ウシα1酸性糖蛋白(α1-acid glycoprotein;α1 AG)の分離精製,性状及び定量について

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Isolation, Characterization, and Quantitative Measurement of Serum α_1-Acid Glycoprotein in Cattle

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ABSTRACT. Bovine α_1-acid glycoprotein (α_1AG) was purified from pooled normal bovine sera by successive ammonium sulfate precipitation, ion-exchange chromatographies and gel filtration. Bovine α_1AG had a molecular weight of 42,000±2,000 and a sedimentation coefficient of 3.4S. It contained 26.6% carbohydrate. Gel isoelectric focusing revealed a microheterogeneity with 7 to 8 bands in a pI range of 3.2 to 3.7. It migrated to the α_1-globulin region upon immunoelectrophoresis. Single radial immunodiffusion was developed for the quantitative measurement of bovine α_1AG in serum. The mean serum value of α_1AG in 152 healthy Holstein cattle (1–12 years old) was 283.2±82.3 μg/m*l*. Elevated values (cut-off value=450μg/m*l*) were observed in cattle with traumatic pericarditis (100%), arthritis (100%), mastitis (91%), pneumonia (70%), and mesenteric liponecrosis (43%).—KEY WORDS: Alpha$_1$-acid glycoprotein, bovine serum, single radial immunodiffusion.

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Alpha$_1$-acid glycoprotein (α_1AG) is one of the most extensively studied glycoproteins isolated from plasma of human and various animals [1, 3, 9, 18, 21]. It is distinguished from other glycoproteins because of its several unique properties, such as very acidic isoelectric points, and a high solubility in water and certain polar organic solvents [18]. Complete amino acid sequence of human α_1AG was determined [16] and the carbohydrate structure was reported [22].

α_1AG increases as one of the acute phase reactant proteins in human and animal sera in conditions such as inflammation [5, 11], neoplasma [6, 20], or trauma [14]. While several investigators have isolated α_1AG from bovine sera [1, 3, 9, 21], little is known about the concentration of serum α_1AG in healthy and diseased cattle.

This paper describes the procedure suitable for obtaining highly purified bovine serum α_1AG and the development of a single radial immunodiffusion (SRID) assay for measuring bovine serum α_1AG. The levels of serum α_1AG were studied in healthy and diseased cattle.

MATERIALS AND METHODS

Serum samples: Total 166 healthy Holstein cattle sera comprising of 95 from bulls aged 1 to 11 years old, 57 from daily cows aged 2 to 12 years old and 14 from calves aged 3 months were used. They were collected at the livestock hygiene service centers or abattoirs in Miyagi and Iwate prefectures. Serum samples were also collected from 59 Holstein cows (1–8 years old) that were diagnosed as having traumatic pericarditis (n=9), mastitis (n=22), arthritis (n=8), pneumonia (n=10), leukemia (n=3) and mesenteric liponecrosis (n=7) by meat-inspecting or practicing veterinarians. Animals were bled from the external jugular vein and sera were stored at −20°C until use.

Isolation of bovine α_1AG: Bovine serum

α_1AG was purified by a procedure described previously [1, 3, 21] with minor modifications. Solid ammonium sulfate was added to a pool of healthy Holstein sera (7.5 litters) to make 60% saturation at pH 7.0. From the supernatant, α_1AG was precipitated by the further addition of solid ammonium sulfate for 90% saturation. The precipitate was dialyzed against 20 mM sodium acetate buffer (pH 4.3) at 4°C, and was applied on a DEAE-cellulose column (6 × 18 cm) equilibrated with the same buffer. A stepwise elution was carried out, and fractions containing α_1AG were eluted with 0.5 M NaCl. The fractions were dialyzed against 20 mM sodium acetate buffer (pH 4.3), and applied on a CM-cellulose column (6 × 18 cm) equilibrated with the same buffer. The pass-through fraction was lyophilized. The resulting fraction (100 mg protein) was dissolved in 2 ml of 20 mM tris-HCl buffer (pH 7.4), and was applied on a Sephacryl S-200 (Pharmacia, Sweden) column (2.6 × 120 cm) at a constant flow rate of 13.2 ml/hr. The second major peak with a molecular weight of about 50,000 was collected, dialyzed against distilled water and lyophilized.

