ISOLATION OF BOVINE β_2-MICROGLOBULIN AND ITS DETERMINATION BY ENZYME LINKED IMMUNOSORBENT ASSAY

F. Hoshi, O. Miyazaki, K. Nakadaka, M. Satho, S. Higuchi, and S. Kawamura.

Department of Veterinary Internal Medicine, School of Veterinary Medicine and Animal Science Kitasato University, Towada-shi, Aomori 034, Japan.

INTRODUCTION

Human β_2-microglobulin (β_2-m) is a single polypeptide chain of 100 amino acids(mol. Wt. = 11.800), first isolated from urine of patients with chronic cadmium poisoning by Berggard and Bearn. It is presents at low concentration in normal human serum, urine and cerebrospinal fluid (2). Human β_2-m is eliminated by glomerular filtration and subsequently reabsorbed almost completely in the proximal tubule cells, where it is metabolized (10). The quantity of human β_2-m excreted into urine reflect proximal tubule disorder, and its concentration in serum reflects glomerular dysfunction (13). In a previous study, we clarified that low moleclar proteins increase at renal dysfunction in bovine urine. but the relationship between this protein, bovine β_2-m and renal dysfunction is unclear (6). Thus, in this study, an attempt was make to isolate and purify bovine β_2-m from urine.

MATERIALS AND METHODS

Animals

Nine healthy females Holstein cows were used. One was used for induction of experimental renal dysfunction by the administration of ethylene glycol (Kantho chemicals, Japan) and the other eight were used to assay urinary β_2-m.

Production of kidney damaged cattle

A healthy female Holstein cow was administrated ethylene glycol to damage the kidneys. Ethylene glycol was injected intravenously for two days at 0.25 ml/body weight kg/day.

Isolation of bovine urinary β_2-m

Fresh urine obtained from ethylene glycol administrated cattle, was dialyzed against distilled water and concentrated urtrafiltration method. This sample was applied onto a DEAE-cellulose (Whatman Bio Systems Ltd, England) column equilibrated with 0.1 M Tris-HCl buffer (pH 8.3). The column was eluted with a step

gradient of sodium chloride (0, 0.1 or 2.0 M). Fractions rich in low molecular weight proteins were corrected and applied by gel filtration of Sephadex G-75 (Pharmacia fine chemicals, Uppsala, Sweden) column. Fraction containing β_2-m was further purified by high performance liquid chromatography (HPLC) on a Bio-Sil SEC-125 column (Bio-Rad Co. Ltd.). Obtained fraction was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and determined the amino acid composition to identify β_2-m.

Analysis of amino acid composition

Amino acid composition analysis was we carried out using approximately 10 pM proteins hydrolyzed by 250 μl 6N HCl at 130°C for 4 hr in evacuated sealed tubes (12). Hydrolyzed protein was dissolved in lithium citrate buffer (pH 2.2). Amino acids were analyzed by HPLC on Li type positive ion exchange column (Polyspher AA-T, Cica-Merk,Japan).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out by the modified method of Laemmli (7). The stacking gel contained 3.0% acrylamide, 0.2% N,N'-methylene-bis-acrlamide (Bis) and 0.1% SDS in 0.125 M Tris-HCl buffer (pH6.8). The separating gel contained 17.5% acrylamide 0.2% Bis and 0.1% SDS in 0.375 M Tris-HCl buffer (pH8.8). Samples were mixed with 1% SDS, 4.7% 2-mercaptoetanole, 19% Glycerol, 0.27% Bromophenol blue and 0.125 M Tris-HCl buffer (pH6.8). The mixture was heated for 5 minute at 95°C. The following molecular mass standards (Merk, munchen) were used: Ovotransferrin (78 kDa), albumin (66.3 kDa), ovalbumin (42.7 kDa), carboanhydrase (30 kDa), myoglobin (16.9 kDa) and cytochrome C (12.3 kDa). The gels were stained with Coomassie brilliant blue R-250 according to Fairbanks et al (4).

