The Isolation and Partial Characterization of Ribonuclease A from *Bison bison*

By GORDON R. STEWART* and KENNETH J. STEVENSON†

Department of Chemistry, University of Calgary, Calgary T2N 1N4, Alberta, Canada

(Received 20 March 1973)

1. Bison ribonuclease was isolated from pancreas glands of *Bison bison* by acid extraction, $(\text{NH}_4)_2\text{SO}_4$ fractionation, affinity chromatography on Sepharose-5'-(4-aminophenylphosphoryl)uridine 2',3'-phosphate and ion-exchange chromatography on Bio-Rex-70. 2. The selectivity of the affinity column towards bison ribonuclease in heterogeneous protein solutions was greatly improved by employing piperazine buffers at pH 5.3, which decreased non-specific interactions of other proteins. Rapid desorption from the affinity column was obtained with sodium phosphate buffer (pH 3). 3. Bison ribonuclease has a total amino acid content very similar to ox ribonuclease. Inactivation of bison ribonuclease with iodoacetic acid leads to the formation of 0.62 residues of π -carboxymethylhistidine and 0.36 residues of τ -carboxymethylhistidine. The amino acid composition of peptides isolated from diagonal peptide 'maps' and also of peptides isolated after pH 1.6 and 2.4 two-dimensional high-voltage electrophoresis of a digest of bison ribonuclease labelled with pyridoxal 5-phosphate indicates that there is complete homology between ox and bison ribonucleases. 4. The Schiff-base attachment site of pyridoxal 5-phosphate was identified as lysine-41 by NaBH_4 reduction followed by peptide isolation.

Barnard (1969) has extensively reviewed various comparative aspects and biological function of ribonucleases. When comparisons were made amongst ribonucleases isolated from pancreas glands of the vertebrates, large variations in amino acid composition and partial amino acid sequence information indicated that the ox (Smyth *et al.*, 1963), rat and horse (Beintema & Gruber, 1967) and sheep (Aqvist & Anfinsen, 1959; Anfinsen *et al.*, 1959) had undergone rapid evolutionary change. The components of their ribonuclease active sites, however, were largely conserved when compared with other segments of their structure.

With the availability of pancreas glands from the Plains Buffalo (*Bison bison bison*), an opportunity existed to isolate and characterize pancreatic ribonuclease from this species of the Bovidae family which is very closely related to domestic beef cattle. Such a study was further facilitated by the developments in selective enzyme isolation through affinity chromatography (Cuatrecasas, 1970; Wilchek & Gorecki, 1969).

Experimental

Materials

Sepharose-4B (Agarose; lot 4763) and Sephadex G-25 were purchased from Pharmacia, Montreal,

* Present address: Department of Biochemistry, Dalhousie University, Halifax, Nova Scotia, Canada.

† To whom reprint requests should be addressed.

Canada. NH_2pPUP -Sepharose* was prepared in the laboratory (see under 'Methods') or was purchased from Miles-Yeda Ltd., Rehovoth, Israel (lot 20013). Bovine (ox) ribonuclease A (lot 99B-8020), pyridoxal 5-phosphate, pepsin and ribonucleic acid (type VI) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Chymotrypsin α was purchased from Worthington Biochemical Corp. (Winley-Morris), Montreal, Canada. Porcine trypsin Novo was from the Enzyme Development Corp., Wall Street, New York, N.Y., U.S.A. Iodoacetic acid was high-purity material from British Drug Houses Ltd., Poole, Dorset, U.K. Bio-Rex-70 was purchased from Bio-Rad Laboratories, Richmond, Calif.,

* Abbreviations: RNAase, ribonuclease A (ox or bison as indicated); NH_2pPUP , 5'-(4-aminophenylphosphoryl)uridine 2',3'-phosphate; NH_2pPUP -Sepharose, Sepharose to which 5'-(4-aminophenylphosphoryl)uridine 2',3'-phosphate has been covalently attached through the amino group; bison RNAase-pyridoxal 5-phosphate, bison ribonuclease to which pyridoxal 5-phosphate has been covalently attached by NaBH_4 reduction of the intermediate Schiff base; CmCys, S-carboxymethylcysteine; His(Cm)RNAase, ribonuclease A (ox or bison) inactivated with iodoacetate by modification of histidine-12 and histidine-119; Hse, homoserine; Hse<, homoserine lactone; Cys(O_3H), cysteic acid; Met(O_2), methionine sulphone; Dnp-Lys, N^ε-2,4-dinitrophenyl-lysine; $m_1^{2\%}$, the mobility of a peptide or amino acid relative to serine during high-voltage electrophoresis at pH 1.6. Similar notations denote the mobility of a peptide relative to an amino acid during high-voltage electrophoresis at a particular pH.

U.S.A. Piperazine was obtained from Fisher Scientific Co., Pittsburgh, Pa., U.S.A. CNBr and cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate were purchased from Pierce Chemicals, Rockford, Ill., U.S.A.

Methods

Synthesis of 5'-(4-aminophenylphosphoryl)uridine 2',3'-phosphate. The synthesis of NH_2pPUP was carried out essentially as described by Wilchek & Gorecki (1969). Characterization on silica gel t.l.c. by using the detection of phosphate-containing compounds as described by Rosenberg (1959), indicated an R_F value of 0.03, compared with the published R_F value of 0.12 in propan-1-ol-aq. NH_3 (sp.gr. 0.88)-water (7:1:2, by vol.). However, the biological specificity of NH_2pPUP attached to Sepharose was identical with commercially available NH_2pPUP -Sepharose.

Coupling of NH_2pPUP to Sepharose-4B. The activation of 100 ml of washed, settled Sepharose-4B with 10 g of CNBr was carried out as suggested by Wilchek & Gorecki (1969), Cuatrecasas (1970) and Cuatrecasas & Anfinsen (1971). NH_2pPUP (210 mg, 0.35 mmol) in 15 ml of 0.1 M- NaHCO_3 was coupled to the activated Sepharose in 50% yield as determined spectrophotometrically at 260 nm. The NH_2pPUP -Sepharose so formed contained about $1.8 \mu\text{mol}$ of $\text{NH}_2\text{pPUP}/\text{ml}$ of settled Sepharose.

