Isolation and Purification of a Low Molecular Weight Protein from Bovine Urine

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ABSTRACT. A low molecular weight protein was separated from urine samples obtained from a heifer with spontaneous renal disease and from cows with CaNa_2EDTA -induced renal dysfunction. The molecular weight and electrophoretic mobility of the separated protein were examined. The low molecular weight protein collected by gel filtration chromatography was further separated into two fractions by ion exchange chromatography using DEAE-cellulose. One of the two fractions, the lowest molecular weight protein showed a single band in SDS-PAGE, and its molecular weight was approximately 12,000. An antiserum against this protein formed a single precipitin line with the urine from cows with experimentally induced renal dysfunction and a heifer with spontaneous renal disease by the double immunodiffusion technique. However, the antiserum did not form any precipitin line with the concentrated urine of healthy cow and human β_2 -microglobulin. In cellulose acetate membrane electrophoresis, this protein migrated in the same position as that of serum γ -globulin from healthy cow.—**KEY WORDS:** cow, heifer, urinary protein.

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Low molecular weight proteins (under 50,000) such as β_2 -microglobulin, retinol-binding protein, ribonuclease and free light chain have been detected in urine from human and some kind of animals with renal disease, especially with renal tubular damages [3, 4, 8, 10, 12, 14]. These proteins can easily pass through the glomerular capillaries, however, almost all of them are reabsorbed through the renal tubules [8, 16]. There are many renal function tests for diagnosis of renal disease in cattle, however, few effective methods is available for renal tubular functions. Therefore, it is necessary for understanding on renal tubular function to isolate the low molecular weight proteins from bovine urine.

In the present study, we attempted to separate and purify the low molecular weight protein in the urine of cattle with renal dysfunction.

MATERIALS AND METHODS

Animals: Three female cattle were used in this study. One was a Japanese Shorthorn heifer with spontaneous renal disorder (2-year-old, body weight; 430 kg), and other two were health Holstein cows (5- and 6-year-old, body weight; 480 and 570 kg). The latter two cows were used for experimental induction of renal dysfunction by administration of CaNa_2EDTA (32 mg/kg, iv).

Collection and storage of urine samples: About five liter urine samples were collected from cattle using a catheter and each sample was immediately centrifuged at 2,000 rpm for 5 min. Sodium azide was added to the urine in order to obtain a final concentration of 0.1% and urine pH was adjusted to 7.0 using 0.5N sodium hydroxide. Urinary protein concentrations were determined by the biuret method according

to Piscator's procedure [13]. Protein in the samples was concentrated about 10-fold by ultrafiltration with dialysis tubing (Spectrapor 1, Spectrum Medical Industries, Inc., Los Angeles, U.S.A.), and the concentrates were stored at -70°C until use.

Isolation and purification of low molecular weight protein: The isolation and purification of low molecular weight protein in urine were carried out according to the method described by Ohsawa and Kimura [11]. The concentrated and frozen urine was dissolved at 4°C . The protein in the samples was precipitated by 50 w/v% ammonium sulfate, of which pH was adjusted to 7.0 by the adding of 25% ammonia solution. The precipitate was immediately dialyzed against 0.1 M Tris-HCl buffer (pH 8.0) for 24 hr and concentrated with the ultrafiltration by use of Ultra Holder of mixing flow type (Model UHP-76, Advantico Toyo Ltd., Tokyo, Japan) in order to obtain a protein concentration of 10 mg/ml or higher. The protein was gelfiltrated on Sephadex G-100 (Pharmacia Fine Chemicals Inc., Uppsala, Sweden) column with 0.1 M Tris-HCl buffer (pH 8.0) containing 1.0 M NaCl. Subsequently, the low molecular fraction was concentrated with the ultrafiltration to get a protein concentration of 1 mg/ml or higher and was dialyzed against 0.01 M phosphate buffer, pH 7.5. The low molecular protein, furthermore, was applied to a DEAE-cellulose (Nakarai Chemicals, Ltd., Kyoto, Japan) column, of which gradient was equilibrated with 0.01 M phosphate buffer, pH 7.5. After the complete washing of the column with the same buffer containing 0.2 M NaCl, a stepwise gradient of 0 to 150 $\mu\text{Eq/ml}$ chloride ion in the buffer was introduced. Protein concentration of the fraction was estimated by measuring the absorbance at 280 nm.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): SDS-PAGE was followed according to the

method by Weber and Osborn [17], using a separating gel containing 10% acrylamide, 0.12% N,N'-methylene-bis-acrylamide (Bis) and 0.1% SDS in 0.357 M Tris-HCl buffer (Tris, pH 8.9). Samples were mixed with 0.125 M Tris (pH 6.8) containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue, and the mixture was boiled for 3 min. The marker proteins used for determining molecular weight were as follows; egg white lysozyme (molecular weight 14,400, Wako Pure Chemical Industries, LTD., Tokyo, Japan), myoglobin from equine muscle (18,000, Sigma Chemical Inc., Missouri, U.S.A.) and pepsin (35,000, Sigma Chemical Inc.).

