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New Approach for Natural Products Screening by Real-time Monitoring of Hemoglobin Hydrolysis using Quartz Crystal Microbalance

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Graphical abstract

Highlights

- The methodology allows to monitor the Hb hydrolysis by cathepsin D in real-time.
- The label-free approach greatly simplifies the procedure.
- The methodology allows QCM screening of NPs products inhibitors in real-time.

- K_d and k_{cat} are followed through the mass changes (frequency variation) on QCM.

Abstract

The hemoglobin (Hb) released from erythrocytes is a primary nutritive component for many blood-feeding parasites. The aspartic protease cathepsin D is a hemoglobinase that is involved in the Hb degradation process and is considered an interesting target for chemotherapy intervention. However, traditional enzymatic assays for studying Hb degradation utilize spectrophotometric techniques, which do not allow real-time monitoring and can present serious interference problems. Herein, we describe a biosensor using simple approach for the real-time monitoring of Hb hydrolysis as well as an efficient screening method for natural products as enzymatic inhibitors using a quartz crystal microbalance (QCM) technique. Hemoglobin was anchored on the quartz crystal surface using mixed self-assembled monolayers. The addition of the enzyme caused a mass change (frequency shift) due to Hb hydrolysis, which was monitored in real time. From the frequency change patterns of the Hb-functionalized QCM, we evaluated the enzymatic reaction by determining the kinetic parameters of product formation (k_{cat}). The QCM enzymatic assay using immobilized human Hb was shown to be an excellent approach for screening possible inhibitors in complex mixtures, opening up a new avenue for the discovery of novel inhibitors.

Keywords: Quartz Crystal Microbalance, hemoglobin hydrolysis, cathepsin D, real-time monitoring.

1. Introduction

Hemoglobin (Hb) digestion is an essential process for the survival of blood-feeding parasites, such as schistosomes, blood-feeding ticks, and plasmodium species, among others [1-3]. In most cases, Hb hydrolysis is used by parasites primarily to provide the amino acids needed for the synthesis of their own proteins, which is fundamental for parasite development [4]. In the digestion process, proteases, such as cysteine, aspartic, and metalloproteases, play important roles [5-10].

In schistosomes, the proteins ingested in blood by the parasite, including Hb, are sources of nutrients, and the process of digestion involves a range of gut-associated proteases. These peptidases are thought to be related to the proteolysis of ingested host proteins and include orthologs of mammalian endopeptidases, such as cathepsins B, D and L, and exopeptidases, such as cathepsin C [11,12].

Several studies of cathepsin D-like (CD), belonging to a Clan AA aspartic protease that is present in both *Schistosoma japonicum* (SjCD, U90750) and *Schistosoma mansoni* (SmCD, U60995), have shown differences in human Hb preferences hydrolysis sites between parasites and human proteases [11,13,14]. In addition, the existence and proper functioning of these proteases are crucial for the maintenance and survival of parasites, making hemoglobinases important alternative targets for schistosomiasis chemotherapy [15,16].

Natural products (NPs) and their synthetic derivatives have been widely used in drug discovery because of their structural diversity and their highly selective and specific biological activities [17-19]._ENREF_17 _ENREF_17_ENREF_17_ENREF_17 Numerous NPs, semi-synthetic NPs and NP-derived compounds are undergoing clinical testing [20-22]. Worldwide, between 2005 and 2010 alone, nineteen new NP-based drugs were approved for marketing [23].

_ENREF_20 Cragg and Newman continuously present updates on NP drugs based on traditional medicine both as pharmaceutical agents and/or as leads for bioactive molecules against serious diseases [18,24]. It is anticipated that nature will continue to be a major source of new structural leads, and effective drug development will depend on multidisciplinary collaborations. A wide number of studies, including *in vitro*, *in vivo* and enzymatic experiments, have reported effective activity in NP extracts and have isolated compounds from them [25-28].

Compound screening using natural products represents an important source of new enzymatic inhibitors. Most enzymatic assays are performed using spectrophotometric or radioactive techniques. Among assays using human Hb as the substrate and proteases as the target, absorbance or fluorescence techniques are most commonly used [2,15,29]. However, compounds can be discarded due to the presence of absorbance or fluorescence at the same wavelength as the product obtained from hydrolysis. This problem has led to false results due to the complexity of crude plant extracts, which include secondary metabolites. Furthermore, compounds that have a structure similar to that of the cleavage product can also be discarded [30]. This limitation prompted us to study new techniques for the development of assays free of interferences using Hb as a substrate, with the possibility of studying enzymatic kinetic parameters in real time. Here, we propose a simple, focused and effective screening approach for enzymatic assays of NPs using an electrogravimetric technique.

