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.

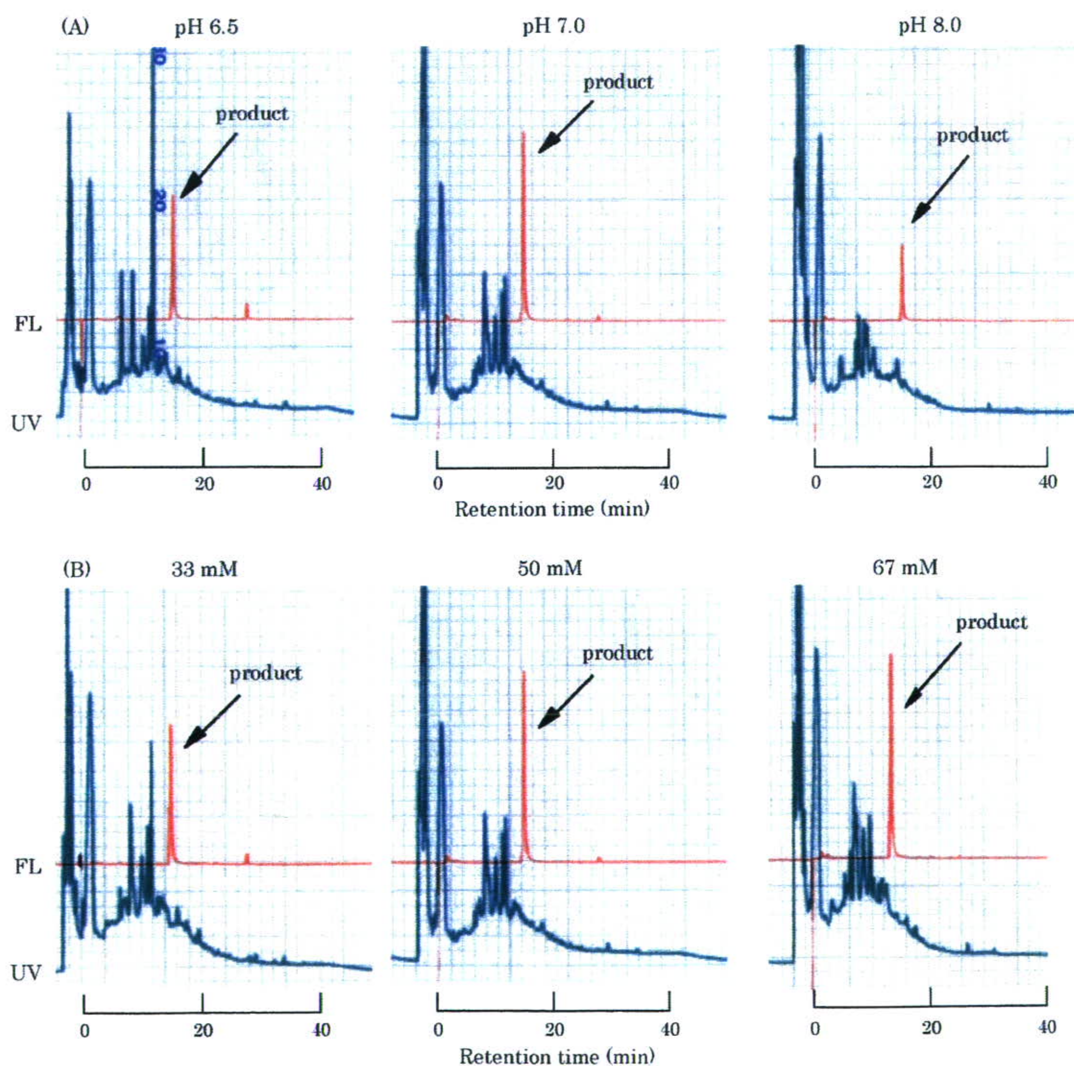

Fig. 3 – RPLC chromatograms obtained with different pHs (A) of 50 mM borate and concentrations (B) of borate at pH 7.0 for the fluorescence reaction. RPLC conditions were the same as those for Fig. 2. *Reaction conditions*: 0.033 mM Phe-NH_2, 3.3 mM catechol, 2.0 mM $NaIO_4$, 0–67 mM borate (pH 6.5–8.0),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

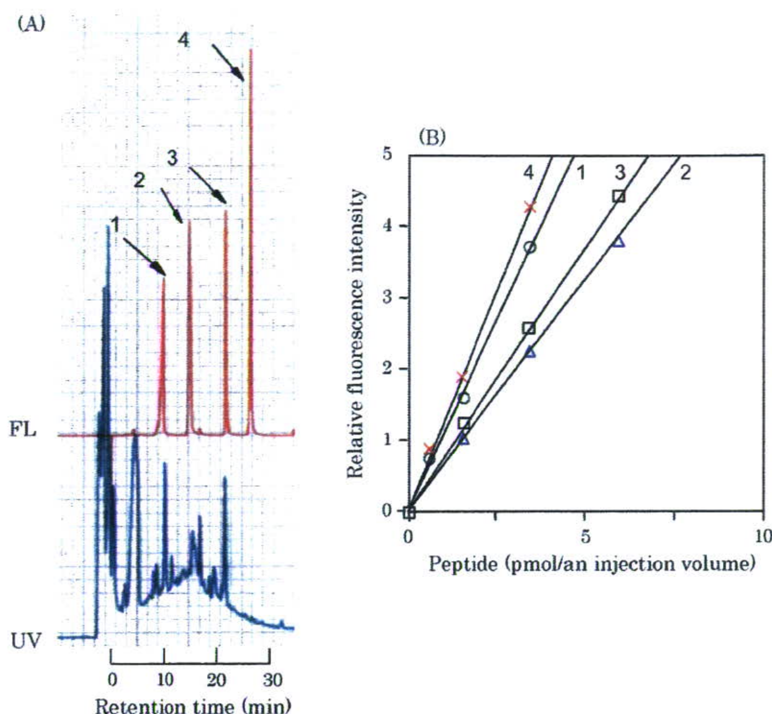

Fig. 4 – RPLC chromatograms (A) obtained with a fluorescence reaction mixture containing four peptides and their calibration curves (B). *RPLC conditions*: mobile phase = CH_3OH (0–80%, v/v) + H_2O (95–15%) + 250 mM borate, pH 7.0 (5%) for 40 min; injection volume = 40 μl; detection conditions were the 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.

