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

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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 FLUOROSCIFIC 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 FLUOROSCIFIC 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.

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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 efficacy5–7.

Up to now, several different methods have been developed for the assay of HIV-PR activity. Billich and coworkers8 proposed a chromatographic assay with ultraviolet (UV) detection for HIV-PR activity using eight synthetic substrates, Stebbins and Deback9 reported a colorimetric assay using two nonenzymatic reactions in a microtiter plate for the high-throughput screening of HIV-PR inhibitors, and Fuse and coworkers10 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 assay11–13 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 fluorescence compound by a novel fluorogenic reaction14 using catechol, borate, and NaClO3. 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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**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)benzenesulfonfonyl]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-NN-dimethylaminobenzene-4′-sulfonic acid; RSD, relative standard deviation.
Materials and methods

Materials

Catechol, H$_2$BO$_3$, and NaIO$_4$ were purchased from Wako Pure Chemicals (Osaka, Japan). Catechol (2.5 mM), H$_2$BO$_3$ (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’s modified Eagle’s medium (DMEM) and fetal bovine serum (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 incubated at 37°C 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). Sense and antisense primers contain a restriction enzyme site (underlined) and a translational stop codon (italic bold). The PCR product was digested by 4-sulfonic acid (EDANS) in the substrate was quenched in advance. Free EDANS was used as a standard material to determine the product.

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 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$_3$BO$_3$–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 icer water bath to stabilize the fluorescent product. The fluorescence (FL) intensity of the product was measured in a 10 × 3-mm quartz cell with a spectrophotometer (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)aminophenyl]-sulfonic acid (EDANS) in the substrate was quenched in advance by 4-N,N-dimethylaminobenzene-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 manufacturer’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 spectrophotometer 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.
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, NaIO₄, 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 NaIO₄, 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 and slightly unstable at room temperature. Therefore, the reaction mixture was cooled in an iced water 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, r² = 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.

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.
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 IC50 of pepstatin A is $10 \times 10^{-6}$ M. Therefore, the proposed method is specific for the assay of HIV-PR activity.

**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 ($n = 10$)
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.

