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A heterogeneous enzymatic assay for quantification of Plasmepsin II activity and the evaluation of its inhibitors

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Abstract

The emergence and worldwide spreading of *Plasmodium falciparum* strains that shown to be resistant to traditional drugs is considered a very serious health problem, given the high mortality and morbidity rate of Malaria. In the search for new drugs against this parasite, Hb hydrolyzing enzymes, such as Plasmepsin II (Plm II), have been classified as very promising targets for therapeutic attacks. In this work, it is developed a cheap and high-throughput heterogeneous enzymatic assay for measuring Plasmepsin II activity in order to use it as a tool in the discovery of new inhibitors of this enzyme. In this assay, Plasmepsin II acts upon a solid-phase bound synthetic peptide (DU2) whose sequence comprises the cleavage site F_{33}-L_{34} present in Hb α-chain. The peptide surface density is quantified by means of a classical ELISA-based procedure. In order to estimate the kinetic constants of the system and to quantify both, enzymatic and inhibitory activity, it was used a model for the kinetics of enzyme quasi-saturable systems previously developed by our group, that fitted very well to the experimental data. It was used Pepstatin as a model inhibitor of Plasmepsin II and the resulting dose–response relation agreed with the expected behavior for the Pepstatin–Plasmepsin II pair under the employed experimental conditions.

Keywords: Solid-phase; Plasmepsin II inhibitors; Malaria protease; Heterogeneous enzymatic assay

Abbreviations: ELISA, enzyme-linked immunosorbent assay; DMF, dimethylformamide; TFA, trifluoroacetic acid; DIEA, diisopropylethylamine; DIC, diisopropylcarbodiimide; OHBt, 1-hydroxybenzotriazol; RP-HPLC, reverse-phase high-pressure liquid chromatography; PBS, phosphate-buffered saline; DTT, dithiotreitol; EDTA, ethylendiaminetetraacetic acid; MALDI-TOF MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry

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1. Introduction

Malaria is the most important parasitic disease worldwide and exhibits the highest mortality and morbidity rate of any infection except tuberculosis. More than 300 million new infections and up to 2 million deaths occur every year [1,2]. In fact, the affection is endemic in more than a hundred countries, threatening around 2400 million people (around 1/3 of total human population). Although four species of Plasmodium are able to infect humans, *Plasmodium falciparum* (*Pf*) is the most virulent, provoking 90% of malarial deaths and the most severe clinical symptoms [1].

Successful therapy regimes allowed controlling malaria in the last five decades of the 20th century. Cloroquine has been the most effective drug against *Pf* and one of the most ever used in all human history. However, in many geographical regions, the parasite has became almost insensitive to this compound and new anti-malarial drugs are urgently needed [3–5]. Thus, there is taking place an intensive search for inhibitors against a variety of parasite targets among which hemoglobin hydrolyzing enzymes have been focused as very promising targets [6]. Most of these enzymes are aspartyl proteases called Plasmepsins (Plm) and among them, Plm I and Plm II are the most important and best characterized [7–10]. They occur in parasite digestive vacuole and seem to be essential in the first stages of hemoglobin (Hb) degradation. Plm II has been recently identified as a promissory therapeutic target and nanomolar concentrations of potent inhibitors of this enzyme have proven effective for killing *Pf* in parasite cultures and animal models [10–12]. Furthermore, some inhibitors of Plm II can also inhibit Plm I, acting upon both enzymes in vitro [13,14].

The search for new inhibitors depends on high-throughput screening programs for the evaluation of the most attractive candidates. Most of the Plm II activity assays developed up to now rely on peptidic substrates whose sequences reproduce the Hb α-chain F_{33}-L_{34} cleavage site recognized by these enzymes (Plm I and Plm II) [15]. Continuous spectrophotometric and fluorometric assays have been very useful [10,15], although they are rather expensive while the former is also rather insensitive. An Anson assay has been adapted for measuring the activity of Plm II using C^{14}-labeled Hb, but its intrinsic low throughput and the handle of radioactive materials constitute a problem for its large-scale use [16].