Preparation of 2-aminoethanol-Sepharose. Washed Sepharose-4B (35 ml) was suspended in 70 ml of water, $150 \mu\text{l}$ (122 mg) of 2-aminoethanol was added and the pH was adjusted to 4.7 with HCl. Cyclohexyl-3-(2-morpholinoethyl)carbodi-imide metho-*p*-toluenesulphonate (500 mg, 1.18 mmol) was introduced and the coupling reaction was allowed to continue at 25°C for 48 h with stirring. 2-Aminoethanol-Sepharose was washed with water.

Assay of ribonuclease activity. Ribonuclease activity was determined by the method of Kunitz (1946). The ribonucleic acid employed in the assay was first dialysed against several changes of 0.1 M-KCl for 48 h, precipitated from solution with ethanol, collected by centrifugation and air-dried. A standard curve for RNAase activity was obtained with solutions containing 2–8 μg of ox RNAase/ml.

Fractionation of RNAase from *Bison bison pancreas* glands. The procedures of McDonald (1956) were followed in part. Pancreas glands were obtained from *Bison bison* that had been recently slaughtered during a herd reduction programme in Elk Island National Park, Alberta, Canada. The glands were removed in an abattoir and quickly frozen on solid CO_2 . They were kept at -20°C until required. Eleven pancreas glands were thawed in 0.125 M- H_2SO_4 in the cold-room overnight. Fat and connective tissue were removed and the glands were minced in an electric meat

grinder to yield a volume of 2 litres. Cold 0.125 M- H_2SO_4 (4 litres) was added and the suspension was stirred overnight in the cold-room. The suspension was strained through cheesecloth and the residue was resuspended in 1 litre of 0.125 M- H_2SO_4 and further extracted for 2 h. The extracts were strained through cheesecloth again, then pooled to yield a volume of 5 litres with an absorbancy at 280 nm of 0.30 for a 1:400 dilution with water.

By using a nomogram for obtaining amounts of $(\text{NH}_4)_2\text{SO}_4$ salt to be added (Dixon, 1953), fractionation of the H_2SO_4 extract was made between 0–20%, 20–40%, 40–65% and 65–85% saturation. The residue remaining from the latter fractionation (the only material used in these studies) was dissolved in 100 ml of water, subjected to Millipore filtration to remove particulate matter and desalted on a column (4 cm $\times$ 77 cm) of Sephadex G-25 (medium grade) at 4°C with water as eluant. Protein fractions were pooled and freeze-dried. The freeze-dried material was dissolved in 0.025 M-piperazine adjusted to pH 5.3 with 0.5 M-HCl and was applied to a NH_2pPUP -Sepharose affinity column as outlined in Fig. 3. Elution of material absorbing at 280 nm was continued with piperazine-HCl buffer until the absorbancy reading approached 0.03 when the piperazine-HCl was replaced with 0.25 M-sodium phosphate buffer, pH 3, to desorb the affinity-bound bison ribonuclease A.

Preparation of bison carboxymethyl-RNAase. Bison RNAase purified by Bio-Rex-70 chromatography was desalted on Sephadex G-25 and freeze-dried. Inhibition by iodoacetic acid was performed as outlined by Crestfield *et al.* (1963a). To a solution of bison RNAase (40 mg, $2.8 \mu\text{mol}$ in 3 ml of water) at pH 5.5 was added 1 ml of iodoacetic acid (5.3 mg, $28.6 \mu\text{mol}$) previously adjusted to pH 5.5. The inhibition was conducted in a pH-stat at 25°C under N_2 and in the dark with the pH being maintained at pH 5.5 by the addition of 0.1 M-NaOH. Assays performed during the inhibition indicated that 37% of the activity remained after 7 h, 21% remained after 28 h and zero activity after 70 h. The reaction mixture was desalted on a column (2 cm $\times$ 82 cm) of Sephadex G-25 (fine grade) equilibrated and eluted with 50 mM-acetic acid. The column was wrapped with aluminium foil to exclude light. Monitoring the effluent by 280 nm absorbancy and conductivity measurements indicated one E_{280} peak eluting just after the void volume with very low conductivity and a second E_{280} peak eluting at twice the void volume with high conductivity. The fractions corresponding to each peak were freeze-dried. The early eluting peak was subsequently identified as His(Cm)RNAase (25 mg), whereas the retarded peak contained iodoacetic acid and salts.

Preparation of bison RNAase labelled covalently with pyridoxal 5-phosphate. Bison RNAase (20 mg) was treated with 0.69 mg of pyridoxal 5-phosphate in

Fig. 1. Elution profiles of chymotrypsin α and ox ribonuclease on unsubstituted Sepharose-4B

(a) Chymotrypsin α (4mg) was chromatographed on a column (0.9cm $\times$ 5cm) of unsubstituted Sepharose-4B equilibrated and eluted with 0.02M-sodium acetate buffer, pH 5.2, at 25°C. The flow rate was 30ml/h with 1ml fractions being collected. The arrow ($\downarrow$) indicates where elution with 0.2M-acetic acid commenced. (b) Chymotrypsin α (4mg) was chromatographed as in (a) but the column was equilibrated with 0.025M-piperazine-HCl buffer, pH 5.3, at 25°C. The arrow ($\downarrow$) indicates where elution with 0.2M-acetic acid commenced. (c) Ox ribonuclease (4.2mg) was chromatographed on a column (0.9cm $\times$ 5cm) of unsubstituted Sepharose-4B equilibrated and eluted with 0.02M-sodium acetate buffer, pH 5.2, at 25°C. The flow rate was 30ml/h with 1ml fractions being collected. The arrow ($\downarrow$) indicates where elution with 0.2M-acetic acid containing 0.2M-KCl commenced. (d) Ox ribonuclease A (4mg) was chromatographed on a column (0.9cm $\times$ 5cm) of Sepharose-4B equilibrated and eluted with 0.025M-piperazine-HCl buffer, pH 5.3, at 25°C. The flow rate was 26ml/h with 1ml fractions being collected. The arrow ($\downarrow$) indicates where elution with 0.2M-acetic acid commenced.

