Ribonuclease A Digestion by Proteinase K

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The digestion of ribonuclease A by proteinase K yielded one major degradation product only, which could not be distinguished from ribonuclease S by electrophoretical and immunological methods. This component (ribonuclease K) possessing full catalytic activity was characterized to be (1–20/21–124) ribonuclease A. Combined action of proteinase K and trypsin on ribonuclease A leads to a significant increase of the inactivation rate which may be useful in the isolation of mRNA from polysomes.

Introduction

Proteinase K (M_r 18000) is isolated from the culture medium of the fungus *Tritrirachium album* (Limber) and is extremely stable against self inactivation [1]. Cleavage of performic acid oxidized insulin B-chain by proteinase K showed that its cleavage specifity is releated to that of the subtilisins [2]. Because of its rapid "ribonuclease inactivating capacity" proteinase K is frequently used in ribonucleic acid isolation [3]. However, despite of its common use no detailed investigations about the action of proteinase K on ribonuclease A have been presented.

Experimental

The hydrolysis of ribonuclease A by proteinase K at pH 8 and 25 °C was followed by the pH-stat-method. In analytical experiments 9 mg ribonuclease A (Sigma) were dissolved in 5 ml CO_2-free water and the pH was adjusted to pH 8 with 0.1 N NaOH. The reaction was started by the addition of 0.12 mg proteinase K (Merck). At different times 20 μl aliquots were withdrawn from the digestion mixture and assayed for residual ribonuclease activity and trypsin sensitivity [4]. To prepare ribonuclease K, a sample of 250 mg RNase was digested with proteinase K (5 mg) at 2 °C. After hydrolysis of 1,3 peptide bonds the action of the endopeptidase was stopped by the addition of PMSF (1000-fold molar excess over proteinase K). Alkali uptake at pH 8 was converted to bonds broken on the assumption that 0.91 mol of base is taken up per mole peptide bonds split [5].

Ribonuclease A activity was measured at 284 nm and 25 °C using cytidine-2′, 3′-cyclic phosphate as substrate as described by Crook *et al.* [6]. Isolation of the major digestion component was carried out essentially in the same manner as described for RNase S [7, 8]. The amino acid compositions are derived from triplicate analysis of 0.2 mg protein/peptide hydrolysed in 6 N HCl for 24 and 96 h. Methionine and cysteine were determined after performic acid oxidation [9].

COOH-terminal amino acid residues were evaluated by digestion of 100 nmol protein/peptide with DFP-treated carboxypeptidase A at pH 8. At different times 100 μl samples were withdrawn and the reaction stopped by boiling. The solution was evaporated to dryness and the released amino acids were analysed on the amino acid analyser. Blanks were performed on solutions identical with digestion mixtures but carboxypeptidase or the substrate (vice versa) was omitted. Norleucine was used throughout as internal standard.

Results and Discussion

Firstly ribonuclease A digestion by proteinase K has been studied at pH 8.0 and various incubation conditions. The inactivation as function of time and base consumption (Fig. 1) indicates that cytidine 2′(3′) monophosphoric acid, a known inhibitor, slightly prevents ribonuclease inactivation. However, the disappearance of native RNase A, taken as the amount of trypsin resistant activity is not effected. According to Fig. 1 B the disappearance of the total ribonuclease activity is correlated to the bonds split per molecule, regardless whether inhi-

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Abbreviations: PMSF, phenylmethanesulfonylfluoride. Proteolytic degradation products of ribonuclease A were designated according to the proposed nomenclature (7).

