Two distinct secretory ribonucleases from human cerebrum: purification, characterization and relationships to other ribonucleases

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Two RNAases from human cerebrum were purified to an electrophoretically homogeneous state and their molecular masses were 22.0 kDa (tentatively called RNAase HB-1) and 19.0 kDa (RNAase HB-2). Analyses of the amino acid compositions, N-terminal amino acid sequences and catalytic properties of these enzymes provided strong evidence that they were strictly related to the secretory (sec) RNAases, such as the pancreatic enzyme, very similar immunologically to urinary sec RNAase, but clearly distinguishable from urinary non-secretory (nonsec) RNAase. There were several differences between HB-1 and HB-2, namely their immunological reactivities with specific antibodies, heat-stabilities, attached carbohydrate moieties and molecular masses. In particular, HB-2 appeared to be non-glycosylated, in view of its lack of affinity for several conjugated

lectins, the absence of hexosamine and no change in electrophoretic mobility before and after peptide:*N*-glycosidase F digestion, whereas HB-1 and human sec RNAases purified from kidney, pancreas and urine all appeared to be glycosylated, as they moved to the same position as HB-2 when electrophoresed after glycosidase digestion. An antibody against urinary sec RNAase inhibited 75% and 20% of the total activity of the crude cerebral extract against RNA at pH 8.0 and 6.0 respectively, whereas an antibody against urinary nonsec RNAase had no such inhibitory effect. These findings suggest that yet another type(s) of cerebral RNAase, which is unable to cross-react immunologically with sec and nonsec RNAases, may exist. Two RNAases corresponding to HB-1 and HB-2 were identified in fresh cerebrospinal fluid.

INTRODUCTION

Pathophysiological changes in the central nervous system, such as anoxia [1], ischaemia [1], hyperphenylalaninaemia [2], nerve regeneration [3], morphine-dependence development [4] and Alzheimer's disease [5,6], have been found to affect RNAase activity in the brain. In particular, Sajdel-Sulkowska and Marotta [5] suggested that the lower than normal total RNA and mRNA contents in post-mortem cerebral tissue from patients with Alzheimer's disease [7] may be due to significant increases in alkaline RNAase activity resulting from changes in an enzyme–RNAase inhibitor system in the patients' brains. Recently, other groups [8–10] demonstrated that the total and free alkaline RNAase levels in the affected brain areas of patients with Alzheimer's disease did not differ significantly from those of carefully age-matched controls. In previous studies [1–6,8–10], the total and free RNAase activities in crude extracts from whole brains or discrete brain regions were evaluated and obviously reflected the combined activities of several enzymes. Therefore, in order to determine changes in cerebral RNAase activity and elucidate their pathophysiological significance, the isolation and characterization of cerebral RNAase and its inhibitor in the pure state would appear to be essential. However, technical limitations accompanied by the difficulty in obtaining source brains and their low cerebral RNAase content have hindered such purification and detailed characterization. Protein sequence studies have yielded evidence that an ever-increasing number of RNAase superfamily proteins possess diverse biological actions, including angiogenic, anti-tumour, immunosuppressive, neurotoxic and sialic acid-binding lectin properties [11,12]. Among these pro-

teins, human eosinophil-derived neurotoxins, which cause nervous tissue degeneration [13,14], have been demonstrated to be virtually identical with human liver RNAase [15]. To our knowledge, no RNAase isolated from human brain has been purified to homogeneity and characterized in its pure state. Human RNAases are distributed widely in various organs and body fluids. The pyrimidine-specific neutral and alkaline RNAases have been classified into two distinct classes: secretory (sec) and non-secretory (nonsec) [16]. The RNAase activities in body fluids were expected to serve as diagnostic markers for various diseases [17,18], but their molecular multiplicity [19–21] has made it difficult to establish definite correlations between RNAase activity fluctuations and disease severity. Elucidation of the biochemical and immunological properties of brain RNAases is of great interest, as they are involved in cerebral nucleic acid metabolism, which may be associated with specific brain function.

In the present paper, we describe the isolation and characterization of two cerebral RNAases, which were compared enzymologically and immunologically with sec and nonsec RNAases purified from cerebrospinal fluid, urine, pancreas, kidney and spleen.

MATERIALS AND METHODS

Materials and proteins

All the chemicals used were of reagent grade or the purest grade available commercially. Agarose–hexane-adenosine 5'-phosphate (AMP–agarose), poly(I), Sephadex G-75 and G-25 and molecular-mass calibration kits were purchased from Pharmacia LKB (Uppsala, Sweden); cellulose phosphate P-11 and

Abbreviations used: AMP–agarose, agarose–hexane-adenosine 5'-phosphate; APUP–agarose, agarose–5'-(4-aminophenylphosphoryl)uridine 2'(3')-phosphate; Con A, concanavalin A; LCA, lentil lectin; nonsec, non-secretory; PMSF, phenylmethanesulphonyl fluoride; PNGase F, peptide:*N*-glycosidase F; poly(G)–agarose, poly(G) linked to agarose; RCA₁₂₀, castor bean lectin; sec, secretory; WGA, wheatgerm lectin.

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Table 1 Details of human brain samples and their RNAase activities

All of the brain samples were proved to be histologically normal. RNAase activities were measured at pH 8.0 using yeast RNA as the substrate as described in the text. All assays were performed at least in quadruplicate, and the results are means $\pm$ S.D.