The quartz crystal microbalance (QCM) provides a means to measure Hb hydrolysis in real time. The QCM technique is based on variations in the quartz crystal frequency related to the amount of mass deposited on the quartz crystal surface. QCM approaches for investigating biomolecular interactions offer high sensitivity and real-time analysis [31]. The QCM technique also offers rapid, cost-effective and reliable

results. Furthermore, QCM is able to measure tiny mass changes in real time through the label-free detection of molecules [32]. Recently, QCM has been used to determine the kinetic constants of numerous biomolecules [33-36],_ENREF_33 including enzymes such as endo- and exo-type proteases [37-39] and β-amylase [40], with the intention of developing reliable protocols and methods to measure enzymatic activity [32].

Usually, enzymatic reactions performed in bulk solution are kinetically studied using the Michaelis–Menten equation assuming a steady-state approach, in which the concentration of the enzyme–substrate complex (ES) is approximated to be constant during the reaction because of the relative difficulty of detecting this complex, as shown in Eq. 1.

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftarrows}} ES \xrightarrow{k_{cat}} P \qquad (1)$$

By monitoring the enzymatic reaction, it is possible to obtain the initial rate (v_0) and values of k_{cat} and K_m, where K_m is a complex value composed of k_1 (k_{on}), k_2 (k_{off}) and k_3 (k_{cat}), as demonstrated in Eq. 1. However, these latter constants are usually not followed independently, limiting the analysis of the reaction progress. In general, enzymes interact in specific and particular ways with their respective substrates. The dissociation constant $K_d = k_{off} / k_{on}$, a parameter not obtained in real-time solution reactions, represents the rates of binding and release of the enzyme and provides a greater understanding of enzyme-substrate interactions.

To ensure the above condition for the kinetic model, it is possible to design the biosensor or QCM functionalized surface strategically to satisfy the model's boundary condition. By using a protein-immobilized QCM, these kinetic parameters (K_d and k_{cat})

can be followed in real-time through the mass changes (frequency variation) on the quartz crystal as a function of time. Thus, if the formation and decomposition of the ES complex can be followed directly during the reaction, then more accurate kinetic constants might be obtained.

In this study, we developed a new approach for inhibitory assays based on the use of a QCM to monitor human Hb hydrolysis in real-time. For this, human Hb was covalently immobilized on the quartz surface, and the hydrolysis process was evaluated by measuring the frequency as a function of time after the enzyme bovine cathepsin D (CatD), either inhibited or not inhibited by extracts of plants or pure compounds, was injected. We discuss the necessary conditions for assays using an immobilized substrate based on previous reports of Hb in solution assays and provide an analysis of the kinetic parameters (K_d and k_{cat}) obtained by QCM. The method proved to be useful for the screening of compounds from NPs without interference. The QCM assays showed the ability to identify potential inhibitors in complex matrices, opening up a new avenue for the screening of plant extracts that has not been proposed hitherto.

2. Materials and Methods

2.1 General Methods

The CatD and human Hb used in this work were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The solvents used in the chromatographic process were HPLC grade from J.T. BAKER, TEDIA and PANREAC. 1D and 2D NMR experiments were performed on a Bruker DRX 400 (9.4 Tesla) instrument using deuterated solvents from Aldrich Chemical Company, Acros Organics, and Cambridge Isotope Laboratories, Inc. The reagents used in the immobilization process were from

Sigma and/or Fluka. The quartz crystal used in this study was 16 mm in diameter, AT-cut, and had a fundamental frequency of 5 MHz (the polished quartz crystals were provided by the QCM Stanford company with gold electrodes). The measurements were performed using a QCM 200 Quartz Crystal Microbalance (Stanford). The degradation of Hb by CatD in solution was recorded using a Hewlett Packard model HP8454 UV-visible spectrophotometer. SDS-Tricine-PAGE was performed in a Bio-Rad Mini Protean cell.[41,42]

2.2 *Plant Extract Preparation and Compound Isolation*

Stems from *Almeidea sp.*, belonging to the Rutaceae family, were collected and ground in a mill. The material was macerated in solvents of different polarities, including hexane (Hex) and dichloromethane (DCM), to extract the corresponding crude extracts after solvent evaporation a Rotavapor (Büchi® R-200). Bio-guided enzymatic assays were performed with the *Almeidea sp.* DCM (A2CD) and *Almeidea sp.* Hex (A2CH) crude dried extracts. To isolate the alkaloid evolitrine, the DCM extract was subjected to normal-phase chromatography under gradient elution, starting with 100% DCM as the mobile phase and increasing the polarity with acetone; methanol was used to wash the column. The fractions were analyzed by analytical thin layer chromatography (TLC), and fraction 23 was subjected to a second normal phase flash chromatography (isocratic elution with 3:1 hexane/acetone). Some fractions were analyzed by TLC and ^{1}H NMR. To confirm the structure of the alkaloid evolitrine, 1D and 2D NMR experiments were performed.

