THE PREPARATION AND PRIMARY STRUCTURE OF S-PEPTIDES FROM DIFFERENT PANCREATIC RIBONUCLEASES

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1. Introduction

In 1955, Richards [1] described the isolation of 'an active intermediate produced during the digestion of ribonuclease by subtilisin'. The characterisation and separation of the non-covalently linked components was described 4 years later [2]. Ribonuclease S* possesses full enzymatic activity and the same holds for the enzyme reconstituted from S-peptide and S-protein. The involvement of S-peptide residues in the binding of S-peptide to S-protein and in the enzymatic activity of the reconstituted RNase S' has been studied by using synthetic S-peptide analogs [3,4] the cleavage by subtilisin takes place in an external loop. Klee [5] and Gold [6] did not succeed in cleaving the RNases from rat and snapping turtle with subtilisin. In this study, we present the successful cleavage with subtilisin Carlsberg of the RNase from goat, brindled gnu, giraffe, reindeer, dromedary, and red kangaroo and the isolation of the corresponding S-peptides. Differences in the observed behaviour are compared with predicted differences in conformation.

2. Materials and methods

Bovine pancreatic ribonuclease was obtained from Miles–Seravac Ltd. (Maidenhead). All other ribonucleases used in this study (goat, giraffe, gnu, reindeer, dromedary, kangaroo, lesser rorqual, pig, and horse) were isolated according to Wierenga et al. [7] and rat RNase, according to Beintema et al. [8]. Subtilopeptidase A (Subtilisin Carlsberg) was a gift from Novo Industri (Copenhagen). Sephadex G-50 (fine) was purchased from Pharmacia (Uppsala). All other reagents were analytical grade products from Merck AG (Darmstadt).

Amino acid analysis, high-voltage paper electrophoresis, dansylation, and dansyl–Edman degradation were performed as described earlier [7, 9].

2.1. *Preparation of S-peptides*

Four mg of ribonuclease in 200 μl 0.1 M Tris–HCl pH 8.0 was treated with 0.04 mg (0.16% solution in buffer) of subtilopeptidase A at 0°C. After 60–120 min of digestion, 250 μl of 0.5 N HCl was added. The mixture was applied to a Sephadex G-50 (fine) column (1 × 100 cm) and eluted with 0.05 N HCl. The absorbance of the effluent was measured at 280 and 220 nm. The S-peptide peak was lyophilised and further purified by preparative paper electrophoresis at pH 3.5.

3. Results and discussion

The S-peptides from reindeer, kangaroo and dromedary RNase could be obtained easily, using a digestion time of 90 min. The elution patterns were more or less identical (fig. 1A). Digestion times had to be 1 hr for goat and gnu RNases and 2 hr for giraffe

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* Abbrevations:
RNase S – subtilisin-modified ribonuclease; S-peptide – the 20-redidue N-terminal peptide obtained from RNase S; S-protein – the protein component obtained from RNase S; RNase S' – the reconstituted enzyme obtained by mixing equimolar amounts of S-peptide and S-protein.

