PRIMARY STRUCTURE OF RIBONUCLEASE FROM *BACILLUS INTERMEDIUS* 7P

G. A. APHANASENKO, S. M. DUDKIN, L. B. KAMINIR, I. B. LESHCHINSKAYA*, E. S. SEVERIN

Institute of Molecular Biology, USSR Academy of Sciences, Moscow, 117984 and **Kazan State University, Kazan, 420008, USSR*

Received 10 October 1978

1. Introduction

Studies on the relation between the structure and function of RNases have been carried out mainly with RNase A [1] and to a lesser extent with RNase T_1 [2]. Bacterial RNases have been significantly less studied in this respect. Up to now, the primary structure and some physico-chemical properties of only one bacterial RNase, namely the RNase of *B. amyloliquefaciens*, have been established [3–5].

The purpose of this study was to determine the primary structure of the extracellular RNase of *Bacillus intermedius*, strain 7P. The RNase catalyses the breakdown of RNA to oligonucleotides terminating in 3′-phosphate and is mainly specific towards the purine bases of the RNA molecule [6]. Nucleoside-2′·3′-phosphates and dinucleoside phosphates are completely resistant to the action of this enzyme. The large scale preparation of the homogeneous RNase and some characteristics of this enzyme were described in [7].

2. Materials and methods

The amino acid composition of the protein and peptides was determined using a Bio-Cal BC-201 amino acid analyser (GFR). The amino acid sequence of the N-terminal part of the protein was established on a Beckman 890C automatic sequenator. PTH derivatives of the amino acids were determined by thin-layer chromatography on polyamide plates [8] with subsequent scanning on a PMQ-II spectrophotometer (Opton, GFR) and processing of results according to [9], by gas-liquid chromatography [10], and by amino acid analysis after hydrolysis of PTH derivatives of amino acids with 57% HI [11].

The sequence of peptides was determined by the dansyl-Edman technique and using carboxypeptidases A, B and C and leucine aminopeptidase. The kinetics of the exopeptidase degradation of peptides were studied by means of the quantitative method of amino acid analysis based on their dansylation, followed by thin-layer chromatography on polyamide plates [12]. DNS-amino acids were scanned using an automatic system with a Saratov minicomputer [13].

Hydrolysis of the protein with the protease from the V8 strain of *Staphylococcus aureus* was performed in 0.1 M NH_4HCO_3 (pH 8.0) at 37°C for 15 h [14] at an enzyme–substrate ratio 1:30. Cleavage of the RNase tryptophanyl bonds was carried out with *N*-chlorosuccinimide according to [15].

3. Results and discussion

RNase of *B. intermedius*, strain 7P, has mol. wt ~12 300 and contains 109 amino acid residues [7].

The native protein was examined by automatic phenyl isothiocyanate degradation, and 40 out of the first 50 residues were unequivocally identified. The remaining residues were identified upon analysis of the peptides.

The mixture of peptides obtained after hydrolysis of RNase with *Staphylococcus aureus* protease was fractionated by gel filtration on a Sephadex G-50 (fine) column in 10% acetic acid, containing 8 M urea. The resulting fractions were further purified by ion-exchange chromatography on an SP-Sephadex