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References

[1] E. Liaudet-Coopman, M. Beaujouin, D. Derozo, M. Garcia, M. Gloudu-Lassie, V. Laurent-Matha, C. Prebois, H. Rochefort, F. Vignon, D. Cathepsin, Newly discovered functions of a long-standing aspartic protease in cancer and apoptosis, Cancer Lett. 237 (2006) 167–179.
[2] I. Ventosio, J. Navarro, M.A. Muñoz, L. Carrasco, Involvement of HIV-1 protease in virus-induced cell killing, Antiviral Res. 66 (2005) 47–55.
[3] P.H. Liang, Characterization and inhibition of SARS coronavirus main protease, Curr. Top. Med. Chem. 6 (2006) 361–376.
[4] D. Yu, Y. Sakurai, C.H. Chen, P.R. Chang, L. Huang, Y. Kashiwada, K.J. Lee, Anti-AIDS agents 69, moronic acid, and other triterpene derivatives as novel potent anti-HIV agents, J. Med. Chem. 49 (2006) 5462–5469.
[5] A.D. Frankel, J.A. Yong, HIV-1: fifteen proteins and an RNA, Annu. Rev. Biochem. 67 (1998) 1–25.
[6] D.D. Richman, HIV chemotheraphy, Nature 410 (2001) 995–1001.
[7] T. Ridky, J. Leis, Development of drug resistance to HIV-1 protease inhibitors, J. Biol. Chem. 270 (1995) 29621–29623.
[8] S. Billich, K. Marie-Theres, J. Hansen, P. Strop, J. Sedlacek, R. Mertz, K. Moelling, Synthetic peptides as substrates and inhibitors of human immune deficiency virus-1 protease, J. Biol. Chem. 263 (1988) 17905–17908.
[9] J. Stebbins, C. Debouck, A microtiter colorimetric assay for the HIV-1 protease 1, Anal. Biochem. 248 (1997) 246–250.
[10] T. Fuse, K. Watanabe, K. Kitazato, N. Kobayashi, Establishment of a new cell line inducibly expressing HIV-1 protease for performing safe and highly sensitive screening of HIV protease inhibitors, Microbes Infect. 8 (2006) 1783–1789.
[11] S. Fournout, F. Roquet, S.L. Salhi, R. Seyer, V. Valverde, J.M. Masson, P. Jouin, B. Pau, M. Nicolas, V. Hanin, Development and standardization of an immunoquantified solid phase assay for HIV-1 aspartyl protease activity and its application to the evaluation of inhibitors, Anal. Chem. 69 (1997) 1746–1752.
[12] O.A. Gutiérrez, E. Salas, Y. Hernández, E.A. Lissi, G. Castrillo, O. Reyes, H. Garay, A. Aguilar, B. García, A. Otero, M.A. Chavez, C.A. Duarte, An immunoenzymatic solid-phase assay for quantitative determination of HIV-1 protease activity, Anal. Biochem. 307 (2002) 18–24.
[13] P. Bagossi, J. Kádas, G. Miklóssy, P. Boross, I.T. Weber, J. Tózsvér, Development of a microtiter plate fluorescent assay for inhibition studies on the HIV-1 and HIV-2 proteases, J. Virol. Methods 119 (2004) 87–93.
[14] T. Kabashima, Z.Q. Yu, C.H. Tang, Y. Nakagawa, K. Okumura, T. Shibata, J.Z. Lu, M. Kai, A selective fluorescence reaction for maturation of the HTLV-1 and HIV proteases, J. Biol. Chem. 270 (1995) 29621–29623.
[15] S. Billich, K. Marie-Theres, J. Hansen, P. Strop, J. Sedlacek, R. Mertz, K. Moelling, Synthetic peptides as substrates and inhibitors of human immune deficiency virus-1 protease, J. Biol. Chem. 263 (1988) 17905–17908.
[16] J. Stebbins, C. Debouck, A microtiter colorimetric assay for the HIV-1 protease 1, Anal. Biochem. 248 (1997) 246–250.
[17] T. Fuse, K. Watanabe, K. Kitazato, N. Kobayashi, Establishment of a new cell line inducibly expressing HIV-1 protease for performing safe and highly sensitive screening of HIV protease inhibitors, Microbes Infect. 8 (2006) 1783–1789.
[18] S. Fournout, F. Roquet, S.L. Salhi, R. Seyer, V. Valverde, J.M. Masson, P. Jouin, B. Pau, M. Nicolas, V. Hanin, Development and standardization of an immunoquantified solid phase assay for HIV-1 aspartyl protease activity and its application to the evaluation of inhibitors, Anal. Chem. 69 (1997) 1746–1752.
[19] O.A. Gutiérrez, E. Salas, Y. Hernández, E.A. Lissi, G. Castrillo, O. Reyes, H. Garay, A. Aguilar, B. García, A. Otero, M.A. Chavez, C.A. Duarte, An immunoenzymatic solid-phase assay for quantitative determination of HIV-1 protease activity, Anal. Biochem. 307 (2002) 18–24.
[20] P. Bagossi, J. Kádas, G. Miklóssy, P. Boross, I.T. Weber, J. Tózsvér, Development of a microtiter plate fluorescent assay for inhibition studies on the HIV-1 and HIV-2 proteases, J. Virol. Methods 119 (2004) 87–93.
[21] T. Kabashima, Z.Q. Yu, C.H. Tang, Y. Nakagawa, K. Okumura, T. Shibata, J.Z. Lu, M. Kai, A selective fluorescence reaction for maturation of the HTLV-1 and HIV proteases, J. Biol. Chem. 270 (1995) 29621–29623.
[22] S. Billich, K. Marie-Theres, J. Hansen, P. Strop, J. Sedlacek, R. Mertz, K. Moelling, Synthetic peptides as substrates and inhibitors of human immune deficiency virus-1 protease, J. Biol. Chem. 263 (1988) 17905–17908.
[23] J. Stebbins, C. Debouck, A microtiter colorimetric assay for the HIV-1 protease 1, Anal. Biochem. 248 (1997) 246–250.
[24] T. Fuse, K. Watanabe, K. Kitazato, N. Kobayashi, Establishment of a new cell line inducibly expressing HIV-1 protease for performing safe and highly sensitive screening of HIV protease inhibitors, Microbes Infect. 8 (2006) 1783–1789.
[25] S. Fournout, F. Roquet, S.L. Salhi, R. Seyer, V. Valverde, J.M. Masson, P. Jouin, B. Pau, M. Nicolas, V. Hanin, Development and standardization of an immunoquantified solid phase assay for HIV-1 aspartyl protease activity and its application to the evaluation of inhibitors, Anal. Chem. 69 (1997) 1746–1752.
[26] O.A. Gutiérrez, E. Salas, Y. Hernández, E.A. Lissi, G. Castrillo, O. Reyes, H. Garay, A. Aguilar, B. García, A. Otero, M.A. Chavez, C.A. Duarte, An immunoenzymatic solid-phase assay for quantitative determination of HIV-1 protease activity, Anal. Biochem. 307 (2002) 18–24.
[27] P. Bagossi, J. Kádas, G. Miklóssy, P. Boross, I.T. Weber, J. Tózsvér, Development of a microtiter plate fluorescent assay for inhibition studies on the HIV-1 and HIV-2 proteases, J. Virol. Methods 119 (2004) 87–93.
[28] T. Kabashima, Z.Q. Yu, C.H. Tang, Y. Nakagawa, K. Okumura, T. Shibata, J.Z. Lu, M. Kai, A selective fluorescence reaction for maturation of the HTLV-1 and HIV proteases, J. Biol. Chem. 270 (1995) 29621–29623.
[29] S. Billich, K. Marie-Theres, J. Hansen, P. Strop, J. Sedlacek, R. Mertz, K. Moelling, Synthetic peptides as substrates and inhibitors of human immune deficiency virus-1 protease, J. Biol. Chem. 263 (1988) 17905–17908.
[30] J. Stebbins, C. Debouck, A microtiter colorimetric assay for the HIV-1 protease 1, Anal. Biochem. 248 (1997) 246–250.