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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 $10^{15}\,M^{-1}$.

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

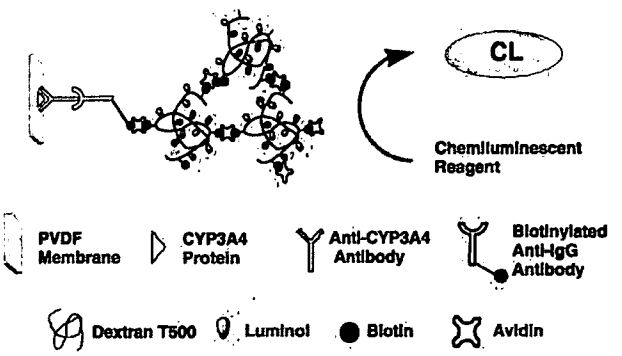
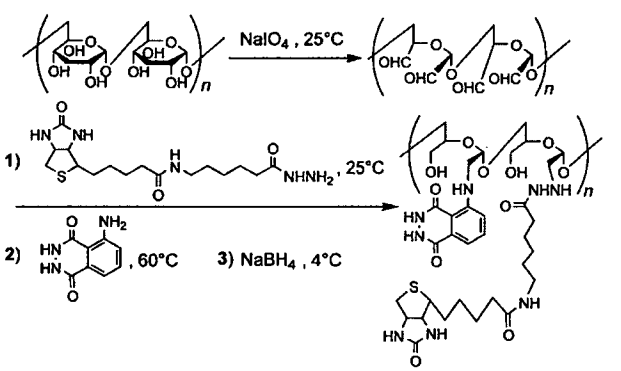


Figure 1. Detection of CYP3A4 (cytochrome P450, family 3, subfamily A, polypeptide 4) on a PVDF membrane with a polymeric dextran-based chemiluminescent probe. IgG = immunoglobulin G.

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

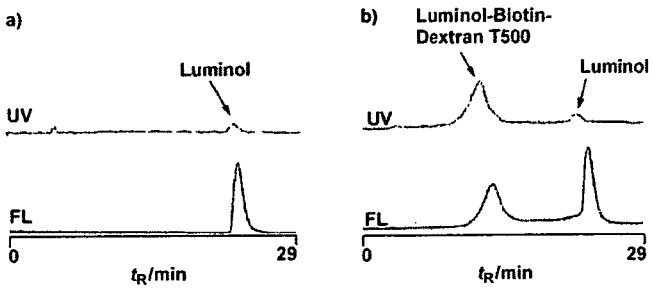

Figure 2. GFLC of dextran-based chemiluminescent probe: a) luminol (0.011 mg mL^{-1}); b) (Lu)560-(biotin)34-(Glc)3100 (1.0 mg mL^{-1}). GFLC conditions: injection volume, 10 mL; column, TSK gel T2000SW; eluent, 0.1% (v/v) aqueous solution of trifluoroacetic acid; flow rate, 1.0 mL min^{-1}; UV detection,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

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.

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.

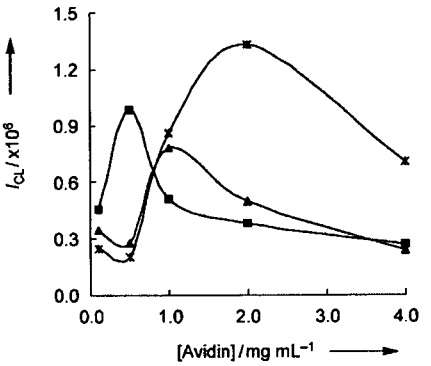

Figure 3. Effect of the concentration of avidin on the formation of an extending polymeric framework of the dextran-based chemiluminescent probe. CYP3A4 protein (750 fmol per spot) was employed on a PVDF membrane. The (Lu)560-(biotin)34-(Glc)3100 probe at 0.5 (■), 1 (▲), or 2 mg mL^{-1} (×) was mixed 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

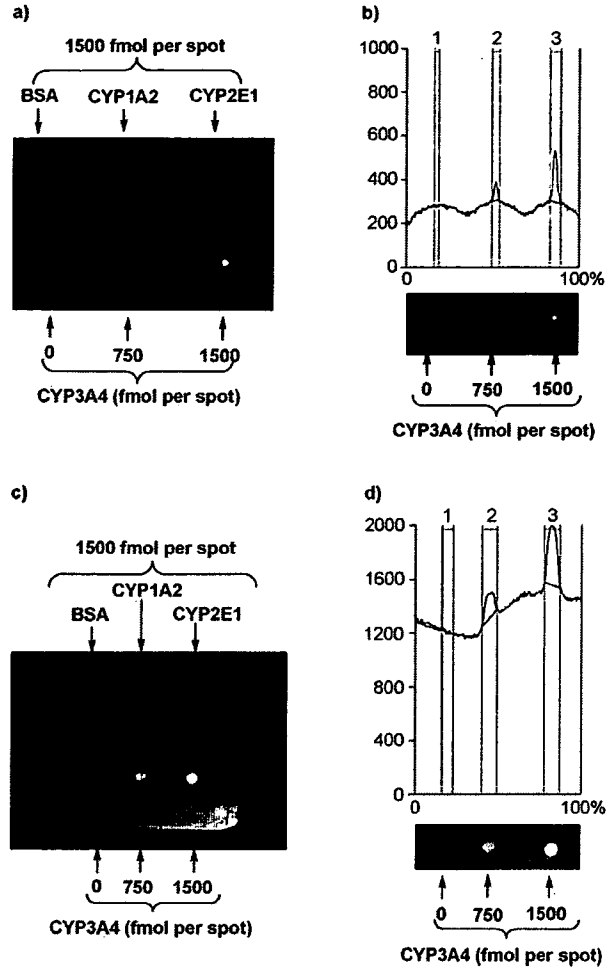

Figure 4. CL images of CYP3A4 on a PVDF membrane detected by a, b) a polymeric dextran-based probe and c, d) an enzyme-labeled avidin probe (see Experimental Section for details). BSA = bovine serum albumin.

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.

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

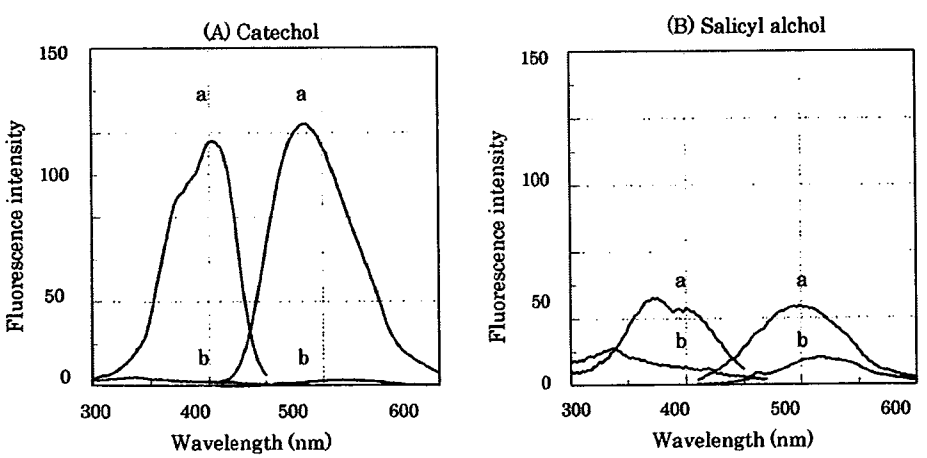

Fig. 1 – Excitation and emission spectra of the reaction mixture of Phe-NH_2 (a) and its reagent blank (b). Phe-NH_2 (0.33 mM) or water were reacted with 3.3 mM catechol (A) or 3.3 mM salicyl alchol (B) in the presence of 2.0 mM $NaIO_4$ and 50 mM borate (pH 7.0) at 120 °C for 20 min.

