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Short Communication

Determination of 5-methylcytosine content of four Cucurbitaceae species using high-performance liquid chromatography[a]

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ABSTRACT

A rapid and sensitive procedure for the determination of methylcytosine in cucurbit DNAs using reversed-phase high-performance liquid chromatography is described. The results showed *ca.* 3.2–5.6% of methylcytosine in total DNA, *i.e.*, about 20–30% of the cytosine residues appeared to be methylated. These results are discussed taking into consideration the influence of degree of methylation on the G + C determination of DNAs.

INTRODUCTION

In plants, considerable importance has been attached to the methylation of DNAs, probably owing to its implication in regulation of replication, gene expression, differentiation, etc. [1]. It has been reported that the extent of methylation of cytosine residues in the total DNA interferes in the G + C determination by either thermal denaturation or caesium chloride density gradient methods. This is because the 5-methylcytosine (m^5C)–guanine (G) pair is comparatively more stable than the CG pair and the presence of m^5C in the DNA lowers the buoyant density [2,3]. Several methods, *viz.*, gas chromatography [4], time-resolved fluoroimmunoassay (TR-FIA) employing europium-labelled antigen as tracer [5] and high-performance liquid chromatography (HPLC) [6–8] have been reported for the determination of the degree of methylation in DNAs. Of these methods, HPLC has become widely used in the analysis of bases, nucleosides and nucleotides in DNA or RNA, probably owing to its ease of operation [7,8].

In this paper, we report a sensitive HPLC method for the detection and determination of m^5C residues from four cucurbit species DNAs.

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EXPERIMENTAL

Apparatus

An LKB (Uppsala, Sweden) Model 2150 solvent-delivery system equipped with a Rheodyne universal injector and an LKB Model 2158 Uvicord SD detector operating at 254 nm were used for routine HPLC analysis. A reversed-phase Ultropac LiChrosorb RP-18 (5 μm) column (250 × 4 mm I.D.) was obtained from LKB.

Preparation of the standard mixture

Stock solutions (2 mg/ml) of standard bases, namely adenine, guanine, thymine, cytosine and 5-methylcytosine (Sigma, St. Louis, MO, U.S.A.) were prepared in filtered water. For adenine, guanine and thymine 1 *M* potassium hydroxide was added (if necessary) for complete dissolution. The standard mixture was prepared by mixing all the bases in equal amounts and suitably diluted with water before use.

DNA isolation and sample preparation

Nuclear DNAs from seedlings of sponge gourd (*Luffa cylindrica*), ridge gourd (*L. accutangula*) and ash gourd (*Bennincasa hispida*) and fruits of ivy gourd (*Coccinia indica*) and *Escherichia coli* were isolated according to Ranjekar *et al.* [9]. Calf thymus and salmon sperm DNAs were obtained from Sigma.

DNA hydrolysis was carried out by incubating 50 μg of DNA with 300 μl of 70% perchloric acid at 100°C for 1 h. After hydrolysis, the excess of perchloric acid was removed as potassium perchlorate by stepwise neutralization with 10 *M* potassium hydroxide solution (40–60 μl each time) [10]. The samples were centrifuged to remove the potassium perchlorate. The final volume of the supernatant was 250 μl, of which 5–10 μl (1–2 μg of hydrolysed DNA) were used for the base analysis.

Chromatographic procedure

The mobile phase, consisting of 0.4 *M* ammonium dihydrogenphosphate buffer (pH 4.3) containing 5% (v/v) of methanol was used at an ambient temperature (20–25°C) and a flow-rate of 0.5 ml/min. A 2–10-μl volume of sample/standard was injected on to the column and the resolution was monitored at 254 nm. The amount under each peak was calculated on the basis of the total area occupied by each peak of the sample and standard. For m^5C the range of linearity of the method was from 0.1 to 1.4 μg.

The relative standard errors were calculated from the results for three different hydrolysates of the same DNA samples and each of the hydrolysates was run in duplicate on the column.

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC of the standard base mixture, commercial DNA hydrolysates and four cucurbit DNA hydrolysates. All five bases were well separated and eluted within 24 min; m^5C was eluted immediately after cytosine.

Table I summarizes the G + C contents of DNAs determined by HPLC and the thermal denaturation method. The G + C contents of DNAs from calf thymus, salmon sperm and *E. coli* determined under our experimental conditions compare well