ELISA-based approaches have been also used for screening protease inhibitors. These methods are generally heterogeneous enzymatic assays given that the substrate is bound to a solid phase while the enzyme is free in solution [17–19]. The Plm II activity assay developed in this work belongs to this kind of assay. An N-terminal biotinilated synthetic peptide (DU2) that comprise a Plm II (and Plm I) cleavage sequence was bound to streptavidin-coated microtiter plates. The bound peptide can be quantitated by an immunoenzymatic procedure (ELISA) that includes a monoclonal antibody (Mab 332) against the peptide C-terminal. The incubation of the bound peptide with a solution of active Plm II resulted in a signal decrease as the peptide was hydrolyzed and the released C-terminal segment washed away.

For quantifying Plm II activity it was used a model that describes the kinetic of enzyme quasi-saturable systems (EQSS). In these systems, there is a very low amount of substrate (quite below the K_m), while there are no restrictions to the amount of enzyme. EQSS distinctive behavior is that the enzyme displaced to the solid phase to form the intermediate complex is negligible compared to the total substrate concentration [18].

Using pepstatin as a model compound it was assessed the ability of assay for detecting inhibitors of Plm II. Pepstatin, is a well known generic inhibitor of aspartic proteinases and has a very high affinity for Plm II ($K_i = 10^{-12}$ mol/l) [8].

2. Materials and methods

2.1. Materials

Most chemicals were purchased from Sigma Chemical (USA). Plasmid pET3aPlmII, containing a copy of the gene encoding Plm II, was kindly provided by Dr. Colin Berry, University of Wales College of Cardiff, UK. Mab332 was obtained by Dr. Emilio Carpio, School of Medicine, Sancti Spiritus, Cuba.

2.2. Methods

2.2.1. Synthesis of the biotinilated peptidic substrate (DU2): functionality of the Plm II cleavage sequence in DU2

The peptide DU2 was synthesized using manual parallel Fmoc solid-phase chemistry on a Rink Amide MBHA resin (0.54 mmol/g, 0.1 mmol scale). DIC/HOBt activation used more than a three-fold molar excess of Fmoc-amino acids in DMF at each coupling cycle. As a rule, no more than 2 h were needed for the completion of the coupling reaction. Biotin was coupled to the N-terminal of the peptide using TBTU/HOBt activation and 1.5 equivalents of DIEA.

The peptide was purified up to 99% by RP-HPLC on a Pharmacia LKB device with a Vydac C-18 column (25 mm × 250 mm). The absorbance was monitored at 226 nm. The peptide molecular mass was verified by MALDI-TOF MS.

On the other hand, a typical homogeneous enzymatic assay was used to check the functionality of the Plm II cleavage site in DU2. Five hundred microliter of a mixture containing DU2 50 μg/ml and 250 nmol/l of Plm II were incubated for 2 h at 37 °C in BAc (100 mmol/l sodium acetate of pH 5). After this time, reaction was stopped by adding 500 μl of 0.1% (v/v) trifluoroacetic acid (TFA). A sample of identical composition was used as control at t_0, but denaturant agent was added before the enzyme. Both mixtures were analyzed by RP-HPLC on a Pharmacia LKB device. Two hundred microliter of each sample (corresponding to 5 μg of DU2) were injected to a C-18 column (4.6 mm × 100 mm, Vydac, USA) and eluted using a linear acetonitrile (ACN) gradient in 0.1% TFA (0–60% ACN in 60 min). Absorbance was monitored at 226 nm. The identity of eluted peaks was assessed by MALDI-TOF.

2.2.2. Plasmepsin II expression, purification and refolding

Recombinant Plm II was obtained from *E. coli* inclusion bodies as described by Hill et al. [20]. In brief, *E coli* BL21(DE3) pLysS transformed with the recombinant plasmid pET3aPlmII that contains a copy of the gene encoding the last 48 residues of the pro region and the mature Plm II were grown in LB medium supplemented with ampicillin and induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG). Three hours after induction the cells were harvested by centrifugation, resuspended in 50 mmol/l Tris–HCl, pH 7.2, 0.15 mol/l NaCl buffer and then lyzed by freezing–thawing process and addition of lysozyme.

