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Selective and facile assay of human immunodeficiency virus protease activity by a novel fluorogenic reaction

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ABSTRACT

A highly selective and facile assay of human immunodeficiency virus protease (HIV-PR) has been required for the screening of medicinal inhibitors and also for classifying the subtypes of HIV in the therapeutic treatment of acquired immune deficiency syndrome (AIDS). This article describes a novel assay method of HIV-PR based on the selective fluorogenic reaction of peptides. A peptide fragment generated from a substrate by the enzymatic digestion with HIV-PR could be selectively quantified by the spectrofluorometric detection after the fluorogenic reaction with catechol in the presence of sodium periodate and sodium borate (pH 7.0). This assay system uses an N-terminal acetyl peptide as the substrate and crude extracts from *Escherichia coli* expressing recombinant HIV-PR. The activity obtained by the proposed assay correlated with that obtained by a conventional HIV-PR assay based on fluorescence resonance energy transfer detection.

Viral proteases play a major role in the maturation of functional proteins and, thus, serve as antiviral targets in many infectious diseases of viral etiology such as acquired immune deficiency syndrome (AIDS)[1] and severe acute respiratory syndrome (SARS) [1–4]. Human immunodeficiency virus protease (HIV-PR), an aspartic protease, is responsible for the processing of the polyproteins Gag and Gag–Pol into the viral structural and functional proteins during the maturation. Therefore, HIV-PR is a major indicator not only for the design of anti-AIDS drugs but also for therapeutic efficacy [5–7].

Up to now, several different methods have been developed for the assay of HIV-PR activity. Billich and coworkers [8] proposed a chromatographic assay with ultraviolet (UV) detection for HIV-PR activity using eight synthetic substrates, Stebbins and Debouck [9] reported a colorimetric assay using two nonenzymatic reactions in a microtiter plate for the high-throughput screening of HIV-PR inhibitors, and Fuse and coworkers [10] developed a new cell line expressing HIV-PR with an induction reagent. Currently solid-phase immunoassay, enzyme-linked immunosorbent assay (ELISA), and fluorescence resonance energy transfer (FRET)-based assay [11–13] have been reported. Although these methods are rather simple and sensitive for the HIV-PR assay, either enzyme or fluorophore labeling of the antibodies or the substrates is required for facile detection and, thus, each assay results in a great expense. In current reports, HIV-PR activity in clinical specimens such as blood of AIDS patients has not been assayed by those methods. In general, the diagnosis of AIDS has been performed by genetic detection of HIV with polymerase chain reaction (PCR) for the sample amplification because of its extremely high sensitivity.

Recent studies led to the development of a novel assay method for the determination of HIV-PR activity. In this proposed method, a free peptide was generated from an N-terminal acetyl peptide as the substrate by the enzymatic reaction with HIV-PR, and then the product was selectively and sensitively converted into a fluorescent compound by a novel fluorogenic reaction [14] using catechol, borate, and $NaIO_4$. In the current study, the fluorogenic and enzymatic reactions were optimized for the development of an inexpensive and sensitive spectrofluorometric assay of HIV-PR activity. This assay method was evaluated by several protease inhibitors of HIV-PR activity and by comparison with a commercial HIV-PR assay kit.

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[1] *Abbreviations used:* AIDS, acquired immune deficiency syndrome; SARS, severe acute respiratory syndrome; HIV-PR, human immunodeficiency virus protease; UV, ultraviolet; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer; PCR, polymerase chain reaction; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Ac-RKILFLDG, acetyl-Arg-Lys-Ile-Leu-Phe-Leu-Asp-Gly; FLDG, Phe-Leu-Asp-Gly; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl-β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; FL, fluorescence; EDANS, 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid; DABSYL, 4-*N*,*N*-dimethylaminoazobenzene-4′-sulfonic acid; RSD, relative standard deviation.

