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ORIGINAL ARTICLE

Determination of Micro-Quantities of DNA Using DNAse and Fluorescence of Hoechst 33258 and Light-Scattering

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Abstract

The DNA hydrolysis by deoxyribonuclease (DNAse I) in aqueous solution was studied, using fluorescence spectroscopy and high-sensitive light-scattering detection. Specific hydrolysis of high-polymer DNA or fragmented DNA by the enzyme led to a strong decrease in the fluorescence of the Hoechst dye. The hydrolysis of mitochondrial DNA was accompanied by a decrease in the fluorescence of the dye only in 1.6 times. Hydrolysis within minutes and even hours led to appearance of large polynucleotide fragments, but not to short oligonucleotides, that was confirmed using polarized fluorescence and highly sensitive measurement of light-scattering. At the moment of the time of formation of a complex between DNA and DNAse I, a strong light-scattering occurred, which then dropped sharply during hydrolysis of high-molecular DNA, and slowly decreased during hydrolysis of fragmented DNA. The proposed methods can be applied for selective detection of trace amounts of various types of DNA, as well as for studying their physic-chemical properties.

Keywords Hoechst 33258 · DNAse I · DNA hydrolysis · Fluorescence · Light scattering · DNA sensor

Detection of micro-quantities of DNA in various biological samples is an important applied task [1]. One of the most sensitive instrumental methods is fluorescence analysis [2]. It is not accident that specific fluorescent dyes are often used for the DNA detection [3]. A number of methods for the rapid estimation of "total microbial contaminations" in liquid media using fluorescent nucleotide-specific dyes for food, drugs, etc. are well-known [4].

Among the fluorescent dyes, one of the most successful is Hoechst 33258, which used for the quantitative determination of DNA in biological materials [5]. When binding with DNA or RNA takes place, fluorescence quantum yield of the dye greatly increases due to by enhancing in the lifetime of the excited state [6].

In oligonucleotides, the Hoechst 33258 dye is predominantly inserted into regions with AT sequences [7, 8]. It is considered that Hoechst derivative bisbenzimidazole, a Hoechst derivative, binds to the AT sites in the minor groove of DNA, and can also interact well with GC-rich sites in both DNA and RNA [9, 10]. Hoechst 33342, more hydrophobic dye than Hoechst 33258, perfectly binds to hairpin oligonucleotides and untwisted sites of DNA [11]. According to Labarca et al. [5] there is a close correlation between the chain length of the DNA molecule and Hoechst binding.

The aim of our work is to develop highly sensitive methods of fluorescence spectroscopy and light-scattering for determination of micro-quantities of three types of DNA preparations: high polymer DNA, fragmented DNA and mitochondrial DNA (mtDNA) using specific hydrolysis by DNAse I, which cleaves the phosphor-diester bond between 3'-OH group and 5 'PO4-residue of a neighboring nucleotide [12]. It is interesting to conduct a comparative study of DNAse I hydrolysis of these three preparations of DNA.

Methods

DNAse I - deoxyribonuclease from the bovine pancreas ("Reachim"), high-molecular DNA from red blood cells

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of chickens ("Reanal"), fragmented Salmon sperm DNA ("Invitrogen") and mitochondrial DNA from the liver of Wistar rats were used in our work. Solutions of these substances were purified from particles by filtration through 0.45-μm Millipore filters.

A fresh aqueous solution of Hoechst 33258 was prepared each time before the experiment. First, the dye solution in isopropanol was diluted 100 times with water. Then, the resulting solution was added to the sample at a final concentration of Hoechst 2×10^{-8} M. This low concentration was chosen accordingly to recommendations in [13] for applying the dye to small amounts of DNA. Concentrations of DNA, DNAse I, and Hoechst 33258 in solutions were determined with a 5400-UV spectrophotometer ("Prom-Ecolab.") at 260 nm, 280 nm, and 350 nm, respectively.

Formation of the DNA complex with Hoechst and subsequent hydrolysis by DNAse I were carried out in aqueous solutions at pH = 7.

Fluorescence of a stained DNA was detected in 1 cm quartz cuvettes on a Cary spectrofluorimeter (at 356 nm excitation) as Hoechst emission spectra in the range 400–600 nm or as the intensity at the maximum of spectrum (450–470 nm, depending on conditions). The DNA hydrolysis reaction was also detected on the SLM-4800 spectrofluorimeter (with polarization prisms) by decreasing in the degree of polarization of the Hoechst fluorescence.