For refolding and purification, insoluble material from cell lysate (containing the recombinant enzyme) was pelleted by centrifugation (100000 × *g*, 15 min, 4 °C) and resuspended in 0.1 mol/l Tris–HCl, pH 11.0, 50 mmol/l β-mercaptoethanol buffer. After two centrifugation–resuspention cycles, the washed pellet was resuspended in 6 mol/l urea, 0.1 mol/l Tris–HCl, pH 8.0, 1 mmol/l glycine, 1 mmol/l EDTA, 50 mmol/l β-mercaptoethanol buffer for solubilization of recombinant Plm II. Residual insoluble material was removed by centrifugation and supernatant was then rapidly 200-fold diluted in 10 mmol/l Tris–HCl, pH 8.5 buffer, allowing refolding of the recombinant protein. After concentrating the sample (cut-off: 10 kDa), it was subject to an anionic Q-sepharose ion-exchange chromatography as the final purification step [21]. Recombinant Plm II was kept at 4 °C (do not freeze) in 10 mmol/l Tris–HCl, pH 8.5 buffer for conservation. Using this protocol, ca. 15 mg of Plm II were obtained per liter of medium. Before use, the enzyme was pre-activated by a 9/10 dilution in 10× BAc followed by 30 min incubation at 37 °C.

2.3. Immunoenzymatic quantification of DU2 by ELISA

In general, all reagents were added in 100 μl per well volumes, unless something different is specified, and each incubation step is followed by plate washing (six times) with 0.05% Tween 20 in distilled water.

Nunc Maxisorp 96-well microtiter plates (NUNC, Denmark) were coated with streptavidin (STA) 5 μg/ml in carbonate/bicarbonate buffer pH 9.6 by incubating at 4 °C O.N. or 2 h at 37 °C. Plates were rinsed and it was added a blocking solution (BS) containing BSA 0.02 g/ml and Tween 0.5% in PBS. After 1 h incubation, plates were washed and 1.5-fold serial dilutions of DU2 (0.04–5 ng/ml) in BS were added (1 h, 37 °C). Following plate wash of a mouse monoclonal antibody (Mab332, against DU2 C-terminal) at 2.5 μg/ml in BS, were added and incubated for 1 h at 37 °C. Later on, anti-mouse IgG horseradish peroxidase (HRPO) conjugate diluted 1:6000 on BS were added to each well. Finally, after washing the plate,

colorimetric reaction was started by adding 0.5 mg/ml *O*-phenylene-diamine (OPD) and H_2O_2 0.05% (v/v) in a buffer containing citric acid 27.3 mmol/l, Na_2HPO_4 52 mmol/l pH 5.5. After 15 min at room temperature, 50 μl per well of 3 mol/l H_2SO_4 were added for stopping the colorimetric reaction. Absorbances at 492 nm were read on a Sensident Scan (Merck, Germany) microtiter plate reader.

2.4. Heterogeneous enzymatic assay for Plm II

For heterogeneous enzymatic assays STA coated microtiter plates were incubated with a 100 μl per well solution of 1.25 ng/ml DU2 in BS. This substrate coating concentration was used in all experiments with Plm II. After washing, 100 μl per well of two-fold serial dilutions (69–1110 nmol/l) of Plm II in BAc were added to the plates. The enzymatic reaction (37 °C) was stopped after 2 h by washing the plate, except for kinetic experiments where stopping occurred at 20, 40, 60, 80, 100 and 120 min. The quantitation of the remaining substrate surface density was done by means of the immunoenzymatic procedure already described. The hydrolysis extension was expressed as degradation fraction (DG):

$$\mathrm{DG} = \frac{(\mathrm{OD_C} - \mathrm{OD_M})}{(\mathrm{OD_C} - \mathrm{OD_O})} \quad (1)$$

where OD_O and OD_C account for the optical densities (492 nm) of a control with no substrate at all (unspecific contributions to the signal) and a control where all substrate is intact (no enzyme), respectively. OD_M stands for the optical density in those wells where the immobilized substrate is incubated with a given enzyme concentration ($[E_0]$) for a reaction time equal to t.