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Materials and methods

Materials

Catechol, H_3BO_3, and $NaIO_4$ were purchased from Wako Pure Chemicals (Osaka, Japan). Catechol (2.5 mM), H_3BO_3 (300 mM), and $NaIO_4$ (1.0 mM) were dissolved in water. Proteinase K and protease inhibitors such as pepstatin A, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), E-64, aprotinin, and bestatin were purchased from Merck Calbiochem (Mannheim, Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco–Invitrogen (Grand Island, NY, USA). Sense and antisense primers were purchased from Sigma–Genosys Japan (Ishikari, Japan). BamHI and HindIII were purchased from New England Biolabs (Beverly, MA, USA). Acetyl-Arg-Lys-Ile-Leu-Phe-Leu-Asp-Gly (Ac-RKILFLDG) and Phe-Leu-Asp-Gly (FLDG) were purchased from Sigma–Genosys Japan. The peptides were dissolved in water or 50% aqueous solution of 2-methoxyethanol (Wako Pure Chemicals), and their stock solutions (2.5 mM) were kept at −20 °C.

Preparation of recombinant HIV-PR

E-PR293 cells [10] containing the HIV-PR gene were cultured at 37 °C for 72 h in DMEM supplemented with 10% FBS at 5% CO_2. After being cultured, the cells were collected by centrifugation and then washed with 0.9% NaCl. The cell suspension in a lysis buffer (10 mM Tris–HCl [pH 8.0], 0.1 mg/ml proteinase K, and 0.1% sodium dodecyl sulfate [SDS]) was incubated at 37 °C for 3 h. The cell lysate was mixed with an equal volume of a solution of phenol, chloroform, and isoamyl alcohol (25:24:1), and the mixture was gently shaken for 30 min. After centrifugation at 12,000*g* for 15 min, chromosomal DNA in the upper phase was collected by ethanol precipitation and resolved in TE buffer (10 mM Tris–HCl [pH 7.5] and 1 mM ethylenediaminetetraacetic acid [EDTA]–2Na). The HIV-PR gene was amplified by PCR using the sense primer 5′-TTTGGATCCCCTCAGATCACTCCTTGGCAG-3′ and the antisense primer 5′-AGGAAGCTT***TTA***AAAATTTAAAGTGCAGCC-3′. The primers contain a restriction enzyme site (underlined) and a translational stop codon (italic bold). The PCR product was digested with BamHI and HindIII and was inserted into the restriction sites of the same enzyme on pMal–c2X vector (New England Biolabs). The constructed plasmid (pHIV-PR) was transformed into *Escherichia coli* DH5α cells by culturing at 37 °C in Luria–Bertani broth (1% NaCl, 1% tryptone, and 0.5% Bacto yeast extract) containing 50 μg/ml ampicillin. The growing cell numbers were monitored by measuring the absorbance at 600 nm ($A \sim 0.8$) before the induction with 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After adding IPTG, the transformed cells were cultured at 37 °C for 3 h. The cells were then washed with 1× phosphate-buffered saline (PBS) and resuspended in 5 ml of the enzyme reaction buffer (50 mM sodium acetate [pH 5.5], 1.0 M NaCl, 1.0 mM EDTA, and 2.5% glycerol. The transformed cells were thereafter disrupted by repeated sonication on ice using an ultrasonic disruptor (model 300 V/T, BioLogics, Manassas, VA, USA). Recombinant HIV-PR in the cell lysate was used for the following enzymatic reaction.

Spectrofluorometric assay of HIV-PR activity

A portion of 20 μl (amounts of total proteins, 4–8 μg) of the cell lysate was mixed with 74 μl of the enzyme reaction buffer (pH 5.5), and the enzymatic reaction was initiated by adding 6 μl of 2.5 mM Ac-RKILFLDG as a substrate. The reaction mixture was incubated at 37 °C for 2 h, and then the pH of the reaction mixture was adjusted to approximately 7.0 with 5 μl of 0.1 M sodium hydroxide. A lysate of *E. coli* cells lacking the HIV-PR protein was used as a negative control. The product FLDG was determined by the following fluorogenic reaction. The whole mixture (105 μl) of the enzymatic reaction was successively mixed with 100 μl of 2.5 mM catechol, 50 μl of 0.3 M H_3BO_3–NaOH (pH 7.0), and 50 μl of 1.0 mM $NaIO_4$, and immediately the mixture was heated at 100 °C for 10 min. After heating, the reaction mixture was cooled in an icewater bath to stabilize the fluorescent product. The fluorescence (FL) intensity of the product was measured in a 10 × 3-mm quartz cell with a spectrofluorometer (FP-6300, Jasco, Tokyo, Japan) at 500 nm (emission) with excitation at 400 nm. All measurements were performed in triplicate for each sample.