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

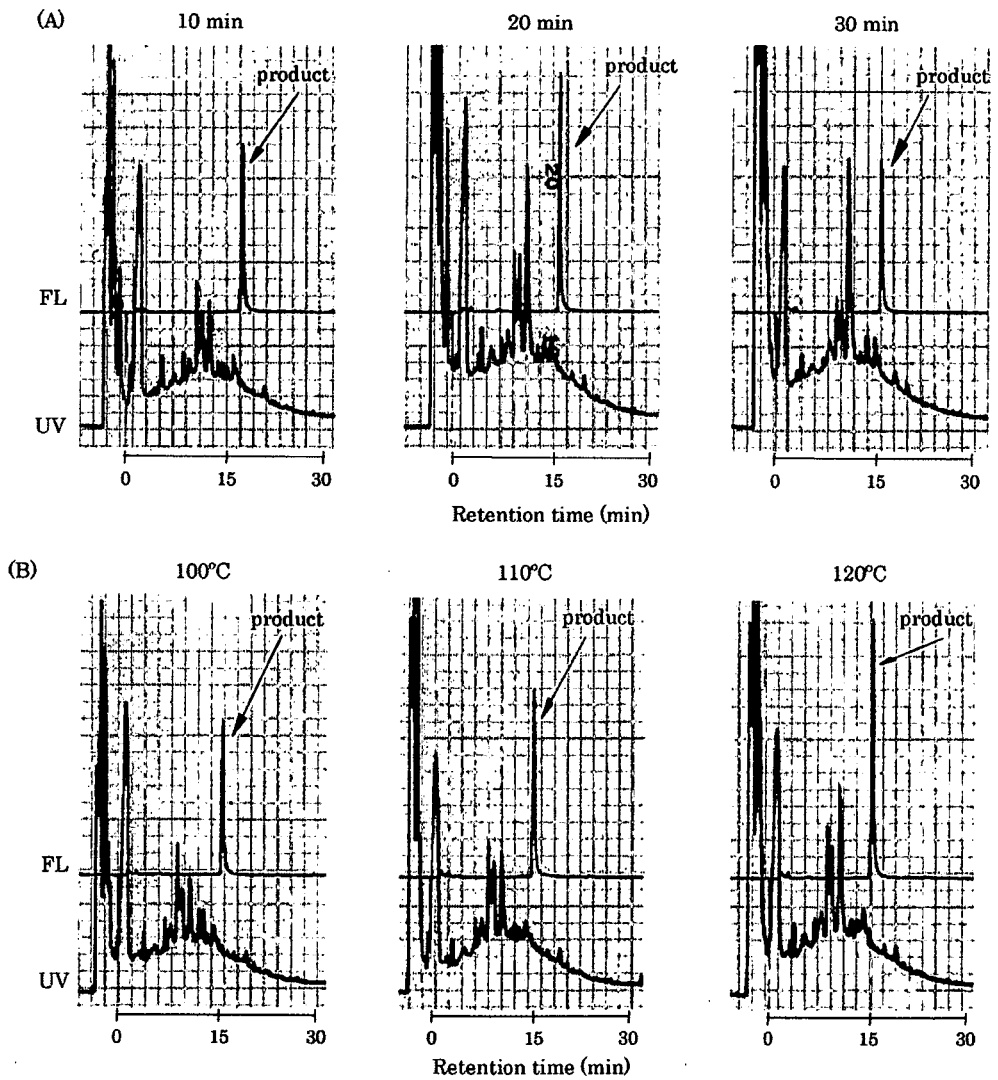

Fig. 2 – RPLC chromatograms obtained with different times (A) and temperatures (B) for the fluorescence reaction. *RPLC conditions*: mobile phase = CH_3OH (15–90%, v/v) + H_2O (80–5%) + 250 mM borate, pH 7.0 (5%) for 40 min; injection volume = 40 μl; FL and UV mean the fluorescence detection at 400 nm (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).

the product from Phe-NH_2 with three oxidation agents (sodium periodate, potassium periodate and potassium ferricyanide) were almost the same because the product structure might not be changed by these agents. On the other hand, potassium iodate, sodium perchlorate, or potassium permanganate did not lead to the formation of fluorescent product, probably because their optimum concentrations were different from that of sodium periodate. In addition, non-fluorescence was observed in the absence of sodium periodate, and the intensity with 4.0 mM periodate was decreased to be approximately 10% of that with 2.0 mM periodate. The optimum concentration of sodium periodate was found to be 2.0 mM as the final concentration in the reaction mixture.

Other reaction conditions were investigated by RPLC. As shown in Fig. 2A, the fluorescence reaction was carried out at 120 °C for different reaction times (0–30 min). It was found that the maximum yield of the fluorescent product from Phe-NH_2 was observed at 20 min. The test tube should be sealed with a screw cap and the highest yield of fluorescent product was obtained at 120 °C (Fig. 2B).

The pH of the sodium borate solution also affected the formation of the fluorescent product. The pH 7.0 gave the greatest yield of fluorescent product, and at pH 6.5 and 8.0, fluorescent products were decreased to less than 65 and 40%, respectively (Fig. 3A). At pH 7.0, the reaction was carried out under various concentrations of the borate (33–67 mM). Fig. 3B shows that the fluorescence intensity is almost the same either with 50 or 67 mM borate. In the presence of 33 mM borate, slightly smaller fluorescent peak was observed, however, the fluorescent peak was not observed without borate.

On the basis of the above results, the optimum conditions for the fluorescence reaction with Phe-NH_2 was selected as follows; 3.3 mM catechol, 2.0 mM sodium periodate, and 50 mM sodium borate (pH 7.0), 120 °C of the reaction temperature and 20 min of reaction time, respectively.

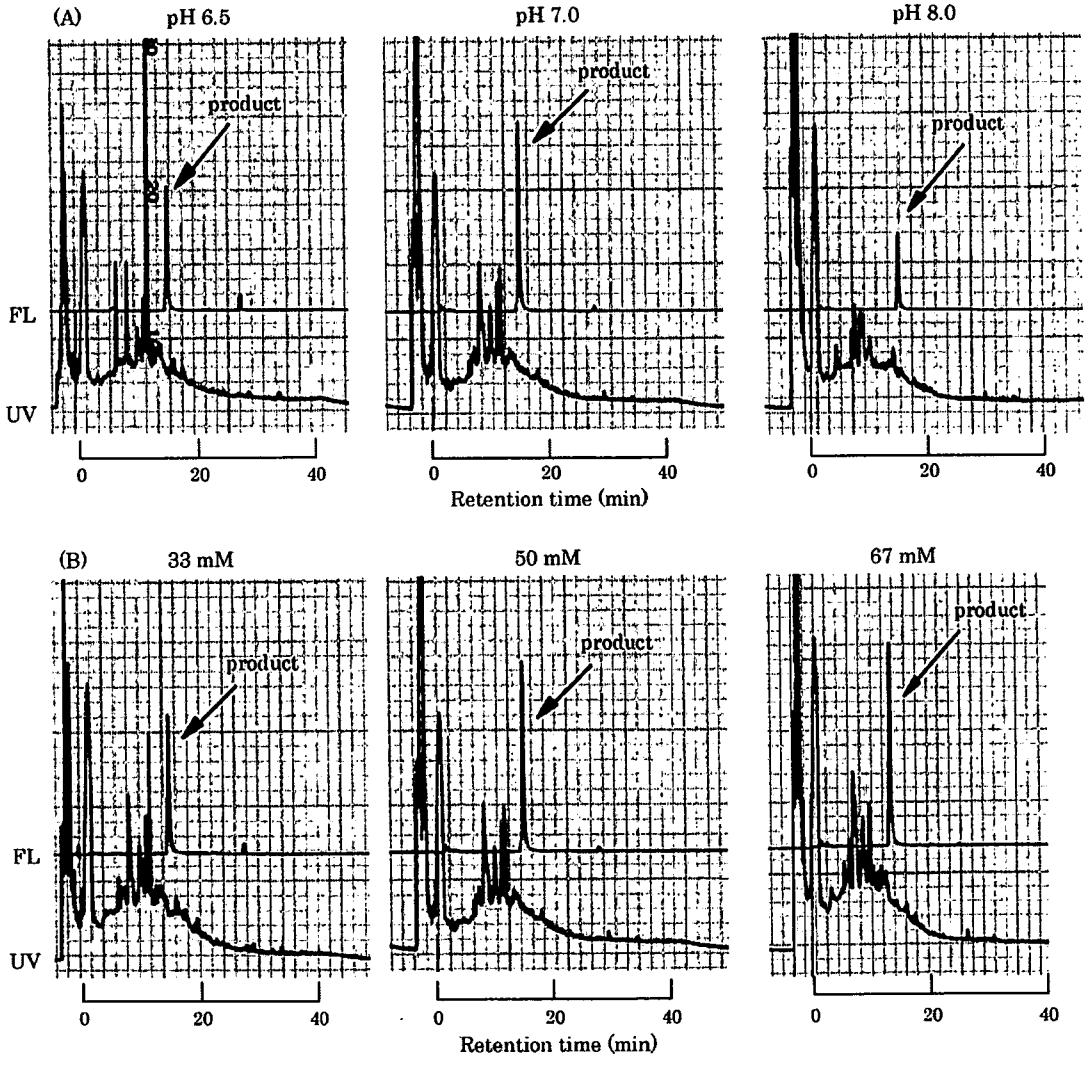

Fig. 3 – RPLC chromatograms obtained with different pHs (A) of 50 mM borate and concentrations (B) of borate at pH 7.0 for the fluorescence reaction. RPLC conditions were the same as those for Fig. 2. Reaction conditions: 0.033 mM Phe-NH_2, 3.3 mM catechol, 2.0 mM $NaIO_4$, 0–67 mM borate (pH 6.5–8.0), 120 °C, 20 min.

