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Synthesis and characterization of fluorogenic peptide substrate of HIV-1 protease based on fluorescence resonance energy transfer

Synthesis of fluorogenic peptide substrate of HIV-1 protease Dns-SQNYPIVWL which corresponds to the p17/p24 cleavage site for HIV-1 protease have been performed. This fluorogenic substrate was based on the fluorescence resonance energy transfer between donor — Trp residue, and acceptor — dansyl group in the intact peptide. Hydrolysis of substrate by recombinant HIV-1 protease resulted in the time-dependent increase of Trp fluorescence and decrease of dansyl fluorescence measured at 350 and 500 nm, respectively, due to the break of resonance energy transfer between donor and acceptor fluorophors. Hydrolysis of fluorogenic peptide substrate was studied also by reversed phase HPLC and two peptide fragments after cleavage of substrate have been detected. Kinetic constants of hydrolysis for this fluorogenic peptide substrate by HIV-1 protease were calculated from Lineweaver — Burk plots: $K_M = 29\ \mu M$, $k_{cat} = 5.4\ s^{-1}$ and $k_{cat}/K_M = 180\ 000\ M^{-1}s^{-1}$.

Introduction. Human immunodeficiency virus protease (HIV-1 protease) performs the proteolytic processing of viral *gag* and *gag-pol* polyproteins precursors during the replication cycle of retrovirus [1, 2]. It has been shown using site directed mutagenesis and also by use of specific inhibitors of HIV-1 protease that disruption of this function produces morphologically immature and noninfectious viral particles [3]. Thus, HIV-1 protease represents an important therapeutic target in the development of treatment for AIDS [4]. The number of specific inhibitors of HIV-1 protease have been synthesized and studied and results are summarized in reviews [5, 6]. The antivirus synergy between HIV-1 protease inhibitors and nucleoside analogue inhibitors of HIV reverse transcriptase (AZT, ddC) has been also revealed [7].

Evaluation of efficiency of novel HIV-1 protease inhibitors requires the rapid and sensitive method of measurement of protease activity. Recently chromophoric peptide substrate for the spectrophotometric assay of HIV-1 protease have been reported [8], which exhibits an absorbance increase upon its hydrolysis by protease. Fluorogenic peptide substrates of HIV-1 protease [9—11] are most efficient and sensitive in the monitoring of specific hydrolysis reaction.

In this work we report the synthesis and characterization of fluorogenic peptide substrate of HIV-1 protease based on fluorescence resonance energy transfer (FRET). Resonance energy transfer between donor and acceptor chromophores is widely used as a tool for the measurement of intramolecular distances in biopolymers [12] and also to monitor the enzyme activity [9—11, 13]. Our substrate was based on the FRET between two fluorescent probes, tryptophan and dansyl group, introduced into the oligopeptide substrate, which corresponds to the *p17/p24* cleavage site for HIV-1 protease.

Materials and Methods. *Purification of HIV-1 protease.* The expression system in *Escherichia coli* strain AR58 was as previously described by Meek et al. [14]. Cells (300 *g*) were thawed in 1500 ml of 50 mM Tris buffer containing

5 mM EDTA, 10 mM DTT and 1 mM PMSF, pH 8.0 and lysed with one pass through a Rainie cell disrupter at 6000 psi. Following centrifugation of the lysate at 30.000 × *g* for 60 min, the supernatant was drawn off and solid ammonium sulfate was added slowly to 35 % final concentration. After stirring for 60 min at 4 °C, the mixture was allowed to settle for 30 min prior to centrifugation at 30.000 × *g* for 60 min. The pellet was resuspended in a minimal volume of Superose 12 chromatography buffer (50 mM Tris buffer containing 5 mM EDTA, 10 mM DTT, 200 mM NaCl and 10 % glycerol, pH 8.0) and dialyzed overnight at 4 °C against the same buffer. The dialysate was then clarified by centrifugation at 30.000 × *g* for 30 min and applied to a Superose 12 column (5 liters). Fractions were collected and assayed for HIV-1 protease by reversed phase HPLC (RP-HPLC) and peak activity fractions were pooled, made 40 % v/v with glycerol and stored at −70 °C. The protein was judged to be greater than 95 % pure by SDS-PAGE and RP-HPLC analysis. Protease activity assay was conducted according to [14] with substrate Ac-RASQNY·PVV-NH_2.

Synthesis of fluorogenic peptide substrate. Protected amino acids Boc-Leu, Boc-Ile, Boc-Val, Boc-Pro, Boc-Asn, Boc-Gln, N(α)-Boc-O-benzyl-L-Ser, N(α)-Boc-O-benzyl-L-Tyr and N(α)-Boc-(indolylformyl)-L-Trp were synthesized as described [15].

