THE PRIMARY STRUCTURE OF GIRAFFE PANCREATIC RIBONUCLEASE

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1. Introduction

To date the primary structure of the pancreatic ribonucleases from cow [1], rat [2], pig [3, 4], horse [5], red deer, roe deer [6], sheep [7], and goat [8] have been determined. Here we present the isolation and determination of the primary structure of giraffe pancreatic ribonuclease. Giraffe pancreatic ribonuclease is a glycoprotein and together with its primary structure we have determined the composition of its carbohydrate chain.

2. Materials and methods

Sephadex G-25 and G-50 were products of Pharmacia (Uppsala) and Aminex A-5 of Biorad Laboratories (Richmond). Trypsin (pig, 3x cryst. batch UK 6) was a product of Miles-Seravac (Maidenhead), and thermolysin (3x cryst. A grade lot 100 939) of Calbiochem (San Diego). Carboxypeptidases A and B (DFP treated) and α-chymotrypsin (bovine pancreas, 3x cryst.) were products from Worthington Biochemical Corporation (New Jersey). Subtilisin Carlsberg was a gift of Novo Industri (Copenhagen). Ethyleneimine was from Fluka AG (Buchs). Trifluoroacetic acid (TFA) and phenylisothiocyanate (PITC) were sequanal grade products of Pierce Chemical Co. (Rockford).

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

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2.1. *Isolation of giraffe ribonuclease*

Giraffe ribonuclease was isolated according to Wierenga et al. [4] using ammonium sulfate precipitation between 50 and 90% saturation. The sediment after the ammonium sulfate precipitation was dissolved in 0.23 M sodium acetate (pH 5.2) and desalted by gel filtration; the peak containing ribonuclease activity was applied in four batches to the affinity column. In fig. 1 a typical elution pattern is shown.

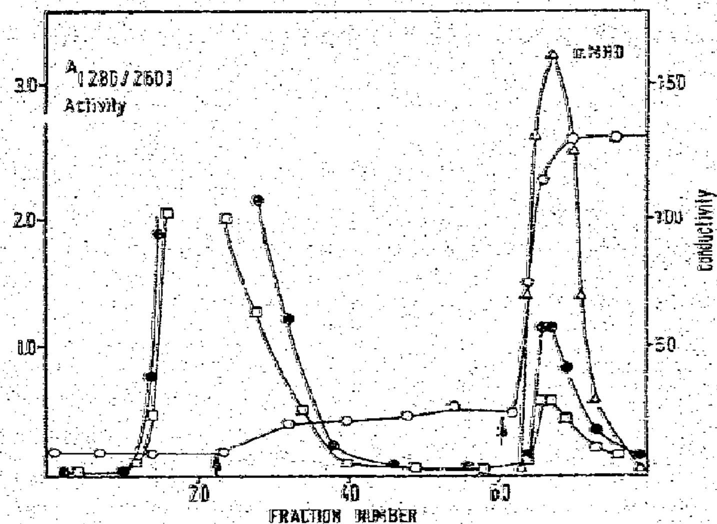

Fig. 1. Affinity chromatography of giraffe pancreatic ribonuclease. See ref. [4] for the exact conditions. Instead of a linear gradient of sodium chloride in the elution buffer, the concentration of sodium chloride was raised stepwise from 0.2 M to 4.0 M. (●—●—●) A_{280}, (□—□—□) A_{260}, (○—○—○) conductivity, (△—△—△) enzyme activity. The arrows indicate the additions of sodium chloride to the elution buffer. In this case we obtained 93 mg of pure giraffe pancreatic ribonuclease.

Enzymatic activities were determined according to Campagne and Gruber [9]. Carbohydrate analysis according to Winzler [10] was performed on the fractions of the gel filtration on Sephadex G-25 after the affinity chromatography.

2.2. *Enzymatic cleavages*

All proteolytic digestions were performed at pH 8.0 and 37°C for 2 hr unless stated otherwise. Aminoethylation and digestion with trypsin were performed as described [6]. Thermolysin digestion of 20 mg oxidised giraffe pancreatic ribonuclease was performed in 0.1 M ammonium carbonate (E:S = 1:40 by weight). Subtilisin digestion was performed as described [8, 11]. The S-peptide was purified by high-voltage paper electrophoresis at pH 3.4 [11]. Incubation of 0.7 mg giraffe ribonuclease with a mixture of carboxypeptidase A and B was performed in an *N*-ethylmorpholine acetate buffer containing 0.1% sodium lauryl sulfate. Peptides 11–26 and 40–58 were digested with thermolysin for 3 hr in a molar ratio of 1:200. Peptide 111–124 was digested with chymotrypsin in 0.2 M ammonium acetate in a molar ratio of 1:800.