3.2. *Specificity and reactivity*

We measured fluorescence intensities produced from various bio-substances such as amino acids, sugars, and polyamines under the recommended conditions for the present fluorescence reaction. All free amino acids (20 kinds), sugars (glucose and ribose), polyamines (spermine and cadaverine), and nucleic acid bases (adenine, thymine, guanine, and cytosine) did not produce any fluorescences.

On the other hand, the fluorescences were yielded when various amino acid amides and dipeptides were reacted (Table 1). Among these amino acid amides, however, Thr-NH_2 and Ser-NH_2 could not generate the fluorescence under the recommended reaction conditions. Hydroxyl group in these amino acid amides might form an intermolecular hydrogen bond with amino or amide group, which reduced the reactivity to catechol. Pro-NH_2 produced the strong fluorescence, while Pro-containing dipeptides such as Pro-Pro and Pro-Gly yielded weak or non-fluorescence. The nitrogen atom in the pyrrolidine ring of the Pro-containing peptide might become a tertiary amine after binding to catechol, which resulted in the complete loss of nucleophilicity for binding to borate. In the case of the reaction with Pro-NH_2, however, its fluorescent product might be formed by the different mechanism from that with other peptides. These results suggested that the reactivity of the peptides depends on the species of the N-terminal amino acid in the peptide molecule.

Table 1 – Relative fluorescence intensity produced from amino acid amides and dipeptides

Substance	Fluorescence intensity (%)
Phe-NH_2	100
Gly-NH_2	17
Ala-NH_2	139
Val-NH_2	150
Leu-NH_2	286
Ile-NH_2	143
Met-NH_2	143
Tyr-NH_2	22
Trp-NH_2	21
Glu-NH_2	133
Asp-NH_2	135
Asn-NH_2	71
Gln-NH_2	81
Lys-NH_2	19
His-NH_2	19
Arg-NH_2	79
Thr-NH_2	0
Ser-NH_2	0
Pro-NH_2	429
Pro-Pro	0
Pro-Gly	9
Glu-Val	48
Tyr-Gly	100
Trp-Leu	13
Gly-Gly	104
Val-Gly	248

The substance (0.33 mM) was reacted according to the procedure described in Section 2, and the generated fluorescence was measured with a spectrofluorimeter set up at 400 and 500 nm for the excitation and emission, respectively.

Table 2 – Retention time and relative fluorescence intensity of each fluorescent product yielded with peptides and amino acid amides

Target analyte (0.033 mM)	Retention time (min)	Relative fluorescent intensity (cm)
Arg-NH_2	3.6	1.8
Gln-NH_2	5.8	1.7
Met-NH_2	14.4	1.7
Ala-NH_2	15.0	1.8
Tyr-NH_2	18.8	1.4
Phe-NH_2	21.6	1.4
Gly-Gly	2.6	9.2
Val-Gly	6.4	40.5
Leu-Gly	9.2	82.8
Phe-Gly	10.8	39.8
Gly-Leu-Ala	11.2	5.7
Ala-Leu-Gly	13.4	55.1
Ala-Leu-Ala	13.6	39.8
Trp-Gly-Gly	14.4	23.4
Tyr-Gly-Gly-Phe	15.6	95.9
Tyr-Gly-Gly-Phe-Leu	20.4	58.7
Leu-Leu-Leu	25.0	100.0

RPLC conditions: mobile phase = CH_3OH (0–80%, v/v) + H_2O (95–15%) + 250 mM sodium borate, pH 7.0 (5%) for 40 min; injection volume = 40 μl.

The separation of the fluorescent derivatives from several amino acid amides and peptides was investigated by RPLC, and the results are shown in Table 2. Each single fluorescent product was observed. The peak heights of the fluorescent products from the peptides were higher than those for amino acid amides. The peptides such as Leu-Gly, Tyr-Gly-Gly-Phe and Leu-Leu-Leu, which have Leu and Tyr at the N-terminus, generated strong fluorescences, while Gly-Gly and Gly-Leu-Ala produced relatively weak fluorescences than other peptides. However, the present fluorescence reaction is fairly specific for most of the peptides.

3.3. *Quantification and detection limit by RPLC with fluorescence detection*

When the reaction mixture of four peptides of Leu-Gly, Ala-Leu-Gly, Tyr-Gly-Gly-Phe-Leu and Leu-Leu-Leu was subjected to RPLC, their fluorescent products were mutually separated within 40 min by gradient elution of methanol in mobile phase, and then detected as shown in Fig. 4A. The borate (pH 7.0) in the mobile phase was an important factor for the fluorescence detection of these products. When the mobile phase without the borate was used, the fluorescent peaks were not observed in the chromatogram. The result indicates that the fluorescent product was formed as a borate complex, since without the borate anion the product did not emit the fluorescence.

The calibration graphs of four peptides were linear with sufficient correlation coefficients of 0.990–1.000 as shown in Fig. 4B. The lower detection limit (signal per noise ratio, S/N = 3) was 0.5–1.0 pmol per an injection volume (40 μl) for the tested peptides. The sensitivity of this RPLC method was

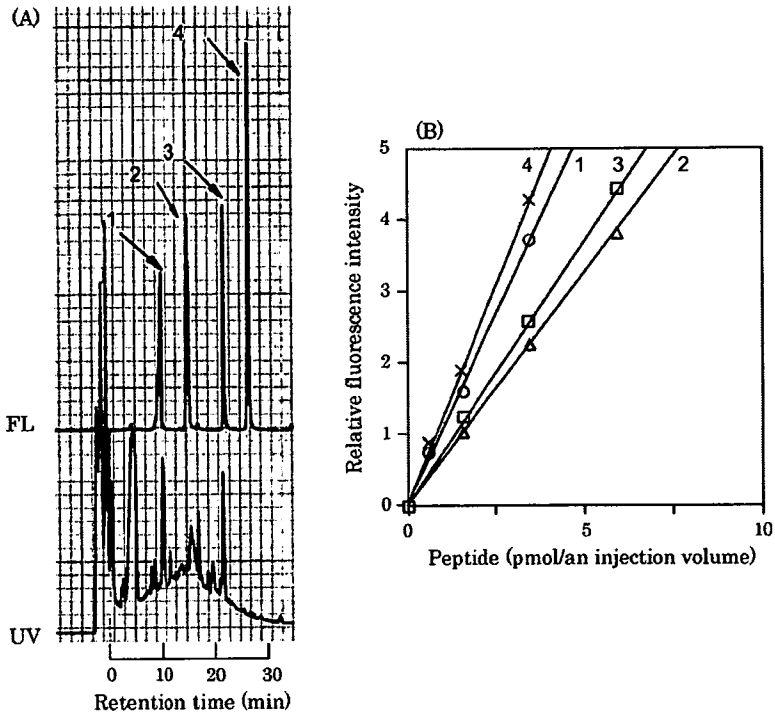

Fig. 4 – RPLC chromatograms (A) obtained with a fluorescence reaction mixture containing four peptides and their calibration curves (B). RPLC conditions: mobile phase = CH_3OH (0–80%, v/v) + H_2O (95–15%) + 250 mM borate, pH 7.0 (5%) for 40 min; injection volume = 40 μl; detection conditions were the same as those for Fig. 2. Reaction conditions: 25–150 nM (1.0–6.0 pmol/40 μl injected), 0.033 mM or 0.067 mM peptides, 3.3 mM catechol, 2.0 mM $NaIO_4$, 50 mM sodium borate (pH 7.0), 120 °C, 20 min. Peaks (concentration): 1 = Leu-Gly (0.033 mM), 2 = Ala-Leu-Gly (0.067 mM), 3 = Tyr-Gly-Gly-Phe-Leu (0.067 mM), 4 = Leu-Leu-Leu (0.067 mM).

10–100 times higher than that by the conventional RPLC method with UV detection.

