AFFINITY CHROMATOGRAPHY OF PORCINE PANCREATIC RIBONUCLEASE AND REINVESTIGATION OF THE N-TERMINAL AMINO ACID SEQUENCE

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1. Introduction

In 1969 Wilchek et al. [1] described the affinity chromatography of bovine ribonuclease A using Agarose to which a strong competitive inhibitor of ribonuclease, 5′-(4-aminophenyl)-uridine-(2′,3′) phosphate (APUP) was coupled. On this adsorbent binding occurred at low ionic strength and neutral pH; desorption was achieved with 2 M acetic acid. We were unable to repeat their results, but could obtain desorption of ribonuclease with 4 M NaCl. Also, with crude pancreatic extracts, we could only achieve satisfactory binding of the enzyme at an ionic strength of 0.2, which largely prevents aspecific interactions. In this way we have already isolated pure ribonucleases from more than fifteen mammalian species in sufficient amounts for amino acid sequence studies.

In this article we describe the purification of porcine ribonuclease. Jackson and Hirs [2] have determined the amino acid sequence of this enzyme. In the N-terminal sequence they found glutamine at the positions 2 and 9, where, in other pancreatic ribonucleases, normally glutamic acid is found, (except for rat [3] which contains lysine at position 9). The side chain of glutamic acid 9 has no distinct function in the structure of bovine ribonuclease, but glutamic acid 2 may form a salt brdige with the side chain of arginine 10 [4], both "constant" residues in pancreatic ribonucleases. Marchiori et al. [5] and Hofmann et al. [4, 6] have demonstrated in their synthetic S-peptide studies that if this ion pair cannot be formed, the resulting partially synthetic ribonucleases S′ are less active. Therefore, we reinvestigated the state of amidation of glutamic acid residues in the N-terminal part of porcine ribonuclease.

2. Materials and methods

Sephadex G-25 was a product of Pharmacia (Uppsala). 100 ml Agarose-APUP was obtained from the research division of Miles Laboratories Inc. (Kankakee, Ill.). Cyanogen bromide (CNBr) was purchased from Fluka AG (Buchs SG, Switzerland). Trypsin from pig (3 × cryst.) and bovine ribonuclease were from Miles-Seravac Ltd. (Maidenhead, England), aminopeptide (hog kidney) from P.L. Biochemicals (Milwaukee, Wisc.). Ribonucleic acid was prepared from yeast according to Crestfield et al. [7]. Ribonuclease activity was measured according to Campagne and Gruber [8] with yeast RNA as a substrate. DEAE-cellulose was bought from W. and R. Balston Ltd. (Maidstone, England) or from Schleicher and Schüll (Dassel, W. Germany).

All other chemicals were analytical grade products from Merck AG (Darmstadt, W. Germany).

2.1. *Isolation of porcine ribonuclease*

Centrifugations were carried out for 2 hr (3900 *g*, 0°, International PR-2). The pancreatic tissue freed from fat and connective tissue was homogenised in a blender in a threefold (v/w) volume of cold 0.125 M H_2SO_4 for 3 min and stirred overnight at 0°. After centrifugation, the residue was washed twice with 0.125 M H_2SO_4. To the combined supernatants a fourfold volume of acetone (−20°) was added. The mixture was stirred for 1 hr and left overnight. After decantation the sediment was centrifuged. The pellet was washed twice (1 hr) with an acetone–water mixture (4:1, the acetone for the last wash was distilled twice over $KMnO_4$) and dried in vacuo over concentrated H_2SO_4. The soluble components were extracted

