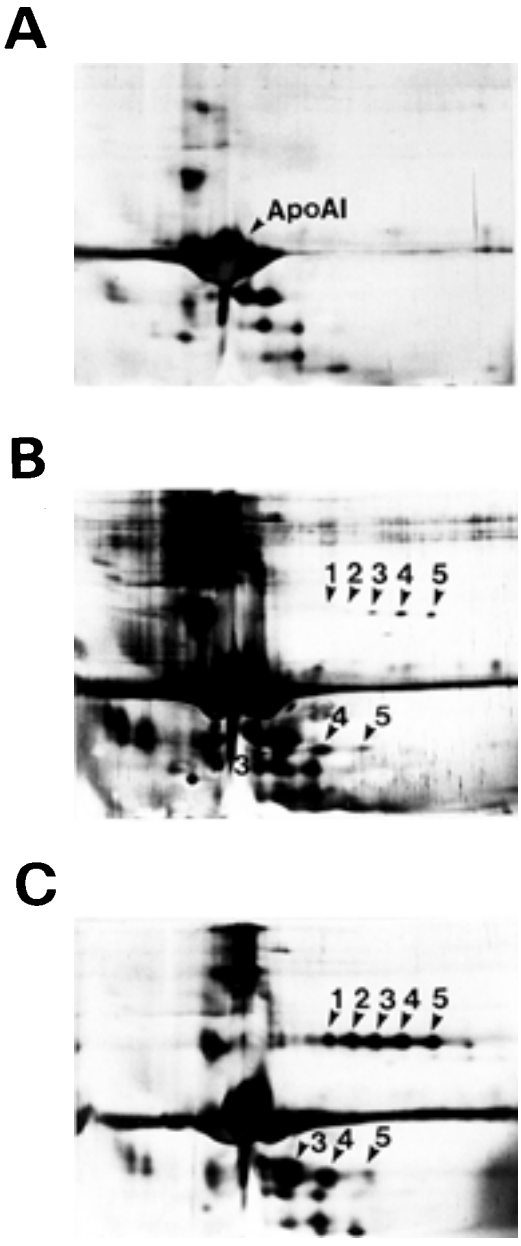

Fig. 5. Two-dimensional electrophoresis of Hp-negative HDL (A), Hp-positive HDL (B) and Hp-positive HDL with purified Hp added (C). Hp (2.38 μg) and 50 μl of undiluted HDL fractions from cow sera were applied. Hp was detected by silver staining.

swered. One possible way to elucidate the functional role of Hp is to examine its concentration in the lipoprotein fractions during the peripartum period. During this period, Hp is induced [19]. Cows during this period are particularly susceptible to infectious diseases such as mastitis attributable to fatty liver developed during the nonlactating stage [6]. In cows with fatty liver, concentrations of lipoprotein lipids and apoproteins including apoA-I are decreased [14]. The lipoprotein Hp, singly or in combination with Hb and/or apoA-I, may act as a bacteriostat [3] or, although controversial, an inhibitor for neutrophil functions [16]. The elucidation of the functional relevance of lipoprotein Hp may shed light on the

mechanism of the cross talk between the acute-phase response and lipid metabolism.

In conclusion, the Hp ELISA based on the affinity for Hb was shown to be useful to evaluate Hp concentration in the lipoprotein fractions, and 0.2 to 0.3% of total serum Hp was distributed in the fractions, particularly in HDL and VHDL fractions. The HDL Hp was suggested to be derived from the lipoprotein-deficient fraction, because of the indistinguishable pI values of α- and β-chains from those of serum Hp.

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