Mammalian ribonucleases

The absence of a glycosylated Asn-Pro-Thr sequence in horse ribonuclease and the presence of tryptophan at position 39 in horse and dromedary ribonuclease

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Parts of the amino acid sequences of horse and dromedary pancreatic ribonuclease were reinvestigated. The sequence of residues 21–25 in horse ribonuclease is Ser-Asn-Pro-Thr-Tyr or Ser-Asn-Ser-Thr-Tyr. The asparagine in the latter sequence is glycosylated. Horse ribonuclease possesses four additional amino acid residues at the C-terminus, like a number of other ribonucleases. Position 39 in horse and dromedary ribonuclease is not deleted but is occupied by tryptophan.

Ribonuclease *Glycoprotein* *Tryptophan* *Stop codon*

1. INTRODUCTION

Horse ribonuclease is a heavily glycosylated protein with carbohydrate attached to two or three different sites on the molecule [1]. All molecules were found to have carbohydrate attached to the sequences Asn-Met-Thr (positions 34–36) and Asn-Ile-Thr (62–64), but only part of them have carbohydrate attached to a third site near residue 21. Only the carbohydrate-free peptide with this site had been sequenced and found to have the sequence Asn-Pro-Thr (21–23).

There was no indication that the carbohydrate-free and glycosylated peptides might differ in sequence and because of scarcity of material the glycopeptide was not sequenced [1,2].

Since these studies on horse pancreatic ribonuclease, knowledge of glycoprotein structure and synthesis has increased tremendously [3]. One aspect is the structural requirements, in addition to an Asn-X-Ser/Thr sequence, of the glycosylation site for accepting carbohydrate. Using synthetic peptides it has been found that the presence of proline at position X prevents carbohydrate attachment [4]. No glycosylated Asn-Pro-Ser/Thr sequences have been found in nature, except the supposed one in horse ribonuclease. Since the experimental evidence for the latter sequence was not strong, it was decided that it should be reinvestigated.

During these studies some other deviations from earlier work were found. One of these is the presence in the sequence of tryptophan at position 39 instead of a deleted residue. Since a deletion at the same position had been found in dromedary ribonuclease [5], the possible presence of tryptophan in the latter sequence was also investigated.

2. MATERIALS AND METHODS

Horse and dromedary ribonucleases were isolated by extraction with 0.125 M sulfuric acid and affinity chromatography [5,6]. Trypsin (treated with L-1-tosylamide-2-phenylethylchloromethyl ketone, TPCK) and α-chymotrypsin were from Worthington (Freehold, NJ), and subtilisin Carlsberg from Fluka (Buchs, Switzerland).

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Horse ribonuclease (10 mg) was treated with 125 mg CNBr in 250 μl of 70% trifluoroacetic acid for 20 h. A few small peptides, including the glycopeptide with Asn-34, were separated from larger fragments by gel filtration on a column (1 × 100 cm) of Sephadex G-50 (fine) in 0.2 M acetic acid. The first peak was oxidized with performic acid, lyophilized and digested with 50 μg TPCK-treated trypsin at 37°C in 500 μl of 0.2 M ammonium bicarbonate for 2 h, followed by the addition of 50 μg chymotrypsin and continuation of the incubation for another 2 h (Ox-peptides). Another batch of horse ribonuclease (10 mg) was reduced and carboxymethylated [7], and digested with 100 μg TPCK-treated trypsin at 37°C in 1 ml of 0.2 M ammonium bicarbonate for 4 h, followed by the addition of 100 μg chymotrypsin and continuation of the incubation for another 4 h (R-peptides).

Peptides were isolated by gel filtration on Sephadex G-25 (fig.1) followed by reversed-phase high-performance liquid chromatography (HPLC) using a Nucleosil 100 C_{18} column (300 × 4.6 mm) with a gradient of 0–67% acetonitrile in 0.1% trifluoroacetic acid in 60 min at a flow rate of 1.0 ml/min (fig.2). Peptide Ox1,2 (100 nmol) was digested with 50 μg chymotrypsin at 37°C in 500 μl of 0.2 M ammonium bicarbonate for 2 h, followed by the addition of 50 μg subtilisin and continuation of the incubation for 3 h. Peptide Ox1,2S1 was isolated from the digest by HPLC. The second peak of fig.2 (peptide R1, 30 nmol) was digested with 30 μg subtilisin at 37°C in 300 μl ammonium bicarbonate for 4 h. Peptides R1S2 and R1S3 were isolated by HPLC.