In addition, formation of a DNA complex with DNAse I and the subsequent DNA hydrolysis were detected by changes in light-scattering (without the dye) at crossed monochromators of the spectrofluorometer. Such method is a thousand times more sensitive than any photometric detection of light-scattering. The excitation monochromator was setting at 400 nm (the slit was 2.5 nm), where there was no any absorption bands. The emitting monochromator was setting at 401 nm (the slit was 5 nm), so that the exciting light can a bit penetrate into the detection channel.

Aqueous solutions of DNA were mixed with Hoechst solution and, after a few minutes, the enzyme (DNAse I) was added. Next, we observed the process of hydrolysis during several hours at room temperature. The concentration of DNA (as nucleotides) was varied from 10^{-7} to 5×10^{-6} M. The concentration of DNAse I was taken excessive to reach a maximum rate of hydrolysis and it was $16{,}8 \times 10^{-5}$ M everywhere.

Mitochondria were obtained from liver of a rat by a standard method. Next, mtDNA was isolated by alkaline extraction (without phenol) accordingly to method [14] with modifications. Optical density of the mtDNA solution at 260 nm was 0.51 per 1 cm, and at 280 nm - 0.39, i.e. DNA prevailed over the protein in the sample.

A weak Hoechst 33258 fluorescence in the mtDNA preparation was detected in a mirco-cuvette as emission

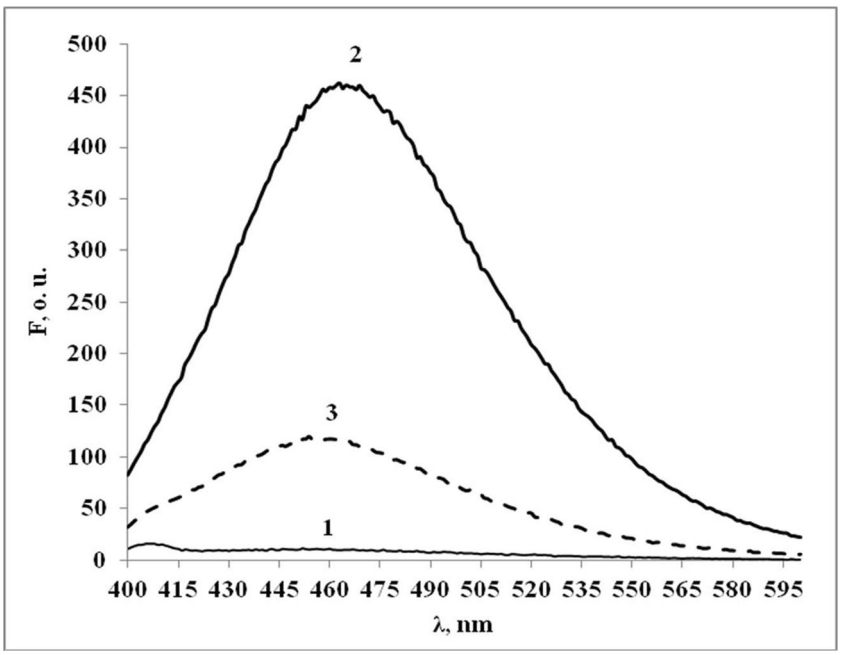

Fig. 1 Fluorescence spectra of Hoechst (2×10^{-8} M) in water (1), the Hoechst complex with high-molecular DNA (5×10^{-6} M) (2) and Hoechst with DNA after 3 h of hydrolysis by DNAse I (3)

Table 1 Fluorescence intensity of Hoechst complexes with preparations of high-molecular weight DNA and fragmented DNA

DNA concentration	The fluorescence intensity, o.u.	
	High-molecular weight DNA	Fragmented DNA
2×10^{-7}M	385	113
5×10^{-6}M	461	507
5×10^{-6}M after 3 h of hydrolysis by DNAse I	120	110

spectra in the range of 400–600 nm (excitation was 350 nm).

Results and Discussion

In aqueous solutions at neutral pH of 7 and low ionic strength, a multiple increase in the fluorescence of Hoechst 33258 is observed upon binding with high molecular weight DNA (Fig. 1). The increase in the fluorescence intensity of Hoechst in the presence of large quantities of this DNA (5×10^{-6}M) was not proportional to its concentration (Table 1), since in the case of a low concentration of DNA (2×10^{-7}M) the dye (2×10^{-8} M) was bound almost totally.