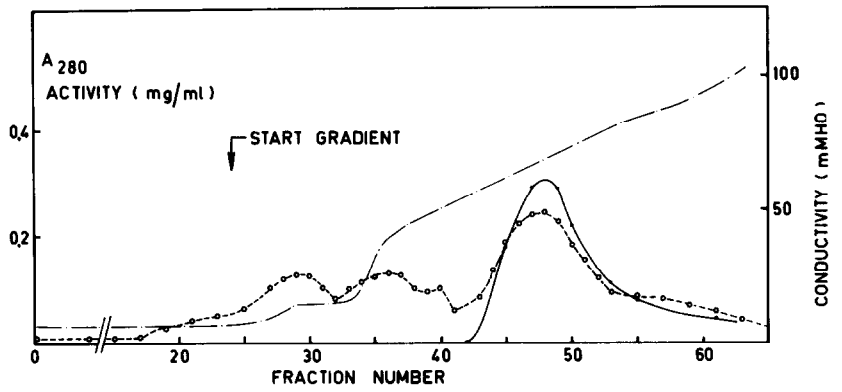

Fig. 1. Rechromatography of porcine ribonuclease on an Agarose-APUP column (14 × 3 cm). Elution with a linear gradient of 0.2 M NaCl–6 M NaCl in 0.23 M sodium acetate pH 5.2. Flow rate 120 ml/hr; 10 ml fractions. (○- -○- -○) A_{280}; (●—●—●) enzymatic activity; (·—·—·) conductivity.

from the dry acetone powder by stirring for 16 hr with 300 ml 0.23 M sodium acetate buffer pH 5.6 (per 100 g pancreatic tissue) followed by centrifugation. This procedure was repeated (1 hr extraction, 200 ml buffer per 100 g tissue). The combined extracts were filtered and stirred for 2 hr with DEAE-cellulose (55 g dry weight in 200 ml acetate buffer per 100 g tissue) equilibrated with 0.23 M sodium acetate pH 5.6, to remove (poly)nucleotides and negatively charged proteins. The DEAE-cellulose was removed by suction on a glass filter and washed with 300 ml sodium acetate buffer per 100 g tissue. The filtrate and the wash were combined and the pH was lowered to 5.2. The solution containing ribonuclease was applied to an Agarose-APUP column (14 × 3 cm) equilibrated with 0.23 M sodium acetate pH 5.2 at a flow rate of 120 ml/hr, followed by 50 ml of 0.23 M sodium acetate pH 5.2. Aspecifically bound material was removed with 0.23 M sodium acetate pH 5.2 containing 0.2 M NaCl until the absorbance at 280 nm of the effluent was below 0.020. The enzyme was desorbed with 0.23 M sodium acetate buffer pH 5.2 containing 4 M NaCl. The course of the chromatography was followed by measurement of the absorbances at 260 and 280 nm, the conductivity and the ribonuclease activity of the fractions. After gel filtration on Sephadex G-25 in 0.1 M acetic acid (40 ml/hr) the product was still contaminated with other material and therefore a second chromatography on the Agarose-APUP column was performed. This time the ribonuclease was eluted with a linear gradient of 0.2 M NaCl–6 M NaCl in 0.23 M sodium acetate pH 5.2 (fig. 1), pooled, desalted by gel filtration and lyphilized.

Carbohydrate analyses according to Winzler [9] of the last gel filtration were performed on fractions on Sephadex G-25. The results were expressed as mannose equivalents per mg ribonuclease.

Electrophoresis in polyacrylamide gel was performed on 10% gels in β-alanine acetate buffer pH 4.5. Gels were stained in 0.5% Amido Black in 7% acetic acid, followed by destaining in 7% acetic acid. Duplicate gels were incubated with yeast RNA and stained with 0.2% toluidine blue according to Wilson [10].

Dansylation was carried out according to Gros and Labouesse [11]. Identification of dansyl amino acids was achieved by thin-layer chromatography on polyamide sheets [12] using a standard mixture of dansyl amino acids [13].

Amino acid analyses were performed on a TSM-1 amino acid analyser (Technicon). Hydrolysates were obtained after hydrolysis for 21 hr at 111° in 1 ml twice-distilled HCl (6 N) in sealed and evacuated tubes.

