Urinalysis for detection of chemically induced renal damage (3) — Establishment and application of radioimmunoassay for lysozyme of rat urine

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Abstract. The radioimmunoassay (RIA) as a high sensitive detection method for rat lysozyme (LZM) was established and applied to determine LZM excretion in urine from rats treated with tubulotoxic chemicals in order to establish a sensitive method of detecting minor renal damage. Rat LZM which showed a single protein band on sodium dodecylsulfate polyacrylamide gel electrophoresis was purified by ion-exchange chromatography and gel filtration. The assay sensitivity of the established RIA using the purified rat LZM was 4-256 ng/ml rat LZM and was about 20 times the turbidity method. The concentration of LZM in normal rat urine was 76.2 ± 6.0 ng/ml (mean ± SE, n = 50) using the RIA. In urine containing more than 100 ng/ml LZM, a high correlation between the values determined by the RIA and those by the turbidity method was observed. However, egg white LZM, human urinary LZM and guinea pig urinary LZM were not detectable by the RIA. Using the RIA, it was ascertained that urinary LZM excretion began to increase on day 5 in rats treated with gentamicin (15 or 30 mg/kg/day sc for 17 days), during the 6-9 h period in the rats treated with mercuric chloride (1 mg/kg sc), and during the 0-3 h period in the rats treated with p-aminophenol (1 mmol/kg sc). These significant increases in LZM excretion were not detectable by the turbidity method; therefore, it was concluded that this RIA for rat LZM was very useful for detection of minor renal damage.

Key words: Lysozyme — Renal damage — Urinalysis — Radioimmunoassay — Rat

Introduction

Chemically induced renal damage has been verified by physiological function tests, blood analysis and urinalysis (Diezi and Biollaz 1979; Berndt 1981). Recently, a great deal of interest has been shown in the use of urinary enzymes for the detection of renal damage in an early phase (Raab 1972; Piperno 1981; Price 1982; Takahashi and Ohata 1982).

One of these urinary enzymes, lysozyme (LZM), has a low molecular weight and can be filtered through the glomerulus. However, little activity can be detected in urine obtained from normal animals because the LZM is reabsorbed into the proximal tubular cells (Christensen and Maunsbach 1974; Cojocel and Baumann 1983). Therefore, lysozymuria has been used as a sensitive indicator of experimental tubulonephritis (Evan and Dail 1974; Ngaha and Plummer 1977). The turbidity method, using Micrococcus luteus as its substrate, has been used for the detection of enzymatic activity; however, the sensitivity is low and normal levels or a slight increase in urinary activity, which is an important indicator of tubulonephritis in the early phase of renal damage, cannot be detected (Evan and Dail 1974; Ohata et al. 1987a).

In this study, we attempted to establish a radioimmunoassay (RIA) as a highly sensitive detection method for rat LZM and to apply this method to determining LZM excretion in urine from rats treated with gentamicin, p-aminophenol and mercuric chloride as tubulotoxic chemicals. This method could, in turn, detect minor renal damage by examining urinary enzymes.

Materials and methods

Materials

Mercuric chloride (HgCl₂) was obtained from Iwai Chemical Co., gentamicin (GM) was obtained from Shionogi Seiyaku Co. and p-aminophenol (PAP) was purchased from Tokyo Kasei Co. All other chemicals were of a commercially available analytical grade.

Animals

Male Wistar rats (9–12 weeks of age) were used throughout this study. The animals were housed in a temperature (23–25°C), humidity (50–70%), and light-cycle (12 h light, 12 h dark) controlled room with free access to standard rat chow and water.

Lysozyme (LZM, EC 3.2.1.17) assay by the turbidity method

LZM activity was measured by lysis of a suspension of Micrococcus luteus cells (Miles Laboratories Inc.) as described in the previous report (Ohata et al. 1987a).

Purification of rat LZM

LZM was purified from the urine of rats treated with p-aminophenol, a tubulotoxic chemical, as follows. The rats were injected sc with p-aminophenol for 2 days in doses of 1 mmol/kg/day and housed in metabolic cages...
(Nippon Clea). The urine was collected in tubes cooled by Coolnit (CML-III, Taiyo Scientific Industrial Co.) to 4–8°C for 48 h after the first injection. Sodium azide was added to about 20% of the pooled urine at a final concentration of 0.1%. An ammonium sulfate fraction (20–80% w/v) of the urine was applied to a CM Sephadex C-50 (Pharmacia Fine Chemicals) column, 3 × 30 cm, equilibrated with a 0.05 M Tris-Cl buffer (pH 8.0) and eluted with stepwise increase of NaCl concentration in the buffer at 4°C. The eluted enzyme fraction was concentrated and applied to a Sephadex G-100 (Pharmacia Fine Chemicals) column, 2.5 × 55 cm, equilibrated with a 0.05 M Tris-HCl buffer (pH 8.0) containing 0.9% NaCl and eluted with the same buffer at 4°C. The eluted enzyme fractions were concentrated and applied to a Sephadex G-50 (Pharmacia Fine Chemicals) column, 1.6 × 90 cm, equilibrated with the same buffer at 4°C. The eluted enzyme fractions were concentrated and dialyzed against a 10 mM Tris-Cl buffer (pH 7.4) containing 0.15 M NaCl. Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of each fraction was performed in 10% polyacrylamide gels according to the method of Fairbanks et al. (1971).

