Biochem. J. (1988) 255, 501–505 (Printed in Great Britain)

Differences in glycosylation pattern of human secretory ribonucleases

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The major secretory ribonuclease (RNase) of human urine (RNase HU_A) was isolated and sequenced by automatic Edman degradation and analysis of peptides and glycopeptides. The isolated enzyme was shown to be free of other urine RNase activities by SDS/polyacrylamide-gel electrophoresis and activity staining. It is a glycoprotein 128 amino acids long, differing from human pancreatic RNase in the presence of an additional threonine residue at the *C*-terminus. It differs from the pancreatic enzyme in its glycosylation pattern as well, and contains about 45 sugar residues. Each of the three Asn-Xaa-Ser/Thr sequences (Asn-34, Asn-76, Asn-88) is glycosylated with a complex-type oligosaccharide chain. Glycosylation at Asn-88 has not been observed previously in mammalian secretory RNases. Preliminary sequence data on the major RNase of human seminal plasma have revealed no difference between it and the major urinary enzyme; their similarities include the presence of threonine at the *C*-terminus. The glycosylation pattern of human seminal RNase is very similar to that of the pancreatic enzyme. The structural differences between the secretory RNases from human pancreas, urine and seminal plasma must originate from organ-specific post-translational modifications of the one primary gene product. Detailed characterization of peptides and the results of gel filtration of tryptic and tryptic/chymotryptic digests of performic acid-oxidized RNase have been deposited as Supplementary Publication SUP 50146 (4 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1988) **249**, 5.

INTRODUCTION

The acid- and heat-stable ribonucleases (RNases) of human origin have been grouped into two broad classes by Sierakowska & Shugar (1977), namely 'secretory' and 'non-secretory', the former resembling pancreatic RNase, with high pH optimum and its preference for poly(C) as substrate, and the latter resembling granulocytic RNase, with a lower pH optimum and a preference for poly(U) as substrate. Human urine, a convenient source, contains both types of RNase. Their isolation and characterization has been described by a number of investigators (Cranston *et al.*, 1980; Iwama *et al.*, 1981; Rabin & Tattrie, 1982).

The major RNase of human urine, namely RNase HU_A, is of the secretory type and constitutes more than 60% of the assayed activity at pH 8.5 (Sugiyama *et al.*, 1981). Two minor ribonucleases (RNases HU_B and HU_C) also are of the secretory type, whereas a fourth (RNase HU_D) is of the non-secretory type. The primary structure of the latter enzyme has been determined recently. It was found to show a low, but significant, degree of homology with the secretory RNases of mammals and turtle and with human angiogenin (Beintema *et al.*, 1988).

Human secretory RNases from different organs are very similar in immunological properties (Weickmann & Glitz, 1982; Morita *et al.*, 1986) and have amino acid compositions very similar to that of the sequenced pancreatic enzyme (Beintema *et al.*, 1984*b*). A significant difference, however, is the presence of much carbohydrate in the secretory RNase from human urine and of relatively little carbohydrate in the enzymes from pancreas and seminal plasma.

It has been suggested that the serum and urine enzymes are of pancreatic origin and that elevated levels of urine RNases may be diagnostic of pancreatic cancer (Reddi & Holland, 1976; Nakane *et al.*, 1979). However, the latter suggestion has not been confirmed by other workers in the field (Peterson, 1979; Corbishley *et al.*, 1982).

Here we present a comparison of the primary structures of the major RNase components of human urine and seminal plasma with that of the pancreatic enzyme.

Preliminary accounts of this work have already appeared in abstract form (Beintema *et al.*, 1984*a*, 1985).

Abbreviations used: RNase, ribonuclease [in the present paper RNase is used rather than RNAase in order to accord with the nomenclature of Sugiyama *et al.* (1981)]; Tos-Phe-CH_2Cl, tosylphenylalanylchloromethane ('TPCK').

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MATERIALS AND METHODS

Trypsin (treated with Tos-Phe-CH_2Cl) and α-chymotrypsin were from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.), thermolysin was from Sigma (St. Louis, MO, U.S.A.) and trypsin was from Miles–Seravac (Maidenhead, Berks., U.K.). Other materials used were as described previously (Gaastra *et al.*, 1978; Beintema *et al.*, 1984*b*).

