A Novel Fluorogenic Substrate for Horseradish Peroxidase : Efficient Detection of Membrane-Bound Nucleic Acids and Simultaneous Detection of DNAs

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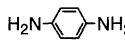
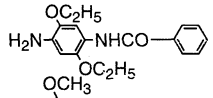
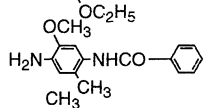
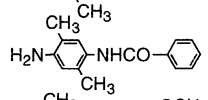
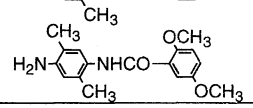
Aniline derivatives were found to be transformed to fluorescent compounds on treatment with horseradish peroxidase/H_2O_2. DNA was detectable with high resolution in the horseradish peroxidase-linked immunosorbent fluorescence assay of the membrane-bound DNA using one of the aniline derivatives. A new method for a simultaneous detection of two different DNA sequences was also achieved.

There is an increasing demand for the non-radioactive nucleic acid probe in the detection of specific complementary nucleic acid sequences. Nonradioactive labeling techniques so far reported involve colorimetric and fluorescence detections which lack the sensitivity required, *e.g.*, for the detection of a single copy gene in human genomic DNA in the absence of the polymerase chain reaction amplification technique.[1] Although fluorescence assays of the DNA sequence have been studied[2], there are few reports on the fluorescence immunoassay, *e.g.*, the europium[3] and terbium chelates[4] are used as fluorochromes to detect DNA hybrids in solutions. All these methods are based on the use of water-soluble fluorescent compounds. Recently, we reported the first successful study on a fluorescence immunoassay for detection of membrane-bound nucleic acids using phosphates derived from hydroxynaphthalenecarboxamide[5] and fluorescein derivatives[6] as fluorogenic substrates for alkaline phosphatase. Nucleic acids could be detected by the fluorescence immunoassay with comparable or even better sensitivity than the luminescence detection method.[7] There is a demand for the detection of multiple target DNA sequences on single Southern and northern blots as well as on single metaphase preparations. We have already developed a sequential detection of two different DNA sequences by using the complementary probes bound to two different antigens, biotin and digoxigenin.[8] The simultaneous detection of two different DNA sequences can be realized by using two different kinds of enzymes, *e.g.*, alkaline phosphatase and horseradish peroxidase. We now wish to report a highly sensitive detection of DNA on a nylon membrane by the use of aniline derivatives newly found as fluorogenic substrates for horseradish peroxidase as well as a simultaneous detection of two different DNAs.

We searched for compounds as a substrate for horseradish peroxidase by the spot test and found some aniline derivatives reactive toward the enzyme. Thus, a series of different amounts of horseradish peroxidase was spotted on a nitrocellulose or nylon membrane, and incubated with aniline derivatives in the presence of H_2O_2. The results using some of the aniline derivatives are listed in Table 1. Compounds **1**, **2**, and **3** precipitated and stained the spot brown or purple after incubation with horseradish peroxidase. It is surprising that only compounds **4**, **5**, and **6**, among the aniline derivatives tested, yielded fluorescent compounds by peroxidase oxidation and showed fluorescence on the spot. Fluorescence signals on the membrane were measured by an epifluorescence detector.[9] When the nitrocellulose membrane was used, horseradish peroxidase could be detected to the amount of 0.01 μg in all cases. On the other hand, the nylon membrane-bound horseradish peroxidase was detectable to the amount of 0.01 μg only by using compounds **1** and **4**. In contrast to the low substantivity in the case of compound **1**, compound **4** gave distinguishably clear spots with high fluorescence and without significant diffusion. In addition, as can be seen in the cases of compounds **4**, **5**, and **6**, a slight change in structures has a great influence on the sensitivity mainly due to the change in substantivity. In order to confirm the

Table 1. Detection of Membrane-Bound Horseradish Peroxidase Using Aniline Derivatives **1-6**

No.	Compound	nitrocellulose membrane		nylon membrane		color / fluorescence
		detection limit, μg	substantivity	detection limit, μg	substantivity	
1		0.01	very low	0.01	very low	brown / none
2		0.01	moderate	1	moderate	brown / none
3		0.01	high	0.1	high	purple / none
4		0.01	high	0.01	high	yellow / fluorescence
5		0.01	moderate	0.1	moderate	yellow / fluorescence
6		0.01	moderate	0.1	moderate	yellow / fluorescence

structure of the fluorescent compound formed by oxidation with horseradish peroxidase, **4** was incubated with horseradish peroxidase and 0.003% H_2O_2 in a phosphate buffer at pH 7.2 for 5 min. The isolated fluorescent compound **7** was a dimeric azo compound,[10] which showed an excitation maximum at 312 nm and an emission maximum at 440 nm in dimethylformamide. This is, to our knowledge, the first fluorescent compound which has an azo moiety in the structure.

