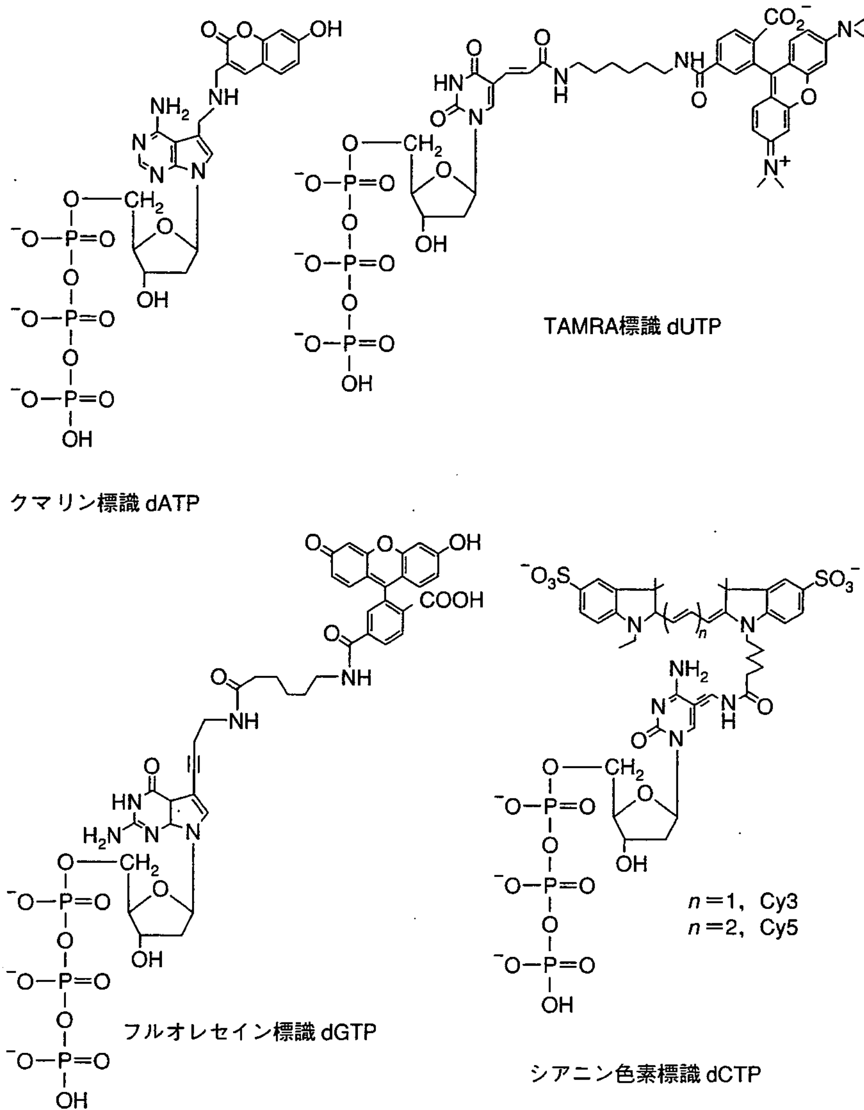

図 16.7 蛍光標識ヌクレオチド

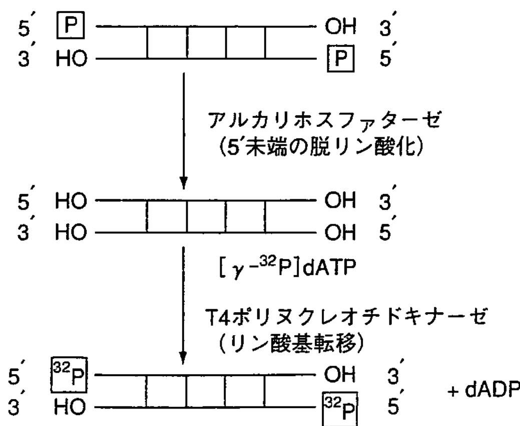

図 16.8 二本鎖 DNA の 5′ 末端放射性同位体標識法
Ⓟはヌクレオチド末端にある糖のリン酸エステル基(糖-OPO_3H^-)を表す.

(TdT)が用いられる. TdT は, 3′末端のヒドロキシル基にヌクレオチドを添加していく酵素で, 反応時に蛍光標識ヌクレオチドを加えることで, 3′末端に蛍光標識ヌクレオチドが付加される. このとき, 被標識 DNA の 3′末端の形状(一本鎖, 二本鎖など)により効率が異なる.

16.4.3　ニックトランスレーション法(図16.9)

二本鎖DNAの最も一般的な標識法として知られている．この方法は，二本鎖DNAの一方の鎖に，任意の箇所にDNase Iによって切れ目(nick：ニックとよばれる)を入れると，DNAポリメラーゼIは，ニックを認識して，そのエキソヌクレアーゼ活性により，ニックの5′端のヌクレオチドを除去すると同時に，本来のポリメラーゼ活性により，3′端から相補鎖DNAを鋳型に修復する．この際に，蛍光標識したヌクレオチドを加えておくと，修復する部位に蛍光標識ヌクレオチドが取り込まれ，蛍光標識DNAを得ることができる．

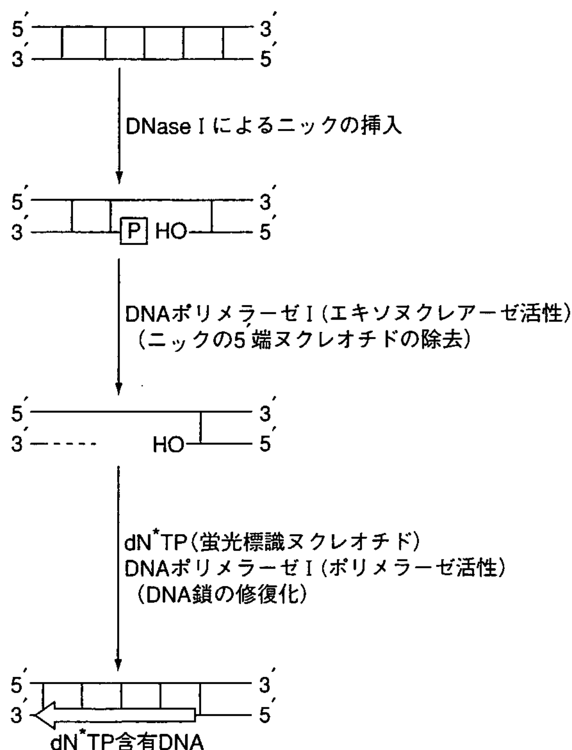

図16.9　ニックトランスレーション法による二本鎖DNAの蛍光標識法

16.4.4　ランダムプライマー伸長法(図16.10)

本法は，ニックトランスレーション法に代わる標識DNAの作製法として多用される．標識したいDNAを加熱して一本鎖にした後，ランダムな配列をもつオリゴヌクレオチドを加えると，その一部がDNAと結合(アニーリング)する．これをプライマーとして，Klenowフラグメント(ポリメラーゼ活性のみをもつ酵素)を作用させると，その伸長反応により，相補鎖DNAを合成する．このときに，蛍光標識ヌクレオチドを加えておくと，蛍光標識された相補鎖DNAが合成される．合成された標識DNAは，元のDNAよりサイズが短く，長さも不均一となる．標識効率は，ニックトランスレーション法より高い．

16.4.5　微粒子によるDNAの標識

ナノテクノロジーの発展に伴い，ナノメートルサイズの微粒子(ナノ粒子)をDNAの標識に利用する方法が開発されている．DNA標識用のナノ粒子としては，

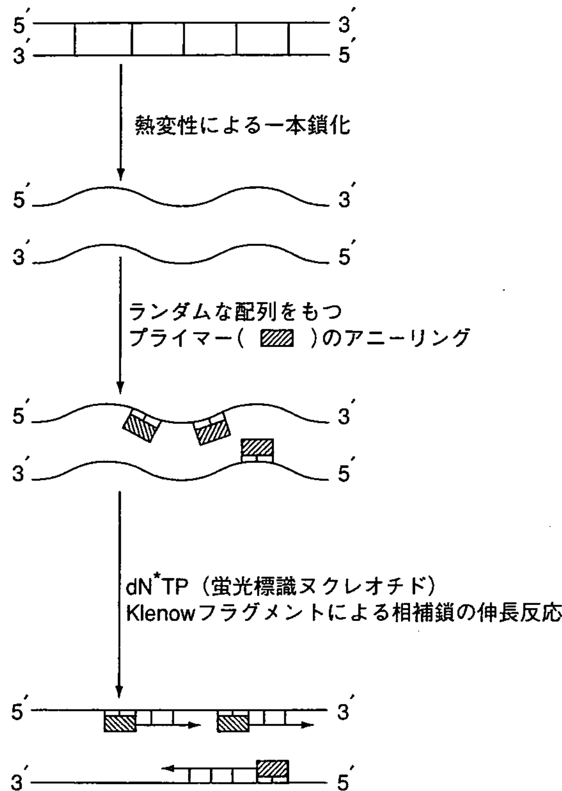

図 16.10 ラムダムプライマー伸長法による DNA の蛍光標識法

量子ドットや金微粒子が利用されており，一塩基多型の検出などに応用されている(16.5.4 参照)．量子ドットは，直径数 nm の半導体素材からなるナノクリスタルで，粒径のサイズにより蛍光波長が異なり，従来の蛍光色素と比較して，光安定性がよい(退色が遅い)，蛍光が強い，すべての色を一つの光源で励起できる，などの利点がある．核酸だけでなく，タンパク質の標識物質としても使用されており，細胞の蛍光染色などにも利用される．

16.4.6 そのほかの DNA 標識方法

DNA の標識としては，上記の方法以外にも，反応性の高い官能基を利用して，化学的に標識する方法も開発されている．官能基として，ソラーレン(psoralen)，ヒドラジド基などをもつビオチン誘導体などが合成され，核酸の標識に利用されている．

