Studies on Radioactive Iodination of Fluorescein Isothiocyanate

KAORU SAGISAKA and HIROKO IWABUCHI

Department of Legal Medicine, School of Medicine, Iwate Medical University, Morioka*

SAGISAKA, K. and IWABUCHI, H. *Studies on Radioactive Iodination of Fluorescein Isothiocyanate*. Tohoku J. exp. Med., 1971, **105** (4), 397-403 — The present paper deals with radioactive iodination of fluorescein isothiocyanate (FITC) in chloroform. In the procedure, FITC in phosphate buffer at pH 7.0 was added to radioactive iodine at molecular ratio 1:1.5 or more. Some derivatives containing various numbers of iodine were prepared at the reaction in accordance with the molecular ratio. Starch-gel electrophoresis, thin layer chromatography, spectrophotometric analysis, infrared spectra, elementary analysis and nuclear magnetic resonance of the products showed that several derivatives incorporated with various numbers of iodine were synthesized and that isothiocyanate group of most of the derivatives was not separated at the reaction. ——— radioactive iodination; fluorescein isothiocyanate

The immunofluorescein technique developed first by Coons *et al.* (1942) has been widely applied in studies of antigen-antibody reactions. In the technique, antibody or antigen labeled with fluorescent dyes, usually fluorescein isothiocyanate (FITC), is traced with a fluorescence microscopy. A number of merits of the technique have been pointed out. For example, the dyes couple easily with proteins, and the immunological activities of the proteins are not changed by the labeling. On the other hand, it has been noted that the techniques have some disadvantages in quantitative analysis and in observation of the changes of antigen and antibody reactions with time. It is regarded that these hard points in the technique may be supplemented partly by radioisotope labeling method. From this point of view, Schneck *et al.* (1966) synthesized radioactive FITC from ^{14}C -labeled benzene. The procedure to incorporate radioactive iodine into FITC is described in this paper.

MATERIALS AND METHODS

Main reagents used were as follows; iodine, chloroform, ethyl acetate, hydrochloride (reagent grade), FITC (Tokyo Kasei Co., Tokyo) and radioactive sodium iodide (Dainabot Radioisotope Lab. Ltd., Tokyo).

Substitution of ^{131}I of radioactive sodium iodide for molecular iodine: In many experiments, 2 mCi of sodium iodide in 0.05% sodium sulphite were added to 2 ml of distilled water. The solution was mixed with 2 mg of I_2 in 6.2 ml of chloroform and stirred for 5

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* Director: Prof. S. Katsura.

TABLE 1. *Iodination procedure of FITC*

Mixture of A and B		
shaking for 10 min at room temperature on a magnetic stirrer		
centrifuged 1,000 rpm for 5 min		
Precipitate (P ₁) between water and chloroform layer		Aqueous layer (S ₁)
dissolved in 0.01 M phosphate buffer, pH 7.0		washed with chloroform 3 times, centrifuged 1,000 rpm 10 min
centrifuged 1,000 rpm for 10 min		Aqueous layer (S ₂)
		add same volumes of ethyl acetate and 0.1 N HCl
		shaking for 5 min. at room temperature
		centrifuged 1,000 rpm 10 min
Precipitate (P ₂)	Supernatant (S ₅)	Ethyl acetate layer (S ₃)
dissolved in ethylalcohol		washed with 0.1 N HCl 3 times
evaporated in vacuo	same procedure as for aqueous layer	Ethyl acetate layer (S ₄)
		dehydrated with Na ₂ SO ₄
		evaporated in vacuo
P ₃	S ₆	I-FITC
A: FITC as 0.1% solution in 0.01 M phosphate buffer pH 7.0		
B: Radioactive iodine as 0.05% solution in chloroform		

TABLE 2. *Elementary analysis of I-FITC prepared at the molecular ratio 1:1.5*

Analysis	Sample (mg)	CO ₂ mg	H ₂ O mg	C %	H %
H and C	3.535	5.747	0.874	44.36	2.77
		(T+t ₂)°C	PmmHg	N ₂ ml	N%
N	2.968	296.8	754.9	37.5	1.44

minutes. After centrifugation at 1,000 rpm for 5 minutes, an aqueous layer was discarded. Then a chloroform layer was washed with aqua dist. 3 times.

Iodination of FITC: Procedures of the iodination were shown in Table 1. In many experiments, the molecular ratio of FITC to iodine was 1:1.5. When the ratio was less than 1.5, some FITC which was not iodinated was electrophoretically observed.