Detection of serum α_1AG: Serum α_1AG and α_1AG at each purification step were identified by the thin layer gel isoelectric focusing (G-IEF) technique as described [19]. Neocarzinostatin (Kayaku Co., Tokyo) with pI 3.3 was used as pI marker.

Physicochemical analysis of α_1AG: The molecular weight of purified α_1AG was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The $S_{20w}°$ value for α_1AG was determined by the sucrose gradient method. The amino acid content of purified α_1AG was analyzed by amino acid analyzer (Nippon-Bunko, Tokyo) after hydrolysis in 6N HCl for 6 hr at 110°C. The carbohydrate content of α_1AG was determined by using gas chromatographic method [4] after methanolysis for 24 hr at 90°C. Protein concentration was determined by the Folin-Ciocalteu method using bovine serum albumin as the standard.

Preparation of antisera: Rabbits were immunized with purified bovine α_1AG emulsified with Freund's incomplete adjuvant.

Determination of serum α_1AG: Serum α_1AG was determined by SRID method of Mancini [12] with minor modifications: agarose gel containing anti-bovine α_1AG rabbit sera was prepared on a plastic container and 2.5 mm-diameter wells were punched out. Samples were applied in each well (5 μl/well). After 24 hr at room temperature in a humid chamber, the diameter of the precipitin ring was measured at 0.1-mm accuracy with a calibrated digital viewer.

RESULTS

Detection of bovine serum α_1AG: Cattle sera were analyzed by G-IEF at a pH range of 2.5 to 5. As shown in Fig. 1, several distinct protein bands, ranging from pH 3.2 to 3.7, were seen in sera from cattle with various diseases. Even though the intensity of staining varied, it was evident that 7 to 8 distinct bands of such acidic proteins were observed. The concentrations of these acidic proteins were much higher in sera from animals with inflammation (Fig. 1, lane 7 to 9), leukemia (lane 10 to 12) or mastitis (lane 13 to 14) than in those from normal animals (lane 3 to 6).

Purification of bovine α_1AG: α_1AG was purified as described in Materials and Methods. The yield in each purification step was 70% for ammonium sulfate, 37% for DEAE-cellulose, 30% for CM-cellulose and 28% for gel filtration. The final yield of α_1AG was 270 mg from 7.5 *l* of pooled normal sera. The purified α_1AG was subjected to the subsequent experiments.

Physicochemical properties of bovine

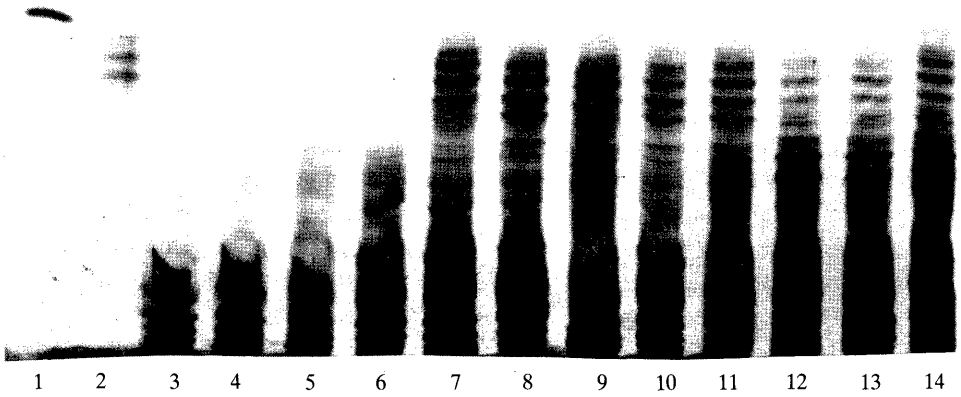

Fig. 1. Isoelectric focusing in polyacrylamide gel (pH range of 2.5 to 5). Lane 1, marker (pI 3.3); lane 2, purified bovine α_1AG; lane 3 to 6, normal bovine serum; lane 7 to 9, inflammation; lane 10 to 12, leukemia; lane 13 and 14, mastitis. Anode is at the top.

