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Note

Specificity of deoxyribonuclease hydrolysis determined by high-performance liquid anion-exchange chromatography

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In the study of deoxyribonucleases (DNases) the discrimination between endo- and exo-nucleases and between 3′- and 5′-monoester formers is of particular interest for the characterization of different enzymatic activities. The methods commonly used for this purpose, conventional anion-exchange column chromatography[1,2] or the application of special substrates such as closed circular DNA[2,3], are in general time-consuming and expensive or require the preparation of large amounts of highly purified enzyme activities. Furthermore, the accurate determination of whether an endonuclease represents a 3′- or a 5′-monoester former requires re-incubation of its primary hydrolysis products (mainly consisting of oligodeoxyribonucleotides) with snake venom (PDase I) or spleen (PDase II) phosphodiesterase[4]. The degradation products are commonly analysed by two-dimensional thin-layer chromatography[1,5]. Provided the oligodeoxyribonucleotides contain 5′- or 3′-phosphate end-groups, PDase I and PDase II preferentially degrade these compounds to deoxyribonucleoside 5′- or 3′-phosphomonoesters, respectively. There exists, however, a weak activity with either enzyme, especially with PDase I[6], which uses oligodeoxyribonucleotides without any phosphate end-groups or even those with 3′-phosphate end-groups as a substrate. Thus, kinetic studies of the hydrolysis process are required which should be fast, inexpensive and feasible with minute amounts of hydrolysis products.

High-performance liquid chromatography (HPLC) has become a routinely used means for the separation, identification and quantitation of a variety of compounds[7,8], in particular of those found in the extracts of biological materials or in biological fluids[9]. If combined with highly sensitive UV detectors, HPLC permits the accurate determination of less than $10 \cdot 10^{-12}$ mol of UV-absorbing compounds[10,11].

We have developed an HPLC anion-exchange separation method for deoxyribonucleoside 3′- or 5′-monophosphates suitable for the fast and accurate determination of picomole amounts of nucleotides. We have applied this method in the identification of DNA breakdown products formed by minute, but highly purified DNase activities from L5178Y mouse lymphoma cells.

EXPERIMENTAL

DNase I (EC 3.1.4.5) (2000 U/mg), snake venom PDase (PDase I, EC 3.1.4.1) (1.5 U/mg) and calf spleen PDase (PDase II, EC 3.1.4.18) (2.0 U/mg) were obtained from Boehringer-Mannheim (Tutzing, G.F.R.), DNase II (EC 3.1.4.6) (200 U/mg)

was from Serva (Heidelberg, G.F.R.). Herring sperm DNA[12] was a gift from H. Mack (Illertissen, G.F.R.). All reagents were of the highest analytical grade supplied by Serva or Merck (Darmstadt, G.F.R.).

DNase activities from L5178Y mouse lymphoma cells (referred to as DNase 4.5 and DNase 7.0) were prepared as described previously[13].

For enzymatic hydrolysis, 0.2 mg of DNA was either (i) dissolved in 1.0 ml of sodium acetate (pH 5.0)–1.5 m*M* EDTA and incubated with DNase II, PDase II and the L5178Y cell DNases, or (ii) dissolved in 1.0 ml of Tris–HCl (pH 7.4)–10 m*M* $MgCl_2$–1 m*M* $CaCl_2$ with DNase I and PDase I as first enzymes in hydrolysis. For a second incubation period, 0.2 ml of the initial incubate was mixed with 0.8 ml of sodium acetate (pH 5.0)–1.5 m*M* EDTA or with 0.8 ml of Tris–HCl (pH 7.4)–10 m*M* $MgCl_2$–1 m*M* $CaCl_2$ containing PDase II or PDase I, respectively. After various intervals (Table I), 0.2 ml of each digest were withdrawn and diluted with 0.8 ml of 0.25 *M* potassium phosphate (pH 3.5). 50 μl-aliquots of these solutions were then analyzed by HPLC.

TABLE I

DEOXYRIBONUCLEOSIDE 3'- AND 5'-MONOPHOSPHATES RELEASED FROM DNA BY VARIOUS NUCLEASES

The values of determinations in triplicate are given as a percentage of total degradation products that can be expected.

Sample No.	*Enzyme 1*	*Incubation time (h)*	*Enzyme 2*	*Incubation time (h)*	*Mononucleotides*	
					3'	*5'*
1	DNA				0	0
2	DNA + DNase I	4			0	0
3	DNA + DNase I	48			0	0
4	DNA + DNase II	4			0	0
5	DNA + DNase II	48			40 ± 1	0
6	DNA + PDase I	2			0	0
7	DNA + PDase I	4			0	0
8	DNA + PDase II	2			0	0
9	DNA + PDase II	4			0	0
10	DNA + DNase I	4	PDase I	1	0	34 ± 10
11	DNA + DNase I	4	PDase I	2	0	81 ± 5
12	DNA + DNase I	4	PDase I	4	0	100
13	DNA + DNase I	4	PDase II	4	0	0
14	DNA + DNase II	4	PDase I	4	0	0
15	DNA + DNase II	4	PDase II	1	7 ± 7	0
16	DNA + DNase II	4	PDase II	2	47 ± 5	0
17	DNA + DNase II	4	PDase II	4	100	0

Nucleotide separations were carried out in a Hewlett-Packard 1080B liquid chromatograph, equipped with a variable-wavelength UV detector operating at 254 nm, an automatic sampling system and a Whatman Partisil PBS 10/25 SAX high performance anion-exchange column which was eluted under isocratic conditions with 37.5 m*M* potassium phosphate (pH 3.5) at a flow-velocity of 6.0 cm min^{-1} (flow-rate 60 ml h^{-1}) at ambient temperature.

