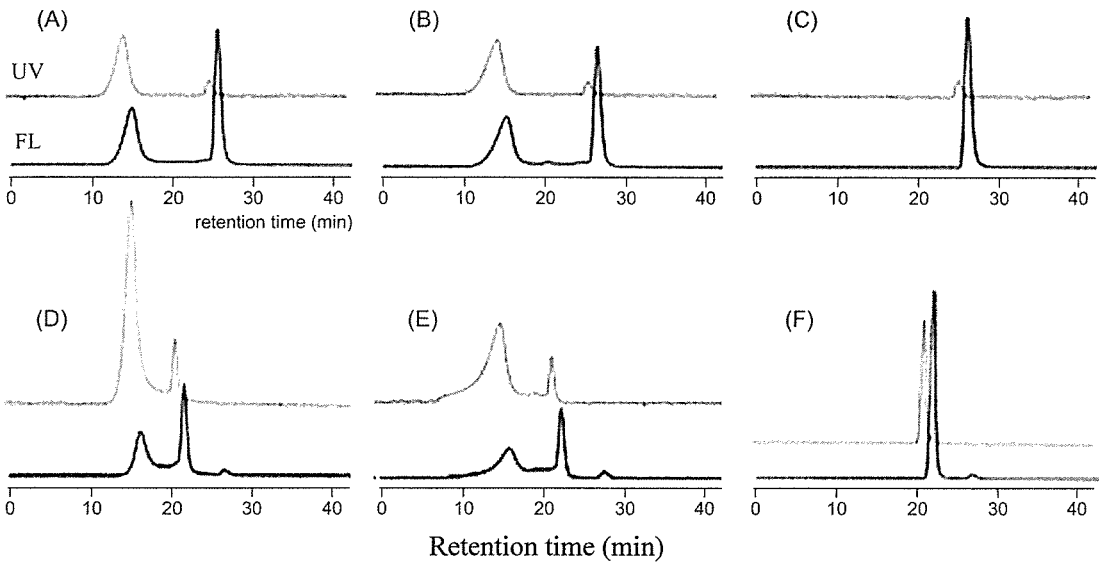

Figure 2. GFLC chromatograms of dextran-based CL probes. Samples: (A), 1.0 mg/mL Dex2000-$(Lu)_{2627}$-$(Bi)_{385}$; (B), 1.0 mg/mL Dex50-$(Lu)_{559}$-$(Bi)_{34}$; (C), 0.011 mg/mL Lu; (D), 1.0 mg/mL Dex2000-$(ILu)_{3262}$-$(Bi)_{275}$; (E), 1.0 mg/mL Dex500-$(ILu)_{321}$-$(Bi)_{15}$; (F), 0.011 mg/mL ILu. Injection volume: 10 μL. For other chromatographic conditions, see in Materials and Methods.

The purity of synthesized dextran-based CL probes was analyzed by gel-filtration liquid chromatography (GFLC). Figure 2 shows the GFLC profile of the Lu- or ILu-modified dextran-based CL probes. Two peaks appeared in each chromatogram, broad peaks eluted at the retention time of 14-15 min were the Lu- or ILu-modified dextran-based macromolecular compounds. Sharp peaks eluted at 27 min and 22 min were due to unbound Lu and ILu, respectively. It demonstrates that the isolated probes contained a small amount of free Lu or ILu. The incorporated ratio (w/w) of Lu or ILu molecules into the probe was calculated from the area of each UV peak in the GFLC chromatogram. The results indicate that 99-91 % of total UV absorption were corresponding to Lu and ILu bound to dextran, respectively. However, the small amounts of free Lu and ILu contaminated in the probes could be removed by washing the

membrane with 50-80 % methanol when the probes were used for the DNA detection on a nylon membrane. Therefore the synthesized probes were used for the DNA detection on a solid-phase membrane without further purification.

Luminescent properties of dextran-based CL probe

In our preliminary study on CL detection of the Lu- or ILu-modified dextran probes on a nylon membrane, we found that the CL intensity of Lu-modified dextran probes was approximately 10 times higher than ILu-modified dextran probes. Therefore UV absorption, fluorescence (FL) and CL properties of Lu-modified dextran probes were evaluated (Table 2). The values of molar extinction coefficient (ε) for each probe were almost in proportion to the number of Lu moieties introduced into the dextran molecule, whilst the FL intensity was out of proportion. It is important to note that the more a probe contains Lu molecules, the higher the probe produces CL upon activation, in a proportional manner.

Table 2. UV, FL and IL intensity of luminol-modified dextran probes

	Probe	UV		FL		CL
		ε	λ_{max} (nm)	RFI[a]	Ex/Em (nm)	
A	Dex170-$(Lu)_{323}$	2.3×10^6	295	16	370/430	32,100
B	Dex170-$(Lu)_{320}$- $(Bi)_{24}$	3.3×10^6	295	14	370/430	48,900
C	Dex800-$(Lu)_{1792}$	16.3×10^6	295	127	370/430	118,000
D	Dex800-$(Lu)_{1803}$- $(Bi)_{104}$	19.0×10^6	295	69	370/430	246,000
E	Dex2000-$(Lu)_{3706}$	17.9×10^6	295	81	370/430	379,000
F	Free Lu	8.4×10^3	295	0.65	370/410	1,800

[a]Relative fluorescent intensity. UV absorption and FL of 1.0 nM each sample were measured, and 50 pM each was used for CL measurement.