The addition of DNAse I resulted in a gradual decrease in the fluorescence of the dye because of the hydrolysis of high-molecular weight DNA (Fig. 1) to fragments. In this experiment, the maximum of spectrum was shifted to the shortwave side, which suggests that the dye falls into a more hydrophobic environment. Because (можно заменить вроде) the dye in high-molecular DNA simply adheres on the surface, but when these DNA is hydrolyzed to large fragments, the dye gets into more hydrophobic regions, which have become available.

Even after 3 h, the fluorescence intensity of the dye did not return to the level of free dye in water. This suggests that the hydrolysis goes exactly to the polynucleotide fragments, but not to short oligonucleotides or individual nucleotides, since the dye slightly goes into the aqueous phase.

Enzymatic hydrolysis, compared with acid or alkaline, has a more selective effect and splits bonds only between certain nucleotides, resulting in the formation of polynucleotides, and then – long oligonucleotides (much more time is required to achieve a higher degree of hydrolysis) [15].

Hydrolysis of the preparation of *fragmented* DNA was also carried out in an aqueous solution at room temperature with the addition of the same concentration of DNAse I

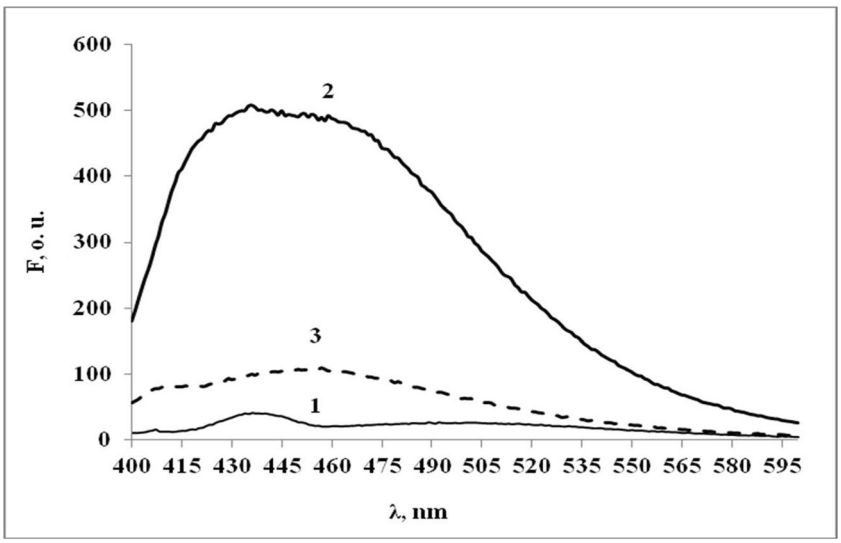

Fig. 2 Fluorescence spectra of Hoechst (2×10^{-8} M) in water (1), the Hoechst complex with fragmented DNA (5×10^{-6} M) (2) and Hoechst with DNA after 3 h of hydrolysis with DNAse I (3)

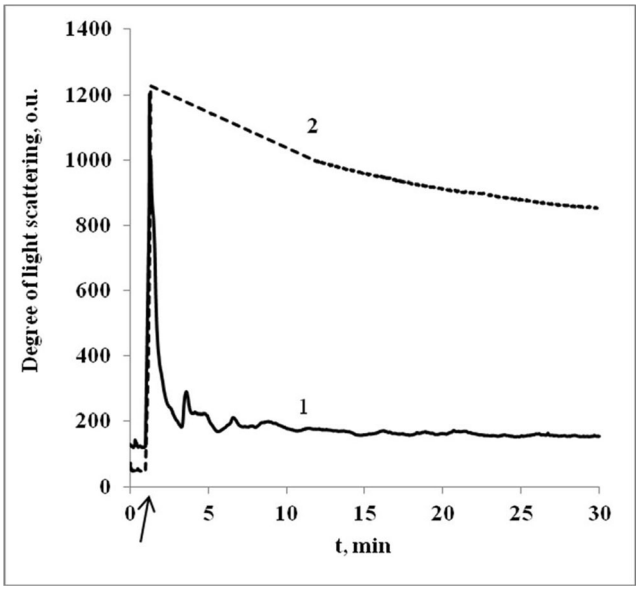

Fig. 3 Changes in the light scattering of high molecular DNA (1) and fragmented DNA (2) at a concentration of 5×10^{-6} M during the hydrolysis. The arrow indicates the moment of addition of DNAse I. The excitation monochromator is 400 nm, the slit is 2.5 nm, the emitting monochromator is 401 nm, the slit is 5 nm. The intensities at 1 min are normalized