In case that OD is linearly related to surface substrate density, DG is related to the amount of the enzyme and the reaction time by [19]:

$$\mathrm{DG} = 1 - \frac{[\bar{S}^{\mathrm{SP}}]}{[\bar{S}_0{}^{\mathrm{SP}}]} = 1 - \mathrm{e}^{-k_2 \cdot ([E_0]/(K_m + [E_0])) \cdot t} \quad (2)$$

$[\bar{S}^{SP}]$ is the total substrate concentration (bound and not-bound to the enzyme) while $[\bar{S}_0^{\mathrm{SP}}] = [S_0^{\mathrm{SP}}]$, the initial substrate concentration. K_m and k_2 are the Michaelis and the catalytic constants of the system, respectively (it has been assumed the existence of just one type of intermediate complex).

2.5. Inhibition assay

A fixed concentration of Plm II (370 nM) was incubated with 1.5-fold serial dilutions of pepstatin (0–733 nM) in BAc for 30 min at room temperature. After that, 100 μl of the mixture were added to STA-DU2 coated well and incubated for 2 h at 37 °C. The inhibitory activity (IA (%)) was computed by using the following equation derived from Eq. (2) [19]:

$$\mathrm{IA}\,(\%) = \left(1 - \frac{\ln(1 - \mathrm{DG}_i)(k_2 t + \ln(1 - \mathrm{DG}_0))}{\ln(1 - \mathrm{DG}_0)(k_2 t + \ln(1 - \mathrm{DG}_i))}\right) \times 100 \quad (3)$$

where DG_0 stands for control experiments with no inhibitor and DG_i for experiments where a given amount of Pepstatin is pre-incubated with the enzyme.

3. Results and discussion

The essential characteristics of the developed assay are shown in Fig. 1. The peptide sequence includes three functional modules: (1) 'anchor' module, i.e., biotin; (2) 'cleavage' module, from L_3 to P_{11}, reproduce the cleavage site F_{33}-L_{34} present in the Hb α-chain; and (3) 'immuno-recognition' module from R_{12} to C-terminal end that is specifically recognized by the monoclonal antibody Mab332. Two β-alanine residues were introduced between first and second modules in order to reduce possible interactions of Mab332 and Plm II with blocking elements, although the effect of this addition was not studied. The modular design of the substrate allows adapting the assay to other proteolytic enzymes by changing the cleavage sequence. In fact, the assay developed in this work was derived in this way from a similar one previously developed for HIV-PR [19].

The properties of the synthetic peptide DU2 matched with those expected from its structure. The mass spectrometric analysis of DU2 rendered 3066 Da, like the one calculated from its chemical composition ($C_{139}H_{221}N_{37}O_{39}S$).

DU2 was hydrolyzed by Plm II (Fig. 2). Profile A (reaction time: 0) shows just one significant peak (I: 43% ACN). The MALDI-TOF determined molecular