**Anti-rat LZM serum**

Purified rat LZM (0.5 mg/0.3 ml) was mixed with the same volume of Freund’s complete adjuvant (Difco Laboratories) to form a water-in-oil emulsion. The emulsion was injected four times at intervals of 3 weeks into the hind footpads and the dorsal hypodermis of rabbits. Ten days after the final injection, serum was obtained from the blood of the rabbits and the dorsal hypodermis of rabbits. Ten days after the final injection, serum was obtained from the blood of the rabbits. Anti-rat LZM serum developed a single precipitin line when tested against the purified LZM in Ouchterlony analysis. An identical precipitin line was produced by testing the antiserum against the purified protein in a similar manner.

**Labelling of rat LZM by 125I**

125I-Labelled rat LZM was prepared according to the chloramine-T method (Greenwood et al. 1963). Purified rat LZM (3 μg/50 μl) of a 0.05 M sodium phosphate buffer, pH 7.4, buffer A) was mixed with 2 mCi Na125I (Amer sham IMS30) and with chloramine-T (50 μg/20 μl buffer A) and incubated for 20 s at room temperature. Then sodium metabisulfite (100 μg/50 μl buffer A), potassium iodide (20 μg/50 μl buffer A) and 0.5 ml buffer A containing 0.15 M NaCl and 0.2% bovine serum albumin (buffer B) were added to the reaction medium. 125I-labelled rat LZM was separated from the reaction medium by gel filtration on the Sephadex G-25 (Pharmacia Fine Chemicals) column (1.5 × 30 cm) equilibrated with buffer B and stored at −20°C.

**RIA for rat LZM**

The RIA for rat LZM was performed according to the method of Yuzuriha et al. (1978). 125I-labelled rat LZM solution (10000 cpm/0.1 ml), 0.1 ml rat LZM standard solution (4–256 ng/ml) or 0.1 ml of samples, and 0.1 ml of anti-rat LZM serum (1:10000 dilution) were mixed and incubated for 2 h at 37°C. Then 0.5 ml Dextran T-70 (Pharmacia Fine Chemicals)-treated charcoal was added and centrifuged for 15 min at 3000 r.p.m. Radioactivities of the supernatant and the sediment were determined with an Auto-gamma Spectrometer model 5130 (Packard Instrument Company Inc.). The preparation and the dilution of materials were made in buffer B. All tests were performed in duplicate.

**Treatments and urine collection**

**Renal damage caused by low doses of nephrotoxic chemicals.** GM was injected sc for 17 days in doses of 15 mg/kg/day (GM-L) and 30 mg/kg/day (GM-H). PAP was injected sc for 3 days in doses of 0.25 mmol (27.5 mg)/kg/day (PAP-L) and 0.5 mmol (55 mg)/kg/day (PAP-H). HgCl2 was injected sc for 9 days in doses of 0.25 mg/kg/day. The rats were housed singly in metabolic cages. The urine was collected in tubes cooled by Coolnit to 4–8°C. In the GM-treated groups, 24 h urine samples were collected on days 1, 3, 5, 7, 9, 11, 13, 15 and 17. In the PAP-treated groups, the urine samples were collected during the periods 0–10 and 10–24 h after the first injection, and then 24 h urine samples were collected on days 2 and 3. In the HgCl2-treated group, 24 h urine samples were collected on days 1, 2, 3, 5, 7 and 9. The control animals were injected sc with saline (2 ml/kg/day) in each experiment.

**Renal damage in the early phase.** HgCl2 and PAP were injected individually sc in doses of 1 mg/kg and 1 mmol (110 mg)/kg, respectively. The urine samples in both groups were collected during the periods 0–3, 3–6, 6–9, 9–12, and 12–24 h after injection. In these groups, 24 h urine samples were also collected before the first injection as a control.

**Other analytical methods.** Urinalysis was performed after the urine samples were dialyzed with Spectropor 3 (Spectrum Medical Industries Inc.) against over 50 vol deionized water for 3 h at 4°C. The concentration of protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. Activity of lactate dehydrogenase (LDH, EC 1.1.1.27) in urine was measured as described in the previous report (Ohata et al. 1987a). Urinary excretions of each substance were expressed as activity or amount per h per kg body weight.

**Results**

**Purification of rat LZM**

Figure 1 shows the elution profile of rat urine LZM on the final purification step, Sephadex G-50 column chromatography. The enzyme fraction was eluted as a single peak and SDS-PAGE of the peak revealed a single protein band. The specific activity of purified rat LZM was 10000 times as much as that of the original urine and was 1.52 mg of egg white LZM per mg protein of rat LZM. Anti-rat LZM serum developed a single precipitin line when tested against the purified LZM in Ouchterlony analysis. An identical precipitin line was produced by testing the antiserum against the ammonium sulfate fraction of the urine.

**Establishment of RIA for rat LZM**

The standard curve of the RIA for rat LZM is shown in Fig. 2. The ratios of 125I-labelled rat LZM bound to the antibody were 62 and 14% in 4 and 256 ng/ml LZM, respectively. The coefficient of the variation of the RIA was 5.4–12.3, and it was lower than those of the turbidity method in the same range (Table 1). When the concentration of LZM in the urine samples was measured by RIA after diluting the urine samples (containing LZM 100–250 ng/ml) with buffer B at 1:1 to 1:7, good linearity