Porcine ribonuclease (40 mg) was cleaved with CNBr according to D'Alessio et al. [14]. The products were separated on a Sephadex G-25 column (200 × 0.9 cm) in 0.1 M acetic acid (flow rate 8 ml/hr; 2.3 ml fractions). The absorbance of the effluent was measured at 280 and 220 nm. The position of the N-terminal peptide was determined by paper electrophoresis at pH 3.5 of an aliquot from each fraction eluting after V_0.

CNBr peptide 1–13 was lyophilised and dissolved in 2 ml 0.1 M *N*-ethylmorpholine acetate buffer pH 8.0

and digested with 0.4 mg trypsin at 37° for 3 hr. The tryptic peptides 1–6, 8–10, 7–10 and free lysine were characterised in the same way as CNBr peptide 1–13.

Electrophoresis was performed at pH 3.5 (2500 V; 70 min) in an apparatus from Gilson Medical Electronics. Pyridine–acetic acid–water (1:10:90; v/v/v) was used for moistening of the paper and the same buffer in the proportion 1:10:290 (v/v/v) was used in the tank. For amide determination according to Offord [15], electrophoresis at pH 6.5 (4400 V; 45 min) was performed in a flat-plate apparatus (Shandon) using pyridine–acetic acid–water (25:1:225; v/v/v).

The presence of amide groups was also determined by incubation of about 50 μmole of peptide with 50 μg aminopeptidase in 150 μl 0.1 M *N*-ethylmorpholine acetate buffer pH 8.0 at 37°. During the incubation 7 μl samples were withdrawn from the mixture, acidified with a drop of acetic acid and analysed for neutral and acidic amino acids on a TSM-1 amino acid analyser. Glutamine eluted at the position of threonine.

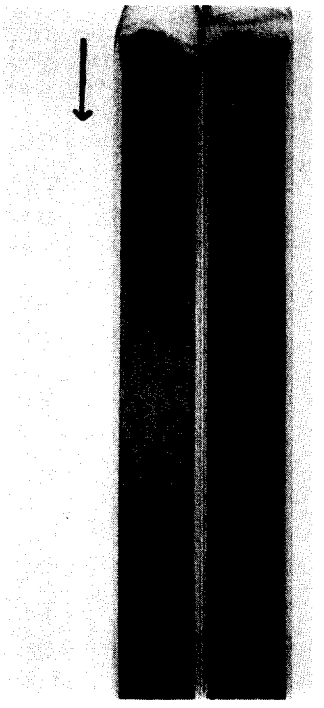

Fig. 2. Polyacrylamide gel electrophoresis of purified porcine pancreatic ribonuclease at pH 4.5. Left gel stained for protein with 0.5 % Amido Black; right gel incubated with RNA and stained with 0.2% toluidine blue (clear zones indicate bands with ribonuclease activity).

3. Results

We isolated 50 mg porcine ribonuclease from 300 g pancreatic tissue. Determination of the N-terminal residue yielded only lysine. The sugar content turned out to be 15% expressed as mannose equivalents, which is the lower limit of the real sugar content because several sugars do not react with the orcinol reagent. After polyacrylamide gel electrophoresis many proteins bands were visible, all enzymatically active (fig. 2). This heterogeneity of porcine ribonuclease has also been observed by others and is caused by differences in glycosidation [16].

After CNBr cleavage of 40 mg ribonuclease we obtained 650 nmoles of the N-terminal peptide 1–13 (table 1). Tryptic cleavage of this peptide yielded 250 nmoles of the N-terminal peptide 1–6 and 150 nmoles of peptide 8–10. Free lysine and peptide 7–10 were also obtained (table 1). After dansylation the expected N-terminal amino acids were found. As a consequence of the conversion of the N-terminal

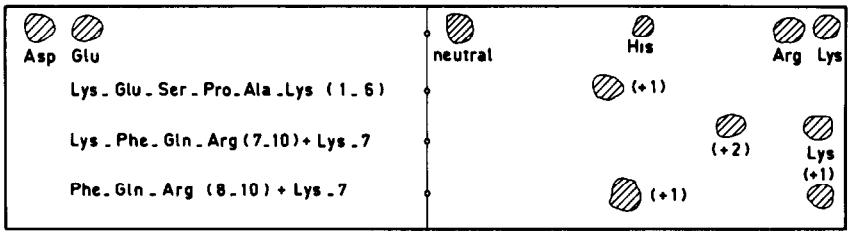

Fig. 3. Ninhydrin-stained peptide pattern obtained after paper electrophoresis at pH 6.5 of tryptic peptides from the N-terminal CNBr peptide of porcine ribonuclease. Charges of the peptides are indicated in the figure.

