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CHARACTERIZATION OF A RIBONUCLEASE FROM *ANACYSTIS NIDULANS* INFECTED WITH CYANOPHAGE AS-1

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Abstract— In *Anacystis nidulans* the ribonuclease (RNase) activity is very low but is greatly increased upon phage-infection. A RNase was isolated and purified over 300-fold from *A. nidulans* cells infected by cyanophage AS-1. The enzyme did not attack single- or double-stranded DNA, was inactive on *p*-nitrophenyl phosphate or bis-*p*-nitrophenyl phosphate as substrates, and had neither 3′- nor 5′-nucleotidase activity. The approximate MW of the enzyme was 12000. Maximal enzyme activity was at pH 7.5. No absolute requirement for metal ions was observed, but Fe^{3+} stimulated and Co^{2+} and Ni^{2+} inhibited enzyme activity. The enzyme is an endonuclease which, upon exhaustive hydrolysis, produces mainly oligonucleotides (average chain-length: 3) with 3′-P termini. Analysis of the base composition of these oligonucleotides and determination of their 3′-terminal nucleosides, together with the investigation of the rate of hydrolysis of synthetic polyribonucleotides, have shown that the enzyme has a relative specificity for uridylic acid.

INTRODUCTION

In view of the probable role of blue-green algae in the evolution of photosynthetic eukaryotes, a wide range of biochemical properties of blue-green algae, including protein synthesis, nucleic acid metabolism, protein and nucleic acid sequences, photosynthesis, and enzyme properties have been studied in detail [1]. However, little attention has been devoted to the nucleolytic enzymes of blue-green algae. Apparently, the extremely low nucleolytic enzyme levels found in the simpler forms of the blue-green algae, e.g. *Anacystis nidulans* [2], hampered this approach. We have recently shown that the low nuclease levels are not 'obligatory' characteristics of the unicellular blue-green algae because after cyanophage infection the deoxyribonuclease (DNase) and ribonuclease (RNase) activities increase dramatically in the cells of *A. nidulans* [3]. This observation made the isolation and characterization of a RNase from *A. nidulans* possible. The present communication describes the purification and properties of this enzyme.

RESULTS

A. nidulans cells from the late logarithmic phase were infected by cyanophage AS-1 [4] at a multiplicity of infection of 3. Since the increase in RNase activity in the infected cells occurs late (*ca* 5 hr after infection) but prior to lysis [3], crude extracts were prepared from *Anacystis* cells 5–6 hr after infection. A 7-step procedure was applied for the purification of an RNase from the crude extract (see Experimental). More than 300-fold purification of the enzyme was obtained (Table 1). The purification of RNase was difficult, not only because of the comparatively low activity even in the infected cells, but also because of the tendency of the proteins of *Anacystis* to form aggregates in extracts, a phenomenon studied in great detail in connection with the purification and characterization of phycobiliproteins from algae [5, 6]. Chromatography on DEAE-cellulose at pH 6.8 of the crude extract, obtained after removal of the nucleic acids in a polyethylene glycol + dextran two phase system, resolved the RNA-splitting activity of the preparation into several peaks. However, it is possible that not all these peaks represent different, individual enzymes. This is suggested by the fact that further purification on DEAE-cellulose at pH 7.5 of the pooled fractions corresponding to the largest RNase peak (eluting at 0.35 M NaCl) resulted in the separation of the peak into 3 fractions, one of which eluted with the front.

After a second rechromatography, under identical conditions, the RNA-splitting activity eluting at 0.35 M NaCl became entirely free of DNA-splitting contamination. However, the peak of RNA-splitting activity was again split and a substantial portion of the RNase activity migrated again with the front. Fractions belonging to the peak of RNase activity eluting at 0.35 M were used as a purified RNase preparation which had the following properties. The enzyme degraded neither native nor denatured DNA. Synthetic homopolymers were hydrolysed by the enzyme preparation in the order poly(U) > poly(C) > poly(I) > poly(A). The enzyme preparation was inactive on *p*-nitrophenyl phosphate and bis-*p*-nitrophenyl phosphate as substrates and had neither 3′- nor 5′-nucleotidase activity. As assayed by gel filtration, the enzyme had an approximate MW of 12 400.