Dromedary ribonuclease (10 mg) was reduced and carboxymethylated [7] and digested with 100 μg trypsin-TPCK at 37°C in 1 ml of 0.2 M ammonium bicarbonate for 3 h (D-peptides). Peptides were isolated by gel filtration on Sephadex G-25 (fig.3) followed by reversed-phase HPLC. Peptide D2 (residues 67–85, 80 nmol) was digested with 20 μg chymotrypsin at 37°C in 200 μl of 0.2 M ammonium bicarbonate for 5 h. Peptide D2C1 was isolated by HPLC.

Amino acid analyses were performed with a Kontron Liquimat III amino acid analyzer. Tryptophan was determined after hydrolysis with 3 M mercaptoethanesulfonic acid for 72 h [8]. Amino acid sequences of peptides were determined by automatic Edman degradation or dansyl-Edman degradation. In a single instance degradation with dimethylaminoazobenzene isothiocyanate (DABITC) was used [9]. The absence of amide groups in peptide Ox4b was concluded from its electrophoretic mobility at pH 6.5 [10]. Quantitative analysis of monosaccharides released from glycopeptides after methanolysis was done as described by Kamerling et al. [11]. Other methods followed previous descriptions [5,12,13].

3. RESULTS AND DISCUSSION

3.1. *Horse ribonuclease*

3.1.1. Positions 21–24

Sequenator runs on purified horse ribonuclease suggested the presence of the sequence Asn-Pro-Thr-Tyr (positions 22–25) rather than the sequence Asn-Pro-Thr-Asn-Tyr (positions 21–25) found earlier [1]. Sequence studies on carbohydrate-free peptides from this region of the molecule, obtained from the digest of the CNBr-treated and performic acid-oxidized protein, confirmed these observations (table 1, fig.4).

3.1.2. Glycopeptides

Horse ribonuclease was eluted as a broad peak from the affinity column, with the front of the peak containing more carbohydrate than the tail. Fig.1 presents the gel filtration pattern of the peptides obtained after digestion of the reduced and carboxymethylated protein with trypsin and chymotrypsin. Peak A contained the glycopeptides, which were separated from each other by HPLC (fig.2).

The glycopeptide with Asn-62 was obtained in a high yield from digests of both the CNBr-treated and oxidized, and the reduced and carboxymethylated protein (peak 1 in fig.2). A small amount of carbohydrate-free peptide was isolated from a digest made from ribonuclease which eluted late from the affinity column. This peptide eluted at a position corresponding to peak D in fig.1 during gel filtration.

The glycopeptide in the second peak of fig.2 was not pure. However, after digestion with subtilisin and HPLC, pure peptides were obtained, including the glycopeptide R1S3 (table 1, fig.4). This peptide has the same sequence as the corresponding carbohydrate-free peptide Ox1,2S1, except for the re-

