

Isozymes of Human Urine Ribonuclease Demonstrated by Isoelectric Focusing¹

JOHN M. THOMAS² and M. E. HODES, Department of Medical Genetics
Indiana University School of Medicine, Indianapolis, Indiana 46223

Abstract

Ribonuclease (RNase) from human urine demonstrates a multiplicity of bands of activity following isoelectric focusing on thin layer polyacrylamide gel (IFPAG) and a negative-staining zymogram technique. Most of the activity focuses at a pH of 3.5-4.0 on the pH gradient, but numerous other bands of activity are located throughout the isoelectric spectrum, extending to about pH 9.5 at the cathode. The urine RNase banding patterns on IFPAG have exhibited uncontrollable variation in different urine specimens from the same individual and in the same specimen stored for different periods of time. Treatment of urine with neuraminidase reduces the complex and variable banding pattern to a single major band which focuses at a pH of about 9.5 (on a broad range "pH 3.5-10" gradient). Thus sialic acid residues apparently account for a series of transitional isozymes, the most acidic of which has an isoelectric point of up to 6 pH units lower than the desialyated enzyme. A two-stage method for alkaline range IFPAG, previously developed in this laboratory, with supplementation of the commercial ampholytes with arginine to extend the pH gradient in the alkaline range, has demonstrated 2 bands of RNase activity in neuraminidase-treated urine which focus at pH 9.5-10.

Introduction

The existence of charge isomers (isozymes) of human urine ribonuclease (RNase) was first demonstrated in 1958 using ion exchange chromatography (9). Subsequently, charge heterogeneity of urine RNase was observed in several investigations employing ion exchange chromatography (4, 8, 19, 20, 21, 28, 42) and electrophoresis (16, 33). Since this previous work did not include a search for variation in isozyme pattern among individuals, it is not known whether this enzyme(s) is polymorphic. In an effort to detect a genetic polymorphism in RNase from human urine, we have employed isoelectric focusing in polyacrylamide gel (IFPAG), a technique which appears to offer greater resolving power than traditional electrophoretic techniques (31). This communication describes the complex isozyme pattern of human urine RNase demonstrated by IFPAG, its variability in specimens from a single individual, and the need to remove sialic acid residues from this glycoprotein before attempting to evaluate a potential polymorphism.

¹This Research was supported in part by the Indiana University Human Genetics Center, PHS GM 21054.

²J.M.T. was supported by National Institutes of Health Training Grant PHS GM 1056.

Materials and Methods

Urine from a single individual was collected over thymol in a plastic cup and either frozen or held at 4° for about 2 hours before processing. The fluid was dialyzed in Spectrapor 1 membrane tubing (M. W. cutoff: 6,000-8,000) against deionized water for 24 hours at 4°. The dialyzed urine was lyophilized and either stored at -20° or reconstituted for immediate analysis with deionized water at one-twentieth the original volume.

One concentrated urine specimen was treated with neuraminidase (Sigma, Type VI, from *Clostridium perfringens*) at 0.1 u/ml (0.05 mg/ml) in 0.1 M sodium acetate buffer, pH 5.0, and dialyzed against this buffer for 24 hours at room temperature and against deionized water for 24 hours at 4°. Controls for the neuraminidase treatment included a aliquot of concentrated urine treated with buffer only and dialyzed as above, and another aliquot stored at 4° for the 48 hour period.

Equipment, materials, and the general procedure for IFPAG in our laboratory have been described elsewhere (36). The composition of the thin layer polyacrylamide gels (1.5 mm thick and 19 cm long) used in this study was as follows: 3.64% acrylamide (T = 4%), 0.36% N,N'-methylenebisacrylamide (C = 9%), 20% glycerol, 0.8% Ampholine pH 3.5-10, 0.1% Ampholine pH 2.5-4, 0.1% Ampholine pH 9-11, 0.05% L-arginine (free base), 0.00005% riboflavin. The electrolyte solutions were 1.0% Ampholine pH 2.5-4:20% glycerol for the anode and 0.2% L-arginine: 20% glycerol for the cathode. Samples were applied as described previously (36) at 10 cm from the cathodic end of the gel. The electrofocusing runs were started at 800 V in a constant power mode so that the voltage increased to 2000 V prior to the end of the run, which lasted for 8 hours.