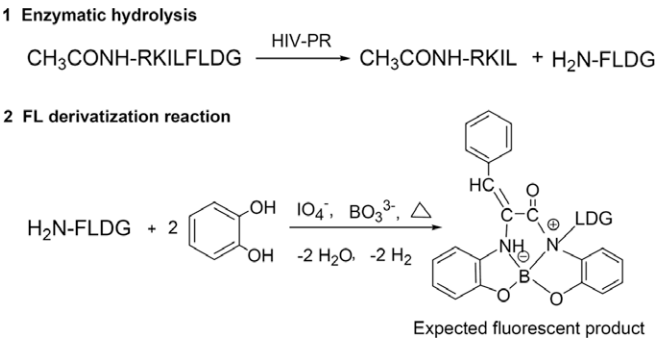
Assay of HIV-PR activity by a conventional FRET-based method

A commercially available kit, the SensoLyte 490 HIV-1 Protease Assay Kit (AnaSpec, San Jose, CA, USA), was used to compare the findings with the proposed assay. The detection system of this kit was based on FRET spectrofluorometry using an FL-labeled peptide (DABSYL-Abu-Ser-Gln-Tyr-Pro-Ile-Val-Gln-EDANS) as the FRET substrate. The FL of 5-[(2-aminoethyl)amino]naphthalene-1-sulfonic acid (EDANS) in the substrate was quenched in advance by 4-*N*,*N*-dimethylaminoazobenzene-4′-sulfonic acid (DABSYL) in the same substrate, although the FL of EDANS at 490 nm (emission)/340 nm (excitation) was recovered by cleaving into two separated fragments of the FRET substrate by HIV-PR. This enzyme reaction with HIV-PR in the crude extract was performed according to the manufacture's instruction. The cell lysate (50 μl) containing the recombinant HIV-PR was mixed with 50 μl of the assay buffer and 100 μl of the FRET substrate. The reaction mixture was incubated at 37 °C for 2 h, and then the FL of the product was detected with a spectrofluorometer at 490 nm (emission)/340 nm (excitation). Free EDANS was used as a standard material to determine the product.

Results and discussion

Assay principle and optimum conditions of the fluorogenic reaction

A novel fluorogenic reaction [14] with catechol, borate, and $NaIO_4$ was used for the assay of HIV-PR activity in this study (Scheme 1). This reaction could provide fluorescent derivatives for synthetic peptides. However, N-terminal acetyl peptides did not form such a fluorescent peptide. Therefore, an N-terminal acetyl octapeptide, Ac-RKILFLDG, containing an HIV-PR cleavage site was employed as the substrate. It was not necessary to remove excess substrate and acetyl product (Ac-RKIL) from the reaction



Scheme 1. Schematic protocol for the spectrofluorometric assay of HIV-PR activity. The N-terminal acetyl octapeptide as the substrate was hydrolyzed by HIV-PR in a lysate of cells. The generated peptide was selectively converted to the fluorescent compound by a novel fluorogenic reaction with catechol, $NaIO_4$, and orate.

mixture after the enzymatic hydrolysis because no FL signals from these peptides were produced by this reaction, whereas another product FLDG was selectively reacted. Therefore, the enzyme activity of HIV-PR could be readily assayed by measuring the FL intensity of the FLDG derivative.

The fluorogenic reaction conditions were optimized by using the synthetic peptide, FLDG. The fluorogenic reaction was carried out for different reaction times (5–15 min) at 100 °C and pH 7.0 (Fig. 1A). The maximum yield of the fluorescent product was observed at 10 min. When the fluorogenic reaction was carried out for 10 min at pH 7.0 under the different temperatures of 80 to 120 °C (Fig. 1B), the fluorescent derivative was produced by the high temperatures of the reaction. However, the FL intensity was gradually decreased at a higher temperature than 100 °C, probably due to instability of the fluorescent product at the higher temperatures. The FL production was also greatly affected by pH of sodium borate solution. For the pH examination, the fluorogenic reaction was carried out at 100 °C for 10 min (Fig. 1C). The signal could not be detected at a lower pH than pH 6.0; however, the maximum signal was obtained at approximately pH 7.0. The concentrations of catechol, $NaIO_4$, and sodium borate at pH 7.0 were individually optimized, and then the maximum signal was obtained by the use of 0.83 mM catechol, 0.17 mM $NaIO_4$, and 50 mM borate (pH 7.0) at 100 °C for 10 min in the aqueous reaction mixture.