2.3 Compound Characterization

Please see the supplementary data for detailed characterization data for the alkaloid evolitrine by 1D and 2D NMR experiments.

2.4 Hemoglobin Degradation by SDS-Tricine-PAGE

The digestion of human Hb (2.0 mg mL^{-1} in H2O) was performed with CatD (7 µL; 0.7 mg mL^{-1} in H_2O) in 0.05 mol L^{-1} sodium formate buffer (pH 3.8) [43] in a total volume of 100 µL for different periods of time (1 h, 2 h, 3 h and 20 h) at room temperature. Two blank solutions were prepared using human Hb (final concentration of 1.5 mg mL^{-1} in H_2O) or CatD (final concentration of 0.05 mg mL^{-1} in H_2O). Following incubation, 20 µL of each reaction was added to a reaction buffer (0.188 mol L^{-1} Tris-HCl, pH 6.8, with 30% (w/v) glycerol, 15% (w/v) β-mercaptoethanol, 6% (w/v) SDS and 0.01% (w/v) bromophenol blue. After denaturation, the samples were heated to 95 °C, and the supernatant was applied to the SDS-Tricine-PAGE gel according to the literature procedure [41,42].

2.5 Human Hb Immobilization on the Quartz Crystal

Prior to the experiments, the quartz electrode surface was cleaned with a solution of freshly prepared 2:1 H_2SO_4 (conc.):H_2O_2 30% (v/v), rinsed with water and dried in air. The typical methodology employed for Hb immobilization was based on self-assembled monolayer on the gold surface of a piezoelectric transducer quartz crystal [44,45]. The cleaned piezoelectric quartz crystal was incubated for 4 h in a solution containing two thiols, 11-mecaptoundecanoic acid and 2-mercaptoethanol, $1.0x10^{-4}$ mol L^{-1} and $1.0x10^{-2}$ mol L^{-1} respectively using ethanol as solvent. The crystal was then washed with ethanol and water. Thereafter, the quartz crystal was incubated for 1.5 h in a MES-buffered solution (pH 5.0) containing both EDC and NHS at concentrations of $1.0x10^{-2}$ and $2.0x10^{-2}$ mol L^{-1}, respectively, followed by washing with MES-buffered

solution. Finally, the crystal was exposed to 200 µL of human Hb solution (concentration of 1.5 mg mL^{-1} in 0.1 mol L^{-1} phosphate saline buffer, pH 7.2 - PBS) overnight and then rinsed with PBS. The Hb-modified crystal surface was exposed to 0.1 mol L^{-1} ethanolamine solution for 30 minutes and washed with milli-Q water. An ethanolamine solution was used after the protein immobilization to react with the remaining carboxyl groups. The crystal was then placed in a QCM open cell with one face in contact with the solution and the other exposed to air.

2.6 Enzymatic assays in QCM

The reactions were performed adding 20 µL of bovine cathepsin D (CatD) enzyme solution in a human hemoglobin-immobilized quartz crystal in a QCM open cell. All assays required pre-stabilization of the functionalized Hb quartz crystal with 924 µL of formate buffer (0.05 mol L^{-1}, pH 3.8) [43], 45 µL of DMSO and 6 µL of BSA solution (2% w/v H_2O) using constant temperature of 25°C. Volumes and concentrations used are presented in Table 1:

As a positive control, we used CatD inhibited with pepstatin A, a commercial inhibitor of aspartic proteases. The inhibition assays were performed using the hexane and dichloromethane extracts of *Almeidea sp.* (A2CH and A2CD, respectively) and the pure alkaloid compound evolitrine. CatD was incubated for 10 min with the extract/inhibitor compound before addition to the quartz crystal functionalized with Hb. The inhibitor was solubilized in dimethyl sulfoxide (DMSO) prior to use. The injection volume was 20 µL (for the blank, positive and negative controls and the inhibitory assays), and the results of the enzymatic assays were obtained after 5 hours of reaction.