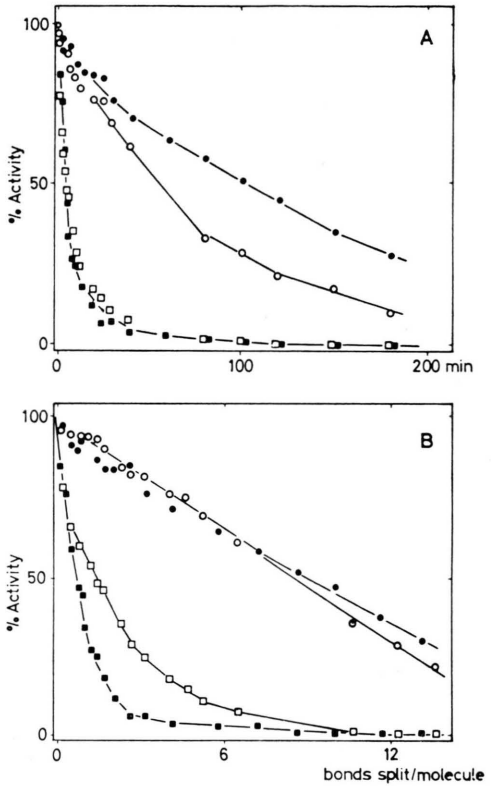


Fig. 1. Residual ribonuclease activity as function of incubation time (A) and of the bonds cleaved per molecule (B). Digestion was performed at 25 °C and pH 8.0 with the aid of a pH-stat (cf. methods) using 1.8 mg/ml RNase A and 0.027 mg/ml proteinase K. Activity without further treatment —○—○—; activity after incubation with trypsin —□—□—; without inhibitor — open symbols; digestion in the presence of a 110-fold molar excess of cytidine 2′ (3′) monophosphoric acid — closed symbols.

bitor is present or absent. However, the degradation of native ribonuclease A increases in the presence of inhibitor indicating that the initial cleavage of RNase (under formation of an active trypsin sensitive cleavage product) becomes more specific. In other words, further degradation and inactivation of the formed trypsin sensitive moiety is reduced and the formation of this product (these products) is only hardly effected if inhibitor is present.

The yield of trypsin sensitive activity increases in the presence of inhibitor if the temperature is lowered or ribonuclease concentration raised up from 1.8 mg/ml to 50 mg/ml or even higher. Addition of S-peptide during different digestion stages prior to activity measurement raises the activity indicating that at least a part of inactivation is due to the degradation of an S-peptide like product.

Different stages of digestion up to a base consumption corresponding to about 16 bonds cleaved per molecule obtained by incubation without and in the presence of inhibitor were analysed by chromatographic, electrophoretic and immunological methods using commercially available RNase A, RN-ase S, S-protein and S-peptide (Sigma) as references. In all cases no significant amount of any active degradation product differing from RNase S could be detected.

On foregoing ribonuclease digestion the amount of small inactive peptides increases, as already shown in detail for the digestion of ribonuclease A with subtilisin [5].

Thus the digestion of ribonuclease A by proteinase K proceeds in a way very similar or even identical with the subtilisin or elastase [4]. To prove identity we isolated RNase K following the recommended procedures used for RNase S purification which were also successfully applied for RNase E isolation of ribonuclease digested with elastase [4, 7, 8]. A 250 mg sample of RNase A was digested at 2 °C with proteinase K and the whole digestion mixture was applied on an ion exchange column (Amberlite CG 50 II). The trypsin sensitive peak (pool II) was lyophilized and desalted on Sephadex G 25. 80% of the initial activity was recovered by this procedure and the product — RNase K — was used for final characterization (Table I). Chromatography of RNase K on Sephadex G 75 using 30% acetic acid as eluant resulted in the separation of K-protein and K-peptide (recovery: 75% K-protein and 12% K-peptide of the applied RNase K weight bases).

The separated components K-peptide and K-protein were each completely inactive. However, full catalytic activity was restored if both components were combined in more than equimolar concentrations prior to activity measurement. The amino acid compositions of both fractions are in good agreement with those of ribonuclease S-protein and S-peptide (Table II) [8, 10]. Values in brackets refer to the minor component of RNase S, which is believed to be a mixture of 1 – 20/21 – 124 RNase and 1 – 21/22 – 124 RNase. The amount of the minor component 1 – 21/22 – 124 RNase differs if subtilisins of different strains of Bacillus subtilis are used for digestion. Nevertheless the behaviour

Table I. Recovery of RNase K of a preparative scale digest of RNase A (250 mg).