α_1AG: The pI value of purified α_1AG had a range between pI 3.2 to 3.7 with 7 to 8 bands by G-IEF (Fig. 1, lane 2). The purified α_1AG migrated in SDS-PAGE as a single protein band and the molecular weight of α_1AG was determined as 42,000±2,000 (data not shown). The S_{20w}° value for α_1AG was 3.4S. Quantitative amino acid composition of bovine α_1AG was indicated in Table 1. The glutamic acid, tyrosine, aspartic acid and leucine contents were higher than other amino acids. The sugar content of bovine α_1AG was 26.6% (Table 1).

In addition, purified bovine α_1AG was not precipitated with 20% perchloric acid, 5% sulfosalicylic acid, 5% trichrloroacetic acid and distilled water as described previously [18].

Serological analysis of anti-bovine α_1AG serum: Antisera to bovine α_1AG obtained from rabbits were analyzed by double immunodiffusion and immunoelectrophoresis. After reaction of purified α_1AG and anti-bovine α_1AG, a single precipitin line was observed with a high intensity and this line was fused with that from bovine whole serum. Immunoelectrophoresis showed the same mobility for purified α_1AG and that in normal bovine serum and gave a single precipitin arc in the α_1-globulin region against anti-α_1AG (Fig. 2). Antisera obtained here contained no contaminating antibodies directed to other serum proteins that could be detected by the electrophoretic method. Commercially available antisera against bovine whole serum did not react with purified α_1AG (Fig. 2).

Measurement of α_1AG in cattle sera:

1) Normal serum α_1AG values

To determine the amount of serum α_1AG in cattle, SRID method was developed. The calibration curve was essentially linear between 50 and 1,500 μg/ml of purified bovine α_1AG. The assay had an intra-assay coefficient of variation under 3% and inter-assay coefficient of variation under 5%.

In order to determine the normal α_1AG range, biochemical parameters of cattle sera were first analyzed, and those with abnormal values were excluded. Serum α_1AG

Table 1. Amino acid and carbohydrate compositions of bovine α_1AG

Amino acid	mol/mol[a)]
Aspartic acid	18.0
Threonine	9.6
Serine	8.5
Glutamic acid	34.9
Proline	5.4
Glycine	3.9
Alanine	10.6
Cystine	5.2
Valine	6.5
Methionine	3.0
Isoleucine	11.0
Leucine	15.0
Tyrosine	18.4
Phenylalanine	13.6
Histidine	4.2
Lysine	14.6
Arginine	11.2
Tryptophane	N.T.

Carbohydrate contents (%)

	Present study	General value[b)]
Hexoses	9.8	11.3–18.6
Hexosamine	8.5	7.9–12.5
Fucose	0	0.4–0.9
Sialic acid	8.3	7.2–16.2
Total	26.6	26.8–47.5

a) mol/mol, assuming the mol. wt. of 42,000. b) references 1, 3, and 9. N. T.: not tested.

