Sensitive, hydrosoluble, macromolecular fluorogenic substrates for human immunodeficiency virus 1 proteinase

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Hydrosoluble macromolecular fluorogenic substrates specific for the human immunodeficiency virus 1 (HIV-1) proteinase have been prepared. The fluoresceinyl peptide Ftc-ε-Ahx-Ser-Phe-Asn-Phe-Pro-Gln-Ile-Thr-$(Gly)_n$, corresponding to the first cleavage site of HIV-1 *gag–pol* native precursor was linked to a water-soluble neutral $(Lys)_n$ derivative. The ε-aminohexanoyl residue (ε-Ahx) and the glycyl sequence were added in order to improve the stability of the substrate and the accessibility of the cleavage site to the HIV-1 proteinase respectively. This macromolecular peptidic-substrate conjugate is significantly more water-soluble than the free peptide itself on a substrate molar concentration basis. The assay is based on the quantitative precipitation of the polymeric material by adding propan-2-ol whereas the fluorescent peptide moiety released upon proteolysis remains soluble in the supernatant. The proteinase activity is assessed by measuring the fluorescence of the supernatant. This assay allows the detection of a few fmol of HIV-1 proteinase, even in the presence of cell culture media, plasma or cell lysate and it gives accurate results within a large proteinase concentration range. The hydrosoluble macromolecular substrate is also suitable for determining the HIV-1 proteinase activity using 96-well microplates, allowing us to test accurately and rapidly numerous enzyme samples and/or the potency of new proteinase inhibitors.

INTRODUCTION

Numerous assays suitable to quantify the activity of endoproteinases have been described. Various sensitive fluorogenic amide substrates such as peptidyl 7-amino-4-methylcoumarin (AMC) [1], or peptidyl 3-amino-9-ethylcarbazole (AEC) [2] are commonly used to monitor the activity of proteinases. These acylated aromatic amides have a low fluorescence quantum yield while the corresponding free aromatic amine, released upon proteolysis, has a relatively high fluorescence quantum yield. Unfortunately, this type of method cannot be used for assaying retroviral endoproteinases such as human immunodeficiency virus (HIV) proteinase as well as other proteinases such as collagenases or cathepsin D. Indeed, these enzymes hydrolyse a peptide linkage between two specific amino-acid residues. Among these endoproteinases, HIV-1 proteinase is a highly specific enzyme responsible for the proteolytic processing of the polyproteins which represent the primary translation products of the *gag* and *pol* genes [3], and its activity is an essential step in the maturation of infectious viral particles [4]. HIV-1 proteinase, a member of the aspartic proteinase family, is therefore a potential target for the development of inhibitors for anti-HIV therapy. Recently, various assays have been developed, in order to characterize the HIV-1 proteinase properties and to test putative inhibitory compounds. Hirel et al. [5] developed a two-step assay using a Suc-peptidyl-AMC substrate: the HIV-1 proteinase cleaves the fluorogenic substrate into two pieces, then the released peptidyl-AMC moiety is exhaustively hydrolysed by aminopeptidase M, leading to the release of free AMC, the fluorescence of which is finally detected. Other methods make use of radiolabelled [6], chromogenic [7–9] or fluorescent substrates analysed by h.p.l.c. [10] as well as intramolecular quenched fluorogenic substrates [11,12].

Recently, we described another fluorogenic method based on the use of a macromolecular substrate in which several *N*-fluoresceinylated peptidic substrates, specific for a given proteinase, were grafted by their C-terminal group on to a $(Lys)_n$ derivative. On such a polymer, ε-amino groups of lysine side-chains were partly substituted by peptidic substrates, while the other amino groups of $(Lys)_n$ were neutralized by N-acylation with gluconolactone, leading to formation of neutral, highly water-soluble residues [13]. Upon proteolysis, the polymeric material is quantitatively precipitated by adding propan-2-ol, whereas the fluorescent product released remains soluble in the supernatant and its concentration is determined with a spectrofluorimeter [14]. This approach can be used with any endoproteinases under the condition that the peptidic moieties linked to the macromolecular carrier have a sequence recognized as a specific substrate by the endoproteinase to be assayed. In this paper, the HIV-1 proteinase specific sequence Ser-Phe-Asn-Phe-Pro-Gln-Ile-Thr, corresponding to the first cleavage site of the *gag–pol* native precursor [15], has been selected, and the