The zymogram method for detecting RNase activity in polyacrylamide gels was a modification of the techniques of Wolf (41) and Wilson (40). Following IFPAG the gel was soaked twice (15 minutes each time) in a solution of 0.1 M sodium phosphate buffer, pH 6.0 or 8.0, with agitation at room temperature, and again in 0.05 M buffer. The gel was incubated in a solution of 1.0 mg/ml RNA (Calbiochem, purified from *Torula*, B grade, M. W. 20,000-30,000) in 0.05 M sodium phosphate buffer, pH 6.0 or 8.0, at 37° for 1 hour. Subsequently, the solution was removed, the gel was rinsed in 0.05 M buffer for 2 minutes, and the incubation was continued (after removal of buffer) in a humidified chamber at 37° for an additional hour. The gel was fixed in 5% acetic acid at 4° overnight, stained in a solution of 0.2% toluidine blue in 0.5% acetic acid over a one-day period.

Results

Examples of the isozyme patterns of RNase from human urine following IFPAG in a broad range pH gradient are illustrated in (Fig. 1). With this negative-staining zymogram technique, bands of RNase activity appear as clear areas against a darkly stained RNA background. Numerous bands of activity are located throughout the isoelectric spectrum. In any given sample, most of the activity focuses near the anode at a pH of 3.5-4.0 on the pH gradient. The remainder of the banding pattern shows much variation with different urine

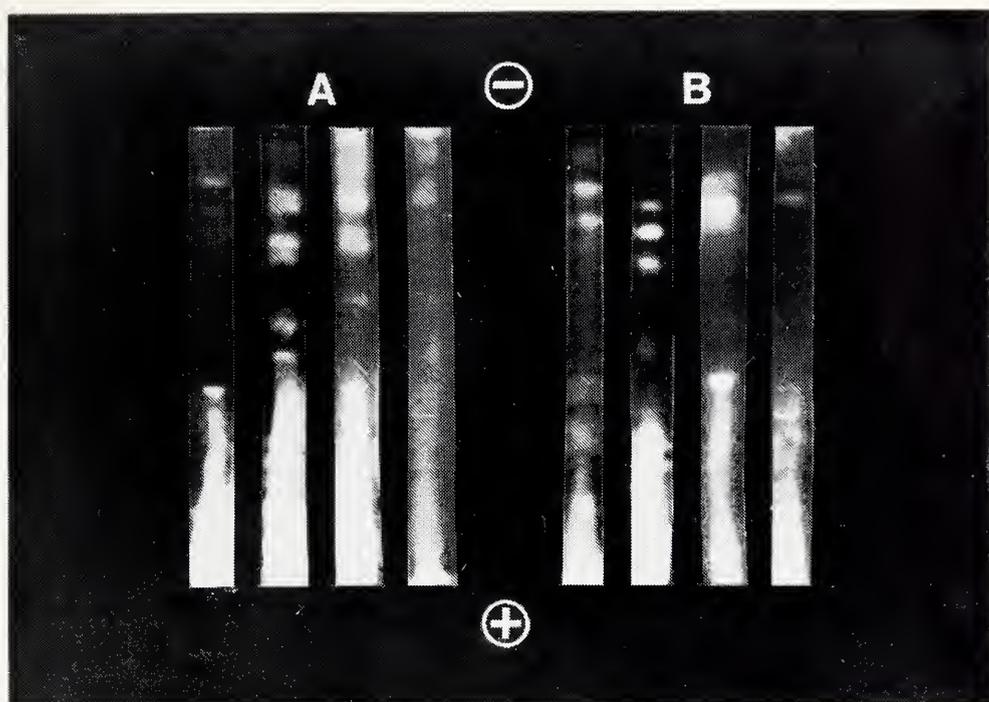


FIGURE 1. Examples of the IFPAG patterns of the isozymes of human urine RNase, with equilibration and incubation at pH 6.0 (A) and at pH 8.0 (B). The pH gradient extends from about pH 3.0 at the anode to about pH 9.7 at the cathode. For each strip 40 μ l of 20-fold concentrated urine was applied. This amount results in excessive activity in the acidic region, but enables the development of minor bands in the alkaline region. The first three strips in each group are patterns from the same specimen stored (lyophilized) for various periods of time, and the last strip in each group is the pattern from a different specimen.