CL-imaging detection of telomere DNA (Figure 1)

The target 60mer telomere DNA was blotted on a nylon membrane and hybridized with a biotinylated 18mer telomere cDNA (b-cDNA) by incubating the membrane in b-cDNA solution. Different amounts of either Dex2000-$(Lu)_{2627}$-$(Bi)_{385}$ or avidin were used to optimize the detection conditions. The results are shown in Figure 3A and 3B. Regardless of the amount of avidin, the CL intensity related to the detection of the target DNA was enhanced as the increased amount of the probe. However, the background signal on the membrane also increased dramatically, which makes it impossible to distinguish the desired signal from the target DNA. This was caused by a non-specific binding of the probe to the membrane, suggesting that limited amount of the probe should be used in order to suppress the background. On the other hand, CL intensity reached the maximum at a particular concentration of avidin when a constant concentration of the probes was used. As a large excess of avidin existed in the chain conjugate mixture, the probe molecules were captured by free avidin and was not involved in a conjugation formation of b-cDNA.

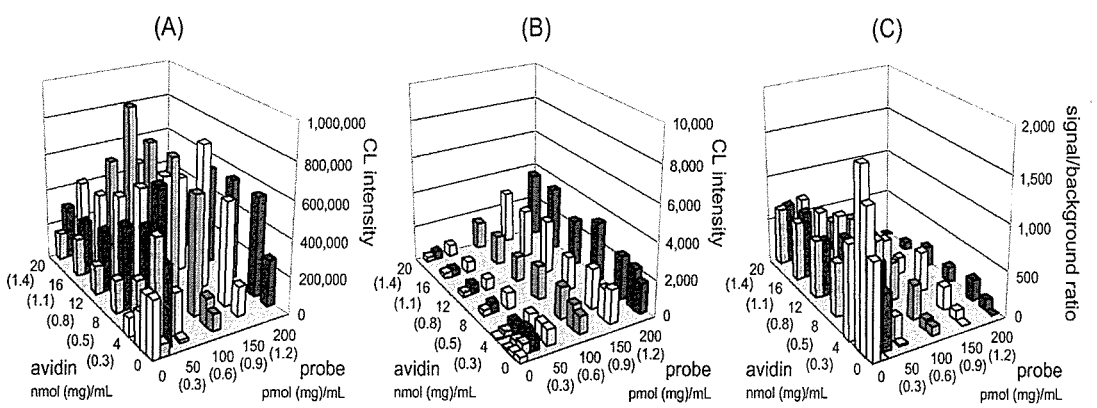

Figure 3. Three dimensional graphs of the CL-imaging efficiency with the probe Dex2000-$(Lu)_{2627}$-$(Bi)_{385}$ at different concentrations of the probe and avidin. (A): CL intensity of telomere DNA. (B): Intensity of background signal without telomere DNA. (C): The ratio of the detection signal from DNA versus the background.

In order to establish the best combination between the amounts of probe and avidin for

depicting the maximal CL intensity with the minimal background, we calculated the DNA signal/background ratio (Figure 3C). The results indicated higher signal/background ratios in the low concentration range of the probe, despite the fact that extremely high signals were observed for the DNA detection at the high concentration of the probe. The sufficient signal/background ratio was attained in the combination of 0.08 mg/mL probe and 0.14 mg/mL avidin. This combination enabled the effective and quantitative detection of the telomere DNA with low background signal.

Comparative study with HRP detection

The efficiency of our method for the CL detection of telomere DNA on a nylon membrane using the Lu-modified dextran probe of Dex2000-$(Lu)_{2627}$-$(Bi)_{385}$ was compared with a conventional enzymatic CL detection system using a HRP-avidin probe (Figure 4). The 18mer b-cDNA was used as a capture DNA, and several different DNA sequences were used to investigate the specificity of detection; 18 mer telomere cDNA (not target), 42mer telomere cDNA (not target), 30mer NF- κB ssDNA (not target), 30mer NF- κB cDNA (not target) and 60mer telomere DNA (target). The results show that our method and HRP method both allowed the sequence-specific detection for the target telomere DNA. Different amounts of the target DNA were successfully detected by use of the Lu-modified dextran probe for the detection time of 2-6 min (Figure 4 (A), (f)-(h)), showing that the target telomere DNA of as low as 0.2 picomole amount was visualized clearly with a small background signal. On the other hand, a relatively high background was observed by use of the HRP-avidin probe, although the detection signals were reasonably intensive (Figure 4 (B), (f)-(h)). In addition, a good reproducibility could not be obtained by the enzymatic detection because of an unstable and irregular luminescence signal from background. This phenomenon was observed more conspicuously when the detection time was elongated to 6 min. An extremely high background significantly interrupted the detection of the desired CL

spots (Figure 4 (B), right), while the dextran-based CL probe gave a good contrast (Figure 4 (A), right). Thus, the dextran probe affords the highly sensitive detection of the target DNA, with a low and steady background and high reproducibility.