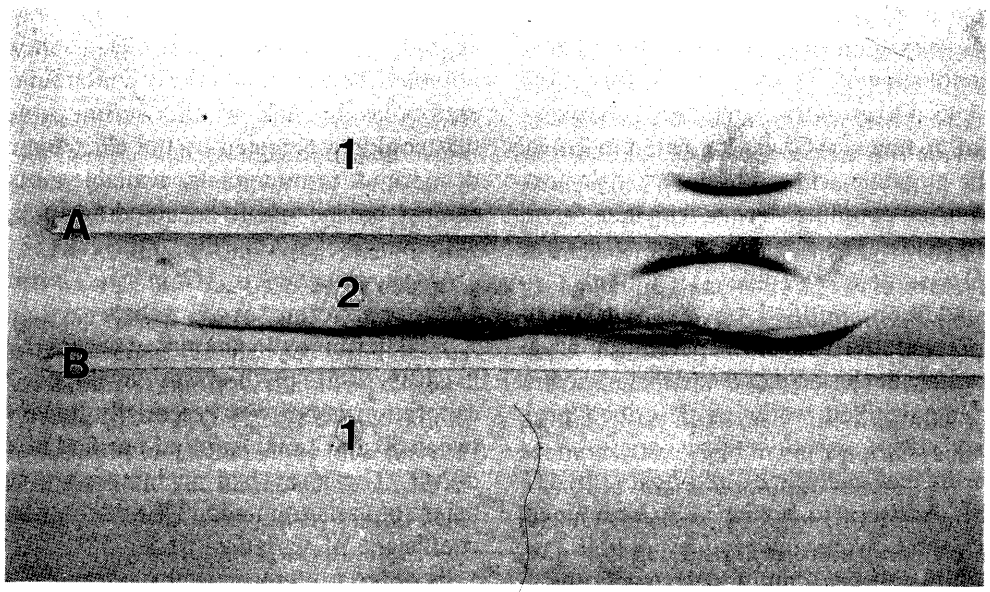

Fig. 2. Immunoelectrophoretic analysis of bovine α_1AG and anti-bovine α_1AG serum. 1, purified bovine α_1AG; 2, normal bovine serum; A, anti-bovine α_1AG serum; B, anti-bovine whole serum. After reaction, the glass plate was washed, dried and stained with Coomassie Brilliant Blue.

levels of 166 healthy cattle were thus determined. The mean value of 95 bulls (1–10 years old), 57 cows (2–12 years old) and 14 calves (3 month) were 262.9±77.1 μg/ml (range of 119–493 μg/ml), 327.3±74.7 μg/ml (range of 140–520 μg/ml), and 365.1±51.0 μg/ml (range of 313–478 μg/ml), respectively. The sex and age differences of serum α_1AG in these healthy cattle were shown in Fig. 3 and Fig. 4. Mean value of bulls by age group were 296.4±80.4 (1 year, n=28), 289.0±73.6 (2 years, n=16), 234.5±67.6 (3 years, n=20), 221.0±63.3 (4 years, n=17), 265.0±62.4 (5 years, n=4), 290.0±46.9 (6 years, n=5) and 250.0±14.0 (7 years, n=2). Similarly, mean value of cows were 341.5±76.8 (2 years, n=13), 305.4±73.1 (3 years, n=14), 309.1±93.5 (4 years, n=11), 270.0±59.4 (5 years, n=5), 233.8±37.1 (6 years, n=4), 325.0±41.5 (7 years, n=5), 315.0±60.6 (8 years, n=3) and 330.0±127.3 (≧10 years, n=2). There was no significant difference in bulls and cows ($P>0.05$). The sex distribution for serum α_1AG level was also relatively uniform in these cattle. The mean values of bulls and cows aged 1–12 years old was 283.2±82.3 μg/ml (n=152), and the mean plus 2 SD was 448 μg/ml. The cut-off value was therefore set at 450 μg/ml and those higher than 451 μg/ml were regarded as α_1AG abnormal.

2) Serum α_1AG levels in cattle with diseases

Serum α_1AG concentrations of 56 cattle with various diseases were determined (Fig. 5). The serum α_1AG levels were significantly higher than those of healthy controls. The incidence of α_1AG abnormal cattle was 100% (9/9) for traumatic pericarditis (range of 535–3,925 μg/ml), 100% (8/8) for arthritis

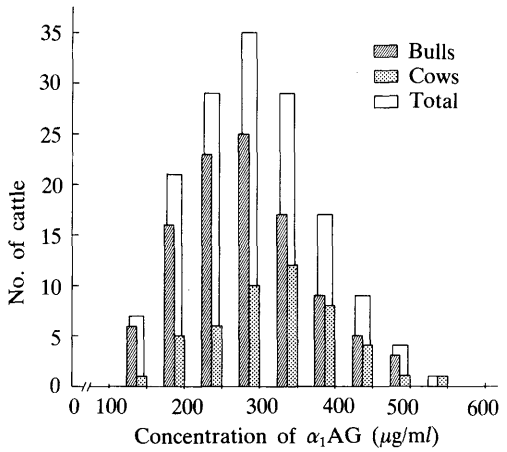

Fig. 3. Distribution of serum α_1AG values in healthy cattle.