Abbreviations used: AMC, 7-amino-4-methylcoumarin; AEC, 3-amino-9-ethylcarbazole; HIV-1, human immunodeficiency virus 1; Suc-, succinyl; Ftc-, fluoresceinylthiocarbamyl; ε-Ahx, ε-aminohexanoyl; **S**-, the peptidic sequence ε-Ahx-Ser-Phe-Asn-Phe-Pro-Gln-Ile-Thr; **P**-, the peptidic sequence ε-Ahx-Ser-Phe-Asn-Phe; 1-FITC, fluoresceinylisothiocyanate isomer 1; HOBt, *N*-hydroxybenzotriazole; DIEA, diisopropylethylamine; BOP, reagent, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; TBTU, 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; PITC, phenylisothiocyanate; DMF, dimethylformamide; GlcA, gluconoyl; Sta, statyl.

Unless otherwise stated, all amino acids and their derivatives are of the L-configuration. The nomenclature system of Schechter and Berger [23] *i.e.* P_2-P_1*P_1'-P_2' is used to depict amino acid residues adjacent to the residues in the P_1 and P_1' positions which constitute the scissile peptide bond (indicated by an asterisk).

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synthesis and the properties of such a polymeric fluorogenic substrate are reported.

MATERIALS AND METHODS

Compounds

Recombinant HIV-1 proteinase from *Escherichia coli* was provided by Dr. Ph-H. Hirel (Rhône–Poulenc–Rorer, Centre de Recherche de Vitry-Alfortville, France). The specific activity of this preparation, before dilution, was 0.41 μmol/min per ml ($k_{cat.} = 0.15\ s^{-1}$) as assessed by the method using Suc-Thr-Leu-Asn-Phe-Pro-Ile-Ser-AMC as substrate [5]. The concentrated aspartic proteinase (1 mg/ml) was stored at −80 °C in 0.5 mM EDTA/0.1 M NaCl/0.05 M sodium phosphate buffer, pH 8.0, containing 10% (w/v) $(NH_4)_2SO_4$ and 50% (v/v) glycerol. Fluoresceinylisothiocyanate isomer I (I-FITC) was purchased from Molecular Probes (Junction City, OR, U.S.A.); $(Lys)_n \cdot HBr$ (M_r: 40000) and pepstatin A were from Bachem (Feinchemikalien, Bubendorf, Switzerland); substituted $(Lys)_n$ derivatives [$(GlcA)_{149}$-,$(Gly\text{-}Gly)_{42}$-$(Lys)_n$] were prepared as previously described [13]; *t*-butyloxycarbonyl amino acids, *t*-butyloxycarbonyl statine and *N*-hydroxybenzotriazole (HOBt) were from Novabiochem (Laufelfingen, Switzerland); Boc-Gly-phenylacetamidomethyl (PAM) resin was from Neosystem (Strasbourg, France), diisopropylethylamine (DIEA) was from Aldrich (Strasbourg, France); benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate (BOP reagent) and 2-(1-*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were from Richelieu Biotechnologies (Saint-Hyacinthe, QC, Canada); δ-gluconolactone was from Serva (Heidelberg, Germany); $ZnCl_2$ was from Merck (Darmstadt, Germany); RPMI 1640 and AIM V media (research grade) were from Gibco Ltd. (Paisley, Scotland, U.K.); fetal bovine serum was from Serovial (Vogelgrun, France). Amino-acid analyses were performed with a Waters 991 h.p.l.c. amino-acid-analysis system using the phenylisothiocyanate (PITC) post-derivatization method.

Cellular lysate

Whole blood was drawn from healthy donors (Centre de transfusion sanguine, Orléans, France) on citrate as anticoagulant. After centrifugation (300 ***g***, for 15 min, to remove the platelet-rich plasma), mononuclear leucocytes were isolated using centrifugation on Ficoll Paque (Pharmacia, France). Cells (5×10^6/ml) in RPMI supplemented with 5% (v/v) decomplemented fetal bovine serum were further plated on Petri dishes precoated with fetal bovine serum. After incubation for 60 min at 37 °C in a humidified atmosphere (5% CO_2, 95% air) non-adherent cells were removed by three vigorous washes and adherent cells (1×10^9 cells) were harvested by scraping with a rubber policeman, washed twice with a phosphate-buffered saline solution (PBS). Cells were resuspended in 10 mM Tris/HCl, pH 7.4, containing 25 mM KCl, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ and ruptured with a Potter–Elvehjem homogenizer.