4ml of 0.05M-Tris adjusted to pH 7.0 with 0.5M-acetic acid as described by Means & Feeney (1971). NaBH_4 was added until the yellow colour of the solution disappeared. The coupling was repeated with 0.54mg of pyridoxal 5-phosphate followed by reduction with NaBH_4 . The reaction products were separated on a column (2cm $\times$ 82cm) of Sephadex G-25 (fine grade) equilibrated and eluted with 50mM-acetic acid at 25°C. The flow rate was 30ml/h with a fraction size of 4ml. The elution pattern was moni-

tored at 280nm to detect protein and at 324nm to detect the pyridoxal 5-phosphate chromophore. A peak with fractions possessing similar absorbancy readings at 280nm and 324nm eluted at about 1.2 void volumes and was collected and freeze-dried (recovery 16mg). A spectrum of this material showed the absorbancy maxima at 270nm and 328nm expected for a pyridoxal 5-phosphate-RNAase. Based on an extinction coefficient at 324nm of 7700 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$ for reduced pyridoxal 5-phosphate

attached covalently to RNAase, the ratio of pyridoxal 5-phosphate to bison RNAase was calculated to be 1.28. A second peak with very high 324nm and low 280nm absorbancy was eluted with about 2 void volumes of acetic acid. This material contained salts and reduced pyridoxal 5-phosphate. Fractions of a third peak possessing high 290nm and low 324nm absorbancy readings were pooled and freeze-dried but yielded an oily residue. The nature of this material is unknown.

High-voltage electrophoresis. The vertical strip high-voltage electrophoresis apparatus was similar to that described by Michl (1951) as modified by Ryle *et al.* (1955). The buffer systems and coolants used were as described by Smillie & Hartley (1966). The two-dimensional high-voltage electrophoresis isolation of

the pyridoxal 5-phosphate-labelled peptide was carried out in pH1.6 buffer (8.6% formic acid) and pH2.4 buffer (4% formic acid, 0.58% pyridine) (K. J. Stevenson, unpublished work). Peptides were detected with the cadmium-ninhydrin dip reagent of Heilmann *et al.* (1957) by developing guide strips of chromatograms, and amino acids were detected with a dip reagent consisting of 0.7 ml of collidine in 100 ml of 0.5% ninhydrin in acetone. The presence of tryptophan-containing peptides was investigated with Ehrlich reagent (*p*-dimethylaminobenzaldehyde) as described by Bailey (1968). The suitability of the Ehrlich reagent was confirmed by obtaining positive results with peptides known to contain tryptophan. Visual dye markers were used throughout high-voltage electrophoresis work as described by Stevenson

Fig. 2. Elution profiles of ox ribonuclease and chymotrypsin α on NH_2pPUP -Sepharose

(a) Ox ribonuclease A (4mg) was chromatographed on a column (0.9cm $\times$ 20.5cm) of NH_2pPUP -Sepharose in 0.02M-sodium acetate buffer, pH5.2, at 25°C. The flow rate was 26ml/h with fraction size of 1 ml. The arrow ($\downarrow^1$) indicates elution with 0.2M-acetic acid, the arrow ($\downarrow^2$) elution with 0.2M-acetic acid containing 0.2M-KCl and the arrow ($\downarrow^3$) elution with 0.2M-KCl adjusted to pH2.0 with HCl. (b) Chymotrypsin α (4mg) was chromatographed on NH_2pPUP -Sepharose as outlined in (a). (c) Ox ribonuclease A (4mg) was chromatographed on a column (0.9cm $\times$ 4.5cm) of NH_2pPUP -Sepharose in 0.025M-piperazine-HCl buffer, pH5.3, at 25°C. The flow rate was 26ml/h with fraction size of 1 ml. The arrows ($\downarrow^1$), ($\downarrow^2$) and ($\downarrow^3$) indicate elutions as outlined in (a). (d) Chymotrypsin α (4mg) was chromatographed on a column (0.9cm $\times$ 5cm) of NH_2pPUP -Sepharose in 0.025M-piperazine-HCl buffer, pH5.3, at 25°C. The flow rate was 26ml/h with fraction size of 1 ml. The arrow ($\downarrow$) indicates elution with 0.2M-acetic acid.

(1971). Homoserine lactone was prepared by heating homoserine in 6M-HCl for 2h at 110°C in a sealed, evacuated tube. Carboxymethylhistidine derivatives were synthesized as outlined by Crestfield *et al.* (1963a) and were separated on a column (1.2cm × 30cm) of Dowex 50 (X8) in the pyridinium form. A gradient of 200ml of 0.1M-pyridine-acetic acid, pH3.2, and 100ml of 2.1M-pyridine-acetic acid, pH6.0, was employed. Final purification was obtained on high-voltage electrophoresis at pH6.5 (cf. Fig. 4).

Diagonal peptide 'mapping' of bison and ox ribonuclease. Diagonal peptide 'mapping' was carried out as described by Brown & Hartley (1966). Bison RNAase (20mg) was digested with 2mg of pepsin in 5ml of 5% (v/v) formic acid at 28°C for 16h. The digest was spotted on Whatman 3MM paper as a 25cm band at a distance of 12cm from the anode and was subjected to high-voltage electrophoresis at pH3.5 and 3kV for 1h. A 3cm side-strip from the initial chromatograms was placed in a desiccator and oxidized by performic acid vapour. The diagonal peptide 'map' of peptides containing cysteic acid was formed by subjecting the oxidized side-strip to high-voltage electrophoresis at pH3.5 and 3kV for 1h (cf. Fig. 5). This 'map' served as the basis for the subsequent isolation of peptides containing cysteic acid from the initial high-voltage run at pH3.5. Cysteic acid-containing peptides were purified by high-voltage electrophoresis at pH3.5 based on the diagonal peptide 'map' obtained at that pH (cf. Fig. 5). All of the peptides were cleanly separated from one another and only peptide B4 was subjected to further purification by high-voltage electrophoresis at pH2.0 (3kV for 3h).

Isolation of pyridoxal 5-phosphate-labelled peptide from bison RNAase-pyridoxal 5-phosphate. After performic acid oxidation (Hirs, 1956) of bison RNAase-pyridoxal 5-phosphate, the freeze-dried material (14.5mg) was digested with porcine trypsin in a pH-stat at pH8 and 25°C for 16h. The digest was applied as a 19cm band at a distance of 10cm from the anode on Whatman 3MM paper and subjected to high-voltage electrophoresis at pH1.6 (2kV, 1h). A major fluorescent ninhydrin-positive band was observed with a mobility of $m_{r,6}^{ser} = 0.36$. Other minor fluorescent bands were also noted. The major fluorescent band was cut out, sewn on to a second sheet of 3MM paper at a distance of 12cm from the anode and subjected to high-voltage electrophoresis at pH2.4 (2kV, 1h). A single fluorescent peptide with an orange, changing to pink, cadmium-ninhydrin colour was obtained with a mobility of $m_{r,4}^{ser} = 0.55$. The peptide was eluted with 1.3ml of 10mM-acetic acid and the absorbancies of this solution were 0.40 and 0.65 at 280nm and 324nm respectively.

