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Determination of DNA base composition by reversed-phase high-performance liquid chromatography

(Enzymatic hydrolysis of DNA; nucleoside; nuclease P1; bacterial alkaline phosphatase)

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1. SUMMARY

DNA base composition was determined by reversed-phase high-performance liquid chromatography (HPLC). DNA was hydrolysed into nucleosides with nuclease P1 and bacterial alkaline phosphatase. The mixture of nucleosides was applied to HPLC without any further purification. One determination by chromatography needed 2 μg of hydrolysed nucleosides and took only 8 min. The relative standard error of nucleoside analysis was less than 1%. The system described here gives a direct and precise method for determining DNA base composition.

2. INTRODUCTION

DNA base composition is an important criterion in microbial taxonomy, and direct or indirect methods of determining guanine + cytosine (G + C) content have been reported [1–7]. Among them, the *t*m method [2], that is, indirect determination, is most popular because of its better reproducibility than direct methods. But the accuracy of the *t*m method is not so good as its reproducibility because different research groups assumed different values of G + C content for essentially the same DNAs, e.g. 50.1 (G + C) mol% was assumed for DNA from *Escherichia coli* K-12 in [2] and 51.0 (G + C) mol% in [8]. This problem is inevitable for an indirect determination.

Recently, reversed-phase HPLC has been applied to analysis of nucleosides from DNA or RNA [9–11], and complete hydrolysis of tRNA into nucleosides with nuclease P1 and bacterial alkaline phosphatase was studied by Gehrke et al. [12].

We have developed a direct method to determine DNA base composition by reversed-phase HPLC after complete enzymic hydrolysis of DNA into nucleosides.

3. MATERIALS AND METHODS

3.1. Bacterial strains and media

The bacterial strains used are listed in Table 1. All strains except *E. coli* IAM1264 and IAM1268 are the type strains. The media used were: nutrient broth (Kyokuto, Tokyo, Japan); marine agar (Difco); and YM broth for *Rhizobium* strains containing 5 g of mannitol, 5 g of lactose, 0.5 g of yeast extract (Difco), 0.5 g of K_2HPO_4, 0.2 g of NaCl, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of $FeCl_3 \cdot 6H_2O$, and 1 litre of dis-

tilled water, pH 7.0. Bacterial cells were harvested in the exponential phase of growth and washed twice by centrifuging with saline-EDTA buffer (0.15 M NaCl and 0.1 M EDTA, pH 8.0).

3.2. Isolation of DNA

DNA was isolated by the phenol method [13] with some modifications. For enzymic hydrolysis of RNA, ribonuclease T_1 (Sigma) was used together with ribonuclease A (Sigma).

3.3. Enzymic hydrolysis of DNA

DNA was hydrolysed into nucleosides with nuclease P1 (Yamasa, Chiba, Japan, EC 3.1.30.1) and bacterial alkaline phosphatase (Sigma, EC 3.1.3.1). This is the modified method to hydrolyse tRNA of Gehrke et al. [12]. DNA was dissolved in distilled water (1 mg/ml). Then the DNA solution was heated at 100 °C for 5 min, and cooled rapidly in an ice bath. The denatured DNA solution (10 μl) was mixed with 10 μl of nuclease P1 solution (0.1 mg dissolved in 1 ml of 40 mM sodium acetate buffer containing 2 mM $ZnSO_4$, pH 5.3), and incubated at 50 °C for 1 h. Then, 10 μl of bacterial alkaline phosphatase, 2.4 units/ml of 0.1 M Tris–HCl buffer, pH 8.1, was added to the

Table 1

Base compositions of DNAs determined by HPLC method and by *t*m method

Source of DNA [a]	G+C mol% determined by	
	HPLC	*t*m
Alcaligenes faecalis IAM12369^T [b]	56.6±0.1 [c]	58.0 [d]
Alcaligenes ruhlandii IAM 12600^T	68.8±0.5	69.2 [d]
Alteromonas espejiana IAM12640^T	41.0±0.1	43.1' [e]
Alteromonas haloplanktis IAM12915^T	42.1±0.3	41.5 [f]
Alteromonas macleodii IAM12920^T	45.3±0.4	46.4 [f]
Alteromonas rubra IAM12643^T	49.3±0.4	48.6 [f]
Arthrobacter globiformis JCM1332^T	65.9±0.4	66.5 [g]
Bradyrhizobium japonicum IAM12608^T	63.5±0.2	64.4 [h]
Escherichia coli IAM1264 (= strain K-12)	51.6±0.2	51.0 [i]
Escherichia coli IAM1268 (= strain B)	51.7±0.2	52.2 [f]
Micrococcus luteus IAM1056^T	73.6±0.2	73.5 [h]
Oerskovia turbata KCC A-160^T	72.3±0.2	71.5 [j]
Oerskovia xanthineolytica KCC A-164^T	74.2±0.3	75.5 [j]
Pseudomonas acidovorans IAM12409^T	67.1±0.2	66.6 [f]
Pseudomonas aeruginosa IAM1514^T	66.4±0.1	66.8 [f]
Pseudomonas palleronii DSM63^T	67.3±0.1	65.7 [f]
Rhizobium leguminosarum IAM12609^T	60.8±0.2	60.2 [h]
Rhizobium lupini IAM12610^T	64.6±0.3	64.8 [h]
Rhizobium meliloti IAM12611^T	62.5±0.3	61.8 [h]
Rhizobium phaseoli IAM12612^T	62.0±0.4	61.9 [h]
Rhizobium trifolii IAM12613^T	61.4±0.3	61.7 [h]
Xanthomonas maltophilia IAM12423^T	66.5±0.2	65.5 [f]