($16{,}8 \times 10^{-5}$ M). In the case of fragmented DNA, the fluorescence intensity during hydrolysis by DNAse I fell without any spectrum shift. This suggests that the resulting small fragments almost do not differ in their physico-chemical properties from the original large macromolecules. The fluorescence intensity of the dye did not return completely to the level of free dye in water (Fig. 2), i.e. hydrolysis goes to polynucleotide fragments, but not to short oligonucleotides or individual nucleotides (the dye does not go completely into water).

The rate of hydrolysis of preparations of high polymer DNA and fragmented DNA was not too different. The fluorescence intensity of the Hoechst complex with high polymer DNA and fragmented DNA at a concentration of 5×10^{-6} M fell down during 4 h in 4 and 5 times, respectively, i.e. the rate of hydrolysis of such polymeric substrates is almost not limited by their length.

Hydrolysis of high molecular weight DNA or fragmented DNA by the enzyme can be detected also by changing in light-scattering, without any dye. Solution of DNA has some light scattering, which can be detected on a spectrofluorometer with crossed monochromators (this method is a thousand times more sensitive than photometry) [16].

DNAse I was added to high-molecular or fragmented DNA after one minute of registration. When a complex of high molecular DNA with DNAse I was formed, light scattering instantly jumped by an order of magnitude (Fig. 3). This means that a tight complex of DNA with the enzyme has been formed. This complex has a bigger size and more density, than a single DNA and single enzyme. And right after formation of the complex, at the beginning of hydrolysis, light-scattering quickly fell almost to the initial level during a minute (Fig. 3). This means that a rapid hydrolysis of DNA into several fragments was occurred (Fig. 4).

Light-scattering of complexes of fragmented DNA with the enzyme during hydrolysis decreases more slowly and in less degree (Fig. 3). This is due to the fact that the deep fragmentation of fragmented DNA requires much more time. Therefore, this method can be used to distinguish these two types of DNA from each other.

To verify the obtained data, we also used the method of polarization fluorimetry. The excitation wavelength was taken not at the absorption maximum of the dye, but at 397 nm in the long-wavelength region, so that the degree

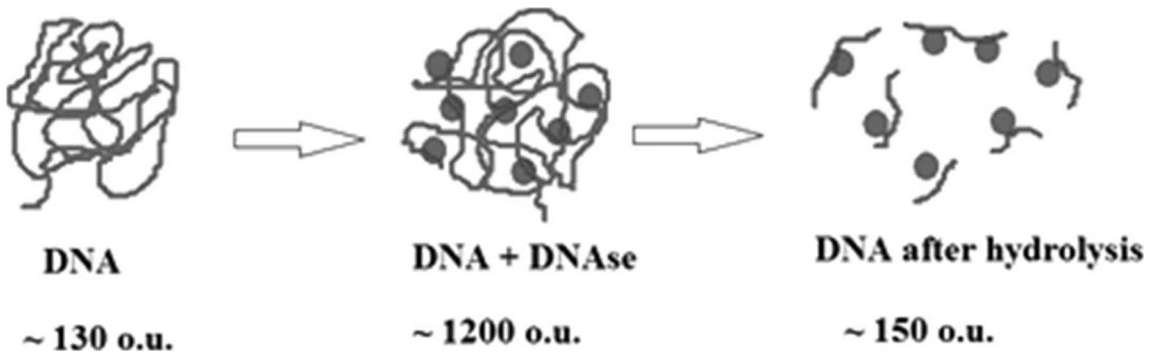

Fig. 4 Scheme of the first stages of hydrolysis of high molecular DNA by DNAse I (the numbers indicate the intensity of light-scattering)

Table 2 The degree of polarization (P) of the fluorescence of the Hoechst complex with DNA before and after hydrolysis (excitation - 397 nm, emission - 497 nm)