Table 1
Amino acid compositions of the N-terminal CNBr peptide of porcine ribonuclease and of the peptides obtained after gel filtration on Sephadex G-25 of the tryptic digest of this N-terminal peptide.

Peptide or peptide mixt.	1–13	Lit.*	1–6	Lit.*	7–10 + 11–13 + Lys-7	8–10 + Lys-7
Aspartic acid	0.3		0.1		0.1	0.1
Serine	1.2	(1)	1.1	(1)	0.3	0.4
Glutamic acid	2.8	(3)	1.1	(1)	3.8**	1.2
Proline	1.1	(1)	0.8	(1)		
Glycine	0.3		0.2		0.2	0.4
Alanine	1.1	(1)	1.0	(1)		
Phenylalanine	0.8	(1)			1.0	0.8
Histidine	1.1	(1)			1.6	0.3
Lysine	2.9	(3)	2.0	(2)	2.8	1.0
Arginine	1.2	(1)			1.0	1.0
Homoserine lactone	+	(1)			+	
N-terminal residue(s)	Not determined		Lys		Lys	Lys + Phe

* Theoretical values from [2] are given in brackets.
** Glutamic acid + homoserine.

glutamine to pyrrolidone carboxylic acid, peptide 11–13 did not interfere since it is dansyl- and ninhydrin-negative.

Determination of the charge of the peptides according to Offord [16] revealed a charge of +1 for peptide 1–6 and peptide 8–10, whilst peptide 7–10 had a charge of +2 (fig. 3). These results indicated that the amino acid residue in position 2 of the polypeptide chain of porcine ribonuclease is glutamic acid and not glutamine as found by Jackson and Hirs [2], and confirmed the presence of glutamine in position 9.

An aminopeptidase digestion confirmed these results. With peptide 1–6, amino acid analysis showed a peak at the position of glutamic acid; whereas in the case of peptide 8–10 a peak appeared at the position of glutamine.

4. Discussion

With the method described, ribonucleases from pancreatic tissue of bison, camel, dromedary, European elk, fallow deer, giraffe, goat, kangaroo, mouse, impala, okapi, pig, reindeer, sheep, Thomson's gazelle, wildebeest and the lesser rorqual have been isolated in our laboratory.

Usually the ribonucleases were sufficiently pure after only one chromatography on the affinity column. Both stepwise elution with 4 M NaCl in 0.23 M sodium acetate buffer and the described gradient elution have been used, the latter method usually giving better results. In most cases a modified procedure was used for the first steps of the isolation: following acidic extraction, the ribonuclease was precipitated with ammonium sulfate between 50 and 90% saturation. After centrifugation the sediment was extracted with small volumes of 0.23 M sodium acetate pH 5.2 until no ribonuclease could be detected in the extract. The combined extracts were desalted by gel chromatography over a Sephadex G-25 column (50 × 3 cm; flow rate 40 ml/hr) in 0.23 M sodium acetate pH 5.2. Fractions containing ribonuclease were directly applied to the Agarose-APUP column.

The presence of glutamic acid at position 2 in porcine ribonuclease is in agreement with the importance of the salt bridge between Glu-2 and Arg-10 for enzymic activity [4–6]. In having glutamine at position 9, however, porcine ribonuclease differs from most other ribonucleases. However, recently we found that in chinchilla ribonuclease position 9 is also occupied by a glutamine (Van den Berg and Beintema, unpublished).

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