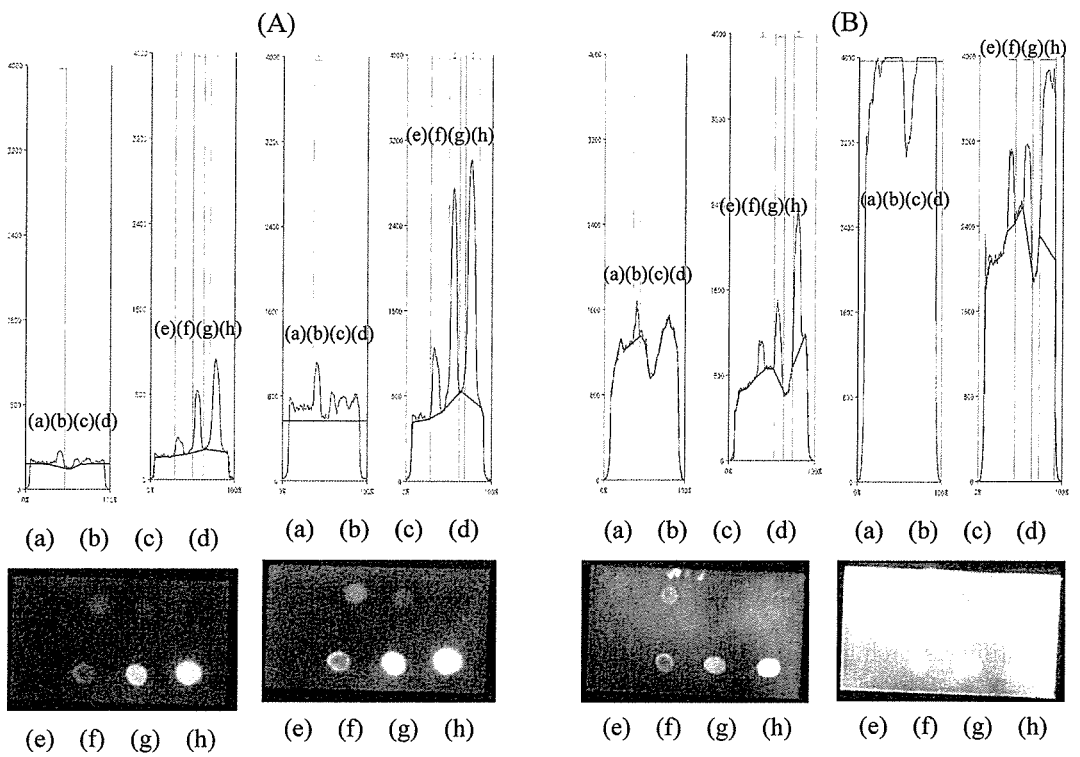

Figure 4. CL-imaging and its specificity for the detection of telomere DNAs using (A) Dex2000-$(Lu)_{2627}$-$(Bi)_{385}$ and (B) HRP-avidin probes. Left and right data of (A) and (B) were obtained by different detection times of 2 min and 6 min, respectively. Samples: (a), 18mer telomere cDNA (1.6 pmol); (b), 42mer telomere cDNA (1.6 pmol); (c), 30mer NF-κB binding site (1.6 pmol); (d), 30mer NF-κB cDNA (1.6 pmol); (e), EDTA (2 nmol); (f), 60mer telomere DNA (200 fmol); (g), 60mer telomere DNA (800 fmol); (h), 60mer telomere DNA (1.6 pmol).

CONCLUSIONS

We aimed to develop the current method for the microanalysis of a target DNA with non-enzymatic CL probe. We newly designed the CL probe based on dextran molecule, in which many Lu and Bi molecules are covalently introduced. Several dextran-based CL probes were

synthesized with changing the size of dextran in the presence of Lu or ILu, and Bi. The synthesis was carried out by oxidation and reductive *N*-alkylation reactions. Among the synthesized compounds, we found that the Lu-modified dextranT2000 probe produces sufficient intensity of chemiluminescence. This probe could conjugate with free avidin to form the non-covalent probe-chained assembly. The method for the DNA detection using our dextran-based CL probe was compared with that using a HRP-labelled avidin probe. The results indicated that our probe gives a low background signal with a high signal from the target DNA. Therefore, this newly developed luminol-modified macromolecular probe provides one of promising techniques for sensitive luminescence-imaging of biologically important materials.

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REFERENCES

1. Barany,F. (1991) Genetic disease detection and DNA amplification using cloned thermostable ligase. *Proc. Natl. Acad. Sci.,USA.*, **88**, 189-193.
2. Tyagi,S. and Kramer,F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. *Nat.Biotechnol.*, **14**, 303-308.
3. Tyagi,S., Bratu,D.P. and Kramer,F.R. (1998) Multicolor molecular beacons for allele discrimination. *Nat.Biotechnol.*, **16**, 49-53.
4. Kostrikis,L.G., Tyagi,S., Mhlanga,M.M., Ho,D.D. and Kramer,F.R. (1998) Spectral genotyping of human alleles. *Science*, **279**, 1228-1229.
5. Cheng,J., Zhang,Y. and Li,Q. (2004) Real-time PCR genotyping using displacing probes.

Nucleic Acids Res., **32**, e61.

6. Leone,G.., van Schijndel,H., van Gemen,B., Kramer,F.R. and Schoen,C.D. (1998) Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Res.*, **26**, 2150-2155.
7. Nuzzo,R.G.. and Allara,D.L. (1983) Adsorption of bifunctional organic disulfideson gold sufaces. *J. Am. Chem. Soc.*, **105**, 4481-4483.
8. Wu,Z.S., Jiang,J.H., Fu,L., Shen,G.L. and Yu,R.Q. (2006) Optical detection of DNA hybridization based on fluorescence quenching of tagged oligonucleotide probes by gold nanoparticle. *Anal. Biochem.*, **353**, 22-29.
9. Wang,H., Zhang,C., Li,Y. and Qi,H. (2006) Electrogenerated chemiluminescence detection for deoxyribonucleic acid hybridization based on gold nanoparticles carrying multiple probes. *Anal. Chim. Acta*, **575**, 205-211.
10. Taton,T.A., Mirkin,C.A. and Letsinger,R.L. (2000) Scanometric DNA array detection with nanoparticle probes. *Science*, **289**, 1757-1760.
11. Moller,R., Csaki,A., Kohler,J.M. and Fritzsche,W. (2000) DNA probes on chip surfaces studied by scanning force microscopy using specific binding of colloidal gold. *Nucleic Acids Res.*, **28**, e91.
12. Maruyama,T., Shinohara,T., Hosogi,T., Ichinose,H., Kamiya,N. and Goto,M. (2006) Masking oligonucleotide improve sensitivity of mutation detection based on guanine quenching. *Anal Biochem.*, **354**, 8-14.
13. Marras,S.A., Kramer,F.R. and Tyagi,S. (2002) Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. *Nucleic Acids Res.*, **30**, e122.
14. Masuko,M., Ohtani,H., Ebata,K. and Shimadzu,A. (1998) Optimization of excimer-forming two-probe nucleic acid hybridization method with pyrene as a fluorophore. *Nucleic Acids*

Res., **26**, 5409-5416.

