— **Article** —

Spectrofluorometric determination of DNA with resorcinol and propionaldehyde

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Abstract In A highly sensitive spectrofluorometric method for the determination of DNA is developed. The proposed method is based on measuring a fluorescence product provided by condensation reaction between resorcinol and propionaldehyde in the presence of trace amounts of DNA. Beer's law is obeyed in the concentration range of 0.5 — 100 pg cm^{-3} DNA (Herring Sperm). The procedure is about 10^4 times more sensitive than the conventional fluorometries.

Key words — spectrofluorometry, DNA determination, resorcinol, propionaldehyde

Introduction

Recently, the study of the relationship between genes and disease has progressed. Identification of causal genes in diseases such as autoimmune diseases, Huntington's chorea and familial adenomatous polyposis has been completed; hence, the risk of the disease has been predictable prior to the disease onset. It has been considered that the pre-onset evaluation (genetic diagnosis) of many other diseases including diabetes mellitus or cancers would be possible in the future as well as the drug development based on genetic information, regenerative medicine and genetic treatment due to the progress of genetic technology. Moreover, DNA tests have been widely used not only in the criminal cases but also in the civil cases. The application of DNA analysis[1, 2] in various fields other than *in vivo* settings, such as the test for genetically modified food, has been also developed as well.

The analysis or quantification of DNA or RNA, such as a quantitative detection of virus genome, has been possible by using PCR (polymerase chain reaction) method[3] which enables amplification of the small amount of DNA. However, PCR method requires a complicated procedure and has some disadvantages; the amount of PCR amplified products does not reflect the amount of the original template DNA due to the problems such as a plateau effect or the difference in amplification efficiency between the tubes, including the efficiency in annealing or DNA (chain) synthesis.[4] Therefore development of the highly sensitive DNA analysis that can detect a very small amount of DNA has been desired without using PCR method.

On the other hand, Baeyer[5] synthesized fluorescein, a strong fluorescent substance, by using the condensation reaction between resorcinol and phthalic anhydride in

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the presence of zinc chloride as a condensing agent, as shown in Fig 1. Besides, we established[6] the simple and sensitive fluorophotometric determination of aldehyde by utilizing condensation reaction with resorcinol.

Therefore, it is presumed that it is possible to develop a fluorophotmetry of DNA by utilizing the condensation reaction between resorcinol and aldehyde in the presence of DNA containing pyrophosphate residues as a condensing agent,

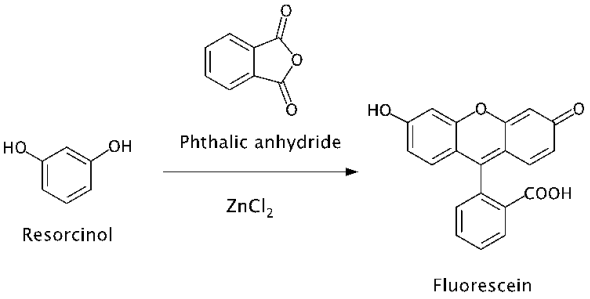

Fig. 1 Fluoresein synthesis scheme

Materials and Methods

Apparatus

A Hitachi fluorescence spectrophotometer, Model F-4500, with 1.0-cm matched silica cells was used for fluorescence intensity and fluorescence spectra. pH measurements were carried out with a Horiba (F-11) pH meter equipped with a calomel glass electrode.

Reagents and Solutions

A stock solution of DNA was prepared by dissolving DNA (Sigma Chemical Co.) in water, and the working solution was prepared by dilution of this stock solution in methanol as required. A 1.0×10^{-1} mol dm^{-3} resorcinol solution, a 5.0×10^{-2} mol dm^{-3} propionaldehyde solution and a 1.0×10^{-1} mol dm^{-3} tetrabuthylammonium hydroxide (TBAH) solution were prepared in methanol. All other chemicals used were analytical reagent grade unless otherwise stated. Distilled methanol was used in the preparation of all solutions.

