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New detection method for ribonuclease 2 (RNase 2) using immunoblotting with specific antibody*

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Summary. A ribonuclease (RNase) was isolated from the urine of a 35-year-old male and purified to electrophoretic homogeneity. The enzyme was tentatively designated RNase 2. A rabbit antibody produced by injection of the purified RNase 2 was able to distinguish RNase 2 from another type of RNase coexisting in body fluids. With this antibody it was possible to detect RNase 2 isozymes in human serum and urine without difficulty using isoelectric focusing or sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by immunoblotting. Both RNase 2 in serum and urine seemed to exist in multiple forms with regard to their molecular masses and pI values. This technique may prove to be useful in genetic and forensic studies of RNase polymorphism.

Key words: Ribonuclease – Isozyme – Immunoblotting – (Human serum) – (Human urine)

Zusammenfassung. Eine Ribonuclease (RNase) wurde aus Urin eines gesunden Mannes (35 Jahre) isoliert und bis zur elektrophoretischen Homogenität gereinigt. Das Enzym wurde versuchsweise "RNase 2" genannt. Ein beim Kaninchen hergestellter Antikörper gegen RNase 2 gestattete die Unterscheidung von RNase 2 und einer anderen in Körperflüssigkeiten vorkommenden RNase. Nach Polyacrylamidgel-isoelektrischer Fokussierung oder Natrium Dodecylsulfat-Polyacrylamidgel-Elektrophorese und Immunoblotting mit dem Antikörper war der Nachweis von RNase 2-Isozymen sowohl im Urin als auch in Serum möglich. RNase 2 scheint in verschiedenen Formen in Bezug auf das Molekulargewicht und den isoelektrischen Punkt (pI)

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im Urin und Serum vorzukommen. Weitere Untersuchungen zum RNase-Polymorphismus stehen noch aus, könnten aber nützliche Anwendungen in der Forensik aufdecken.

Schlüsselwörter: Ribonuclease – Isozym – Immunfärbung – (Humanserum) – (Humanurin)

Introduction

Human ribonuclease (RNase) is widely distributed in various organs and body fluids, including serum, urine, saliva and cerebrospinal fluid. RNases are classified on the basis of their catalytic and serological properties into two distinct types, secretory and nonsecretory RNases [1]. Recently, the existence of genetic polymorphism in serum RNase was elucidated for the first time in our laboratory [2, 3] employing both polyacrylamide gel isoelectric focusing (IEF-PAGE) and immunoblotting with a specific antibody. This lead to the evaluation of RNase as a useful identification marker in the forensic field. It is also known the RNase is very resistant to heating and other physical factors and its content in serum and urine is high, providing a potentially valuable marker in blood and urine stains for identification tests. Secretory RNase is present in serum and urine in a higher concentration than nonsecretory RNase, but a specific detection method for the enzyme has so far been unavailable.

The combination of IEF-PAGE and immunoblotting with a specific antibody has been used as a valuable technique for investigating possible heterogeneity and new genetic polymorphisms of pepsinogen [4, 5], deoxyribonuclease [6, 7] and a urinary glycoprotein, GP 43 [8]. Accordingly, immunological detection of the secretory RNase in body fluids seems most suitable for genetic or forensic distinction between the two RNases coexisting in body fluids [1].

In the present paper, we describe a purification procedure for the secretory type of urinary RNase, RNase 2 and a new detection method for the isozymes of RNase 2 utilizing IEF-PAGE or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting with an antibody specific to the enzyme.

Materials and methods

Twenty liters of human urine was collected from a healthy 35-year-old male and concentrated using a Pericon cassette (Millipore, Bedford, USA) with a PTGC membrane. The urine samples for analysis were concentrated by ultrafiltration, dialyzed against 0.1% glycine and then lyophilized according to the method described previously [9]. CM-cellulose (CM-52) and phosphocellulose (P11) were purchased from Whatman (Maidstone, UK); SP-Sephadex C-50, Sephadex G-75 and molecular weight calibration kits from Pharmacia (Uppsala, Sweden); Bio-Gel P-100 from Bio-Rad (Richmond, USA). Other chemicals used were of reagent grade.

