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Difference in the Primary Structures of Ribonuclease F_1 Isoforms

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The carboxy terminal asparagine residue of ribonuclease F_1 has been shown to be replaced by an aspartic acid residue in the isoform, ribonuclease F_2. A new name ribonuclease F_1' has been proposed for this isoform. The previously reported primary structure of ribonuclease F_1 has been corrected at two positions.

RNase[1)] F_1 is a guanine specific RNase excreted by the fungus, ***Fusarium moniliforme***.[2)] Sequence analysis has revealed that it belongs to the RNase T_1 family,[3)] whose members are under active investigation from structural and functional points of view. In the course of purification of RNase F_1, a minor form named RNase F_2 was copurified.[4)] They were separable on ion exchange column chromatography or electrophoresis, RNase F_2 being more acidic than RNase F_1. In fact, they differed in isoelectric points: 4.10 for RNase F_1 and 3.96 for RNase F_2. Otherwise, practically no difference has been found between them with respect to enzymatic and physicochemical properties. This has led to the hypothesis that RNase F_2 has the same primary structure as RNase F_1 but one less amide group. However, the exact position of deamidation has remained to be determined.

In this study, we have determined the deamidation position along the following strategy. RNase F_1 or F_2 was reduced and carboxymethylated and the product, RCM-RNase F_1 or F_2, was digested with a protease. The resulting peptides were separated by anion exchange HPLC capable of separating charge isomers. The isolated peptdes were identified on the basis of their amino acid compositions[5)] and a pair of peptides, one from RNase F_1 and the other from RNase F_2, were selected which had the same amino acid composition but different retention times. These peptides were subfragmented and the above procedures were repeated until peptides short enough to pinpoint the deamidation position were obtained. Peptides were named according to the following principle. Those from RNases F_1 and F_2 were prefixed with F_1 and F_2, respectively. The following symbols were used to refer to the peptides obtained by digestion with the proteases shown: T (trypsin), Sp (***Staphylococcus aureus*** V8 protease), and Th (thermolysin). The symbol was followed by a number indicating the sequence alignment. Sometimes, more than one peptides with the same composition but different retention times were obtained from a single form of RNase, the fastest eluting (thus the least acidic) peptide being always the most abundant. In such cases, the least acidic peptide was assumed to be the

original present in the protein and named according to the principle. The minor peptide was given the same name but with a suffixed prime like T2', because it was probably produced from the original by deamidation during the separation.

RNases F_1 and F_2 were purified as described previously,[4] reduced and carboxymethylated by the known method.[6] RCM-RNase F_1 or F_2 was digested with trypsin as reported,[3] yielding peptides T1 (pyroGlu 1-Arg 17), T2 (Ala 18-Lys 62), T3 (Ser 63-Arg 76), and T4 (Val 77-Asn 106). They were separated by HPLC using a Toso HLC-803A chromatograph under the following conditions: column, Asahipak ES-502N 7.6 mm (i.d.) x 100 mm; eluent, 0.3 M (1 M = 1 mol dm^{-3}) or 0.5 M NH_4HCO_3; flow rate, 1.0 ml/min; detection, absorbance at 230 nm. As shown in Table 1, only the pair F_1-T4 versus F_2-T4 differed in their retention times and thus was analyzed further.

Table 1. Retention times of tryptic peptides on anion exchange HPLC with 0.3 M NH_4HCO_3 eluent

	Retention time/min	
Peptide	F_1-	F_2-
T1	2.8	2.8
T2	43 (8.6)[a]	43 (8.6)[a]
T2'	85 (10.9)[a]	85 (10.9)[a]
T3	3.2	3.2
T4	4.6	5.7
T4'	5.9	7.9

a) Values with 0.5 M NH_4HCO_3 eluent.