例題 16.2 DNA の末端標識法とインターナルラベリング法の特徴について述べよ．

解 末端標識法は，短い DNA や一本鎖 DNA，RNA の標識に適しているが，インターナルラベリング法は，比較的長い DNA にしか適用できない．標識効率に関しては，インターナルラベリング法が末端標識法より高い．

16.5　標識核酸を用いる分析法

16.5.1　DNAの塩基配列決定

DNAの塩基配列決定法としては，ジデオキシヌクレオチドを用いるジデオキシ法(サンガー法)と化学反応を利用して決定するマキサム・ギルバート法が考案されているが，簡易性や結果の解釈の容易さから，現在ではジデオキシ法が広く普及している．ジデオキシ法は，当初，ラジオアイソトープを使用して行われていたが，さまざまな蛍光ヌクレオチドが開発され，検出感度の上昇とともに，現在では蛍光検出法が主流となっている．ポリメラーゼ連鎖反応(polymerase chain reaction；PCR)やキャピラリー電気泳動と組み合わせることで，多くの試料を短時間に一度に処理できるようになり，生物のゲノム解析に利用されるなど，さまざまな分野で広く使用されている．

16.5.2　DNAとタンパク質の結合性の評価

転写調節やDNAの複製・修復にかかわるタンパク質は，DNAと結合し，その役割を果たしている．これらの中で，転写因子のような特定の塩基配列に結合するタンパク質は，疾病と深くかかわっており，これらの機能解析が盛んに行われている．これらタンパク質のDNA結合能を調べる方法として，標識DNAを用いたゲルシフトアッセイがある．図16.11に示すように，蛍光標識DNAとタンパク質の複合体が形成されると分子量が大きくなり，これを未変性条件下でのゲル電気泳動の蛍光バンドのシフトとして検出する方法である．この方法は，あるタンパク質が，どのような配列をもつDNAと結合できるか，また逆に，ある特定の配列をもつDNAにどのようなタンパク質が結合するかを調べるのに用いる．

タンパク質やDNAは，SDS(ドデシル硫酸ナトリウム)や尿素などにより変性する．このため，複合体が解離しないように，SDSや尿素などの変性剤を含まない条件(未変性条件下)でゲル電気泳動を行う必要がある．

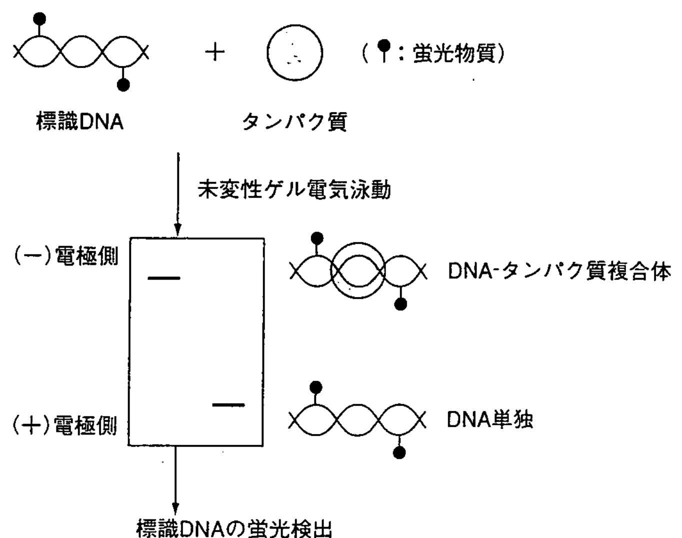

図16.11　蛍光標識DNAを用いたDNAとタンパク質の結合性の評価法

16.5.3　リアルタイム PCR

PCR は，容易に DNA を増幅できるので，試料中に含まれる微量 DNA の検出などに用いられている．この PCR を試料中の DNA の定量に応用したものがリアルタイム PCR(real-time PCR)である．リアルタイム PCR には，いくつかの方法があるが，図 16.12 には，両末端に蛍光団と蛍光消去剤(クエンチャー)が標識されている DNA プローブ(TaqMan プローブとよぶ)を用いた方法を示している．PCR のアニーリングステップ中に，標識プローブと PCR プライマーは標的配列にアニールするが，蛍光団とクエンチャーが接近しているため，蛍光は発しない(図 16.12 a)．次の PCR の伸長反応では，Taq DNA ポリメラーゼのエキソヌクレアーゼ活性により蛍光団が結合したヌクレオチドは分解され，蛍光団とクエンチャーは分離されるので，遊離した蛍光団のシグナルが測定される(図 16.12 b)．この結果，PCR 産物の蓄積量に比例した蛍光シグナルが検出される．定量操作としては，濃度既知の DNA を用いて PCR を行い，一定の PCR 産物に達するサイクル数を調べ，これとの比較により未知 DNA の濃度を求める．リアルタイム PCR は，遺伝子発現解析，ウイルスの定量，対立遺伝子の判別解析などにも利用される．

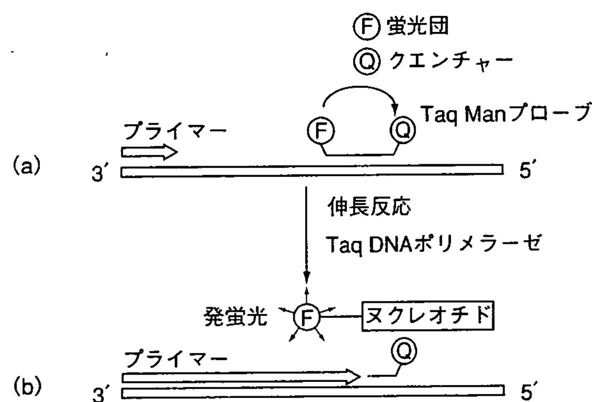

図 16.12　蛍光プローブ(Taq Man プローブ)によるリアルタイム PCR

16.5.4　一塩基多型の検出

一塩基多型(single nucleotide polymorphisms ; SNPs)とは，遺伝子の塩基配列における個人差のことで，一つの塩基が別の塩基に変異していることを指し，ヒトでは数百万個存在すると推定されている．SNPs には，病気や薬効，副作用と密接に関連している場合が多い．この分析手法としてハイブリダイゼーションを利用したものがある(図 16.13 a)．この方法は，一本鎖オリゴヌクレオチドを膜に固定化し，これと試料中の蛍光標識 DNA の間で二本鎖を形成(ハイブリダイズ)させる．完全相補と不完全相補の二本鎖では，熱安定性が異なるため，反応温度を設定することで，完全相補の二本鎖のみを蛍光検出できる．この原理は，PCR と組み合わせた DNA チップ検出に適応されている．

このほかに，ナノ粒子による標識 DNA の応用が試みられている．金微粒子は，溶液中に分散している場合，溶液の色は赤色であるが，これらが凝集すると溶液の色は青色に変化する．金微粒子の表面に一本鎖オリゴヌクレオチドを結合させ，

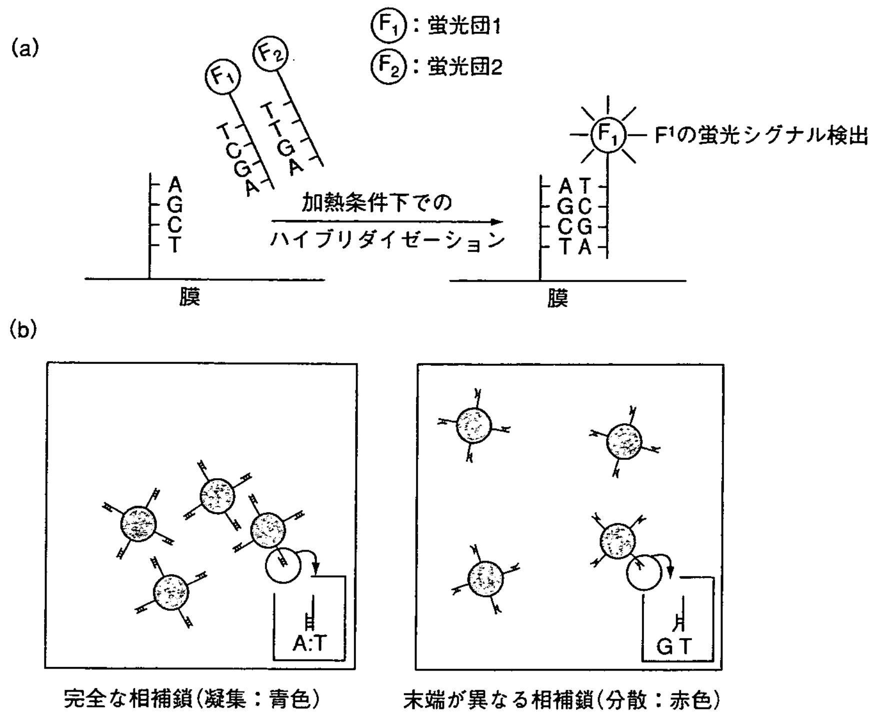

図 16.13 (a) 蛍光団, (b) 金微粒子標識 DNA による SNPs の検出

これに完全な相補鎖をもつ試料を加えると，金微粒子-オリゴヌクレオチド複合体は凝集し，溶液の色は青色になる．しかし，末端が一塩基異なる試料を加えた場合，凝集は起こらず，溶液の色は赤色のままである(図 16.13 b)．この方法は，測定器具を必要とせず，比色検出が目視できるため，SNPs の検出に有望と考えられている．

章末問題

問題 16.1 蛍光，酵素，放射性同位体標識の長所と短所について，それぞれ述べよ．
問題 16.2 標識タンパク質を用いた実験例をあげよ．
問題 16.3 標識核酸を用いた実験例をあげよ．

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Facile determination of DNA-binding nuclear factor-κB by chemiluminescence detection