15. Paris,P.L., Langenhan,J.M. and Kool,E.T. (1998) Probing DNA sequences in solution with a monomer-excimer fluorescence color change. *Nucleic Acids Res.*, **26**, 3789-3793.
16. Okamoto,A., Kanatani,K. and Saito, I. (2004) Pyrene-labeled base-discriminating fluorescent DNA probes for homogeneous typing. *J. Am. Chem. Soc.*, **126**, 4820-4827.
17. Thorpe,G.H., Kricka,L.J., Moseley,S.B. and Whitehead,T.P. (1985) Phenols as enhancers of the chemiluminescent horseradish peroxidase-luminol-hydrogen peroxide reaction: application in luminescence-monitored enzyme immunoassays. *Clin. Chem.*, **31**, 1335-1341.
18. Thorpe,G.H. and Kricka,L.J. (1986) Enhanced chemiluminescent reactions catalyzed by horseradish peroxidase. *Methods Enzymol.*, **133**, 331-353.
19. Collins,M.L., Irvine,B., Tyner,D., Fine,E., Zayati,C., Chang,C., Horn,T., Ahle,D., Detmer,J., Shen,L.P., Kolberg,J., Bushnell,S., Urdea,M.S. and Ho,D.D. (1997) A branched DNA signal amplification assay for quantification of nucleic acid targets below 100 molecules/ml. *Nucl. Acids Res.*, **25**, 2979-2984.
20. Klevan,L., Horton,L., Carson,D.P. and Eisenberg,A.J. (1995) Chemiluminescent detection of DNA probes in forensic analysis. *Electrophoresis*, **16**, 1553-1558.
21. Danner,S.A. *et al.* (1995) A Short-Term Study of the Safety, Pharmacokinetics, and Efficacy of Ritonavir, an Inhibitor of HIV-1 Protease. *N. Engl. J. Med.*, **333**, 1528–1533.
22. Markowitz,M. *et al.* (1995) A Preliminary Study of Ritonavir, an Inhibitor of HIV-1 Protease, to Treat HIV-1 Infection. *N. Engl. J. Med.*, **333**, 1534–1539.
23. Onto,E., Mizokami,M., Nakano,T., Terashima,H., Nojiri,O., Sakakibara,K., Mizuno,M., Ogino,M., Nakamura,M,, Matsumoto,Y., Miyata,K. and Lau,J.Y.N. (1994) Serum hepatitis C virus RNA level as a predictor of subsequent response to interferon-α therapy in Japanese patients with chronic hepatitis C. *J. Med. Virol.*, **44**, 410–414.
24. Lau,J.Y.N., Davis,G.L., Kniffen,J., Qian,K.-P., Urdea,M.S., Chan,C.S., Neuwald,P.D.,

Wilber,J.C. and Mizokami,M. (1993) Significance of serum hepatitis C virus RNA levels in chronic hepatitis C. *Lancet*, **341**, 1501–1504.

25. Yamada,G., Takatani,M., Kishi,F., Takahashi,M., Doi,T., Tsuji,T., Shin,S., Tanno,M., Urdea,M.S. and Kolberg,J.A. (1995) Efficacy of interferon alfa therapy in chronic hepatitis C patients depends primarily on hepatitis C virus RNA level. *Hepatology,* **22**, 1351–1354.
26. Lu,J., Lau,C., Morizono,M., Ohta,K. and Kai,M. (2001) A chemiluminescence reaction between hydrogen peroxide and acetonitrile and its applications. *Anal. Chem.*, **73**, 5979-5983.
27. Yamaguchi,M., Yoshida,H. and Nohta,H. (2002) Luminol-type chemiluminescence derivatization reagents for liquid chromatography and capillary electrophoresis. *J. Chromatogr. A*, **950**, 1-19.
28. Fukushima,T., Usui,N., Santa,T. and Imai,K. (2003) Recent progress in derivatization methods for LC and CE analysis. *J. Pharm. Biomed. Anal.*, **30**, 1655-1687.
29. Ishida,J., Takada,M., Hara,S., Sasamoto,K., Kina,K., and Yamaguchi,M. (1995) Development of a novel chemiluminescent probe, 4-(5',6'-Dimethoxybenzothiazolyl) phthalhydrazide. *Analytica Chimica Acta*, **309**, 211-219.
30. Hanaoka,S., Lin,J.M. and Yamada,M. (2000) Chemiluminescence behavior of the decomposition of hydrogen peroxide catalyzed by copper(II)–amino acid complexes and its application to the determination of tryptophan and phenylalanine. *Analytica Chimica Acta*, **409**, 65-73.
31. Lu,C., Lin,J.M., Huie,C.W. and Yamada,M. (2003) Simultaneous determination of copper(II) and cobalt(II) by ion chromatography coupled with chemiluminescent detection. *Anal. Sci.*, **19**, 557-561.
32. Zui,O.V. and Birks,J.W. (2000) Trace analysis of phosphorus in water by sorption preconcentration and luminol chemiluminescence. *Anal. Chem.*, **72**, 1699-1703.

33. He,D., Zhang,Z. and He,C. (2006) Investigation on the interaction between dihydroxybenzene and Fe^{3+}-H_2O_2-Rh6G system based on enhancing chemiluminescence. *Luminescence*, **21**, 15-19.

34. Kyaw,T., Fujiwara,T., Inoue,H., Okamoto,Y. and Kumamaru,T. (1998) Reversed Micellar Mediated Luminol Chemiluminescence Detection of Iron(II, III) Combined with On-Line Solvent Extraction Using 8-Quinolinol. *Anal. Sci.*, **14**, 203-207.