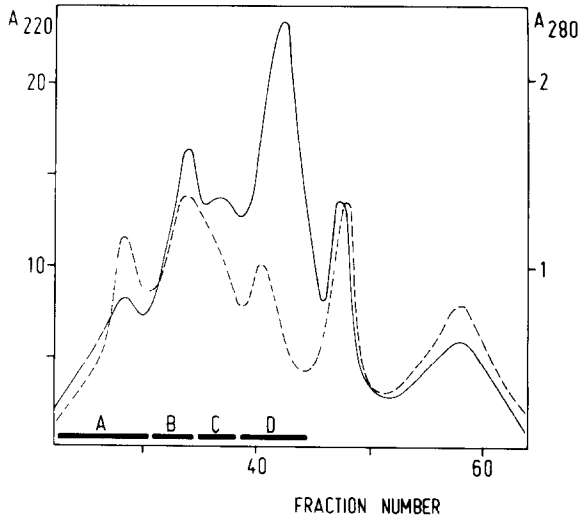
Table 1

Amino acid compositions of peptides

Peptide	Ox1,2	Ox1,2S1	Ox1	R1S2	R1S3	R2	R3	Ox4a	Ox4b	D1	D2C1
Cysteine[a]	1.0(1)						0.8(1)			1.9(2)	1.1(1)
Aspartic acid	3.2(3)	1.1(1)	2.3(2)	0.9(1)	1.3(1)	1.0(1)	1.0(1)	1.3(1)	1.2(1)	3.3(3)	2.2(2)
Threonine	2.0(2)	1.0(1)	1.9(2)	0.9(1)	1.1(1)	0.9(1)	1.1(1)	1.1(1)		2.1(2)	2.8(3)
Serine	4.9(5)	2.0(2)	5.0(5)	3.1(3)	2.7(3)	0.2		2.3(2)	1.0(1)	2.1(2)	2.1(2)
Glutamic acid	1.1(1)			0.2	0.4	1.0(1)	0.2	1.1(1)	0.9(1)	5.2(5)	1.7(2)
Proline	1.0(1)	1.0(1)	1.0(1)							1.1(1)	
Glycine	1.0(1)	0.1	1.0(1)	1.0(1)	0.6	1.0(1)		0.2		1.1(1)	1.2(1)
Alanine				0.3				0.7(1)	1.1(1)	1.2(1)	
Valine				0.1				1.5(2)	1.8(2)	2.9(3)	
Methionine[b]	0.8(1)			0.1		1.1(1)				1.1(1)	0.9(1)
Glucosamine					2.5	2.3	2.6				
Isoleucine							1.1(1)			0.5(1)	
Leucine										1.0(1)	
Tyrosine	0.9(1)	0.9(1)	0.8(1)		0.8(1)						
Phenylalanine										1.1(1)	
Lysine							1.2(1)			1.6(2)	
Histidine				0.1						0.8(1)	1.0(1)
Arginine						0.2					
Tryptophan[c]						0.8(1)				0.5(1)	
Position in sequence	14–29	20–25	14–25	14–19	20–25	34–39	62–66	121–128	121–126	34–61	67–79
Sephadex pool (fig.1 or 3)	'B'	('B')	'D'	(A)	(A)	A		'C'	'D'	A	(B)
Amount (nmol)	140	57	11	22	10	150	200	27	40	100	22

Amino acid analysis of peptides were performed on samples hydrolyzed in 0.25 ml of 6 M HCl at about 110°C in evacuated sealed tubes. Values were not corrected for hydrolysis losses. The composition of each peptide as derived from this and previous studies [1,2,5] is shown within parentheses. Peptides were isolated from digests by gel filtration and HPLC. Sephadex pools are indicated. 'B', etc., pool corresponding with pool B from fig.1; (A), etc., peptide formed by secondary digestion and isolated by HPLC. The peptide nomenclature is explained in section 2

[a]Cysteic acid (Ox-peptides) or carboxymethylcysteine (R- and D-peptides)
[b]Homoserine + homoserine lactone (Ox-peptides) or methionine (R- and D-peptides)
[c]Tryptophan determined after hydrolysis with 3 M mercaptoethanesulfonic acid



placement of proline by serine. Apparently, the reason that only part of the horse ribonuclease molecules are glycosylated in this stretch of the sequence is not the presence of a less active glycosylating system in horse pancreas. Instead, it has a structural basis in the amino acid sequence and is in agreement with results obtained from in vitro glycosylation studies on synthetic peptides [4].

←

Fig.1. Gel filtration of the tryptic and chymotryptic digest of reduced and carboxymethylated horse ribonuclease on a column (0.5 × 100 cm) of Sephadex G-25 (superfine). Elution was carried out with 0.2 M acetic acid at 2 ml/h; 0.3-ml fractions were collected. Fractions were pooled as indicated by bars. (——) A_{220}; (– – –) A_{280}.

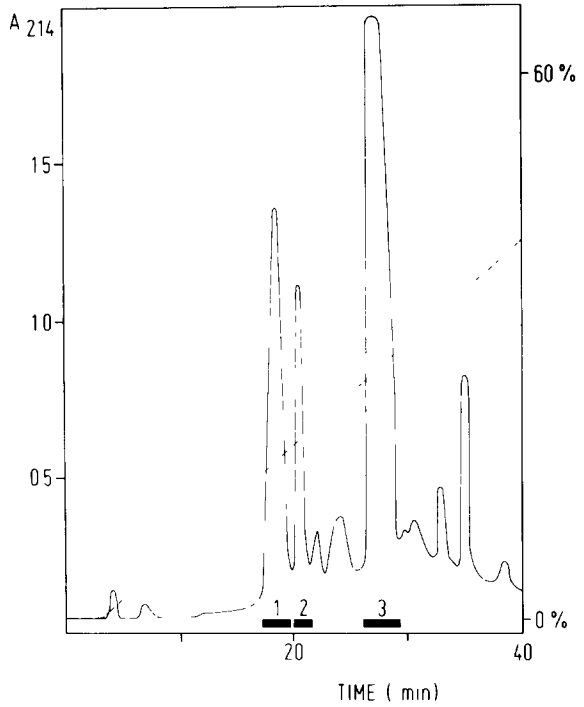


Fig.2. Reversed-phase high-performance liquid chromatography of pool A of fig.1 performed as described in section 2. (——) A_{214}, (– – –) acetonitrile gradient. The eluate was pooled as indicated by bars.