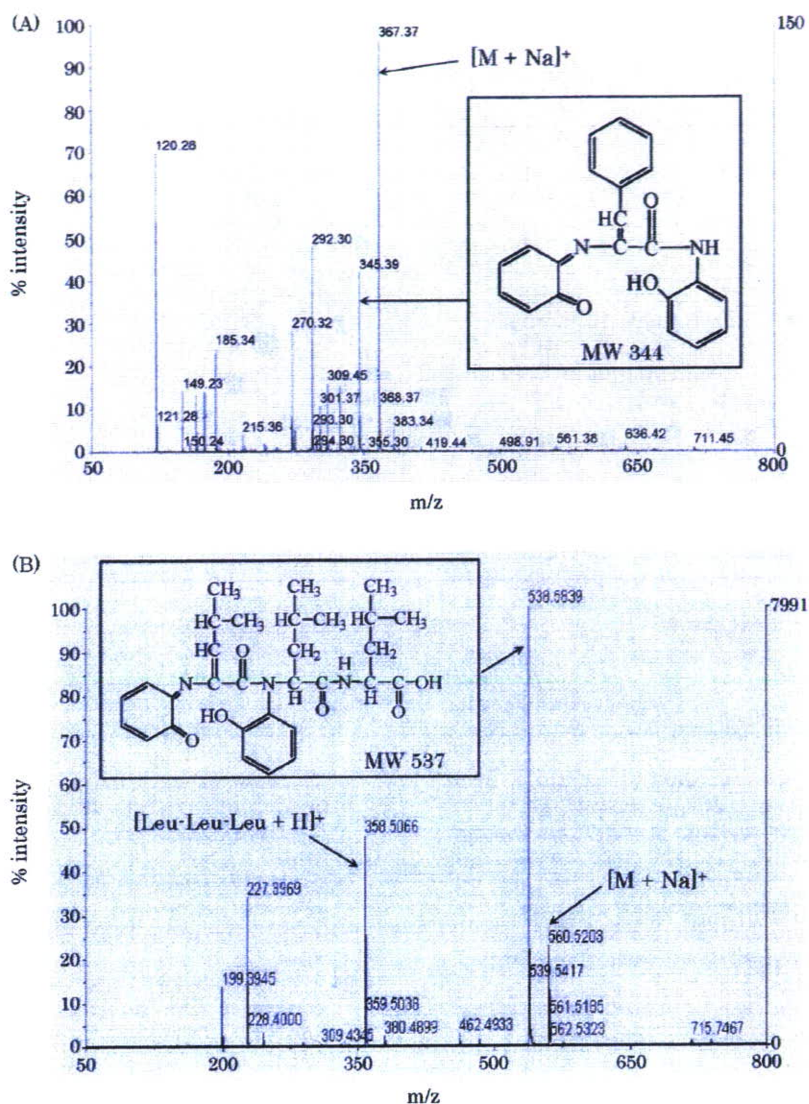

Fig. 5 – Positive ion ESI mass spectra of fluorescent products from Phe-NH_2 (A) and Leu-Leu-Leu (B).

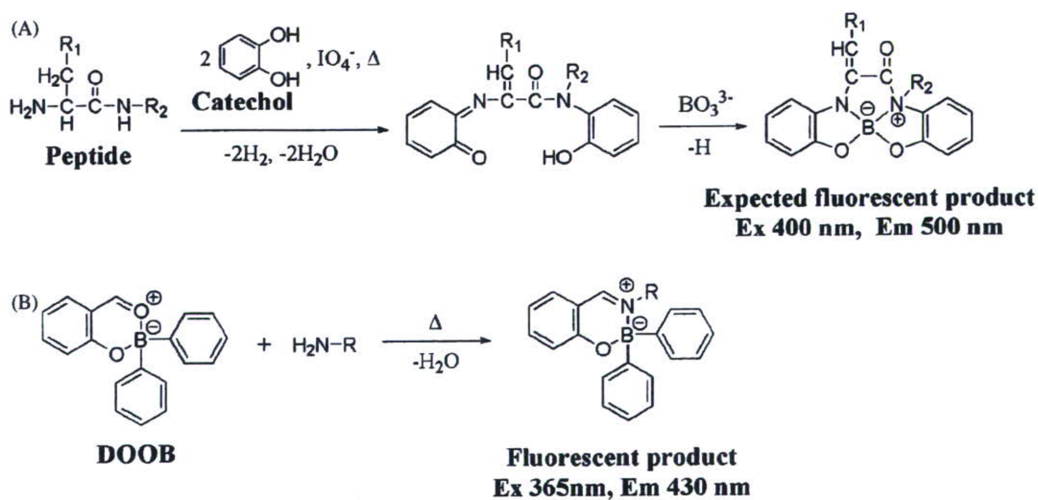

Fig. 6 – Possible chemical structure of the fluorescent product formed by the present reaction of a peptide with catechol, periodate and borate (A), and a fluorescent product [18] formed by a similar reaction of amine with DOOB (B).

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.
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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 byproducts 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

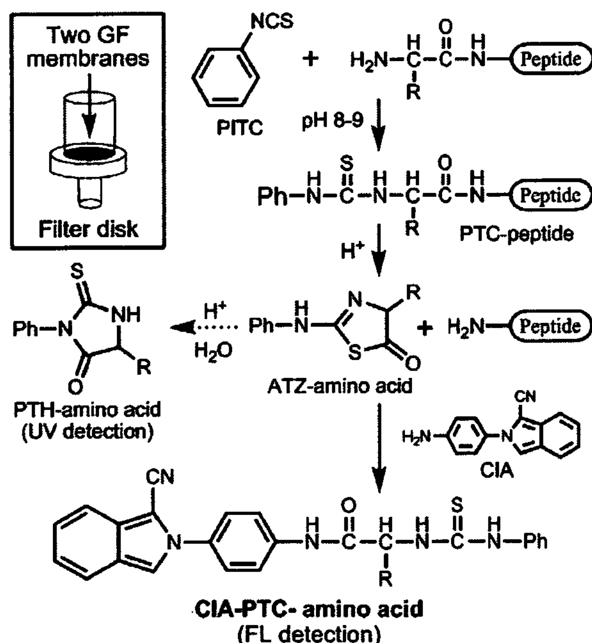

Fig. 1. Reaction scheme for the sensitization of ATZ–amino acid derivatives with CIA. The inset shows the design of a filter disk of polypropylene type supplied with a polytetrafluoroethene filter. Two GF membranes were set in the disk as depicted.