Our previous article [14] using an amino acid amide, phenylalanine amide, reported a reaction mechanism for this fluorogenic reaction in which a high production yield of the fluorescent derivative of phenylalanine amide was obtained with the reaction time of 20 min at 120 °C. When this fluorogenic reaction with the peptide (FLDG) was carried out at 120 °C for 20 min, however, the production yield of the fluorescent derivative of the peptide was approximately 65% of that at 100 °C for 10 min. The different conditions between the peptide and the amino acid amide might be caused by the stability of the fluorescent products given that the product of the amino acid amide was more stable than that of the peptide. The stability of the product of the peptide was investigated by measuring the FL intensity after the fluorogenic reaction. The product was stable at 0 to 4 °C for at least 48 h, although the product was unstable at 100 °C and slightly unstable at room temperature. Therefore, the reaction mixture was cooled in an icewater bath after the fluorogenic reaction until detection.

A standard curve for the synthetic peptide of FLDG was made by this fluorogenic reaction with the peptide in the range from 0 to 30 μM in the enzymatic reaction mixture in the absence of the substrate and HIV-PR to determine the concentration of FLDG generated by the enzymatic hydrolysis with HIV-PR. As shown in Fig. 1D, a linear relationship was observed between the FL signal and the peptide concentration ($y = 3.647x + 1.08$, $r^2 = 0.990$, where x and y indicate the concentration of the peptide and FL intensity, respectively). Therefore, the close correlation between the FLDG concentration and the FL intensity makes it possible to carry out the quantitative assay of HIV-PR activity.