35. Kyaw,T., Kumooka,S., Okamoto,Y., Fujiwara,T. and Kumamaru,T. (1999) Reversed Micellar-Mediated Luminol Chemiluminescence Reaction with Bis(acetylacetonato)oxovanadium(IV). *Anal. Sci.*, **15**, 293-297.

36. O'Sullivan,D.W., Hanson,Jr.A.K. and Kester,D.R., (1995) Stopped flow luminol chemiluminescence determination of Fe(II) and reducible iron in seawater at subnanomolar levels. *Mar. Chem.*, **49**, 65-77.

37. Parejo,I., Petrakis,C. and Kefalas,P. (2000) A transition metal enhanced luminol chemiluminescence in the presence of a chelator. *J. Pharmacol. Toxicol. Methods*, **43**, 183-90.

38. Dodeigne,C., Thunus,L. and Lejeune,R. (2000) Chemiluminescence as diagnostic tool. A review. *Talanta*, **51**, 415-439.

39. González-García,M.B., Fernández-Sánchez,C. and Costa-García,A. (2000) Colloidal gold as an electrochemical label of streptavidin–biotin interaction. *Biosens. Bioelectron.*, **15**, 315-21.

40. Sakahara,H. and Saga,T. (1999) Avidin–biotin system for delivery of diagnostic agents. *Adv. Drug Deliv. Rev.*, **37**, 89-101.

41. Wilchek,M. and Bayer,E.A. (1988) The avidin-biotin complex in bioanalytical applications. *Anal. Biochem.*, **171**, 1-32.

42. Yoshitani,N. and Takasaki,S. (2000) Microscale Synthesis of Dextran-Based Multivalent N-Linked Oligosaccharide Probes. *Anal. Biochem.*, **277**, 127-134.

A Selective Fluorescence Reaction for Peptides and Chromatographic Analysis

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A selective fluorescence reaction is proposed for the quantitative determination of peptides by reversed-phase liquid chromatography. After each peptide is heated at 120°C for 20 min in a neutral aqueous medium (pH 7.0) containing catechol, sodium periodate, and sodium borate, a single product is formed with an intense fluorescence. Moreover, the fluorescent products of four peptides such as Leu-Gly, Ala-Leu-Gly, Tyr-Gly-Gly-Phe-Leu, and Leu-Leu-Leu can be 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 fluorescence. The lower limit (S/N=3) of the detection is 0.7-3.0 pmol per an injection volume (40 μL) for the tested peptides. In addition, the fluorescent products of Phe-NH_2 and Leu-Leu-Leu are identified by electrospray ionization-time of flight-mass spectrometry for the elucidation of their chemical structures.

Peptides are biological molecules of paramount importance as either drugs for therapeutic treatment or disease markers for clinical diagnosis, or the analytes 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] or 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 atrial natriuretic peptide was also found to play an important role for homeostatic control of body water, sodium 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 reversed-phase liquid chromatography (RPLC) and then detected by their absorption at an ultra violet (UV) wavelength (210-300 nm). It is well know that the detection limit of the RPLC-UV method is generally at the nmol level, due to that peptides have a small molar extinction coefficient. In addition, there are also highly sensitive fluorescence methods for the detection of peptides, such as the employment of *o*-phthalaldehyde or fluorescamine, however their selectivity is poor. These fluorescent reagents not only react with primary amino groups in peptide molecules, but also react with amines, amino acids and nucleic acids, etc, which may also exist in a real sample.[12,13] Therefore, a sensitive and selective method for the detection of peptides would be highly desired.

Previously, we reported the fluorogenic reactions of Arg-containing peptides[14-19] and N-terminal Tyr-containing peptides,[20-22] and the concentrations of those peptides *in vivo* were measured by RPLC on the basis of their fluorogenic reactions.[23-27] Herein, we found that the peptides reacted with catechol in the presence of sodium periodate, leading to a fluorescent product in a neutral borate aqueous solution. In this paper, the conditions of this fluorogenic reaction were first optimized for peptides and RPLC was studied for the separation and detection of the fluorescent products for simultaneous and quantitative

determinations of three peptides. Second, we presumed the chemical structures of their fluorescent products by the analysis with an electrospray ionization-time of flight-mass spectrometry (ESI-TOF-MS).

EXPERIMENTAL SECTION

Chemicals. Catechol, boric acid, and sodium periodate as guaranteed reagents were purchased from Wako 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 chemicals. Catechol (50 mM), boric acid (0.3 M), and sodium periodate (20 mM) were dissolved in water and various pHs of the borate solution were adjusted with sodium hydroxide. Amino acid amides and peptides were dissolved in water or 50 % aqueous solution of 2-methoxyethanol (Methyl Cellosolve, Wako chemicals), and their stock solutions (0.5-5.0 mM) were kept at -20°C or 4°C. For each experiment, the stock solution was diluted with water, and desired concentration solution was prepared.

Apparatus and Experimental Conditions. The RPLC system consisted of a PU-2089 type gradient pump (Jasco, Tokyo, Japan), Lichrospher RP-18e column (125 x 4.0 mm i.d., pore size 5 □m, Merck, Darmstadt, Germany), UV-2070 plus type intelligent UV/VIS Detector (Jasco), and fluorescence spectrometer RF-530 (Shimadzu, Kyoto, Japan). For the separation of the fluorescent derivatives of peptides on the reversed-phase column, gradient elution from 15 to 75 % (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^{-1}. The fluorescence intensity of the column elute was monitored at 500 nm (emission) with excitation at 400 nm, and UV absorption was monitored at 254 nm. Uncorrected fluorescence excitation and emission spectra, and intensities were measured with a FP-6300 spectrofluorometer (Jasco) in 10 x 10 mm quartz cells.