Peptide synthesis

Peptides were prepared by solid-phase synthesis using a PAM resin and Boc-amino acids according to Merrifield [16], on a Vega 250 coupler (DuPont Company, Wilmington, DE, U.S.A.) and cleaved from the resin by anhydrous liquid HF at 0 °C. All peptides were purified to homogeneity by h.p.l.c. on a reversed-phase C_{18} column (Vydac, Hesperia, CA, U.S.A.) and characterized by amino-acid analysis and ^{252}Cf-plasma desorption m.s.

Preparation of fluoresceinylthiocarbamyl (Ftc) peptidic substrates

To 100 mg (74 μmol) of crude peptide ε-Ahx-Ser-Phe-Asn-Phe-Pro-Gln-Ile-Thr-Gly-Gly-Gly-Gly-Gly ($SGly_5$) dissolved in 2 ml of dimethylformamide, 26 μl (148 μmol) of DIEA, and 43 mg (111 μmol) of FITC were added. After 30 min stirring, the starting peptide was quantitatively converted into Ftc-$SGly_5$ ($R_F = 0.25$) as shown upon t.l.c. analysis in a solvent A (butanol/acetic acid/H_2O; 4/1/1, by vol.). The fluoresceinyl-peptide was precipitated in cold ethyl ether and purified by semi-preparative h.p.l.c. on a reverse-phase C_{18} column eluted with CH_3CN/H_2O (74/26, v/v) containing 13 mM ammonium acetate (yield = 30%).

Ftc-SGly and Ftc-ε-Ahx-Ser-Phe-Asn-Phe (Ftc-**P**) were prepared with the same procedure. Reactions were monitored by t.l.c. in the same solvent A [R_F (Ftc-SGly) = 0.46; R_F (Ftc-**P**) = 0.67].

Preparation of $(Lys)_n$ conjugates

Preparation of (Ftc-$\mathbf{S}Gly_3)_6$-$(Lys)_n$ conjugate

Samples (18 mg; 0.28 μmol) of substituted $(Lys)_n$ derivative, of formula $(GlcA)_{149}$-,$(Gly\text{-}Gly)_{42}$-$(Lys)_n$, were dissolved in 500 μl of DMF; 8 mg (5.3 μmol) of Ftc-SGly, 3.5 mg (7.95 μmol) of BOP reagent, and 2.76 μl (15.9 μmol) of DIEA were added to the polymer solution. After stirring for 1 h the polymer conjugate was quantitatively precipitated in nine volumes of propan-2-ol, washed twice with propan-2-ol and dried. The macromolecular substrate obtained contains six fluorescent peptidic substrates as determined by amino-acid analysis (coupling yield: 32%). The 36 remaining free α-amino groups (unsubstituted glycyl residues) were acetylated by acetic anhydride in order to obtain a neutral conjugate as previously described [14]. The formula of this macromolecular substrate is $GlcA_{149}$,(Ftc-S-Gly-Gly-Gly)$_6$-,(Ac-Gly-Gly$(Lys)_n$ and will be further abbreviated in the text as (Ftc-$SGly_3)_6$-$(Lys)_n$.

Preparation of (Ftc-$\mathbf{S}Gly_5)_7$-$(Lys)_n$ conjugate

The peptide containing five glycyl residues was directly coupled to the ε-amino groups of $(Lys)_n$. To 13 mg (7.5 μmol) of Ftc-$SGly_5$ dissolved in 450 μl of DMF, 2.6 μl (150 μmol) of DIEA and 3.6 mg (11.5 μmol) of TBTU were added; the fluoresceinylated peptide active ester, as ascertained by t.l.c. analysis, was added dropwise to a solution of 30 mg (0.75 μmol) of $(Lys)_n \cdot HBr$ in 250 μl of H_2O/DMF (4/1, v/v) containing 1.3 μl of DIEA (7.5 μmol), under stirring. After 1 h, the polymer conjugate was quantitatively precipitated by adding nine volumes of propan-2-ol and washed twice with propan-2-ol. The precipitate (0.75 μmol) was immediately solubilized in 600 μl of DMF/H_2O (5/1; v/v) then 74 μl (427 μmol) of DIEA and 76 mg (427 μmol) of δ-gluconolactone were added in order to acylate the remaining free ε-amino groups of the $(Lys)_n$. After 48 h of stirring at room temperature, t.l.c. analysis in the system solvent B (chloroform/methanol/H_2O; 6/6/1, by vol.) showed a complete substitution of $(Lys)_n$ ε-amino groups by *N*-gluconoyl groups. The gluconoylated polymer was precipitated in nine volumes of propan-2-ol, washed twice with propan-2-ol and freeze-dried (42 mg, yield: 86%). From amino-acid analysis, it was established that seven peptidic substrates were coupled per macromolecule of $(Lys)_n$ (coupling yield: 70%). The formula of