Amino acid analyses. The amino acid analyses of RNAase A from *Bison bison* were performed by

hydrolysing 3.0mg samples of RNAase in 1ml of 6M-HCl at 110°C for 18, 24, 48 and 72h. To each test-tube was added 59.2µg of norleucine to act as an internal standard (Walsh & Brown, 1962). The acid hydrolysates were rapidly evaporated to dryness on a Evapomix and were analysed on a Beckman 120C amino acid analyser by the methods of Spackman *et al.* (1958).

The quantitation of cystine as cysteic acid after performic acid oxidation by the method of Hirs (1956) was determined on a Phoenix M-7800 amino acid analyser.

The quantitation of *N*^ε-carboxymethylhistidine and *N*^ε-carboxymethylhistidine was carried out on performic acid-oxidized bison His(Cm)RNAase by the method of Crestfield *et al.* (1963a) employing the Phoenix analyser. Performic oxidation was necessary

Fig. 3. Selective isolation of bison ribonuclease from a crude pancreatic extract by affinity chromatography on NH_2pPUP -Sephacrose

The sample was 200mg of a crude desalted preparation derived from bison pancreas glands (see the Experimental section). Affinity chromatography was conducted on a column (0.9cm × 13.3cm) of NH_2pPUP -Sephacrose equilibrated and eluted with 0.025M-piperazine-HCl buffer, pH5.3, at 25°C. The flow rate was 30ml/h with 5ml fractions being collected. The arrow (↓) indicates where elution commenced with 0.25M-sodium phosphate buffer, pH3.

to transform cystine into cysteic acid, thus eliminating the overlap on the elution profile between cystine and N^{ϵ} -carboxymethylhistidine.

Results and Discussion

Isolation of bison ribonuclease

During the application of affinity chromatography on NH_2pPUP -Sephacrose for the selective isolation of bison RNAase by the method of Wilchek & Gorecki (1969), difficulties were encountered in trying to obtain successful application of this elegant technique for enzyme purification. The areas of concern were ultimately traced to (1) the existence of negatively charged species (likely carboxyl groups, inherent in the native unsubstituted Sepharose-4B) and (2) the inability of 0.2M-acetic acid to remove the adsorbed RNAase from the NH_2pPUP -Sephacrose affinity column.

To obtain evidence for the presence of low concentrations of carboxyl groups in native Sepharose-4B, ox RNAase and bovine chymotrypsin α were chromatographed separately in the presence of 0.02M-

acetic acid adjusted to pH 5.2 with 1M-NaOH and 0.025M-piperazine-HCl buffer, pH 5.3, as shown in Fig. 1. Equilibration and elution of unsubstituted Sepharose with the acetate buffer caused both proteins to be weakly adsorbed to the column as indicated by the lengthy tailing of the peaks (Figs. 1a and 1c). After the stepwise addition of 0.2M-acetic acid, to desorb any protein material not eluted with acetate buffer, portions of chymotrypsin α and ox RNAase were further desorbed from the Sepharose as shown in Figs. 1(a) and 1(c), respectively. The results clearly suggest the existence of non-specific interactions between the Sepharose matrix and protein molecules. The nature of these interactions is predominantly ionic and probably involves carboxyl groups on Sepharose (Porath *et al.*, 1971). The existence of these negatively charged species was supported by the observation that pepsin, an acidic protein with a pI of 2, passed unretarded through Sepharose under conditions similar to Fig. 1(a). Moreover, Sepharose, in which the majority of the carboxyl groups had been

Table 1. Amino acid composition of bison ribonuclease A

The value for alanine is arbitrarily set at 12.0 residues, for an average of three analyses per hydrolysis time. The values for ox were reported by Smyth *et al.* (1963).

Amino acid	Hydrolysis time	...	Amino acid composition				Average value	Integral value	Ox
			18h	24h	48h	72h			
Lysine			10.4	11.1	10.7	10.5	10.7	11	10
Histidine			3.9	4.2	4.3	4.1	4.1	4	4
Arginine			4.1	4.1	4.3	4.1	4.1	4	4
Aspartic acid			15.3	15.4	15.4	15.3	15.4	15	15
Threonine			9.3	9.5	8.9	8.9	9.8*	10	10
Serine			13.1	12.1	11.5	8.9	14.6*	15	15
Glutamic acid			12.2	11.9	12.3	12.2	12.2	12	12
Proline			4.4	3.8	4.1	4.1	4.1	4	4
Glycine			3.2	3.2	3.2	3.2	3.2	3	3
Alanine			12.0	12.0	12.0	12.0	12.0	12	12
Half-cystine			9.4	9.7	9.3	9.0	9.4	9	8
(Cysteic acid)			—	—	—	—	(7.0)§	(8)	(8)
Valine			8.7	9.1	9.3	9.2	9.2	9	9
Methionine			4.2	4.1	4.2	4.1	4.2	4	4
(Methionine sulphone)			—	—	—	—	(4.0)§	(4)	(4)
Isoleucine			2.2	2.7	3.0	3.0	3.0†	3	3
Leucine			2.2	2.2	2.2	2.2	2.2	2	2
Tyrosine			6.0	6.1	6.0	5.9	6.0	6	6
Phenylalanine			3.2	3.2	3.3	3.4	3.3	3	3
Tryptophan			—	—	—	—	0	0‡	0

* Extrapolated to zero time.

† Value for the 72h hydrolysis time was taken.

‡ Based on Ehrlich's reagent test for tryptophan on two-dimensional peptide 'maps'.

§ Average of three analyses for oxidized bison RNAase. Cysteic acid was corrected for 94% recovery (Moore, 1963).

transformed into uncharged $-\text{CO}-\text{NH}-\text{CH}_2-\text{CH}_2\text{OH}$ moieties by covalent attachment of 2-amino-ethanol (see under 'Methods'), allowed both chymotrypsin α and ox RNAase to pass through essentially unretarded. This was in contrast with the elution profiles of these enzymes on native (unsubstituted) Sepharose (cf. Figs. 1*a* and 1*c*).

