CRYSTALLIZATION AND PRELIMINARY X-RAY INVESTIGATION OF THE *SERRATIA MARCESCENS* NUCLEASE

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The *Serratia marcescens* endonuclease (EC 3.1.4.9) splits DNA and RNA with the formation of 5'-phosphorylated oligonucleotides. The endonuclease is the object of detailed investigations, being conducted in a number of scientific centers. At present, however, the results of various authors on the characteristics of the enzyme and the study of its properties are largely contradictory. On the one hand, preparations of *S. marcescens* nuclease differing in certain physico-chemical properties and structural parameters have been isolated from various strains of bacteria [1-5]. On the other hand (at least in the case of nucleases secreted by the strains W 225 and SM6), there is weighty evidence for complete identity of the structures of the proteins [6, 7]. The ambiguity of the conclusions of individual publications evidently may be associated with the absence of sufficient amounts of highly purified protein. Inevitable consequence of this is the need to search for strains of bacteria with increased expression of nuclease and, to develop effective methods of isolation of preparative amounts of the enzyme.

We attempted to purify the enzyme expressed by *S. marcescens* bacteria strain B10 M1 producing a considerable amount of nuclease activity compared with the original material of so far investigated bacterial strains (Bu 211, ATCC 9986 and 24) [8].

We report here the isolation of two isoforms of *S. marcescens* nuclease, nucleases $S.m_1$ and $S.m_2$, in electrophoretically and structurally homogeneous states, the crystallization of the nuclease $S.m_1$, the preparation of its heavy atom derivatives and the results of the preliminary X-ray diffraction analysis of the protein.

The crude preparations of *S. marcescens* nuclease were obtained by means of ligand-exchange chromatography on the iminodiacetate-agarose

in Cu^{2+}-form (Table 1), based upon an interaction of protein histidine residues with chelated transition metal ions, and then by ion-exchange chromatography of the nuclease on phosphocellulose [9]. After this two-steps purification procedure the enzyme showed a specific activity of $5x10^6$ U/mg. The nuclease was free from phosphatase, phosphomonoesterase and phosphodiesterase activities. SDS-gel electrophoresis indicated purity of the protein according to the molecular weight (figure 1, N2). At the same time there was some evidence for structural heterogeneity of the enzyme. Non-denaturating electrophoresis of the purified nuclease showed two separate protein bands containing active enzymes (figure 1, N3). Further electrofocusing of the protein yielded two active peaks with isoelectric points of 7.1 and 6.7. Analysis of the N-terminus of the protein sample indicated the coexistence of two different forms of N-terminal amino acid residues, Glu and Asp. Eventually, we tried to crystallize the *S. marcescens* nuclease after the above described purification procedure and obtained a lot of small crystals in the protein drop (the size less than 0.1 mm), which were not suitable for X-ray diffraction analysis (figure 2).

We succeeded in separating two electrophoretically different nucleases by ion-exchange chromatography on DEAE-Toyopearl 650S (figure 3). These isozymes, nucleases $S.m_1$ and $S.m_2$, showed the same isoelectric points and N-terminal amino acid residues as the initial enzyme components and the same specific activities as unfractionated nuclease.

The structure analysis of nucleases $S.m_1$ and $S.m_2$ showed a high degree of similarity both to each other and to *S. marcescens* nucleases from strains W 225 and SM6 [10], (figure 4). In contrast to the nuclease $S.m_1$, the nuclease $S.m_2$ contains a supplement tripeptide segment Asp-Thr-Leu in the N-terminal portion of the molecule. The difference found in the N-terminal portion of nuclease isoforms, containing a negatively charged dicarboxylic acid residue, can explain the corresponding differences in chromatographic behaviour of the isoforms and values of their isoelectric points.

Table 1. Purification scheme of *Serratia marcescens* nuclease.