RNase activity was assayed by measuring the formation of acid-soluble oligonucleotides with hydrolysis of RNA using 0.2 *M* Tris-HCl buffer, pH 7.5, according to the procedure described previously [3]. The protein concentration was determined by the method of Lowry et al. [10] using bovine serum albumin as a standard.

Antibodies specific to the purified RNase 2 were obtained as described previously [11]. A total of 0.3 mg of the purified RNase 2 was injected into a Japanese white rabbit with Freund's complete adjuvant.

The molecular masses of both native and denatured enzyme were determined mainly according to the method of Yasuda et al. [3, 12]. Amino acid analysis, carbohydrate analysis and determination of the N-terminal amino acid sequence of the enzyme were performed as described previously [13, 14].

IEF-PAGE for detection of RNase 2 isozymes was carried out essentially as described in our previous paper [7]. Gels measuring 0.5 × 90 × 120 mm were prepared using the following components: 1.4 ml acrylamide-bisacrylamide (19.4% w/v, 0.6% w/v), 2.3 ml sucrose-glycerin (20% w/v, 10% v/v), 1 ml distilled water, 95 mg urea, 200 µl Ampholine 6-8 (LKB, Bromma, Sweden), 80 µl Ampholine 5-7 (LKB), 5 µl *N,N,N',N'*-tetramethylethylenediamine and 40 µl 1.2% ammonium persulfate. Wicks were formed from strips of filter paper #526 (Toyo, Tokyo, Japan) and soaked in the electrode solution: 0.5 *M* H_3PO_4 at the anode and 0.5 *M* NaOH at the cathode. Samples were applied to the gel with a plastic applicator 1.5 cm from the cathode wick. A Multiphore apparatus (LKB) was employed to run the gel at 5 W for 4 h. SDS-PAGE was performed in 12.5% polyacrylamide gel according to the method of Laemmli [15]. Samples were dissolved in buffer containing 10 m*M* Tris-HCl buffer, pH 6.8, 10% glycerol, 2% SDS, 0.2% 2-mercaptoethanol and a trace of bromophenol blue. Electrophoresis was performed for 80 min at a constant current of 20 mA.

After IEF-PAGE, the isozymes was transferred onto a Durapore strip (Millipore) essentially according to the method described in our previous papers [4–8]. In the case of SDS-PAGE, transfer was carried out by electroblotting using a KS-8453 apparatus (Marysol, Tokyo, Japan). After transfer, electrophoretic patterns of RNase 2 were detected by immunoblotting using the RNase 2-specific antibody and peroxidase-labeled goat anti-rabbit immunoglobulin (Bio-Rad), as described previously [6, 7].

Results

Purification of RNase 2

The following operations were performed at 0–4°C. About 500 ml of the concentrate derived from 20 liters of urine was adjusted to pH 3.0 with concentrated HCl and mixed with about 250 ml of SP-Sephadex C-50 pre-equilibrated with 50 m*M* sodium citrate buffer, pH 3.0, containing 0.1 *M* NaCl. After 2 h, this suspension was packed into a column (3.2 × 40 cm) and then washed with 500 ml of the equilibration buffer. The RNases adsorbed on the column were eluted with 0.1 *M* Tris-HCl buffer, pH 7.5, containing 0.5 *M* NaCl. The RNase-active fractions were pooled and dialyzed against 5 m*M* potassium phosphate buffer, pH 6.0. The dialyzate was applied to a phosphocellulose column (1.6 × 29 cm) pre-equilibrated with the same buffer. The column was washed with 100 ml of the equilibration buffer and then eluted with 600 ml of a linear NaCl gradient (0–1 *M*) in the same buffer. The RNase eluted at a broad range of NaCl concentrations from 0.4 to 0.8 *M*, was pooled, dialyzed against distilled water and then concentrated to about 5 ml in vacuo. The concentrate was applied to a Bio-Gel P-100 column (1.6 × 100 cm) pre-equilibrated with 10 m*M* Tris-HCl buffer, pH 7.5, containing 0.2 *M* NaCl. As shown in Fig. 1, the RNase activity was separated into two fractions, the former of which was pooled and dialyzed against 5 m*M* Tris-HCl buffer, pH 8.0. The dialyzate was applied to a CM-cellulose column (1.6 × 16 cm) pre-equilibrated with the same buffer. The column was washed with 50 ml of the equilibration buffer and then eluted with 400 ml of a linear NaCl gradient (0–150 m*M*) in the same buffer. The adsorbed RNase was eluted at NaCl concentrations ranging from 40 to 80 m*M*. These fractions were pooled and concentrated in vacuo. This gave a single band on SDS-PAGE by protein staining, but several bands on IEF-PAGE. In order to obtain a single