3.4. *Structure of the fluorescent product with ESI-TOF/MS*

To estimate the chemical structures of the fluorescent products from Phe-NH_2 and Leu-Leu-Leu, the fluorescent products were first isolated by RPLC, respectively. Then, positive ion mass spectra of these fluorescent fractions were measured by ESI-TOF/MS. In both MS spectra of the products from Phe-NH_2 and Leu-Leu-Leu, the mass signals corresponding to the reaction products were observed at $m/z = 345$ (Fig. 5A) and at $m/z = 538$ (Fig. 5B) as their $[M + H]^+$ ions, respectively, involving two molecules of catechol in the peptide molecule. However, it was difficult for the fluorescent product to be detected as a borate complex by the positive ion ESI-TOF/MS. The borate complex might be dissociated in the ion source of ESI-TOF/MS.

We estimated that the boron atom in the fluorescent product coordinates to hydroxyl groups of two catechol molecules, and the N-terminal amino and imino groups in the peptide molecule for emitting the fluorescence, as shown in Fig. 6A. The chemical structure of the fluorescent product was also elucidated on the basis of a similar fluorescent product (Fig. 6B) which is formed by the reaction of a primary amine with 2,2-diphenyl-1-oxa-3-oxonia-2-boratanaphthalene (DOOB) [18].

4. Discussion

Herein a novel fluorescence reaction for peptides was developed and then applied to RPLC for the quantitative detection of the peptides. Peptides first reacted with catechol and periodate, and then the fluorescent product might be formed as a borate complex in the presence of borate anions at pH 7.0. Most of the peptides could be sensitively detected by RPLC, showing a detection limit at picomole level per an injection volume. This sensitivity is 10–100 times higher than that with UV detection for the peptides, and shows almost the same level as that of the other fluorescence reaction with OPA or fluorescamine. However, the proposed reaction was fairly specific for peptides, since any fluorescent compounds were not produced from other bio-substrates such as amino acids, sugars, polyamines, and nucleic acid bases. Therefore, this reaction will be a convenient tool for the sensitive detection of peptides, in particular, composed of aliphatic amino acids in complex matrices such as cell, blood, and tissue.

In addition, we measured the mass spectra of the isolated fluorescent products due to Phe-NH_2 and Leu-Leu-Leu by ESI-TOF/MS for the elucidation of their chemical structures. The data suggested that the two molecules of catechol in the fluorescent product are involved, and a fluorescent boron complex is formed by coordinate bonds to the amino and imino groups in the N-terminal amino acid of the peptide, and the hydroxyl group in catechol.

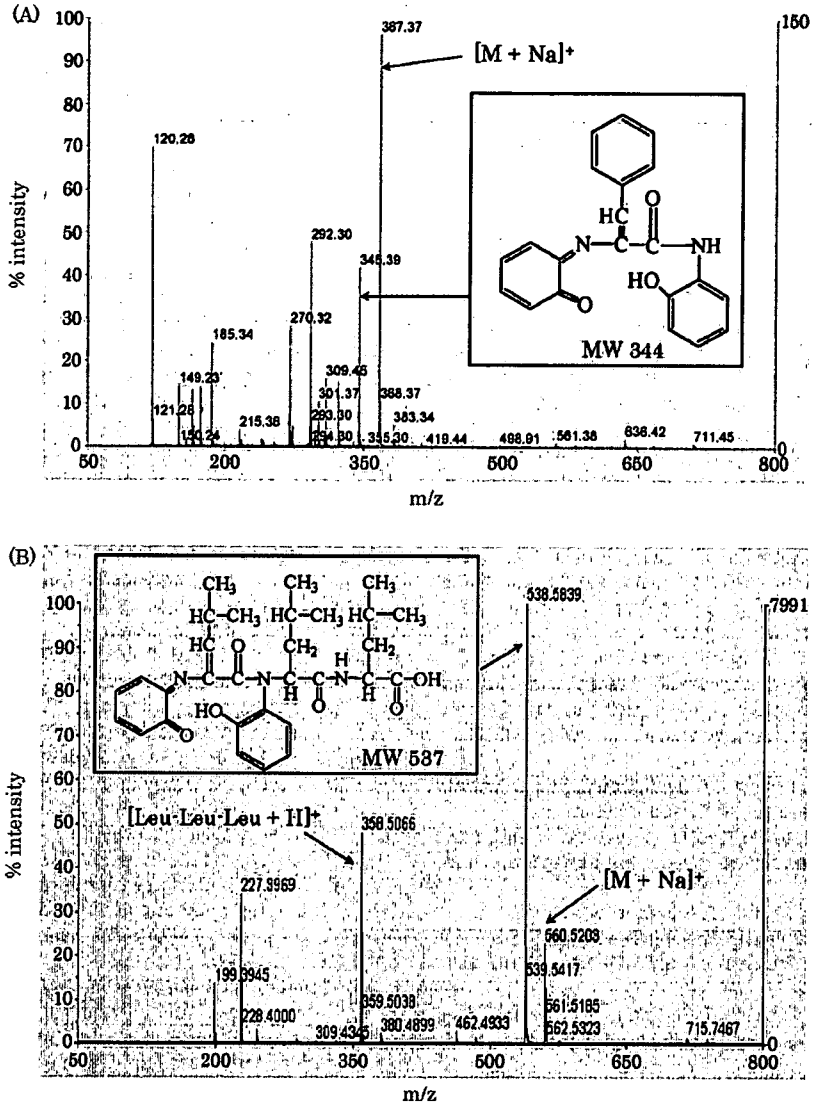

Fig. 5 – Positive ion ESI mass spectra of fluorescent products from Phe-NH_2 (A) and Leu-Leu-Leu (B).

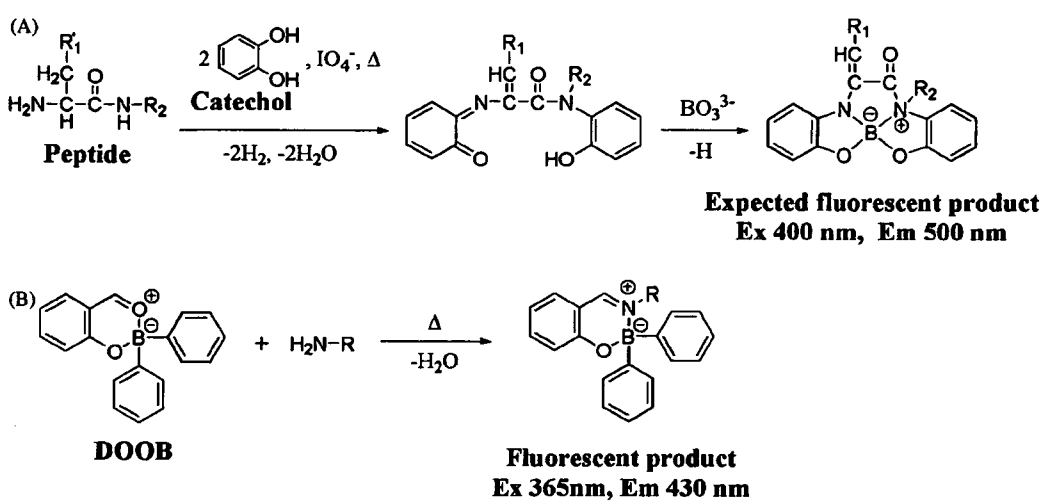

Fig. 6 – Possible chemical structure of the fluorescent product formed by the present reaction of a peptide with catechol, periodate and borate (A), and a fluorescent product [18] formed by a similar reaction of amine with DOOB (B).

Acknowledgements

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Notes & Tips

Fluorescence detection of amino acids in the postcleavage conversions for manual sequencing of a peptide

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Abstract

A modified Edman degradation method where fluorescent derivatives of amino acids were generated from the postcleavage products of a peptide is described. In the method, the target peptide was applied onto double glass fiber membranes in a small filter disk (4 mm i.d.) and then treated with small amounts of reagents for the manual sequencing of the peptide. The anilinothiazolinone (ATZ) of N-terminus amino acid residue after the isolation from the solid-phase membranes was reacted with a primary amine, 4-(1′-cyanoisoindolyl)aniline (CIA), to form a more stable and sensitive fluorescent derivative, phenylthiocarbamoyl–CIA. An average yield of 85% was obtained in neutral pH conditions for the CIA reaction. The ATZ–CIA–amino acids were separated by reversed-phase liquid chromatography and detected by fluorometry. The lower limits of the detection for amino acids after the Edman degradation were 0.16 to 0.52 pmol (signal/noise ratio = 3) on the column. The sensitivity was approximately 10 times higher than ultraviolet absorbance detection of phenylthiohydantoin products in the conventional Edman degradation. The suitability of the method was demonstrated by the sensitive manual sequencing of insulin chain B composed of 30 amino acids.