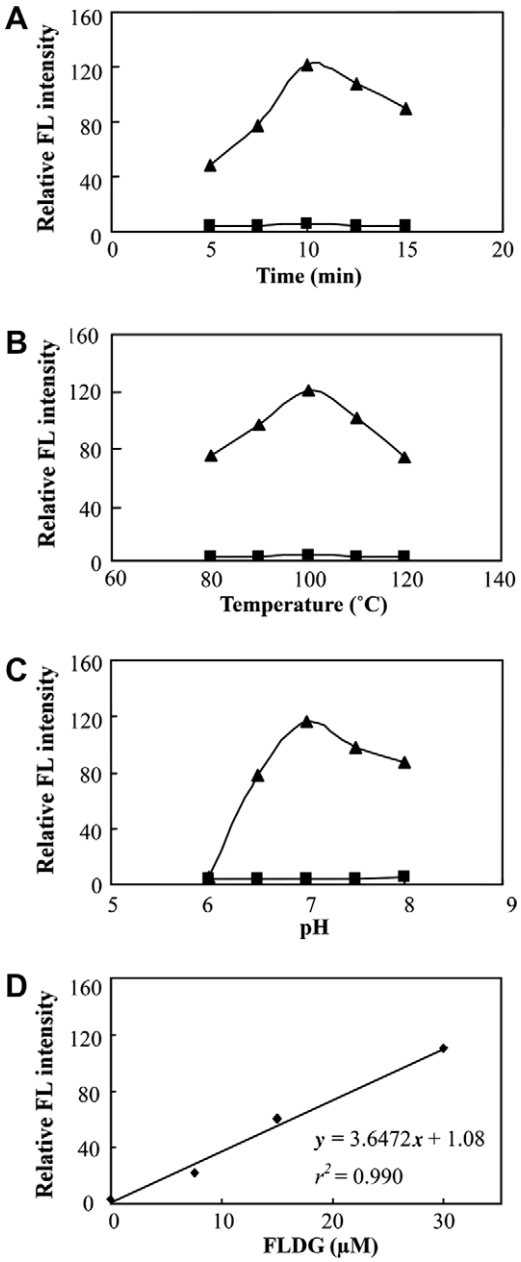


Fig. 1. (A–C) Effects of reaction time (A), reaction temperature (B), and pH (C) on the fluorogenic reaction of FLDG (▲) and H_2O as its blank (■). (D) Calibration curve for the concentration of synthetic FLDG. A portion of 6 μl of 500 μM FLDG (the experiments of panels A, B, and C) or of 125 to 500 μM FLDG (the experiment of panel D) was mixed with 94 μl of H_2O, and then 100 μl of 2.5 mM catechol, 50 μl of 1.0 mM $NaIO_4$, and 50 μl of 0.3 M H_3BO_3–NaOH (pH 7.0) (the experiments of panels A, B, and D) or of 0.3 M H_3BO_3–NaOH (pH 6.0–8.0) (the experiment of panel C) was added to the peptide solution, and immediately the mixture was heated at 100 °C (the experiments of panels A, C, and D) or at 80 to 120 °C (the experiment of panel B) for 10 min (the experiments of panels B, C, and D) or for 5 to 15 min (the experiment of panel A). The fluorescent detection of the FLDG derivative was carried out as described in Materials and methods.

Assay of HIV-PR activity

A lysate specimen of *E. coli* expressing recombinant HIV-PR was used as a model source of HIV-PR in this study. The influence of pH in the enzymatic reaction mixture was first studied on HIV-PR activity by the use of Ac-RKILFLDG as a substrate. As shown in Fig. 2A, HIV-PR activity was greatly influenced by the pH. The enzymatic hydrolysis of the substrate decreased to below pH 4.0 and reached a maximum yield at pH 5.5. The optimum pH value for the enzymatic reaction with HIV-PR was 5.5, and this result was consistent with the previous report [9]. Therefore, the pH after the enzymatic reaction must be changed to 7.0 because the fluorogenic reaction effectively occurs at approximately pH 7.0.

HIV-PR in the cell lysate was reacted with the substrate at 37 °C for different incubation times (0–3 h) to investigate the incubation time of the enzymatic reaction. As shown in Fig. 2B, the product amount increased by prolonging the incubation time. For convenience, an incubation time of 2 h was set up in the current assay.

The enzymatic reaction was performed with various concentrations of the substrate in the presence of 0 to 16 μg of total proteins in the cell lysate to determine the optimum concentration of the substrate (Fig. 3A). The product amount increased proportionally

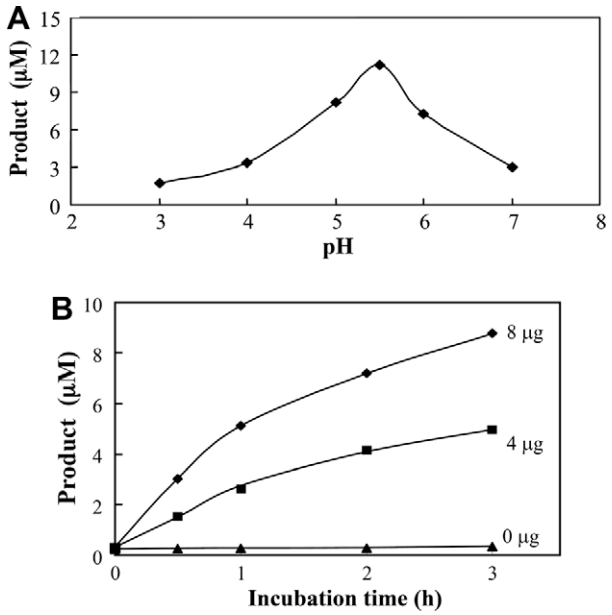


Fig. 2. Effects of pH of the enzymatic reaction (A) and incubation time (B) on HIV-PR activity. The enzymatic reaction was carried out at 37 °C with 16 μg (A) and 0, 4, or 8 μg (B) of total proteins in the mixture, respectively.

up to 150 μM substrate in the presence of 0 to 8 μg of the total proteins in the lysate. The concentration of the substrate was expressed as the final concentration in the enzymatic reaction mixture. The enzymatic production of FLDG was also proportional to the amount of the total proteins in the cell lysate (Fig. 3B). Its correlation coefficient (r^2) was 0.968. These results indicate that HIV-PR activity in the crude extract can be determined by this novel spectrofluorometric method.