Sample no.	Age (years)	Sex	Post-mortem interval (h)	Storage interval (years)	Cause of death	RNAase activity (units/g of tissue)		
						HB-1 fraction	HB-2 fraction	HB-1/HB-2
1	41	Male	3	0.3	Loss of blood	2.31 $\pm$ 0.12	1.52 $\pm$ 0.02	1.52
2	26	Female	17	2.9	Loss of blood	2.58 $\pm$ 0.02	2.28 $\pm$ 0.40	1.13
3	47	Male	6	5.4	Myocardial infarction	2.53 $\pm$ 0.20	3.16 $\pm$ 0.21	0.80
4	29	Male	14	1.8	Acute pancreatitis	3.57 $\pm$ 0.10	2.88 $\pm$ 0.04	1.24
5	46	Female	19	2.7	Asphyxia by hanging	1.93 $\pm$ 0.07	1.54 $\pm$ 0.09	1.26

agarose-5'-(4-aminophenylphosphoryl)uridine 2'(3')-phosphate (APUP-agarose) were from Whatman (Maidstone, Kent, UK) and ICN Immuno Biologicals (Lisle, NY, U.S.A.) respectively; phenylmethanesulphonyl fluoride (PMSF), poly(G) linked to agarose [poly(G)-agarose], spermine and spermidine were from Sigma Chemicals Co. (St Louis, MO, U.S.A.); heparin-Actigel and yeast RNA were from Sterogen Biochemicals (San Gabriel, CA, U.S.A.) and Kojin (Tokyo, Japan) respectively; the synthetic homoribopolymers, poly(A), poly(C) and poly(U) and agaroses conjugated with concanavalin A (Con A), lentil lectin (LCA), castor bean lectin (RCA₁₂₀) and wheatgerm lectin (WGA) were from Seikagaku Kogyo (Tokyo, Japan).

RNAases were purified from human kidney, pancreas and urine, as described previously [22–24]. Human placental RNAase inhibitor and the recombinant peptide: *N*-glycosidase F (PNGase F) were obtained from Toyobo Co., Ltd. (Osaka, Japan) and New England Biolabs (Beverly, MA, U.S.A.) respectively. Three discrete specific antibodies against human urinary sec RNAase and two synthetic peptides, KESRAKKFQRQHMDS and PYVPVHFDASVEDST, which corresponded to the amino acid sequences of the N- and C-terminal 15 residues of the enzymes respectively, were prepared as described in previous papers [20,24]. Antibodies against the nonsec RNAases purified from human kidney, spleen and urine were raised in rabbits, as described previously [23,25,26]. Each IgG fraction was purified using DEAE-Affi-Gel Blue (Bio-Rad, Richmond, CA, U.S.A.) and used as the relevant antibody.

RNAase activity assay

The RNAase activity assay was carried out using the method of Uchida and Egami [27] with some modifications. Samples (50 μ l) of the required substrate solution, 1.2% (w/v) yeast RNA or 0.4% (w/v) poly(C), were added to 150 μ l of reaction mixture, which comprised 50 mM Tris/HCl buffer, pH 8.0, 5 mM EDTA and the required RNAase enzyme preparation. Each mixture was incubated at 37 °C for the desired time and the reaction was terminated by adding 5 μ l of 1% (w/v) BSA and 50 μ l of 25% (w/v) perchloric acid, which contained 0.75% (w/v) uranyl acetate. The mixture was centrifuged at 1600 *g* for 5 min; a 100 μ l portion of the supernatant was added to 2.5 ml of distilled water and the absorbance of the resulting solution was measured at 260 and 280 nm for the RNA and poly(C) substrates respectively. One unit of RNAase activity was defined, using RNA as the

substrate, as the amount that increased the absorbance by 1.0 per 15 min [26].

Purification of enzymes from human cerebrum

Human cerebra were obtained at autopsy and stored at –80 °C before use. The sample background details are summarized in Table 1. All the subsequent procedures were carried out at 0–5 °C.

Step 1

Approximately 1 kg of cerebrum (sample no. 1) was partially thawed overnight at 4 °C, cut into small pieces, washed thoroughly with cold saline to remove excess blood, suspended in about 800 ml of 0.25 M H₂SO₄ containing 1 mM MgCl₂ and homogenized using an Ultraturrax (IKA-WERK, Staufen, Germany). The homogenate was centrifuged at 12000 *g* for 30 min. The supernatant was decanted and stored, and the pellet was resuspended in the same buffer and centrifuged under the same conditions. This supernatant was combined with the first and the pH was adjusted to 7.0 with NH₄OH (crude extract).

Step 2

The crude extract was fractionated with (NH₄)₂SO₄. The precipitate that formed at a saturation level of between 0.3 and 0.9 after standing for at least 3 days was collected, dissolved in 25 mM potassium phosphate buffer, pH 6.0, containing 1.0 mM PMSF (buffer I) and then dialysed against this buffer.

Step 3

The material from step 2 was centrifuged at 12000 *g* for 20 min, the supernatant was applied to a cellulose phosphate P-11 column (3.2 cm $\times$ 40 cm) pre-equilibrated with buffer I and the adsorbed materials were eluted with a 2-litre linear NaCl gradient (0–2.0 M) in buffer I. The RNAase activities were eluted as two main peaks at a broad range of NaCl concentrations between 0.7 and 1.1 M (Figure 1). When subjected to SDS/PAGE, the first peak produced one major band with the fastest mobility and several minor bands with slower mobilities. The final portion comprising two-thirds of the first peak only was collected and called HB-1. The second peak was named HB-2. Each peak was collected

Figure 1 Cellulose phosphate P-11 column chromatography of the RNAase-active fractions from human cerebrum

The materials containing the RNAase activities after $(\text{NH}_4)_2\text{SO}_4$ fractionation were subjected to chromatography on a cellulose phosphate P-11 column, and 26 ml portions of each were collected at a flow rate of 25 ml/h. The horizontal bars indicate the two RNAase-active fractions, nos. 32–39 and 40–47, which were designated HB-1 and HB-2 respectively. ●, RNAase activity; —, A_{280} ; — · —, [NaCl]. Inset: SDS/PAGE patterns of the major fractions eluted from the column. Samples of each fraction were electrophoresed through a 13.5% polyacrylamide gel containing 0.2 mg/ml poly(C), and the RNAase activities were visualized by activity staining, as described in the text. The numbers indicate the fractions analysed. The cathode is at the top.

separately and dialysed against 25 mM Tris/HCl buffer, pH 7.0 (buffer II).