Step	Volume (ml)	A_{280}	Spec. activ. (U/ml 10^{-4})	Purific. level	Yield (%)
Culture fluid	10000	9.50	2.7	1.0	100
Cu^{2+}-IDA-agarose	180	9.80	116.0	42.3	78
Phosphocellulose	85	0.98	185.0	66.3	59
DEADE-Toyopearl					
nuclease $S.m_1$	45	0.18	44.5	88.0	5
nuclease $S.m_2$	50	0.95	256.6	96.0	33

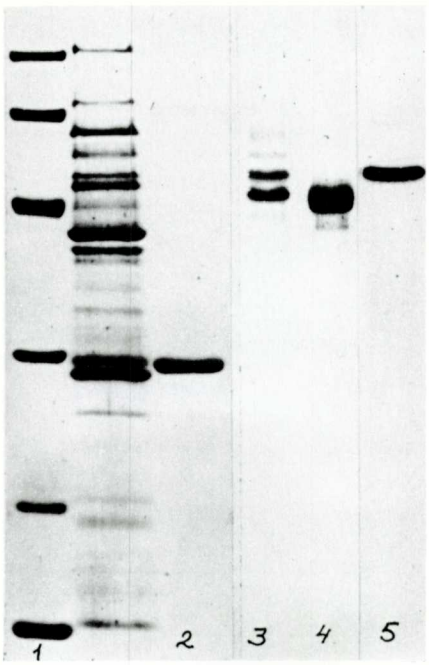

Fig. 1. PAG electrophoresis under denaturation conditions (1-2) and without denaturants (3-5): 1 – protein markers, 2, 3 – nuclease sample after phosphocellulose chromatography, 4 – nuclease $S.m_2$, 5 – nuclease $S.m_1$.

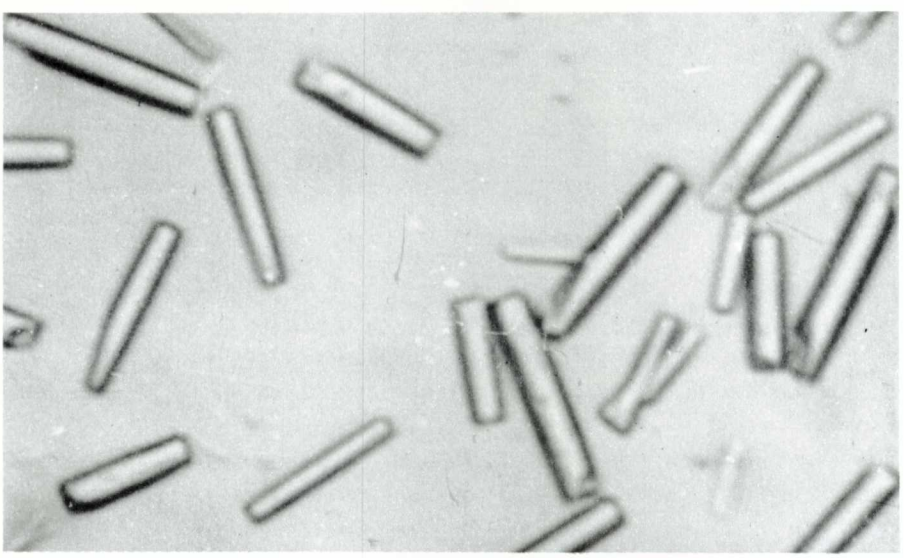

Fig. 2. Crystals of *S.m* nuclease after two-steps purification procedure.