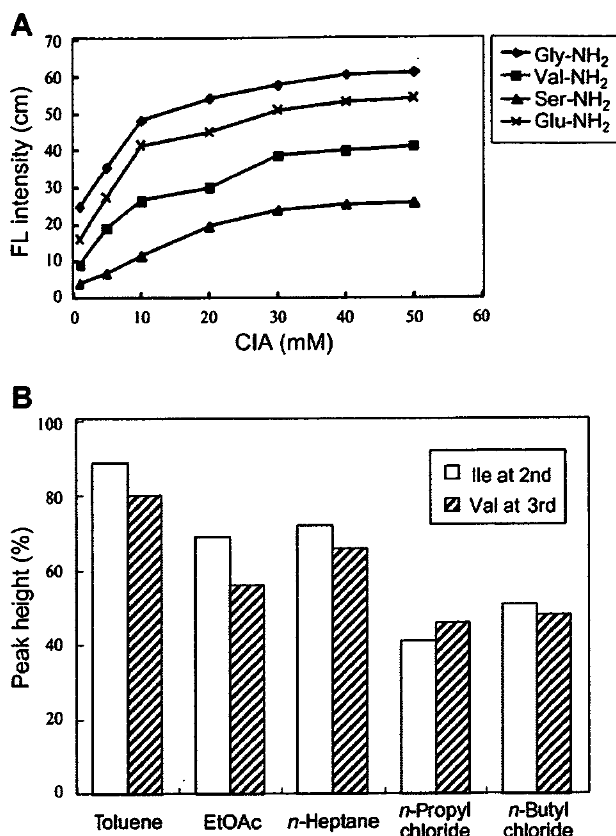

Fig. 2. (A) Effect of CIA concentration on the formation of PTC–CIA–amino acids in the derivatization reaction at 80 °C for 15 min. A portion (20 µl) of ATZ–amino acid in the eluate from membranes was reacted with 20 µl of 1 to 50 mM CIA in CH_3CN. FL intensity was based on peak heights of PTC–CIA derivatives. 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.

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.

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-trimethoxyphenylglyoxal (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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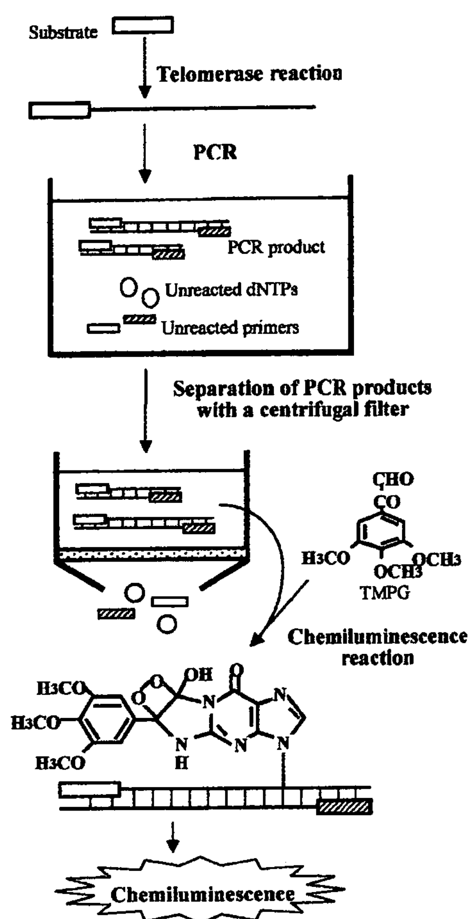

Fig. 1 Schematic protocol for the assay of telomerase activity by means of the CL reaction with TMPG. Telomerase synthesizes telomeric repeats from the substrate, and then the telomerase product is amplified by PCR with forward (the substrate) and reverse primers. The PCR reaction mixture is transferred 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).

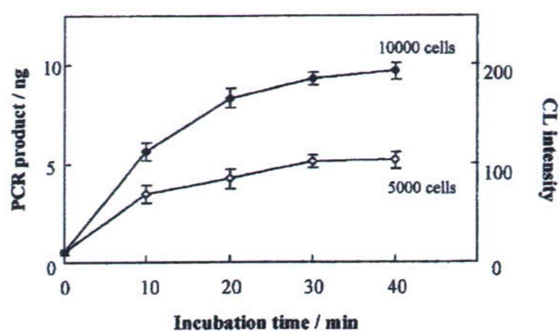

Fig. 2 Effect of the incubation time on the telomerase activity. Telomerase in the lysate of HeLa cells (5000 or 10000 cells) was reacted at 30°C for various times (0 - 40 min), and then the telomerase product was amplified by PCR. The Y-axis represents the produced amount of the DNA products (left side)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

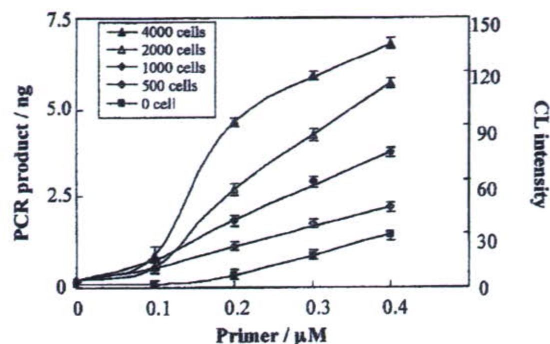

Fig. 3 Influence of various concentrations of primers. The lysate of HeLa cells varied from 500 to 4000 cells was incubated with 0 - 0.4 μM substrate. The telomerase products were amplified by PCR with 50 μM dNTP and various concentrations of primers (0 - 0.4 μM) (n = 3 each).

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 Amplifluor® 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, Amplifluor® 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.

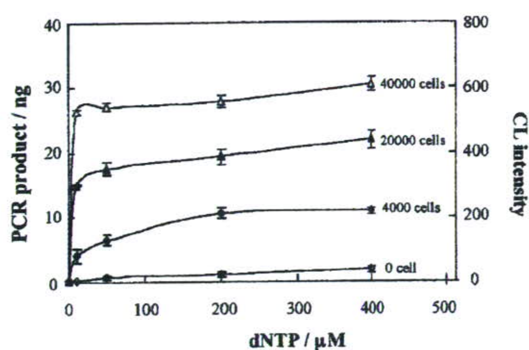

Fig. 4 Influence of various concentration of dNTP. The lysate of HeLa cells (4000, 20000, and 40000) was incubated with 0.2 µM substrate. The telomerase products were amplified by PCR in the presence of various concentrations of dNTP (0 - 400 µM) (n = 3 each).