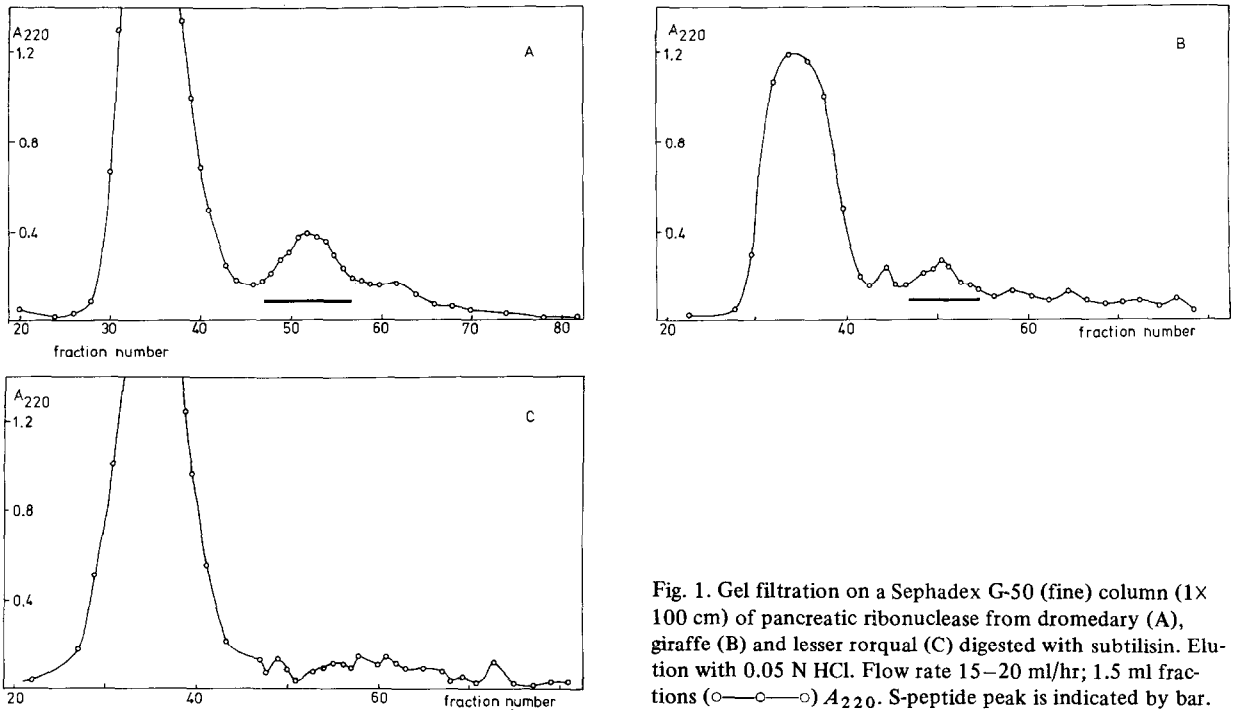

Fig. 1. Gel filtration on a Sephadex G-50 (fine) column (1× 100 cm) of pancreatic ribonuclease from dromedary (A), giraffe (B) and lesser rorqual (C) digested with subtilisin. Elution with 0.05 N HCl. Flow rate 15–20 ml/hr; 1.5 ml fractions (o—o—o) A_{220}. S-peptide peak is indicated by bar.

Table 1

Amino acid compositions of S-peptides obtained after cleavage of ribonucleases with subtilisin and subsequent gel filtration on Sephadex G-50 (fine). Nearest integers are given in brackets.

	Goat	Cow	Gnu	Giraffe	Reindeer	Dromedary	Kangaroo
Lys	2.0 (2)	2.1 (2)	1.8 (2)	2.1 (2)	2.0 (2)	1.1 (1)	0.8 (1)
His	0.9 (1)	1.0 (1)	0.9 (1)	1.1 (1)	0.9 (1)	1.0 (1)	1.2 (1)
Arg	0.8 (1)	1.0 (1)	1.2 (1)	0.8 (1)	1.1 (1)	0.8 (1)	0.7 (1)
Asp*	1.4 (1)	1.0 (1)	1.5 (1)	1.3 (1)	1.2 (1)	1.4 (1)	1.8 (2)
Thr	1.0 (1)	1.9 (2)	1.0 (1)	0.8 (1)		1.1 (1)	3.0 (3)
Ser	5.3 (5)	2.8 (3)	5.0 (5)	4.2 (4)	3.8 (4)	4.9 (5)	1.9 (2)
Glu	2.8 (3)	2.8 (3)	3.1 (3)	3.1 (3)	2.9 (3)	4.0 (4)	5.3 (5)
Pro					2.0 (2)		1.3 (1)
Gly**	0.6 (–)	0.3 (–)	0.3 (–)	0.7 (–)	0.4 (–)	0.4 (–)	
Ala	4.0 (4)	4.6 (5)	4.0 (4)	2.9 (3)	4.0 (4)	2.2 (2)	2.0 (2)
Val		0.2 (–)		0.3 (–)			
Met	0.9 (1)	0.6 (1)	0.7 (1)		1.0 (1)	1.0 (1)	1.3 (1)
Ile				1.0 (1)			
Leu				0.2 (–)			
Tyr				0.4 (–)		1.0 (1)	
Phe	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	0.8 (1)
Total residues	20	20	20	18	20	19	20