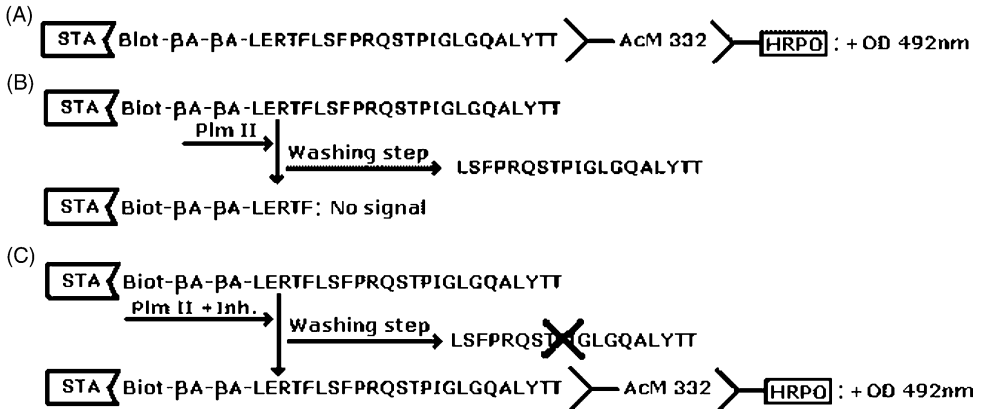

Fig. 1. Schematic representation of Plm II assay. (A) Streptavidin coated plates specifically binds biotinilated peptide DU2. (B) The LERTFLSFP sequence is recognized and hydrolyzed by Plm II at the F–L bond, releasing the C-terminal epitope (RQSTPIGLGQALTYTT) which is washed away. It results in signal reduction (compared to (A)). (C) The pre-incubation of the enzyme with an inhibitor (Pepstatin) precludes hydrolysis and signal recovering (compared to (B)). Total inhibition of Plm II converges to (A).

weight for this peak (3066.6 g/mol) corresponds to unprocessed DU2. On the other hand, the profile B (2 h of reaction time) shows two new peaks (II: 34% ACN and III: 38% ACN) indicating the occurrence of a unique proteolytic event in the proper site as suggested by the molecular weight of II (1016 Da) and III (2050 Da).

The use of STA coated microtiter plates lowered the detection limit for DU2 by a factor of around 1000 when compared to the direct binding of this peptide to the plastic surface (data not shown). This results point to a specific recognition of DU2 by the STA coating, as well as some homogenizing effect of this coating over the orientation of bound DU2 that generate a more uniform and better oriented DU2 population.

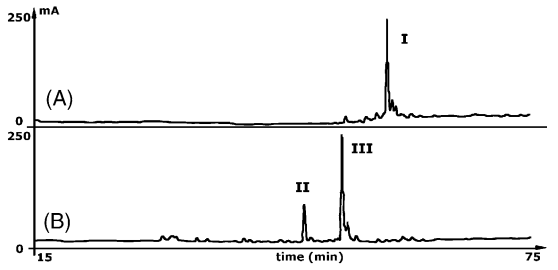

Fig. 2. RP-HPLC chromatographic profiles of mixtures of DU2 and Plm II. (A) reaction time: 0; Peak I corresponds to intact DU2. (B) Reaction time: 2 h; Peaks II and III correspond to expected cleavage products as determined by MALDI-TOF MS.

Fig. 3 shows a calibration curve for DU2 ($OD_{492\,nm}$ versus [DU2]) (A). The immuno-quantification of DU2 yields a signal linearly related to peptide concentrations up to 1.25 ng/ml (40 fmol per well). Higher peptide concentrations resulted in a reduced steepness, typical of saturated systems. One reason of this deviation is the depletion of the biotin binding sites in the STA coating, as pointed out by the quantification of remaining free peptide (B) of the previous calibration curve experiment (after equilibrium was achieved in 1 h incubation) (A) by means of the same immunoassay. Since 1.25 ng/ml rendered the highest OD in the linear segment,

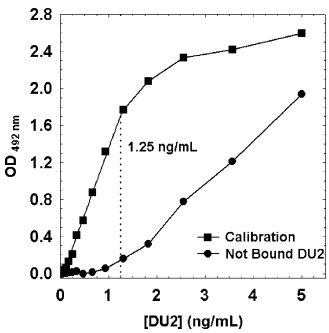

Fig. 3. DU2 calibration curve (squares) and quantification of not bound DU2 (circles) (residuals from calibration). Saturation of DU2 binding sites in STA coating contributes to the lost of linearity in the calibration curve for [DU2] >1.25 ng/ml. Each point accounts for the mean of triplicates.