Step 4

The dialysed HB-1 and HB-2 solutions were centrifuged separately and each supernatant was applied to a heparin–Actigel column (1.6 cm × 15 cm) pre-equilibrated with buffer II, and the adsorbed materials were eluted with a 700 ml linear NaCl gradient (0–1.0 M) in buffer II. The active fractions of each, which were eluted at around 0.5 M NaCl, were pooled and concentrated with poly(ethylene glycol) 6000.

Step 5

Each concentrate from step 4 was dialysed against 25 mM Tris/HCl buffer, pH 7.5, containing 0.25 M NaCl (buffer III) and subjected to gel filtration on a Sephadex G-75 column (1.6 cm × 100 cm) pre-equilibrated with buffer III and eluted with this buffer at a flow rate of 6 ml/h. The RNAase-active fractions of HB-1 and HB-2, which were detected at elution volumes of between 310 and 350 ml and 340 and 380 ml respectively, were pooled and dialysed against 20 mM sodium acetate buffer, pH 5.0 (buffer IV).

Step 6

The dialysed materials from step 4 were applied to an APUP–agarose column (1.0 cm × 10 cm) pre-equilibrated with buffer IV and eluted with a 400 ml linear NaCl gradient (0–1.2 M) in this buffer. The HB-1 and HB-2 RNAase-active fractions were eluted at around 0.6 M NaCl and those for each were pooled and

dialysed against 20 mM sodium phosphate buffer, pH 7.5 (buffer V).

Step 7a

The dialysed HB-1 material from step 6 was applied to a poly(G)–agarose column (1.0 cm × 10 cm) pre-equilibrated with buffer V and eluted with a 200 ml linear NaCl gradient (0–1.2 M) in this buffer. The HB-1 RNAase-active fraction was eluted at around 0.6 M NaCl and dialysed against buffer IV.

Step 7b

The dialysed HB-2 material from step 6 was applied to a poly(G)–agarose column, as described for step 7a, which was washed with 100 ml of buffer IV and eluted with 1.0 M NaCl in this buffer. The peak fractions showing RNAase activity were pooled and concentrated by ultrafiltration.

Step 8

The dialysed solution from step 7a was applied to an AMP–agarose column (1.0 cm × 5.0 cm) pre-equilibrated with buffer IV and eluted with a 110 ml linear NaCl gradient (0–0.75 M) in this buffer. The RNAase-active fractions were pooled and concentrated by ultrafiltration. The RNAases obtained after steps 8 and 7b were used as purified HB-1 and HB-2 respectively.

Electrophoresis and detection of RNAase

SDS/PAGE was performed using 13.5% polyacrylamide gel [28], and immunoblotting was carried out as described in previous papers [29,30]. In order to detect RNAase by activity staining, poly(C) (final concentration of 0.2 mg/ml) was added to the separating gel solution before polymerization [19,31]. After the electrophoretic run, the gel was incubated for the desired times in 50 mM Tris/HCl buffer, pH 8.0, containing 5 mM EDTA, at 37 °C and then stained with 0.2% (w/v) Toluidine Blue (Eastman Kodak Co., Rochester, NY, U.S.A.) in this buffer.

Protein characterization

A portion of each purified RNAase was desalted on a Sephadex G-25 column pre-equilibrated with 0.2 M acetic acid and then lyophilized. Edman degradation of native RNAases (about 10 µg) was carried out on an automated gas-phase protein sequencer (Applied Biosystems 477A, Foster City, MI, U.S.A.) and the phenylthiohydantoin derivatives were identified using an h.p.l.c. attached to the sequencer. The amino acids and amino sugars obtained by hydrolysing each enzyme (5–10 µg) in 5.7 M HCl at 110 °C for 20 h (amino acids) and 4.0 M HCl at 110 °C for 4 h (amino sugars) were converted into phenylthiocarbamoyl derivatives using a Waters PICO-TAG work station (Millipore Corp.) and the derivatives were separated and quantified using h.p.l.c. according to the manufacturer's instructions. The protein concentrations were determined with a Bio-Rad protein assay kit using BSA as the standard.

Inhibitory effects of specific antibodies and placental RNAase inhibitor on the enzyme activity

Incubation mixtures (50 µl) comprising the required purified enzyme (1.0 or 0.3 units) or crude extract with the same activity, the desired amounts of the required antibodies and 20 mM PBS, pH 6.8, were kept at 4 °C overnight, then centrifuged at 2000 g

Figure 2 Elution profile of human cerebrospinal fluid subjected to cellulose phosphate P-11 column chromatography

Cerebrospinal fluid (5 ml) was subjected to cellulose phosphate chromatography, and 4 ml portions of each were collected at a flow rate of 10 ml/h. Their absorbances at 280 nm were below the sensitivity of the instrument used. The RNAase-active fractions, nos. 45–50 and 51–56, were designated CSF-1 and CSF-2 respectively and are indicated by the horizontal bars. ●, RNAase activity; —, [NaCl]. Inset: SDS/PAGE patterns of the major fractions eluted from the column. The RNAase activities in each fraction were detected as described in the text; the numbers indicate the fractions analysed. The upper and lower arrows indicate the positions corresponding to cerebral HB-1 and HB-2 respectively. The cathode is at the top.

for 30 min, and the RNAase activity remaining in each supernatant was measured at pH 6.0 and 8.0 using yeast RNA and poly(C) as substrates. The placental RNAase inhibitor (10 units) was diluted serially with 50 mM KCl, 5 mM dithiothreitol and 50% (v/v) glycerol in 20 mM Tris/HCl buffer, pH 7.5. Samples (25 μ l) of the diluted inhibitor were added to the reaction mixtures, which comprised 75 μ l of 0.1 M Tris/HCl buffer, pH 7.5, containing 10 mM EDTA, 35 μ l of 5 mM dithiothreitol and 20 μ l of required purified enzyme (1.0 or 0.3 unit), left to stand at 37 °C for 30 min and the RNAase activity in each mixture was determined, as described above.