Influence of inhibitors on HIV-PR activity

There is growing interest in developing specific inhibitors of HIV-PR as possible therapeutic agents that can be used for the treatment of AIDS. A number of commercially available inhibitors have been investigated for their characterization of HIV-PR activity. As shown in Fig. 4A, the residual activity of HIV-PR was 70% to 80% of its initial activity at a high concentration of 1.0 mM inhibitors such as AEBSF, E-64, bestatin, and aprotinin. However, the residual activity of HIV-PR was less than 5% at 1.0 mM pepstatin A, which was one of the specific inhibitors for aspartic proteases such as HIV-PR [15–17]. The activity of HIV-PR in the cell lysate was strongly decreased with increasing concentrations of pepstatin A and was completely inhibited with 100 μM pepstatin A. The data indicated that the IC_{50} of pepstatin A is 10×10^{-6} M. Therefore, the proposed method is specific for the assay of HIV-PR activity.

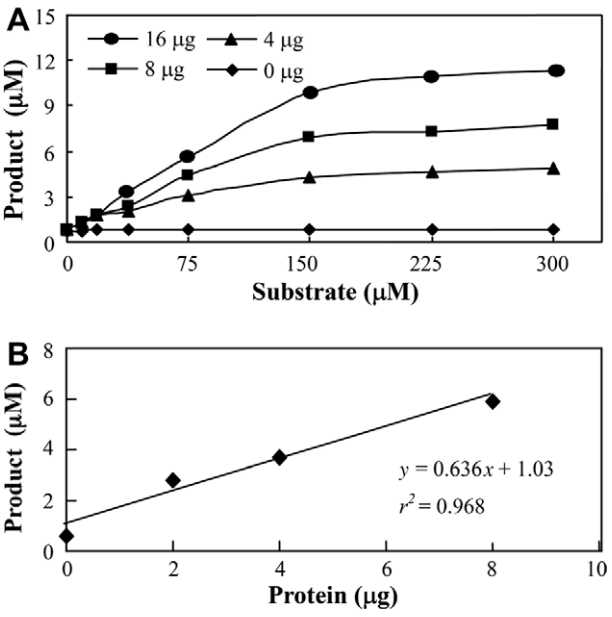


Fig. 3. Influences of substrate concentration in the presence of 0 to 16 μg of total proteins (A) and of the protein amount in the presence of 150 μM substrate in the enzymatic reaction mixture (B).

Comparison of proposed method with other method

A commercial HIV-PR assay kit was employed to determine HIV-PR activity in the same lysate so as to evaluate the reliability of the proposed spectrofluorometric method. As shown in Fig. 4B, the amount of the enzymatic product measured by the proposed method was approximately 10 times larger than that measured by the commercial kit, and a good correlation ($r^2 = 0.991$) of each amount of the enzymatic products was obtained by both methods. Therefore, this proposed method possesses sufficient reliability to be used for the assay of HIV-PR activity.

Precision of repeated assays

In the proposed assay method, the enzymatic and fluorogenic reactions should be performed at pHs 5.5 and 7.0, respectively, as described above. A sodium hydroxide solution was used only initially for the adjustment of the pH of the reaction mixture to 7.0 from 5.5 after the enzymatic reaction. In this case, the activity values that were obtained by individually repeated assays ($n = 10$)