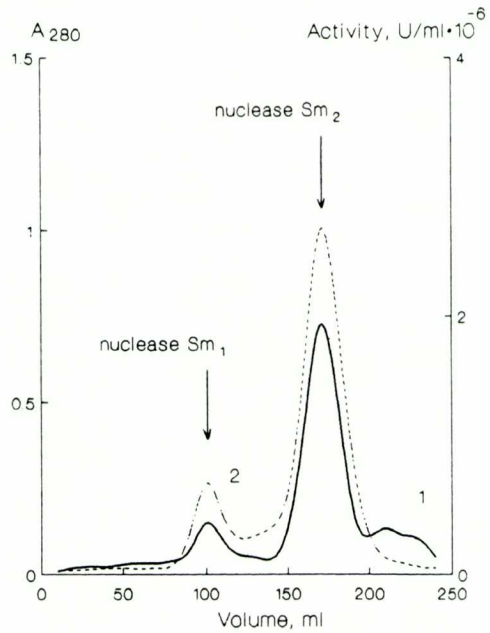

Fig. 3. Chromatographic separation (1 – A_{280}, 2 – endonuclease activity) of the nucleases $S.m_1$ and $S.m_2$ on a column with DEAE – Toyopearl 650S.

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SM6   MET-ARG-PHE-ASN-ASN-LYS-ASN-VAL-GLU-LEU-GLU-ALA-LEU-LEU-PHE-
Sm1
Sm2

SM6   -ALA-ALA-GLN-ALA-SER-ALA-ASP-THR-LEU-GLU-SER-ILE-ASP-ASN-CYS-
S.m1                                      GLU-SER-ILE-ASP-ASN-CYS-
S.m2                                ASP-THR-LEU-GLU-SER-ILE-ASP-ASN-CYS-

SM6   -ALA-VAL-GLY-CYS-PRO-THR-GLY-GLY-SER-SER-ASN-VAL-SER-ILE-VAL-
S.m1  -ALA-VAL-GLY-CYS-PRO-THR-GLY-GLY-SER-SER-ASN-VAL-SER-ILE-VAL-
S.m2  -ALA-VAL-GLY-CYS-PRO-THR-GLY-GLY-SER-SER-ASN-VAL-SER-ILE-VAL-

SM6   -ARG-HIS-ALA-TYR-THR-LEU-ASN-ASN-ASN-SER-THR-THR-LYS-PHE-ALA-
S.m1  -ARG-HIS-ALA-TYR-THR-LEU-ASN-ASN-ASN-SER-THR-THR-LYS-PHE-ALA-
S.m2  -ARG-HIS-ALA-TYR-THR-LEU-ASN-ASN-ASN-SER-THR-THR-LYS-PHE-ALA-
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Fig. 4. N-terminal region of amino acid sequences of nucleases $S.m_1$, $S.m_2$ and SM6.

A general prediction of the distribution of secondary structure elements of the proteins of this type applying a modified Chou- Fasman method [11] indicates that *S. marcescens* nucleases are α/β-proteins, which contain one large and three small regions identified as α-helices and six β-pleated structural conformations located between these α-helices (figure 5).

The isolation of two isoforms of the protein in a homogeneous state made it possible to carry out a successful crystallization and to obtain highly ordered crystals of *S. marcescens* nuclease suitable for X-ray analysis. These crystals were grown at 4°C from 10 mM TRIS-HCl buffer, pH 8.3 in "hanging" or "sitting" drops as well as by a microdialysis method. The protein concentration varied between 1 and 3.5% and different precipitants were applied.

Crystals of the best quality and maximum geometrical sizes were obtained by the vapour diffusion technique in 10 µl drops with 1% protein solution and ammonium sulphate as a precipitant (1.2– 1.6M). Two crystal habits were found to be present in the same solution: prolongated (rod-like) and rhombic prisms (figure 6,a,b), the rhombic crystals being usually observed in the presence of a small amount of dioxane (2– 3%). Crystals of both forms having the approximate dimensions of 0.7x0.3x0.2 mm (rod-like) and 0.4x0.4x0.2 mm (rhombic prisms) were suitable for X-ray analysis. Crystals