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Abstract

A simple, rapid, and sensitive method for the assay of a sequence-specific DNA-binding protein, nuclear factor-κB (NF-κB), has been developed by using a DNA-detectable chemiluminogenic reagent and a centrifugal filter that distinguishes different molecular sizes. After the formation of a complex between NF-κB and DNA, the unbound DNA is separated from the complex by the centrifugal filter. The amount of the bound NF-κB is estimated by chemiluminescence detection of the bound DNA. This detection is performed within 2 min at room temperature by the use of a chemiluminogenic reagent, 3′,4′,5′-trimethoxyphenylglyoxal, which selectively recognizes guanine moiety in oligonucleotides or DNAs. This method does not require any labeled probes or antibodies and can determine a concentration as low as 5 nM of DNA-binding NF-κB. The sensitivity is nearly the same as that of other methods such as gel shift assay using fluorescence-labeled probes and enzyme-linked immunosorbent assay. Therefore, the current method provides a convenient tool for surveying various DNA-binding proteins.
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Keywords: NF-κB; Chemiluminescence detection; DNA-binding protein; TMPG

Sequence-specific DNA-binding proteins play critical roles in the biology of cells. These proteins are involved in the regulation of gene transcription and DNA replication, recombination, repair, and restriction [1–4]. Among the various sequence-specific DNA-binding proteins, transcription factors have been estimated to constitute 6 to 7% of all proteins expressed by eukaryotic genomes, which are responsible for the transfer of biological information from genes to proteins [5,6]. These transcription factors have become potential targets in medical diagnosis and drug development because of their pivotal roles in the pathways and networks involving the regulation of gene expression [7].

A prominent protocol for the determination of various sequence-specific DNA-binding proteins has attracted considerable attention from many research fields such as genomics and proteomics. However, the conventional methods for the detection of those proteins, including gel shift assay, DNA footprinting assay, and enzyme-linked immunosorbent assay (ELISA[1]), usually are time-consuming. In addition, they require labeled probes and/or specific antibodies for the detection of the DNA-binding protein in combination with electrophoresis for separation.

In this article, we describe a convenient method for the determination of sequence-specific DNA-binding nuclear factor-κB (NF-κB). NF-κB is a ubiquitous redox-sensitive transcription factor that responds to pro-inflammation

[1] *Abbreviations used:* ELISA, enzyme-linked immunosorbent assay; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α; TMPG, 3′,4′,5′-trimethoxyphenylglyoxal; dsDNA, double-stranded DNA; SDS, sodium dodecyl sulfate; ChRE, carbohydrate response element; dI–dC, poly(deoxyinosinic deoxycytidylic) acid; NMWL, nominal molecular weight limit; TPA, tetra-*n*-propyl ammonium phosphate; DMF, *N*,*N*-dimethylformamide; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; DMSO, dimethyl sulfoxide; MW, molecular weight; BSA, bovine serum albumin; FRET, fluorescence resonance energy transfer.

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caused by cytokines and oxidative stress [8,9]. By the activation with tumor necrosis factor-α (TNF-α), NF-κB translocates into the nucleus, where it binds to specific nucleotide sequences [8,9]. The cellular response of NF-κB to inflammatory and stress signals has been implicated in disease conditions such as atherosclerosis, cancer, diabetes, and Alzheimer's disease [10]. For the sensitive and facile detection of NF-κB bound to DNA, we employed 3′,4′,5′-trimethoxyphenylglyoxal (TMPG) as the chemiluminogenic reagent. TMPG reacts specifically with guanine moiety in DNA to form a chemiluminescent product [11]. The reaction conditions were first improved for the rapid detection of DNA with TMPG within 2 min in a neutral medium, and then a facile method was developed for the quantitative and sensitive determination of the DNA-binding NF-κB by the chemiluminescent detection with the TMPG reaction.

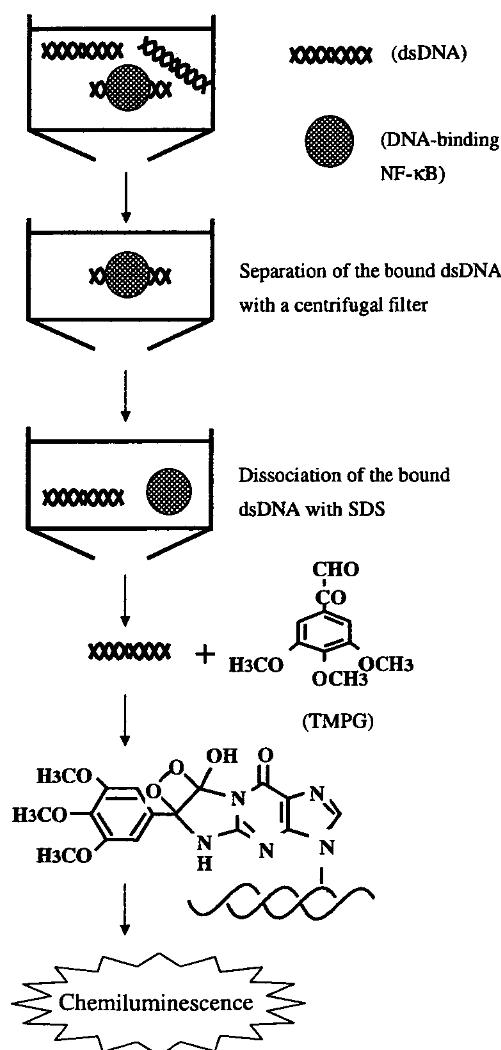

Fig. 1. Schematic protocol for the assay of DNA-binding NF-κB by means of chemiluminescence reaction with TMPG.

Fig. 1 illustrates our protocol proposed for the determination of the DNA-binding NF-κB. In this assay, the bound and unbound double-stranded DNAs (dsDNAs) that contain 14 guanine bases after the formation of a complex between NF-κB and dsDNA were readily separated with a centrifugal filter that was able to remove molecular sizes lower than 100,000 Da. The bound dsDNA, which was dissociated with sodium dodecyl sulfate (SDS), was filtrated. The amount of the DNA in the filtrate was then detected by the chemiluminescence reaction with TMPG. The proposed method does not require any labeled probes or specific antibodies, and it allows the analysis of the interaction between DNAs and proteins by using conventional inexpensive instruments.

Materials and methods

Materials and reagents

Purified recombinant human NF-κB p50 was purchased from Promega (Madison, WI, USA). A dsDNA, dsDNA/NF-κB with the sequences 5′-AGTTGAGGGGACTTTCCCAACTAGGAATCT-3′ and 3′-TCAACTCCCCTGAAAGGGTTGATCCTTAGA-5′, was used for binding to the protein, NF-κB. The underlined sequence represents the protein-binding site [8]. Another dsDNA, dsDNA/ChRE with the sequences 5′-GGGCGCACGTGGCACTCACGTGGTTCC-3′ and 3′-CCCGCGTGCACCGTGAGTGCACCAAGG-5′ (which binds to a carbohydrate response element (ChRE)-binding protein [12]), was used as the negative control for dsDNA/NF-κB. Those single-stranded oligonucleotides and a 60-mer oligonucleotides, 5′-$(TTAGGG)_{10}$-3′, were products obtained from Sigma–Genosys Japan (Ishikari, Japan). Poly(deoxyinosinic deoxycytidylic) acid (dI–dC) was purchased from Roche (Mannheim, Germany) and was used to suppress nonspecific binding of the protein. Nuclear extracts of HeLa cells were purchased from Active Motif (Carlsbad, CA, USA). The concentration of whole proteins in each extract was 2.5 g/L based on information provided by the supplier. Centrifugal filter devices (Ultra-free MC 100,000 nominal molecular weight limit [NMWL] filter units) were purchased from Millipore (Bedford, MA, USA).

TMPG was synthesized according to previously reported conditions [13], but with a slight modification as follows. To a stirred solution of selenium dioxide (45 mmol) in dioxane (40 ml) was added 3,4,5-trimethoxyacetophenone (50 mmol) at 40 °C. The mixture was refluxed for 2 h, and selenium dioxide (45 mmol) was then added to the reaction mixture. After being refluxed for 3 h, the mixture was filtrated to remove an insoluble selenium. The filtrate was mixed with 240 ml of H_2O and then kept at 4 °C for approximately 15 h. The formed precipitates were recrystallized from water to give colorless needles (melting point 101–102 °C, yield 60–70%).

Chemiluminescence reaction of DNA with TMPG

The TMPG reaction was carried out as follows. A portion (20 μl) of a sample in H_2O was mixed with 10 μl of 0.1 M tetra-*n*-propyl ammonium phosphate (TPA) solution (pH 7.0) in a glass tube, and then a 200-μl portion of 10 mM TMPG in *N,N*-dimethylformamide (DMF) was added to start the chemiluminescence reaction. Chemiluminescent intensity was measured with a luminescence reader (BLR-201, Aloka, Tokyo, Japan) for 2 min at room temperature (24–26 °C) after the addition of TMPG.

Assay of DNA-binding NF-κB

A single-stranded oligonucleotide and its complementary oligonucleotide were mixed at an equimolar concentration (4.0 μM each) to form dsDNA. The mixture was heated at 95 °C for 5 min and cooled slowly to room temperature. NF-κB (0–100 nM) was mixed with 1.0 μM dsDNA/NF-κB and 0.25 g/L dI–dC in 40 μl of a binding buffer (10 mM Tris–HCl [pH 7.5], 0.5 mM ethylenediaminetetraacetic acid [EDTA], 0.5 mM dithiothreitol, and 10% glycerol) and then reacted at room temperature for 30 min.

The reaction mixture (20 μl) was transferred to a centrifugal filter device and centrifuged at 2000 *g* for 2 min to separate free dsDNA from NF-κB-binding dsDNA. The filter was washed twice with 100 μl of the binding buffer containing 0.01% Triton X-100 by centrifugation at 2000 *g* for 10 min. A portion (40 μl) of 0.1% SDS solution was added to the filter device, which was then incubated at 37 °C for 10 min. The filter device was centrifuged at 4000 *g* for 10 min, and a 20 μl portion of the filtrate was used to react with TMPG to determine the amount of DNA-binding NF-κB.