Cellulose acetate membrane electrophoresis (CAME): CAME was performed by a modification of the method described by Kawamura *et al.* [9] using Separax (Jookoo Sangyo, Tokyo, Japan).

Antiserum: Rabbit antiserum against the low molecular weight protein was prepared by the method of Arimura *et al.* [2]. Two mg of low molecular weight protein dissolved in 0.3 ml of physiological saline was added to 0.6 ml of 50% polyvinyl pyrrolidone (PVP). The mixture was gently stirred for 2 hr at room temperature. Rabbits were injected intracutaneously with an emulsion of the mixture and an equal volume of Freund's complete adjuvant.

Double immunodiffusion (DID): DID was performed on glass plates coated with 3 ml of 1% agar gel in 0.15 M phosphate buffer solution (pH 7.2).

RESULTS

Separation and purification of urinary low molecular weight protein: Urine samples obtained from healthy cows, cows with experimentally induced renal dysfunction and a heifer with spontaneous renal disorder were concentrated. In Sephadex G-100 gelfiltration (GF) chromatography, the

Fig. 1. Sephadex G-100 column chromatogram of urinary protein precipitated by ammonium sulfate. The column was equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 1.0 M NaCl. UPNC, upCERD and upCSRD show the concentrated urinary proteins from a healthy cow, a cow with experimentally induced renal dysfunction and a heifer with spontaneously renal disorder, respectively. P-5 peak was used for separation of low molecular weight protein.

Fig. 2. DEAE-cellulose column chromatogram of the low molecular weight protein (P-5 fraction shown in Fig. 1). Elution buffer: stepwise gradient of chloride ions from 0 to 150 $\mu\text{Eq/ml}$ in 0.01 M PBS (pH 7.5). upCERD and upCSRD show each the concentrated urinary proteins from a cow experimentally induced renal dysfunction and a heifer with spontaneous renal disorder, respectively.

healthy cow's urinary globulin concentrated by ammonium sulfate was fractionated at three peaks (P-1 to P-3). Whereas, other two peaks (P-4 and P-5) were moreover detected in the globulin from cows with experimentally induced renal dysfunction and a heifer with spontaneous renal dysfunction (Fig. 1). The P-5 fraction, the lowest molecular weight protein, was collected and concentrated. And then, P-5 was chromatographed on DEAE-cellulose columns with the stepwise gradient of chloride ions. As shown in Fig. 2, chromatogram of P-5 fraction was further separated into two fractions (P-5a and P-5b).

SDS-PAGE: The fractions separated by

Fig. 3. SDS-polyacrylamide gel electrophoresis of urinary proteins. Lanes: 1; original urine, 2-6; P-1 to P-5 fractions by Sephadex G-100 column chromatography, 7-8; P-5a and P-5b fractions separated by DEAE-cellulose column chromatography, 9-11; molecular mass markers used pepsin (MW; 35,000), myoglobin (18,000) and lysozyme (14,400).

Fig. 4. Diagrammatic representation of each bands as shown in Fig. 3.

the chromatography of Sephadex G-100 and DEAE-cellulose were individually subjected to SDS-PAGE. The lowest molecular weight protein (P-5b) fraction obtained by DEAE-cellulose column showed a single peak, as shown in Figs. 3 and 4. The molecular weight of P-5b fraction was determined from molecular weight markers. Estimated molecular weight of P-5b fraction was 12,000.

Cellulose acetate membrane electrophoretograms of P-5b fraction and a serum of healthy cow are presented in Fig. 5. The P-5b migrated in the same position as that of serum γ -globulin.

Specificity of antiserum against P-5b: Antisera against the lowest molecular weight fraction (P-5b) were produced in three rabbits. As shown in Fig. 6, the specificity of the antiserum against P-5b (anti P-5b) was evaluated by double immunodiffusion tests. The P-5b fraction reacted with rabbit anti P-5b, but not with anti-human β_2 -microglobulin (Sigma) (Fig. 6-a). Double immunodiffusion test was also performed between the anti P-5b and following five substances; the concentrated urine samples from (1) healthy cows, (2) cows with experimentally induced renal

Fig. 5. Electrophoretic profile of P-5b fraction from cow experimentally induced renal dysfunction on cellulose acetate membrane electrophoresis.

Fig. 6. Double immunodiffusion (Ouchterlony test).

6-a: The purified P-5b produced a precipitin line with anti P-5b, but not with anti human β_2 -microglobulin.