this macromolecular substrate is $(GlcA)_{184}$-,(Ftc-S-Gly-Gly-Gly-Gly-Gly)$_7$-$(Lys)_n$ and will be abbreviated in the text by (Ftc-$SGly_5)_7$-$(Lys)_n$.

Proteinase assay

Basic assay

The polymeric substrate at a concentration of from 0.05 to 1.5 mg per assay, corresponding to a final concentration of 0.1–3 mM peptidic substrate, was incubated with 0.025–2.0 μg of HIV-1 proteinase (23 nM–1.8 μM final concentration) in a 50 μl reaction volume of 100 mM sodium acetate buffer, pH 5.0, containing 14.7 μM BSA. After incubation for 1 h at 37 °C, the reaction was stopped by adding 5 μl of 1 M NaOH. Then, the samples containing an initial substrate concentration higher than 0.25 mM were adjusted to a final peptidic substrate concentration of 0.25 mM (equivalent to a 36 μM polymer concentration), and nine volumes of propan-2-ol were added in order to quantitatively precipitate the polymeric material, whereas the released fluorescent peptide remained soluble. The mixture was quickly vortex-mixed, then centrifuged for 1 min in a microfuge (10000 *g*), and finally the organic supernatant fraction was diluted 2-fold, with 0.1 M NaOH. After transfer of the solution into a 96-well black fluoroplate (Labsystem, Helsinki, Finland), fluorescence was recorded on a Titertek fluoroscan II microplate fluorimeter (Labsystem, Helsinki, Finland) against a blank lacking HIV-1 proteinase. Fluorescein fluorescence was measured at 535 nm upon excitation at 485 nm. For each concentration of polymeric substrate, an assay without enzyme was performed in order to take into account the non-specific fluorescence. The relative fluorescence was converted into fluoresceinyl peptide concentrations (mM) using a serial dilution of known concentrations of Ftc-**P**, the fluoresceinylated proteinase released moiety.

Microplate assay

For the determination of IC_{50} values of inhibitors, a microplate assay was developed: 10 μl of 0.2 mM peptidic substrate (equivalent to a 28 μM polymer concentration) and 5 μl of variable concentrations of inhibitor were incubated for 1 h at 37 °C with 5 μl of proteinase (equivalent to 40 ng of enzyme) in a 20 μl total volume of 100 mM sodium acetate buffer, pH 5.0, containing 14.7 μM BSA. Alternatively, for comparative purposes with other works [8,11], an assay with the above buffer supplemented with 1 M NaCl was conducted. The reaction was stopped by precipitating the polymeric material in 200 μl of 12.5 mM NaOH in propan-2-ol. After mixing, the samples were centrifuged for 10 min at 230 *g*, and 100 μl of supernatant was diluted 2-fold with 0.1 M NaOH in a 96-well white fluoroplate (Labsystem, Helsinki, Finland) and fluorescence was measured as described above.

RESULTS AND DISCUSSION

The new fluorescent assay for endoproteinases which is described here is based on the quantitative precipitation of a macromolecular carrier bearing several *N*-fluoresceinyl peptide moieties, the sequence of which is specific for a given enzyme. The validity of this new method was previously checked using pig pancreatic elastase [14]. Here hydrosoluble macromolecular fluorogenic substrates are used to assay the HIV-1 proteinase.