When a general rule of nature has been discovered, there are sometimes conflicting observations from earlier work. Such exceptions may be real or they may be caused by experimental errors, and it is generally useful to reinvestigate such cases. Our earlier finding that horse ribonuclease contains a glycosylated Asn-Pro-Thr sequence is an example. Another instance is the sequence Asn-Ser-Gly with attached carbohydrate in phosvitin, which had been found by Shainkin and Perlmann [14] before the Asn-X-Ser/Thr sequon rule was formulated by Marshall [15]. The recently published amino acid sequence of phosvitin derived from its DNA sequence presents the sequence Asn-Gly-Ser [16], which is in agreement with the sequon rule of carbohydrate attachment.

Although pig and horse ribonuclease are both glycosylated in the so-called S-peptide loop, we now know that the exact positions are not identical: in pig ribonuclease Asn-21 is glycosylated, while in horse ribonuclease it is Asn-22.

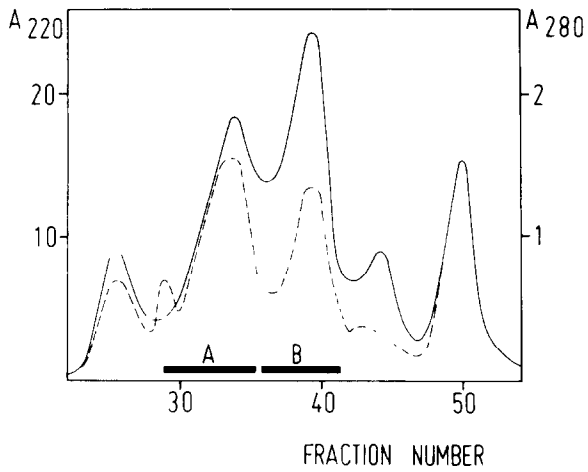


Fig.3. Gel filtration of the tryptic digest of reduced and carboxymethylated dromedary ribonuclease on a column of Sephadex G-25. See legend to fig.1 for other details.

3.1.3. Position 39

A glycopeptide with Asn-34 could be isolated by gel filtration of the CNBr digest before performic acid oxidation and further digestion. No corresponding carbohydrate-free peptide was found.

The glycopeptide with Asn-34 from the reduced and carboxymethylated digest eluted rather late from the HPLC column and had a high UV absorbancy (peak 3 in fig.2). These properties and the amino acid analysis obtained after hydrolysis with HCl made the deletion of residue 39 proposed in [1] suspect. Amino acid analysis after hydrolysis with 3 M mercaptoethanesulfonic acid (table 1) and sequence determination with identification of the dansylated derivative after 5 Edman steps without hydrolysis confirmed that position 39 is occupied by tryptophan (fig.4).

3.1.4. Carbohydrate composition of glycopeptides

The carbohydrate compositions of the glycopeptides R1–R3 are presented in table 2. Rather similar values were obtained for the 3 peptides.

3.1.5. C-terminus

The first ribonuclease we investigated which has additional residues at its C-terminus was the enzyme from horse. The C-terminal sequence Gln-Thr (positions 125–126) was derived from analysis of peptides from the C-terminal region of the

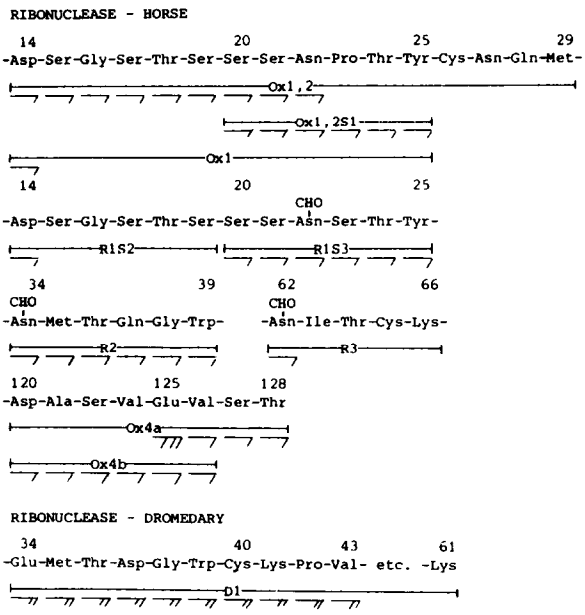


Fig.4. Amino acid sequence of the peptides used to correct the sequences of horse and dromedary ribonuclease. The peptide nomenclature is explained in section 2. (|—|) Peptides, (—7) identified as dansyl amino acid, (—77) identified as phenylthiohydantoin derivative during automatic Edman degradation, (—777) identified as dimethylaminoazobenzene thiohydantoin derivative. The C-terminal residues of peptides Ox4a and Ox4b were identified by analysis of both hydrolyzed and non-hydrolyzed samples of the dansylation reaction products.