ESI-TOF-MS of the eluates from the fluorescent peaks was analyzed with Mariner (Applied Biosystems, 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, quadropole DC potential = 5 mV, deflection voltage = 0 mV, einzel lens potential = -25 mV, quadropole RF voltage = 700-1000 mV, and quadropole 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 sec/spectrum, curtain gas flow rate = 0.2 L min^{-1}, nebulizer gas flow rate = 1.0 L min^{-1}, (5) infusion injection = 10 μL min^{-1}.

Typical Procedure of Fluorogenic Reaction. A 500-μL portion of 1.0 mM each amino acid amide or peptide (final concentration in the reaction mixture, 0.3 mM) was placed in a test tube, to which were added 500 μL of 10 mM catechol (final conc. 3.3 mM), 250 μL of 12 mM sodium periodate (final conc. 2.0 mM), and 250 μL of 0.3 M sodium borate (pH 7.0) (final conc. 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.

RESULTS AND DISCUSSION

Optimum Conditions for Fluorescent Reaction. Phe-NH_2 was reacted with catechol or salicyl alcohol in the presence of sodium periodate and sodium borate, and then the fluorescence spectra of the reaction mixtures were measured. As shown in Figure 1, the excitation and emission maxima for the fluorescent products were observed around at 400 nm and 500 nm, respectively. Fluorescence intensity from the reaction mixture using catechol was about three times higher than that from the reaction mixture using salicyl alcohol. In addition, the background was much higher by using salicyl alcohol than catechol. Moreover, no fluorescent product was observed by using resorcinol or phenol instead of catechol.

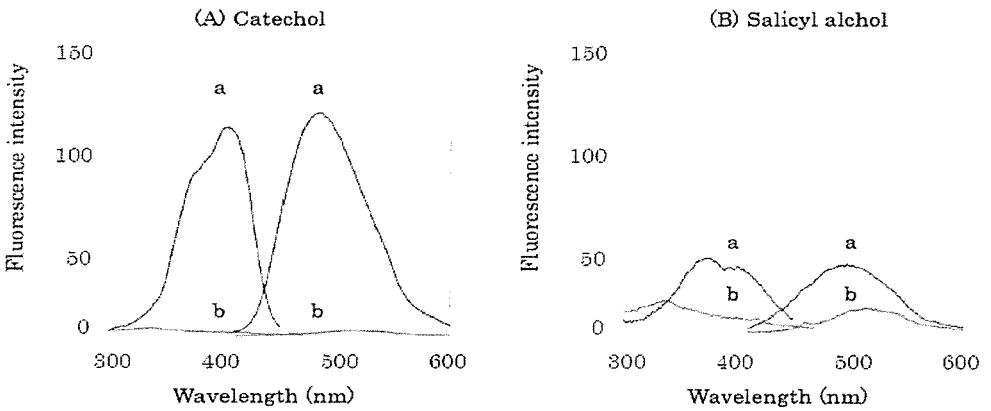

Figure 1. Excitation and emission spectra of the reaction mixture of Phe-NH_2 (a) and its reagent blank (b). Portions (0.5 mL) of 10 mM Phe-NH_2 or water were reacted with 3.3 mM catechol (A) or 3.3 mM salicyl alchol (B) in the presence of 2.0 mM $NaIO_4$ and 50 mM Na borate (pH 7.0) at 120°C for 20 min.

The effect of the oxidizing agent was further examined by using various oxidizing 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. On the other hand, the employment of potassium iodate, sodium perchlorate, or potassium permanganate did not lead to the formation of fluorescent product from Phe-NH_2. The optimum concentration of sodium periodate was found to be 2.0 mM as the final concentration in the reaction mixture. In addition, no fluorescence was observed in the absence of sodium periodate, and with the periodate concentration of 4.0 mM, the intensity was decreased to be approximately 10 % of that with 2.0 mM periodate.

Other reaction conditions were investigated by using RPLC. As shown in Figure 2A, the fluorescent 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 (Figure 2B).