DNA-binding NF-κB in nuclear extracts of HeLa cells, which were incubated in advance for 30 min in the presence or absence of 20 ng/ml TNF-α, was determined as described above. For this sample preparation, the extract solution containing 2.5 g/L whole proteins was diluted with H_2O to adjust the protein concentration to 0.31 g/L in the binding reaction mixture (40 μl).

Gel shift assay

A gel shift assay was performed as described previously [12] by using fluorescein isothiocyanate (FITC)-labeled oligonucleotides. NF-κB (0–50 nM) was mixed with 250 nM FITC-labeled dsDNA/NF-κB and reacted in the binding buffer. The reaction mixture (20 μl) was loaded onto a 4.5% polyacrylamide gel (75 × 90 mm), and then free dsDNA and dsDNA-binding NF-κB in the mixture were separated by electrophoresis at 60 V for approximately 3 h. The separated components on the gel were detected by a fluorescence imaging analyzer (FLA5000, Fujifilm, Tokyo, Japan).

Results and discussion

Chemiluminescence reaction of DNA with TMPG

We previously reported that TMPG could react with guanine moieties in a DNA molecule to form a product that emits chemiluminescence [11]. However, a long incubation time (20 min) in a medium containing sodium phosphate buffer (pH 6.5) and dimethyl sulfoxide (DMSO) is required to produce the chemiluminescent product. To develop a more facile procedure, we optimized the conditions of the TMPG reaction by using DMF instead of DMSO and using a tetra-alkyl ammonium salt instead of sodium phosphate buffer (pH 6.5). As a result, we found that DMF and TPA at pH 7.0 were effective not only for rapid formation of the chemiluminescent product but also for fast emission of the chemiluminescence.

Fig. 2A shows the emission profile of the chemiluminescence produced from dsDNA/NF-κB depending on the time after mixing with TMPG in the presence of DMF and TPA. The chemiluminescence reached the maximum at 20 s and then rapidly decreased, and no chemiluminescence was observed in the absence of dsDNA. There was a good correlation between the chemiluminescent intensity and the amount of dsDNA/NF-κB that contains 14 guanine bases in the molecule. dsDNA/NF-κB at 5 nM could be detected within 2 min by the modified reaction conditions with TMPG. This TMPG reaction did not give any signal for proteins and oligonucleotides such as dI–dC because there is no guanine moiety in its molecule and thus it permitted the selective detection of guanine-containing DNAs and oligonucleotides such as 5′-$(TTAGGG)_{10}$-3′, as shown in Fig. 2B. There are 14, 19, and 30 guanine moieties in dsDNA/NF-κB, dsDNA/ChRE, and 5′-$(TTAGGG)_{10}$-3′, respectively. Compared with the chemiluminescent intensity from these guanine-containing DNAs, the intensity was enhanced according to the number of guanine bases. There was a good relationship between chemiluminescent intensity and the number of guanine bases, although the intensity was not exactly proportional to guanine contents. Therefore, the lower limit of detection was dependent on the guanine contents in the DNA molecule. The results indicate that the TMPG reaction is preferable for the sensitive detection of specific protein-binding DNA containing many guanine bases in the molecule, and thus dI–dC is usable as a suppressor for the nonspecific binding of protein to DNA.

Assay of DNA-binding NF-κB

To verify the proposed assay (Fig. 1), we first prepared dsDNA/NF-κB (double-stranded molecular weight [MW] 18,400 Da) that can bind specifically to the dimer proteins of NF-κB (dimer MW 98,800 Da) [8–10]. We also used dsDNA/ChRE (double-stranded MW 16,600 Da) that binds specifically to a ChRE protein [12] as the negative control. The NF-κB protein at various concentrations

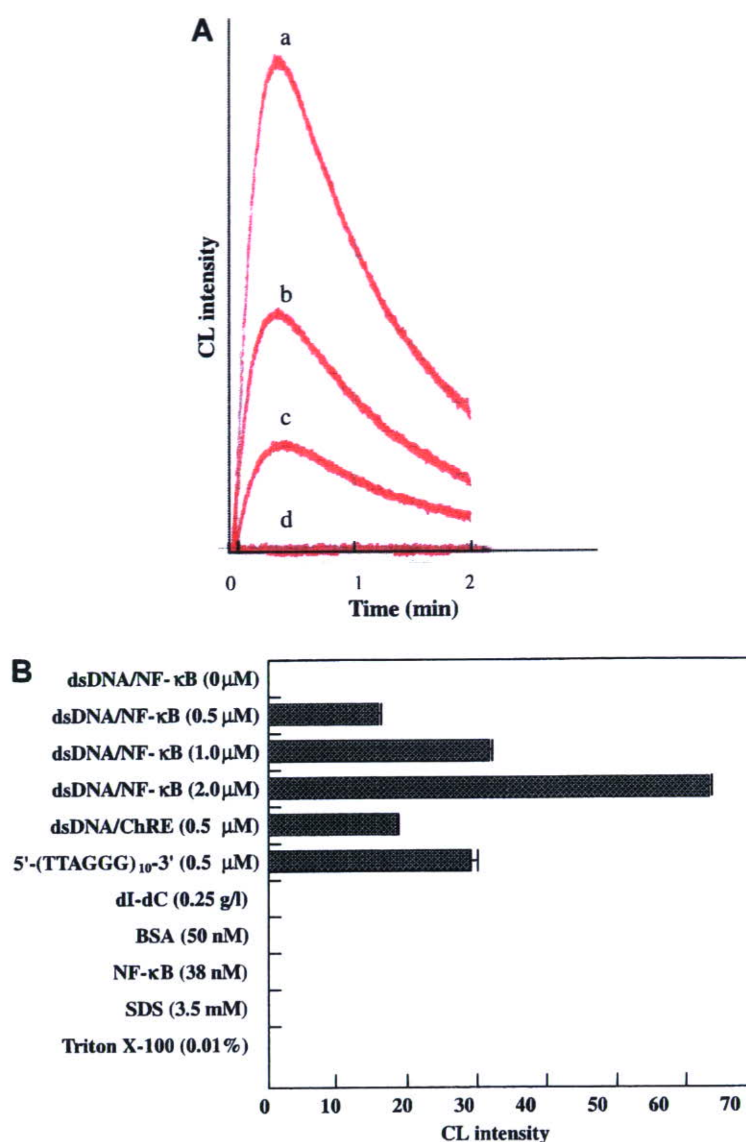

Fig. 2. Chemiluminescence (CL) reactions of DNAs with TMPG. (A) Different concentrations of dsDNA/NF-κB at 2.0 μM (a), 1.0 μM (b), 0.5 μM (c), and 0 μM (d) were reacted individually with TMPG, and their chemiluminescent emissions were recorded for 2 min. (B) dsDNAs, oligonucleotides, and proteins were reacted individually with TMPG, and their total chemiluminescent intensities for 2 min were measured ($n = 3$ each).

was reacted individually with 1.0 μM dsDNA/NF-κB. After the separation of the unbound dsDNA/NF-κB in the reaction mixture by a centrifugal filter device, the dsDNA bound to NF-κB was released from the complex in the filter device. This centrifugal filter device was equipped with an ultrafiltration membrane for the removal of particulates less than MW 100,000 Da. After the centrifugation, free dsDNA in the filtrate was detected by the TMPG reaction.

The chemiluminescent intensity from the bound dsDNA/NF-κB was increased by increasing the concentration of NF-κB (Fig. 3). The dissociation of the bound dsDNA from the DNA–protein complex was performed by SDS treatment. SDS at a concentration of 0.5 to 0.05% (w/v) gave a maximum and constant intensity of chemiluminescence due to the free dsDNA. However, the chemiluminescent intensity was decreased drastically by the use of 1.0% SDS because SDS at a concentration greater than 1.0% inhibited the TMPG reaction. On the other hand, when water was used instead of SDS solution for the dissociation, the chemiluminescent intensity from the free dsDNA could not be observed. These results indicate that 0.1% SDS solution could quantitatively dissociate the bound dsDNA from the DNA–protein complex. The calibration graph showed a linear relation between the chemiluminescent intensity and the concentration of 6.3 to 50 nM NF-κB, indicating a linear correlation coefficient (r^2) of 0.997; the linear equation was $y = 0.117x + 1.72$, with x and y indicating the concentration of NF-κB and the intensity, respectively. The lower limit of detection

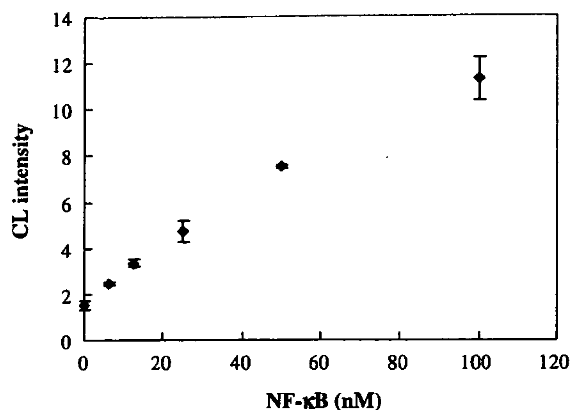

Fig. 3. Calibration curve for the protein of NF-κB bound to dsDNA/NF-κB. Various concentrations of NF-κB (0, 6.3, 12.5, 25, 50, and 100 nM) were reacted individually with 1.0 μM dsDNA/NF-κB in a 40 μl binding reaction mixture ($n = 4$ each). CL, chemiluminescence.