We next studied the DNA detection using the substrates for horseradish peroxidase bound to a DNA probe through the digoxigenin-antidigoxigenin antibody. They were subjected to the spot test.[11] Among the compounds tested, compound **4** again gave distinguishably clear spots without diffusion and nonspecific adsorption. λDNA was detectable to the amount of 0.4 pg. It should be noted that **4** is non-fluorescent and, thus, a highly efficient off-and-on system for fluorescence could be attained before and after oxidation with horseradish peroxidase.

DNA could be detected to the amount of 0.7 pg (2.5×10^{-19} mol)[12] as orange fluorescence (λex = 298 and 418 nm, λem = 530 nm) in Southern blot hybridization[13] using **4** as shown in Figure 1. Next, we examined a simultaneous detection of two different DNA sequences using the fluorogenic substrate **4** for horseradish peroxidase and our fluorogenic substrate, phosphorylated hydroxynaphthalenecarboxamide,[5] for alkaline phosphatase. Horseradish peroxidase is bound to the *Eco* RI-digested λDNA probes through digoxigenin-antidigoxigenin antibodies, whereas alkaline phosphatase is bound to the *Eco* RI-digested Col EI and the *Eco* RI-digested pBR322 probes through fluorescein-antifluorescein antibodies.[14] As shown in Figure 2, the *Eco* RI-digested λDNA appeared as five orange bands of 21, 7.4, 5.8/5.6, 4.9 and 3.5 kbs, where bands of 5.8 and 5.6 kbs appeared as an unseparated band. A mixture of the *Eco* RI-digested Col EI and the *Eco* RI-digested pBR322 appeared as two bluish-green bands of 6.6 and 4.4 kbs. Thus, two different target DNAs could be detected simultaneously as orange and bluish-green signals of fluorescence under a ultraviolet light (312 nm).

In summary, the present study provides a new fluorescence assay of membrane-bound DNAs using a new fluorogenic substrate for horseradish peroxidase which enables the simultaneous detection of two different DNAs.

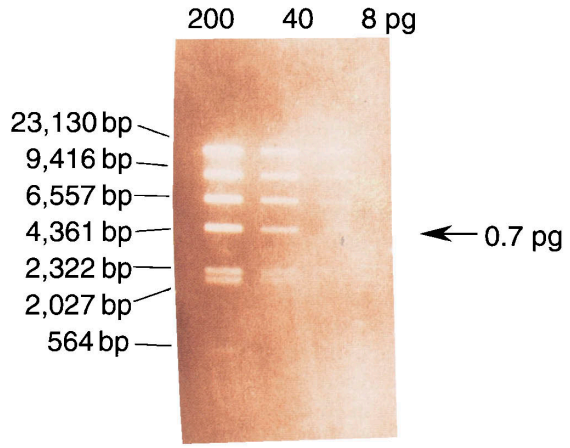


Figure 1. Southern blot hybridization. A dilution series of *Hind* III-digested λDNA was electrophoresed, transferred onto a nylon membrane, hybridized with the digoxigenin-labeled *Hind* III-digested λDNA, and incubated with horseradish peroxidase-labeled anti-digoxigenin antibodies. Fluorescence detection was carried out with compound **4**.

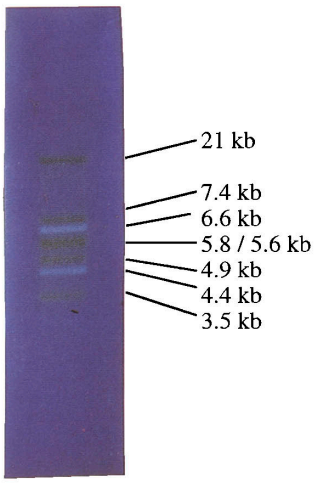


Figure 2. A simultaneous detection of two different DNAs on a single blot. The orange fluorescent bands for the *Eco* RI-digested λDNA, and the bluish-green fluorescent bands for the *Eco* RI-digested Col EI and the *Eco* RI-digested pBR 322 were derived from **4** / horseradish peroxidase and HNPP / alkaline phosphatase, respectively .