specimens from the same individual and with the same specimen stored for different periods of time. Variation is especially evident in the alkaline region. This variation appears to be uncontrollable and can not be attributed to the initial freezing of a urine specimen or the length of storage of a lyophilized sample.

Treatment of urine with neuraminidase, which removes sialic acid residues from glycoproteins, has a dramatic effect, as illustrated in (Fig. 2). The complex and variable banding pattern is reduced to a single band which focuses at a pH of about 9.5.

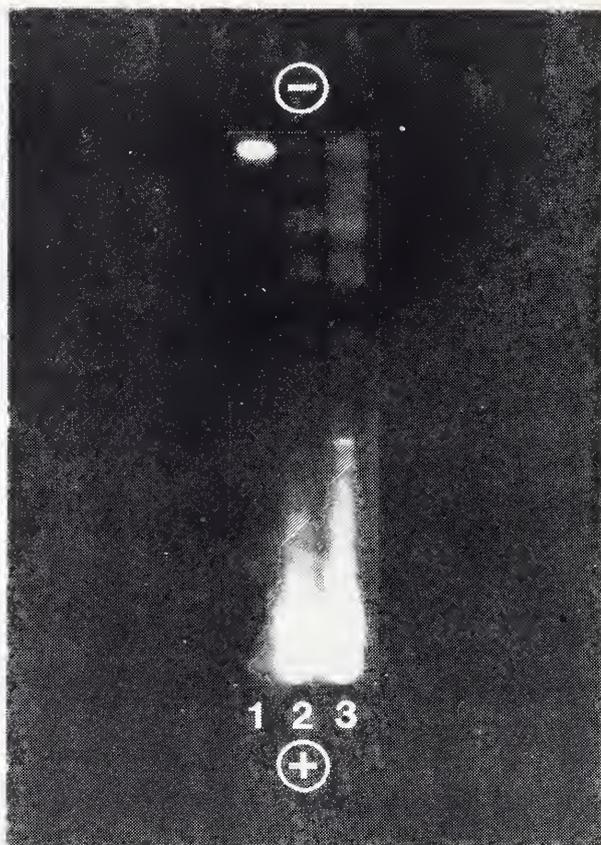


FIGURE 2. IFPAG patterns of the isozymes of human urine RNase, with and without treatment of the urine with neuraminidase. Equilibration and incubation were at pH 6.0. The pH gradient is the same as in (Fig. 1). 1): 40 μ l of 20-fold concentrated urine sample treated with neuraminidase (diluted 1:2 for treatment). 2): 40 μ l of 20-fold concentrated urine sample treated with buffer only (diluted 1:2 for treatment). 3): 20 μ l of 20-fold concentrated urine sample stored at 4° (not diluted).

Discussion

The present demonstration of the isozymes of human urine RNase by IFPAG has been accomplished by certain modifications of the method which deserve mention. Since the urine RNase isozymes can focus at very low or very high pH's, the commonly-used "pH 3.5-10" Ampholine must be supplemented with Ampholines of pH 2.5-4 and pH 9-11 in order to extend the gradient at the extremes. To enable the retention of the most alkaline band on the gel, it is supplemented with arginine (pI 10.7), which also comprises the cathodic electrolyte. Following IFPAG, the gel must be extensively equilibrated with a buffered solution to overcome the buffering capacity of the Ampholines and establish a constant pH throughout the gel. To accomplish this, the gel was soaked in buffer for a much longer period than that recommended by Wilson (40). Also, incubation of the gel after removal of the substrate solution was found to be necessary to detect minor bands of activity. As have other investigators (30, 39, 40), we have determined that the negative-staining zymogram may be non-specific for RNase, since concentrated zones of a protein (e.g., albumin in serum) may yield a false-positive band. This possibility is excluded from our analysis of urine, since there are no detectable protein bands after the application of urine at this level of total urinary protein.