The peptidic sequence Ser-Phe-Asn-Phe-Pro-Gln-Ile-Thr which corresponds to a known cleavage site in the HIV-1 *gag–pol* precursor, one of the most efficient substrates of the HIV-1 proteinase [15], was selected and substituted at both N- and C-terminal ends, leading to specific fluorescent peptidic substrates: Ftc-SGly and Ftc-$SGly_5$. As the hydrolysis buffer, in the case of HIV-1 proteinase, is mildly acidic (pH 5.0), the ϵ-aminohexanoyl group (ϵ-Ahx) between the fluorophore and the peptidic sequence was added in order to prevent the release of the fluoresceinyl-thiocarbamyl moiety as fluoresceinylthiohydantoin by Edman reaction. Furthermore, the ϵ-Ahx residue works as a spacer arm between the peptidic substrate and fluorescein, avoiding a steric hindrance in the interaction of the fluoresceinylated substrate with the catalytic site of the HIV-1 proteinase. A first macromolecular substrate was synthesized, in which the Ftc-SGly peptidic sequence was grafted on to the α-amino groups of glycylglycyl moieties linked to the $(Lys)_n$ derivative, prepared as described previously [13,14]. The macromolecular substrate (Ftc-$SGly_3)_6$-$(Lys)_n$ prepared that way contained six fluoresceinylated substrates linked to $(Lys)_n$ through triglycyl bridges used as spacer arms. The structure of the macromolecular substrate (Ftc-$SGly_3)_6$-$(Lys)_n$ is shown in Figure 1. The coupling yield of the peptide on to the polymer being low (32%), another approach leading to a macromolecular substrate with a pentaglycyl spacer arm was developed. The Ftc-$SGly_5$ peptide was directly grafted on to the ϵ-amino groups of the lysine side-chains of $(Lys)_n$. This second method allowed preparation of the macromolecular substrate (Ftc-$SGly_5)_7$-$(Lys)_n$ with a better coupling yield (70%).

The kinetics of the cleavage reaction by HIV-1 proteinase of (Ftc-$SGly_5)_7$-$(Lys)_n$ were compared with those of the micromolecular substrate Ftc-SGly determined by h.p.l.c. (Figure 2). The substrate hydrolysis of the macromolecular substrate is linearly related to the enzyme concentration in a much larger range than in the case of the micromolecular substrate. This new assay is therefore suitable for determining an enzyme concentration from a few nM to 0.2 μM.

Kinetic constants of HIV-1 proteinase were determined with (Ftc-$SGly_3)_6$-$(Lys)_n$ and (Ftc-$SGly_5)_7$-$(Lys)_n$: incubations were carried out for 1 h at 37 °C with 100 ng of enzyme and different concentrations of the fluorescent macromolecular substrate. As shown in Table 1, (Ftc-$SGly_5)_7$-$(Lys)_n$ ($k_{cat.}/K_m = 1100$) is a

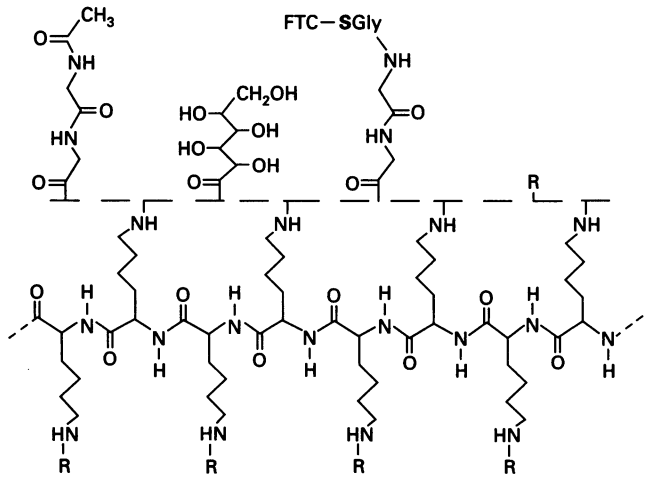

Figure 1 Schematic representation of a macromolecular fluorogenic substrate of HIV-1 proteinase constituted of gluconoylated $(Lys)_n$ (190 lysine residues) substituted with glycylglycyl spacer arms on which were grafted a fluorescent decapeptide (Ftc-SGly) and acetyl residues (Ac-)

Each substituent (R: GlcA, Ac-GlyGly or Ftc-SGlyGlyGly) was randomly linked to the ϵ-amino groups of the $(Lys)_n$ side-chains.