Synthesis of fluorogenic peptide substrate of DnsSerGlnAsnTyrProIleVal-TrpLeu-OH was performed by peptide synthesizer using Merrifield resin (chloromethylated copolymer of styrol and divinylbenzole, particle size of 200—400 mesh and active chlorine content of 0.7 mmole per 1 g of resin) obtained from Fluka (Switzerland). For synthesis convenience N(α)-Boc-Leu has been attached to the polymer as C-terminal amino acid by means of potassium salt in the presence of 18-crown-6-ether as catalyst [16].

H_2N-SerGlnAsnTyrProIleValTrpLeu-resin was dansylated by Dns-Cl. Dansylated peptide was isolated by pass of bromide hydrogen for 90 min through the resin suspension in trifluoroacetic acid (TFA), containing thioanizole. Formylation protection of Trp was removed by 0.1 M triethylamine in H_2O for 10 min at 0 °C. The crude peptide (52 mg) was then purified to homogeneity by RP-HPLC.

HPLC. Substrate purification, as well as the analysis of peptide fragments after cleavage of substrate by HIV-1 protease were performed on a Gold System (Beckman), using either Ultrasphere C-18 column (10 × 250 mm) or Ultrasphere C-18 IP column (4.6 × 45 mm). Linear elution gradient of 0 to 80 % acetonitrile in 0.1% TFA was used in HPLC experiments.

Fluorescence measurements. All measurements were performed on the Hitachi Model 850 fluorescence spectrophotometer (Japan), with a thermostated cell compartment at 20 °C. Fluorescence measurements were performed in 0.5 × 0.5 cm^2 quartz microcuvette.

Fluorogenic peptide substrate Dns-SQNYPIVWL-OH was dissolved in 50 % isopropanol. Then peptide solution was diluted by addition of reaction buffer, containing 0.05 M sodium acetate, pH 5.5, 0.01 M dithiotreitol and 10 % glycerol.

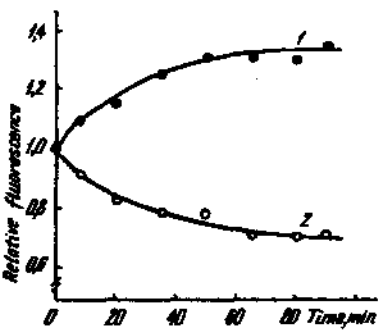
Kinetic analysis. Kinetic constants were determined from the fluorescence assay data of hydrolysis of fluorogenic substrate by HIV-1 protease. Initial rate of hydrolysis was calculated according to [17] as: $v = F_t[S]/(F-F_0)t$, where v — initial rate of hydrolysis (μmole/min), $[S]$ — concentration of substrate (μmole), F_t — fluorescence intensity of substrate at the time t (t — time of hydrolysis, min), F — fluorescence intensity after complete hydrolysis and F_0 — initial fluorescence intensity. The kinetic parameters were obtained from the Lineweaver — Burk plots after linearization procedure performed using "Enzfitter" program.

Results. *Design of structure of fluorogenic substrate.* The amino acid sequence which corresponds to the *p17/p24* cleavage site for HIV-1 protease was selected. This sequence was selected because it had been shown to be a better substrate for cleavage by HIV-1 protease than synthetic peptides based on other cleavage sites [18].

Trp residue was introduced into C-terminus of oligopeptide as a donor fluorophor (λ_{max} at 355 nm) and dansyl group at N-terminus served as an acceptor fluorophor (λ_{max} at 520 nm). Substrate design was based on the FRET between donor and acceptor groups in the intact peptide. Proteolytical cleavage of substrate by HIV-1 protease at the Tyr-Pro site should release two fluorophor-peptide fragments and break resonance energy transfer between Trp and Dns with concomitant changes of fluorescence intensities both of donor and acceptor fluorophors.

Fluorescence properties of substrate. Fluorescence of fluorogenic substrate was excited at 290 nm and corrected emission spectra were recorded. Two emission bands at 355 and 520 nm (Trp and Dns emissions) were observed in the spectra. The ratio of fluorescence intensity of Dns to Trp fluorescence was about 1.8—2.0 in the intact substrate.

Hydrolysis of substrate by HIV-1 protease. Addition of HIV-1 protease ($1.9 \cdot 10^{-7}$ M) to substrate solution in the reaction buffer resulted in the changes of intensities of both fluorescence emission bands. Time-dependent increase of relative emission intensity of Trp fluorescence and decrease of dansyl fluorescence measured at 350 and 500 nm, respectively, are shown in Figure. These fluorescence changes were apparently due to the break of FRET between donor and acceptor fluorophors.