The amino acid composition and sequence analysis of peptides or proteins are essential for the investigation of structure–function relationships because the secondary and tertiary structure of proteins, which are necessary for analyzing biological functions, can be estimated from the amino acid sequences. The knowledge of protein structure is vital for the design of pharmaceutical peptide drugs. Recent advances in the drug designs include amino acid engineering where the primary sequence of the peptide or protein is modified, ranging from a single residue to a large-scale manipulation of the amino acid sequence [1–3]. The Edman degradation principle is a well-known method for direct peptide or protein sequencing determination. In this method, phenylisothiocyanate (PITC)[1] is first coupled with N terminus of the peptide. Treatment of the product with anhydrous acid removes the N-terminal amino acid residue as an unstable anilinothiazolinone (ATZ), which is then isomerized to a stable and ultraviolet (UV) active phenylthiohydantoin (PTH) by the treatment with hydrous acid. The PTH derivatives are finally separated by reversed-phase liquid chromatography (RPLC) at the end of the degradation. However, a large amount of sample has been required due to the low sensitivity for the UV detection of PTH derivatives. Although numerous techniques have been described for generating fluorescent

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[1] *Abbreviations used:* PITC, phenylisothiocyanate; ATZ, anilinothiazolinone; UV, ultraviolet; PTH, phenylthiohydantoin; RPLC, reversed-phase liquid chromatography; CIA, 4-(1′-cyanoisoindolyl)aniline; GF, glass fiber; PTC, phenylthiocarbamoyl; TFA, trifluoroacetic acid; DMF, *N,N*-dimethylformamide; FL, fluorescence; SDS, sodium dodecyl sulfate; S/N, signal/noise ratio; RSD, relative standard deviation.

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amino acid derivatives to enhance the sensitivity of thiohydantoin–amino acids [4–6], these reagents are not routinely used because the coupling yield of peptides with the fluorescent reagents is poorer than PITC and/or their by-products interfere with the sensitive detection of amino acids.

Here we describe a highly sensitive manual RPLC method based on the Edman principle employing a fluorescent amine, 4-(1′-cyanoisoindolyl)aniline (CIA) [7]. The target peptide was absorbed on double glass fiber (GF) membranes in a small filter disk (4 mm i.d.) to improve its retention ability without polybrene or covalent attachment of peptides [8–10], and this small disk made the treatment with small amounts of reagents possible. ATZ–amino acid derivatives were reacted with CIA to form highly sensitive fluorescent derivatives, CIA–phenylthiocarbamoyl (PTC)–amino acids (Fig. 1). The product was stable and identified as the final luminescent product instead of the conventional PTH–UV detectable product.

The reaction of ATZ-amino acid with CIA proceeded smoothly in organic solvents such as CH_3CN and *N,N*-dimethylformamide (DMF) but was deteriorated in CH_3OH, $CHCl_3$, and *n*-butyl chloride, showing only 20 to 35% of the yield obtained with CH_3CN or DMF. CH_3CN was selected due to its high volatility. The influence of concentration on the product yield was then investigated with various CIA concentrations of 1 to 50 mM (Fig. 2A). There was a rapid increase in the production of CIA–PTC–amino acid up to 30 mM, and thereafter only a gradual increase was observed. Thus, the concentration of 30 mM was conveniently selected. Under the optimal conditions, the CIA reaction mixture theoretically contains 3.3 nmol of ATZ–amino acid and 600 nmol of CIA, and the relative yields of CIA–PTC derivative to the corresponding ATZ–amino acids were 93% for ATZ–Gly, 90% for ATZ–Val, 88% for ATZ–Ser, and 69% for ATZ–Glu.

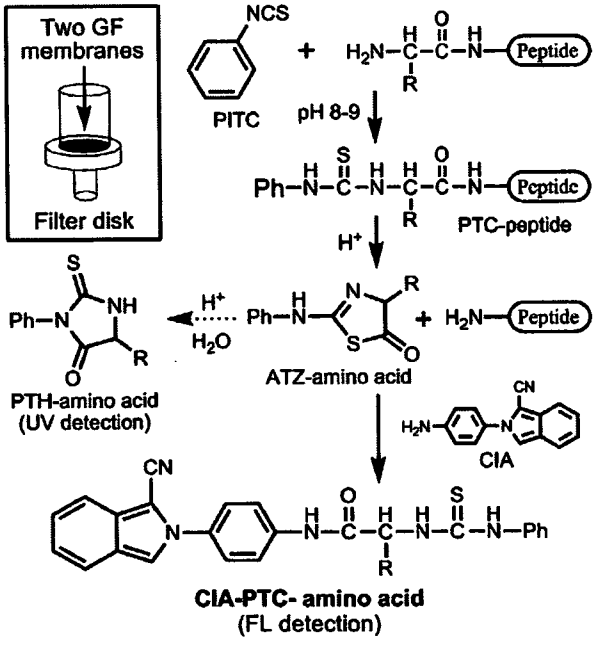

Fig. 1. Reaction scheme for the sensitization of ATZ–amino acid derivatives with CIA. The inset shows the design of a filter disk of polypropylene type supplied with a polytetrafluoroethene filter. Two GF membranes were set in the disk as depicted.

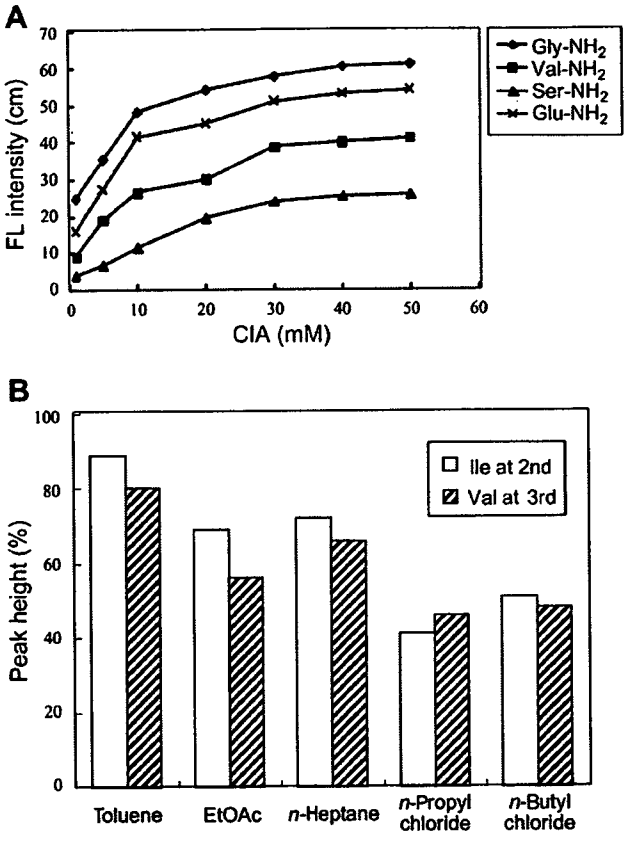

Fig. 2. (A) Effect of CIA concentration on the formation of PTC–CIA–amino acids in the derivatization reaction at 80 °C for 15 min. A portion (20 μl) of ATZ–amino acid in the eluate from membranes was reacted with 20 μl of 1 to 50 mM CIA in CH_3CN. FL intensity was based on peak heights of PTC–CIA derivatives. Each point represents the mean value of three replicate experiments. (B) Effect of solvents for washing after the PITC coupling and the TFA cleavage on the production of PTC–CIA–amino acid of insulin chain A at the 2nd cycle (Ile) and the 3rd cycle (Val). Peak heights obtained from Ile and Val amides, respectively, were taken as a reference (100%). Each point represents the mean value of three replicate experiments.

The retention time and relative fluorescence (FL) intensities were measured for the CIA–PTC derivatives of 20 amino acids (5.0 nmol each) (see Supplementary Table 1). We used a reversed-phase column (250 × 4.6 mm i.d.) maintaining the column temperature at 40 °C. Most of the CIA–PTC derivatives were separated by isocratic elution with 55% CH_3CN containing 12 mM acetic acid and 0.014% (w/v) sodium dodecyl sulfate (SDS). The isomeric amino acids of Leu and Ile could not be separated. Some amino acids (e.g., Arg, Cys, Ser) gave comparatively less intensity. The lower limits of detection for the amino acids were 0.16 to 0.52 pmol (signal/noise ratio [S/N] = 3) on the column. Their CIA–PTC derivatives were stable for the

separation period. There was also no trace of degradation of labile amino acids such as Ser and Thr, which have a tendency to undergo degradation in aqueous acidic conditions required for PTH conversion.