2.3. *Isolation of peptides*

The tryptic digest was dissolved in 0.1 M acetic acid and fractionated on a column of Sephadex G-25 (fine) (200 × 0.9 cm, 7 ml/hr). 110 fractions of 2.5 ml were collected, pooled as indicated in fig. 2 and lyophilised after determination of the absorbances at 280 and 220 nm. Aliquots of the fractions were tested for the presence of carbohydrate [10] and electrophorated at pH 3.4 [4, 6]. Purification of peptides by high-voltage paper electrophoresis on Whatman 3 MM at pH 3.4 and elution of peptides was done as described by Ambler [12]. In some cases it was necessary to separate a peptide mixture at a different pH [8]. In table 1 the fractionation procedures used for the purification of the different tryptic pools and peptides obtained are indicated. The peptides obtained after the digestions with thermolysin and chymotrypsin were purified by high-voltage paper electrophoresis.

Ion-exchange chromatographies on Aminex A-5, amino acid analysis, polyacrylamide gel electrophoresis and column electrophoresis were performed as described earlier [4–6].

Quantitative analysis of the monosaccharides released from the glycopeptides after methanolysis, was performed essentially according to Clamp et al. [13].

2.4. *Edman degradation and determination of amide positions*

Dansyl–Edman degradation was performed as described by Gray and Smith [14]. Charges of peptides

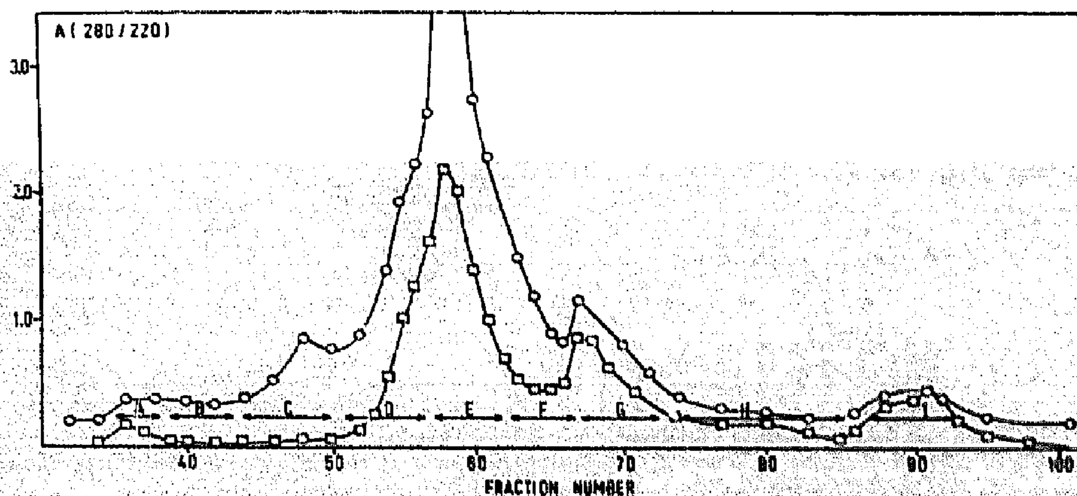

Fig. 2. Gel filtration on Sephadex G-25 (fine) (0.9 × 200 cm) of a tryptic digest of giraffe ribonuclease. Flow rate 7 ml/hr. 2.5 ml fractions were collected. Fractions were pooled as indicated by horizontal bars and lyophilised. (○–○–○) A_{220}, (□–□–□) A_{280}.

Table 1
Fractionation procedures used for the purification of the peptides obtained after tryptic digestion of aminoethylated giraffe ribonuclease

Fractionation	Peptide zone from Sephadex G-25								
	A	B	C	D	E	F	G	H	I
Further fractionation	None	Column electrophoresis		Paper electrophoresis	Aminex		Paper electrophoresis		
Peptides obtained	None	40–58		11–26	1–7		8–10	None	73–76
		34–59*		111–124	27–33		66		
						59–61	85		
						62–65	92–95		
					67–72				
					96–104	73–85			
					105–110	80–84			
					121–124	80–85			
						86–91			

* Glycopeptides.

were determined according to Offord [15]. Automatic Edman degradation of oxidised giraffe ribonuclease was performed in a Beckman 890 C sequencer using a standard protein program. PTH amino acids were identified by gas chromatography.

3. Results

From 340 gram of pancreatic tissue we isolated 256 mg giraffe pancreatic ribonuclease. Determination of the N-terminal residue yielded only lysine. Upon polyacrylamide gel electrophoresis three protein bands were visible, all enzymatically active (as tested by the method of Wilson [16]).

Preliminary data on kinetic experiments with giraffe pancreatic ribonuclease show no significant differences between the kinetic parameters of bovine and giraffe ribonuclease.