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Table 1. Purification of a nuclease from *Anacystis nidulans* infected by cyanophage AS-1

Fraction	Volume (ml)	RNA-splitting activity (units*)	Total protein (mg)	Specific activity (unit/mg protein)*	Degree of purification	Yield (%)
Crude extract†	114	285	994.0	0.15	1.0	100
Dextran-PEG-treatment followed by $(NH_4)_2SO_4$-precipitation	110	181	303.0	0.30	2.0	64
DEAE-cellulose chromatography, pH 6.8	42	356	24.0	7.40	51.0	125
DEAE-cellulose chromatography, pH 7.5	45	225	5.4	20.85	143.8	79
DEAE-cellulose rechromatography, pH 7.5	36	235	2.4	49.00	337.9	82

* 1 enzyme unit is defined as the amount of enzyme which gives an absorbance change of 1 per hr under the assay conditions.

† Prepared from 21 g (packed wet wt) of cells 6 hr after infection.

The optimum of enzyme activity was at pH 7.5. The enzyme appeared to have no requirement for metal ions (10^{-2}–10^{-3} M EDTA had no significant effect). Cu^{2+} and Ni^{2+} (10^{-2}–10^{-3} M) were inhibitory, while Fe^{3+} (10^{-2}–10^{-3} M) stimulated the enzyme.

As shown by gel filtration on Sephadex G-50 of RNA hydrolysates obtained after various times of hydrolysis, the enzyme is an endonuclease which releases only small amounts of mononucleotides even after hydrolysis for 144 hr. This was also shown by the chromatographic analysis of the hydrolysis products obtained after exhaustive digestion of RNA. The majority of the products eluted from a DEAE-cellulose column at relatively high NH_4HCO_3 concentrations, known to elute oligonucleotides under the conditions used. The average chain length determined from total hydrolysates was found to be 3. The mononucleotides and nucleosides represented only a relatively small fraction (30%).

The oligonucleotides liberated by the enzyme had 3′-P termini. This was shown in enzymatic hydrolysates of poly(U) subjected to alkali hydrolysis which yielded only one compound identified on the basis of its chromatographic and spectral properties as 3′-UMP.

The base specificity of the enzyme was investigated by 3 methods. All of them were suggestive of a relative specificity of the enzyme for uridylic acid; (a) the base composition of the RNA used as substrate was compared with that of some short-chain oligonucleotide breakdown products (di- to tetranucleotides). As an example, it is shown in Table 2 that the base composition of the RNA did not correspond to the base composition of an oligonucleotide fraction isolated from the breakdown-products. Prominent was the relatively high percentage of U in the oligonucleotide(s). (b) The determination of the 3′-terminal nucleosides, obtained from oligonucleotides by treatment with phosphatase followed by alkaline hydrolysis, showed a relative preponderance of uridine. (c) Of the synthetic polyribonucleotides tested, poly(U) was split at the highest rate.

Table 2. Nucleotide composition of the RNA used as substrate, and that of an oligonucleotide fraction separated from the RNA-hydrolysate

Nucleotide	Percentage in the total of the nucleotides of	
	RNA	Oligonucleotide fraction
AMP	31.8	14.9
GMP	28.0	26.3
CMP	19.3	15.2
UMP	20.9	43.6

DISCUSSION

Only one report is available on the purification and properties of a nuclease from blue-green algae. The *A. nidulans* RNase, the isolation and characterization of which have been reported, has unusual specificity: it cleaves phosphodiester linkages in RNA which is methylated in the 2′-hydroxy position of ribose [7]. *Anacystis* appears to contain another RNA-splitting enzyme, termed 'RNase β' by Norton and Roth [7], which has not been characterized in detail. The RNase reported in the present paper is not identical with these nucleolytic enzymes, although it shares some common properties with 'RNase β'. Fe^{3+} activates both RNases considerably and both enzymes split poly(U) more actively than poly(A) and poly(C). However, the products of 'RNase β' are terminated in 5′-phosphates whereas those of the enzyme described in the present communication are terminated in 3′-phosphates. Moreover, important properties of 'RNase β' which would make a better comparison with other RNases possible (e.g. MW; analysis of the breakdown products) have not been described.