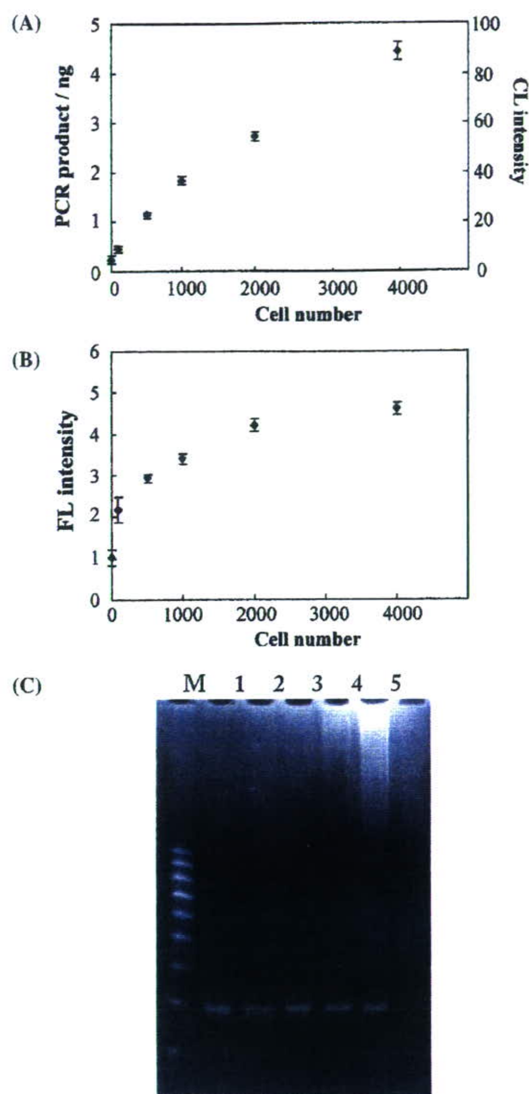

Fig. 5 Telomerase activity in HeLa cell lysates measured by the proposed CL method (A), FRET method (B) (n = 5 each) and the electrophoresis method with ethidium bromide staining (C). In the figure of (C), lane M represents 10 bp markers, and lanes 1 - 5 represent the cell numbers of 0, 1000, 5000, 10000,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

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 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

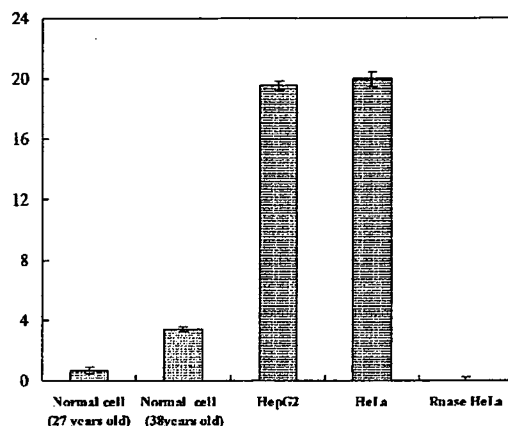

Fig. 6 Telomerase activities in several human cells. The cell lysates of oral cavity cells from two human specimens (male at 38 years old, and female at 27 years old) and two cell lines of HeLa and HepG2 were used for the assay of the telomerase activity under the recommended conditions (n = 3 each). The specific activity of telomerase was represented as the amount of the amplified products that were equivalent to telomerase products generated for 1 h of the enzymatic reaction per one of cells. In the experiment for the right column, the lysates of HeLa cells were treated by 0.01 unit ribonuclease at 37°C for 30 min before the telomerase reaction.

sensitively detected by the TMPG reaction. This method could distinguish the telomerase activities in immortal cells from those in normal somatic cells with low activity of telomerase.

Our method has the following advantages compared with other methods,[16,22] although the separation and CL reaction are required for the telomerase product: 1) our assay can be applied to any type of telomerase assay based on telomeric repeat amplification protocol, 2) this method shows higher sensitivity for the telomerase assay, and thus can determine a very low telomerase activity over a wide range, and 3) it is rapid and simple. Thus, this proposed method would be a convenient tool for the determination of telomerase activity.

Acknowledgements

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A chemiluminescence derivatization method for detecting nucleic acids and DNA probes using a trimethoxyphenylglyoxal reagent that recognizes guanine

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Abstract

A chemiluminescence derivatization method using a novel reagent, 3′,4′,5′-trimethoxyphenylglyoxal (TMPG) was studied for the quantitative detection of nucleic acids and deoxyguanylic acid oligomers on a nylon membrane. In the method, TMPG rapidly converted guanine of a nucleic acid to a chemiluminescent derivative in an alkaline phosphate solution at a room temperature. A cooled, charge-coupled device camera was used to detect the chemiluminescence emitted at room temperature from the derivatives on the membrane after the TMPG reaction. The detection signal was increased by increasing the amount of deoxyguanylic acid in the analyte molecule. When a DNA probe having a 5′-$(G)_{15}TT(G)_{15}TT$ deoxyoligomer at its 3′ terminus was hybridized with a target complementary DNA fragment on the membrane, an increased chemiluminescence from the hybrid was detected by the proposed method. The lower limit of detection is approximately 1.0 pmol of the target DNA fragment bound to the probe in the hybridization assay. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Chemiluminescence; Derivatization; Guanine; Nucleic acid; DNA probe; Trimethoxyphenylglyoxal

1. Introduction

Non-radiochemical methods utilizing a fluorescent or chemiluminescent reagent for detecting DNA probes or primers are very desirable [1]. They allow the development of powerful analytical techniques for obtaining information on gene structure and function. Recent advances include the use of laser-induced fluorescent dyes such as fluorescein analogues, which have been used to replace radioisotope-labelled oligodeoxyribonucleotides in automated DNA sequence analysis [2,3] and hybridization assays [4]. Another recently developed reagent was a chemiluminescent substrate of alkaline phosphatase, 3-(2′-spiroadamanatane)-4-methoxy-4-(3″phosphoryloxy)phenyl-1,2-dioetane (AMPPD) [5–7]. When alkaline phosphatase attached to a DNA probe or primer is allowed to react with AMPPD, a signal amplification technique can detect an increased chemiluminescence depending on the enzymatic reaction time. On the other hand, we