	% activity RNase A [a]	RNase K [b]
initial activity	100	0
after proteinase K digest-ion (1,3 bonds cleaved)	3	82
pool I of ion exchanger chromatography (RNase A)	3	0
pool II two of ion exchanger chromatography (RNase K)	0	93
RNase K (desalted and twice lyophilised)	0	80

[a] Determined as the trypsin resistant part of the activity.
[b] *i. e.* the trypsin sensitive activity.

of both components is identical. A similar heterogeneity has been described for RNase E [4]. The authors conclude that elastase acts on two bonds, between residues 19/20 and 20/21 resulting in the liberation of one alanine yielding 1 – 19/21 – 124 RNase E.

In the case of K-peptide, carboxypeptidase A digestion proved that it is solely composed of residues 1 – 20 of RNase A (Table III). The amount of liberated amino acid residues (2 Ala, 2 Ser, 1 Thr) is in good agreement with the carboxyl-terminal sequence of S-peptide. The uncertainty due to the somewhat small amount of alanine in the isolated K-peptide (Table II) could thus be clarified. Probably no cleavages between bond 19 and 20 occurred during ribonuclease digestion with proteinase K. A final proof of homogeneity was given by chromatography of the isolated peptide component on DOWEX 50 WX 2. The isolated K-peptide eluted as a single homogeneous peak, while commercially available S-peptide contained the minor peptides 1 – 20/21 and minor impurities. The homogeneity of K-protein was ascertained by performic acid oxidation. No low molecular weight peptides were liberated, as could be expected if cleavage between disulfide bridges occurred. By carboxypeptidase A digestion of the K-protein only the carboxyl-terminal amino acid residues of ribonuclease A were liberated.

Table II. Amino acid composition of K-protein and K-peptide compared with S-protein and S-peptide.

	S-protein	K-protein	S-peptide	K-peptide
Aspartic acid	14	14.2	1	1.1
Threonine	8	8.4	2	2.0
Serine	12 (11)	11.9	3 (4)	2.9
Glutamic acid	9	9.3	3	3.0
Proline	4	4.0		—
Glycine	3	3.1		—
Alanine	7	7.2	5	4.6
Half-cystine	8	7.8		
Valine	9	8.8		—
Methionine	3	3.0	1	1.0
Isoleucine	3	1.9 §		—
Leucine	2	2.1		—
Tyrosine	6	6.1		—
Phenylalanine	2	2.1	1	1.1
Histidine	3	2.9	1	1.0
Lysine	8	8.3	2	2.1
Arginine	3	3.2	1	1.0

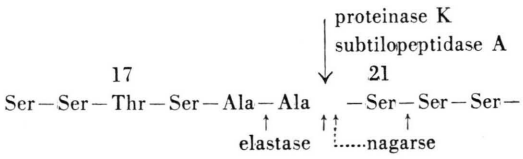
§ 24 Stunden Hydrolyse

Table III. Carboxypeptidase A digestion of K-peptide *.

Time of incubation [min]	mol amino acid liberated/mol K-peptide			
	5	30	90	300
Alanine	1.58	2.04	1.99	2.00
Serine	0.19	1.61	1.85	2.03
Threonine	0.07	0.84	1.10	1.05

* K-peptide: carboxypeptidase A 80:1 (w/w)

The cleavage pattern of the different protease acting about residue 20 of ribonuclease A can be summarized:



In view of the use of proteinase K for the isolation of ribonucleic acids [3] it was found that combined action of proteinase K and trypsin leads to a significant increase of the inactivation rate of ribonuclease A (Fig. 1).

We conclude that proteinase K and the subtilisins act on native protein substrates in the same way. It seems justified in all cases where the stability of subtilisin offers problems during degradation of native proteins to substitute it by the extremely stable proteinase K.

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