References and Notes

1 R. K. Saiki, S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim, *Science*, **230**, 1350 (1985).
2 For example, see J. Ju, I. Kheterpal, J. R. Scherer, C.Ruman, C. W. Fuller, A. N. Glazer, and R. A. Mathies, *Anal. Biochem.*, **231**, 131 (1995) and references cited therein.
3 Y. X. Ci, Y. Z. Li, and X. J. Liu, *Anal. Chem.*, **67**, 1785 (1995).
4 T. K. Christopoulos and E. P. Diamandis, *Anal. Chem.*, **64**, 342 (1992).
5 S. Fujita, M. Momiyama, Y. Kondoh, N. Kagiyama, S. H. Hori, and T. Toru, *Anal. Chem.*, **66**, 1347 (1994).
6 a) S. Fujita, N. Kagiyama, M. Momiyama, and T. Toru, *Chem. Lett.*, **1996**, 1073; b) S. Fujita, T. Toru, Y. Kitagawa, N. Kagiyama, and M. Momiyama, *Anal. Chim. Acta*, **339**, 289 (1997).
7 S. Beck, T. O'Keeffe, J. M., Coull, and H. Koster, *Nucleic Acids Res.*, **17**, 5115 (1989); G. An, G. Luo, R. W. Veltri, and S. M. O'Hara, *Biotechniques*, **20**, 342 (1996).
8 S. Fujita, T. Toru, Y. Kondoh, M. Momiyama, N. Kagiyama, and S. H. Hori, *Acta Histochem. Cytochem.*, **30**, 165 (1997).
9 Fluorometric detection was carried out with a fluorescence detector Epilight-UV-FA-1100 (Aisin Cosmos R & D).
10 Formation of the benzidine azo dimer in benzidine oxidation by horseradish peroxidase/H_2O_2, see; P. D. Josephy, T. E. Eling, and R. P. Mason, *J. Biol. Chem.*, **258**, 5561 (1983).
11 The spot test was carried out as described previously, see ref. 6b.
12 The operation transferring the target DNA to membrane and hybridizing the probe to target DNA may bring about a reduction of the sensitivity of Southern blot hybridization in comparison with the results in the spot test. Other known methods are much less sensitive than our method; ALP-BCIP/NBT; detection limit, ca, 5pg pBR 322 DNA (size 2kb); see J. D. Norton, J. Connor, and R. J. Avery, *Nucleic Acids Res.*, **12**, 3445 (1984). POD-Luminol method; detection limit, ca, 5pg; see C. Kessler, *Nonisotopic DNA Probe Techniques*, Academic Press (1992).
13 The procedure for the southern blot hybridization is essentially the same as described previously, see ref. 6b.
14 Simultaneous detection of two different target DNAs on a single blot was carried out using two fluorogenic substrates **4** and phosphorylated N-(2-biphenyl)-3-hydroxy-2-naphthalenecarboxamide (HNPP). The *Eco* RI-digested λDNA and a mixture of the *Eco* RI-digested Col EI and the *Eco* RI-digested pBR322 were electrophoresed, transferred onto a nylon membrane and hybridized with the *Eco* RI-digested λDNA and a mixture derived from Col EI and pBR322 which were labeled with digoxigenin (DIG) and fluorescein, respectively. Then, horseradish peroxidase-conjugated anti-DIG antibody (Boehringer Mannheim) and alkaline phosphatase-conjugated anti-fluorescein antibody (Vector Laboratories Inc.) were coupled with hybrid DNAs. Reactions for both enzymes were carried out at room temperature with 2 mM of **4**, 0.003% H_2O_2, and 80 μM of HNPP (Aisin Cosmos R&D) in a 0.1 M tris buffer (pH 7.5) containing 0.1 M NaCl and 10mM $MgCl_2$. After incubation for 2h, the membrane was washed with distilled water and dried in the air. Orange and bluish-green signals appeared under irradiation of ultraviolet light (312 nm) and were recorded with a 35 mm color film (Kodak) using a cut filter (400 nm).