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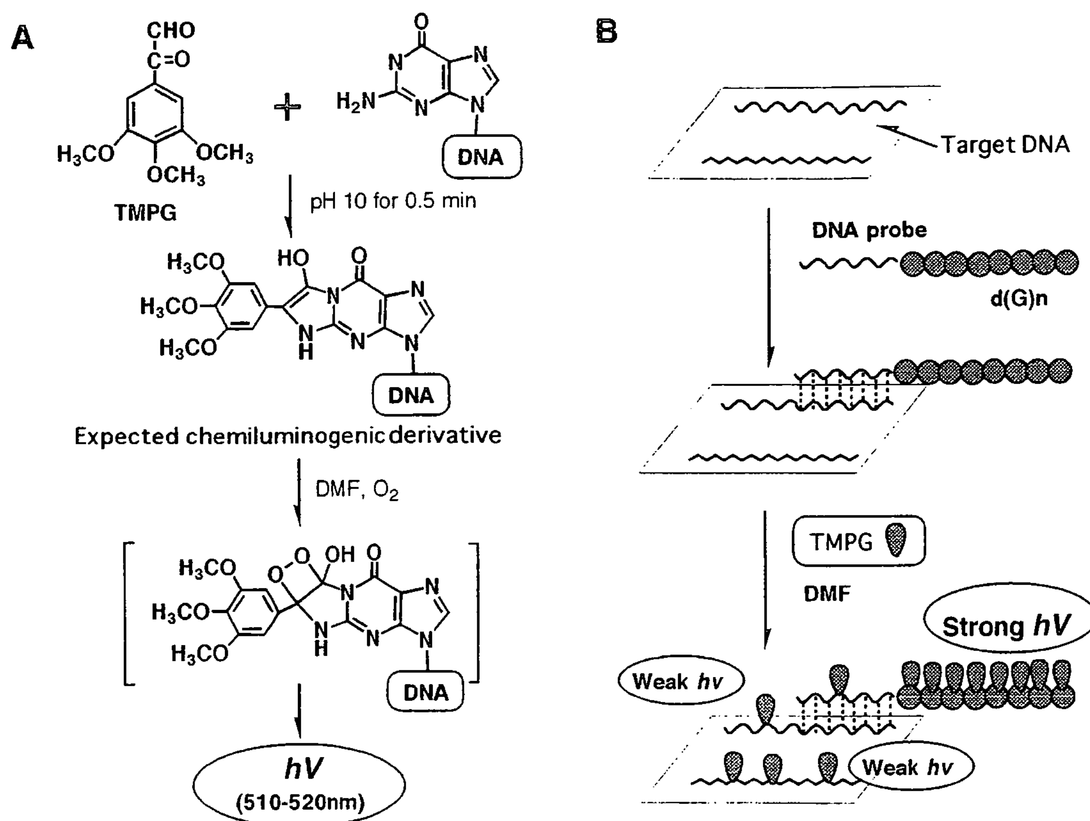

Fig. 1. (A) A possible pathway for the derivatization and chemiluminescence rections of nucleic acids with TMPG. The chemical structure of the product was considered on the basis of a reaction product between methylglyoxal and guanine reported by Shapiro et al. [12]. (B) Schematic illustration of chemiluminescence detection in a hybridization assay of a DNA fragment bound to a probe with a $d(G)_n$ tail. $h\nu$ is the chemiluminescence light emitted by the products of the reaction between guanine and TMPG.

have recently found that phenylglyoxal (PGO) reacts with guanine nucleotides to produce fluorescent derivatives [8,9], which emit chemiluminescence in a weakly alkaline solution [10].

In this paper, we found that 3′,4′,5′-trimethoxyphenylglyoxal (TMPG), one of PGO analogues, was more useful for the chemiluminescence detection of guanine bases in nucleic acids. We then studied a chemiluminescene derivatization method (Fig. 1(A)) for detecting DNA and polydeoxyguanylic acids ($d(G)_n$) on a blotting membrane. The chemiluminescene detection was performed by a densitometric luminometer equipped with a cooled, charge-coupled device (CCD) camera. This method was further applied to the detection of a hybrid with a DNA probe having a $d(G)_n$ tail in a hybridization system (Fig. 1(B)). The sensitivity of the proposed method should be increased by guanine-repeated amplification.

2. Experimental

2.1. Materials

The nucleic acid bases, nucleocides, nucleotides and salmon testes DNA were obtained from Seikagaku-Kogyo (Tokyo, Japan) and Sigma (St. Louis, MO, USA). TMPG and its analogues were synthesized using a previously described method for tryptophan analysis [11]. Phenylglyoxal monohydrate was purchased from Sigma–Aldrich Japan (Tokyo, Japan). The deoxyguanylic acid oligomers, DNA fragments and probes were synthetic products from Toowa–Gohsei (Tokyo, Japan). Other reagents were of the highest purity available. Nylon membranes (Clear Blot Membrane-*N* AE-6650) were obtained from Atto (Tokyo, Japan).

2.2. Solution reaction and detection with a photon-counting luminometer

A portion (100 µl) of an analyte dissolved in H_2O was mixed with 100 µl each of 50 mM sodium phosphate buffer (pH 6.5) and 0.2 M TMPG dissolved in dimethyl sulfoxide (DMSO). Water, instead of the sample, was used for a blank. The mixture was then warmed at 37°C for 20 min to produce the derivatives. In succession, 20 µl of 0.2 M sodium phosphate (pH 8.0), 20 µl of 0.25 M H_2O_2 and 160 µl of dimethylformamide (DMF) were added to 40 µl of the derivative mixture. After mixing for about 1 s, the chemiluminescence intensity in the mixture was measured at ambient temperature (26°C) for 0.5 min by a photon-counting computer-controlled Lumat LB-9051 luminometer (Berthold Japan, Tokyo, Japan), using 75×12 mm round-bottom glass tubes.

2.3. Membrane reaction and detection with a CCD camera

Each sample in H_2O (2 µl) was dotted on a nylon membrane. The spots were dried with an electric drier for a few minutes. The membrane was moistened with 0.2 M sodium phosphate aqueous solution (pH 10) for a few seconds, and then immersed in a 0.3 M TMPG solution for 0.5 min at ambient temperature (26°C). The moist membrane was then dipped in DMF for a few seconds, and quickly placed in a densitometric luminometer. The chemiluminescence image was detected in a 0.5 min exposure at ambient temperature (26°C) by a Lumino CCD AE-6930 densitograph (Atto, Tokyo, Japan) and processed in a computer using Densitometer Analyst version 4.0 software.