6-b: The anti P-5b produced only one precipitin line against upCERD and upCSR D but not against upNC and human β_2 -microglobulin.

upNC, upCERD and upCSR D are the same in the Fig. 1, aP-5b; anti P-5b serum. H β_2 -m; human β_2 -microglobulin, aH β_2 -m; anti human β_2 -microglobulin.

dysfunction, (3) a heifer with spontaneous renal disorder, (4) human β_2 -microglobulin (Sigma), and (5) PBS (Fig. 6-b). A single precipitin line was formed between the anti P-5b and each of the urine from cows with experimentally induced renal dysfunction and a heifer with spontaneous renal disorder, while no line formation was observed between the anti P-5b and each of the urine from healthy cows and human β_2 -microglobulin.

DISCUSSION

Since no active transportation mechanism is involved in the filtration of the body fluid by the glomerulus, whether or not a substance passes out of the glomerular capillaries is simply dependent on its molecular size [12]. Low molecular weight proteins (<50,000) which readily transverse the glomerulus are reabsorbed from or metabolized in the proximal renal tubules, thus most of them are not detected in the urine. It is generally known that disorder of the renal tubules causes inhibition of the reabsorption of low molecular weight proteins, resulting in their excretion in the urine [6]. Accordingly, it is thought that an increase in excretion of low molecular weight proteins in the urine should be able to apply as an index of renal tubule dysfunction [12]. However, there are no reports on the diagnostic significance of low molecular weight proteins in bovine urine.

A low molecular weight protein (P-5b fraction) in bovine urine was purified as a single peak by ion exchange chromatography using DEAE-cellulose, SDS-PAGE and CAME. And the estimated molecular weight of P-5b fraction was 12,000, and the molecular weight and chromatogram of DEAE-cellulose were similar to the those of human β_2 -microglobulin [3, 4, 11].

In double immunodiffusion test of the present study, antiserum against the lowest molecular weight protein (P-5b) did not react with the concentrated urine from healthy cow, while it formed a single precipitin line with the concentrated urine from cows with renal dysfunction. These results clearly indicates that P-5b fraction is a pure protein component which is not contained or is limited infinitesimally in the urine of healthy cow.

β_2 -microglobulin has been separated from urine and serum of human [3, 11], rat [10], mouse [1], guinea pig [14], rabbit [5] and

dog [15]. It has also been reported that human β_2 -microglobulin migrates to the serum β_2 -globulin position by CAME, while rat β_2 -microglobulin migrates to serum γ -globulin position [10]. In the present study, the lowest molecular weight protein had the same molecular weight as that of bovine lactoferrin [7], and it migrated to the serum γ -globulin position. Furthermore, this protein was different from other low molecular weight proteins with respect to molecular weight, i.e., lysozyme (MW 14,600), retinol-binding protein (21,000), *I*-chain monomer (22,000) and α_1 -microglobulin (33,000) [16], but was similar to human β_2 -microglobulin (11,800). Authors estimated that, from the results of molecular weight and chromatography, the lowest molecular weight protein from the urines of cattle with experimental or spontaneous renal dysfunction is extremely similar to human β_2 -microglobulin. However, for identification of this low molecular weight protein, further investigation will be necessary to clarify its physical and chemical properties such as sedimentation and absorption coefficient, amino acid and carbohydrate composition, etc.

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要 約

ウシ尿中低分子蛋白の分離および精製：川村清市・井上平太・小田高司・伊藤直之・樋口誠一（北里大学獣医畜産学部獣医内科学教室）——CaNa₂EDTAの静脈注射により作出した腎機能障害牛および腎障害自然発症牛の尿中から低分子蛋白を分離精製した。即ち、これらのウシの濃縮した蛋白陽性尿から塩析法によりグロブリン分画を分離し、これを Sephadex G-100カラムを用いたゲル濾過法で5つに分画した。これらのうち、最小分子量を示した分画をさらに DEAE-cellulose を用いたイオン交換カラムクロマトグラフィーにより2つに分画した。このうちの1分画は、SDS-PAGEにより単一のピークを示し、かつ分子量が12,000と推定され、ヒト β_2 -microglobulin (MW 11,800)と類似した分子量であった。一方、この分画は、セルロースアセテート膜電気泳動法により正常ウシ血清の γ -グロブリン位に移動し、さらに、ゲル内沈降反応において、それに対する抗血清との間に1本の沈降線を形成したが、抗ヒト β_2 -microglobulin との間には沈降線が形成されなかった。また、この抗血清と実験的腎障害牛および腎障害自然発症牛の各濃縮尿との間には、沈降線が形成されたが、正常牛の濃縮尿およびヒト β_2 -microglobulin とは沈降線を形成しなかった。