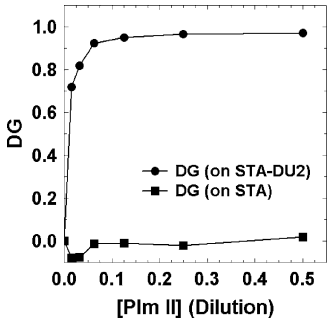

Fig. 4. Effect of Plm II on STA coating functionality. The incubation of STA–DU2 (12.5 ng/ml) plates with Plm II (2 h, 37 °C) results in a [Plm II]-dependent reduction of the signal (circles). The pre-treatment of STA coated plates with Plm II (2 h, 37 °C) before incubation with DU2 (12.5 ng/ml) did not affect the DU2 binding capacity of STA coating as judged from the very low DG (squares). Each point accounts for the mean of triplicates.

this concentration was selected for enzymatic and inhibition assays.

The incubation of STA-DU2 coated plates with increasing Plm II concentrations for 2 h resulted in decreasing signals, transformed into DG by using Eq. (1)). A parallel assay was performed by incubation of STA coated plates with the same enzyme concentrations used in the previous assay and under identical conditions. After adding DU2 (1.25 ng/ml) to Plm II–treated STA coated plates, no significant degradation (DG $\sim$ 0) was observed at any of the assayed [Plm II] (Fig. 4). This suggests that Plm II does not process STA under assayed conditions, or if it does, this processing does not affect its capacity to bind DU2.

The gathered evidence points to the occurrence of a unique Plm II catalyzed hydrolytic event on a likely uniform solid-phase bound DU2 population as the cause of signal reduction.

This fact makes clearer the physical meaning of the kinetic constants estimated by fitting Eq. (2) to the progress curves obtained for different enzyme concentrations and reaction time (Fig. 5).

For a kinetical description of this enzymatic transformation, peptide degradation produced by the Plm II concentrations used before, was followed for a period of time ranging from 0 to 2 h. As expected, higher reaction times and enzyme concentrations produce higher DU2 degradation ratios, which correspond to lower signals (Fig. 5A). Taking into account the value of the determination coefficient ($r^2 = 0.98$) and the distribution of observed versus estimated points around the identity line (Fig. 5B), the quality of this fitting can be considered rather high, despite the existence of a moderated dispersion.

Estimated values for K_m and k_2 were 1.6 μmol/l and $3.5 \times 10^{-3}\,s^{-1}$, respectively. When these parameters are compared to those obtained in a classical homogeneous assay for this enzyme and a substrate with the same cleavage site under very similar reaction conditions ($K_m = 0.96$ μmol/l and $k_2 = 1.4 \times 10\,s^{-1}$) [15], K_m is of the same order but k_2 is four orders lower in the heterogeneous assay. Even when these experiments have been carried out by different groups under

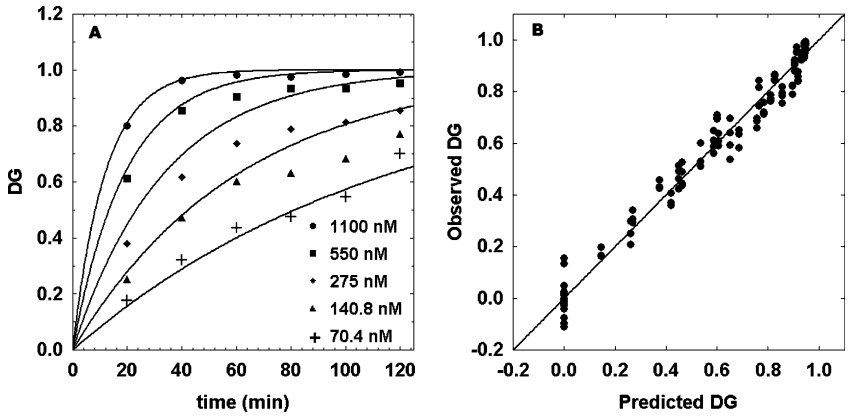

Fig. 5. (A) Kinetics of immobilized DU2 hydrolysis at different Plm II concentrations. The solid curves correspond to the fitting of Eq. (2) to experimental data. Each point accounts for the mean of triplicates. (B) Experimental vs. predicted values of DG.