2.4. Hybridization with a DNA probe on a membrane

DNA fragments and a $d(G)_{30}$ probe (10 pmol each per 2 µl) were spotted on a nylon membrane. After baking with an electric drier, the membrane was immersed in a hybridization buffer consisting of 60 mM sodium citrate buffer (pH 7.0) and 0.6 M NaCl in the presence or absence of 50 nM of the probe at 42°C for 2 h. The membrane was then washed in a mixture of 0.1% sodium dodecyl sulfate, 30 mM sodium citrate buffer (pH 7.0) and 0.3 M NaCl for 1 min. Then the membrane was washed with H_2O for 1 min. The membrane was placed in the TMPG solution, and the chemiluminescence images were detected with a CCD camera in the same manner as described above.

3. Results and discussion

3.1. Conditions for the reaction with TMPG

We first studied the reactivities of the PGO analogues such as 4′-methoxyphenylglyoxal (MPG), 3′,4′-dimethoxyphenylglyoxal (DMPG), TMPG and 3′,4′-methylenedioxyphenylglyoxal (MDPG) in order to find a promising chemiluminogenic reagent for a nucleic acid. These arylglyoxals as well as PGO produced chemiluminescence with guanine under neutral reaction conditions, but not with the other nucleic acid bases and nucleotides (e.g., adenine, cytosine, uracil, thymine, adenosine-5′-monophosphate (AMP), cytidine-5′-monophosphate (CMP)). Of these reagents, TMPG produced the most intense chemiluminescence for salmon testes DNA (Fig. 2). The chemiluminescent derivative was produced by the reaction at 37°C with TMPG in sodium phosphate buffer of pH 6.5 for 20 min or at pH 7.5 for 2 min under almost the same conditions as those [10] described for the PGO reaction.

The derivative in the reaction mixture emitted chemiluminescence more intensely by oxidation with H_2O_2 in the presence of DMF (Fig. 3(A)). The maximum number of photons was counted 10 s after the addition of DMF and H_2O_2. Generally, chemiluminescence occurs in dioxetane formation of the luminogenic molecule by oxidation [7]. In this study, we found that the resulting chemiluminogenic derivative of DNA bound to a nylon membrane emits a strong chemiluminescence in DMF without H_2O_2 (Fig. 3(B)). The results suggest that the derivative bound to the membrane is more liable to oxidation. Therefore, we examined the reaction conditions for producing the derivative of TMPG with the membrane-bound samples of salmon testes DNA and a deoxyguanylic acid 20 oligomer ($d(G)_{20}$).

The nucleic acids on the membrane reacted rapidly (30–60 s) with TMPG at pH 9–10, as shown in Fig. 4. Consequently, we developed a quick and easy procedure described in Section 2 to detect the luminescence

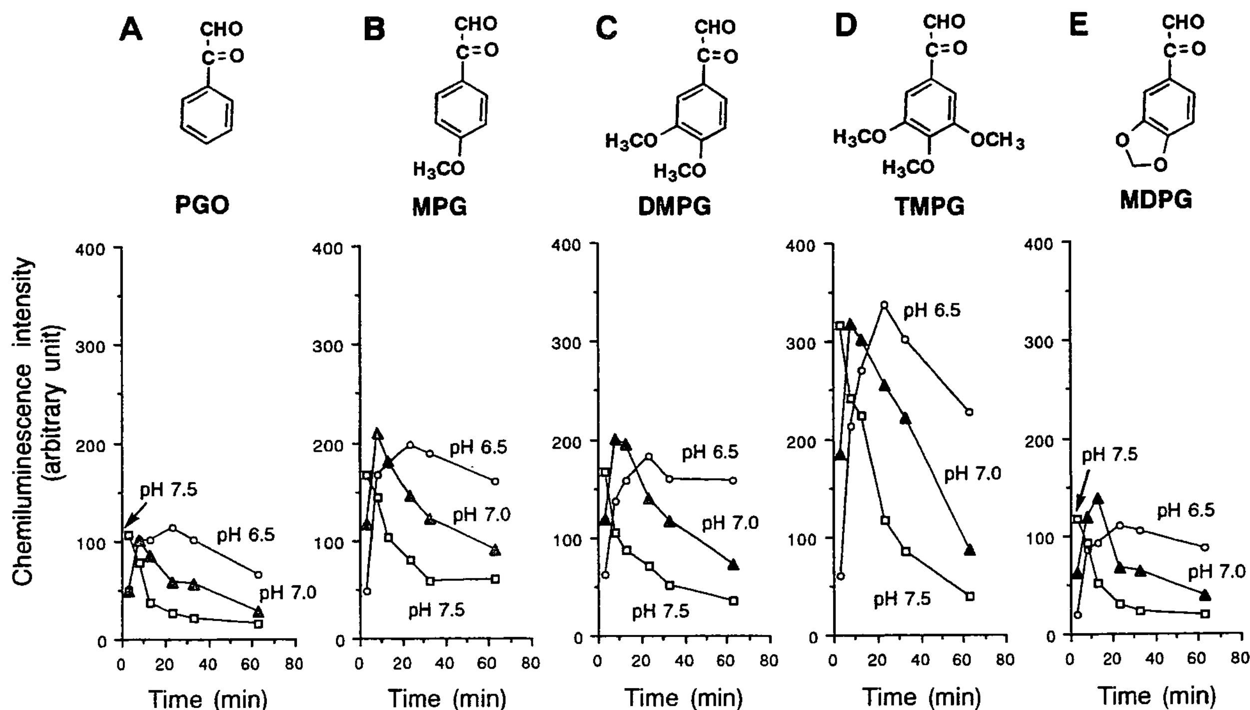

Fig. 2. Effects of the reaction time and the pH of the sodium phosphate buffer, on the production of luminescent DNA derivatives by arylglyoxals in the reaction mixtures. Arylglyoxals: (A) PGO, (B) MPG, (C) DMPG, (D) TMPG, and (E) MDPG. A portion (100 μl) of 30 μg/ml salmon testes DNA in a glass tube was treated according to the procedure recommended for the solution reaction, except that the PGO analogues and 50 mM sodium phosphate buffers at pH 6.5–7.5 were used for various reaction times.