Piperazine-HCl buffer was substituted for sodium acetate buffer in an attempt to eliminate non-specific interactions between the suspected negatively charged groups on the Sepharose matrix and protein molecules under study. The positively charged heterocyclic ring of piperazine was considered to be a more effective competitor than the sodium ion for these groups. This view was based on observations that pyridinium ions effectively decreased tailing of chromatographic peaks eluting from sulphonate polystyrene resins (Dixon, 1966). Sodium ions were much less effective at decreasing tailing than were pyridinium ions at similar concentrations.

The rationale for employing a piperazine-HCl buffer was supported by the data in Figs. 1(*b*) and 1(*d*). Both chymotrypsin α and RNAase A passed unretarded through Sepharose when this buffer was employed, but were weakly adsorbed when sodium acetate buffer was used. The sum of the concentrations of monovalent and divalent piperazinium ions (10mM and 15mM respectively) was about twice the concentration of the sodium ion (14mM) present in the acetate buffer. The greater effectiveness of piperazine-HCl buffer in competing for the negative charges on Sepharose was due, not only to the higher total concentration of cations present, but also to the probable non-ionic interactions of the monovalent and divalent piperazinium ions with the Sepharose matrix.

Control affinity-chromatography runs were performed on NH_2pPUP -Sepharose with ox RNAase A as the specific enzyme and bovine chymotrypsin as a non-specific enzyme. RNAase was adsorbed to

Fig. 4. Chromatogram of a qualitative amino acid analysis of an acid hydrolysate of bison *His(Cm)RNAase*

High-voltage electrophoresis was conducted at pH 6.5 and 3 kV, until the dye marker Orange G (OG) migrated 19 cm towards the anode. Amino acid and dye markers are indicated by S; XCFF refers to Xylene Cyano FF. A denotes $N^{\pi,\pi}$ -dicarboxymethylhistidine, B denotes N^{π} -carboxymethylhistidine, C denotes N^{π} -carboxymethylhistidine and M denotes a mixture of the three carboxymethylhistidine derivatives.

Fig. 5. Diagonal peptide 'map' of a pepsin digest of bison ribonuclease

High-voltage electrophoresis was conducted at pH 3.5 and 3 kV for 1 h in both dimensions. N denotes a mixture of amino acids and visual dye markers. MG and CV denote Methyl Green and Crystal Violet respectively. XCF refers to Xylene Cyano FF and A1–A5, B1–B5 and C1 are peptides (see Table 3).

NH₂pPUP–Sephacrose when either acetate buffer or piperazine buffer was used. However, desorption of RNAase from the affinity column was very difficult, particularly when the enzyme had been applied with acetate buffer. As shown in Fig. 2(a), the stepwise addition of 0.2M-acetic acid failed to desorb the enzyme initially applied in acetate buffer and the addition of 0.2M-acetic acid containing 0.2M-KCl was required to remove, albeit slowly, the RNAase from the column. Additional RNAase was removed when the more rigorous eluent, 0.2M-KCl adjusted to pH 2.0 with HCl, was employed. The use of piperazine buffer to replace acetate buffer did improve the elution profile: during desorption, about one-half of the RNAase was eluted with 0.2M-acetic acid and the remaining material bound on the column was desorbed with 0.2M-acetic acid containing 0.2M-KCl (Fig. 2c). However, satisfactory desorption was not obtained with this system.

Substantial non-specific adsorption of chymotrypsin α occurred when acetate buffer was used during chromatography on NH₂pPUP–Sephacrose. In this

system, marked retardation was observed in the presence of acetate buffer and the complete desorption of chymotrypsin α required potent elution systems (Fig. 2b). By employing piperazine buffer to apply the enzyme, all of the chymotrypsin α passed through the NH₂pPUP–Sephacrose column unretarded (Fig. 2d) in marked contrast with the experiments employing acetate buffer. The work with chymotrypsin α served to indicate the importance of masking charged carboxyl groups on the Sepharose matrix and masking the negatively charged phosphate moiety introduced with NH₂pPUP to improve the biological specificity of the affinity column employed.

The disparity between the present data and the results of Wilchek & Gorecki (1969) is undoubtedly due to the increase in the column size (up to 13-fold) used in these studies, reflecting the difficulties that can arise through scaling-up of procedures.

After experiments with various reagents, desorption of affinity-bound RNAase from NH₂pPUP–Sephacrose was ultimately obtained quickly and completely through the replacement of the acetic acid and

Table 2. Amino acid composition of peptides isolated by diagonal peptide 'mapping' of bison ribonuclease A

The numbering of the peptides is explained in Fig. 5. Peptide B4 was further purified on high-voltage electrophoresis at pH2.0 (3 kV for 3 h).

Peptide	Cadmium-ninhydrin colour	Composition
A1 (minor)	Yellow → red	Cys(O ₃ H) (1.0), Asp (1.0)*, Thr (1.2), Ser (3.4), Glu (0.9), Pro (+)†, Gly (1.2), Ala (0.9), Tyr (0.2), Lys (0.6), Arg (0.5)
A2 (major)	Red	Cys(O ₃ H) (1.6), Asp (1.9), Thr (2.3), Ser (2.4), Glu (1.0)*, Pro (+)†, Gly (1.0), Ala (0.8), Ile (1.0), Tyr (0.9), Lys (0.9), Arg (1.0)
A4 (major)	Red	Cys(O ₃ H) (0.8), Asp (1.9), Ser (2.6), Glu (1.0)*, Ala (0.8), Met(O ₂) (1.2), Tyr (0.9)
A5 (minor)	Red	Cys(O ₃ H) (0.8), Asp (1.1), Ser (0.3), Glu (0.9), Met(O ₂) (1.0)*, Tyr (0.8)
B1 (intermediate)	Red	Cys(O ₃ H) (0.5), Asp (1.7), Thr (0.7), Ser (1.0), Glu (2.0)*, Gly (0.8), Ala (0.9), Val (1.0), Tyr (1.1), Lys (0.3)
B3 (intermediate)	Red	Cys(O ₃ H) (1.8), Asp (3.0)*, Thr (0.9), Ser (2.0), Glu (3.1), Gly (1.4), Ala (2.1), Val (2.4), Tyr (1.2), Lys (0.9)
B4 (major)	Yellow → orange	Cys(O ₃ H) (0.8), Asp (1.0)*, Glu (1.0), Pro (+)†, Gly (1.1), Val (1.5), Tyr (1.0), Phe (1.2), His (1.0)
B5 (major)	Red	Cys(O ₃ H) (2.5), Asp (3.4), Thr (1.3), Ser (2.3), Glu (3.4), Gly (1.0), Ala (2.0)*, Val (2.2), Tyr (1.6), Lys (0.3)
C1 (major)	Yellow → orange	Thr (0.9), Ser (0.8), Met(O ₂) (1.0)*

* Arbitrarily taken as 1.0, 2.0 or 3.0 residues as indicated with recoveries of other amino acids relative to this amino acid.