RNase HU_A was isolated from urine. Human urine (25 litres), collected from healthy volunteers, was concentrated 14-fold by dialysis against poly(ethylene glycol) (Carbowax 6000) at 4 °C. The concentrated urine was dialysed against water and made 5 mM with respect to sodium phosphate, pH 6.7. Phosphocellulose fractionation (Yamanaka *et al.*, 1977) was used for further concentration and partial purification. The sample was applied in 0.01 M-sodium phosphate, pH 6.7, and eluted with 2.0 M-NaCl in the same buffer. Fractions enriched in RNases were identified by polyacrylamide-gel electrophoresis and activity staining (Blank & Dekker, 1981).

The urine RNase concentrate was dialysed and chromatographed on a phosphocellulose column (1.7 cm × 33.5 cm) as described by Yamanaka *et al.* (1977), using a linear gradient of 0–1.2 M-NaCl in a total of 2 litres. Fractions (3.6 ml each) were collected. RNase activity was eluted as a single asymmetric peak when assayed at pH 8.5. SDS/polyacrylamide-gel electrophoresis and activity staining of individual column fractions showed RNase HU_A to be the dominant activity eluted in the peak tubes. It was followed by RNases HU_B and HU_D and, finally, RNase HU_C, which was eluted as the trailing edge of the peak.

For purification of RNase HU_A, fractions 53–63 were pooled, dialysed against equilibration buffer and rechromatographed on another phosphocellulose column (1.2 cm × 16.7 cm) using a shallower gradient of 0–0.6 M-NaCl in 1.8 litres of buffer. Fractions (3.8 ml each) were collected. Fractions 99–104 were pooled, dialysed, freeze-dried and dissolved in 3 ml of 0.02 M-Tris/HCl/0.4 M-NaCl, pH 8.5. The solution was loaded on a Sephadex G-100 column (2.2 cm × 64 cm). The column was eluted with the same buffer at a rate of 1 ml/min. Fractions (1.5 ml each) were collected. Fractions 60–89 were pooled, dialysed, freeze-dried and dissolved in 1.5 ml of 0.1 M-Tris/HCl/0.6% SDS, pH 7.4. The solution was loaded on a Sephadex G-150 column (2.2 cm × 100 cm), equilibrated with 0.1 M-Tris·HCl/0.1% SDS, pH 7.4. The column was eluted with the same buffer at a rate of 0.2 ml/min. Fractions (2 ml each) were collected. Fractions 121–134 were pooled and dialysed extensively, first against 0.1 M-NaCl and finally against water. Electrophoresis in SDS/polyacrylamide gel with embedded RNA (Blank & Dekker, 1981) showed a single broad band upon activity staining. Electrophoresis of a reduced and denatured sample also gave a single broad band after Coomassie Brilliant Blue staining for protein.

Human seminal RNase was isolated as described by De Prisco *et al.* (1984).

RNase samples used for sequence analysis were desalted by gel filtration on 0.5 cm × 7 cm (Pasteur pipette) columns of Sephadex G-25 with 0.2 M-acetic acid and freeze-dried.

Automatic Edman degradation on 2 mg of urine RNase was performed as described previously (Beintema *et al.*, 1984*b*). The phenylthiohydantoin derivatives of amino acids were identified by h.p.l.c. (Vereijken *et al.*, 1980).

Urine RNase (3 mg) was oxidized with performic acid and digested with 30 μg of Tos-Phe-CH_2Cl-treated trypsin at 37 °C in 300 μl of 0.2 M-NH_4HCO_3 for 3.5 h. Peptides T1–T16 were isolated by gel filtration on Sephadex G-25, followed by high-voltage paper electrophoresis at pH 3.5 or by h.p.l.c. on a Nucleosil 10 C_{18} column (300 mm × 4.6 mm) with a linear gradient from 0 to 67% acetonitrile in 0.1% trifluoroacetic acid over 60 min at a flow rate of 1.0 ml/min (peptide elution was monitored by absorbance at 214 nm).