	P	*P* after hydrolysis
Hoechst in water	0,17	–
Hoechst + high molecular DNA	0,41	0,172
Hoechst + fragmented DNA	0,42	0,23

The time after completion of the hydrolysis of high polymer DNA was 1 day, and of fragmented DNA was 1 h

of polarization (P) was as so high as possible [16]. When Hoechst binds to DNA, we observed a significant increase in P. So, the *P* value of the dye in the high-molecular DNA preparation was reached 0.41 and in the preparation of fragmented DNA - 0.42. Such high P means that Hoechst, having life-time $\tau = 2$ ns in DNA [1, 6, 16], practically does not have rotational mobility (due to the short Hoechst lifetime in water of 0.2 ns, the molecule does not have time to turn). The addition of DNAse I had a little effect on P in the first hour of hydrolysis of high polymer DNA. This can be explained by the fact that DNA hydrolysis occurs first into large fragments with low rotational mobility.

Only the next stages of hydrolysis (after a few hours) led to a noticeable decrease in P (Table 2). In the case of a fragmented DNA preparation, the *P* value after 1-h hydrolysis fell to 0.23, that is not much higher than the P of free Hoechst in water (Table 2).

In the mtDNA preparation, the Hoechst fluorescence intensity was too low even at 5×10^{-6}M of nucleotides. This DNA is circular and twisted, and therefore it binds the dye poorly. Dye binding was observed only at a very high concentration of mtDNA - at 2.5×10^{-3}M of nucleotides. The hydrolysis of such large amount of DNA by small amount of DNAse I (16×10^{-5} M) reached 24 h. About of 40% of mtDNA was hydrolyzed during this time (Fig. 5). Thus, using Hoechst and DNAse I for hydrolysis, it is still possible to estimate the amount of mtDNA if it is large enough.

The methods which we have developed for determining DNA with Hoechst 33258 fluorescence and light scattering, using DNAse I as a selective "sensor", can be, in principal, widely applied to determine the DNA content in biological samples, including nuclei DNA and mtDNA. These methods are not less sensitive than the method of two-photon correlation spectroscopy of nucleic acids labeled covalently with two fluorophores [17]. Our methods have a number of important advantages: a) there is no need for a time-consuming procedure for covalent DNA labeling with dyes; b) no correlation microscope and complicated two-photon spectroscopy technique are needed; c) the method can be used not only in vitro, but also for living cells and cell organelles

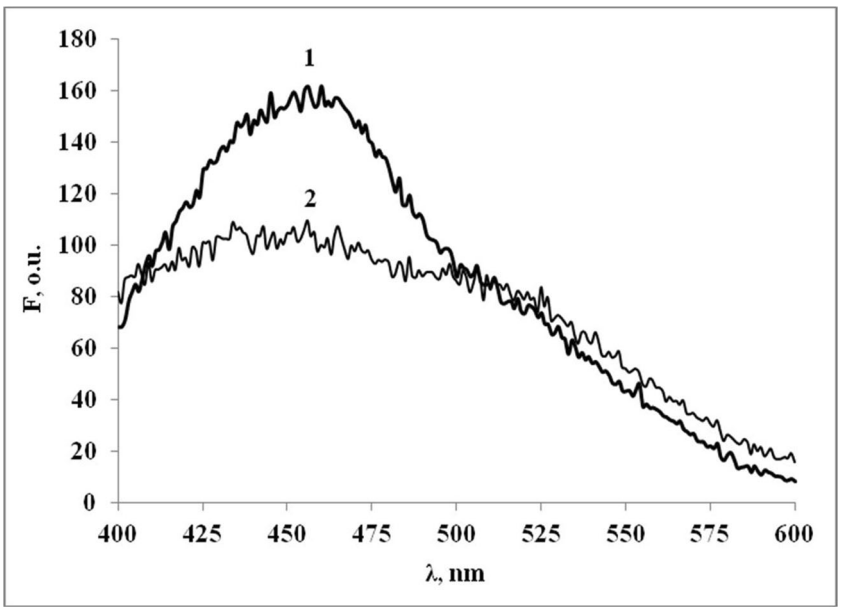

Fig. 5 The Hoechst fluorescence spectra in mtDNA before (1) and after hydrolysis by DNAse I (2). The spectra are presented minus the contribution of light scattering at the used wavelengths

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