Thin layer chromatography (TLC): Silica Gel G (E. Merk AG. Darmstadt) was coated on glass plates 20×5 cm in size, and 25 μ in thickness. A drop of the sample dissolved in chloroform was applied in the origin. The plates were developed by ascending method using the following solvent: buthanol, H₂O, ethanol and conc. NH₄OH in the proportion of 200:80:40:2. After developing, the thin layers were examined under ultraviolet ray.

Spectrophotometric analysis: The absorption spectra of FITC and its derivatives over the range from 250 to 600 mμ were obtained using Hitachi 101. The samples were measured at a concentration of approximately 10 μg/ml in 0.01 sodium phosphate buffer, pH 7.0.

Starch-gel electrophoresis: Discontinuous starch-gel electrophoresis was carried out using buffers (gel buffer: boric acid-lithium hydroxide, Tris-citric acid, pH 8.1; bridge buffer:

barbital-barbital sodium, pH 8.6, $\mu=0.05$) and a thin gel block $10 \times 20 \times 0.3$ cm in size for 5 hours at 5.5 v/cm. The mixture of human albumin prepared by DEAE-cellulose column chromatography (Sober *et al.*, 1956) with a small amount of bromophenol blue was simultaneously applied. After electrophoresis, the gel was cut into 0.5 cm segments, and radioactivities of the segments were counted with a well type scintillation counter (Aloka TDC-6). The mobilities of samples were expressed as ratio to the human albumin.

Infrared (IR) spectra: IR spectra measurements were made using a spectrophotometer (Hitachi EP₁-G₂) with microtablets of potassium bromide which contained the dyes at approximately 1%.

Elementary analysis: The analysis of I-FITC prepared at the molecular ratio of FITC and iodine 1:1.5 was performed. The material was re-crystallized repeatedly with acetone before the analysis.

Nuclear magnetic resonance (NMR): NMR spectra of the sample from the same lot examined in the elementary analysis and FITC were recorded using a spectrometer (Hitachi H-60).

RESULTS

After the substitution, measurements of radioactivities of the aqueous and chloroform layers showed that about 95% of radioactive iodine changed into molecular forms. The fluorescence of FITC became obscure, and pink color was revealed with the progress of the iodination of FITC. When the ratio of FITC and iodine in molarity was high, the color of iodine in chloroform did not disappear because of remaining of molecular iodine in chloroform. At the time when ethyl acetate and 0.1 N HCl were added to S₂, the product became colorless and transparent. Once ethyl acetate was evaporated *in vacuo*, a light crimson of I-FITC and S₆ and a deep crimson of P₃ were brought out again. When the iodination was done at the ratio of FITC and iodine 1:2 or more, a relatively large amount of an insoluble residue (P₁) was noted between chloroform and aqueous layer. Applying ultraviolet ray to the products in the iodination, FITC emitted extremely strong fluorescence, I-FITC and S₆ very slight, but P₃ no fluorescence. I-FITC and S₆ were very soluble in organic solvents and alkaline buffers and slightly in distilled water. But the solubility of P₃ was considerably low.

A neat, strongly fluorescent spot of FITC, two or three spots of I-FITC and S₆ synthesized at the molarity 1:1.5 were observed in the TLC. However, P₃ showed an indistinct, undeveloped spot at the origin. The R_f values of the spots were as follows: FITC was 0.43, and I-FITC and S₆ were 0.38 and 0.28, respectively. A chloroform layer of the solvent disturbed frequently the patterns of the chromatographies, and each shave of the spots had radioactivity.

Absorption spectra indicated, as shown in Fig. 1, that the absorption peaks of FITC, I-FITC and P₃ were at 490, 505 and 510 m μ , respectively. The shapes of spectra of I-FITC and P₃ were essentially identical with that of FITC.

Each of the products had different mobilities in starch-gel electrophoresis. FITC had a neat band, proportional mobility of which was 0.95. The mobilities of I-FITC and S₆ produced at relatively low molecular ratios were 0.88 and 0.58

Fig. 1. Absorption spectra of FITC, I-FITC and P_3 .

Fig. 2. An autoradiography of ^{131}I -FITC and ^{131}I -labeled human albumin after the gel electrophoresis. The labeling of human albumin was carried out by the method of Hunter and Greenwood (1962). After electrophoresis, the gel is contacted with a X-ray film and exposed for 1 day. Then, the film is developed and fixed. Above, ^{131}I -FITC; below, ^{131}I -labeled human albumin.