It is important to wash the membranes after each reaction because the excess reagents, as well as by-products, interfere with the continuous sequencing procedures. As shown in Fig. 2B, washing with toluene gave the highest yield of the CIA products from the amino acid residues at the 2nd and 3rd cycles in the sequencing of the peptide insulin. The production of the CIA derivatives after washing the membrane with *n*-propyl chloride or *n*-butyl chloride was decreased because the PITC-coupled peptide was eluted with these solvents. Despite the fact that toluene was the most effective solvent to retain the peptides on the membranes, ethyl acetate was selected for the extraction of ATZ–amino acid due to its high ability to remove impurities stuck on the membranes. In addition, washing the membrane with toluene before the start of the next cycle resulted in an increased yield for coupling with PITC.

Cleavage of the PTC–peptide was performed with anhydrous TFA. It was found that the direct addition of the TFA liquid onto membrane resulted in increasing the formation of unfavorable TFA-caused artifact. This artifact disturbed the reaction with CIA and next coupling with PITC. Therefore, we used the gas-phase cleavage reaction for sequencing. This gas-phase reaction was effective at decreasing the artifacts and also had the advantage of quicker drying of the GF membranes after the TFA treatment. Thus, the unstable ATZ derivatives could be extracted immediately for the CIA reaction. For the efficient cleavage with TFA vapor, the temperature was increased to 80 °C. From the chromatogram obtained, the cleavage was nearly complete after 5 min. A long reaction time was accompanied by a decrease of the CIA products that may be attributed to preconversion of ATZ intermediates.

The suitability of the method was examined by sequencing of an insulin peptide (see Supplementary Fig. 1). The amino acid sequence of insulin chain B used in this study is FVNQHLCGSHLVEALYLVCGERGFFYTPKA, in which all cysteine residues were commercially oxidized. Under the described conditions, sequencing was successfully achieved up to 20 cycles. Beyond 20 cycles, identification of amino acids became difficult because of low peak height of the corresponding CIA–PTC derivatives. This might be caused by continuous loss of peptide fragments from the membrane during washing and extraction processes at each cycle. Most of the CIA–PTC–amino acids from the peptide could be identified on the chromatograms, including oxidized Cys (Cys-SO_3H), although Tyr (16th cycle), Ala (14th cycle), and His (5th and 10th cycles) derivatives coeluted with by-product peaks. The reproducibility of the peak heights of the CIA–PTC–amino acids was examined for the first three cycles. The peak heights corresponding to Phe, Val, and Gln at the 1st, 2nd, and 4th cycles showed relative standard deviations (RSDs) of 3.3 to 4.1% for three repeated analyses.

In summary, a manual method with modified Edman degradation procedures has been developed for peptide sequencing on solid-phase GF membranes in a small filter disk. The reaction of ATZ–amino acids with CIA gave highly fluorescent and stable products for all amino acid residues and gave approximately 10-fold higher sensitivity than did the conventional UV detection. However, it should be noted that the repeated use of the filter disk made of polypropylene may reduce its strength and, thus, may contribute to generation of hindering contaminants. We are investigating the use of Teflon or glass-type disks that are stronger and resistant to heat and most acidic and basic organic solvents.

Acknowledgments

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Appendix A. Supplementary data

Supplementary material for this article is available in the online version at doi:10.1016/j.ab.2007.12.003.

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Facile Assay of Telomerase Activity Utilizing a DNA-Detectable Chemiluminogenic Reagent

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Telomerase shows increased activity in most human cancers and germ line cells, but not in normal human somatic cells. We describe a novel chemiluminescence method for the facile assay of telomerase activity in human cells. The telomerase substrate was incubated with the cell lysate containing various amounts of telomerase, and then the telomerase product was amplified by the polymerase-chained reaction (PCR). The PCR products were separated from the excess substrate, primer and deoxyribonucleotide triphosphates by a centrifugal filter, which distinguished different molecular sizes. The isolated products were reacted with a DNA-detectable chemiluminogenic reagent, 3,4,5-trimethoxyphenylglyoxal. The proposed assay method gave linearity for the telomerase activity in 100 to 10000 cells ($r^2 = 0.997$), and allowed the assay not only of lower activity, but also of higher activity of telomerase without the requirement of any special labeled-PCR primers in the assay system.

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Introduction

A chromosome end in almost all eukaryotes is capped with a telomeric repeat that is composed of tandem copies of a simple DNA sequence, $(TTAGGG)_n$. The telomeric repeat and its associated proteins form a telomere moiety in the chromosome, which protects against cell degradation, such as apoptosis.[1] Telomerase, a unique ribonucleic protein enzyme, synthesizes the telomeric repeat onto the chromosomal end using a segment of its RNA component as an internal substrate.[2,3] The telomeric repeat is progressively lost at a rate of 30 - 120 bp with each replication cycle of the cell.[4] The telomerase counteracts this shortening of the telomere moiety, and thus shows increased activity in more than 80% of all human cancers.[5] In addition, the telomerase is reactivated to proliferate and to bypass the Hayflick limit[6] in human fibroblasts,[7] mammary epithelial cells,[8] and keratinocytes.[9] Therefore, the telomerase is an interesting target for the diagnosis, prognosis, and treatment of cancer and other diseases associated with aging.[10-12]

The telomeric repeat amplification protocol (TRAP) is usable for the detection of telomerase activity, in which the telomerase-enzymatic reaction is performed, and subsequently the telomerase product is amplified by PCR. In TRAP, the telomerase substrate should be designed not only as the specific substrate of the telomerase, but also as the forward primer for the PCR amplification. The conventional TRAP methods for the assay of telomerase activity employed a radio-labeled primer[13] or a fluorescence-labeled primer[14] in combination with polyacrylamide gel electrophoresis for the separation and detection of the six-base different lengths of the PCR products. Recently, two different fluorescence-tagged primers were used for energy-transferring fluorescence detection without separating the products.[15] In those TRAP assays, however, many short length products are formed by PCR, because hybridization between the forward and reverse primers occurs during PCR.

In the present study, we employed special forward and reverse PCR primers having each of one mismatched base-pair at the 3′-ends in order to prevent the formation of the short-length products.[16] Figure 1 illustrates our protocol for the assay of telomerase activity in human cells. In this assay, the PCR products are readily separated with a centrifugal filter, which is able to remove molecular sizes lower than 50000 Da. The amount of the final PCR products on the filter is determined by the chemiluminogenic reaction with 3,4,5-trimethoxyphenyl-glyoxal (TMPG) at room temperature for a few minutes, using 60 mer oligonucleotide, $(TTAGGG)_{10}$ as a standard material. The TMPG reagent gives chemiluminescent signals selective for the guanine base in DNA, and is quantitatively determinable for the concentration of DNA in the reaction mixture.[17] The present method does not require any labeled probes and primers, and allows a sensitive analysis of the telomerase activity by a conventional chemiluminescence (CL) detector.

Experimental

Chemicals and materials

HeLa and HepG2 cells were purchased from Riken BRC cell bank (Tsukuba, Japan). Dulbecco's modified Eagle's medium was purchased from GIBCO (Grand Island, USA). 3-[(3-Cholamidopropyl)-dimethylammonium]-1-propane-sulfonate (CHAPS) lysis buffer [10 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM bezamidine, 5 mM 2-mercaptoethanol, 0.5% CHAPS, 10% glycerol] was purchase from (Chemicon,

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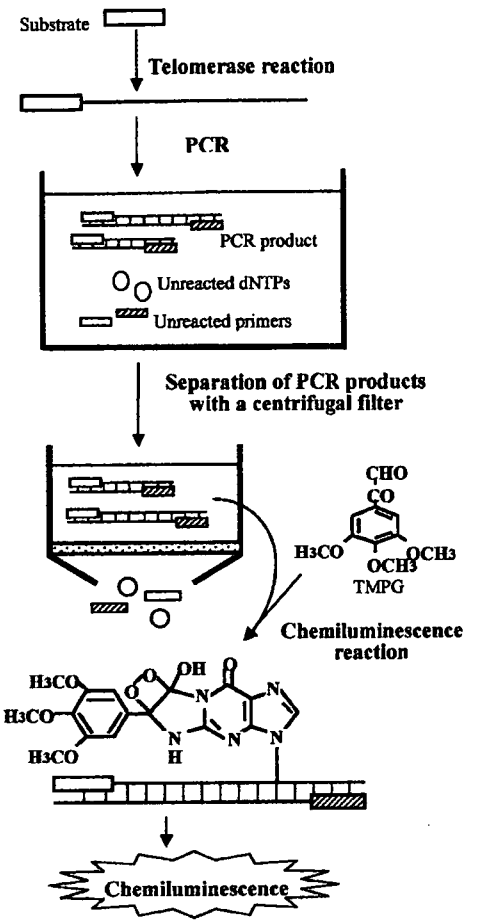

Fig. 1 Schematic protocol for the assay of telomerase activity by means of the CL reaction with TMPG. Telomerase synthesizes telomeric repeats from the substrate, and then the telomerase product is amplified by PCR with forward (the substrate) and reverse primers. The PCR reaction mixture is transferred to a centrifugal filter device to separate the amplified DNA products from unreacted primers and dNTP. The amplified products are solved in 0.1% SDS solution, and the product amount is determined by the chemiluminogenic reaction with TMPG.