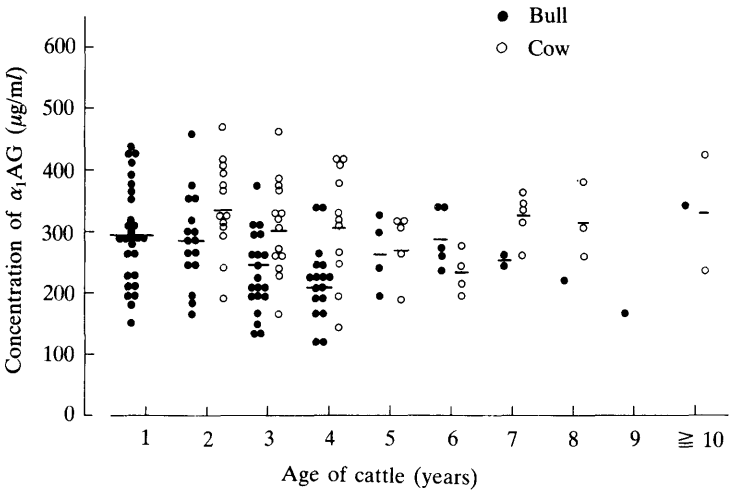

Fig. 4. Distribution of serum α_1AG values by age and sex in healthy cattle. Bars: mean value of serum α_1AG.

(range of 920–2,430 μg/m*l*), 91% (20/22) for mastitis (range of 380–2,725 μg/m*l*), 70% (7/10) for pneumonia (range of 350–3,250 μg/m*l*) and 43% (3/7) for mesenteric liponecrosis (range of 220–1,210 μg/m*l*).

DISCUSSION

There have been many reports on purification of serum α_1AG from various mammals [1, 3, 9, 18, 21]. Recently, Iwata *et al.* [9] reported the purification of α_1AG from bovine sera by salting out, acid precipitation, and ion exchange chromatographies. The purified α_1AG had a molecular weight of 31,000–40,000 (pI 3.5–4.0). In the present study, we purified bovine α_1AG from pooled normal sera by means of ammonium sulfate precipitation, ion-exchange chromatographies, and subsequent gel filtration. Purified bovine α_1AG contains 26.6% carbohydrate and has a molecular weight of 42,000. The amino acid compositions of our bovine α_1AG preparation closely agrees with those of human [18] or animals [1]. Although we observed some differences on the carbohydrate contents. Hexoses was slightly lower than the general values (Table 1). Fucose, which has been regarded very low [7], was not detected in this study. The α_1AG molecule is heterogeneous in both its amino acid composition and sugar contents [18]. Furthermore, G-IEF of purified bovine α_1AG showed a microheterogeneity with 7 to 8 distinct protein bands within a pI range of 3.2 to 3.7. Charge microheterogeneity of this protein is mainly due to sialic acids [17, 18].

The sialic acids and mucoproteins in human serum have been regarded as serum markers for inflammatory digestive disease [15] and malignant tumors [2, 15]. Previously, Motoi *et al.* [13] reported that the serum levels of sialic acids and mucoproteins in cattle with hepatic abscess were much higher than those of healthy controls, and pointed out that the measurements of these serum markers were useful for determination of the prognosis of cattle with inflammatory diseases and also for monitoring the effectiveness of therapy. But, since many glycoproteins contain sialic acids at their terminal portion of carbohydrate chains, no specific sialylglycoprotein in serum is determined by the assay for sialic acids alone.