A second digest of urine RNase (3 mg) was performed by incubating first with 20 μg of trypsin at 37 °C in 200 μl of 0.2 M-NH_4HCO_3 for 2 h, followed by addition of 20 μg of chymotrypsin and continuation of the incubation at 37 °C for another 2 h. Peptides were isolated by gel filtration on Sephadex G-25, followed by h.p.l.c. Tryptic peptides not cleaved by chymotrypsin were indicated with the same symbol (T) as that used for the first digest; chymotryptic cleavage products of three large tryptic peptides were termed T5C1, T5C2, T8C1, T8C2 and T16C1–T16C3. Peptide T8C2 was digested with 15 μg of thermolysin at 37 °C in 100 μl of 0.2 M-NH_4HCO_3 for 6 h. Peptides T8C2H1–T8C2H4 were isolated by h.p.l.c.

The isolated peptides were characterized by dansylation, amino acid analysis, dansyl–Edman degradation and electrophoresis at pH 6.5 for charge determination as described previously (Beintema *et al.*, 1984*b*). Quantitative analysis of monosaccharides released from glycopeptides after methanolysis was done as described by Kamerling *et al.* (1975).

About 1 mg of seminal RNase was used for automatic Edman degradation. Since human seminal RNase, like the pancreatic enzyme (Weickmann *et al.*, 1981), aggregates readily (De Prisco *et al.*, 1984), part of the material did not dissolve in dilute acid. The suspension was centrifuged and the clear supernatant was transferred to the sequencer cup. After the automatic Edman degradation the residue in the sequencer cup was extracted with 70% formic acid (in which the parvalbumin used as carrier did not dissolve). The extract was freeze-dried and, together with the undissolved aggregate, subjected to performic acid oxidation and digestion with 10 μg of trypsin at 37 °C in 100 μg of 0.2 M-NH_4HCO_3 for 2 h, followed by addition of 10 μg of chymotrypsin and continuation of the incubation at 37 °C for another 2 h. The digest was freeze-dried and several peptides were isolated by h.p.l.c.

RESULTS

Urine RNase

The automatic Edman degradation run provided the sequence of the *N*-terminal 25 residues, except for the serine residues and Met-13 (Fig. 1). Tryptic peptides T1–T16 were characterized by dansylation and amino acid analysis (Fig. 1; Table 1 of the Supplementary Publication). The results indicated no differences with the amino acid sequence of human pancreatic RNase (Beintema *et al.*, 1984*b*), except for glycosylation of all Asn-Xaa-Ser/Thr sequences and the possible presence of an additional threonine residue in peptide T-16.