An attempt to separate the different components of giraffe pancreatic ribonuclease by ion-exchange chromatography (CM-32 cellulose) was unsuccessful. However, we isolated three peptides having identical amino acid sequences but different amounts of carbohydrate (table 2). 150 mg of giraffe ribonuclease has been used for the amino acid sequence studies on two tryptic digests of the aminoethylated protein (fig. 3).

Table 2
Carbohydrate composition of the glycopeptides of giraffe ribonuclease. The amount of monosaccharide is given in nmoles per nmole peptide. The peptides are numbered according to increasing electrophoretic mobility at pH 3.4

Monosaccharide	Peptide		
	I	II	III
Fucose	1.73	2.63	0.81
Mannose	5.26	6.24	5.35
Galactose	2.76	3.10	1.10
N-acetylglucosamine	4.37	6.58	2.70
Sialic acid*	3.31	3.31	1.11

* Because of the procedure we used, methanolysis and re-*N*-acetylation, we could not distinguish between *N*-glycolylneuraminic acid and *N*-acetylneuraminic acid.

Since it was obvious from the amino acid composition of the peptides containing them and the known specificity of the cleavage method used, not all C-terminal amino acids of the tryptic peptides have been identified as their DNS derivatives. The locations of the tryptic peptides were inferred from homology with other ribonucleases. Some overlapping peptides were obtained from a thermolysin digest of performic acid-oxidised giraffe ribonuclease. From the amino acid analysis of the S-peptide it was clear that cleavage