* Some values are too high because of baseline shift and, sometimes, traces of methionine sulfoxide.

** Glycine values were sometimes too high because of contamination from the paper electropherogram.

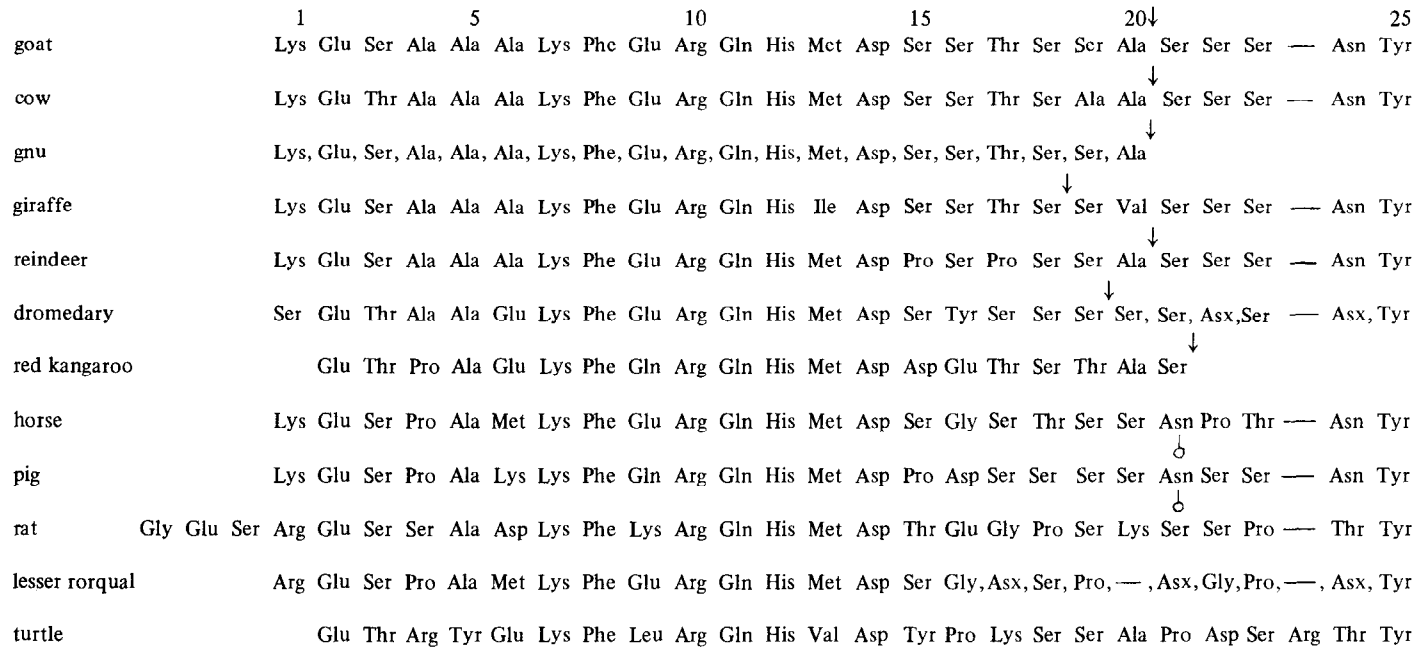

Fig. 2 Primary structures of the N-terminal part of ribonucleases from cow [10], horse [11], pig [12,7], rat [13], turtle [14], red kangaroo [14]. The rest of the sequences is determined in our laboratory and will be published elsewhere. In the ribonucleases that are susceptible to cleavage by subtilisin the cleavage points are indicated by arrows. O– = carbohydrate chain.