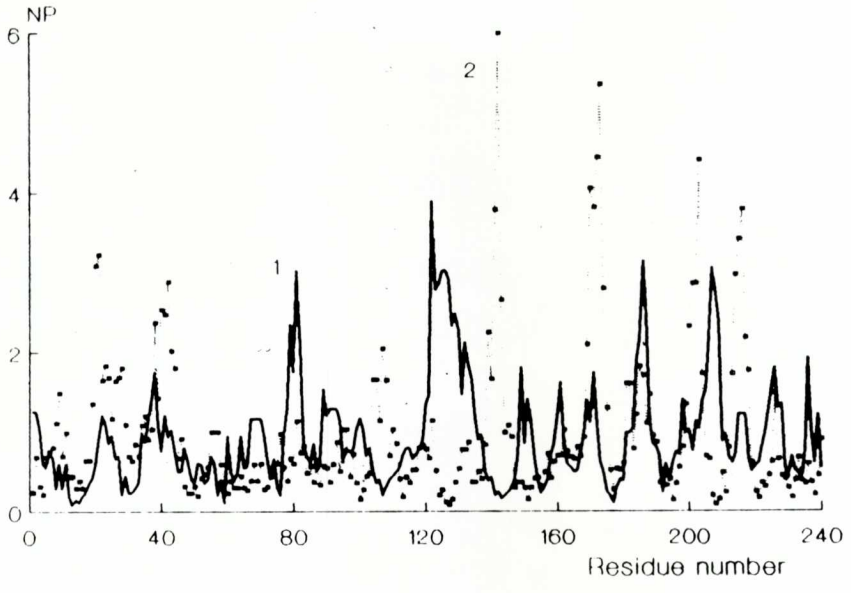

Fig. 5. Prediction of the secondary structure of the nuclease *S.m* strain W 225. NP denotes the multiplicity of the α-helical (1) and β-structure (2) conformational potentials of the hexa- and pentapeptide segments of the protein.

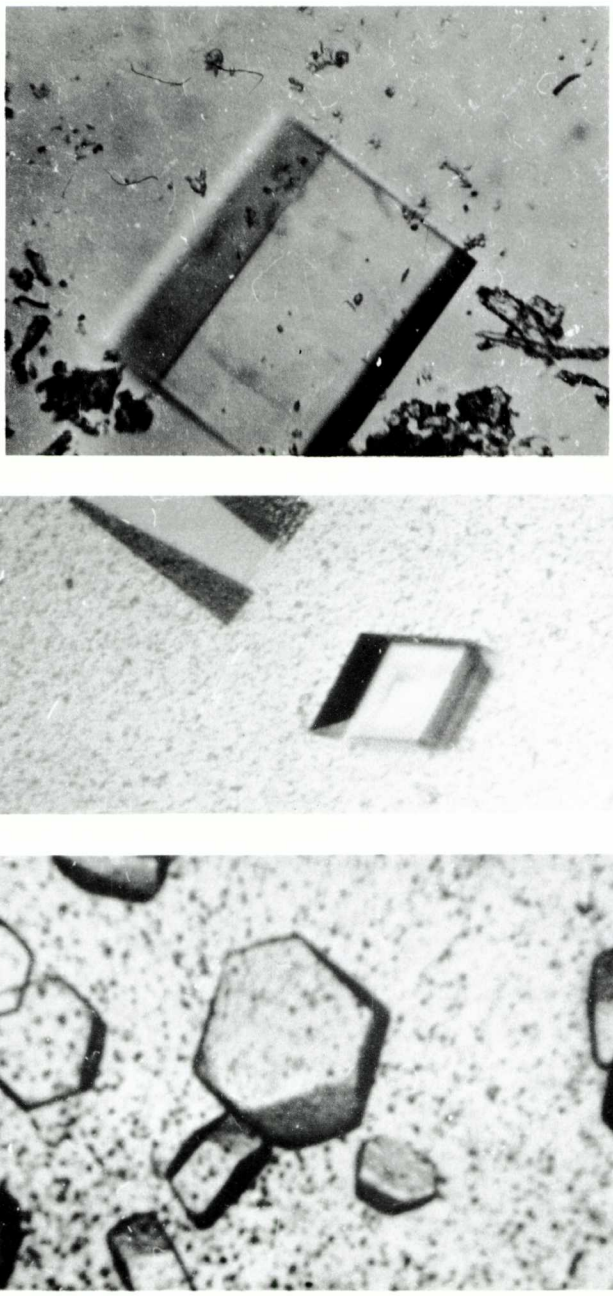

Fig. 6. Photographs of the nuclease $S.m_1$ crystals: a. stick-like, b. rhombic, c. the third form of crystals.

formed within two weeks and reached their maximum size within 1.5 – 2 months.

