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Primary structure and catalytic properties of extracellular ribonuclease of bacillus circulans

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A complete amino acid sequence of extracellular *Bacillus circulans* RNase was established and compared with a structure of *B. amyloliquefaciens* RNase. Gln^{15}, Gly^{65} and Gln^{104} in *B. amyloliquefaciens* RNase were found to be replaced by Leu, Ala and Lys, respectively, in *B. circulans* RNase. Catalytic properties of *B. circulans* RNase were studied.

RNase; *Bacillus circulans*; Sequence; Catalytic property

1. INTRODUCTION

Extracellular microbial RNases form homologous protein family which consists of at least three subgroups, formed by the *Bacillus*, *Streptomyces* and fungal enzymes [1]. The most studied extracellular *Bacillus* RNases are RNase of *B. amyloliquefaciens* (RNase Ba), RNase of *B. intermedius* (RNase Bi) and RNase of *B. thuringiensis* (RNase Bth). The amino acid sequences of these RNases have been established [2–4] and three-dimensional structures of RNases Ba and Bi have been determined [5]. Amino acid sequences of RNases Bi and Bth are identical except for the substitution of Ala^{106} in RNase Bth for Thr^{106} in RNase Bi. The structural similarity of RNases Bi and Ba is about 83%.

Recently, we have isolated a strain producing high levels of extracellular RNase, which was identified according to its morphological and biochemical properties as *B. circulans*, and the procedure of purification of the protein (RNase Bci) has been developed [6]. In this paper, we report the results of determination of the complete primary structure of RNase Bci and some catalytic properties of the enzyme.

2. MATERIALS AND METHODS

Extracellular RNase Bci was isolated from the filtrate of cultural medium of *B. circulans*, strain BCF 247 [6]. RNase Ba was a product of gene-engineering technology [7]. *Staphylococcus aureus* protease V8, polynucleotides, GpC and cyanogen bromide were purchased from Sigma, and the sequence grade chemicals were from Applied Biosystems.

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Abbreviations: RNase Bci, Ba, Bi and Bth, ribonucleases of *Bacillus circulans*, *B. amyloliquefaciens*, *B. intermedius* and *B. thuringiensis* (EC 3.1.27.1), respectively.

Proteolysis of the RNases with *S. aureus* protease was performed in 10 mM potassium-phosphate buffer, pH 7.5, at 37°C for 7 h and an enzyme-to-substrate ratio of 1:50. Subdigestion with cyanogen bromide was made in 1:1 mixture of heptafluorobutyric and 88% formic acid at 25°C in the dark for 24 h and at a 1:1,000 molar peptide/reagent ratio.

Automatic Edman degradation were carried out on a Knauer Model 816 Protein Sequencer, equipped with an on-line Model 120 PTH Applied Biosystem Analyzer.

Molecular mass analyses of RNase Bci and peptides were performed on the time-of-flight spectrometers using methods of electrospray mass spectrometry [8] and plasma-desorption mass spectrometry with a Californium-252 source [9].

3. RESULTS AND DISCUSSION

Determination of the amino acid sequence of RNase Bci was based on the assumption of the high level of similarity between the extracellular *Bacillus* RNases. Only one digestion of the RNase Bci with Glu-specific *S. aureus* protease was used to elucidate the protein primary structure.

According to electrospray mass spectrometry data the relative molecular mass of RNase Bci (M_r 12,384 ± 5) closes to RNase Ba mass (M_r 12,383) calculated from its amino acid sequence [2]. RNases Bci and Ba have the same amino acid sequence for the N-terminal 25 amino acid-long regions with the exception of one substitution of Gln^{15} in RNase Ba for Leu^{15} in RNase Bci [6].

Individual peptides of RNase Bci digestion with *S. aureus* protease (SP-peptides) were isolated using the reversed-phase HPLC. Chromatographic elution patterns of RNases Bci and Ba hydrolysates coincide qualitatively (Fig. 1), and only the positions on chromatograms of the peptides SP-4 differ radically. There are four major components in the fractionating peptide mixtures and this number is in agreement with the spec-

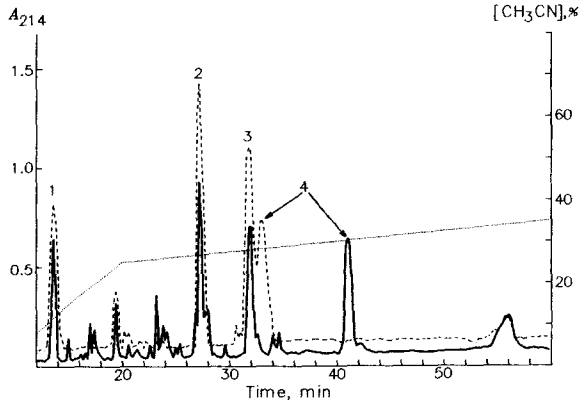

Fig. 1. Reversed-phase HPLC patterns of the RNases Bci (solid line) and Ba (dashed line) digests with *S. aureus* protease. Fractionation on an Aquapore R-300, C8 column (0.4 × 10 cm) with elution (0.7 ml/min) by acetonitrile gradient (dotted line) in 0.1% trifluoracetic acid. The numbers of peptides are shown above peaks.

ificity of the peptide bond hydrolysis by protease and the occurrence of the three glutamic acid residues in the proteins – Glu^{29}, Glu^{60} and Glu^{73} in RNase Ba [2].