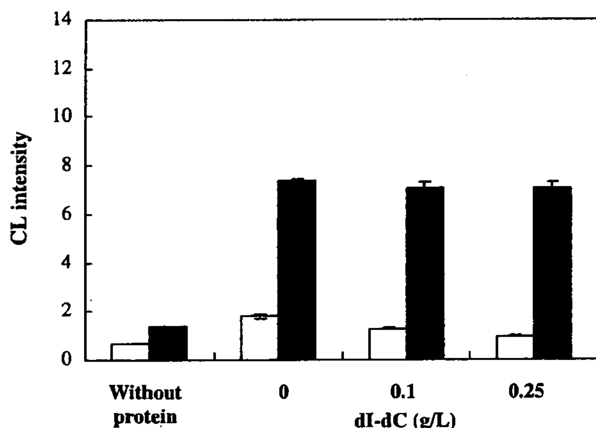

Fig. 4. Effect of dI–dC on the specific binding of NF-κB to dsDNA. NF-κB (50 nM) was reacted with 1.0 μM dsDNA/ChRE (white column) or 1.0 μM dsDNA/NF-κB (black column) in the presence of dI–dC (0, 0.1, or 0.25 g/L) in a 40-μl binding reaction mixture ($n = 4$ each). The left column indicates that the buffer was used instead of NF-κB as a blank for the binding reaction mixture containing 0.25 g/L dI–dC. CL, chemiluminescence.

was approximately 5 nM for NF-κB. The results indicate that the DNA-binding protein was determined sensitively by the proposed assay.

Specificity and assay of DNA-binding NF-κB in nuclear extracts

To evaluate the specific binding of NF-κB to dsDNA/NF-κB, dsDNA/ChRE was used instead of dsDNA/NF-κB for the binding reaction. The dsDNA/ChRE contains an E-box sequence (CACGGG) [14] that corresponds to the consensus binding site for other transcription factors such as upstream stimulatory factors and their related family members [15]. When dsDNA/ChRE was reacted with NF-κB, the chemiluminescent intensity was nearly the same as that obtained without protein (Fig. 4). This result indicates that most of the NF-κB protein did not bind to dsDNA/ChRE. In addition, the chemiluminescent intensity due to the nonspecific binding dsDNA/ChRE decreased in the presence of dI–dC. Therefore, we added 0.25 g/L dI–dC to the reaction mixture so as to suppress the nonspecific binding of NF-κB to other dsDNA. In this case, chemiluminescent intensity due to the specific binding dsDNA/NF-κB did not change in the presence or absence of dI–dC (Fig. 4).

When bovine serum albumin (BSA), instead of NF-κB, was mixed with dsDNA/NF-κB, the chemiluminescent intensity due to the nonspecific binding dsDNA/NF-κB indicated the background value that was obtained without the protein (Fig. 5). These results indicate that the proposed protocol has quite high reliability for the determination of sequence-specific DNA-binding NF-κB.

Conventional methods, such as gel shift assays [16], DNA footprinting [17,18], and ELISA [19], have been used for the detection of specific interaction between DNA and protein in crude nuclear extracts. This prompted us to investigate the applicability of the current method to the determination of DNA-binding NF-κB in a crude nuclear extract of cultured cells. For the assay of the target NF-κB in the nuclear extract of HeLa cells, HeLa cells were stimulated either with or without 20 ng/ml TNF-α. It has been reported that the concentration of NF-κB in the nucleus is induced by the TNF-α stimulation [20]. As shown in Fig. 5, the concentration of DNA-binding NF-κB in the nuclear extract after the stimulation with TNF-α was approximately four times greater than that without the stimulation. According to the calibration curve in Fig. 3, the concentrations of NF-κB in the stimulated and nonstimulated nuclear extracts were estimated as 78 and 20 nM, respectively.

Comparison with gel shift assay

Gel shift assay is one of the most useful methods for the detection of the DNA-binding proteins [16]. Therefore, our assay was compared with the gel shift assay. FITC-labeled dsDNA/NF-κB was used for the gel shift assay. As shown in Fig. 6, 12.5 nM NF-κB was detected at a lower limit of detection by the gel shift assay, although this electrophoresis took approximately 3 h for the assay. By the gel shift assay using radiolabeled DNA probes, the detection limit of NF-κB may be approximately 4 nM based on information provided by the supplier. Thus, the sensitivity of our method is comparable to that of other conventional methods [16–18].

Conclusions

The proposed method was applied to the facile and rapid assay of the DNA-binding NF-κB in crude nuclear extracts of mammalian cells, although similar DNA-binding proteins cannot be distinguished by this method. To

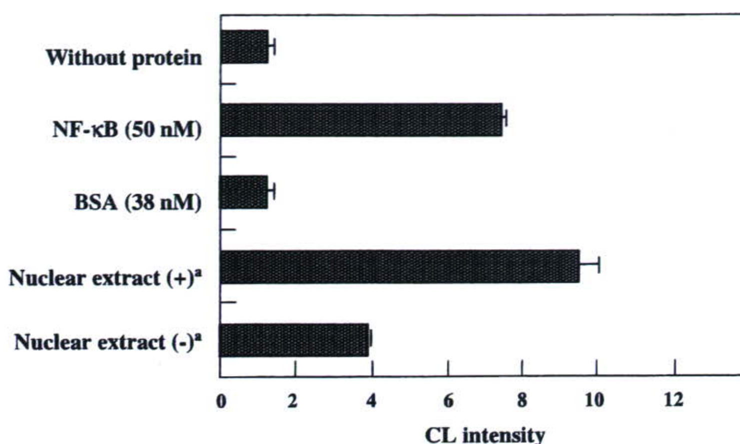

Fig. 5. Assay of DNA-binding NF-κB by the current method. NF-κB (50 nM), BSA (38 nM), and nuclear extracts of HeLa cells were reacted individually with 1.0 μM dsDNA/NF-κB in a 40-μl reaction mixture ($n = 4$ each), and the concentrations of the bound NF-κB were determined by chemiluminescence (CL) detection. [a]HeLa cells were incubated with (+) or without (−) TNF-α before extraction.

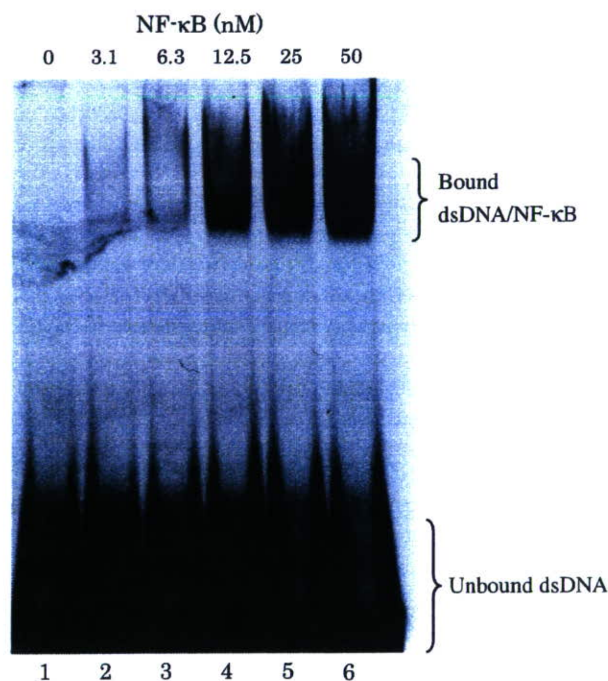

Fig. 6. Fluorescence detection of NF-κB bound to dsDNA/NF-κB by a gel shift assay. The concentrations of NF-κB used for the binding reaction (40 μl) were 0 nM (lane 1), 3.1 nM (lane 2), 6.3 nM (lane 3), 12.5 nM (lane 4), 25 nM (lane 5), and 50 nM (lane 6).

establish the conditions for the proposed method, we examined the reaction of DNA with TMPG and found that TPA and DMF enhanced the progress of the reaction between DNA and TMPG. This TMPG reaction progressed at room temperature and provided strong chemiluminescence within 2 min for guanine-containing DNA. For the assay of the sequence-specific DNA-binding NF-κB, we employed two surface-active agents: Triton X-100 and SDS. Triton X-100 was used to remove the unbound dsDNA from a centrifugal filter, whereas SDS was used to dissociate the bound dsDNA from the DNA–protein complex. We also employed dI–dC to suppress the nonspecific binding of NF-κB to dsDNA. dI–dC at a high concentration could suppress the nonspecific binding without any influence for the detection of the specific binding. Using these reagents, we succeeded in determining the target protein, NF-κB, in a crude nuclear extract of cultured cells.

The detection sensitivity of our method is nearly the same as that of the gel shift assay using a fluorescent probe. In addition, our method does not require any labeled probes or antibodies and can be performed within 1 h for many samples, whereas the gel shift assay and ELISA require several hours. However, the gel shift assay can distinguish more precisely the molecular size of a DNA–protein complex. Recently, several fluorescence resonance energy transfer (FRET) probes have been used for the detection of DNA-binding proteins [21]. Using this method, it is generally difficult to design and prepare the FRET probe, although the method is simple, rapid, and sensitive. The current method may detect many samples at the same time because the simultaneous detection is possible by the use of a microtiter plate. Therefore, this method will be a convenient tool not only for the detection of various DNA-binding proteins but also for the analysis of unknown transcription factors in mammalian cells.

Acknowledgments

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Chemiluminescent Probes

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Dextran-Based Polymeric Chemiluminescent Compounds for the Sensitive Optical Imaging of a Cytochrome P450 Protein on a Solid-Phase Membrane**

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Chemiluminescence (CL) has been exploited within a wide range of applications in many scientific fields.[1] CL imaging represents a promising detection system that is increasingly used for the ultrasensitive quantification and localization of several analytes. Currently, microarray technology has gained in popularity for the analysis of biological samples because of its benefits in the simultaneous detection of multiple analytes.[2] A CL signal is generally measured by a charge-coupled device (CCD) camera and then quantified by imaging software in a computer. For CL imaging, the traditional method usually employs horseradish peroxidase (HRP) or alkaline phosphatase as a signal enzyme, although the reduced stability of the enzyme at room temperature and high background interference limit the applicability of the technique in clinical analyses, especially for serum samples.[3] Thus, the development of a nonenzymatic CL-imaging probe is encouraged.