Another, third form of crystals was obtained in slightly different conditions: the final pH of the solution in the protein drop was higher than in the previous cases (figure 6,c).

Preliminary X-ray investigation was carried out on the rod-like crystals. It was determined from X-ray precession photographs (Fig. 7), that the crystals of *S.m*$_1$ nuclease belong to an orthorhombic space group $p2_12_12_1$ with unit cell dimensions $a = 69.0$, $b = 106.7$, $c = 74.8$ Å; contain two molecules in an asymmetric unit and the value of packing density (V_m) is equal to 2.3 Å^3/Da. The crystals diffract to at least 1.6 Å resolution. The X-ray diffraction experiments were carried out with radiation of $\lambda = 1.54$ Å, monochromatized by a graphite monochromator. The intensities of control reflections decreased by 10% in 3 days.

We attempted to solve the phase problem in our case by the method of isomorphous heavy-atom derivatives, as the structures of homologous objects have not been solved yet. More than 20 heavy-atom compounds were tested in this work. The refinement of unit cell parameters and X-ray diffraction data from the native crystals of nuclease *S.m*$_1$ and crystals of its heavy-atom derivatives were obtained on an automatic Syntex $P2_1$ diffractome-

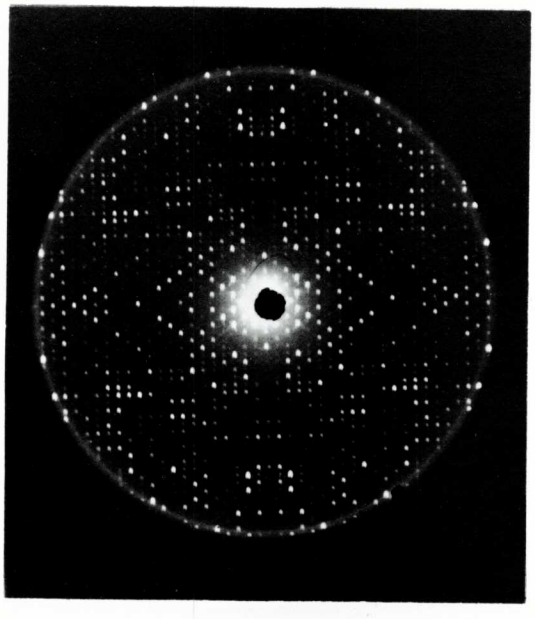

Fig. 7. X-ray diffraction precession photograph of the nuclease *S.m*$_1$ crystals.

ter. X-ray diffraction sets were measured by the ω-scan method. The angle width of intensity peaks, as a rule, did not exceed 0.28°, that also indicated a rather high quality of the crystals.

Among various heavy-atom compounds the Pt- and UO_2- derivatives were found to be the best ones (Tables 2, 3). Both derivatives were obtained by soaking the native nuclease *S.m*$_1$ crystals in 0.5 mM K_2 $Pt(SCN)_4$ solution for 5 days and in 1 mM UO_2 $(Ac)_2$ solution for 120-130 hours, respectively. The medium contrast for the Pt- derivative was 20%, for UO_2- derivative – 16%.

Further crystallographic investigations of nuclease *S.m*$_1$ as well as the crystallization of nuclease *S.m*$_2$ are in progress.

Table 2. Summary of data collection on the Syntex $P2_1$.