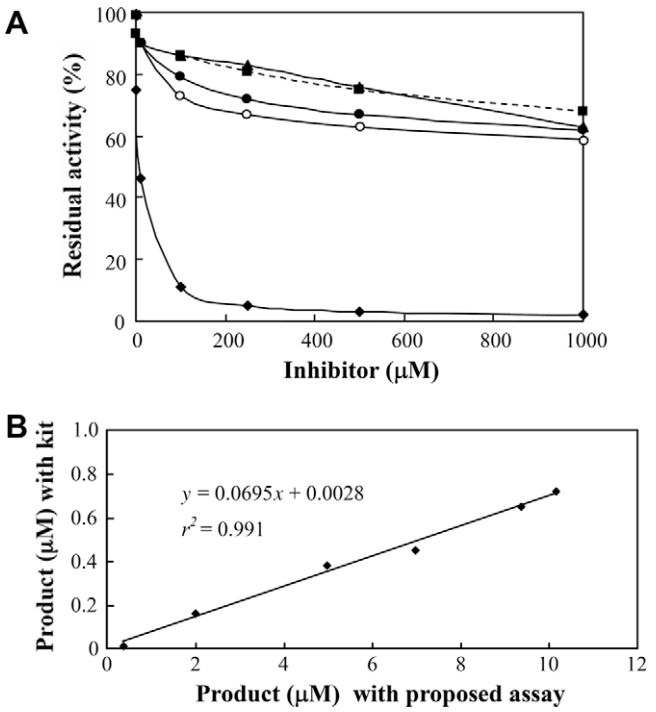


Fig. 4. Specificity and reliability of the proposed spectrofluorometric method. (A) The enzymatic reaction was performed in the presence of various protease inhibitors of pepstatin A (♦), AEBSF (■), E-64 (▲), bestatin (●), and aprotinin (○). (B) The HIV-PR activity in the crude proteins was measured with either the current spectrofluorometric method or a commercial FRET assay kit.

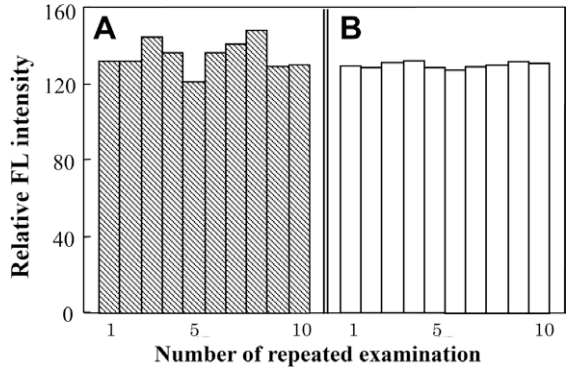


Fig. 5. Activities obtained by 10 repeated assays in the absence (A) or presence (B) of PBS used for the pH adjustment before the fluorogenic reaction. A portion of 20 μl of the HIV-PR solution was mixed with 74 μl of the enzyme reaction buffer (pH 5.5) and 6 μl of 2.5 mM Ac-RKILFLDG, and the mixture was incubated at 37 °C for 2 h. Then the pH of the reaction mixture was adjusted to approximately 7.0 with 5 μl of 0.1 M sodium hydroxide (A) or 20 μl of PBS (pH 7.0) and 5 μl of 0.1 M sodium hydroxide (B). The fluorogenic reaction of the product of FLDG and the fluorescent detection of the FLDG derivative were carried out as described in Materials and methods.

using all of the same reagents within a day indicated a larger error with a 7.35% relative standard deviation (RSD), as shown in Fig. 5A. However, the pH adjustment using the same NaOH solution in the presence of PBS (pH 7.0) gave satisfactory precision with a 1.82% RSD for the assays (Fig. 5B). Because mixing the strong alkaline solution and the acetate buffer (pH 5.5) resulted in varying pHs, it is better to use PBS (pH 7.0) for the pH adjustment. Therefore, PBS that has a buffering strength at pH 7.0 should be used for such pH adjustment.

Conclusions

A facile method for the assay of HIV-PR activity in a crude sample has been successfully developed by using a synthetic acetyl octapeptide as the substrate. This assay method is based on the selective fluorogenic reaction of a peptide fragment cleaved from the substrate with the reagents of catechol, borate, and sodium periodate. The conditions for the enzymatic and fluorogenic reactions were optimized, and thus HIV-PR activity in cell lysate could be readily assayed. This assay was applicable to the evaluation of several protease inhibitors for their effect on the activity of HIV-PR. This proposed spectrofluorometric method is reliable and inexpensive, and it does not require any special modified substrates. It is important to measure HIV-PR activity in the blood of AIDS patients. Unfortunately, no specimens of AIDS patients could be prepared in our laboratory due to the biohazard restriction. Therefore, the application of this method is considered as being able to discriminate HIV activity in the presence of other proteases.

Acknowledgments

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