Mass spectrometric analysis of the RNase Bci SP-peptides, and the results of their sequencing and comparison of the obtained data with those on the structure of the corresponding fragments of RNase Ba (Table I, Fig. 2) permit reconstruction of the RNase Bci polypeptide chain beyond the 10 C-terminal positions.

In order to cover the missing part of the RNase Bci structure required for deciphering of the RNase complete sequence, the additional information was obtained from the results of automatic Edman degradation of the non-fractionated cyanogen bromide cleavage products of the C-terminal SP-3 peptide (SP-CB-peptides) and their mass spectrometric investigation (Table I, Fig. 2). The specific hydrolysis of the peptide by cyanogen bromide at a carboxyl group of Trp^{94} produces two polypeptide chains. Results of Edman sequencing of this mixture -Ala/Leu-Asp/Ile-Ile/Tyr-Asn/Lys-Tyr/Thr-Thr/Thr-Ser/Asp-Gly/His-Phe/Tyr-Arg/Lys-Asn/Thr-Ser/Phe-Asp/Thr-Arg/Lys-Ile/Ile-Leu/Arg-, characterize the partial and full structures of the SP-3-CB-1/SP-3-CB-2 peptides, respectively. Analysis of the sequencing data, together with results of the previously determined structure of the N-terminal part of SP-3 peptide (residues 74–100), allowed to deduce the complete structure of the SP-3-CB-2 peptide (residues 95–110, Table II), and consequently, the C-terminal amino acid sequence of RNase Bci (Fig. 2).

Table I

Mass spectrometric analysis of the RNase Bci peptides

Peptide	Relative molecular mass		Protein chain position
	RNase Bci[a]	RNase Ba[b]	
SP-1	1,485 ± 2	1,472	61–73
SP-2	3,123 ± 9	3,129	30–60
SP-3	4,499 ± 14	4,491	74–110
SP-4	3,333 ± 10	3,345	1–29
SP-3-CB-1	2,657 ± 8[c]	2,481	74–94
SP-3-CB-2	2,187 ± 6[c]	2,028	95–110

[a] Data of plasma-desorption mass spectrometry with ^{252}Cf source.
[b] Calculated from the amino acid sequence [2].
[c] Tyr and Trp residues of the peptides were modified to 3-bromotyrosyl and *N*-acyl-dioxoindolylalanyllactone derivatives [10].

The derived polypeptide chain of RNase Bci contains 110 amino acid residues: 9 Asp, 6 Asn, 9 Thr, 9 Ser, 3 Glu, 2 Gln, 3 Pro, 9 Gly, 9 Ala, 4 Val, 8 Ile, 8 Leu, 7 Tyr, 4 Phe, 2 His, 6 Arg, 9 Lys and 3 Trp. According to the amino acid sequence, the molecular mass of RNase Bci is 12,382 Da. The differences in structures of RNases Bci and Ba were found in three positions in the amino acid sequence: Leu^{15} of RNase Bci is replaced by Gln^{15} in RNase Ba, and Ala^{65} and Lys^{104} are replaced correspondingly by Gly and Gln in RNase Ba. Comparison of the primary structures of RNases Bci and Ba shows that these substitutions are not located at functionally important sites in the enzymes but occupy peripheral parts of the tertiary protein structures [5].

Similar to RNase Ba, RNase Bci belongs to a cyclizing RNase family and hydrolyzes phosphodiester bonds in RNA, poly- and oligoribonucleotides resulting in 3′-nucleoside monophosphates via 2′,3′-cyclophosphate intermediates [6]. These RNases are guanyl-specific towards low molecular weight substrates and do

Fig. 2. Amino acid sequence of RNase Bci. The RNase Ba residues different from those of RNase Bci are shown above the sequence. Localization of peptides of RNase digesting with *S. aureus* protease (SP) and the parts of their structures established by automatic Edman seqencing (solid lines) are shown below the sequence. SP-CB-peptides are the products of SP-3 peptide subfragmentation with BrCN.

Table II

Kinetic parameters of the transesterification reactions (I = 0.2 M, pH 6.2, 25°C)

RNase	Substrate	k_{cat} (s^{-1})	$K_m \cdot 10^4$ (M)	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Bci	poly(I)	870	0.9	$1.0 \cdot 10^7$
	poly(A)	7.8	1.6	$4.9 \cdot 10^4$
	GpC	0.1	5.3	190
Ba	poly(I)	1,200	1.1	$1.1 \cdot 10^7$
	poly(A)	16	4.4	$3.6 \cdot 10^4$
	GpC	0.5	33	160

not cleave cyclophosphates of adenosine, uridine and cytidine. The RNases lose its guanyl specificity when homopolynucleotides are used as substrates and cleave not only poly(I), but also poly(A) and poly(U), although poly(I) is the best substrate (Table II). Thus, in this case the RNases are rather guanyl-preferential.

The similarity of extracellular *Bacillus* RNases strongly argues that the enzymes are derived from a common ancestor.

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