Herein, we report a simple method for synthesizing dextran-based chemiluminescent compounds and their application as CL-labeling macromolecular probes for the sensitive CL imaging of a cytochrome P450 (CYP) protein on a poly(vinylidene difluoride) (PVDF) membrane (Figure 1). The dextran-based chemiluminescent compound was tethered with a small amount of biotin as a linker and a large amount of luminol or isoluminol as CL emitter. Luminol and isoluminol are known for their CL properties, and their mechanistic details have been described.[4] In addition, the avidin–biotin interaction has been recognized in immunohistochemistry, enzyme-linked immunosorbent assay, and molecular biology.[5] The affinity of biotin binding to avidin is extremely high with an association constant of $10^{15}\,\text{M}^{-1}$.

To obtain a good signal strength and high sensitivity, extensive work on the synthesis of luminol and isoluminol derivatives is desirable to search for a novel nonenzymatic probe. We first synthesized luminol- or isoluminol-containing

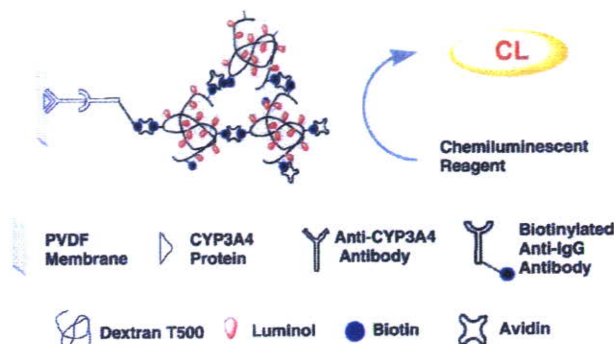
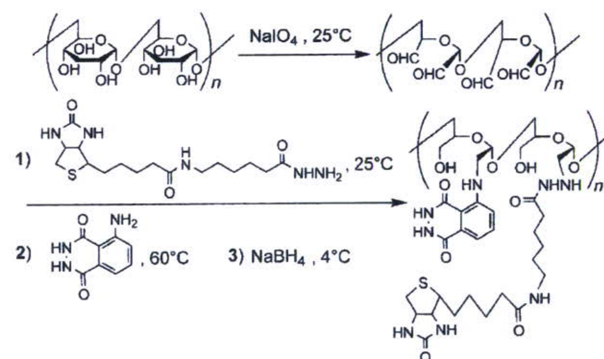


Figure 1. Detection of CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4) on a PVDF membrane with a polymeric dextran-based chemiluminescent probe. IgG = immunoglobulin G.

dextran T500 (average molecular weight 5×10^5 Da) chemiluminescent probes, which were tethered with biotin according to the procedure represented in Scheme 1. Biotin is a key



Scheme 1. Synthesis of dextran-based chemiluminescent compounds; see Experimental Section for details.

molecule that facilitates the extension of the structural framework of the chemiluminescent dextran. According to data from elemental analysis, the atomic composition of one of the synthesized dextran-based chemiluminescent probes was: C 43.0, H 5.8, N 4.4, and S 0.17%, and its molecular weight was approximately 6.3×10^5 Da. The data show that the probe contains 560 luminol units and 34 biotin units in a dextran T500 molecule (3100 glucose units), termed (Lu)560-(biotin)34-(Glc)3100. The increased introduction of luminol or isoluminol gave the probe a higher CL intensity.

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Figure 2a and b show gel-filtration chromatograms of luminol and the dextran T500-based chemiluminescent probe, respectively. Free luminol eluted at a retention time of 25 min, whereas the dextran-based probe was identified at 14 min. A small amount of free luminol (approximately 1%, w/w) was also observed in the probe as an impurity. However, the free luminol did not interfere with the detection of a target protein on a membrane, because the free luminol in the product could be sufficiently removed from the membrane by washing with a mixture of 0.15% Triton X-100 and phosphate-buffered saline (PBS; 10 mM), followed by an aqueous 50–100% methanol solution.

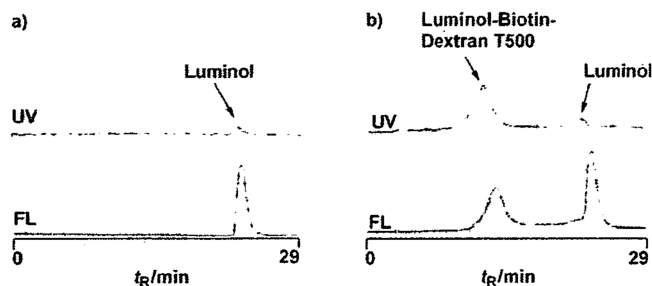

Figure 2. GFLC of dextran-based chemiluminescent probe: a) luminol (0.011 mg mL^{-1}); b) (Lu)560-(biotin)34-(Glc)3100 (1.0 mg mL^{-1}). GFLC conditions: injection volume, 10 mL; column, TSK gel T2000SW; eluent, 0.1% (v/v) aqueous solution of trifluoroacetic acid; flow rate, 1.0 mL min^{-1}; UV detection, $\lambda_{abs}=275$ nm; fluorescence (FL) detection, excitation (mercury lamp)/emission (wavelength cutoff filter) = 254/ > 360 nm.

We previously reported a nonenzymatic procedure that employed a $CH_3CN/Na_2CO_3/H_2O_2$ system for CL with luminol in aqueous solution.[3] In a slight modification of the procedure, the employment of tetrapropylammonium hydroxide (TPA) instead of Na_2CO_3 gave a significant increase of CL intensity (> 20 times). In addition, Kyaw et al. reported that CL intensity could be enhanced by transition-metal catalysis.[6] Therefore, it was interesting to further improve our CL-emitting system by metal catalysis. Encouragingly, the highest CL intensity (> 8 times) from the chemiluminescent probe was observed when the CL-emitting reagents CH_3CN, TPA, and H_2O_2 were mixed with $FeCl_3$ (0.45 mM). The kinetics of this CL reaction was very fast and lasted approximately 80 s, with the most intensive signal 40 s after the start of the reaction. This short measurement time was advantageous for saving computer accumulation of enormous signals of CL imaging in the limited capacity of a hard disk. As little as 1.0 fmol of the dextran-based chemiluminescent probe could be sensitively visualized on a nylon membrane. The CL intensity was directly proportional to the concentration of the chemiluminescent probe ($y=0.1362x+0.0843$, $R^2=0.9917$).

From the Scatchard plot method, the binding constant K_a of the dextran-based probe to avidin on a membrane was 5.1×10^6. The formation of the extending framework for probe-chain assembly depended on the linkage of the biotin moieties of the chemiluminescent probe to four binding sites of avidin. Thus, the conditions for this process were optimized by investigating several concentrations of both avidin and the chemiluminescent probe (Figure 3). The optimal ratio of the dextran-based probe and avidin was 1:1 by weight. At this ratio, the polymeric chemiluminescent probe gave the highest CL intensity for the detection of the target CYP3A4 protein.

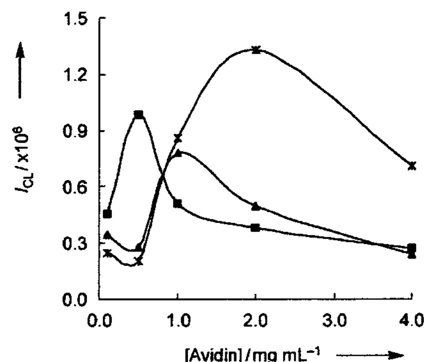

Figure 3. Effect of the concentration of avidin on the formation of an extending polymeric framework of the dextran-based chemiluminescent probe. CYP3A4 protein (750 fmol per spot) was employed on a PVDF membrane. The (Lu)560-(biotin)34-(Glc)3100 probe at 0.5 (■), 1 (▲), or 2 mg mL^{-1} (×) was mixed with avidin (0.1–4 mg mL^{-1}). The detection protocol was almost the same as that used in Figure 4, except that the amounts of avidin and probe were varied.

It is known that the sensitivity of the immunoassay could be greatly improved by attaching a number of chemiluminescent or fluorescent compounds of low molecular weight to a secondary antibody.[7] Thus, we employed this luminol- and biotin-containing dextran-based chemiluminescent macromolecular probe for the sensitive optical imaging of a specific protein on a PVDF membrane by the formation of a probe-chain assembly based on the interaction between avidin and biotin. We set up an immunoassay to detect CYP3A4 protein on a PVDF membrane. This membrane facilitated a higher absorption of proteins than the nylon membrane, and its hydrophobic property minimized the nonspecific interaction between the membrane and the dextran probe.

As shown in Figure 4a and b, at least 190 fmol of CYP3A4 on the PVDF membrane could be selectively detected by our system. The CL intensity was directly proportional to the concentration of CYP3A4 (in femtomoles per spot) on the membrane ($y=949401x+33975$, $R^2=0.9972$). Figure 4c and d show CL imaging data using an enzyme (HRP)-labeled avidin probe. The assay conditions were similar to those of the present system. The dextran-based probe gave lower background CL signals than the protein-based HRP probe. It is suggested that the dextran-based probe is more hydrophilic than the protein-based probe, and thus not readily absorbed on the PVDF membrane.