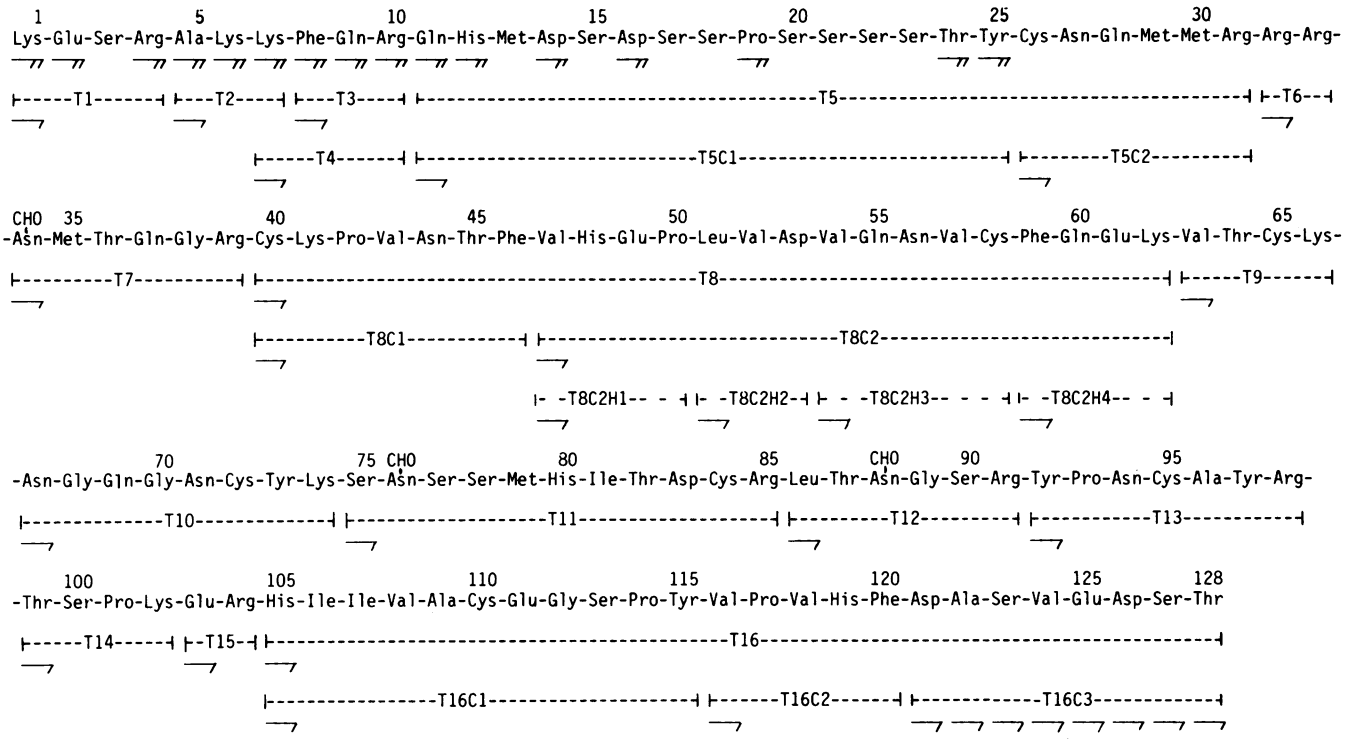

Fig. 1. Amino acid sequence of the major secretory RNase (RNase HU_A) from human urine

The nomenclature of the peptides is explained in the text. ⊢---⊣, Peptides used in reconstruction of the sequence; ⊢ - ⊣, peptides used only for charge determination at pH 6.5; ⁀⁀, identified during automatic Edman degradation as an amino acid phenylthiohydantoin derivative; ⁀, identified as a dansyl-amino acid; CHO, carbohydrate.

Therefore we decided to compare both proteins only by analysing the short peptides from a second digest.

Details of the isolation procedures are presented in Table 1 of the Supplementary Publication, together with the amino acid compositions of the peptides, their recoveries and their charges at pH 6.5. Results of the dansylations and of the dansyl–Edman degradation of peptide T16C3 are shown in Fig. 1.

Since the amino acid compositions of the peptides and other properties point to complete identity of the amino acid sequences of human pancreatic and urine RNase, except for the *C*-terminal residue, only the *C*-terminal

Table 1. Carbohydrate compositions of glycopeptides or the glycosylated components of human urine, pancreatic and seminal RNases

The amount of monosaccharide is given in mol/mol peptide or protein. The amount of *N*-acetylglucosamine has been corrected for incomplete cleavage of the *N*-glycosidic bond during methanolysis. Xylose and glucose probably are contaminants from the isolation procedures. The data for pancreatic RNase are from two forms of the same peptide (Beintema *et al.*, 1984*b*) recalculated relative to 3.0 mol of mannose/mol of peptide, and those of seminal RNase are from the glycosylated component (De Prisco *et al.*, 1984; recalculated to mol/mol of protein). Also included are carbohydrate analyses of the urine enzyme presented by Cranston *et al.* (1980) (recalculated to mol/mol of protein) and by Iwama *et al.* (1981). [a] indicates *N*-acetylglucosamine + *N*-acetylgalactosamine. ' + ' indicates a small amount present.