F_1- or F_2-T4 (40 nmol) was incubated with 0.4 nmol of V8 protease in 1.0 ml of 0.05 M NH_4HCO_3 at 37 °C for 19 h. The resulting peptides were separated by the HPLC described above but 0.2 M NH_4HCO_3 as an eluent. As shown in Fig. 1, F_1- and F_2-T4-Sp2 eluted with different retention times while F_1- and F_2-T4-Sp1 behaved in the same way. F_1- and F_2-T4-Sp2 were sequenced using a gas phase sequencer (Applied Biosystems, model 470A). The phenylthiohydantoin amino acids were identified by C_{18} reversed phase HPLC. The results are shown in Fig. 2. Though F_1-T4-Sp2 was sequenced completely, F_2-T4-Sp2 could be sequenced only until 17th cycle with certainty. However, since Asn 97 and Asn 98 were found in both peptides, the only possibility left was that the C-terminal Asn residue of F_1-T4-Sp2 was replaced by an Asp residue in F_2-T4-Sp2. An unexpected important problem arose at this stage: the sequence of F_1-T4-Sp2 did not accord with the previously reported primary structure of RNase F_1, in which the C-terminal sequence was -Asn-Thr-Gly instead of -Gly-Thr-Asn.

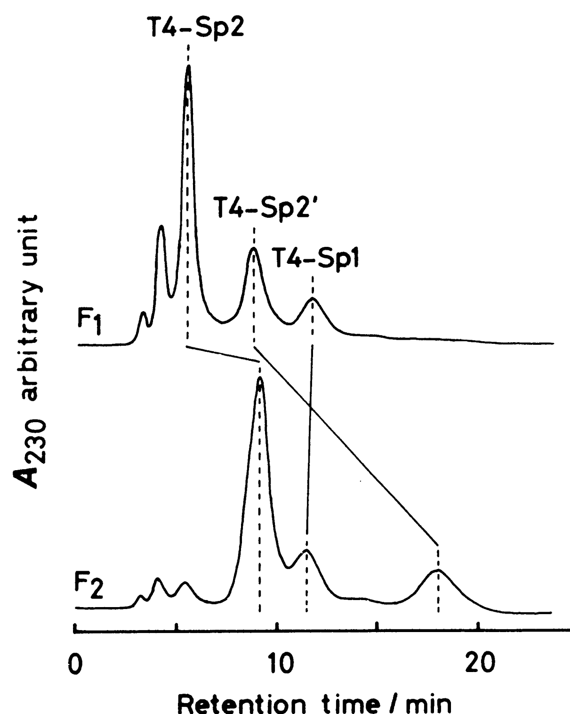

Fig. 1. HPLC separation of V8 protease peptides from F_1- and F_2-T4.

Table 2. Amino acid compositions of thermolysin peptides from F_1- and F_2-T4-Sp2. The results are expressed in mol of residue/mol of peptide with the expected values from the sequence in parentheses.

	F_1-		F_2-	
	T4-Sp2-Th1	T4-Sp2-Th2	T4-Sp2-Th1	T4-Sp2-Th2
Cmc		+ [a] (1)		+ [a] (1)
Asp	2.00 (2)	1.10 (1)	2.58 (2)	1.00 (1)
Thr	1.81 (2)	0.83 (1)	1.96 (2)	0.94 (1)
Ser	1.23 (1)	0.89 (1)	1.45 (1)	1.20 (1)
Gly	2.65 (3)	2.00 (2)	3.00 (3)	2.25 (2)
Ala	3.41 (3)		2.81 (3)	
Val		0.72 (1)		0.92 (1)
Ile	0.98 (1)		1.09 (1)	
Phe		0.73 (1)		0.89 (1)
His	0.87 (1)		1.13 (1)	
Total	13	8	13	8
Yield/nmol	2.0	2.2	1.6	2.6
Retention time/min	6.3	9.4	6.3	9.9

a) Detected as cysteic acid.