In conclusion, dextran-based chemiluminescent compounds containing luminol (or isoluminol) and biotin were successfully synthesized. At least 1 fmol of the chemiluminescent probe on a nylon membrane could be detected by use of the reagents CH_3CN, TPA, and H_2O_2 catalyzed by Fe^{III}. The extending polymeric framework of the dextran-based probe was simply formed by mixing avidin and the probe in a ratio of

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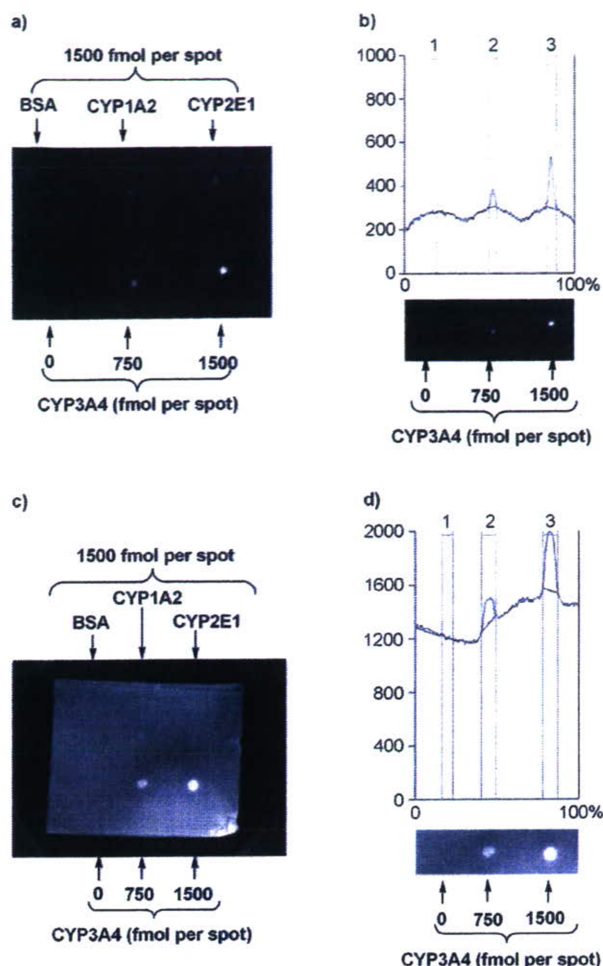

Figure 4. CL images of CYP3A4 on a PVDF membrane detected by a, b) a polymeric dextran-based probe and c, d) an enzyme-labeled avidin probe (see Experimental Section for details). BSA = bovine serum albumin.

1:1 (w/w). The probe-chain assembly produced enhanced the CL intensity, and thus sensitively and selectively detected CYP3A4 at concentrations as low as 190 fmol on a PVDF membrane after binding two kinds of antibody: a specific antibody for CYP3A4 and a biotinylated antibody for IgG. Therefore, this newly developed dextran-based chemiluminescent probe provides one of the most rapid and sensitive detection methods for CL imaging of proteins, and is complementary to the currently available enzymatic CL imaging. Ongoing research aims to extend our developed system to the detection of various proteins on a membrane microchip.

Experimental Section

Synthesis of chemiluminescent compounds: Dextran T500 (400 mg) was dissolved in water (60 mL) and the solution was mixed with sodium periodate (317 mg).[8] After approximately 30% oxidation, the partially oxidized dextran was precipitated with methanol and subsequently dissolved in dimethyl sulfoxide (60 mL). 6-Hydrazidohexyl D-biotinamide (30 mg) was added and the mixture was stirred at room temperature for 3 h. Luminol or isoluminol (80–240 mg) and glacial acetic acid (16 mL) were added and the mixture was stirred overnight at 60°C. The modified dextran was precipitated with methanol and dissolved in ethylene glycol (30 mL). Sodium borohydride (870 mg) was added and the mixture was stirred at 4°C for 4 h. The resultant dextran (approximately 280 mg), which contained luminol (or isoluminol) and biotin, was precipitated with methanol and dried in vacuo. Its purity was checked by gel-filtration liquid chromatography (GFLC). Detection of CYP3A4 with polymeric dextran-based probe: A PVDF membrane was spotted with ethanol followed by BSA and several human recombinant CYP proteins in aqueous solution (2 μL each). After drying, the membrane was incubated at 37°C for 1 h with anti-human CYP3A4 rabbit polyclonal antibody (5.7 $\mu g\,mL^{-1}$) and biotinylated anti-rabbit IgG goat antibody (16.0 $\mu g\,mL^{-1}$) in a probe-chain assembly mixture (2 mL) composed of (Lu)560-(biotin)34-(Glc)3100 probe (4 mg), avidin (4 mg), BSA (6 mg), dextran (6 mg), and PBS (10 mM). The probe assembly mixture was preincubated at 37°C for 1 h. After the reaction, the membrane was washed with a mixture of 0.15% Triton X-100 and 10 mM PBS solution (15 mL × 3) followed by 75% methanol (2 mL). The membrane was dried at 37°C for 10 min in vacuo, then immersed in a CL-emitting solution (700 μL CH_3CN and 300 μL 1.0 M TPA) followed by addition of 30% H_2O_2 (50 μL) and 10 mM $FeCl_3$ (50 μL). The membrane was allowed to stand at room temperature for 3 s before CL detection for 2.0 min with a CCD camera.

Detection of CYP3A4 with enzyme-labeled avidin probe: The spotting of proteins on the membrane was the same as in the experiment with the dextran-based probe. After drying, the membrane was blocked with 5% skimmed milk at 37°C for 1 h, and then incubated at 37°C for 1 h with anti-human CYP3A4 rabbit polyclonal antibody (5.7 $\mu g\,mL^{-1}$), biotinylated anti-rabbit IgG goat antibody (16.0 $\mu g\,mL^{-1}$), and avidin–HRP (0.05 $\mu g\,mL^{-1}$). After the reaction, the membrane was washed with a mixture of 0.15% Triton X-100 and 10 mM PBS (15 mL × 3), and then treated with an enzymatic CL detection kit consisting of H_2O_2, 4-iodophenol, and luminol, which is available as the LumiGLO system before CL detection for 2.0 min with a CCD camera.

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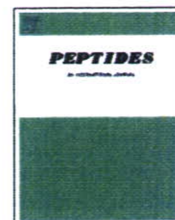

A selective fluorescence reaction for peptides and chromatographic analysis

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ABSTRACT

A novel and selective fluorescence reaction is proposed for the quantitative determination of peptides by reversed-phase liquid chromatography (RPLC). A single fluorescent product was formed when a peptide was heated at 120 °C for 20 min in a neutral aqueous medium (pH 7.0) containing catechol, sodium periodate, and sodium borate. The fluorescent products of four peptides such as Leu-Gly, Ala-Leu-Gly, Tyr-Gly-Gly-Phe-Leu, and Leu-Leu-Leu were easily separated on a reversed-phase column by gradient elution of methanol in a mobile phase containing sodium borate (pH 7.0), and then quantitatively detected by fluorimetry. The lower limits (S/N = 3) of the detection for the tested peptides were 0.5–1.0 pmol per an injection volume (40 μl). In addition, the fluorescent products of phenylalanine amide and Leu-Leu-Leu were identified by electrospray ionization-time of flight-mass spectrometry (ESI-TOF/MS) for the elucidation of their chemical structures.

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1. Introduction

Peptides are biological molecules of paramount importance as either drugs for therapeutic treatment or disease markers for clinical diagnosis, or the target for protein identification in proteome research [1]. In particular, peptide hormones have various biological activities, and thus they play major roles to keep the homeostasis in vivo. Until now, more than 1000 biological active peptides have been isolated from animals or plants, and their activities were analyzed for their roles as neurotransmitters [2], hormones [3,4], or enzyme inhibitors [5]. As recent examples, two new opioid peptides of Tyr-Pro-Trp-Gly-NH_2 (Tyr-W-MIF-1) [6–8] and Tyr-Pro-Trp-Phe-NH_2 (endomorphin-1) [9], which selectively bound to μ-opioid receptor, were isolated in mammalian brain, and a new arterial natriuretic peptide was also found to play an important role for homeostatic control of body water and adiposity in response to high pressure of blood [10,11].

In contrast, the detection technique for the peptides proceeds with comparative slowness in recent years. Generally, peptides are separated by RPLC and then detected by their absorption at an ultra violet (UV) wavelength (210–300 nm). It is well known that the detection limit of the RPLC-UV method shows generally the nmol level, due to that peptides have a small molar extinction coefficient. In addition, there are also sensitive fluorimetric methods for the detection of peptides, utilizing the reagents of o-phthalaldehyde (OPA) [12] and fluorescamine [13], however their selectivity is lower. These

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fluorescent reagents not only react with primary amino groups in peptide molecules, but also react with amines, amino acids and nucleic acids, etc., which exist in many biologic samples [12,13]. Therefore, it is important to develop a novel sensitive and selective reaction for the detection of peptides.

We developed previously two selective fluorescence reactions for Arg-containing peptides with benzoin [14,15] and for N-terminal Tyr-containing peptides with hydroxylamine [16,17]. Recently, we have found that a novel fluorescence reaction for peptides different from the above reactions. In this reaction, a peptide was reacted with catechol in the presence of sodium periodate, and then converted to a fluorescent compound in a neutral borate aqueous solution. In this paper, the reaction conditions were first optimized using an amino acid amide, thereafter the reactivities to peptides were studied, and then the separation and quantitative detection of the fluorescent products from several peptides were investigated by RPLC. Finally, we presumed the chemical structures of their fluorescent products by ESI-TOF/MS.

2. Material and methods

2.1. *Chemicals and reagents*

Catechol, boric acid, and sodium periodate as guaranteed reagents were purchased from Wako pure chemicals (Osaka, Japan). Salicyl alcohol was purchased from Nacalai tesque (Kyoto, Japan). Amino acid amides were obtained from Bachem (Bubendolf, Switzerland). Peptides were purchased from Sigma (St. Louis, MO, USA) and Wako pure chemical. Catechol (50 mM), boric acid (300 mM), and sodium periodate (20 mM) were dissolved in water and various pHs of the borate solution were adjusted with sodium hydroxide. Amino acid amides, peptides, amino acids, and other biologic substances were dissolved in water or 50% aqueous solution of 2-methoxyethanol (Methyl Cellosolve, Wako Pure Chemical), and their stock solutions (0.5–5.0 mM) were kept at −20 or 4 °C. For each experiment, the stock solution was diluted with water at a desired concentration.