To construct a linear curve, 20 µL drops of different concentrations of CatD ($0.76x10^{-8}$, $2.17x10^{-8}$, $4.57x10^{-8}$, $7.61x10^{-8}$, $10.7x10^{-8}$ mol L^{-1} in H_2O) were added to the quartz crystal functionalized with Hb (1.5 $mg.mL^{-1}$). The standard deviation did not exceed 1% and was calculated from the results obtained from triplicate experiments.

2.7 Spectrophotometric Enzymatic Assays

Please see the supplementary data for detailed assays using CatD and Hb performed in UV-visible.

3. Results and discussion

3.1 Time course of Human Hb cleavage by CatD

Before starting the assays using human Hb immobilized on a QCM, it was necessary to analyze the time course of common solution reactions. For this purpose, samples taken at various points throughout the experiment were analyzed in a denaturing polyacrylamide gel in the presence of Tricine (SDS-Tricine-PAGE), as shown in Figure 1.

The time course of Hb degradation demonstrated the gradual hydrolysis of the human Hb substrate after 1, 2, 3 and 20 hours of reaction.

The most common Hb type in adult humans is a tetramer that contains 4 subunit proteins that are structurally similar and approximately the same size. Each subunit has a molecular weight of approximately 16 kDa, for a total molecular weight of approximately 64 kDa and 32 kDa for the Hb dimer.

Since the detection of low molecular weights fragments, resulted from the degradation of Hb tetramer and dimer was necessary, tricine gel was employed for this study.

After 1 hour of reaction, fragments of smaller peptides (~7 kDa) resulting from the cleavage of Hb dimers and monomers (~32 kDa and 16 kDa, respectively) became apparent. The gradual cleavage of Hb as well as the hydrolysis of small peptides (~3 kDa) occurred during the reaction, according to the analysis after 20 hours of reaction. This result was due to the tendency of the enzyme to cleave peptides, including smaller peptides, to produce amino acid residues, which are used for the synthesis of the parasite proteins.

3.2 Immobilization of Human Hb Monitored by QCM

The Hb was immobilized onto self-assembled monolayers (SAMs) consisting of two alkanethiols, i.e. 11-mercaptoudecanoic acid (MUA) and 1-mercaptoethanol (MHO) [46]. The use of mixed SAMs, formed for instance by co-adsorption of mixtures of two thiols, provide a useful methodology for incorporating into a SAM a molecular species whose own physical dimensions would preclude a direct, well-organized assembly [45]. The immobilization was carried out in two steps. First, a mixed SAM was formed with MUA and MHO on the gold surface, followed by activation with a solution containing both N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS), and the remaining groups were blocked with ethanolamine. Then, Hb was linked to the SAM via interactions between the carboxylic acids present on MUA and the amines present in Hb.

The immobilization of human Hb on the quartz crystal was measured by QCM. A schematic diagram of the immobilization of Hb on the SAM surface is illustrated in Figure 2.

The QCM showed real-time variations in oscillation frequency when a PBS solution with Hb was placed on the SAM-functionalized quartz crystal (indicated by the arrow in Figure 2).

Experiments with the addition of the same volume of PBS in place of Hb were performed to confirm that the decrease of frequency observed after the addition of Hb to the quartz crystal resulted from the Hb-SAM interaction. A decrease in frequency was obtained when the solution of PBS was added to the crystal. Therefore, after the system stabilization the frequency increased, reaching values close to initials (see supplementary data for detailed curve with PBS addition). The decrease in the oscillation frequency was related to Hb binding to the self-assembled monolayer immobilized on the transducer surface.

When the Hb solution was added to the crystal, the oscillation frequency decreased and a difference of 345 ± 10 Hz was obtained for three measurements by comparing the initial and final frequency values.

The Sauerbrey equation (2) can be used to quantify the changes in frequency of a quartz crystal and to determine the mass or thickness of a vacuum-deposited metal on the quartz surface [47]. For an AT-cut shear mode QCM:

$$\Delta f = -C_f \Delta m \qquad (2)$$

Where delta *f* is the measured frequency change (Hz), and delta *m* is the mass change (g). The linear sensitivity factor C_f is 56.6 Hz μg^{-1} cm^2 for a 5 MHz AT-cut quartz crystal at room temperature.

According to Sauerbrey equation (2) and considering the observed frequency change (345 ± 10 Hz), the amount of immobilized human Hb was calculated to be estimated of 6 ± 0.2 μg cm^{-2} which represents $3.75x10^{-11}$ mol of immobilized Hb. This value is an estimate considering that the equation is applicable to uniform, rigid, thin-film deposits. However, typically, Sauerbrey equation has been extensively used for experiments involving molecular recognition in biological systems [32-34,38]. QCM attained significance as an analytical device due the relationship between deposit mass and the frequency response.