[a] Abbreviations: DSM, Deutsche Sammlung von Mikroorganismen, Göttingen, F.R.G.; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; JCM, Japan Collection of Microorganisms, the Institute of Physical and Chemical Research, Wako-shi, Saitama, Japan; KCC, Kaken Pharmaceutical Co., Ltd., Tokyo, Japan.

[b] T, type strain.

[c] Values are means of five determinations with standard error.

[d] From [14].

[e] From [15].

[f] From [16].

[g] From [17].

[h] From N. Oishi, unpublished data.

[i] From [8].

[j] From K. Suzuki, unpublished data.

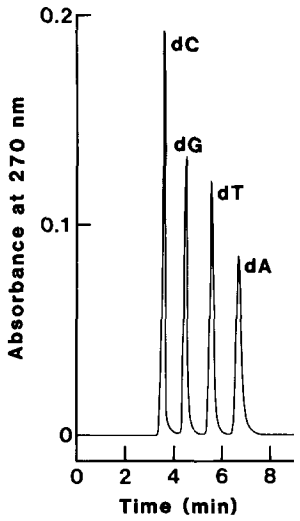

Fig. 1. HPLC chromatogram of a standard mixture of four deoxyribonucleosides: dC, 2′-deoxycytidine; dG, 2′-deoxyguanosine; dT, 2′-deoxythymidine; dA, 2′-deoxyadenosine. HPLC conditions are described in the text.

sample, and incubated at 37 °C for 1 h. The hydrolysate was stored at −20 °C, and 5 μl of the hydrolysate was applied to reversed-phase HPLC.

3.4. Conditions of HPLC

The HPLC system consisted of an LC-4A Liquid Chromatograph (Shimadzu, Kyoto, Japan), RADIAL-PAK C_{18} cartridge installed in Z-MODULE (Waters Associates), Lambda-Max Model 481 LC spectrophotometer (Waters Associates), and a data analyser Chromatopac C-R2AX (Shimadzu). The nucleosides were eluted by a mixture of 0.6 M $NH_4H_2PO_4$ (pH 4.0) and acetonitrile (20 : 1, v/v), at flow rate of 1 ml/min at room temperature. Each nucleoside was detected by its UV absorbance at 270 nm.

3.5. Standard mixture solution of four nucleosides

Four deoxyribonucleosides were obtained as crystals from Sigma. The water content of crystals of nucleosides was calculated from elemental analyses of carbon and nitrogen. Four nucleosides were weighed carefully and dissolved in distilled water, 0.1 mg of each/ml. After five runs of this mixture by HPLC, a molar absorption coefficient for each nucleoside was determined relative to that of 2′-deoxythymidine taken as 1000.

3.6. Calculation of G + C content

Relative amounts of nucleosides were determined on peak areas, which represented integrated absorbance at 270 nm, and on coefficients of relative molar absorption using the following equation: relative amount of nucleoside in mol

$$= \frac{\text{peak area}}{\text{coefficient of relative molar absorption}}$$

DNA base composition was calculated as follows:

$$\text{G + C mol\%} = \frac{\text{Gr + Cr}}{\text{Ar + Gr + Cr + Tr}}$$

(Nr = relative amount of each nucleoside in mol)

4. RESULTS

4.1. Separation of nucleosides on chromatogram

Fig. 1 shows an HPLC chromatogram of a standard mixture. The four nucleoside peaks were well separated and eluted within 8 min.

4.2. G + C mol% of DNA determined by HPLC

Table 1 records G + C contents of DNAs from 22 strains of bacteria. The two strains of *E. coli* showed almost the same values of G + C mol%. The differences of the two values determined by the HPLC method and by the *t*m method were about 1% or less with a few exceptions. The value determined by the HPLC method was the mean of the data of five different hydrolysates of the same DNA sample with the standard error as shown in Table 1. The relative standard error of the HPLC method was less than 1% for all determinations. This shows high reproducibility of enzymic hydrolysis of DNA and reversed-phase HPLC determination.