CA, USA). A ribonuclease inhibitor (RNasin) was purchased from Promega (Madison, USA). The telomere substrate (the forward primer) with a sequence of 5′-GTAAAACGACGGCCA-GTTTGGGGTTGGGGTTGGGG-TTG-3′ and the reverse primer with a sequence of 5′-CAGGAAACAGCTATGACCCCTAA-CCCTAACCCTAA-CCCT-3′, and a standard oligonucleotide (60 mer) with the sequence of 5′-$(TTAGGG)_{10}$-3′ were commercially ordered to Sigma Genosys Japan (Ishikari, Japan). The underlined sequences represent the hybridization sites in PCR. Takara rTaq polymerase was purchased from Takara Bio (Otsu, Japan). Bovine pancreas ribonuclease A was purchased from Nacalai tesque (Kyoto, Japan). Centrifugal filters (Ultra free-MC 50000 NMWL Filter Unit) were purchased from Millipore (Bedford, USA). TMPG was synthesized as follows according to previously reported conditions:[18] 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 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 (mp. 101 - 102°C, yield 60 - 70%).

Sample preparation

Two immortal HeLa or HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 3 - 4 days. Normal somatic cells from oral cavity were collected with a cotton bud and suspended in 21.5 mM phosphate-buffered saline (PBS). The cell number was counted under a microscope with a cell counting chamber. Cells (approximately 40000 cells) were washed two times with 500 μL of PBS and suspended in 100 μL of CHAPS lysis buffer. The cell suspension was incubated for lysis on ice for 30 min and centrifuged at 13400*g* for 20 min at 4°C. Aliquots of the lysate were immediately frozen in liquid nitrogen and stored at −80°C.

Assay procedure of telomerase activity by CL detection

A telomerase reaction and subsequent PCR were carried out in a thermal cycler (PROGRAM TEMP CONTROL SYSTEM PC-708; Astek, Fukuoka, Japan). Twenty microliters of the cell lysate were mixed with 25 μL of a reaction buffer [40 mM Tris-HCl (pH 8.5), 126 mM KCl, 3 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μM dNTP (dATP, dCTP, dGTP and dTTP), 0.1 μg/μL BSA, 1.6 U/μL RNasin and 0.4 μM the telomerase substrate], followed by incubation at 30°C for 30 min. The mixture (45 μL) was heated at 95°C for 5 min to inactivate any telomerase activity, and then mixed with 5 μL of a rTaq mixture [2.5 U rTaq polymerase and 2 μM reverse primer], which was pre-heated at 85°C for 1 min to reduce non-specific amplification during the initial set-up stages of PCR. PCR was carried out under thermal conditions with 25 cycles at 95°C for 30 s, 68°C for 30 s, 72°C for 1 min, and finally at 72°C for 5 min. The reaction mixture (50 μL) was transferred to a centrifugal filter device and centrifuged at 3500*g* for 5 min to separate PCR products from excess dNTP and primers. The filter was washed twice with 100 μL of H_2O by centrifugation at 3500*g* for 5 min. A portion (50 μL) of 0.1% SDS was added onto the filter, which was then incubated at 37°C for 10 min. The product in the solution on the filter was reacted with TMPG and detected with a CL detector (Luminescence Reader AccuFLEX Lumi 400; Aloka, Tokyo, Japan). The TMPG reaction and the CL detection were carried out as follows: A portion (20 μL) of the DNA product solution was mixed with 10 μL of 0.1 M tetra-*n*-propyl ammonium phosphate (pH 7.0) in a glass tube, and then a 200-μL portion of 10 mM TMPG in *N,N*-dimethylformamide was added. After the addition of the TMPG solution, the CL intensity was immediately detected with the CL detector for 2 min at room temperature (24 - 26°C).

An oligonucleotide (60 mer) of $(TTAGGG)_{10}$ was used as a standard material for the determination of the DNA products.

Assay of telomerase activity by a conventional polyacrylamide gel electrophoresis[13]

The telomerase reaction and subsequent PCR were carried out as described above. A portion (20 μL) of the PCR product solution was separated on 8% polyacrylamide gel (75 × 90 mm) at 200 V for approximately 35 min. After electrophoresis, the gel was stained with 5 μg/mL ethidium bromide for 10 min and washed with water, and then the fluorescence image was detected by a gel scanner (DAIANA III; Raytest, Straubenhardt, Germany).

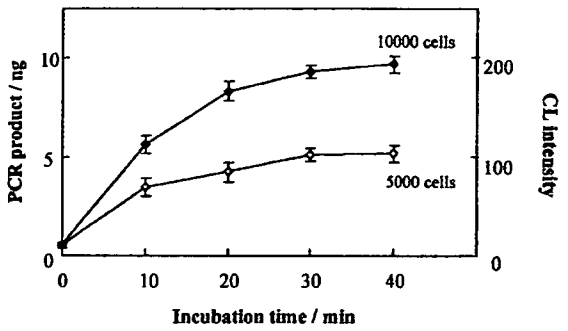

Fig. 2 Effect of the incubation time on the telomerase activity. Telomerase in the lysate of HeLa cells (5000 or 10000 cells) was reacted at 30°C for various times (0 - 40 min), and then the telomerase product was amplified by PCR. The Y-axis represents the produced amount of the DNA products (left side) and chemiluminescence (CL) intensity (right side) (n = 3 each).

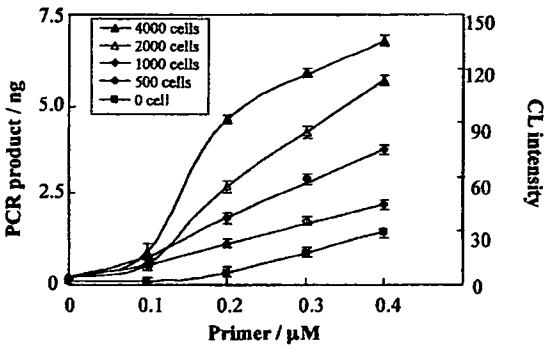

Fig. 3 Influence of various concentrations of primers. The lysate of HeLa cells varied from 500 to 4000 cells was incubated with 0 - 0.4 μM substrate. The telomerase products were amplified by PCR with 50 μM dNTP and various concentrations of primers (0 - 0.4 μM) (n = 3 each).

Assay of telomerase activity by a conventional fluorescence detection[14]

A commercially available kit, TRAPese® XL Telomerase Detection Kit (Chemicon, CA) was used to compare with our proposed assay. The detection system of this kit was based on fluorescence resonance energy transfer (FRET), and Ampliflour® primer was used as a PCR primer. This primer consists of a complementary sequence to the tolomeric repeat sequence at a 3′ end, and a hairpin structure with two fluorescent compounds of fluorescein and 4-*N*,*N*-dimethylaminoazobenzene-4′-sulfonyic acid (DABSYL) at a 5′ end. The fluorescence of fluorescein in the primer was quenched by DABSYL during the enzymatic reaction with telomerase, because fluorescein and DABSYL were in close proximity within 5′ hairpin. When the primer was incorporated into a double-stranded PCR product, the hairpin was unfolded by the polymerase activity. In this extending conformation, the distance between fluorescein and DABSYL was longer, and then the fluorescence signal was increased. The telomerase reaction and subsequent PCR using the lysate of HeLa cells were performed according to the recommended protocol. The cell lysate (2 μL) was mixed with 48 μL of a TRAPeze XL reaction mixture containing substrates, Ampliflour® primer, and rTaq polymerase, followed by incubation at 30°C for 30 min. PCR was carried out under the thermal conditions with 36 cycles at 95°C for 30 s, 59°C for 30 s, 72°C for 1 min, and finally at 72°C for 3 min. After PCR amplification, the PCR solution (20 μL) was diluted to 600 μL with 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 2 mM $MgCl_2$. Then the fluorescence of the product was detected with a spectrofluorometer (FP-6300 Fluorescence Spectrometer; JASCO, Tokyo, Japan) at excitation and emission wavelengths of 495 and 516 nm, respectively.