Most properties of the RNase described in the present paper are shared by a number of RNases isolated from various sources including prokaryotes, lower eukaryotes, unicellular green algae, and higher plants [8–16]. We draw attention only to two properties of the enzyme: (a) the pH optimum of this RNase is 7.5 and (b) it degrades its substrates, upon exhaustive digestion, prefer-

entially to short-chain oligonucleotides. It would be interesting to see if a nuclease possessing these characteristics could be found in the chloroplasts of higher plants. The RNases described from higher plants have pH optima in the acidic range and degrade their substrates, upon exhaustive digestion, preferentially to mononucleotides [13, 14].

EXPERIMENTAL

Cultivation of Anacystis nidulans *and phage infection.* This was carried out as described previously [3].

Enzyme assays. The methods used were described in previous publications [3, 11, 17, 18]. When incubation times longer than 2 hr were needed, the Tris–HCl buffer was replaced by Me arsenate buffer, pH 7.5, to prevent bacterial growth.

Enzyme purification. Extraction (step 1). Frozen cells were ground in a mortar with Al_2O_3 (1:2 w/w) for 30 min. The paste formed was suspended in 3 vol. of 0.05 M Tris–HCl buffer, pH 7.5, containing 1 mM EDTA and 0.01% 2-mercaptoethanol. The extract was centrifuged at 20000 *g* for 20 min. Fresh Al_2O_3 was added to the sediment and the extraction procedure was repeated. The 2 supernatants were combined and centrifuged at 40000 *g* for 30 min. The supernatant was centrifuged at 150000 *g* for 90 min. The resulting cell-free supernatant was used as a crude extract. *Phase separation. (step 2).* To each 10 ml of the crude extract the following were added: 2.5 ml of 20% dextran T-500, 4.8 ml of 30% PEG-6000 and NaCl in a final concn of 3M. The mixture was centrifuged at 10000 *g* for 10 min. Only the top layer was kept (PEG phase). *Removal of polymers by batch-treatment with DEAE (step 3).* DEAE-cellulose (a vol. equal to the PEG-phase fraction obtained in step 2) equilibrated with 60 × vol. of 0.05 M Tris–HCl buffer, pH 7.5, containing 1 mM EDTA plus 0.01% 2-mercaptoethanol was added to the PEG-phase fraction and filtered through a Buchner funnel. The colourless filtrate was discarded.