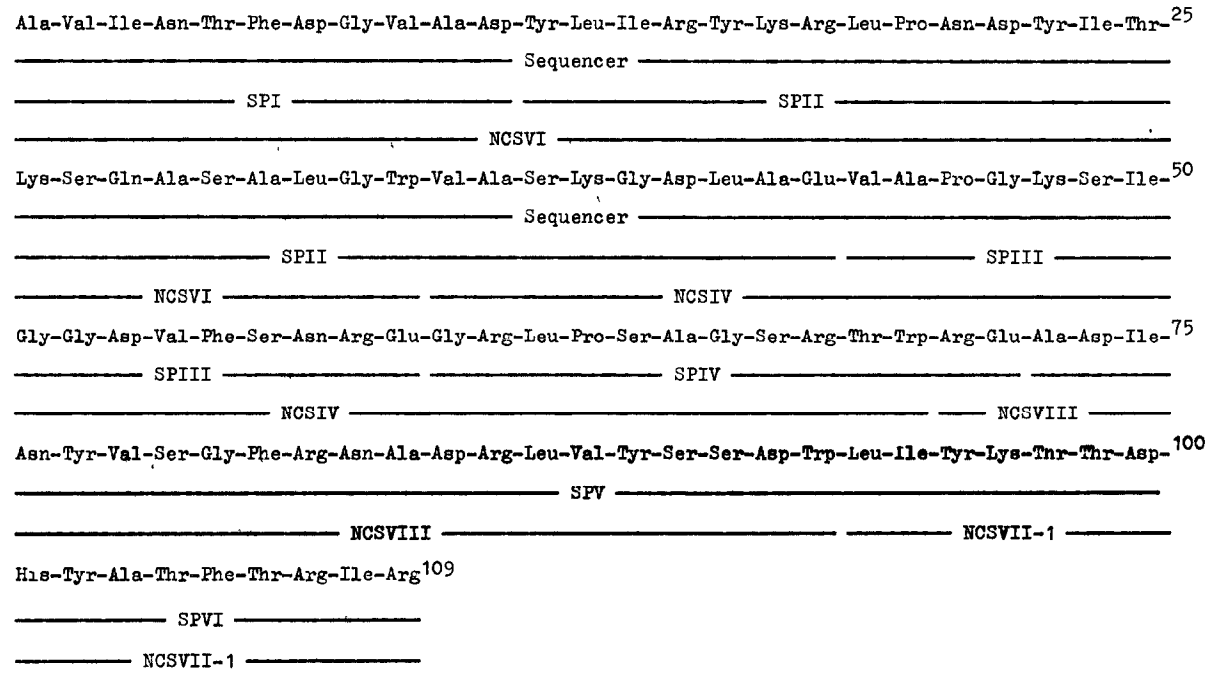

Fig.1 Primary structure of ribonuclease of *B. intermedius*, strain 7P SP and NCS are peptides obtained after cleavage of RNase with the *Staphylococcus aureus* protease and *N*-chlorosuccinimide, respectively

C-25 column, by paper chromatography and high-voltage electrophoresis.

The results obtained upon automatic degradation of the RNase permitted the localization of the peptides SPI and SPII in the protein structure (fig.1). The peptide SPVI is placed at the C-terminus since its C-terminal sequence (. —Arg—Ile—Arg) is identical with that of the intact polypeptide chain. After preliminary analysis of peptides SPIII, SPIV and SPV by Edman degradation technique, they were subjected to tryptic and/or chymotryptic hydrolysis. Sequence determination of these small peptides enabled us to establish the complete primary structure of peptides SPIII, SPIV and SPV.

To obtain overlapping peptides, the protein was cleaved at tryptophanyl residues with *N*-chlorosuccinimide. After treatment with this reagent, the reaction mixture was fractionated on Sephadex G-50 (superfine) (fig.2). Fractions NCSIII, NCSIV, NCSVI and NCSVIII contained pure peptides. Upon fractionation of the peptides of fraction NCSV by ion-exchange chromatography on CM-cellulose, the homogeneous peptides NCSV-1 and NCSV-2 were obtained. The peptide NCSV-1 was identical with NCSVI. Final purification of the peptide NCSVII-1 was achieved by gel filtration of the fraction NCSVII on Bio-Gel P-6.

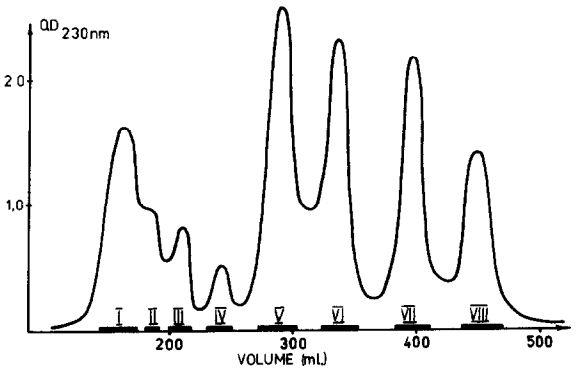

Fig 2 Fractionation of the products of RNase cleavage with *N*-chlorosuccinimide on the column (1 8 × 200 cm) of Sephadex G-50 (superfine), equilibrated and eluted with 0 02 N HCl at 5 ml/h Fractions were pooled as indicated

Peptide NCSIV contained two glutamic acids and overlapped peptides SPII, SPIII and SPIV. This overlapping was demonstrated upon sequence determination of the peptides derived from tryptic and chymotryptic digestions of NCSIV. The overlapping between peptides SPIV and SPV was shown by direct sequencing of NCSVIII. Sequence analysis of the peptide NCSVII-1 structure using Edman degradation and carboxypeptidase A and B digestion has proved that this peptide is C-terminal in the protein molecule and contains the C-terminal region of SPV and the whole peptide SPVI.