Production of anti-bovine β_2-m IgG

Purified β_2-m in 0.5 ml physiological saline was mixed with an equal volume of Freund's complete adjuvant oil and an emulsion was formed. The emulsion was injected intradermally for Japanese White Rabbit (body weight 1.5 kg, male). Injections were conducted six times at 10 days intervals and whole blood was collected at 10 days after the last injection. After whole blood was incubated 37°C for 30 minutes and left at 4°C overnight, serum was separated. Serum was inactivated at 56°C for 30 minutes and precipitated with 40% ammonium sulfate. The IgG was purified by gel chromatography on a Sephadex G-200 column.

Enzyme-linked immunosorbent assay of β_2-m (ELISA)

The principle used was enzyme immunoassay of anti-

gen solid-phase two antibodies. The coating solution was 0.1 M sodium hydrogen carbonate buffer (pH9.8). The washing buffer was 0.05% Tween 20, 25% Block Ace (Dainihon seiyaku, Japan) in 0.02M phosphate buffer solution (PBS) pH 7.4 to which 0.15M sodium chloride. The incubation buffer was 0.02M PBS (pH7.4). The plate was immunoplate CORNING 25801 (Corning, USA). Primary and secondary antibodise were anti-bovine β_2-m from rabbit and alkaline phosphatase labeled anti-rabbit IgG from goat. The procedure was a follows: (a) 96 wells of immunoplate were coated with 100 μl urine or purified bovine β_2-m for standard material. The plates were stored overnight at 4°C. (b) The plates were washed three times with washing solution, 400 μl per well. Residual washing solution was removed by tapping the inverted plates on absorbing paper. (c) 100 μl of primary antibody adjusted concentration of 5 μg/ml, were pipetted into each well and incubated for 2 hour at 4°C. (d) The plates were washed and dried as above. (e) 100 μl of secondary antibody, diluted 1:500 in the incubation buffer were added to each well. The plates were incubated for 2 hour at 4°C. (f) They were washed and dried as above. (g) 200 μl of enzyme substrate were added to each well. The plates were incubated for 15 minute at 37°C. The reaction was stopped by adding 50 μl of 1 M sodium hydroxide. (h) Absorbance of the wells was measured using a microplate reader (Bio-Rad model 3550, USA) at 405 nm. All assays were carried out in duplicate.

Results

Urine from cattle distracted by the administration of ethylene glycol contained much low molecular protein, and this urine was considered usable for isolateing β_2-m. The First step of isolation of β_2-m was carried out with DEAE-cellose column. Each fraction eluted with 0, 0.1 or 2.0 M sodium chloride were contained various proteins, and most low molecular weight proteins were presented in the second fraction (data no shown). Gel filtration was carried out with this fraction, and the chromatogram is shown Fig.1. Each fraction was analyzed by SDS-PAGE. Fraction A and B contained proteins, but all low molecular weight proteins were contained in fraction B. The proteins were further purified by HPLC and analyzed by SDS-PAGE. Molecular weight of this protein, that estimated from relative mobility on SDS-PAGE, was about 11,300 daltons. Amino acid composition was similar to that of bovine β_2-m (5). We identified this protein as bovine β_2-microglobulin and the following experiment of ELISA was conducted.

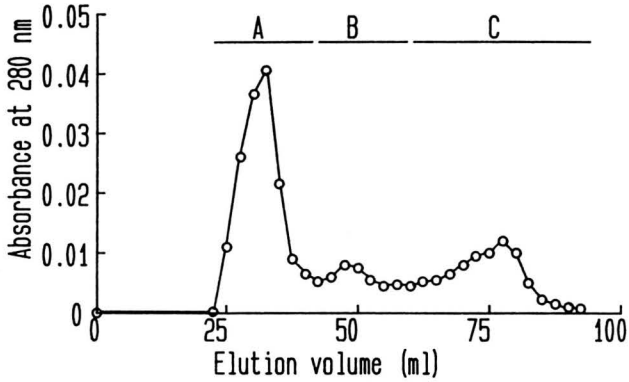

Fig. 1 Chromatography on Sephadex G-75. The column (1.7 x 51 cm) was equilibrated and eluted with 0.1 M Tris-HCl buffer (pH 8.3).