Standard procedure for determining DNA

A 1.0 mL of 1.0×10^{-1} mol dm^{-3} resorcinol solution, 1.0 mL of 5.0×10^{-2} mol dm^{-3} propionaldehyde solution and 0.15 mL of 1.0×10^{-1} mol dm^{-3} TBAH solution were added to the test tube with a stopper. Then, 1.0 mL of DNA containing solution was added and stirred well. As a next step, a semi-micro distillation tube was attached to the test tube with a stopper, and the heating reaction was allowed to occur for 30 minutes at 185℃ in the aluminum block, temperature control unit. The distillation tube was washed well with a certain amount of methanol, and the solution obtained, which contains the methanol, was prepared to be the sample solution with a total amount of 10 mL. Afterward, the fluorescent intensity of the reagent blank similarly solution (B) prepared in the same way and the sample solution (S) were measured at 380 nm of the excitation wavelength and 457 nm of the emission wavelength, and then, the DNA concentration was obtained from the calibration curve from which the value of (S–B) / B was calculated.

Results and Discussion

This time, a condensation reaction of resorcinol derivative and aldehyde derivative was studied systematically. As a consequence, it was found that the condensation reaction was accelerated and that fluorescent substance was produced efficiently in the presence of trace amount of DNA; hence, the spectrofluorometric method for the determination of DNA, using this reaction system, was designed to be discussed.

At first, the following aldehyde groups, the chemical species which goes through the condensation reaction with resorcinol, were assessed: propionaldehyde, furfural, 4-(dimethylamino)- benzaldehyde, *p*-anisaldehyde, *N*-ethylcarbazole-3-carboxyaldehyde, vanillin, piperonal, crotonaldehyde, cinnamicaldehyde, *o*-phthalaldehyde and formylbenzoic acid. As a result, it was found that the fluorescent substance with the greatest fluorescent intensity was produced when propionaldehyde, the aliphatic aldehyde, was used.

Then, the following resorcinol derivatives, the reactive chemical species which goes through the condensation reaction with propionaldehyde, were assessed: resorcinol, γ-resorcylic acid, 4-bromoresorcinol, 2-nitroresorcinol, orcinol, pyrogallol, pyrocatecol and phloroglycinol. In this reaction system, the fluorescent intensity of the condensation product of the sample solution was particularly high when reacted with resorcinol, and the value of [(fluorescent intensity of sample solution – fluorescent intensity of blank solution) / (fluorescent intensity of blank solution)]; namely, (S–B) / B, was also the largest.

It is known that the condensation fluorescent reaction of resorcinol derivative and aldehyde derivative is emphasized in acidic or basic medium[7]; hence, the condensation reactions of resorcinol and propionaldehyde were compared under various conditions and discussed in the presence of DNA. When hydrochloric acid solution, acetic acid solution, sodium bicarbonate solution, ammonia solution, sodium hydroxide solution, potassium hydroxide solution, lithium hydroxide solution, tetrabuthylammonium hydroxide (TBAH) solution, tetramethylammonium hydroxide solution, tetraethyl ammonium hydroxide solution and sodium methoxide solution were assessed, high fluorescent intensity was seen in the strong basic medium. The results of effect of strong bases are given in Table 1. Out of all, the maximum value of (S–B) / B was obtained when TBAH solution was used. Considering the fluorescent intensity of the fluorescent substance being produced or the total amount of the reaction mixture, the optimal concentration of TBAH solution was observed when 0.15 mL of 1.0×10^{-1} mol dm^{-3} TBAH solution was added.

Table 1 Effect of various strong bases

Bases	(S − B) / B
TBAH	7.84
tetraethylammonium hydroxide	4.98
tetramethylammonium hydroxide	0
sodium methoxide	1.36
potassium hydroxide	1.04
sodium hydroxide	0.88
lithium hydroxide	0.03

DNA：10 pg/mL；resorcinol：1.0×10^{-1} mol dm^{-3}；propionaldehyde：5.0×10^{-2} mol dm^{-3}；
bases：X mL of 1.0×10^{-1} mol dm^{-3}：EX 380 nm, EM 457 nm

As for the solvent used for the condensation reaction, the following water-soluble solvents were considered: methanol, ethanol, ethylene glycol and water. The excellent reagent solubility and the maximum (S–B) / B value of the products were observed when methanol was used; therefore, methanol was selected as the reaction solution and as the reagent solvent.