RNase. These elution patterns were comparable to that of giraffe RNase that is shown in fig. 1B. Lesser rorqual, rat, pig, and horse ribonuclease resisted a 120 min subtilisin treatment. The elution pattern obtained after subtilisin digestion of lesser rorqual RNase is shown in fig. 1C.

From the amino acid compositions of the S-peptides (table 1) and sequence information from primary structure studies on the whole ribonuclease molecules – which will be described elsewhere – we derived the primary structure of the isolated S-peptides (fig. 2). This figure also shows the points of cleavage by subtilisin in the RNases attacked. The presence of proline, valine, tyrosine, or glutamic acid in the S-peptide loop does not interfere with the susceptibility to cleavage by subtilisin. However, different sequences are attacked at different positions not easily rationalised from the sequence data. Thus, the change of an alanine in position 20 of goat RNase to a valine in giraffe RNase shifts the cleavage point two residues towards the N-terminus.

The sequences resistant to cleavage by subtilisin are also shown in fig. 2. The reasons for this resistance may be: i) the presence of amino acids incompatible with the substrate binding site of subtilisin; ii) prevention of binding to or hydrolysis by subtilisin due to a different conformation of this part of the RNase, or iii) the presence of carbohydrate attached to Asn 21 in the pig enzyme [15], but not in the horse enzyme [11]. In the latter enzyme, only partial glycosidation is observed.

Empirical methods to predict β-bends using a tetramer [16] or a nonamer [17] correlation have been applied to the sequences known on both sides of the potential cleavage region. The results of the nonamer model (fig. 3) for the rat and turtle enzymes are similar to those for the horse enzyme, whereas the profiles for the cow and reindeer enzymes resemble that of goat RNase. The dromedary profile differs from all others, having a higher-than-average bend probability for all but three of the residues in the region 17–25 (independent of the choice of Asx). The sequences split show a trough in the bend probability around the point of cleavage, whereas bends are predicted in the resistant RNases, with the exception of the pig enzyme. Here, the carbohydrate chain attached to

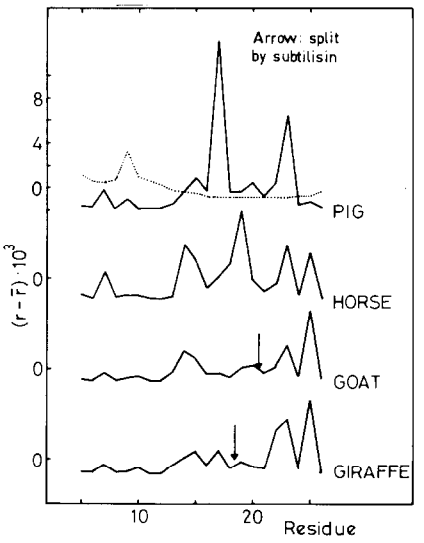

Fig. 3. Bend probability of RNase sequences (r), plotted as $(r-\bar{r})$, where $\bar{r} = (\Sigma r)/n$ [17] A bend is predicted whenever $r > r$. The dotted line represents the helix probability [17] of horse RNase (plotted as $(h-\bar{h})$). The corresponding profiles of the other enzymes looked similar.

Asn 21 may beimportant. The shift of the split in the giraffe enzyme seems to be correlated to the different conformational properties of valine, reflected in the increased bend probability of residues 22 and 23 (fig. 3). The tetramer prediction algorithm showed a similar result (not shown) with the sequences from fig. 2.

We suggest that, in the S-peptide loop, there may exist minor difference in the three-dimensional structure of the different RNases due to slightly different conformational preferences of different amino acids. Such changes may interfere with the attack by subtilisin.

Acknowledgements

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