The DEAE-cellulose was suspended in the same buffer (60 × vol. of the PEG-phase fraction obtained in step 2), stirred for 30 min and washed with the diluted buffer. The filtrate was discarded. To elute the protein the washed DEAE-cellulose cake was suspended in 0.05M Tris–HCl buffer, pH 7.5, supplemented with EDTA and 2-mercaptoethanol and containing 0.5 M NaCl (× 3 the vol. of the PEG-phase fraction), stirred for 30 min and filtered through a Buchner funnel. One additional vol. of the eluting soln was added to the residue, the elution repeated and the filtrates combined. $(NH_4)_2SO_4$ *precipitation (step 4).* $(NH_4)_2SO_4$ was added to the filtrate to 80% satn. The sample was cetrifuged at 25000 *g* for 30 min. The sediment was taken up in 0.01 M Pi buffer, pH 6.8, containing 1 mM EDTA and 0.01% 2-mercaptoethanol (0.7 vol. of the PEG-phase fraction), and was dialysed against 10 l. of the same buffer for 18 hr. The resulting small amount of ppt. was removed by centrifugation. *Chromatography on DEAE-cellulose (step 5).* The dialysate from step 4 was applied to a DEAE-cellulose column. Protein (0.3 g), obtained from step 4 of the purification procedure, was applied to a 2 × 30 cm DEAE-cellulose column equilibrated with 0.01 M Pi buffer, pH 6.8, containing 1 mM EDTA and 0.01% 2-mercaptoethanol. Proteins were eluted with a linear NaCl gradient (0–0.5 M) in the same buffer. Fractions were assayed for *A* at 280 nm, RNA-splitting, DNA-splitting, and denatured DNA-splitting activities. *Chromatography on DEAE-cellulose at pH 7.5 (step 6).* Fractions corresponding to the peaks of RNA-splitting activity were pooled. The largest RNA-splitting peak eluted at 0.35 M NaCl was further purified. The pooled fractions belonging to this peak were dialysed against 0.01 M Tris–HCl buffer containing 1 mM EDTA and 0.01% 2-mercaptoethanol at pH 7.5. The dialysates were loaded on a 2 × 30 cm DEAE-cellulose column equilibrated with the same buffer and the proteins eluted with a linear NaCl gradient of 0.15–0.45 M. *Rechromatography on DEAE-cellulose at pH 7.5 (step 7).* Fractions corresponding to the peak eluted at 0.35 M NaCl, from step 6, were pooled and, after dialysis, rechromatographed on DEAE-cellulose at pH 7.5 as described in step 6. Fractions belong to the peak eluted at 0.35 M NaCl were pooled, dialysed against 0.01 M Tris–HCl buffer containing 1 mM EDTA and 0.01% 2-mercaptoethanol (pH 7.5). Aliquots (6–8 ml) were frozen at −20° and used as a purified RNase prepn.

Determination of enzyme MW. A method based on gel filtration, as described in ref. [19] was adopted. The RNase prepn and reference compounds were chromatographed on a 2.4 × 100 cm Sephadex G-100 column. The marker proteins were identified on the basis of UV absorption (serum albumin), absorption in the visible range (cytochrome-*c*) and by enzyme assay (peroxidase), respectively. The location of RNase activity was identified by the standard RNase assay.

Determination of endonucleolytic or exonucleolytic type of enzyme action. The method of ref. [20] was used. High MW RNA (Calbiochem) was hydrolysed by the RNase prepn as follows: 5 mg RNA, 100 μmol of Me arsenate buffer, pH 7.5, and 0.5 ml of the purified enzyme prepn were incubated at 37°. Aliquots (20 μl) were withdrawn at 0-time as well as after incubation for 48, 96 and 144 hr respectively, and chromatographed on a 1 × 40 cm Sephadex G-50 column. Authentic 3′-AMP was used as a marker for the position of mononucleotides.

Substrate specificity of the enzyme was assayed as described in ref. [21]. The molar extinction coefficients of the homopolymers, the RNA, and the nucleotide residues released were used for the calculation, also according to ref. [21].

Fractionation of the enzymatic breakdown products according to their chain-length. The method of ref. [22] was used. High MW RNA (Calbiochem) (40 mg) was incubated with 4 ml of the purified RNase prepn in the presence of 800 μmol of Me arsenate buffer, pH 7.5, in a final vol. of 8 ml. After incubation for 168 hr, the reaction was stopped by heating to 100° for 10 min. After cooling and adjusting the pH to 8.6 with dil. NH_4OH, the hydrolysate was applied to a 1.3 × 30 cm DEAE-cellulose column. The hydrolysis products were eluted with a concave NH_4HCO_3 gradient obtained from a 4-membered Varigrad system. Chamber 1 contained 300 ml of 1 M NH_4HCO_3 and chambers 2, 3 and 4 each contained 300 ml of 0.01 M NH_4HCO_3. Fractions were collected and the *A* at 260 nm was measured. The fractions corresponding to well defined peaks were combined, desalted and lyophylized.