RESULTS AND DISCUSSION

Under the separation conditions given above, the elution patterns of syn-

thetic mixtures of the major deoxyribonucleoside 5′-monophosphates and of their 3′-isomers were significantly different (Fig. 1b and c, respectively). The total separation time did not exceed 15 min.

DNase I and DNase II both attack DNA endonucleolytically producing oligodeoxyribonucleotides with phosphate groups at the 5′-end[14] or at the 3′-end[15], respectively. Thus, during the early incubation periods, further degradation of these samples with PDase I and PDase II exclusively releases 5′-monophosphates or 3′-monophosphates. The amounts of free nucleotides formed under different incubation conditions (digestion of DNA with DNase I, DNase II, PDase I and PDase II alone or in combination during different time intervals) and the time-dependent release of deoxyribonucleoside 5′- or 3′-monophosphates from DNase I and DNase II hydrolysates by the action of PDases I and II, are listed in Table I.

No free nucleotides were detectable in undigested DNA samples (Fig. 1a; Table I, sample 1). After exhaustive digestion of DNA with DNase I (Table I, samples 2 and 3), PDase I (Table I, samples 6 and 7) or PDase II (Table I, samples 8 and 9), no mononucleotides were detectable. Exhaustive digestion of

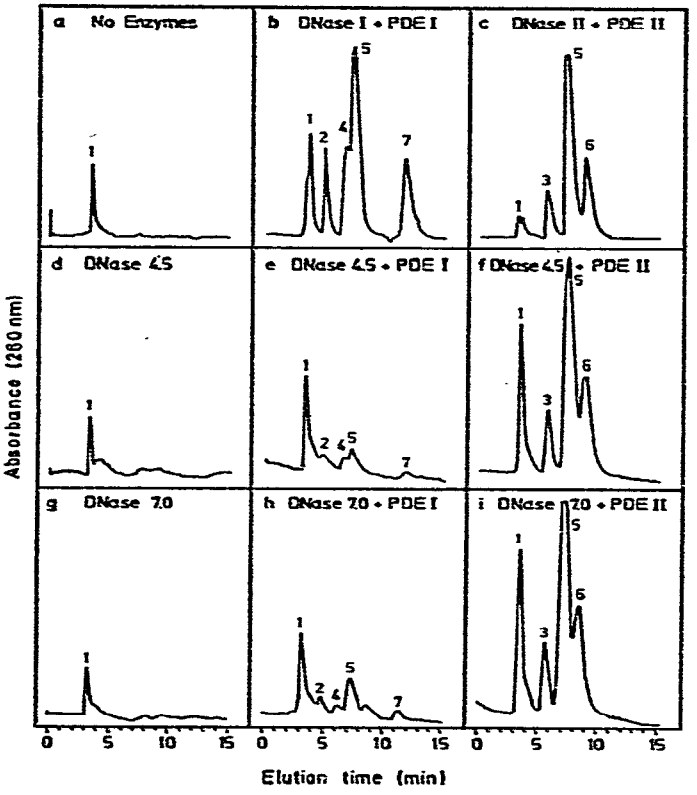

Fig. 1. HPLC elution patterns obtained from separations of DNA hydrolysates on Partisil SAX-column (for separation conditions see text). Peaks: 1 = void; 2 = 5′ dCMP; 3 = 3′ dCMP; 4 = 5′ dTMP; 5 = 5′ dAMP or 3′ dTMP and 3′ dAMP; 6 = 3′ dGMP; 7 = 5′ dGMP. The diagrams correspond to the following samples in Table I: a, 1; b, 10; c, 14. Further diagrams were obtained from digests with L5178Y cell DNases as follows: d, DNA incubated with DNase 4.5; e, DNA incubated with DNase 4.5 and PDase I; f, DNA incubated with DNase 4.5 and PDase II; g, DNA incubated with DNase 7.0; h, DNA incubated with DNase 7.0 and PDase I; i, DNA incubated with DNase 7.0 and PDase II.

DNA with DNase II, however, released 3'-mononucleotides (Table I, sample 5) owing to an exonuclease activity which is normally present in DNase II preparations[15].

Incubating the DNase I digest with PDase I for different time intervals released increasing amounts of 5'-mononucleotides (Table I, samples 10, 11 and 12), whereas with PDase II no 3'-mononucleotides were detectable (Table I, sample 13). The opposite results were obtained with the DNase II digest and PDases I and II (Table I, samples 14, 15, 16 and 17). In general, the DNA of either of the digests was completely degraded into acid-soluble compounds.

These results, obtained with DNA-hydrolysing enzymes whose specificity and mode of action are well known, clearly demonstrate that the analysis of DNA digests with anion-exchange HPLC represents a suitable method for the determination of whether an exo- or an endo-nuclease or whether a 3'- or 5'-monoester former was used for hydrolysis.

We applied the method to the elucidation of the mode of action and the nature of the degradation products of two highly purified acid DNases of L5178Y mouse lymphoma cells. When incubating DNA with either DNase for 48 h, the DNA was completely degraded into acid-soluble products, but no free deoxyribonucleoside 3'- or 5'-monophosphates could be detected (Fig. 1d and g). This implies that both enzymes were endonucleases. Incubation of the digests with PDase I released only minute amounts of 5'-mononucleotides (Fig. 1e and h), whereas on incubation with PDase II the oligodeoxyribonucleotides of the samples were quantitatively degraded to 3'-monophosphates (Fig. 1f and i).

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