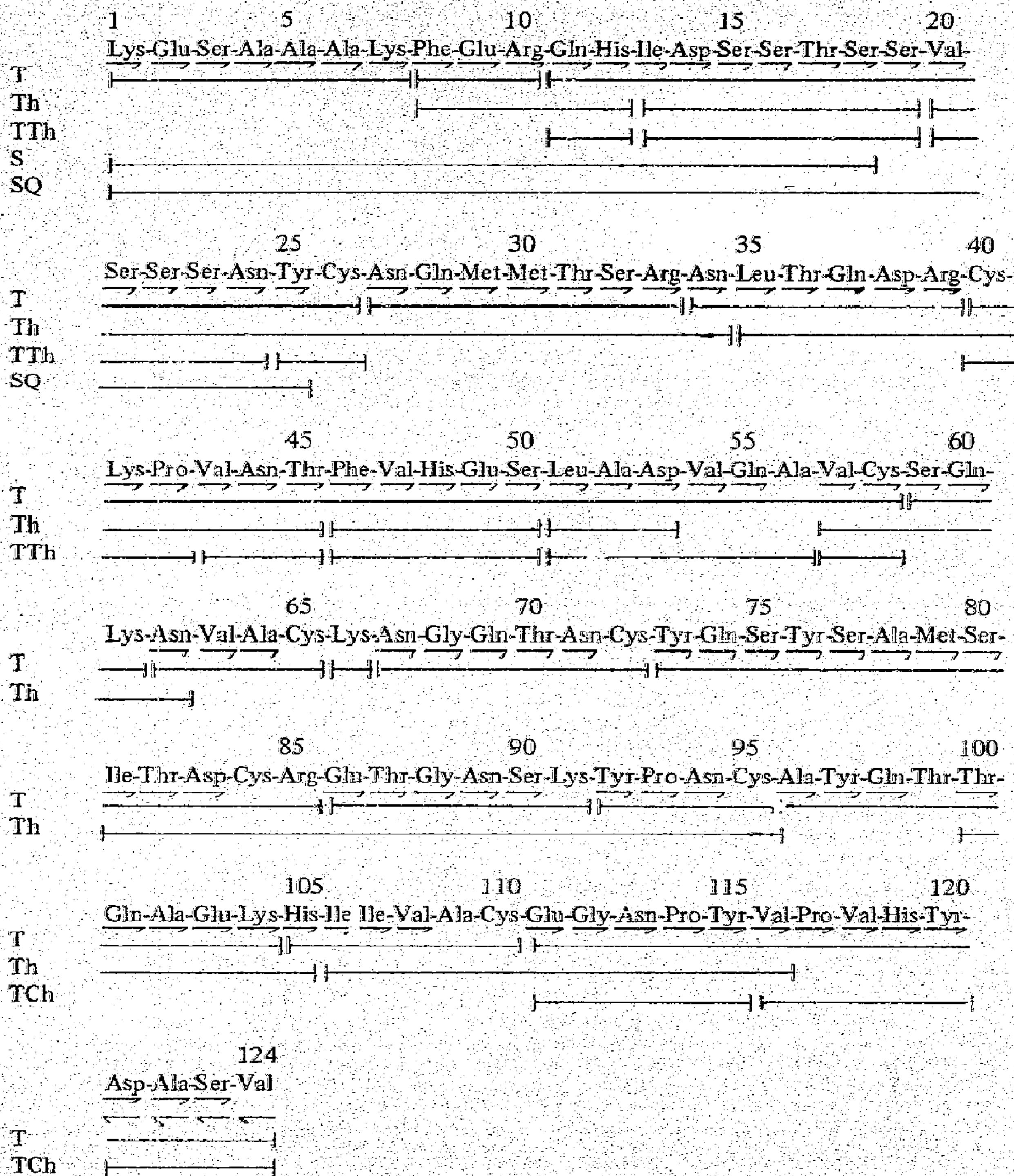

Fig. 3. The primary structure of giraffe pancreatic ribonuclease. The peptide sequences determined with the dansyl–Edman technique or as PTH amino acid are indicated by arrows ⟶, ⟵ determined from digestion by carboxypeptidase. T = peptides from the primary tryptic digests; Th = peptides from the thermolysin digest; TTh = peptides from the digestion of tryptic peptides with thermolysin; TCh = peptides from the digestion of tryptic peptides with chymotrypsin; S = S-peptide; SQ = determined with automatic Edman degradation.

had taken place between amino acid residues 18 and 19 [11]. This is in contrast to the bovine enzyme, where predominantly the bond between residues 20 and 21 is split [17].

4. Discussion

An earlier isolation of giraffe pancreatic ribonuclease from another pancreas using several procedures for chromatography on CM-cellulose [6, 18] was unsuccessful. Probably, this ribonuclease is bound only very weakly to cation exchangers because of the large carbohydrate moiety and the small excess of positive charges (as compared to other ribonucleases).

The percentage of differences between the amino acid sequences of ribonuclease from the giraffe and from the bovids (6–9%) is remarkably less than between the ribonucleases from the deer species and the bovids (13–15%) [6], although the giraffoids are positioned as an early branch of the line to the deer in the phylogenetic tree of the ruminants [19].

Although there are relatively few substitutions between the ribonucleases of giraffe and cow (table 3), there are a few interesting ones: e.g. the substitution of lysine at position 98 (hitherto a constant residue within the artiodactyls), the substitution of methionine at position 13 by isoleucine and the substitution of phenylalanine at position 120 by tyrosine. It was already known from studies with synthetic S-peptides [20, 21] that the hydrophobicity rather than the thioether function of the amino acid residue at position 13 is a fundamental prerequisite for its capability to act as a binding site in the S-peptide/S-protein association of bovine ribonuclease. However, the substitution of methionine 13 has so far only been found in the pancreatic ribonuclease of the snapping turtle [22], a species very distant from the cow. Therefore, it was interesting to find isoleucine at position 13 in a species so much related to the bovidae.

The same holds for the substitution of phenylalanine 120 by tyrosine. This substitution has also been found in the turtle enzyme [23]. In this case, studies with synthetic C-terminal peptides and bovine ribonuclease 1–118 [24] showed the necessity of an aromatic side chain at position 120 for maximum stability of the whole molecule.

The presence of glycosidated ribonuclease components can be correlated with the occurrence of an Asn-X-Thr/Ser sequence. It has been suggested that an apolar residue at position X is generally found in the case of a so-called 'simple'-type carbohydrate chain [3, 25].

However, in giraffe pancreatic ribonuclease we found an apolar residue together with a 'complex'-type of carbohydrate chain (table 2). Similar complexity was found in bovine ribonucleases C and D [26], ovine ribonuclease C [27] and in the fully glycosidated ribonuclease of the European elk (Gita Leijenaar and J. J. Beintema, unpublished). Therefore, we suggest that the complexity of the carbohydrate chain attached to Asn 34 in pancreatic ribonucleases is not only determined by the characteristics of the amino acid residue at position 35 but also by other still unknown factors.

The suggestion of our group [28] that probably all of the enzyme is glycosidated if methionine is present at position 35 and only part of it if position 35 is occupied by leucine, is wrong since we have found that the ribonucleases of giraffe, okapi, and the European elk (Gita Leijenaar and J. J. Beintema, unpublished), which contain leucine at position 35, are fully glycosidated.

We have isolated 8 mg pancreatic ribonuclease of the short-necked relative of the giraffe, the okapi, in the same way as described for giraffe ribonuclease. Because of this small amount we cannot present the complete sequence of okapi ribonuclease. However, we did determine that at position 120 phenylalanine is also substituted by tyrosine but that position 98 is occupied by lysine. We also found three glycopeptides with the same amino acid sequence as that of the three

Table 3
Differences in amino acid sequence between giraffe and bovine pancreatic ribonuclease

Position	3	13	19	20	31	37	78	89	98	103	120
Giraffe	Ser	Ile	Ser	Val	Thr	Gln	Ala	Asn	Gln	Glu	Tyr
Bovine	Thr	Met	Ala	Ala	Lys	Lys	Thr	Ser	Lys	Asn	Phe

giraffe glycopeptides, and with comparable electrophoretic mobilities.

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