Fluorescence intensities changes (excitation wavelength 290 nm) of Dns-SQNYPIVWL-OH (concentration of $2.5 \cdot 10^{-5}$ M) in the course of its hydrolysis by HIV-1 protease. Protease concentration was $1.9 \cdot 10^{-7}$ M. *1* — relative intensity change of tryptophan fluorescence measured at 350 nm; *2* — relative intensity change of dansyl fluorescence measured at 500 nm

Hydrolysis of fluorogenic peptide substrate was studied also by RP-HPLC. Action of HIV-1 protease on Dns-SQNYPIVWL resulted in the appearance of two new peaks with retention times of 12.5 and 15.2 min. These species were characterized by fluorescence spectroscopy and identified as substrate fragments Dns-SQNY and PIVWL. These results suggest that fluorogenic substrate had been cleaved by HIV-1 protease at Tyr-Pro site.

Kinetic parameters of hydrolysis. Kinetic studies of the hydrolysis reaction were performed at nine different concentrations of fluorogenic substrate. The kinetic constants of HIV-1 protease-catalyzed cleavage were calculated from Lineweaver — Burk plots. For this fluorogenic peptide substrate the values $K_M = 29$ μM and $k_{cat} = 5.4$ s^{-1} were obtained. These kinetic constants are close to the values obtained by Matayoshi et al. [9] for the hydrolysis of fluorogenic substrate based on the same *p17/p24* cleavage site ($K_M = 103$ μM and $k_{cat} = 4.9$ s^{-1}, respectively).

Discussion. Different methods have been used for the measurement of proteolytic activity of HIV-proteases, including HPLC analysis of cleavage fragments [14], protein immunoblot analysis, spectrophotometric assay using chromophoric peptide substrate [8]. Most of these methods are time-consuming and unacceptable for screening of large number of HIV-protease inhibitors.

Fluorogenic peptide substrates of HIV-1 protease are based on fluorescence assay of its proteolytic activity. This method is very sensitive and provides an accurate determination of kinetic parameters of hydrolysis reaction.

Fluorogenic peptide substrate of HIV-1 protease Dns-SQNYPIVWL described in this work was based on the FRET between Trp as a donor and Dns as an acceptor molecules. This donor-acceptor pair is very suitable for energy transfer measurements. It is known that FRET measurements are most sensitive to distance variation when donor-acceptor separation is close to their Förster distance [12]. The value of Förster distance using Trp as a donor and Dns as an acceptor of resonance energy transfer R_0 = 21 Å [12], which corresponds well to the length of peptide separating two fluorescent probes in fluorogenic substrate. Our fluorogenic substrate is similar to substrate described by Geoghegan et al. [11] but it has much better ratio k_{cat}/K_M = 180 000 $M^{-1}s^{-1}$. The solubility of this fluorogenic substrate may be increased by addition of other hydrophilic groups into its structure, e.g. by incorporation of Arg residues at the C- and N-ends of substrate.

Fluorogenic peptide substrate of HIV-1 protease based on FRET may be used not only in steady-state fluorescence measurements but also in liquid chromatography assays, microscopy and other biological and clinical applications.

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СИНТЕЗ ТА ХАРАКТЕРИСТИКА ФЛЮОРОГЕННОГО ПЕПТИДНОГО СУБСТРАТУ ВІЛ-1 ПРОТЕАЗИ НА ОСНОВІ ФЛЮОРЕСЦЕНТНОГО РЕЗОНАНСНОГО ПЕРЕНОСУ ЕНЕРГІЇ

Резюме

Проведено хімічний синтез флюорогенного пептидного субстрату ВІЛ-1 протеази, що має структуру Dns-SQNYPIVWL і відповідає сайту розщеплення *p17/p24* у *gag*-поліпротеїні вірусу імунодефіциту людини. Принцип використання даного субстрату базується на резонансному переносі енергії збудження між донором — залишком Trp і акцептором — дансильною групою. Встановлено, що гідроліз флюорогенного пептидного субстрату рекомбінантною ВІЛ-1 протеазою призводить до падіння інтенсивності флюоресценції дансильної групи і одночасного зростання триптофанової флюоресценції внаслідок порушення резонансного переносу енергії між донором і акцептором. За допомогою високоефективної рідинної хроматографії в оберненій фазі зафіксовано появу пептидів, які є продуктами гідролізу субстрату. Визначено кінетичні параметри гідролізу флюорогенного пептидного субстрату ВІЛ-1 протеазою: K_M = 29 мкМ, k_{cat} = = 5,4 c^{-1} та k_{cat}/K_M = 180 000 $M^{-1}c^{-1}$.

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