Results and Discussion

Telomerase assay with CL detection

To develop a facile telomerase-assay method, we utilized a chemiluminogenic reagent, TMPG.[17] About 40 fmol (800 pg)/20 μL DNA with telomeric sequence $(TTAGGG)_{10}$ could be quantified with a good reproducibility by the TMPG reaction at room temperature (24 - 26°C). The molecular sizes of the PCR products after the enzymatic reaction with telomerase were more than 45000 Da, and the molecular sizes of the substrate (= forward primer) and the reverse primer of the proposed method were less than 12000 Da. In the proposed protocol, it was therefore important to separate the final PCR products from excess primers and dGTP used for PCR amplification in order to reduce the background signals. We used an ultrafiltration membrane, which could rapidly and easily separate the final PCR products. In our assay, the telomerase product was amplified by about 2^{25} times by PCR using the substrate (= forward primer) and the reverse primer. Then, the final PCR products were detected with the TMPG reaction.

To investigate the optimum telomerase-incubation time, telomerase in the lysates of HeLa cells (5000 or 10000 cells) was reacted at 30°C for different reaction times (0 - 40 min). As shown in Fig. 2, the CL intensity that was obtained from the final PCR products by the TMPG reaction proportionally increased with the amounts of telomerase until 40 min. For convenience, an incubation time of 30 min was set up in the present assay. The telomerase activity was expressed as the amounts (ng) of the DNA products in the final PCR mixture (50 μL).

For optimizing the reaction conditions of PCR, we examined the influence of the concentrations of the primers and dNTP on the PCR amplification. When the telomerase activity in HeLa cells was measured in the presence of various concentrations of primers, the CL intensity was increased with the concentration of the primers (Fig. 3). At 0.4 μM primers, the CL intensity from the reaction mixture without the cell lysate was increased. This result suggested that a primer/primer complex was formed during PCR, and could not be removed by ultra filtration. However, we could obtain a low background and a good correlation between the cell number and the CL intensity at 0.2 μM primers.

When the primers concentrations were 0.2 μM, the CL intensity due to the final products was almost constant between the concentrations of 50 - 400 μM dNTP (Fig. 4). The background was very low, even when 400 μM dNTP was used. This result indicated that 50 μM dNTP was sufficient for this PCR amplification. However, the CL intensity was not proportional to the cell number when a large number of cells, more than 10000, were used for the telomerase reaction, as shown in Figs. 2 and 4. This would be due to inhibition of the telomerase reaction and the following PCR amplification by endogenous substances in cells. Based on the above results, we selected 0.2 μM primers and 50 μM dNTP for the telomerase assay.

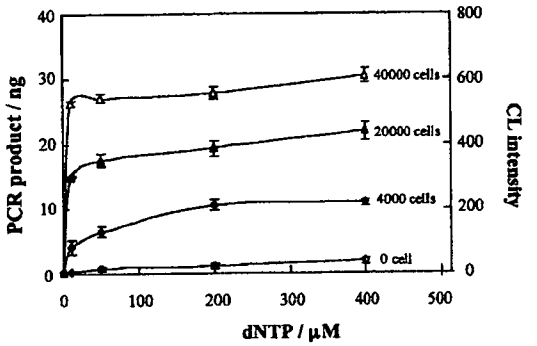

Fig. 4 Influence of various concentration of dNTP. The lysate of HeLa cells (4000, 20000, and 40000) was incubated with 0.2 μM substrate. The telomerase products were amplified by PCR in the presence of various concentrations of dNTP (0 - 400 μM) (n = 3 each).

Comparison with other assay methods

Conventional methods, such as electrophoresis with a fluorescence dye, ethidium bromide, and non-separable fluorescence detection assay based on FRET, were compared with the present method for the assay of the telomerase activity in the cell lysates of HeLa cells. Figure 5A shows the telomerase activity in the lysates of HeLa cells by the proposed CL method. From 100 to 4000 cells, a good correlation between the amount of the final telomerase product and the cell number was obtained by our assay (r^2 = 0.997). The relative standard deviation (RSD) was determined for each lysate as an indicator of the inter-assay variation. The RSD values in 5 different runs were 4.3% for 1000 cells, 4.9% for 500 cells, and 14.9% for 100 cells.

Figure 5B showed the telomerase activity in the same sample from HeLa cells measured by the FRET assay method. The sensitivity of the fluorescence assay was lower because of the high background signal, and the linearity between the fluorescent signal and the cell number in the wide range was not sufficient. However, in our assay, the CL signal gave a more linear relation for a cell number of less than 10000.

On the other hand, the final PCR products using the same substrate and primers as those in the proposed assay method were separated by polyacrylamide gel electrophoresis, and detected with fluorescence staining (Fig. 5C). Molecular sizes of the amplified products were more than 60000 Dalton. This result indicates that the telomerase product was satisfactorily amplified by PCR. However, the detection limit of the staining with ethidium bromide was approximately one-fiftieth lower than that of the present method, and its image intensity from the amplified telomerase products was not proportional to the cell number.

Telomerase activities in various cells

The telomerase activities in lysates from normal human somatic cells and two cell lines of HeLa and HepG2 were assayed by the proposed CL method. The normal cells were obtained from an oral cavity. As shown in Fig. 6, the telomerase activities in the lysates from two cell lines of HeLa and HepG2 (approximately 4000 cells, each) were very much higher than that in the lysate of the normal somatic cells (approximately 4000 cells), because these two cell lines were immortal, and thus their cell division was occurred frequently.[19] Our method could measure the very low activity of telomerase in normal somatic cells from the oral cavity. The telomerase activities in

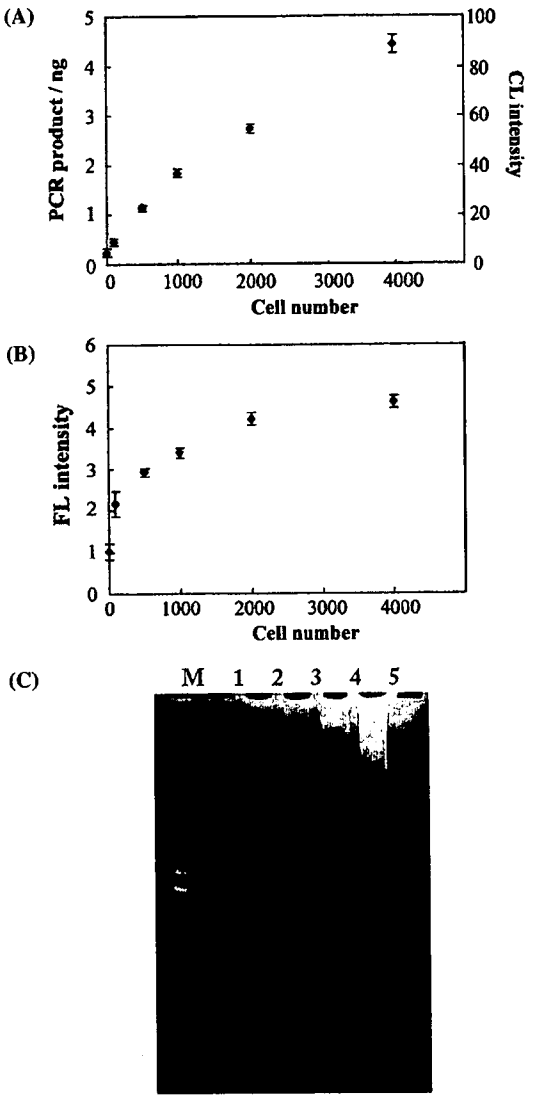

Fig. 5 Telomerase activity in HeLa cell lysates measured by the proposed CL method (A), FRET method (B) (n = 5 each) and the electrophoresis method with ethidium bromide staining (C). In the figure of (C), lane M represents 10 bp markers, and lanes 1 - 5 represent the cell numbers of 0, 1000, 5000, 10000, and 20000, respectively.

the cell lines were not detected when the cell lysate was treated with ribonuclease. The results indicate that the final PCR products were due to the enzymatic reaction with telomerase in the analyzed cells, since ribonuclease decomposes telomerase.[20,21]

Conclusions

We developed here a novel telomerase-assay method based on a chemiluminogenic TMPG reaction. We employed partially mismatched primers, since many longer length PCR products were formed by hybridization between both primers during PCR amplification. The final PCR products could be easily isolated from excess primers and dNTP in the reaction mixture by an ultra-filtration membrane. The isolated final PCR products were simply and rapidly converted to chemical illuminants, and