It should be mentioned that, besides the four peptides discussed, peptide fragments containing uncleaved tryptophanyl bonds were also isolated Thus, according to the results of amino acid analysis and the data on the N-terminal structure of the peptides NCSIII and NCSV-1, they are sums of the peptides NCSIV and NCSVIII, and the peptides NCSVIII and NCSVII-1, respectively. The data from SDS–polyacrylamide gel electrophoresis showed that the fractions NCSI and NCSII contained high-molecular-weight components with alanine and valine as N-terminal amino acids, and were likely to be the peptide fragments 1–70, 1–93, 35–109 and uncleaved protein.

Sequence comparison of the RNase of *B. intermedius*, strain 7P, with RNases of known primary structure from other microorganisms revealed two sequence homologies, namely RNases of *B. amyloliquefaciens* and *Streptomyces erythreus (St.)* [3–5,16]. Unfortunately, data on the structure of the active site of the former, which is most similar to the enzyme under study, are not available. It has been suggested [16] that the active site of the RNase *St.* contains two histidines, glutamic acid and arginine. Results of the comparative analysis of the primary structures of the enzyme under study and RNase *St.* are shown in fig.3. The coinciding amino acids are designated by black rectangles. One can see that these enzymes have three similar fragments localized in the C-terminal region· (52–63), (79–88) and (98–107), the two latter fragments contain arginine and histidine residues which have been implicated in the active site of the RNase *St* (Arg^{77} and His^{92}) [16], RNases T_1, U_1 and U_2 also contain arginine and histidine residues localized in their active sites, approximately in the same positions; in

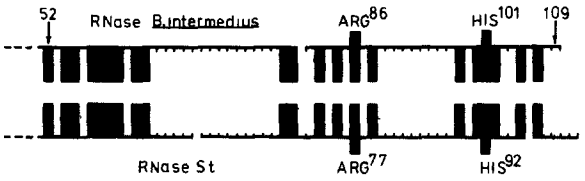

Fig 3 Comparative analysis of the primary structures of RNases of *B intermedius*, strain 7P, and *Streptomyces erythreus* (RNase *St*).

all cases, the distance between the aforementioned arginine and histidine residues being 16–17 amino acid residues [2,17,18].

Studies on the structure of the active site of RNase of *B. intermedius*, strain 7P, using the methods of chemical modification are in progress.

Acknowledgements

The authors would like to express their gratitude to I. A. Golubenko for the help in preparative isolation of the enzyme and V. A. Kulikov for sequence analysis of the protein on sequenator.

References

[1] Richards, F. M and Wyckoff, H W (1971) in The Enzymes 3rd edn (Boyer, P. D. ed) vol. 4, pp 647–806. Academic Press, London, New York
[2] Uchida, T and Egami, F (1971) in The Enzyme 3rd edn (Boyer, P. D ed) vol. 4, pp 205–223, Academic Press, London, New York
[3] Hartley, R W (1968) Biochemistry 7, 2401–2408
[4] Hartley, R W (1970) Biochem. Biophys. Res Commun. 40, 263–270
[5] Hartley, R W. and Barker, E A (1972) Nature New Biol. 235, 15–16
[6] Bulgakova, R Sh , Leshchinskaya, I B , Balaban, N P. and Egorova, G. S (1974) Biokhimiya 39, 299–302
[7] Golubenko, I A., Balaban, N. P , Leshchinskaya, I B , Volkova, T. I., Kleiner, G I , Chepurnova, N K , Aphanasenko, G. A and Dudkin, C M (1978) Biokhimiya in press
[8] Kulbe, K D (1974) Analyt Biochem 59, 564–573
[9] Treiber, L. R (1974) J. Chromatogr 100, 123–135
[10] Pisano, J J and Bronzert, T J (1969) J Biol Chem 244, 5597–5607

[11] Inglis, A., Nicholls, P. and Roxburgh, C. (1971) Aust J Biol. Sci. 24, 1247–1250.
[12] Aphanasenko, G. A., Nisanov, V Ya , Shlyapnikov, S. V., Kaminir, L. B. and Severin, E S. (1977) Biokhimiya 42, 2178–2185.
[13] Nisanov, V. Ya , Aphanasenko, G A and Kamınır, L B. (1978) Prib. Tech Exp 1, 43–45 (Rus.)
[14] Houmard, J. and Drapeau, G R. (1972) Proc Natl Acad. Sci USA 69, 3506–3509.
[15] Lischwe, M. A and Sung, M T (1977) J Biol Chem 252, 4976–4980
[16] Yoshida, N., Sasaki, A , Rashid, M A and Otsuka, H. (1976) FEBS Lett. 64, 122–125
[17] Hashimoto, J and Takahashi, K (1974) J. Biochem 76, 1359–1361.
[18] Sato, S. and Uchida, T. (1975) Biochem J. 145, 353–360