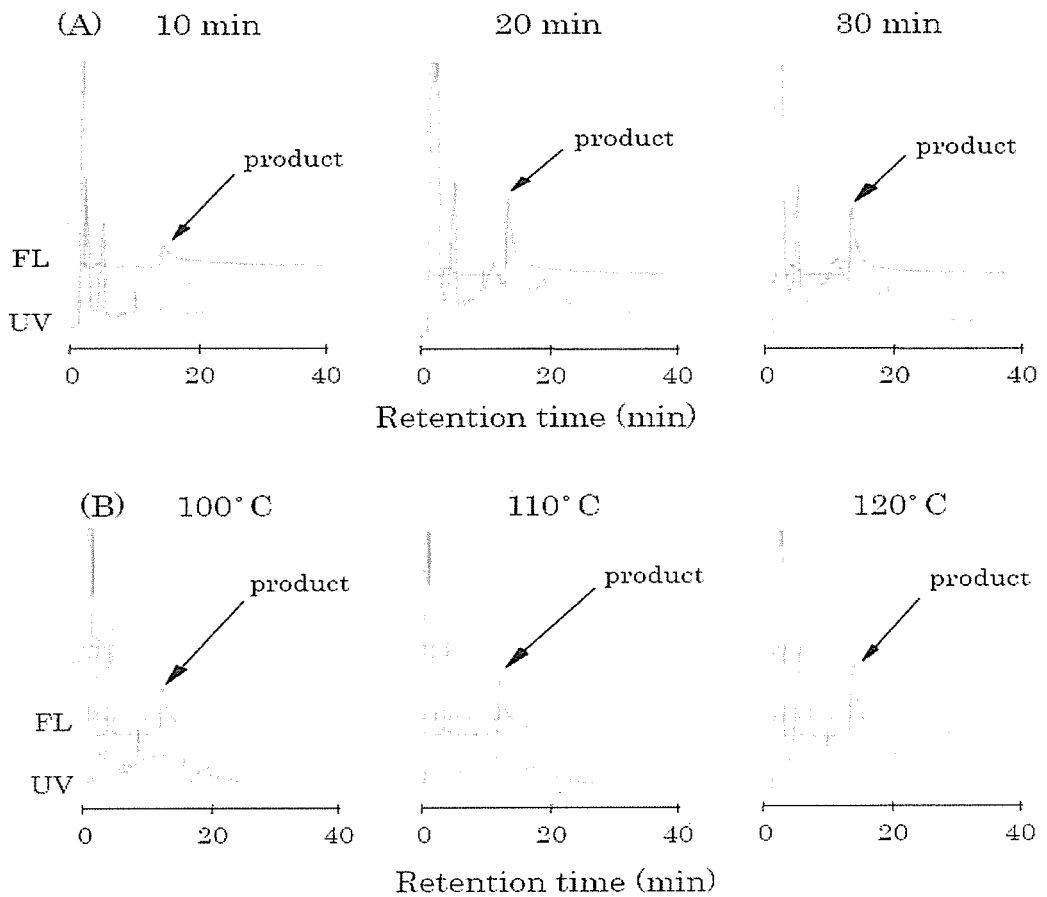

Figure 2. RPLC chromatograms obtained with different reaction times (A) and different reaction temperatures (B). RPLC conditions: mobile phase = MeOH (15 to 75 %, v/v) + H_2O (80 to 20 %) + 0.25 M Na borate, pH 7.0 (5 %) for 40 min; injection volume = 40 μL; and reaction components: 0.3 mM Phe-NH_2, 3.3 mM catechol, 2.0 mM $NaIO_4$, 50 mM sodium borate (pH 7.0).

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, however, at pH 6.0 no fluorescent product was

formed (Figure 3A). At pH 7.0, the reaction was further carried out under various concentrations of sodium borate (17-67 mM). Figure 3B shows that, in the presence of 17 mM sodium borate, no fluorescent peak was observed. The fluorescent intensity was almost the same either with 50 mM sodium borate or with 67 mM sodium borate.

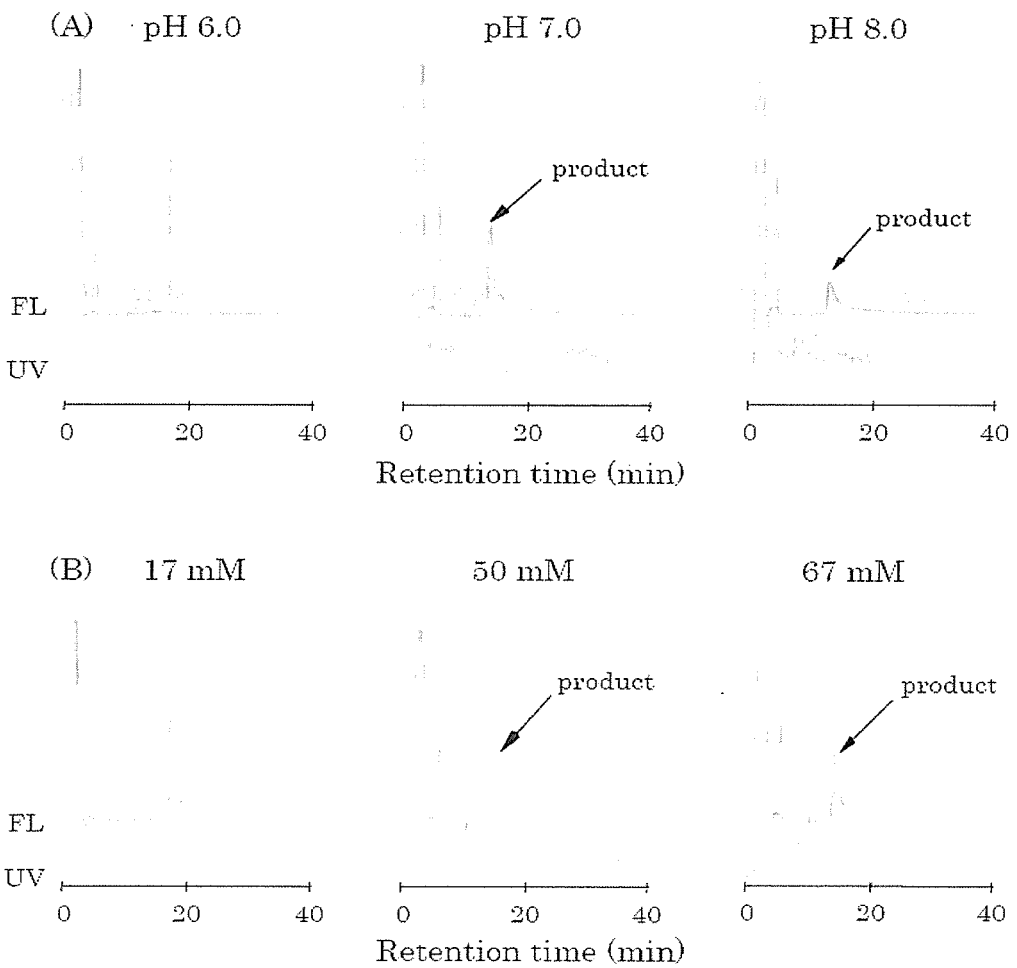

Figure 3. RPLC chromatograms obtained with different pHs (A) and different concentrations of borate (pH 7.0) (B). RPLC conditions were the same as those for Figure 2. Reaction components and conditions: 0.3 mM Phe-NH_2, 3.3 mM catechol, 2.0 mM $NaIO_4$, 50 mM sodium borate (pH 7.0), 120°C, 20 min.

On the basis of the above results, the optimum conditions for the fluorescent reaction 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.