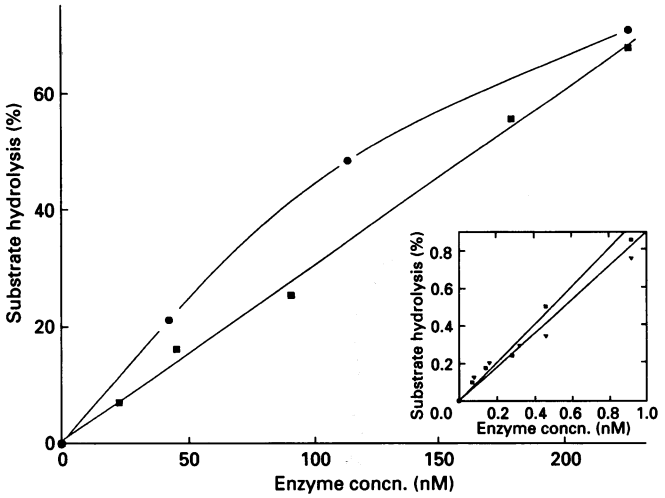

Figure 2 Hydrolysis of Ftc-SGly (●) and (Ftc-SGly$_5$)$_7$-(Lys)$_n$ (■) by HIV-1 proteinase

Peptidic substrate (100 μM) was incubated for 1 h at 37 °C with increasing amounts of proteinase in 50 μl of 14.7 μM BSA, 100 mM sodium acetate buffer, pH 5.0. Inset: hydrolysis of 100 μM (Ftc-SGly$_5$)$_7$-(Lys)$_n$ related to HIV-1 proteinase concentrations from 0.08 to 0.92 nM in 14.7 μM BSA, 100 mM sodium acetate buffer, pH 5.0, either in a 50 μl reaction volume in the basic assay (■), or in a 20 μl reaction volume in the microplate assay (▼).

Table 1 HIV-1 proteinase activity on Suc-Thr-Leu-Asn-Phe-Pro-Ile-Ser-AMC, (Ftc-SGly$_3$)$_6$-(Lys)$_n$ and (Ftc-SGly$_5$)$_7$-(Lys)$_n$

These three experiments were conducted using the same pool of HIV-1 proteinase in 100 mM sodium acetate buffer, pH 5.0, containing 14.7 μM BSA. Results are mean values of experiments in triplicate.

Substrate	K_m (mM)	$k_{cat.}/K_m$ (10^3 l·mol·s^{-1})
Suc-Thr-Leu-Asn-Phe-Pro-Ile-Ser-AMC	2.0	70
(Ftc-SGly$_3$)$_6$-(Lys)$_n$	1.7	600
(Ftc-SGly$_5$)$_7$-(Lys)$_n$	1.2	1100

Table 2 Assay in complex media

Proteinase activity was determined at 37 °C in 14.7 μM BSA/100 mM sodium acetate buffer, pH 5.0, in the absence (check buffer) or presence of different complex media (+), in a 50 μl reaction volume containing 100 μM of peptidic substrate (equivalent to a concentration of 14 μM macromolecular substrate) and 91 nM of HIV-1 proteinase. The proteolytic activity in each medium, expressed as a percentage (%), was calculated relatively to that of HIV-1 proteinase activity in the check buffer, taken as 100%.

Medium	Activity (%)
Check buffer	100
+5% (v/v) Fetal calf serum	96±2
+25% (v/v) Cellular lysate	98±2
+50% (v/v) RPMI 1640	98±2
+50% (v/v) AIM	92±6

slightly better substrate than (Ftc-SGly$_3$)$_6$-(Lys)$_n$ ($k_{cat.}/K_m$ = 600); these two macromolecular substrates are respectively 16-fold and 8-fold better substrates than another fluorogenic substrate Suc-Thr-Leu-Asn-Phe-Pro-Ile-Ser-AMC ($k_{cat.}/K_m$ = 70) [5], the same pool of HIV-1 proteinase being used in these three experiments.

The value obtained for K_m is in the millimolar range, close to that reported by Moore et al. [17] using the synthetic peptide substrate Ac-Ser-Gln-Asn-Tyr-Pro-Val-Val-NH$_2$ specific for HIV-1 proteinase. In a high-ionic-strength buffer, Matayoshi et al. [11], using a fluorogenic substrate (Dabcyl)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-(Edans), and Richards et al. [8], using a chromogenic substrate Lys-Ala-Arg-Val-Leu-NphAla-Glu-Met, found K_m values of 100 μM and 22 μM repectively; using similar conditions, we obtained a better K_m value: 150 μM using the fluorogenic macromolecular substrate (Ftc-SGly$_5$)$_7$-(Lys)$_n$.