(Fig. 2), but the products at the ratio of 1:2 or more had several slowly moving bands, whereas P_3 almost stayed at the origin.

The infrared analysis of FITC and its derivatives showed spectra as follows (Fig. 3): FITC had a strong absorption peak of isothiocyanate with a maximum at $2,050\text{ cm}^{-1}$, sharp ones of lactone carboxyl group at $1,760\text{ cm}^{-1}$ and an aromatic ring system at $1,660\text{ cm}^{-1}$. I-FITC and S_6 had a relatively low peak at $2,050\text{ cm}^{-1}$ and the peaks which were almost the same in sharpness and intensity to those of the FITC at $1,800$ to $1,600\text{ cm}^{-1}$. In the spectrum of P_3 , however, no absorptions of isothiocyanates and aromatic ring groups were observed. The elementary analysis indicated that percentages of C, H and N of I-FITC were 44.36, 2.77 and 1.44, respectively. The proton peak of FITC was observed at 7–9 ppm and that of I-

Fig. 3. Absorption spectra of FITC and products of the iodination I-FITC and P_3 by micro tablet method of potassium bromide.

FITC was at 6–8 ppm in the nuclear magnetic resonance. All spectral patterns of the samples except a proton peak was almost identical.

DISCUSSION

The synthesis of fluorescein and rhodamine isothiocyanate by Riggs *et al.* (1958) offered a mean to obtain the simple conjugations of proteins with the fluorescent markers. These fluoresceins had been widely applied to immunobiology. On the other hand, Moore (1948) reported that radioactive sodium diiodofluorescein was easily prepared from sodium fluorescein with radioactive iodine. Further studies on radioactive sodium diiodofluorescein were described by the previous investigators (Vigne and Fondarai 1957, Kato and Kurata 1965). Present studies showed that the iodination of FITC occurred in the same way as sodium diiodofluorescein. It seemed that the number of iodine atom being incorporated into FITC changed with the variation of molecular ratio between FITC and iodine. It was confirmed by TLC and starch-gel electrophoresis that several derivatives containing various numbers of iodine atom were prepared by the iodination. In view of the fact that a relatively large amount of P_3 was produced in the reaction at the ratio of 1:2 or more, the derivative P_3 might be one which incorporated iodine more than 3 or 4 atoms. Kato and Kurata (1965) reported that tri- or tetra-iodo-fluorescein was synthesized in the iodination of sodium fluorescein, and different colors of these products were observed in the TLC. In the iodination of FITC, the color difference between I-FITC and P_3 was observed distinctly, but several derivatives

of I-FITC did not show clear color difference on TLC. In the absorption spectra, the shifts of maximal absorption peaks of the products to the longer wavelength due to the halogenization were obvious. The maximum absorption of P_3 showing no IR absorption patterns of isothiocyanate group shifted considerably to the longer wavelength. Therefore, it is considered that the more iodine is incorporated into FITC, the more the absorption peak of the product shifts to the longer wavelength. It is essential to identify that an isothiocyanate group conjugating with amino groups of proteins was not separated in the process of iodination. Infrared analysis, which was one of the most available methods for determination of isothiocyanate group (McKinney *et al.* 1964, McKinney *et al.* 1969), showed that I-FITC and S_6 had the maximum absorption peak of isothiocyanate group at $2,050\text{ cm}^{-1}$. The results obtained by the elementary analysis might not be accurate since the sample contained some impurities. The material was so soluble in organic solvents that it was impossible to separate them each other. If I-FITC was the mixture of products which incorporated 1 to 2 iodine atoms, percentages of C, H and N should theoretically be 44.1, 1.65 and 2.45. The values of C and H almost agreed to the values obtained by analysis of the samples. Although the N-value of the the sample was lower than the theoretical one, it still indicates that isothiocyanate group existed in the molecule of I-FITC. It was admitted that iodine atom existed in I-FITC and isothiocyanate group of FITC was not separated by the iodination. NMR showed that proton peaks of FITC and I-FITC were at 7–9 and 6–8 ppm, respectively. Since I-FITC was not regarded as a simple material and a molecular weight of H-ion or I-ion was relatively small compared with those of FITC and I-FITC, the results offered a little information about the molecular structure. But it was conceivable that the shift of proton peak to low magnetic field was due to the influence of iodination. It was concluded that the iodinations of FITC produced several derivatives which incorporated various numbers of iodine, and that isothiocyanate group of most of the derivatives was not separated in the reactions. The studies on the conjugative potencies of the derivatives with serum proteins are now in progress.

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