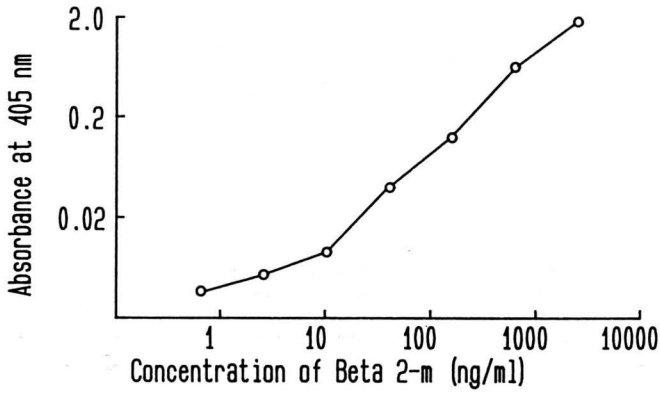

Fig. 2 Standard curve of pure bovine β_2-m. Each open circle is the mean of 5 values.

Standard curve in Fig. 2 was obtained by using the ELISA system. The detectable concentration range was 4.6-5000 ng/ml. Accuracy of the assay was evaluted by analytical recovery of pure bovine β_2-m added at 10 and 50 ng. The recovery rate averaged 106.3% (range 93.9-124.5, n=6). Intra-assay measurement of urinary β_2-m was precise and the coefficient of variation was 2.4% (n=8). By the ELISA system, urinary β_2-m was assayed for eight healthy Holsteins.

DISCUSSION

β_2-microglobulin has been isolated from various body fluids of various spices (1,2,3,8,9,11). Bovine β_2-m was isolated from bovine milk and colostrum by Gloves et al (5) for the first time. In this study, we isolated bovine β_2-m from urine. The urine has advantage of containing only a few kinds of protein. In the modified methods of Globes et al, the first step of isolation was carried out using a DEAE-cellulose column. β_2-m was bound to DEAE-cellulose with 0.1 M Tris-HCl buffer (pH 8.3), and eluted with 0.1 M sodium chloride. The proteins with molecular weights of about 20-30 kDa were removed by this procedure. After gel filtration, a composition of the obtained fraction was found to be about 11 kDa protein but to be slightly contaminated with 30 kDa protein. This protein was finally purified by HPLC. The electrophretic pattern of this protein showed a single somewhat broad band and molecular weight was estimated as about 11.3 kDa by relative mobility. This is similar to the value of Gloves. This protein was further analyzed for amino acid composition, which was found to match reported bovine β_2-m with little difference (5). The difference may depend on hydrolyzing method of protein. The protein was identified as bovine β_2-m.

Measurement of bovine β_2-m was done by the ELISA principled non-competitive solid phase method. This method is most popular for measurement of antibody values. In this study, antigen (β_2-m) was assayed by this method. Its merits of this method were detective specificity is high and quantity of antibody needed to assay is little. However demerit is high background absorbance and the range of detection is limited. The ELISA system developed in this study showed a relatively wide detectable range, high accuracy and excellent reproducibility for measurement of β_2-m in bovine urine. Nevertheless, it was not suitable for measurement of serum β_2-m, because serum contains too much protein to bind a plate. To assay bovine β_2-m in serum, sandwich ELISA system should be developed. The concentrations of β_2-m in healthy Holstein cows as measured in this system were similar reported values for other animals.

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SUMMARY

In the previous study, we clarified that low moleclar proteins increase at renal dysfunction in bovine urine. but the relationship of between this protein, bovine β_2-m and renal dysfunctionremeins unclear. Thus in this study, an attempt was make to isolate and purify bovine β_2-m from urine of bovine treated with ethylene glycol by ion-exchange chromatography and gel chromatography conducted in conjugation. Purified protein was analyzed by SDS-PAGE and amino acid composition analysis and was identified as bovine β_2-m. An enzyme linked immunosorbent assay (ELISA) was developed for the determination of β_2-m in bovine urine. The detectable range was 4.6-5000 ng/ml in this ElISA system. The accuracy of the assay was assessed by adding of 10 or 50 ng pure β_2-m to normal urine. In these experiments, recovery was 99.4% and 113.2% in the urine (n=6). reproducibility was determined in the urine. The intra-day coefficient of variation was 2.4% in the urine (n=8)