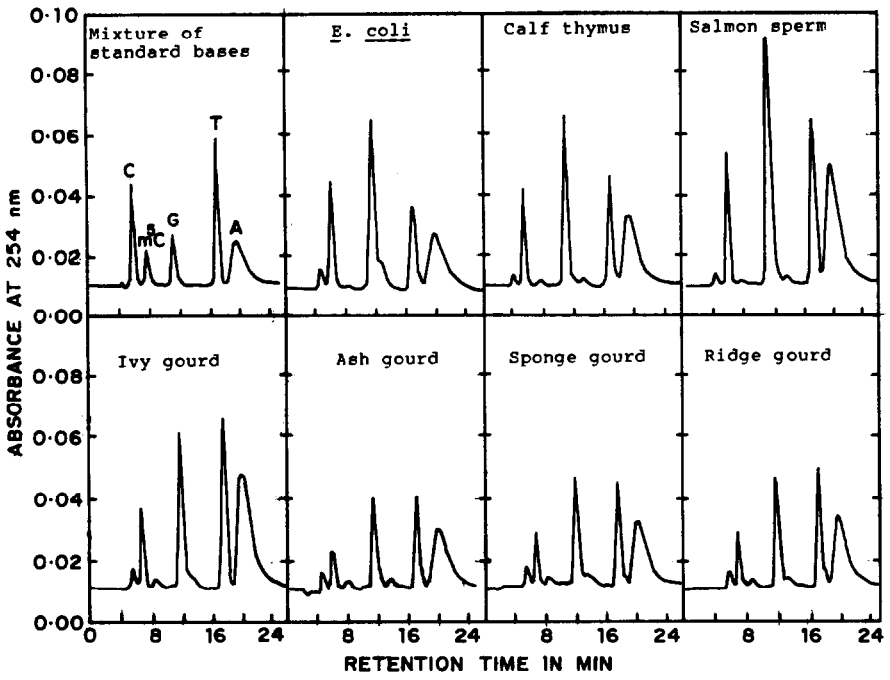

Fig. 1. High-performance liquid chromatography of DNAs. Base compositions of DNAs of *E. coli*, calf thymus, salmon sperm, ivy gourd, ash gourd, sponge gourd and ridge gourd were determined by HPLC with an Ultropac LiChrosorb RP-18 (5 μm) column (250 × 4 mm I.D.). The flow-rate of the eluent [0.4 *M* ammonium dihydrogenphosphate buffer (pH 4.3) containing 5% (v/v) of methanol] was 0.5 ml/min. The effluent was monitored at 254 nm.

TABLE I

BASE COMPOSITION OF DNA DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Source	G + C content from T_m (%)[a]	G + C content from HPLC (%)[b]	m^5C content of total DNA (%)[b]	$\frac{m^5C}{C + m^5C}$ (%)[b]
Ivy gourd (*Coccinia indica*)	34.01	34.89 (± 0.65)	3.24 (± 0.58)	19.18 (± 3.55)
Ash gourd (*Bennincasa hispida*)	36.39	35.57 (± 2.06)	5.57 (± 0.85)	30.93 (± 3.14)
Sponge gourd (*Luffa cylindrica*)	36.83	36.68 (± 0.88)	4.52 (± 1.17)	24.40 (± 3.43)
Ridge gourd (*Luffa accutangula*)	39.94	37.02 (± 1.75)	5.20 (± 1.11)	27.88 (± 2.84)
E. coli	52.54	49.52 (± 0.30)	–	–
Calf thymus	41.19	44.22 (± 1.72)	2.80 (± 0.27)	13.24 (± 1.97)
Salmon sperm	45.50	47.00 (± 1.82)	1.75 (± 0.08)	9.00 (± 1.17)

[a] From ref. 11.

[b] Standard errors in parentheses, as described under Experimental.

with the reported values [11,12]. The cucurbit nuclear genomes showed about 3.2–5.6% of m^5C in total DNA, *i.e.*, about 20–30% of the cytosine residues appeared to be methylated. For sponge gourd and ivy gourd the G + C contents as determined by HPLC agreed well with those determined by thermal denaturation. However, for ash gourd and ridge gourd the G + C contents calculated from melting studies (T_m) were 1–3% higher than the values obtained by HPLC. This may be due to the high cytosine methylation [2]. It has been reported for plant DNAs that the melting point of DNA increases only when *ca.* 30% of the cytosine residues are methylated [6]. For ridge gourd and ash gourd, the discrepancy in the G + C values determined by the two methods (higher values determined by T_m) can be correlated with the higher degree of methylation of cytosine residues. However, it is interesting that in ivy gourd, which has the smallest amount of repeated DNA sequences (more methylated than euchromatin) reported so far in higher plant species, is the least G + C rich among the cucurbits. In addition, probably among all the higher plants it has the smallest amount of m^5C.

Determination of m^5C in DNA by gas chromatography with electron-capture detection involves acid hydrolysis, derivatization, solid-phase extraction, HPLC and gas chromatography with electron-capture detection, whereas time-resolved fluoroimmunoassay (TR-FIA) involves generation of polyclonal anti-5-CH_3-dCytidine antibodies and also tagging of the sample and standard bases with a europium label. Both methods can be applied to the determination of the m^5C content in animal DNAs, which are known to be present in smaller amounts. The HPLC method described by Patel and Gopinathan [7] involves off-line operation to achieve a higher sensitivity of detection. However, the method reported here is highly practical, rapid and sensitive for the determination of m^5C contents in plant DNAs, which are known to contain large amounts [13].

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