non-identical but quite similar conditions, a four orders divergence seems to be too high for being motivated by these small differences. The same situation was described for a similar heterogeneous assay [19]. This fact could has its causes in the existence of partition phenomena which makes the microenvironment for the enzyme at the solid phase not well suited for catalysis by virtue of pH shifts to non optimal values. There could be also a stabilization of the complex (low k_2) by favorable electrostatic interactions with coating and/or blocking elements as well as some distortion of the substrate bound to the solid phase. All this put together, could affect the kinetics constants of the system in such a way that while k_2 gets smaller, K_m remains approximately the same due to compensating effects among k_1, k_{-1} and k_2. Whatever the causes of this phenomena, they must be found out because the lower the k_2 the higher the amount of enzyme needed in order to avoid affecting, the productivity of the assay and such increase affects significantly the assay detection limit of Plm II inhibitors.

The dose–response curve for the evaluation of inhibitory activity of Pepstatin over Plm II is shown in Fig. 6. Increasing Pepstatin concentrations ranging from 0 to 733 nmol/l were incubated with the enzyme. Inhibitory activities were determined by using Eq. (3). Linear curves are typical for enzyme/tight-binding inhibitor systems where $[E_0]/K_i$ ratio is higher than 100 [22] (in the case of this assay, $[E_0]/K_i = 370000$). The linear relation obtained confirms the tight-binding behavior of pepstatin under these conditions and corroborates the validity of Eq. (3) as well as the model from which it derives. Most important of all, this result shows the applicability of the assay for quantitative determination of inhibitory activity. Additionally, this can be used to estimate the total Plm II concentration by extending lineal segment of titration curve to IA (%) = 100: the pepstatin concentration that totally inhibits the active enzyme corresponds to 370 nmol/l.

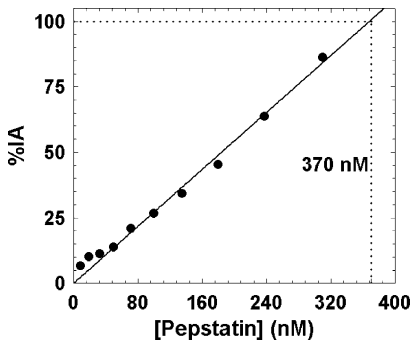

Fig. 6. Dose–response curve for the pair Pepstatin–Plm II (IA (%) vs. [Pepstatin]). The IA (%) was calculated from experimental data (DG) by using Eq. (3). It was just represented the linear segment of the plot. The observed behavior matched the tight-binding interaction between Pepstatin and Plm II under assay conditions. Each point represents the mean of hexaplicates.

This tight-binding behavior for pepstatin arouse from both, the high enzyme concentration (370 nmol/l) and the high stability of Plm II–Pepstatin complex ($K_i = 10^{-12}$ mol/l). Estimation of K_i for this system using this approach requires lower $[E_0]/K_i$ ratios ($[E_0]/K_i < 1$) [22]. The remarkable low value of K_i for Pespstatin–Plm II interaction forces the use of very low Plm II concentrations and it is not practical, given that the very low value of k_2 would make the assays improperly long. However, in the present conditions this assay would be valuable for estimating $K_i > 10^{-7}$ with two added values: (i) K_i estimations do not need to be corrected for substrate competition effects given that this assay belongs to the class of EQSS, where the substrate do not compete; and (ii) the high enzyme concentrations used, allow to discriminate between tight-binding and classical inhibitors given that the demarcation line between both types has been placed around $K_i = 10^{-7}$.

It is remarkable that due to the intrinsic advantages of the ELISA format, this method could be used in high-throughput screening of Plm II inhibitors from both, natural and synthetic sources.

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