The results of treatment of urine with neuraminidase indicate that sialic acid residues apparently account for a series of transitional isozymes of RNase, the most acidic of which has an isoelectric point (pI) of up to 6 pH units lower than the desialydated enzyme. Many hydrolytic enzymes from animal tissues are known to be glycoproteins and contain sialic acid (7, 10, 11, 22, 23, 26). Treatment of these various enzymes with neuraminidase results in shifts from acidic to basic forms, increases in pI's, and reduction or abolition of heterogeneity (7, 10, 22, 23, 26). The reported changes in pI's following neuraminidase treatment of various hydrolytic enzymes vary from about 0.5 to 2.5 pH units (7, 22, 23, 26). Thus the pI increase we have observed for desialydated human urine RNase appears to be unusual. While there are no reports of a human RNase containing bound sialic acid, Reddi (29) suggested that the urine enzyme was a glycoprotein on the basis of a positive stain with periodic acid-Schiff reagent. With respect to nonhuman RNases, one report demonstrated that neuraminidase treatment of the soluble fraction from rat kidney lysosomes altered the electrophoretic mobility of RNase bands to more basic forms (10).

Other reports of the pI's of human RNases are of interest. RNase purified from human urine has been reported to have a pI of 3.5 (28) and 4.1 (29), as determined by isoelectric focusing in a sucrose density gradient. These values agree well with the major area of activity on our IFPAG gels when using untreated urine. RNase purified from human pancreas had a pI of 10.3, as determined by traditional electrophoretic mobility measurements (38). Also, RNases purified from human serum and leukocytes reportedly had pI's above 11.0, as determined by the density gradient focusing technique (2, 3). These basic pI values appear similar to the pI of the desialydated urine RNase.

As mentioned previously, charge heterogeneity of human urine RNase has been observed in several investigations. Human RNase from other fluids or tissues also appeared heterogeneous. Ion exchange chromatography demonstrated heterogeneity of RNase in serum (1, 2, 3, 4, 5, 6, 14, 32, 34), leukocytes (18), pancreas (38), and epidermis (17). Electrophoretic methods demonstrated heterogeneity of RNase in serum (5, 6, 14, 30), leukocytes (13, 27, 30), erythrocytes (30), pancreatic juice (37), liver (30), spleen (12, 13), kidney (30), heart (30), testes (30), and epidermis (15). The degree of heterogeneity observed in these investigations ranged from 2 to 8 peaks or zones of activity. Since most tissue RNases reportedly are present in urine (4, 25), further investigations concerning the sites of origin, relationship and significance of the various human RNases should include the effect of neuraminidase treatment. In addition, the present study has demonstrated that treatment of urine with neuraminidase is requisite for determining possible heterogeneity and genetic polymorphism inherent to the urine RNase protein.

Preliminary results using a two-stage method for alkaline range IFPAG to enhance the resolution, similar to our method described elsewhere (36), indicate that neuraminidase-treated urine shows 2 bands of RNase activity which focus at pH 9.5-10. In this regard, it is of interest that other investigators have determined the existence of two distinct classes of human RNases, both of which occur in urine (4, 24, 25).