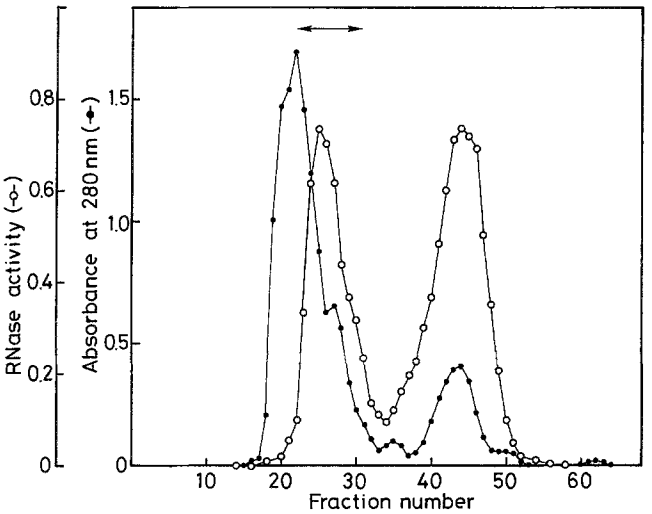

Fig. 1. Bio-Gel P-100 column (1.6 × 100 cm) chromatography of the RNase-active fractions eluted from the phosphocellulose column. The column was eluted with 10 m*M* Tris-HCl buffer, pH 7.5, containing 0.2 *M* NaCl. A 4-ml aliquot of each was collected at a flow rate of 6 ml/h. The *horizontal bar* indicates RNase 2-active fractions, which were pooled and dialyzed for further purification

isozyme of the RNase 2 isozymes eluted from the CM-cellulose column, the concentrate was applied to the first LKB 8101 electrofocusing column (110 ml) in 1% Ampholine (pH 3.5–10), containing a sucrose density gradient (5%–50%). All procedures were carried out according to the method described previously [16]. After electrophoresis 2-ml aliquots were collected and dialyzed against distilled water, and then RNase activity was assayed. The main fractions (pH 6.2–7.6) of the activity were pooled and applied to the second electrofocusing column containing a 1% mixture of an equal volume of Ampholine 5-7 and Ampholine 6-8. The main RNase active fractions (pH 7.2–7.6) were pooled and concentrated in vacuo and then the concentrate was applied to a Sephadex G-75 column (1.6 × 100 cm) pre-equilibrated with 10 m*M* Tris-HCl buffer, pH 7.5, containing 0.2 *M* NaCl. The RNase-active fractions were collected and dialyzed against distilled water and this RNase was designated RNase 2. About 0.8 mg of the purified RNase 2 was obtained from 20 liters of human urine.

Purity and molecular mass of the enzyme

As shown in Fig. 2, the purified enzyme migrated as a single band on SDS-PAGE under reducing conditions. The protein band also showed enzymatic activity on poly(C)-casting gel [17].

The molecular mass of the enzyme was estimated to be 32000 Da by SDS-PAGE under reducing conditions (Fig. 2), while that of the native enzyme was determined to be 38000 Da by gel-filtration.

Biochemical properties of the enzyme

The amino acid composition of the enzyme was quite similar to that of human urinary secretory RNase, termed RNase U_L [18]. Carbohydrate analysis indicated that RNase 2 was composed of 4 fucose, 10 mannose, 9 galactose, 23 glucosamine and 5 sialic acid residues per molecule. Galactosamine was not de-