Affinity of the purified RNAases for lectin columns and digestion with PNGase F

The purified cerebral enzymes (about 0.5 unit) dissolved in 0.5 ml of 20 mM sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl were applied to individual lectin-agarose columns (1 ml each) conjugated with Con A, LCA, RCA₁₂₀ or WGA, which were all pre-equilibrated with this buffer. The columns were washed with 5 ml of this buffer (unbound fractions), and the adsorbed enzyme was eluted from each column with 5 ml of 0.2 M methyl α -D-mannopyranoside (Con A), 0.1 M D-lactose (LCA), 0.2 M D-galactose (RCA₁₂₀) or 0.2 M N-acetyl-D-glucosamine (WGA) in the same buffer (bound fractions). The enzyme activities of the bound and unbound fractions were determined as described above.

The purified enzymes (about 0.1 unit) were dissolved in 50 μ l of 50 mM sodium phosphate buffer, pH 7.5, which contained 0.5% (w/v) SDS and 1% (v/v) 2-mercaptoethanol, heated at 100 °C for 10 min, after which 10 μ l of 5% (v/v) Nonidet P40 in 50 mM sodium phosphate buffer, pH 7.5, was added, followed by 2000 units of PNGase F. The mixture was then incubated at 37 °C for 2 h. The resulting digested RNAases were subjected to

SDS/PAGE using gel containing poly(C) and then detected by activity staining, as described above.

Partial purification of RNAases from human cerebrospinal fluid

Fresh cerebrospinal fluid (5 ml) obtained from a healthy 24-year-old male was mixed with an equal volume of buffer I and then dialysed against this buffer. The dialysed solution was applied to a cellulose phosphate column (1.0 cm $\times$ 20 cm) pre-equilibrated with buffer I, which was eluted with a 300 ml linear NaCl gradient (0–1.75 M) in this buffer. The RNAase activity was recovered as two peaks at a broad NaCl concentration range (0.7 and 1.2 M) (Figure 2). The first (called CSF-1) and the second (CSF-2) peaks were pooled separately and dialysed against buffer IV. The two enzymes were purified separately by successive chromatography on APUP-agarose and poly(G)-agarose columns using the purification procedures described above for the cerebral RNAases. The final RNAase-active fractions were pooled separately, dialysed against 0.1% (w/v) glycine, lyophilized and subjected to further analyses, as described above for HB-1 and HB-2.

RESULTS

Purification of cerebral enzymes

Typical cerebral RNAase purification results are summarized in Table 2. As the RNAase activities in the crude extracts obtained after tissue homogenization in neutral buffer solution were extremely low, as reported for bovine brain [32], and 59–90% of the total activity was associated with an RNAase inhibitor in an inactive complex [9], the initial crude extract was prepared by homogenization in 0.25 M H₂SO₄. The RNAase activities of the five extracts prepared from different cerebra (Table 1) ranged from 0.74 to 1.25 units/mg of protein and RNAase activities of each separated into two peaks, designated HB-1 and HB-2, when subjected to cellulose phosphate chromatography (Figure 1). The HB-1/HB-2 activity ratios of the five preparations showed a narrow distribution range of 0.80 to 1.52 (Table 1).

The purified HB-1 and HB-2 were subjected to SDS/PAGE and detected by protein staining and immunoblotting with anti-(urinary sec RNAase) (Figure 3). Each produced a single band, HB-2, migrating faster than HB-1 and purified urinary sec RNAase.

Protein structure

The respective molecular masses of HB-1 and HB-2 were 22 and 19 kDa by SDS/PAGE, and 14 and 12 kDa by gel filtration, which was carried out as described elsewhere [33]. The secretory RNAase was reported to undergo aggregation-disaggregation reactions under the influence of polyamines [34]. Therefore gel filtration on Sephadex G-75 with eluent containing 0.5 mM spermine gave an acceptable molecular mass for the enzyme corresponding to that estimated by SDS/PAGE (results not shown) as described previously [33].

The amino acid compositions of HB-1 and HB-2 closely resembled those of pancreatic and urinary sec RNAases, but not that of urinary nonsec RNAase (Table 3); HB-1 contained at least 2.0 residues of glucosamine/molecule as a hexosamine, which was calculated on the basis of its molecular mass, 22 kDa, whereas the HB-2 enzyme contained no hexosamine (Table 4). The N-terminal amino acid sequences of HB-1 and HB-2 were identical with those of the sec RNAases obtained from kidney [23], urine [35] and pancreas [36], but very different from those of nonsec RNAases [25,37] (Figure 4): the amino acid residues Arg,