5. DISCUSSION

Various methods of analysis of G + C content have been reported, and the *t*m method is most popular although it is an indirect determination. Direct determination is preferable to indirect because of the definition of 'G + C content'. Ion-exchange HPLC determination of bases after formic acid hydrolysis of DNA has been reported [4], in

which one chromatography run took only 8 min, but the relative standard error of repeated HPLC runs was about 2%, while that of the *t*m determination was about 1% [18]. Another ion-exchange HPLC determination of nucleotides after enzymic hydrolysis of DNA has been reported [7], and its relative standard error was not less than 3%.

In our method, relative standard errors of G + C mol% determination were from 0.1 to 0.9%, which were calculated from the data of five different hydrolysates from the same DNA sample. Therefore, this procedure, consisting of enzymic hydrolysis of DNA and reversed-phase HPLC determination of nucleosides, has high reproducibility.

In quantitative analysis of a mixture of nucleosides by HPLC, use of a standard mixture solution is indispensable, because there are many factors affecting UV absorbance of each nucleoside such as pH of eluent, width of wavelength of a detector, cell shape of a detector, etc. The total of these factors can be determined as a coefficient of relative molar absorption by analysis of a standard mixture solution. Therefore, a standard mixture must be carefully prepared, especially in estimating the water content of nucleoside crystals. This was determined by elemental analysis in our study.

The DNA base compositions determined by the HPLC method showed good agreement with those determined by the *t*m method (Table 1). DNAs from some strains of the genus *Bacillus* had high amounts of modified nucleosides (not shown), and the values of G + C mol% did not agree with those determined by the *t*m method. There may be some bacterial strains in which DNAs represent high fractions of the modified nucleosides, and the values of G + C mol% of such DNAs determined by the *t*m method would be better examined by a direct method.

If the sample DNA was contaminated with RNA, ribonucleosides from the RNA could be detected on the chromatogram. For example, the peak of adenosine would appear between peaks of 2′-deoxythymidine and 2′-deoxyadenosine in this system. The DNA samples used in this study were not contaminated with RNA.

In conclusion, this HPLC method has the following advantages in determining the DNA base composition. (1) The HPLC method is a direct determination. (2) The relative standard error of the HPLC method is $< 1\%$. (3) RNA contamination and modified nucleosides are detected by the HPLC method.

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REFERENCES

[1] Fredericq, E., Oth, A. and Fontaine, F. (1961) J. Mol. Biol. 3, 11–17.
[2] Marmur, J. and Doty, P. (1962) J. Mol. Biol. 5, 109–118.
[3] Schildkraut, C.L., Marmur, J. and Doty, P. (1962) J. Mol. Biol. 4, 430–443.
[4] Ko, C.Y., Johnson, J.L., Barnett, L.B., McNair, H.M. and Vercellotti, J.R. (1977) Anal. Biochem. 80, 183–192.
[5] Kaneko, T. (1977) Agric. Biol. Chem. 41, 2277–2283.
[6] Cashion, P., Holder-Franklin, M.A., McCully, J. and Franklin, M. (1977) Anal. Biochem. 81, 461–466.
[7] Katoh, K., Suzuki-Onozaki, A., Ohta, T., Ebine, H., Kumagai, M., Fujimoto, M. and Kuninaka, A. (1983) Rept. Natl. Food Res. Inst. No. 43, 79–89.
[8] Snell, J.J.S. and Lapage, S.P. (1976) Int. J. Syst. Bacteriol. 26, 451–458.
[9] Zumwalt, R.W., Kuo, K.C.T., Agris, P.F., Ehrlich, M. and Gehrke, C.W. (1982) J. Liq. Chromatogr. 5, 2041–2060.
[10] Buck, M., Connick, M. and Ames, B.N. (1983) Anal. Biochem. 129, 1–13.
[11] Gomes, J.D. and Chang, C.-J. (1983) Anal. Biochem. 129, 387–391.
[12] Gehrke, C.W., Kuo, K.C., McCune, R.A., Gerhardt, K.O. and Agris, P.F. (1982) J. Chromatogr. 230, 297–308.
[13] Saito, H. and Miura, K. (1963) Biochim. Biophys. Acta 72, 619–629.
[14] Yamasato, K., Akagawa, M., Oishi, N. and Kuraishi, H. (1982) J. Gen. Appl. Microbiol. 28, 195–213.
[15] Chan, K.Y., Baumann, L., Garza, M.M. and Baumann, P. (1978) Int. J. Syst. Bacteriol. 28, 217–222.
[16] De Vos, P. and De Ley, J. (1983) Int. J. Syst. Bacteriol. 33, 487–509.
[17] Skyring, G.W. and Quadling, C. (1970) Can. J. Microbiol. 16, 95–106.
[18] De Ley, J. (1970) J. Bacteriol. 101, 738–754.