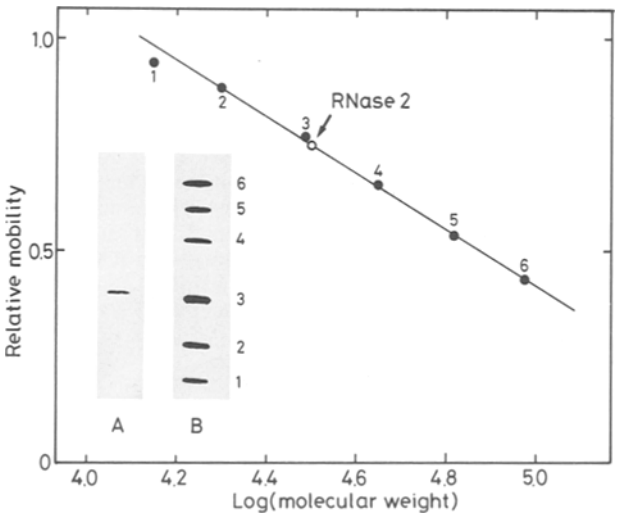

Fig. 2. Determination of the molecular mass of RNase 2 by SDS-PAGE. About 5 μg of the purified RNase 2 was treated with 1% SDS containing 0.1 *M* dithiothreitol at 100°C for 5 min and then subjected to a polyacrylamide gradient gel (4%–20%) using a SDS-PAGE 4/20 plate (Daiichi Pure Chem., Tokyo). The electrophoresis was performed in running buffer (0.025 *M* Tris and 0.192 *M* glycine with 0.1% SDS) at 150 V for 2 h. After electrophoresis, the protein was stained with 0.1% Coomassie brilliant blue R-250. Molecular weight markers were co-migrated (1. α-lactalbumin 14.4 kDa; 2. trypsin inhibitor 20 kDa; 3. carbonic anhydrase 30 kDa; 4. ovalbumin 45 kDa; 5. bovine serum albumin 67 kDa; 6. phosphorylase 94 kDa). The relative mobility of each protein on SDS-PAGE is the ratio of the migration distance of each protein to that of bromophenol blue. *Inset:* SDS-PAGE patterns of the purified RNase 2. *Lane A*, about 5 μg of the purified RNase 2; *lane B*, molecular weight markers

tected as an amino sugar in RNase 2. The amino acid sequence of the N-terminal 16 residues from the purified enzyme is shown below:

1 5 10 15
Lys-Glu-Ser-Arg-Ala-Lys-Lys-Phe-Gln-Arg-Gln-His-Met-Asp-Ser-Asp.

In addition to a great preference for poly (C) as a substrate (data not shown), catalytic properties of the purified RNase 2 satisfied the criteria for secretory RNase as recommended by Sierakowska and Shugar [1]. As for proteochemical and enzymological aspects, the purified RNase 2 was obviously different from RNase 1 of nonsecretory RNase, which has been purified from human urine and well characterized [3]. Furthermore, the antibody specific to RNase 1 did not cross-react with the purified RNase 2.

Specificity of anti-RNase 2 antibody

The IgG fraction purified by the method of Harboe and Ingild [19] was used as the antibody specific to RNase 2. A double diffusion test in agarose gel showed only a single precipitin line formed by the antibody with purified RNase 2, human plasma and concentrated urine and these lines fused with one another. When purified RNase 2 was applied to SDS-PAGE followed by immunoblotting

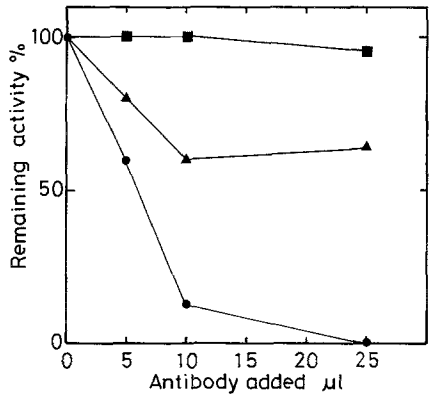

Fig. 3. Effect of anti-RNase 2 antibody on RNase activities of both purified RNase 2 (●) and RNase 1 (■), and concentrated urine (▲). Incubation mixtures containing about one unit of enzyme corresponding to 3.5 µg of purified RNase 2, 2.7 µg of purified RNase 1 or the concentrated urine derived from about 5 ml of the original urine and antibody were stored at 4°C overnight. They were then centrifuged and their RNase activities in the supernatant (5 µl) were determined using the method described in Materials and methods