† Detected on the amino acid analyser but not quantified owing to instrument malfunction.

acetic acid-KCl solutions with 0.25M-phosphoric acid adjusted to pH3.0 with 1M-NaOH. Since phosphate ion at pH5.2 is an effective inhibitor of RNAase (Wyckoff *et al.*, 1967), it was felt that affinity-bound RNAase could be desorbed from NH₂pPUP-Sepharose with 0.25M-sodium phosphate buffer at pH3.0 because of the combined effects of low pH, high ionic strength and competitive binding of the H₂PO₃⁻ ion.

The selective isolation of *Bison bison* RNAase from crude pancreatic extracts is presented in Fig. 3. NH₂pPUP-Sepharose specifically adsorbed RNAase from a heterogeneous mixture of proteins chromatographed in 0.025M-piperazine-HCl buffer, pH5.3. Continual elution with piperazine buffer removed all extraneous proteins but did not lead to removal of bound RNAase. Desorption of the RNAase was accomplished by the stepwise addition of 0.25M-sodium phosphate buffer, pH3.0. The purity of this preparation of bison RNAase was attested to by subsequent ion-exchange chromatography on Bio-Rex-70 (Hirs *et al.*, 1953). Only one major peak possessing RNAase activity was evident and this material, after desalting on Sephadex, was employed in all subsequent characterizations of the enzyme.

Characterization of bison ribonuclease A

The amino acid composition of ribonuclease from *Bison bison* is shown in Table 1 along with the amino

acid composition of ox RNAase. The data are based on assuming a value of 12.0 residues of alanine for bison RNAase and indicate the apparent similarity between the ox and bison enzymes, which are isolated from animals within the Bovidae family. The only amino acid that appears to vary is lysine; there is one more residue present in the bison RNAase than in the ox RNAase. The recoveries of 9.0 residues of half-cystine are believed not to be reliable. However, quantitation of half-cystine as cysteic acid also proved to be unsatisfactory as recoveries were consistently low even with ox RNAase taken as a standard. Certainly, the recoveries of 7.0 residues of cysteic acid in bison RNAase is not a true value, as it implies the presence of a free thiol group. Amino acid analyses of bison His(Cm)RNAase did not reveal the presence of any *S*-carboxymethylcysteine derivatives which, if a cysteine residue had been present and accessible in the native enzyme, would have been formed preferentially when bison RNAase was exposed to iodoacetic acid. The strongest experimental evidence for assigning a value of eight to the half-cystine content of bison RNAase comes from the diagonal peptide 'maps' of ox and bison RNAase prepared in three different high-voltage electrophoresis buffer systems (pH2.0, 3.5 and 6.5). These 'maps' were identical in appearance and make it unlikely that there was an extra disulphide bridge in the bison enzyme. Further support for a half-cystine content of eight residues was obtained by the isolation and

B1	Ox:	Val-Ala-Cys-Lys-Asn-Gly-Gln-Thr-Asn-Cys-Tyr-Gln-Ser	
		O ₃ H ⁶⁵ 70	O ₃ H 75
	Bison:	(Val, Ala, Cys, Lys, Asx, Gly, Glx, Thr, Asx, Cys, Tyr, Glx, Ser)	
		O ₃ H 	O ₃ H
B3	Ox:	Ala-Val-Cys-Ser-Gln-Lys-Asn-Val-Ala-Cys-Lys-Asn-Gly-Gln-Thr-Asn-Cys-Tyr-Gln-Ser	
		⁵⁶ O ₃ H ⁶⁰ 65	⁷⁵ O ₃ H 70
	Bison:	(Ala, Val, Cys, Ser, Glx, ---, Asx, Val, Ala, Cys, Lys, Asx, Gly, Glx, Thr, Asx, Cys, Tyr, Glx, Ser)	
		O ₃ H *	O ₃ H
B4	Ox:	Cys-Glu-Gly-Asn-Pro-Tyr-Val-Pro-Val-His-Phe	
		O ₃ H ¹¹⁰ 115	¹²⁰
	Bison:	(Cys, Glx, Gly, Asx, Pro, Tyr, Val, Pro, Val, His, Phe)	
		O ₃ H 	O ₃ H
B5	Ox:	Gln-Ala-Val-Cys-Ser-Gln-Lys-Asn-Val-Ala-Cys-Lys-Asn-Gly-Gln-Thr-Asn-Cys-Tyr-Gln-Ser-Tyr	
		⁵⁵ O ₃ H ⁶⁰ 65	⁷⁶ O ₃ H 70
	Bison:	(Glx, Ala, Val, Cys, Ser, Glx, ---, Asx, Val, Ala, Cys, Lys, Asx, Gly, Glx, Thr, Asx, Cys, Tyr, Glx, Ser, Tyr)	
		O ₃ H *	O ₃ H
C1	Ox:	Thr-Met-Ser	
		O ₂ 78	⁸⁰
	Bison:	(Thr, Met, Ser)	
		O ₂ 	

*** This deletion is tentative as described in the text.**

characterization of peptides containing cysteic acid (cf. Tables 2 and 3). The absence of tryptophan from bison RNAase is based on the specificity of dimethylaminobenzaldehyde (Ehrlich's reagent) for the indole ring of tryptophan when employed as a detection reagent on two-dimensional peptide 'maps'. The Ehrlich test for tryptophan was always negative.