Data set	Conditions, mM	No. of cryst.	No. of unique refl.	Diffraction limit, Å	$R(I)_{st}$, %	R-fac, %
Native	–	5	10197	2.87	4.8	4.6
$K_2Pt(SCN)_4$	0.5	1	2002	5.2	3.7	20.4
$UO_2(AC)_2$	1.0	1	3780	4.5	4.3	17.3

$$R\text{-fac} = < | |F_{ph}| - |F_p| | > / < |F_p| >$$

Table 3. R-fac values distribution in the resolution areas.

	$K_2Pt(SCN)_4$								
d, Å	74.5	–27.5	–16.9	–12.2	–9.5	–7.8	–6.6	–5.8	–5.1
R-fac, %		23.0	30.5	27.4	23.4	22.5	22.3	21.7	20.2
	$UO_2(Ac)_2$								
d, Å	32.0	–18.1	–12.7	–9.7	–7.9	–6.6	–5.7	–5.0	–4.5
R-fac, %		28.3	16.8	12.0	9.8	8.8	8.6	8.2	7.8

ABSTRACT

Two isoforms of an extracellular endonuclease, nucleases *S.m*$_1$ *and S.m*$_2$ were purified from culture fluid of *Serratia marcescens* strain B10 M1 by the ligand-exchange chromatography on Cu^{2+}-iminodiacetate-agarose and ion-exchange chromatography

on phosphocellulose and DEAE-Toyopearl 650S. The pI-values for nucleases *S.m*$_1$ and *S.m*$_2$ were found to be 7.1 and 6.7, respectively. The amino acid analysis and N-terminal amino acid sequencing of the proteins showed a high degree of homology between the enzymes. Three different forms of nuclease *S.m*$_1$ crystals were obtained from ammonium sulphate solution by a vapour diffusion technique. The crystals used for X-ray analysis belong to the space group $p2_12_12_1$ with unit cell constants a = 69.0, b = 106.7, c = 74.8 Å, contain two molecules in an asymmetric unit, packing density V_m = 2.3 Å^3/Da, and diffract to at least 1.6 Å resolution. The Pt- and UO_2-derivatives of the protein were obtained. Preliminary X-ray investigation of nuclease *S.m*$_1$ crystals was carried out.

REFERENCES

1. NESTLE, M. and ROBERTS, W.K. (1969), J. Biol. Chem., 244, 5213-5218.
2. YONEMURA, K., MATSUMOTO, K. and MAEDA, H. (1983), J. Biochem., 93, 1287-1295.
3. LESCHINSKAYA, I.B., BALABAN, N.,P., EGOROVA, G.S., TANYASHIN, V.I. and TRETIYAK, T.M. (1974), Biokhimiya (USSR), 39, 116-122.
4. FILIMONOVA, M.N., BALABAN, N.P., SHARAPOVA, F.P. and LESCHINSKAYA, I.B. (1980), Biokhimiya (USSR), 45, 2096-2103.
5. FILIMONOVA, M.N., BARATOVA, L.A., VOSPELNIKOVA, N.D., ZHELTOVA, A.O. and LESCHINSKAYA, I.B., (1981), Biokhimiya (USSR), 46, 1660-1665.
6. BIEDERMANN, K., JEPSEN, P.K., RIISE, E. and SVENDSEN, I.B., (1989), Carlsberg Res. Communs., 54, 17-27.
7. BALL, T.K., SAURUGGER, P.N. and BENEDIK, M.J., (1987), Gene, 57, 183-192.
8. YASNOVA, L.N. and PUCHKOVA, L.J., (1976), Microbiologiya (USSR), 45, N6, 979-983.
9. VARLAMOV, V.P., LOPATIN, S.A. and BANNIKOVA, G.E. (1989), *Proc. of the First Intern. Meeting "Structure and Chemistry of Ribonucleases"* (Moscow), 383-391.
10. FILIMONOVA, M.N., DEMENTIEV, A.A., LESCHINSKAYA, I.B., BAKULINA, G.Y. and SHLYAPNIKOV, S.V., (1991), Biokhimiya (USSR), 56, N3, 508-520.
11. DUFTON, H.J. and HIDER, R.C. (1977), J. Mol. Biol., 115, 117-193.