Table 2 Summary of the purification of two RNAases from human cerebrum

The RNAases were purified from about 1 kg of a cerebrum derived from a 41-year-old male (sample no. 1 in Table 1). Protein concentration was determined with a Bio-Rad protein assay kit using BSA as the standard. Enzyme activity was measured at pH 8.0 using yeast RNA as the substrate as described in the text.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Purification (-fold)	Yield (%)
1. Crude extract	8786.2	9593.4	1.092	1	100
2. (NH ₄) ₂ SO ₄ fractionation	1207.7	5726.4	4.753	4.4	59.7
3. Cellulose phosphate					
1st fraction (HB-1)	115.36	2523.2	21.87	20.0	26.5
2nd fraction (HB-2)	46.40	1673.2	36.06	33.0	17.4
4. Heparin-actigel					
HB-1	19.98	2123.2	106.3	97.3	22.1
HB-2	7.20	1354.5	188.1	172.3	14.1
5. Sephadex G-75					
HB-1	1.584	1722.6	1087.5	995.9	18.0
HB-2	0.725	661.9	913.0	836.1	6.9
6. APUP-agarose					
HB-1	0.352	1397.4	3969.6	3635.4	14.6
HB-2	0.192	628.0	3270.8	2995.3	6.6
7. Poly(G)-agarose					
HB-1	0.154	1301.7	8452.6	7740.5	13.6
HB-2	0.040	463.2	11 580.0	10 604.4	4.8
8. AMP-agarose					
HB-1	0.080	946.7	11 833.7	10 836.7	9.9

Figure 3 SDS/PAGE patterns of cerebral RNAases HB-1 and HB-2 detected by (a) protein staining and (b-d) immunoblotting with specific antibodies

(a) Purified HB-1, HB-2 and urinary sec RNAase (about 2 μ g of each) were dissolved in 10 mM Tris/HCl buffer (pH 6.8), which contained glycerol (10%, v/v), SDS (2%, w/v), 25 mM dithiothreitol and a trace of Bromophenol Blue, heated at 100 °C for 5 min and electrophoresed at 200 V for 2 h, after which the proteins were stained with Coomassie Brilliant Blue R-250. (b-d) HB-1 and HB-2 (about 0.5 μ g of each) were subjected to SDS/PAGE in the same manner, after which the enzymes were transferred to a Durapore membrane (Millipore, Bedford, MA, U.S.A.) and visualized by immunoblotting with (b) an anti-(urinary sec RNAase) antibody, (c) a specific antibody against the peptide corresponding to the C-terminal 15 residues of urinary sec RNAase and (d) an anti-(urinary nonsec RNAase) antibody, as described in a previous paper [20]. Lane 1, HB-1; lane 2, HB-2; lane 3, urinary sec RNAase; lane 4, molecular-mass markers: A, phosphorylase b (94 kDa); B, BSA (67 kDa); C, ovalbumin (43 kDa); D, carbonic anhydrase (30 kDa); E, trypsin inhibitor (20 kDa); F, α -lactalbumin (14.4 kDa).

Lys, Gln, Asp and Pro of the HB-1 and HB-2 sequences were replaced with Ala, Ala, Arg, Gly and Ser at positions 4, 6, 9, 16 and 19 in bovine brain RNAase [38].

Catalytic properties

The pH-activity profiles of HB-1 and HB-2 against poly(C) and yeast RNA substrates were bell-shaped with an optimal pH of

Table 3 Amino acid compositions of two RNAases from human cerebrum

Values are averages of triplicate determinations. The composition of sec RNAase was calculated from the sequence from ref. [35]. It is nearly identical with that of human pancreatic RNAase [36]. The composition of nonsec RNAase was calculated from the sequence from ref. [37]. ND, not determined.

Amino acid	Composition (mol%)			
	Cerebral RNAase		Urinary RNAase	
	HB-1	HB-2	Sec	Nonsec
Asp + Asn	10.8	11.5	11.7	15.7
Glu + Gln	10.3	10.0	10.2	10.5
Ser	11.9	10.8	12.4	4.5
Gly	7.4	4.9	3.9	1.5
His	3.2	3.6	3.9	3.8
Arg	6.0	6.4	7.8	6.0
Thr	5.9	6.0	6.3	9.0
Ala	4.8	3.7	3.1	4.5
Pro	6.0	6.1	5.5	9.0
Tyr	2.8	3.2	3.9	3.0
Val	6.9	8.3	7.8	6.8
Met	3.7	3.9	3.9	3.0
Cys	5.2	6.9	6.3	6.0
Ile	2.5	2.2	2.3	5.3
Leu	1.7	1.7	1.6	3.8
Phe	3.3	3.7	3.1	3.8
Lys	7.6	7.0	6.3	3.0
Trp	ND	ND	0	0.8

8.0 and their activities disappeared below pH 5.0 and above pH 10.0. When the ionic strength was adjusted by adding NaCl to 25 mM Tris/HCl buffer, pH 8.0, their activities against poly(C) were affected profoundly: maximal activities were observed at an ionic strength of 0.075 mol/l, which was about three times greater

Table 4 Carbohydrate compositions of RNAases from human cerebrum

Values are averages of duplicate determinations. The values for kidney RNAases are taken from ref. [23], and those for urinary RNAase from ref. [35]. The amino sugars were calculated as the *N*-acetyl form. ND, not detected; NT, not tested.

Carbohydrate component	Composition (residues/molecule)				Urinary RNAase (sec)
	Cerebrum RNAases		Kidney RNAases		
	HB-1	HB-2	HK-2A	HK-2B	
Glucosamine	2.0	ND	1.5	2.3	16.7
Galactosamine	ND	ND	ND	ND	ND
Fucose	NT	NT	1.2	2.5	3.3
Mannose	NT	NT	1.6	3.0	8.9
Galactose	NT	NT	Trace	Trace	10.9

Table 5 Substrate specificity of human cerebral and cerebrospinal RNAases

The reaction mixture (0.2 ml) comprised 0.2 mg of the homoribopolymer substrate indicated, 50 mM Tris/HCl buffer, pH 8.0, and 5 mM EDTA. The other conditions were as described in the text. The activity of each enzyme is expressed relative to that for RNA. The values for urinary RNAases are taken from refs. [26] and [39]. ND, not detected.