Literature Cited

1. AKAGI, K., K. MURAI, N. HIRAO, and M. YAMANAKA. 1976. Purification and properties of alkaline ribonuclease from human serum. *Biochem. Biophys. Acta* **442**:368-378.
2. AKAGI, K., M. YAMANAKA, K. MURAI, and T. OMAE. 1978. Purification and properties of acid ribonucleases in human serum and leukocytes. *Cancer Res.* **38**:2163-2167.
3. AKAGI, K., M. YAMANAKA, K. MURAI, and T. OMAE. 1978. Serum acid ribonuclease in myelogenous leukemia. *Cancer Res.* **38**:2168-2173.
4. BARDON, A., H. SIERAKOWSKA, and D. SHUGAR. 1976. Purification and properties of human acid-thermostable ribonuclease, and diagnosis of childhood pancreatic fibrosis. *Clin. Chim. Acta* **67**:231-243.
5. BLANK, A. and C. A. DEKKER. 1977. Characterization of human serum ribonuclease by RNA-polyacrylamide gel electrophoresis. *Fed. Proc.* **36**:907.
6. BLANK, A. 1978. Analysis of human serum ribonucleases by polyacrylamide gel electrophoresis. *Fed. Proc.* **37**:1639.
7. CAMPBELL, H. D., N. P. B. DUDMAN, and B. ZERNER. 1973. On the multiple forms of acid phosphatase in pig liver. *FEBS Letters* **31**:123-126.
8. DELANEY, R. 1963. Chemical, physical, and enzymic properties of several human ribonucleases. *Biochemistry* **2**:438-444.
9. DICKMAN, S. R., L. H. WHITE, and J. D. MASON. 1958. Human urine ribonuclease. *Arch. Biochem. Biophys.* **74**:476-477.
10. GOLDSTONE, A., P. KONECNY, and H. KOENIG. 1971. Lysosomal hydrolases: conversion of acidic to basic forms by neuraminidase. *FEBS Letters* **13**:68-72.
11. GOLDSTONE, A. and H. KOENIG. 1974. Synthesis and turnover of lysosomal glycoproteins. Relation to the molecular heterogeneity of the lysosomal enzymes. *FEBS Letters* **39**:176-181.
12. HODES, M. E., M. K. SONG, R. C. KARN, G. L. PRAU, and M. Z. HODES. 1976. Isoelectric focusing patterns of several acid phosphohydrolases of beef, pig, and human spleen, *Comp. Biochem. Physiol.* **54B**:155-161.
13. HODES, M. E., M. CRISP, and E. GELB. 1977. Electrophoresis of acid phosphohydrolase isozymes on Cellogel. *Anal. Biochem.* **80**:239-248.
14. KOTTEL, R. H., S. O. HOCH, R. G. PARSONS, and J. A. HOCH. 1978. Serum ribonuclease activity in cancer patients. *Br. J. Cancer* **38**:280-286.
15. LISS, M. and W. F. LEVER. 1962. Purification and characterization of ribonuclease from psoriatic scales. *J. Invest. Dermatol.* **39**:529-535.
16. MAGDON, M., Z. HANICKI, W. PAJDAK, and J. SZNAJD. 1974. Use of the separation of urinary proteins on polyacrylamide gel for clinical diagnosis of multiple myeloma. *Przegl. Lek.* **31**:728-733.
17. MELBYE, S. W., B. A. BRANT, and I. M. FREEDBERG. 1977. Epidermal nucleases. III. The ribonucleases of human epidermis. *Br. J. Dermatol.* **97**:355-364.
18. NASKALSKI, J. and J. SZNAJD. 1967. Ribonucleases of chronic granulocytic leukaemia leucocytes. *Nature* **215**:414-415.
19. NASKALSKI, J. 1972. A comparison of the isoenzymic patterns of urinary ribonucleases in healthy subjects and patients with chronic granulocytic leukemia. *Polish Med. J.* **11**:827-834.
20. NASKALSKI, J. 1972. The properties of ribonucleases isolated from urine of patients with chronic granulocytic leukemia (CGL). I. Urinary alkaline ribonuclease. *Polish Med. J.* **11**:1407-1422.
21. NASKALSKI, J. 1972. The properties of ribonucleases isolated from urine of patients with chronic granulocytic leukemia (CGL). II. Urinary acid ribonuclease. *Polish Med. J.* **11**:1423-1438.
22. NEEDLEMAN, S. B. and H. KOENIG. 1974. Isoelectric-focusing behavior of acid hydrolases in rat kidney lysosomes. Effects of the pH gradient, autolysis and neuraminidase. *Biochim. Biophys. Acta* **379**:43-56.
23. NEEDLEMAN, S. B., H. KOENIG, and A. D. GOLDSTONE. 1975. Changes in electronegativity of lysosomal hydrolases during intracellular transport. An isoelectric-focusing study in subcellular fractions of rat kidney. *Biochim. Biophys. Acta* **379**:57-73.
24. NEUWELT, E. A., M. SCHMUKLER, M. S. NIZIAK, P. B. JEWETT, and C. C. LEVY. 1977. The immunological characterization of several human ribonucleases by using primary binding tests. *Biochem. J.* **163**:419-426.
25. NEUWELT, E. A., M. S. BOGUSKI, J. J. FRANK, K. PROCTER-APPICH, and C. C. LEVY. 1978. Possible sites of origin of human plasma ribonucleases as evidenced by isolation and partial characterization from several human tissues. *Cancer Res.* **38**:88-93.