We have employed the SRID method for determination of serum α_1AG in cattle with and without various diseases. Mean value of serum α_1AG in 152 healthy cattle was 283.2±82.3 μg/m*l* and this value was quite similar to those reported by Iwata *et al.* (0.31±0.09 g/*l*, n=33) [10] and by Itoh *et al.* (284.4±95.5 μg/m*l*, n=147) [8]. This SRID method is exceedingly reproducible and accurate. Furthermore, it is much simpler

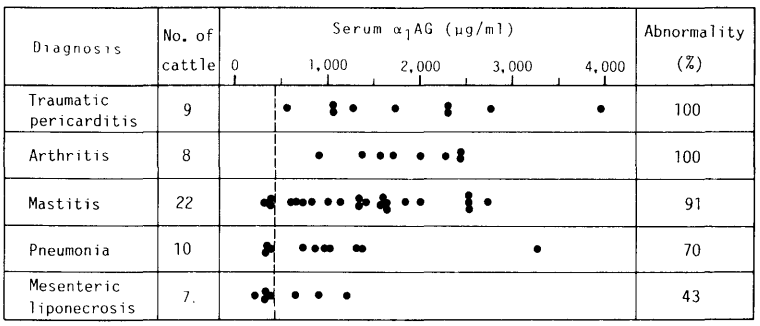

Fig. 5. Serum α_1AG levels in cattle with diseases.
Dashed line denoted the upper limit (450 μg/m*l*) of α_1AG in healthy control.

than G-IEF, rocket immunoelectrophoresis [5], enzyme linked immunosolvent assay [20] or radioimmuno-assay [6].

As is shown in Fig. 5, the greatest increase in serum α_1AG was observed among cattle with traumatic pericarditis (3,925 μg/m*l*) and over 70% cattle with traumatic pericarditis, arthritis, mastitis and pneumonia indicated α_1AG abnormality ($\geqq$451 μg/m*l*). Indeed, previous reports [8, 10] indicated that cows with various diseases such as malignant lymphoma, bovine leukemia, pneumonia, downer cow syndrome or hepatitis indicated remarkably high serum α_1AG values. The amount of circulating α_1AG also seems to correlate with the extent of the disease (Motoi *et al.* unpublished data). Therefore, quantitative measurement of serum α_1AG in cattle may be a useful aid in monitoring the course of various diseases, although an elevation of α_1AG has no specificity for diagnosis of particular diseases. Furthermore, increases in α_1AG without apparent diseases may indicate sub-clinical pathologic conditions.

Evaluation of serum α_1AG may provide one of useful markers for screening of various abnormality. For this purpose, more cases will be investigated at the clinical level by using our SRID method.

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要　約

ウシα_1酸性糖蛋白(α_1-acid glycoprotein: α_1 AG)の分離精製,性状及び定量について：田村啓二・谷津寿郎[1]・伊藤　博[2]・元井葭子[3](細菌化学研究所, [1]宮城県石巻保健所, [2]岩手県盛岡家畜保健衛生所, [3]農林水産省家畜衛生試験場)——ウシ血清より急性期反応蛋白の一つであるα_1 AGの精製を試みた．ウシ血清α_1 AGは分子量42,000±2,000，糖合量26.6%で極めて酸性の等電点(pI. 3.2-3.7)を有し，α_1-グロブリン領域に泳動された．抗ウシα_1 AG血清を用いた免疫拡散法で健康ホルスタイン(年齢1～12歳;雄95例，雌57例)の血清α_1 AGを測定したところ，283.2±82.3μg/ml(平均値±標準偏差)であった．この結果から正常値の上限を450μg/mlと定め，451μg/ml以上を異常値とした．次いで疾病牛の血清α_1 AGを測定したところ，異常値を示したのは創傷性心膜炎で100%(9/9例)，関節炎で100%(8/8例)，乳房炎で91%(20/22例)，肺炎で70%(7/10例)，腸間膜脂肪壊死症で43%(3/7例)であった．