Substrate	Relative activity					
	Cerebrum			Cerebrospinal fluid		Urine
	HB-1	HB-2	Crude extract	CSF-1	CSF-2	Sec RNAase Nonsec RNAase
RNA	1.0	1.0	1.0	1.0	1.0	1.0
Poly(A)	ND	ND	ND	ND	ND	ND
Poly(C)	44.9	45.4	30.6	30.6	42.6	100
Poly(I)	ND	ND	ND	ND	ND	ND
Poly(U)	0.6	0.7	0.3	0.3	0.7	6

	Residue number				
	1	5	10	15	20
Cerebral RNAase					
HB-1	K-E-S-R-A-K-K-F-E-R-E-H-M-D-S-D-S-S-P-(S)				
HB-2	K-E-S-R-A-K-K-F-E-R-E-H-M-D-S-D-S-S-P-(S)-S				
Secretory RNAase					
Kidney	K-E-S-R-A-K-K-F-E-R-E-H-M-D-S-D-S-S-P				
Pancreas	K-E-S-R-A-K-K-F-E-R-E-H-M-D-S-D-S-S-P-S-S-S				
Urine	K-E-S-R-A-K-K-F-E-R-E-H-M-D-S-D-S-S-P-S-S-S				
Non-secretory RNAase					
Urine	K-P-P-Q-F-T-X-A-Q-W-F-E-T-Q-H-I-N-M-T-S-Q-Q				
Spleen	K-P-P-Q-F-T-X-A-Q-W-F-E-T-Q-H-I-X-M-T-S-Q-Q				
Bovine brain RNAase	K-E-S-A-A-A-K-F-R-R-Q-H-M-D-S-G-S-S-S-S-N				

Figure 4 N-Terminal amino acid sequences of human cerebral RNAases HB-1 and HB-2

The equivalent sequences of human pancreatic RNAase [36], human urinary RNAases [35,37], human kidney sec RNAase [23], human spleen RNAase [25] and bovine brain RNAase [38] are also shown. The X symbol indicates the undetermined positions. The residues in parentheses were identified tentatively.

than that at 0.025 mol/l, and the activities were virtually abolished at 0.5 mol/l. The effects of bivalent cations (5 mM as chlorides) and polyamines on the enzyme activities were investigated at pH 8.0 using poly(C) as the substrate. Both enzymes were activated slightly by Ba²⁺, Ca²⁺, Co²⁺ and Mg²⁺, whereas they were inhibited strongly by Cd²⁺, Cu²⁺, Fe²⁺ and Mn²⁺ and abolished by Hg²⁺ and Zn²⁺. Addition of polyamine, spermine or spermidine (in excess of 0.25 mM) elevated the enzyme activities about 2-fold compared with control preparations. The catalytic properties of the RNAases in the crude extract were very similar to those of purified HB-1 and HB-2 and urinary sec RNAase.

The HB-1 and HB-2 enzymes were preincubated at various temperatures for 10 min in 50 mM Tris/HCl buffer, pH 8.0, which contained 5 mM EDTA, and left at 37 °C, after which the residual RNAase activity was measured. The HB-2 enzyme was found to be somewhat more labile than both HB-1 and urinary sec RNAase between 45 and 70 °C: the residual activity of HB-2 was reduced to 50 % at 50 °C, whereas that of the other two enzymes was reduced to 50 % at 60 °C; all three enzyme activities were undetectable above 75 °C.

Synthetic homoribopolymers were used as substrates in place of yeast RNA (Table 5). Both HB-1 and HB-2 exhibited a 45- to 70-fold greater preference for poly(C) than for RNA or poly(U), and failed to hydrolyse purine homoribopolymers under the same conditions. Furthermore the substrate preference of the cerebral crude extract was similar to those of purified HB-1 and HB-2, although the reactivity of the former with RNA was slightly higher than that of HB-1 and HB-2. Therefore the substrate specificities of HB-1 and HB-2 were quite similar and resembled those of urinary and pancreatic sec RNAases [22,39], but differed markedly from that of urinary nonsec RNAase [26].

Immunological properties

The SDS/PAGE pattern of RNAases in the cerebral crude extract (step 3) detected by activity staining was very similar to that detected by immunostaining with the anti-(sec RNAase) antibody. This antibody did not react with any other cerebral proteins separated by SDS/PAGE and demonstrated by protein staining (results not shown). Purified HB-1 and HB-2 each produced a single band on SDS/PAGE when detected with the anti-(sec RNAase) antibody, but not the anti-(nonsec RNAase) antibody (Figure 3). The two enzymes were also stained with two antibodies against the synthetic peptides corresponding to the amino acid sequences of the N- and C-terminal 15 residues of urinary sec RNAase, but not with antibodies against kidney and spleen nonsec RNAases. The anti-(nonsec RNAase) antibody exerted no inhibitory effect on the HB-1 and HB-2 activities, whereas the anti-(sec RNAase) antibody blocked both activities completely, although the amount of the antibody required to inhibit HB-2 activity by 50 % was about 7 and 10 times greater than that for HB-1 and urinary sec RNAase respectively (Figure 5). These results provide strong evidence that both HB-1 and HB-2 are immunologically similar to sec RNAase, despite the small anti-(sec RNAase) antibody affinity difference, but differ from nonsec RNAase.