Specificity and Reactivity. We examined the production of the fluorescent derivatives by using several amino acid amides and peptides at the same concentration as the target analyte, and the results were shown in Table 1. Note that the peak heights of the fluorescent products for 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 amino acids such as Leu and Tyr at the N-terminus, generated strong fluorescence, while Gly-Gly and Gly-Leu-Ala produced relatively lower fluorescence than other peptides. These results suggest that the reactivity of peptides under the present reaction conditions depended on the species of the N-terminal amino acid in the peptide molecule.

Amino acids (Phe and Tyr), sugars (glucose and ribose), polyamines (spermine and cadaverine), and nucleobases (adenine, thymine, guanine, and cytosine) did not produce the fluorescent compounds by the proposed reaction. This indicated that the proposed fluorescent reaction was specific for peptides.

Table 1. Retention time and relative fluorescent intensity of each fluorescent product for different peptides and amino acid amides.

Target analyte	Retention time (min)	Relative fluorescent intensity (cm)
Arg-NH_2	3.6	1.2
Gln-NH_2	5.8	1.1
Met-NH_2	14.4	1.1
Ala-NH_2	15.0	1.2
Tyr-NH_2	18.8	0.6
Phe-NH_2	21.6	0.9
Gly-Gly	2.6	6.1

Val-Gly	6.4	27.0
Leu-Gly	9.2	98.3
Phe-Gly	10.8	26.5
Gly-Leu-Ala	11.2	3.8
Ala-Leu-Gly	13.4	38.2
Ala-Leu-Ala	13.6	26.5
Trp-Gly-Gly	14.4	15.6
Tyr-Gly-Gly-Phe	15.6	95.9
Tyr-Gly-Gly-Phe-Leu	20.4	36.7
Leu-Leu-Leu	25.0	100.0

Quantification and Detection Limit by RPLC with Fluorescence Detection. When the reaction mixture of four peptides (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 Figure 4A. The borate (pH 7.0) in the mobile phase was an important factor for the fluorescent detection of these products. When the mobile phase without the borate was used, the fluorescent peaks were not observed in the chromatogram. This indicated that the fluorescent products are formed as a borate complex and the reaction products without borate anion do not emit the fluorescence.

The calibration graphs of four peptides were linear with sufficient correlation coefficients of 0.990-1.000, as shown in Figure 4B. The lower detection limit (signal per noise ratio, S/N = 3) of the fluorescent RPLC method was 0.7-3.0 pmol per an injection volume (40 μL) for the tested peptides. The sensitivity of this method was 10-100 times higher than that by the conventional RPLC method with UV detection.

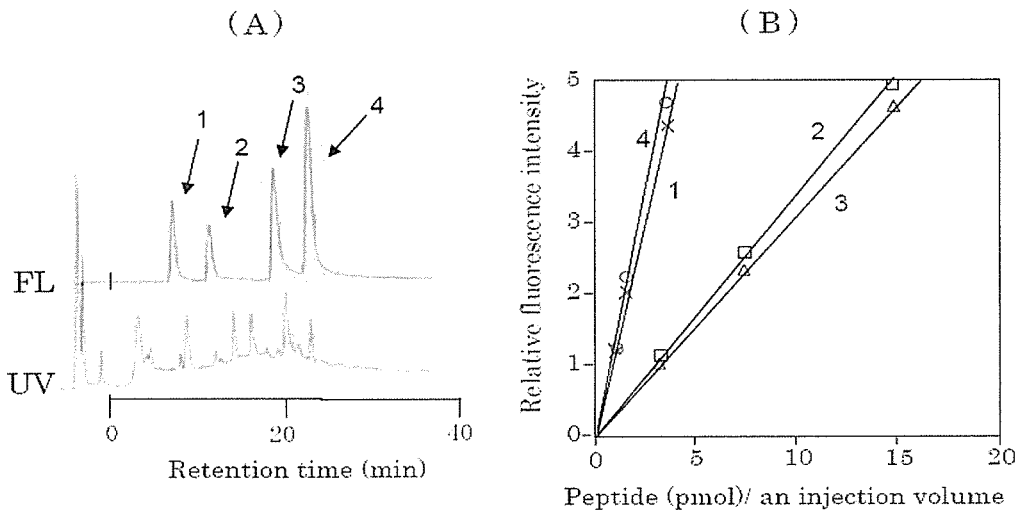

Figure 4. RPLC chromatograms (A) obtained from a mixture of the fluorescent reaction with four peptides by fluorescence (FL) and UV detections, and their calibration curves (B). RPLC conditions: mobile phase = MeOH (0 to 80 %, v/v) + H_2O (95 to 15 %) + 0.25 M sodium borate, pH 7.0 (5 %) for 40 min; injection volume = 40 μL. Reaction components and conditions: 33.3-166.7 μM peptides, 3.3 mM catechol, 2.0 mM $NaIO_4$, 50 mM sodium borate (pH 7.0), 120°C, 20 min. Peaks (concentration) and lines: 1=Leu-Gly (33.3 μM), 2=Ala-Leu-Gly (66.7 μM), 3=Tyr-Gly-Gly-Phe-Leu (166.7 μM), 4=Leu-Leu-Leu (66.7 μM).

Structure of the Fluorescent Product with ESI-TOF-MS. To estimate the chemical structure of the fluorescent products from Phe-NH_2 and Leu-Leu-Leu, the fluorescent products were first isolated by the RPLC, respectively. Then, positive ion mass spectra of these fluorescent fractions were measured by ESI-TOF-MS. In both spectra of Phe-NH_2 and Leu-Leu-Leu, the mass peaks corresponding to the fluorescent products involving two molecules of catechol in the peptide molecule were observed at m/z=345 (Figure 5A) and at m/z=538 (Figure 5B) as their $[M+H]^+$ ions, respectively. However, it was