The extent of modification of the active site of bison RNAase by iodoacetic acid was determined by analysing the acid hydrolysate of three performate-oxidized and two unoxidized His(Cm)RNAase preparations on an amino acid analyser as described by Crestfield *et al.* (1963a). Performic acid oxidation of His(Cm)RNAase transformed half-cystine into cysteic acid thereby allowing *N*^ε-carboxymethylhistidine to be eluted in a position normally occupied by half-cystine. The recoveries of *N*^ε-carboxymethylhistidine and *N*^ε-carboxymethylhistidine were 0.62 and 0.36 residues respectively, by employing the recovery of alanine as 12.0 residues and utilizing an average amino acid integration constant for *N*^ε- and *N*^ε-carboxymethylhistidine. These derivatives were also shown to be present in bison His(Cm)RNAase by subjecting the acid hydrolysate to high-voltage electrophoresis at pH 6.5 in the presence of synthetic carboxymethylhistidine standards (Fig. 4). The data indicate that the modifications of the histidine residues have been mutually exclusive since the net modification of histidine is 0.98 residues. The data support the finding that the bison His(Cm)RNAase was enzymically inactive when assayed against RNA. The distribution of the modifications in *N*^ε- and *N*^ε-

carboxymethylhistidine in bison RNAase is 2:1, whereas Crestfield *et al.* (1963a,b) have reported the distribution in ox RNAase to be 8:1 respectively. Since the inactivation was carried out under identical experimental conditions, the quantitation suggests differences between these two very closely related enzymes. However, more studies are required before differences in active-site reactivity can be clearly ascertained.

Diagonal peptide 'maps' of the disulphide bridges of bison RNAase and ox RNAase were prepared as described by Brown & Hartley (1966) by high-voltage electrophoresis at pH 2.0, 3.5 and 6.5. The diagonal peptide 'maps' of both enzymes prepared at the three pH values were identical in appearance and suggested closely related primary structures adjacent to disulphide bridges. The best separation of the cysteic acid-containing peptides on the diagonal peptide 'maps' was obtained by high-voltage electrophoresis at pH 3.5 (Fig. 5).

The amino acid composition, cadmium-ninhydrin colour and the relative recoveries of cysteic acid-containing peptides from bison RNAase are presented in Table 2. Peptides referred to as 'major peptides' were recovered in about 80 nmol quantities, whereas minor peptides were recovered in 10–25 nmol amounts. Based on the above data, the best positioning of the peptides into the known amino acid sequence of ox RNAase is shown in Table 3. The peptides derived from bison RNAase had extensive homologies with certain amino acid sequences adjacent to disulphide bridges of ox RNAase. The

Table 4. *Peptides isolated from two-dimensional pH 1.6 and pH 2.4 high-voltage electrophoresis of a trypsin digest of bison RNAase-pyridoxal 5-phosphate*

Peptide composition	Peptide sequence alignment
Lys(0.7), Ser(0.9), Arg(1.0) *	<div style="text-align: center;">31 32 33</div> Ox: Lys-Ser-Arg Bison: (Lys, Ser, Arg)
Lys(1.9), Thr(1.2), Glu(0.9), Ala(3.0) *	<div style="text-align: center;">1 7</div> Ox: Lys-Glu-Thr-Ala-Ala-Ala-Lys Bison: (Lys, Glx, Thr, Ala, Ala, Ala, Lys)
Glu(1.0) *, Phe(1.0), Arg(0.9)	<div style="text-align: center;">8 10</div> Ox: Phe-Glu-Arg Bison: (Phe, Glx, Arg)
Asp(1.0) *, Thr(1.0), Leu(1.0), Lys(1.0)	<div style="text-align: center;">35 38</div> Ox: Leu-Thr-Lys-Asp Bison: (Leu, Thr, Lys, Asx)

* Arbitrarily taken as 1.0 or 3.0 residues as indicated with recoveries of other amino acids relative to this amino acid.

Table 5. Characterization of the fluorescent phosphorylpyridoxal peptide from bison RNAase-pyridoxal 5-phosphate

The recovery of the basic compound 'X' was calculated by using an average amino acid integration constant. This compound is the acid-degradation product of ϵ -pyridoxaminolysine, which was eluted from a basic column (0.9cm $\times$ 14cm) of the Phoenix analyser with 0.20M-sodium citrate buffer, pH 5.28, 55°C, at the 50min position, whereas lysine, histidine and arginine were eluted at 49.5, 57.5 and 108 min respectively. The notation Lys indicates

|
PLP

the site of covalent attachment of pyridoxal 5-phosphate to the isolated peptide.

Amino acid composition

Cys(0.3H) (0.6), Asp(1.0)*, Thr(0.9), Pro(0.3), Val(0.6), Phe(0.6), 'X' (0.5)

Sequential alignment

Ox: $\begin{array}{c} \text{O}_3\text{H} \\ | \\ \text{Cys-Lys-Pro-Val-Asn-Thr-Phe} \end{array}$ 40 41 45

Bison: $\begin{array}{c} \text{O}_3\text{H} \\ | \\ (\text{Cys, Lys, Pro, Val, Asx, Thr, Phe}) \\ | \\ \text{PLP} \end{array}$

* Arbitrarily taken as one residue.

recovery of lysine from the amino acid analyses of peptides B1, B3 and B5 was consistently lower than expected if total homology existed between these segments of both ribonucleases. The possibility exists that the lysine residue known to be present at position 61 (or perhaps position 66) in ox RNAase could be deleted in bison RNAase.

The amino acid composition of peptide A4 from bison RNAase, isolated in high yield, can be only accommodated with the known amino acid sequence of residues 20–29 of ox RNAase. Thus, on the basis of peptide characterizations presented, the amino acid sequence adjacent to half-cystine in position 26 in bison RNAase could tentatively be assigned: Ala²⁰-Ser-Ser-Ser-Asn-Tyr-Cys(O₃H)-Asn-Gln-Met(O₂)²⁹. Barnard *et al.* (1972) have reported the amino acid sequence of this region of the bison RNAase to be Ala²⁰-?-Thr-Ala-Asn-Tyr-Cys(O₃H)-Asn-Gln-Met(O₂)²⁹ based on sequential degradation of the oxidized enzyme in a Beckman Protein Sequencer. Identification of each amino acid residue released was determined on its phenylthiohydantoin derivative by g.l.c. (with and without silylation) and also on t.l.c. The uncertainty existing in the nature of the amino acid at position 21 (?) has been suggested to arise from a carbohydrate attachment through an asparagine residue. In this study, the amino acid analyses of pep-

ptide A4 did not indicate the presence of threonine nor did it suggest the existence of an amino sugar. The latter could have been formed during acid hydrolysis and would be expected to be partially stable. The disparity between the data is probably not due to differences in subspecies within the genus *Bison*, since the American or Plains bison (*Bison bison bison*) was undoubtedly utilized by both groups. RNAase from the closely related Wood bison (*Bison bison athabasca*) would not have been studied, since this animal is considered rare with only a few animals existing within the National Parks of Canada.