26. OSTROWSKI, W., Z. WASYL, M. WEBER, M. GUMINSKA, and E. LUCHTER. 1970. The role of neuraminic acid in the heterogeneity of acid phosphomonoesterase from the human prostate gland. *Biochim. Biophys. Acta* **221**:297-306.
27. PAJDAK, W. and J. LISIEWICZ. 1973. Heterogeneity of enzymes in leukaemic eosinophils. *Scand. J. Haematol.* **11**:325-331.
28. RABIN, E. Z. and V. WEINBERGER. 1975. The isolation, purification, and properties of a ribonuclease from normal human urine. *Biochem. Med.* **14**:1-11.
29. REDDI, K. K. 1977. Purification and properties of a ribonuclease in human urine that hydrolyses polycytidylic acid. *Prep. Biochem.* **7**:283-299.
30. RESSLER, N., E. OLIVERO, G. R. THOMPSON, and R. R. JOSEPH. 1966. Investigation of ribonuclease isozymes by an electrophoretic ultra-violet method. *Nature* **210**:695-698.
31. RIGHETTI, P. G. and J. W. DRYSDALE. 1974. Isoelectric focusing in gels. *J. Chromatogr.* **98**:271-321.
32. SCHMUKLER, M., P. B. JEWETT, and C. C. LEVY. 1975. The effects of polyamines on a residue-specific human plasma ribonuclease. *J. Biol. Chem.* **250**:2206-2212.
33. STAMBAUGH, R. L. 1963. Electrophoresis of human urinary ribonuclease. *Fed. Proc.* **22**:493.
34. SZNAJD, J. 1972. Isolation and characterization of serum ribonucleases (RNases) from healthy persons and patients with chronic granulocytic leukemia (CGL). *Folia Med. Cracow* **14**:297-333.
35. SZNAJD, J. and J. W. NASKALSKI. 1973. Ribonuclease from human granulocytes. *Biochim. Biophys. Acta* **302**:282-292.
36. THOMAS, J. M. and M. E. HODES. 1978. Two-stage methods for alkaline range analytical isoelectric focusing in thin layer polyacrylamide gel: application to cationic proteins in serum. *Anal. Biochem.* **90**:596-608.
37. TOURNUT, R., B. J. ALLAN, and T. T. WHITE. 1978. Cancer, pancreatitis, and the detection of the isozymes of DNase, RNase, and amylase. *Clin. Chim. Acta* **88**:345-353.
38. UKITA, T., T. TAKAHASHI, K. WAKU, and O. HOSHINO. 1964. Research on pancreatic ribonuclease. III. Purification and properties of human pancreatic ribonuclease. *J. Biochemistry* **55**:293-302.
39. VAN LOON, L. C. 1975. Polynucleotide-acrylamide gel electrophoresis of soluble nucleases from tobacco leaves. *FEBS Letters* **51**:266-269.
40. WILSON, C. W. 1969. A rapid staining technique for detection of RNase after polyacrylamide gel electrophoresis. *Anal. Biochem.* **31**:506-511.
41. WOLF, G. 1968. Nachweis pflanzlicher Ribonukleasen in Polyacrylamid-Gelen nach Disk-Elektrophorese. *Experientia* **24**:890-891.
42. YAMANAKA, M., K. AKAGI, K. MURAI, N. HIRAO, S. FUJIMI, and T. OMAE. 1977. Purification and properties of urinary alkaline ribonuclease from patients with nephrotic syndrome. *Clin. Chim. Acta* **78**:191-201.