Figure 5 Effects of specific antibodies on the enzymic activities of cerebral RNAases HB-1 and HB-2

The preparation of incubation mixtures containing 0.3 unit of purified enzyme and the residual activity assay are described in the text. The effects of the anti-(urinary sec RNAase) antibody on the activity of HB-1 (○) and HB-2 (●) and of the anti-(urinary nonsec RNAase) antibody on the activity of HB-1 (□) and HB-2 (■) are shown. The activities are expressed as percentages of that in control samples to which no antibody was added. The concentration of antibody was determined on the basis of the weight of the lyophilized IgG fraction.

Figure 6 Inhibitory effects of specific antibodies on the RNAase activities present in human cerebral crude extract

Crude extract (step 1) corresponding to 1 unit of RNAase was incubated with anti-(urinary sec) (●) and -(nonsec RNAase) (○) antibodies. The residual activities were determined under the following conditions: (a) 50 mM Tris/HCl buffer, pH 8.0, using poly(C) as the substrate; (b) 50 mM Tris/HCl buffer, pH 8.0, using yeast RNA as the substrate; (c) 50 mM Mes buffer, pH 6.0, using yeast RNA as the substrate. All the other experimental conditions are described in the text. The effects of the antibodies on the activity against poly(C) at pH 6.0 were virtually identical with those at pH 8.0.

When assayed at pH 8.0, the anti-(sec RNAase) antibody inhibited the total RNAase activity in the crude extract by 100% when poly(C) was used as the substrate and by 75% when yeast RNA was used, whereas the anti-(nonsec RNAase) antibody exhibited no such inhibition with either substrate (Figure 6). At pH 6.0, the anti-(sec RNAase) antibody inhibited the total activity in the extract by 20%, but the anti-(nonsec RNAase) antibody showed no inhibitory activity, with yeast RNA as substrate. These two antibodies inhibited the respective purified sec and nonsec RNAase activities completely. These findings enabled us to confirm that sec RNAase exists in the brain, whereas the type that cross-reacts immunologically with nonsec RNAase is apparently absent.

Table 6 Binding of the cerebral RNAases to lectin-affinity columns

The chromatographic conditions and the activity assay for the bound and unbound fractions are described in the text. Values are averages of triplicate determinations. Binding of RNAase to the lectin-agarose is expressed relative to the initial activity. The values for urinary sec RNAase are taken from ref. [40].

Lectin column	Bound fraction of RNAase (%)		
	HB-1	HB-2	Urinary sec
Con A-agarose	79.0	0	72
LCA-agarose	52.4	0	49
RCA ₁₂₀ -agarose	10.3	0	100
WGA-agarose	20.6	0	0

Figure 7 SDS/PAGE patterns of the secretory RNAases before (lanes 1–4) and after (lanes 5–8) PNGase F digestion

Purified sec RNAases (about 0.05–0.1 unit) from cerebrum, pancreas and urine were subjected to digestion with PNGase F, as described in the text. Both the digested and corresponding undigested enzymes were subjected to SDS/PAGE and then detected by activity staining. Kidney sec RNAase also shifted to the position corresponding to HB-2. Lanes 1 and 6, HB-1; lanes 2 and 7, urinary sec RNAase; lanes 3 and 8, pancreatic RNAase; lanes 4 and 5, HB-2. The cathode is at the top.

Purified HB-1 and HB-2 carbohydrate moieties

The affinities of HB-1 for several conjugated lectins differed from those of urinary sec RNAase (Table 6): the RCA₁₂₀-bound fraction of HB-1 was markedly less and the WGA-bound fraction greater than those of the urinary enzyme. Detailed carbohydrate analysis of RNAase U_L (urinary sec RNAase) revealed that this enzyme was rich in the complex-type sugar chains with *N*-acetyl-lactosamine outer chains, which bound preferentially to RCA₁₂₀ [41]. The small RCA₁₂₀-bound fraction of HB-1 may reflect a paucity of such outer chains in this cerebral enzyme. It is worth noting that HB-2 was not retained by any of the conjugated lectins studied.

Purified HB-1 and HB-2 were digested with PNGase F from *Flavobacterium meningosepticum* [42] and then analysed by SDS/PAGE and activity staining. Before digestion, HB-1, HB-2, pancreatic and urinary sec RNAases displayed different mobilities. The mobility of HB-2 did not change after digestion, which suggested that it may not contain a carbohydrate moiety, which is consistent with its lack of affinity for the conjugated lectins tested. However, HB-1 and the other sec enzymes migrated faster after than before digestion and shifted to new positions corresponding to HB-2 on the gel (Figure 7).

Figure 8 Inhibition of the cerebral RNAase activities by placental RNAase inhibitor

Preparation of incubation mixtures containing 0.3 unit of purified enzyme and the residual activity assay are described in the text. The concentration of the inhibitor is given in the Figure and expressed as inhibitor units. One RNAase inhibitor unit is defined as the amount of protein required to inhibit the activity of 5 ng of RNAase A by 50% [43]. The effects of the inhibitor on the activity of HB-1 (●) and HB-2 (○) are shown. The effect on urinary sec RNAase activity was virtually similar to that of HB-1. The residual activity is expressed as a percentage of the activity of control samples to which no inhibitor was added.

Inhibitory effect of human placental RNAase inhibitor on the HB-1 and HB-2 activities

The placental RNAase inhibitor inhibited the HB-1, HB-2 and urinary sec RNAase activities (Figure 8): those of HB-1 and urinary sec RNAase were blocked to a greater extent (about 2-fold) than that of HB-2.