	Human urine RNase						Human pancreatic RNase (Asn-34)		Human seminal RNase
Monosaccharide	Asn-34	Asn-76	Asn-88	Total	Cranston *et al.* (1980)	Iwama *et al.* (1981)	Peptide A	Peptide B	
Fucose	1.0	1.3	1.0	3.3			4.4	1.8	4.3
Mannose	2.7	3.2	3.0	8.9			3.0	3.0	3.2
Galactose	2.1	6.0	2.7	10.8			4.6	1.7	3.4
Total hexose...				23.0	20.8	25			
N-Acetylglucosamine	3.2	8.0	5.5	16.7	12.8	8.5	5.9	3.6	4.1[a]
Sialic acid	0.7	1.6	1.2	3.5	3.2		–	–	–
Xylose	–	–	–				0.9	0.3	–
Glucose	+	–	+				1.0	0.4	1.6

peptide T16C3 was subjected to dansyl–Edman degradation, with identification of dansylthreonine-128 both before and after acid hydrolysis.

The void-volume peaks A of both gel filtrations (Figs. 1 and 2 of the Supplementary Publication) contained the glycopeptides T7, T11 and T12, with Asn-34, Asn-76 and Asn-88 respectively. No carbohydrate-free form of these peptides was observed. Table 1 of the present paper lists the carbohydrate compositions of these peptides, together with earlier-published compositions of both glycopeptides and whole glycosylated components of human urine, pancreatic and seminal RNases (Cranston *et al.*, 1980; Iwama *et al.*, 1981; Beintema *et al.*, 1984*b*; De Prisco *et al.*, 1984).

Seminal RNase

An automatic Edman degradation on seminal RNase revealed no differences with the *N*-terminal sequences of human pancreatic and urine ribonucleases. The *N*-terminal 25 residues could be identified, except for the serine residues. To obtain additional sequence information, a few peptides were isolated by h.p.l.c. of a tryptic and chymotryptic digest of insoluble aggregate of the protein and the residue from the sequencer cup (see the Materials and methods section). Five peaks from the h.p.l.c. pattern were identified by comparison of their elution positions with those of urine RNase peptides and by dansylation (Table 1). Amino acid analyses of these peptides were of less satisfactory quality. The available data indicate no differences with the urine and pancreatic enzymes. T16C3 contained 1.0 equiv. of threonine, suggesting that the seminal enzyme contains, like the urine enzyme but unlike the pancreatic one, threonine-128 at its *C*-terminus. No glucosamine was found in peptide T7 containing Asn-34.

DISCUSSION

Our results indicate that human pancreatic, urine and seminal-plasma RNases differ only in their *C*-terminal residue and in the glycosylation state of the Asn-Xaa-Ser/Thr sequences.

Cranston *et al.* (1980) isolated a secretory RNase from human urine with a *N*-terminal amino acid sequence of 13 residues identical with that shown in Fig. 1. However, they reported as *C*-terminal sequence:

His-Phe-Asp-Ala-Ser-Val-Ser-Thr

lacking the sequence Glu-Asp we found at positions 125 and 126.

C-Terminus

Most sequenced pancreatic RNases have valine-124 as *C*-terminal residue. However, a number of sequences have four additional residues at the *C*-terminus, and with a few exceptions they have threonine at the *C*-terminal position 128 (Beintema *et al.*, 1986). A second stop codon at position 129 and a codon for threonine at position 128 have been found in the 3′ non-coding nucleotide sequences of rat and mouse pancreatic RNase (MacDonald *et al.*, 1982; C. Schüller, unpublished work), of bovine seminal RNase (Palmieri *et al.*, 1985) and of bovine pancreatic RNase (Carsana *et al.*, 1988).

Serine-127 was identified unambiguously as the *C*-terminal residue in four separate batches of human pancreatic RNase (Beintema *et al.*, 1984*b*; Weickmann *et al.*, 1981). The major secretory RNase of human urine, however, has a *C*-terminal addition of four residues, with threonine at position 128. Probably carboxypeptidase removed the *C*-terminal residue of the pancreatic enzyme. In horse pancreatic RNase predominantly the *C*-terminal sequence Glu-Val (125–126) was found, and a minor component with the sequence Glu-Val-Ser-Thr (125–128) (Beintema, 1985). The removal of residues by carboxypeptidase action may have been stopped by the vicinity of negatively charged residues in both cases (Ambler, 1967).