In order to confirm the deamidation position and to verify the C-terminal sequence, further subfragmentation was performed. F_1- or F_2-T4-Sp2 (5 nmol) was incubated with 25 pmol of thermolysin in 55 µl of 0.1 M Tris-HCl (pH 7.8) containing 10 mM $CaCl_2$ at 37 °C for 3 h. The resulting peptides were separated by C_{18} reversed phase HPLC using a Hitachi 655 system under the following conditions: column, Hitachi #3063, 4 mm (i.d.) x 150 mm; eluent, a linear 0-60% gradient of CH_3CN in 0.1% CF_3COOH; flow rate, 1.0 ml/min; detection, absorbance at 210 nm. The gradient was started 2 min after the injection at a rate of 1% increase/min. The amino acid compositions of the obtained peptides are summarized in Table 2. Unexpectedly, the N-terminal Tyr residue was lacking in F_1- and F_2-T4-Sp2-Th1 as a result of rather unusual cleavage at the N-terminus. F_1- and F_2-T4-Sp2-Th2 were completely sequenced. The results shown in Fig. 2 confirmed that the C-terminal Asn residue in the F_1-peptide is replaced by an Asp residue in the F_2-peptide and that the C-terminal sequence of RNase F_1 is indeed -Gly-Thr-Asn.

Two conclusions can be drawn from the above results. (1) The difference in the primary structures of RNases F_1 and F_2 resides at the C-terminal residue: Asn in RNase F_1 and Asp in RNase F_2. (2) The previously reported primary structure of RNase F_1 has to be corrected at position 104 from Asn to Gly and at position 106 from Gly to Asn. The main reason for the previous error was the difficulty of the determination of the C-terminal residue with carboxypeptidase A. Since peptide T4 was very resistant to the enzyme, it was digested with a large amount of the enzyme for a long period. These unusual conditions might have caused the erroneous results, which escaped correction by the Edman degradation. Multiple forms have also been reported with RNase U_2.[7] A peptide bond rearrangement between Asn 32 and Gly 33 of RNase U_2-A gives RNase U_2-B which has an isopeptide bond through the

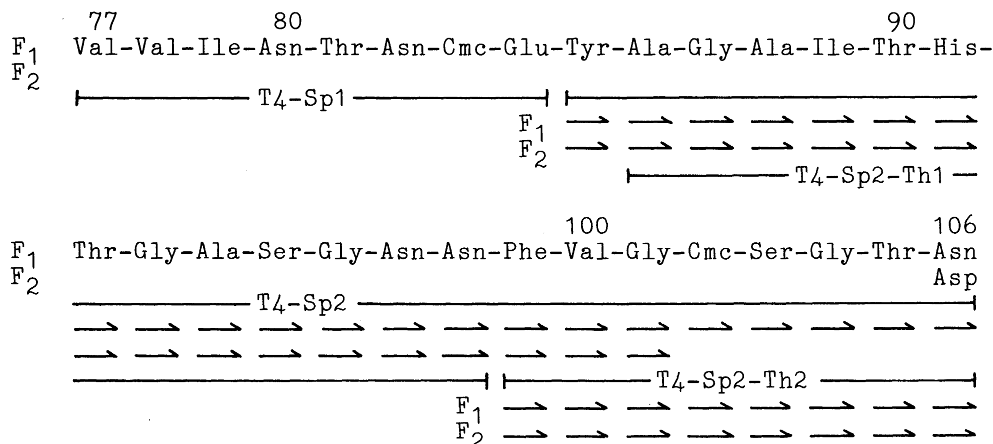

Fig. 2. Primary structures of F_1-T4 and F_2-T4. Only the different residue is shown for F_2-T4. Residue number in the original protein is indicated. The residues identified by Edman degradation are shown by small arrows.

β-carboxyl group.[8] The rearrangement reaction has been supposed to take place spontaneously at alkaline pH. Possibly, in the case of RNase F_1 too, spontaneous deamidation is the cause for occurrence of the isoform rather than presence of different genes. The amide group of Asn residues in proteins is known to be unstable, giving rise to multiple forms through spontaneous deamidation. For example, such a phenomenon has been reported with cytochrome c.[9] As a consequence of this study, we think that the nomenclature of RNase F_2 should be revised, because different suffix numbers had better to be used for different gene products such as RNases T_1 and T_2 or RNases U_1 and U_2. We, therefore, propose a new name RNase F_1' instead of RNase F_2 used hitherto.

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References

1) Abbreviations used are: RNase, ribonuclease; RCM, reduced and carboxymethylated; HPLC, high performance liquid chromatography. The three-letter symbols are used for the amino acids. Cmc stands for carboxymethyl cysteine.
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