For this condensation reaction, the temperature range of 170 – 200℃ and the heating time of 30 minutes were selected as the reaction temperature and the reaction time and assessed considering the rapidity, simplicity and the usefulness of the analytical procedure. As a result, at the heating temperature of 185℃, the maximum fluorescent intensity was obtained with the condensation products and an excellent solubility to the solvent was observed after being placed under the room temperature. Furthermore, when the reaction time of 25 – 40 minutes was set, and the heating time was re-assessed at the reaction temperature of 185℃, the maximum value of (S–B) / B with the condensation products was obtained with 30 minutes of heating. Since it was confirmed that the fluorescent intensity, after being placed under the room temperature, had been nearly consistent over 2 hours, 30 minutes of heating reaction at 185℃ was selected.

Following the above mentioned standard procedure for the determination of DNA, the fluorescent spectrum and the excitation spectrum of the blank solution and

the DNA containing solution were measured. As a result, as described in Figure 2, the maximum emission wavelength seemed to be around 457 nm (380 nm for the excitation wavelength), and the fluorescent intensity was found to be dependent on the amount of DNA. In addition, the photograph of sample and reagent blank solutions is shown in Photo 1.

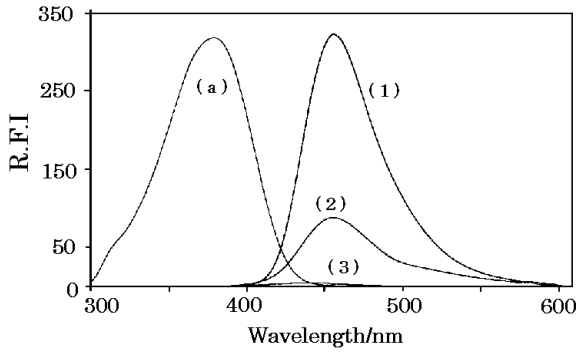

Fig. 2 Fluorescence excitation and emission spectra

Curves (1) and (a), respectively, emission and excitation spectrum in the presence of DNA (10 pg); Curve (2); emission spectrum in the presence of DNA (5 pg); Curve (3); emission spectrum in the absence of DNA (reagent blank)

Reagent Blank Sample (10 pg DNA)

Photo 1. Photograph of sample and reagent blank solutions

The calibration curve for DNA (Herring Sperm) was constructed by the standard procedure. A good linear relationship which shows a sufficiently high correlation with the correlation coefficient of r = 0.999 was obtained within the wide concentration range of 0.5 — 100 pg/mL. This method is about 10^4 times more sensitive than the high sensitive fluorometry using SYBR Green Ⅱ dye[8] or ruthenium complex[9]. Also, the relative standard deviation (RSD) with 10 pg/mL of DNA was 1.22% (n=5), showing excellent reproducibility.

The coexisting substances which may affect the results were studied under the condition with 10 pg/mL of DNA for the purpose of applying this method to the biological samples. Upon the assessment of the coexisting substance, the methanol-soluble substances were mainly studied; no effect was observed when a large amount of ammonium chloride, sodium chloride, sodium nitrate, calcium chloride, magnesium sulfate, zinc acetate, cobalt chloride, glucose, glycine, ascorbic acid, caffeine, sodium citrate and human serum albumin were added. In addition, the effect was hardly seen with any coexisting substances, including copper (II) ion or iron (III) ion; it was confirmed that there was very little effect with the coexisting substances.

In conclusion, it was found that, in the presence of trace amounts of DNA, the condensation reaction between resorcinol and propionaldehyde with heat was accelerated and that the fluorescent substance was efficiently produced; thereby the spectrofluorometric method for the determination of ultra-trace DNA was established. Though it is necessary to isolate and structurally analyze the fluorescent substance produced by this reaction, the reaction scheme can be guessed as shown in Fig. 3.

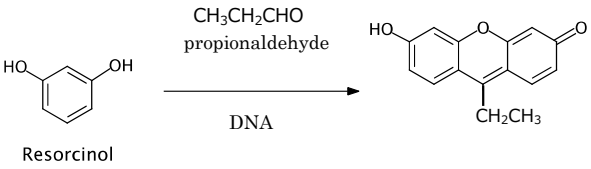

Fig. 3 Product reaction scheme

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