molecule and from degradation of intact ribonuclease with carboxypeptidase A [1,2]. Later, several other ribonucleases with additional residues at the C-terminus were found. However, these ribonucleases all have 4 additional residues, in agreement with a second stop codon at position 129 in the non-coding region of the messenger RNA sequence of rat ribonuclease [17,18].

Here, 4 additional residues, with the sequence Glu-Val-Ser-Thr (125–128), were also found in the sequence of horse ribonuclease (fig.4, table 1).

However, the major peptide isolated now was Asp-Ala-Ser-Val-Glu-Val, besides Asp-Ala-Ser-Val-Glu-Val-Ser-Thr (table 1). Probably, carboxypeptidase activity had removed the two C-terminal residues in most of the ribonuclease molecules. A similar situation was encountered during our studies on human ribonucleases, in which we found the C-terminal sequence Glu-Asp-Ser

Table 2

Carbohydrate compositions of glycopeptides of horse ribonuclease

Monosaccharide	Peptide R1 (Asn-22)	Peptide R2 (Asn-34)	Peptide R3 (Asn-62)
Fucose	0.5	1.0	0.9
Mannose	2.4	3.7	3.9
Galactose	0.5	1.4	0.9
N-Acetylglucosamine	2.2	4.3	3.4
Sialic acid	0.1	0.8	0.4

The amount of monosaccharide is given in mol per mol peptide. The amount of *N*-acetylglucosamine has been corrected for incomplete cleavage of the *N*-glycosidic bond during methanolysis

(125–127) in the pancreatic enzyme [13] and Glu-Asp-Ser-Thr (125–128) in a ribonuclease from urine with otherwise identical sequence [19]. The removal of the two residues in horse ribonuclease and of one residue in human ribonuclease is in agreement with the specificity requirements of carboxypeptidase A [20].

3.2. *Dromedary ribonuclease*

The only other ribonucleases in which we found a deletion at position 39 are those from dromedary [5] and bactrian camel [21].

Amino acid analysis of dromedary ribonuclease after hydrolysis with 3 M mercaptoethanesulfonic acid showed the presence of 0.6 equivalents of tryptophan. A large tryptic peptide was isolated from the reduced and carboxymethylated protein (D1, table 1). This peptide was submitted to automatic Edman degradation and tryptophan was identified at the sixth position (fig.4). Another difference with previous studies was the observation of aspartic acid instead of asparagine at position 37.

Peptide D2C1 (residues 67–79) has also been isolated and analyzed (table 1). This peptide contains one serine less and one threonine more than the published sequence [5]. This result and reexamination of the earlier results [5,21] suggest that position 78 is occupied by threonine instead of serine.

Fig.5A summarizes the corrected amino acid sequence of horse ribonuclease (with the carbohy-

A. RIBONUCLEASE - HORSE

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          5        10        15        20        25        30
  1 K E S P A M K F E R Q H M D S G S T S S S N P T Y C N Q M M
 31 K R R N M T Q G W C K P V N T F V H E P L A D V Q A I C L Q
 61 K N I T C K N G Q S N C Y Q S S S S M H I T D C R L T S G S
 91 K Y P N C A Y Q T S Q K E R H I I V A C E G N P Y V P V H F
121 D A S V E V S T
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B. RIBONUCLEASE - DROMEDARY

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          5        10        15        20        25        30
  1 S E T A A E K F E R Q H M D S Y S S S S S N S N Y C N Q M M
 31 K R R E M T D G W C K P V N T F I H E S L E D V Q A V C S Q
 61 K S V T C K N G Q T N C H Q S S T T M H I T D C R E T G S S
 91 K Y P N C A Y K A S N L K K H I I I A C E G N P Y V P V H F
121 D A S V
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Fig.5. Corrected amino acid sequences. (A) Horse ribonuclease, (B) dromedary ribonuclease.

drate-free Asn-Pro-Thr sequence at positions 22–24) and fig.5B contains the corrected sequence of dromedary ribonuclease. Bactrian camel ribonuclease was no longer available. However, since the sequence of this enzyme had been found to be identical to that of a minor component of dromedary ribonuclease [21], we may assume that the corrections of the latter sequence also are valid for bactrian camel.

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