Support for the published sequence of the 30 *N*-terminal residues of bison RNAase (Barnard *et al.*, 1972) was obtained by the isolation of peptides possessing the amino acid composition equivalent to the sequences Lys¹-Glu-Thr-Ala-Ala-Ala-Lys⁷ and Phe⁸-Glu-Arg¹⁰ (Table 4).

The modification of ox RNAase with pyridoxal 5-phosphate followed by reduction with NaBH₄ was reported by Means & Feeney (1971) to be associated with the covalent attachment to the ϵ -amino group of lysine-7. However, studies by Raetz & Auld (1972) revealed that in addition to lysine-7, lysine-41 was also modified during the reaction of equimolar mixtures of ox RNAase and pyridoxal 5-phosphate. Amino acid analyses of phosphorylpyridoxal pep-

Table 6. *Bison ribonuclease disulphide peptides*

The pairing of the cysteic acid peptides isolated from the pH 3.5 diagonal peptide 'map' (Fig. 5) is based on data in Tables 2 and 3 and on the known order for ox RNAase disulphide bridges: 26–84, 40–95, 58–110 and 65–72. The peptide A_x was not recovered and its proposed sequence is arbitrarily based on the specificity of pepsin. Peptide C1 did not contain cysteic acid but was located below the diagonal as a result of the formation of methionine sulphone, which increased the molecular weight of the peptide by 8% and could have influenced the pK of the α -CO₂H group.

Group A	Peptide
$ \begin{array}{c} \text{Thr}^{36}-\text{Cys}^{40}-\text{Phe}^{46} \\ \\ \text{S} \\ \\ \text{S} \\ \\ \text{Arg}^{85}-\text{Cys}^{95}-\text{Ala}^{96} \end{array} $	A _x
$ \begin{array}{c} \text{Ile}^{81}-\text{Cys}^{84}-\text{Cys}^{95}-\text{Ala}^{96} \\ \\ \text{S} \\ \\ \text{S} \\ \\ \text{Tyr}^{25}-\text{Cys}^{26}-\text{Met}^{29} \end{array} $	A1
$ \begin{array}{c} \text{Ile}^{81}-\text{Cys}^{84}-\text{Cys}^{95}-\text{Ala}^{96} \\ \\ \text{S} \\ \\ \text{S} \\ \\ \text{Tyr}^{25}-\text{Cys}^{26}-\text{Met}^{29} \end{array} $	A2
$ \begin{array}{c} \text{Tyr}^{25}-\text{Cys}^{26}-\text{Met}^{29} \\ \\ \text{S} \\ \\ \text{S} \\ \\ \text{Ala}^{20}-\text{Cys}^{26}-\text{Met}^{29} \end{array} $	A5
$ \begin{array}{c} \text{Ala}^{20}-\text{Cys}^{26}-\text{Met}^{29} \\ \\ \text{S} \\ \\ \text{S} \\ \\ \text{Ala}^{20}-\text{Cys}^{26}-\text{Met}^{29} \end{array} $	A4
Group B	
$ \begin{array}{c} \text{Val}^{63}-\text{Cys}^{165}-\text{Cys}^{172}-\text{Ser}^{75} \\ \quad \\ \text{S} \quad \text{S} \end{array} $	B1
$ \begin{array}{c} \text{Ala}^{56}-\text{Cys}^{58}-\text{Cys}^{165}-\text{Cys}^{172}-\text{Ser}^{75} \\ \quad \quad \\ \text{S} \quad \text{S} \quad \text{S} \end{array} $	B3
$ \begin{array}{c} \text{Gln}^{55}-\text{Cys}^{58}-\text{Cys}^{65}-\text{Cys}^{72}-\text{Tyr}^{76} \\ \quad \quad \\ \text{S} \quad \text{S} \quad \text{S} \end{array} $	B5
$ \begin{array}{c} \text{Cys}^{110}-\text{Phe}^{120} \\ \\ \text{S} \\ \\ \text{S} \\ \\ \text{Cys}^{110}-\text{Phe}^{120} \end{array} $	B4
Group C	
$ \begin{array}{c} \text{Thr}^{78}-\text{Met}^{79}-\text{Ser}^{80} \quad (\text{before oxidation}) \\ \\ \text{O}_2 \\ \\ \text{Thr}^{78}-\text{Met}^{79}-\text{Ser}^{80} \quad (\text{after oxidation}) \end{array} $	C1

tides indicated that lysine-7 and lysine-41 were modified in a ratio of 2:3. Bison RNAase, covalently modified with pyridoxal 5-phosphate under conditions employed by Means & Feeney (1971) for ox RNAase, yielded a single highly fluorescent peptide after two-dimensional high-voltage electrophoresis of a trypsin digest. Amino acid analysis of this peptide was only compatible with the amino acid sequence of ox RNAase adjacent to lysine in position 41 (Table 5).

Other very weakly fluorescent peptides were observed after high-voltage electrophoresis but attempts to isolate these were unsuccessful because of the limited amounts of the peptides.

On the basis of the amino acid composition of peptides isolated from bison RNAase, almost complete homology with the corresponding segments of ox RNAase was observed. Of the 90 residues for which the information exists, 89 appear to be homologous

when side-chain amide groups are assigned on the basis of homology with the ox sequence. The peptides studied from bison RNAase essentially represented amino acid residues adjacent to the disulphide bridges, which tend to be invariant segments of protein structure (Table 6). In addition, the bison and the ox are closely related species within the family Bovidae. The high degree of homology observed between the RNAase enzymes from these species is therefore to be expected.

The authors acknowledge the generous assistance of Dr. L. B. Smillie and Mr. Mike Nattris, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada, for amino acid analyses. The co-operation and assistance of Dr. Ann C. Currier of the Canadian Wildlife Service and the National and Historic Parks Branch of Canada is gratefully acknowledged. This research was supported by the National Research Council of Canada through Grant A 5859.

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