The macromolecular fluorogenic substrates are readily water-soluble at 3 mM and they are at least 5-fold more water-soluble than the fluoresceinylated peptidic substrate Ftc-SGly which is water-soluble at concentrations lower than 0.6 mM. In contrast, intramolecularly quenched fluorescence peptidic substrates are quite hydrophobic and scarcely water-soluble, and in addition their fluorescence is not fully quenched by the chromophore moiety, 60% for the fluorescent substrate described by Geoghegan et al. [12]. On the contrary, the precipitation of the macromolecular fluorescent substrate was quantitative and the method allowed detection of HIV-1 proteinase at a concentration as low as 0.1 nM (Figure 2) i.e. 5 or 2 fmol in the basic or the microplate assays respectively. In the case of the macromolecular fluorogenic substrates described here the specific peptide used is the sequence of the natural cleavage site, whereas in chromogenic substrates the P_1' residue was replaced by *p*-nitrophenylalanine [8]; the nitro function may modify the substrate–proteinase interaction.

The specific proteolysis of macromolecular substrates by HIV-1 proteinase is almost independent of the medium components. For instance, the assay works with acetate buffer containing 5% (v/v) fetal bovine serum, or 25% (v/v) cellular lysate, or 50% (v/v) culture media (RPMI 1640, or AIM) as shown in Table 2.

We have adapted this assay to a microplate assay, the hydrolysis being conducted in a 20 μl reaction volume. Upon addition of propan-2-ol, centrifugation of microplates at 230 ***g*** allowed a quantitative sedimentation of the macromolecular material and an accurate and fast determination of the fluoresceinyl peptide released by using an automatic fluorescence reader machine. The hydrolytic release of the fluorescent peptide from the macromolecular fluorogenic substrate (Ftc-SGly$_5$)$_7$-(Lys)$_n$ by HIV-1 proteinase in the microplate assay is similar to that obtained by using the basic assay (Figure 2). This microplate assay allows a quick determination of the IC_{50} values of inhibitors. The following inhibitors were tested: $ZnCl_2$, pepstatin A, a potent inhibitor of all aspartic proteinases which has been reported to be an inhibitor of HIV-1 proteinase [18], and a pseudopeptidic inhibitor Ac-Ser-Phe-Asn-Sta-Gln-Ile-Thr-Gly-OH. The IC_{50} values of pepstatin A and of the pseudopeptidic inhibitor are 0.8 μM and 2.5 μM respectively, while $ZnCl_2$ has an IC_{50} of 900 μM. In the case of pepstatin A and of the pseudopeptidic inhibitor, both of them being competitive inhibitors, the K_i values determined according to Cha [19] are 0.65 and 2.3 μM respectively; Tomasselli et al. [20], with pepstatin A and another pseudopeptidic inhibitor H-Val-Ser-Gln-Asn-Sta-Ile-Val-OH, obtained similar K_i values of 0.36 and 3.6 μM respectively. This microplate assay, which uses low amounts of substrate, of enzyme and of inhibitor, is well suited to the screening of a large series of putative inhibitors.

Furthermore, upon precipitation of the macromolecular material by addition of propan-2-ol, the released fluoresceinylated peptide moiety, which is soluble in the propan-2-ol/water mixture, can be easily purified from the supernatant, allowing a subsequent easy determination of the site of hydrolysis.

In summary, this new fluorescent proteinase assay is suitable for assaying endoproteinases such as the HIV-1 proteinase for a number of reasons. (i) The method is reproducible, rapid and sensitive: fluorescein being characterized by a very high specific absorbance ($\epsilon = 85200\ M^{-1} \cdot cm^{-1}$ [21]), and by a high quantum yield of 0.92 in 0.1 M NaOH [22]. (ii) Neutral macromolecular substrates, with their numerous *N*-gluconoyl residues, improve the water solubility of hydrophobic peptidic substrates, an important point in order to collect accurate data. (iii) This type of substrate takes into account the nature of P_1'-$P_2' \ldots P_n'$ amino acids which follow the proteolytic site, allowing us to obtain the most specific substrate of a given endoproteinase. (iv) This new sensitive assay may allow quick testing of the potency of inhibitors to block HIV-1 proteinase. (v) This new assay may also be helpful in characterizing new endoproteinases.

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