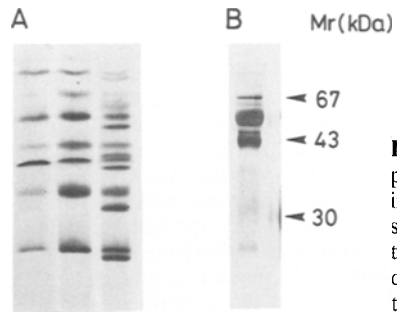

Fig. 4. IEF-PAGE patterns (A) and SDS-PAGE patterns (B) of serum RNase 2 isozymes detected by immunoblotting with anti-RNase 2 antibody. Three serum samples from different individuals were applied to IEF-PAGE. Conditions of electrophoresis and detection of the isozymes were performed according to the method described in Materials and methods

with anti-RNase 2 or protein staining with Coomassie blue, a single band was detected at the corresponding position on the gel. Otherwise, the nonsecretory RNase purified from human urine [3] was not stained by the anti-RNase 2 antibody. Figure 3 shows that anti-RNase 2 antibody completely blocked all the activity of the purified RNase 2 and about 50% of the total activity in the original urine. However, the antibody showed almost no inhibition of the activity of the purified urine nonsecretory RNase indicating that the anti-RNase 2 antibody could immunologically distinguish the RNase 2 from the urine nonsecretory RNase. From these results, it was shown that the anti-RNase 2 antibody was suited for the specific detection of secretory RNase in body fluids.

Detection of RNase 2 isozymes in polyacrylamide gels by immunoblotting with anti-RNase 2 antibody

After IEF-PAGE or SDS-PAGE of serum it was possible to detect RNase 2 isozymes by immunoblotting with anti-RNase 2 antibody. As shown in Fig. 4, RNase 2 isozymes in serum migrated as several discrete bands with different pI values and/or different molecular masses. Similarly, after IEF-PAGE or SDS-PAGE of concentrated urine followed by imunoblotting with anti-RNase 2 antibody, RNase 2 isozymes were separable into several bands (data not shown). These results indicate that RNase 2 in both serum and urine may exist in multiple forms, with regard not only to pI values, but also to molecular masses.

Discussion

One of the RNases, named RNase 2, was isolated from human urine and purified to an electrophoretically homogeneous state using a series of column chromatographies and preparative isoelectric focusings. The purified RNase 2 was found to be a glycoprotein containing 12.5% amino sugar (glucosamine only), 9.7% neutral sugar (fucose, mannose and galactose) and 4.2% sialic acid with a molecular mass of 32000 Da and 38000 Da estimated by SDS-PAGE and gel filtration, respectively. RNase 2 was identified as one of the secretory RNases based on the catalytic properties of the purified enzyme according to the classification of Sierakowska and Shugar [1]. An antibody against the purified RNase 2 reacted with secretory RNase, but not with nonsecretory RNase. At least these two types of RNase are present in serum and urine [20], but it has been impossible to distinguish them from each other with regard to their enzymatic properties, such as substrate specificity, optimal pH, sensitivity to divalent cations etc. In order to increase the value of RNase in body fluids as a useful marker in forensic science, a specific detection method is essential. IEF-PAGE or SDS-PAGE followed by immunoblotting with the antibody specific to RNase 2 was successful in specific detection and good separation of RNase 2 isozymes in body fluids, not only with different pI values, but also with different molecular masses. This newly devised method provides better resolution and more specific detection than previous methods, such as PAGE followed by activity staining [17]. Two different types of isozyme pattern of RNase 2 among sera derived from different individuals were observed, with IEF-PAGE followed by immunoblotting and these patterns were highly reproducible for each individual suggesting the existence of genetic polymorphism of RNase 2. However, in order to elucidate the genetic aspect of the enzyme in detail further analysis will be needed. Approximately 7.2 mg (total of RNase 2 isozymes) of a purified RNase 2 could be obtained from 20 liters of human urine and approximately 2.4 mg of the urine nonsecretory RNase was obtained from 126 liters of urine [3]. Thus, the content of RNase 2 in urine was estimated to be about 20 times that of the other type of RNase. These results may be useful in shedding further light on the forensic application of RNase 2 as a potentially valuable identification marker.

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