Carbohydrate

Human secretory RNase contains three Asn-Xaa-Ser/Thr sequences (Asn positions 34, 76 and 88), with carbohydrate attached to Asn-34 in about half of the molecules in the pancreatic enzyme, but to all three sites in the urine enzyme. The compositions of the carbohydrate moieties in the urine enzyme (Table 1) are typical for acetyl-lactosamine-type chains (Strecker & Montreuil, 1979). The presence of six galactose and eight *N*-acetylglucosamine residues in the carbohydrate moiety attached to Asn-76 indicates the presence of more than one *N*-acetyl-lactosamine unit in at least one of the branches of a tetra-antennary structure (Krusius *et al.*, 1978).

Hitoi *et al.* (1987) determined the structures of carbohydrate moieties of secretory RNases of human urine after quantitative release as oligosaccharides on hydrazinolysis. The compositions of the structures indicated as II, V and VI in Table II of Hitoi *et al.* (1987) strongly resemble those of the moieties attached to Asn-76, Asn-88 and Asn-34 respectively in our study.

The three carbohydrate moieties together contain about 45 sugar residues (Table 1), in agreement with data reported by Cranston *et al.* (1980) and Iwama *et al.* (1981). The carbohydrate-to-protein ratio is about 1:2 on a weight basis. The high carbohydrate content accounts for the relatively high apparent M_r upon SDS/polyacrylamide-gel electrophoresis (31000) and gel filtration (44000) (Sugiyama *et al.*, 1981), and must have a significant influence on other physical properties of the enzyme as well.

Human seminal-plasma RNase is synthesized in the prostate (De Prisco *et al.*, 1984). Its glycosylation characteristics are similar to those of the pancreatic enzyme. About half of the molecules contain carbohydrate with a composition very similar to those of two glycopeptides of the pancreatic enzyme (Table 1). Both enzymes contain rather a lot of fucose.

Interrelationships between the human RNases

Our results indicate that the major secretory RNase present in human pancreas, urine and seminal plasma are very similar. The differences probably originate from post-translational modification of a single primary gene product. Close similarity between human RNases from pancreas, brain, kidney, serum and urine has been found by Weickmann & Glitz (1982) using immunological methods. By contrast, the major secretory RNases from bovine pancreas, seminal plasma and brain are structurally different (Elson & Glitz, 1975; Okazaki *et al.*, 1975; Suzuki *et al.*, 1987).

Yamashita *et al.* (1986) also report organ-specific differences in the carbohydrates attached to human RNases. Morita *et al.* (1986) separated secretory and

non-secretory RNases from several tissues and body fluids by phosphocellulose chromatography and identified them by immunoassay. Similar elution patterns were observed for the major peaks of secretory RNases of urine and kidney, which suggests that kidney may be the origin of the urine RNase.

It has been suggested that the secretory ribonucleases in serum and urine are of pancreatic origin (Reddi & Holland, 1976; Nakane *et al.*, 1979). However, the presence of carbohydrate in urine RNase at sites which are carbohydrate-free in the pancreatic enzyme indicates that the urine enzyme does not originate in the pancreas, as the addition of oligosaccharide moieties to asparagine residues occurs before folding of the nascent polypeptide chain (Beintema *et al.*, 1973, 1976; Hanover & Lennarz, 1981). This suggests that a primary gene product may undergo glycosylation to different extents in different parts of the body. A similar observation has been made by Abe & Liao (1983), who found that bovine deoxyribonuclease is glycosylated differently in the pancreas and the parotid gland.

Many thanks are due to Mr. Peter Jekel and Mr. Piet Wietzes for performing the amino acid analyses and automatic Edman degradations, to Mr. G. J. Gerwig and Dr. J. P. Kamerling (Laboratorium voor Bio-Organische Chemie, Utrecht, The Netherlands) for performing the carbohydrate analyses of the glycopeptides and to Dr. R. N. Campagne for reviewing the manuscript before its submission. This work was supported by a travel grant to J. J. B. from the Collaborative Research Grant Programme of the NATO Scientific Affairs Division.

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Received 19 January 1988/6 May 1988; accepted 1 June 1988