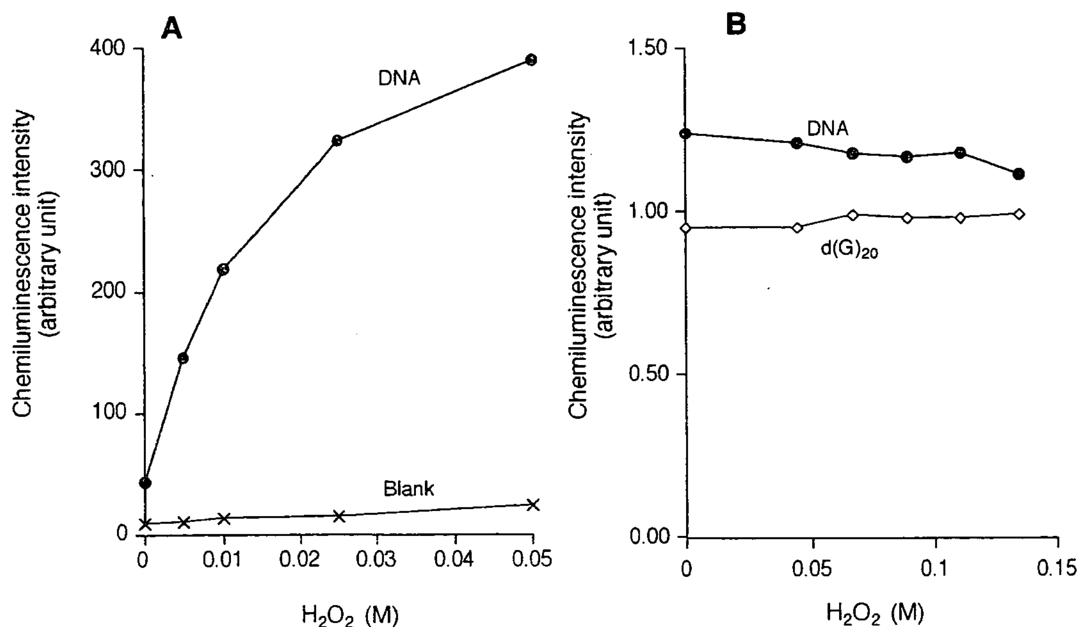

Fig. 3. Effect of H_2O_2 concentration in the oxidation mixture on the chemiluminescence development from the TMPG derivatives of (A) DNA and its blank in the reaction mixture, and of (B) DNA and $d(G)_{20}$ on a nylon membrane. For the experiment of (A), a portion (100 μl) of 30 μg/ml salmon testes DNA or water was treated according to the procedure recommended for the solution reaction, except that 0.05–0.5 M (final conc., 0.005–0.05 M) H_2O_2 aqueous solutions were used. For the experiment of (B), 2 μl each of 0.3 μg/μl DNA and 0.06 μg/μl $d(G)_{20}$ was treated according to the procedure recommended for the membrane reaction, except that DMF solutions containing 10% of 0.5–1.5 M (final conc., 0.05–0.15 M) H_2O_2 were used.

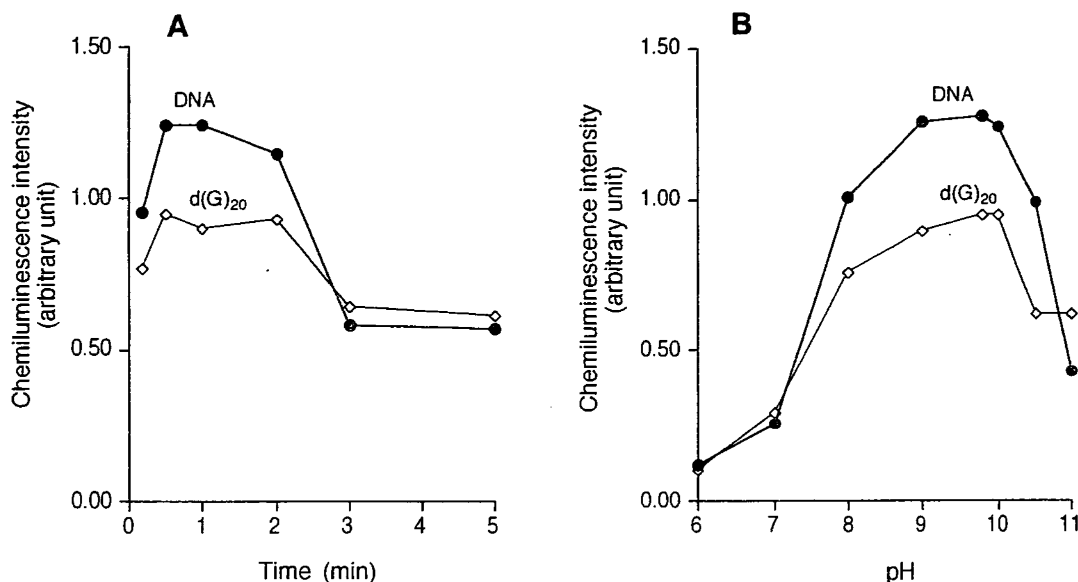

Fig. 4. Effect of (A) the reaction time and (B) the pH of the sodium phosphate solution, on the production of the TMPG derivatives of DNA and $d(G)_{20}$ on a nylon membrane. The samples (2 μl each) of 0.3 μg/μl DNA and 0.06 μg/μl $d(G)_{20}$ were treated according to the procedure recommended for the membrane reaction, except that the reaction time and the pH of 0.2 M sodium phosphate (composition, NaH_2PO_4–Na_2HPO_4–Na_3PO_4) were varied.

of the TMPG derivatives on the membrane, using a densitometric luminometer equipped with a cooled CCD camera. Each sample on the membrane can be processed in about 2 min. This includes 0.5 min for the reaction with TMPG at pH 10 and the 0.5 min exposure time for the detection.

It was reported that aliphatic glyoxals such as methylglyoxal and glyoxal react with guanine base [12]. However, they did not make guanine or DNA luminescent under neutral or alkaline reaction conditions. Recently, PGO and the alkylglyoxals were found to react with adenine base under strongly acidic conditions when heated at 100°C, and to then produce chemiluminescence under alkaline conditions [13,14]. Under the drastic reaction conditions, however, guanine or its containing nucleotides could not be made luminescent by the glyoxals.

3.2. Chemiluminescence signals detected by CCD camera

A method of detecting chemiluminescence with a cooled (−120°C) CCD camera attached to a computer digital evaluation system has recently been adapted for low light imaging of the chemiluminescence, which was emitted from a hybrid of a plasmid DNA (2.5×10^3 bases) as low as sub-attomole (2.5 pg) by an alkaline phosphatase-conjugated probe and AMPPD system [15]. We used a similar CCD camera, but it was cooled at −25°C.

Fig. 5(A) shows the chemiluminescence images and intensities from the reaction mixtures of salmon testes DNA and synthetic oligomers of $d(G)_{20}$, $d(G)_{15}$ and $d(G)_{10}$ with TMPG in the wells of a microtiter plate. The CCD camera could detect the chemiluminescent signal from 300 ng of the DNA in the reaction mixture, which was twice as strong as the background noise. However, a photon-counting luminometer used for Figs. 2 and 3(A) can detect the same reaction mixtures 100-fold more sensitively. Thus, the sensitivity of the used CCD camera is relatively low, though its sensitivity may be improved by cooling the CCD part to increase the signal-to-noise ratio.