The position of the terminal phosphoryl group. Lyophilized samples of well defined peaks, obtained from RNA digests after chromatography on DEAE-cellulose, were taken up in 0.2 ml of 0.3 M KOH and hydrolysed at 37° for 18 hr. To split the eventually formed 2′,3′-cyclic bonds, HCl was added (pH 1) and the mixture was kept at room temp. for 3 hr. Then, the soln was neutralized with KOH and desalted on a Sephadex G-10 column (1 × 30 cm). The combined fractions were lyophylized. The determination of hydrolysis products was performed by 2 methods: (a) a dry sample of the hydrolysate was dissolved in 0.025 M NH_4 formate (pH 5) and loaded on a DEAE-cellulose column (1 × 5 cm) equilibrated with the same soln. Stepwise elution was carried out with 0.025, 0.075 and 0.25 M NH_4 formate, respectively, at pH 5. At these concns of eluent, nucleosides, nucleoside monophosphates,and nucleoside diphosphates are eluted, respectively [23]. (b) Dry samples of the hydrolysate were taken up in 0.1 ml H_2O and chromato-

graphed on Whatman 3MM paper together with reference compounds in 'solvent system I' of Laskowski [24].

Base specificity. Two methods were used: (a) alkaline-hydrolysis of short-chain oligonucleotides and comparison of their base-ratios with that of the non-digested substrate, and (b) end-group analysis of oligonucleotides.

For alkaline hydrolysis, freeze-dried samples of oligonucleotide peaks obtained upon DEAE-cellulose chromatography of the enzymatic RNA digest were taken up in 0.3 M KOH and hydrolysed at 37° for 18 hr. The incubation mixture was treated with HCl as described above, desalted and lyophylized. Samples were dissolved in H_2O and chromatographed together with reference compounds on Whatman 3MM paper previously impregnated with 0.2 M NaPi, pH 7.5. The separation of nucleoside 2',3'-monophosphates was carried out in a solvent system containing *iso* PrOH–NH_4OH (18 M)–H_2O (7:1:3) as described in ref. [25]. The UV absorbing spots were cut out and eluted with 0.01 N HCl. The respective amounts of the nucleotides were estimated at appropriate wavelengths. Alkaline hydrolysis and determination of the base composition of the enzymatically non-digested substrate RNA were performed in the same way.

For the end-group analysis of oligonucleotides, freeze-dried samples of well defined peaks from the enzymatic digest of RNA, corresponding to longer chain-length oligonucleotides were taken up in 0.2 ml glycine–NaOH buffer, pH 10.4, containing 0.01 M $MgCl_2$. Alkaline phosphatase was added to the mixture which was then incubated at 37° for 24 hr to remove the 3'-terminal phosphate residues. Alkaline hydrolysis was then carried out by adding 0.13 ml M KOH to the samples and incubating them at 37° for 18 hr to release the 3'-terminal nucleosides. The pH was reduced with HOAc to 8.6 and the samples diluted with H_2O to 5 ml and loaded on a Dowex 1 × 2 column to separate the nucleosides (by elution with H_2O and 0.1 M NH_4OAc pH 6.2) as described in ref. [26].

Determination of the average chain-length of the digestion products. Aliquots of the enzymatic digest were treated with alkaline phosphatase as described above. The dephosphorylated chains were subjected to alkaline hydrolysis.

After desalting and freeze-drying, the samples were chromatographed on Whatman 3MM paper in solvent system I of ref. [24]. The spots were eluted by 0.01 M HCl and the amounts of nucleosides and nucleotides determined spectrophotometrically. The average chain-length was calculated from the nucleotide/nucleoside-ratio.

Estimation of protein content. Protein content in crude extracts was determined by the method of ref. [27]. In the later stages of purification, the protein concn was calculated from the *A* of the soln at 280 nm.

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