2.2. *Apparatus and experimental conditions*

The RPLC system consisted of a gradient pump (PU-2089 type; Jasco, Tokyo, Japan), a reversed-phase column (Lichrospher RP-18e type; 125 mm × 4.0 mm i.d., pore size 5 μm, Merck, Darmstadt, Germany), an absorbance detector (UV-2070 plus type intelligent UV/VIS detector; Jasco), and a fluorescence spectrometer (RF-10AXL type; Shimadzu, Kyoto, Japan). For the separation of the fluorescent derivatives of peptides on the reversed-phase column, gradient elution from 15 to 90% (v/v), or from 0 to 80% of methanol in aqueous mobile phase containing 12.5 mM sodium borate (pH 7.0) was carried out during 40 min at a constant flow-rate of 0.5 ml/min. The fluorescence intensity in the column eluate was monitored at 500 nm (emission) with excitation at 400 nm, and its UV absorption was monitored at 254 nm. Uncorrected fluorescence excitation and emission spectra, and intensities were measured with a spectrofluorimeter (FP-6300 type; Jasco) in 10 mm × 10 mm quartz cells.

The fluorescent products were analyzed with ESI-TOF/MS (Mariner type; Applied Biosystems, Foster, CA, USA). Operating conditions of the ESI-TOF/MS interface in the positive-ion mode were as follows: (1) source setting; spray tip potential = 4000–5000 mV, (2) interface settings; nozzle potential = 100 mV, skimmer 1st potential = 11 mV, quadrupole dc potential = 5 mV, deflection voltage = 0 mV, einzel lens potential = −25 mV, quadrupole RF voltage = 700–1000 mV, and quadrupole temperature = 140 °C, (3) analyzer settings; push pulse potential = 490 mV, pull pulse potential = 200 mV, pull bias potential = 2 mV, acceleration potential = 4000 mV, reflector potential = 1500 mV, and detector voltage = 2300 mV, (4) spectrum acquisition settings; 3.0 s/spectrum, curtain gas flow rate = 0.2 l/min, nebulizer gas flow rate = 1.0 l/min, (5) infusion injection = 10 μl/min.

2.3. *Typical procedure for fluorescence reaction*

A 0.5-ml portion of 1.0 or 0.1 mM each amino acid amide or peptide (final concentration in the reaction mixture, 0.33 or 0.033 mM) was placed in a test tube, to which were added

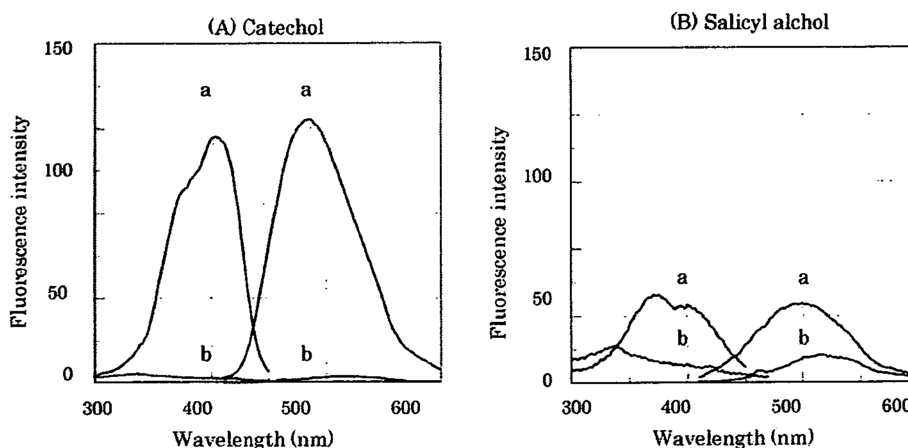

Fig. 1 – Excitation and emission spectra of the reaction mixture of Phe-NH_2 (a) and its reagent blank (b). Phe-NH_2 (0.33 mM) or water were reacted with 3.3 mM catechol (A) or 3.3 mM salicyl alchol (B) in the presence of 2.0 mM $NaIO_4$ and 50 mM borate (pH 7.0) at 120 °C for 20 min.

0.5 ml of 10 mM catechol (final concentration, 3.3 mM), 0.25 ml of 12 mM sodium periodate (final concentration, 2.0 mM), and 0.25 ml of 300 mM sodium borate (pH 7.0) (final concentration, 50 mM). The mixture was heated at 120 °C for 20 min. The reagent-blank solution was prepared in the same way except that the sample solution was replaced with water.

3. Results

3.1. *Optimum conditions for fluorescence reaction*

Phenylalanine amide (Phe-NH_2) was reacted with catechol or its analogue, salicyl alcohol in the presence of periodates and borates, and then the fluorescence spectra of the reaction mixtures were measured. As shown in Fig. 1, the excitation and emission maxima of the produced fluorescence were observed at 400 and 500 nm, respectively. The fluorescence produced by the reaction with catechol was about three times higher than that by the reaction with salicyl alcohol. In addition, the background became much higher by using salicyl alcohol than catechol. Non-fluorescence was produced by using other analogues such as resorcinol or phenol instead of catechol.

The oxidizing effect was examined by using various agents such as sodium periodate, potassium periodate, potassium iodate, sodium perchlorate, potassium ferricyanide, and potassium permanganate at each 2.0 mM final concentration. When potassium periodate or potassium ferricyanide was employed, fluorescence intensity from Phe-NH_2 was decreased to 50 or 20%, respectively, as compared with that obtained with sodium periodate. The fluorescence spectra of

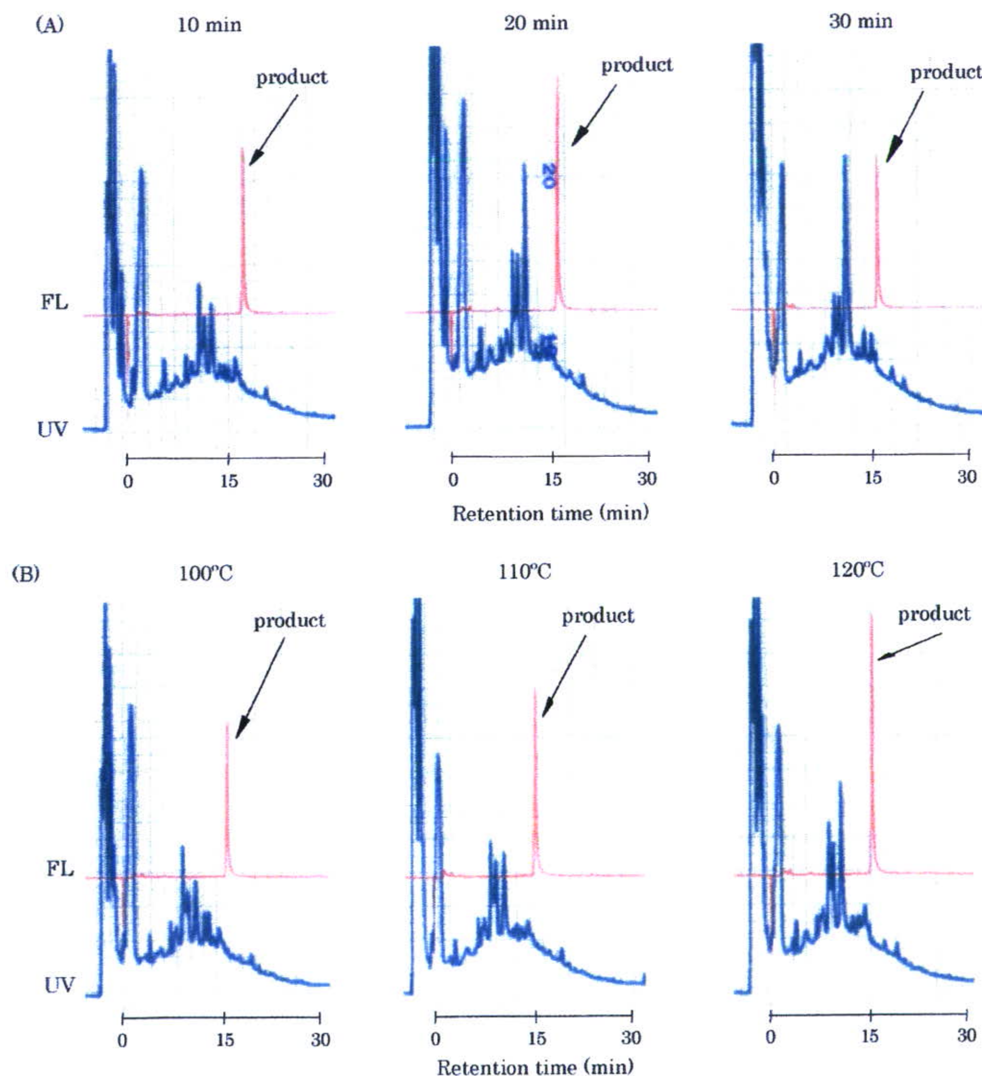

Fig. 2 – RPLC chromatograms obtained with different times (A) and temperatures (B) for the fluorescence reaction. ***RPLC conditions*: mobile phase = CH_3OH (15–90%, v/v) + H_2O (80–5%) + 250 mM borate, pH 7.0 (5%) for 40 min; injection volume = 40 μl; FL and UV mean the fluorescence detection at 400 nm (excitation) and 500 nm (emission), and the ultraviolet absorption detection at 254 nm, respectively. *Reaction components*: 0.033 mM Phe-NH_2, 3.3 mM catechol, 2.0 mM $NaIO_4$, 50 mM borate (pH 7.0).**