The CCD camera at −25°C was used for the detection of the chemiluminescences emitted from the samples on a nylon membrane after the TMPG reaction. The detection signal (Fig. 5(B)) for the membrane-bound samples was nearly 80 times greater than that (Fig. 5(A)) for the microtiter samples. In this case, the detector response (Fig. 5(A)) for the latter samples was gained 50 times, so that the background intensity for the titerplate was higher than that for the

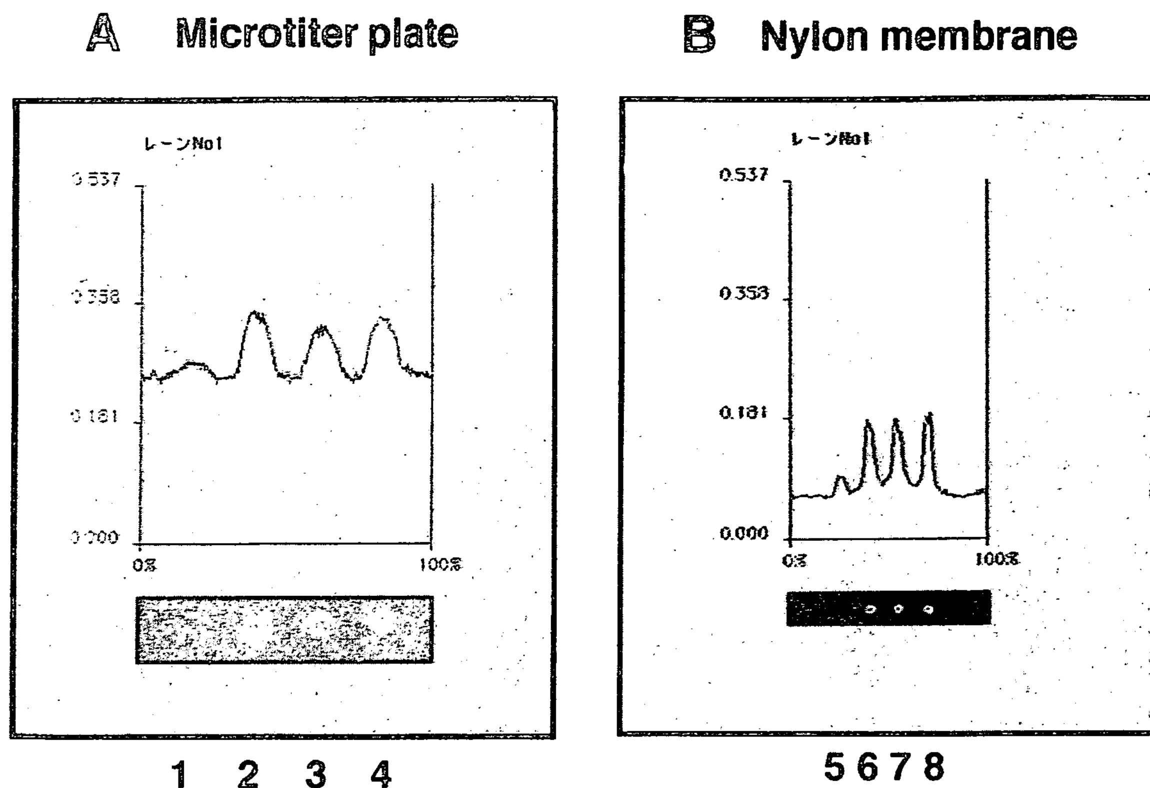

Fig. 5. Detection of the chemiluminescent TMPG derivatives of DNA, $d(G)_{20}$, $d(G)_{15}$ and $d(G)_{10}$ in (A) the wells of a microtiter plate, or on (B) a nylon membrane, by a cooled CCD camera. Samples [ng per well or spot]: (1) salmon testes DNA [670], (2) $d(G)_{20}$ [830], (3) $d(G)_{15}$ [810], (4) $d(G)_{10}$ [750], (5) salmon testes DNA [100], (6) $d(G)_{20}$ [120], (7) $d(G)_{15}$ [120], and (8) $d(G)_{10}$ [110]. The samples in the wells or on the membrane were tested according to the procedures recommended for the solution and membrane reactions, respectively, and their produced chemiluminescences were detected by the CCD camera.

membrane. For the same mass concentration (700–800 ng per well or 100–120 ng per spot) of the analytes, the chemiluminescence produce by $d(G)_{10-20}$ was almost as intense, and approximately 4 times greater than that of salmon testes DNA (Fig. 5), because the guanine content in the DNA molecule is probably one fourth. The detection limits at a signal-to-noise ratio of 2 on the membrane were approximately 1 ng for the $d(G)_{10-20}$ (molecular weight, 3230–6522) and 4 ng for the DNA (genome size, about 3×10^{9} base-pair molecular weight, about 2×10^{12}). These mass amounts correspond to 0.15–0.3 pmol for the $d(G)_{10-20}$ and approximately 2 zmol for the DNA as a genome. Therefore, the presented method gives a high sensitivity for the nucleic acid containing a large amount of guanine base in the molecule.

Quantitative measurements were performed on the membrane using salmon testes DNA and $d(G)_{10-20}$. The calibration graph for each sample, as shown in Fig. 6, is linear with a correlation coefficient (r) between 0.9867 and 0.9959. The relative standard deviation (RSD) of the chemiluminescence intensity, based on the integral area, was 7.8% when 0.5 μg DNA (n=15) was repeatedly reacted with TMPG. At an equimolar concentration of 4–16 pmol, the $d(G)_{20}$ oligomer produced twice the signal intensity of the $d(G)_{10}$ oligomer (Fig. 6(B)). Therefore, the detection signal for $d(G)_n$ can be increased by increasing the guanine content of the nucleic acid molecule.

Fig. 7 shows the comparing of the data of the calibration graph of DNA (0–0.2 μg) obtained by the present chemiluminescence method and a popular fluorescent method with ethidium bromide (EBr). The EBr reagent is an intercalator that binds to double-stranded DNA, and gives an increased fluorescence intensity by increasing the concentration of DNA. Nevertheless, we used two different concentrations of EBr for the determination of DNA, a good linearity of the graph was not obtained, and the blank fluores-