Partial purification and characterization of RNAases from human cerebrospinal fluid

The RNAase activity present in cerebrospinal fluid was resolved into two distinct peaks, a major and a minor, by cellulose phosphate column chromatography (Figure 2) and showed a similar elution profile to that reported previously [44]. The two peaks, which contained RNAases with the same mobilities as those of HB-1 and HB-2 when subjected to SDS/PAGE, were pooled separately for further purification and designated CSF-1 and CSF-2, the yields of which were estimated to be 44% and 10% respectively. Their catalytic properties, pH- and bivalent-cation-dependencies were almost identical with those of HB-1 and HB-2. With respect to their substrate specificities (Table 5), CSF-1 and CSF-2 both exhibited a greater preference for poly(C) than for poly(U) or RNA, to a similar extent to the cerebral RNAases, and both CSF enzymes failed to hydrolyse poly(I) and poly(G) under the assay conditions used. The enzyme activities of both CSF-1 and CSF-2 were blocked by the anti-(sec RNAase), but not the anti-(nonsec RNAase), antibody. Unfortunately, the amounts of the purified CSF enzymes obtained were too small for further analyses. However, our results led us to conclude that CSF-1 and CSF-2 fit into the sec RNAase category and correspond to HB-1 and HB-2 respectively.

DISCUSSION

Among human organs, the brain is known to be the most susceptible to post-mortem autolysis and it remains to be

excluded that HB-2, with its lower molecular mass, is derived from HB-1 as a result of modification by proteases and/or glycosidases involved in post-mortem changes. However, analysis of five brain samples from different subjects demonstrated that the total RNAase activity, content ratios, cellulose phosphate chromatography elution profiles and SDS/PAGE and isoelectric focusing isoform patterns (results not shown) of HB-1 and HB-2 were almost identical and showed no relationship to the time that elapsed between death and autopsy or the cause of death (Table 1). The specific activity variations (0.74–1.25 units/mg of protein) in the crude extracts were not wide compared with those in serum [45] and other human tissues [46] and it was not necessary to consider the agonal state with our preparations, which has been suggested to be one of the most important factors affecting RNA integrity in post-mortem brain tissues [8]. The CSF-1 and CSF-2 enzymes were isolated from fresh cerebrospinal fluid obtained from a healthy donor and corresponded to the cerebral HB-1 and HB-2 RNAases respectively. Therefore these findings suggest that HB-1 and HB-2 reflect the state of the cerebral enzymes present in living cells and fluid.

The catalytic properties of HB-1 and HB-2, which are characterized by a high optimal pH and preference for poly(C) as a substrate, closely resembled those of urinary sec RNAase, which is the most extensively studied of sec RNAases [22–24,33,39]. Additional similarities were as follows: immunological cross-reactivity with and inhibition by the anti-(sec RNAase) antibody (Figures 3 and 5); their amino acid compositions (Table 3) and their N-terminal amino acid sequences (Figure 4). These enzymological, proteochemical and immunological similarities suggest strongly that these two cerebral enzymes belong to the sec, and not the nonsec, RNAases which are represented by the spleen [25] and liver [47] enzymes.

Several differences between HB-1 and HB-2 were observed: molecular mass, heat-stability, reactivity with the anti-(sec RNAase) antibody (Figure 5), inhibition by placental RNAase inhibitor (Figure 8) and affinity for conjugated lectins (Table 6). Furthermore, after digestion with PNGase F glycosidase, the individual electrophoretic mobilities of HB-1, pancreatic and urinary sec RNAases changed and were identical with that of HB-2 (Figure 7). Therefore the differences between HB-1 and HB-2 appear to be due to the absence of a carbohydrate moiety in the latter, rather than differences between their polypeptide moieties, that is, the differences between them are probably small. Human sec RNAases have been demonstrated to contain three Asn-Xaa-Ser/Thr sequences (Asn positions 34, 76 and 88) which may be attached to the carbohydrate moiety [35]. However, its lack of hexosamine (Table 4), affinity for conjugated lectins (Table 6) and electrophoretic shift after glycosidase digestion (Figure 7) suggest strongly that HB-2 has no carbohydrate moiety and thus may be the first carbohydrate-free RNAase in a pure form discovered. Beintema et al. [35] and Yamashita et al. [40] demonstrated organ-specific differences between the carbohydrate moieties attached to human RNAase. Close immunological similarities among human RNAases present in crude extracts from pancreas, brain, kidney, serum and urine were demonstrated by Weickmann and Glitz [48]. Therefore it appears a feasible postulate that a primary gene product may undergo glycosylation to a different extent and/or in a different manner in the brain from that in other organs.

The RNAase activity of the cerebral crude extract against poly(C) appears to be derived mainly from HB-1 and HB-2 (Figure 6a). The activities of the crude extract remaining after precipitation with anti-(sec RNAase) antibody, which were measured at pH 6.0 and 8.0 using yeast RNA as a substrate, were about 80% and 25% of the initial activity respectively (Figures

6b and 6c). This incomplete inhibition of the activities by this antibody suggests that there may be yet another type of RNAase, which is unlike the sec RNAases. Postek et al. [49] found a guanylyl(3'-5')adenosine-specific RNAase in mouse brain. However, the anti-(nonsec RNAase) antibody failed to block the cerebral extract RNAase activity against RNA. As liver RNAase, a typical nonsec RNAase, has been shown to be identical with eosinophil-derived neurotoxin [15], which induces nervous tissue degeneration, the absence of nonsec RNAase in the cerebral extract is not surprising.

Recently, Sorrentino et al. [15] demonstrated that the Con A-unbound fraction, which was probably non-glycosylated, of human pancreatic RNAase induced neurological abnormalities (Gordon phenomenon), whereas the Con A-bound glycosylated fractions did not. As HB-2 was non-glycosylated and virtually identical with the non-glycosylated pancreatic enzyme, the presence of this type of enzyme in the brain is somewhat surprising.

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