3.3 Cleavage of Immobilized Human Hb by CatD monitored by QCM

The enzymatic reaction comprising the cleavage of the Human Hb immobilized on the quartz crystal by CatD was monitored by varying the frequency of the quartz crystal (Fig. 3). After stabilizing the frequency signal by placing the crystal in a QCM cell containing formate buffer (0.05 mol L^{-1}, pH 3.8, 24 °C), 20 μL of enzyme solution was carefully added to the open cell.

Figure 4 shows the real-time variation in the oscillation frequency as a function of time for different CatD concentrations ($0.76x10^{-8}$, $2.17x10^{-8}$, $4.57x10^{-8}$, $7.61x10^{-8}$, $10.7x10^{-8}$ mol L^{-1}). The enzymatic cleavage of the Hb anchored to the gold surface was monitored directly from the changes in the mass-sensitive resonance frequency, and the ladle-shaped curve observed indicates successful Hb hydrolysis by CatD.

When the described volume of enzyme solution (indicated by the arrow) was added to the cell containing the Hb-functionalized quartz surface, an initial frequency decrease (i.e., mass increase) due to the binding of CatD to the Hb substrate, indicating the formation of the ES complex, which is characterized by a dissociation constant of K_d

= k_{off} / k_{on}. The ES complex formation is also confirmed by the hydrolysis of the substrate, which is only possible if such interaction occur. Immediately, the frequency began to gradually increase (i.e., mass decrease) due to the hydrolysis of the substrate on the QCM, making it possible to obtain the catalytic constant k_{cat}. Finally, the frequency reached constant values. These behaviors suggest that CatD has a high ability to bind to the substrate and a slow hydrolysis rate, as identified by the long reaction times observed using SDS-Tricine-PAGE.

Increased concentrations of CatD produced increases in the rate of frequency change, allowing the hydrolysis constants to be obtained. Data were acquired for 5 hours based on the Hb degradation reaction times established by SDS-Tricine-PAGE. However, after only 2 hours, we could clearly observe a tendency of stabilization of the signal, indicating that, at this point, it was possible to confirm whether inhibition had occurred in the case of inhibitor screening.

The hemoglobin hydrolysis rate was determined based on the slope of the linear part of the kinetic curves for frequency vs. time, which varied as a function of enzyme activity. The reaction was analyzed by determining the apparent hydrolysis rate constant (k_{app}), which was assumed to occur in a single turnover reaction. This parameter can be determined from the curve fittings of Eqs. (3) – (7), considering [E] = $[E]_0$ and [S] = $[S]_0 - [ES]$ [40].

$$k_{app} = \frac{d[P]}{dt} = k_{cat}[ES] \qquad (3)$$

$$[P] = [S]_0(1 - e^{-\alpha k_{cat} t}) \qquad (4)$$

$$\alpha = \frac{[E]_0}{[E]_0 + K_d} \quad (5)$$

$$k_{app} = \frac{d[P]}{dt} = \alpha\, k_{cat} = \frac{k_{cat}[E]_0}{[E]_0 + K_d} \quad (6)$$

$$\frac{1}{k_{app}} = \frac{1}{k_{cat}} + \frac{K_d}{k_{cat}[E]_0} \quad (7)$$

As observed, the linear correlation between $1/k_{app}$ and $1/[E]_0$ presented in Eq. 7 made it possible to determine a dissociation constant (K_d) of 2.67 x 10^{-9} M and a catalytic hydrolysis rate (k_{cat}) of 1.228 x 10^{-4} s^{-1}.

Michaelis–Menten kinetics in bulk solutions usually do not reveal the dissociation constant (K_d) because of difficult to detect the concentration of the ES complex and also it is not possible to detect it in real-time. However, the formation of the ES complex is observed on QCM method in real-time and reflects the real dissociation constant, not being an estimative or an approximation. Furthermore, QCM also provides a useful tool for characterizing kinetic parameters such as k_{cat} in enzyme reactions.

These values are extremely important for understanding enzyme-substrate affinity. One example is a study carried out with endo- and exo-type proteases in which the enzyme reactions were characterized using a 27 MHz quartz crystal microbalance. It was observed that the k_{cat} of 1.1 s^{-1} for the exo-type carboxypeptidase P was larger than the k_{cat} of 0.08 s^{-1} for the endo-type subtilisin, and the value of K_d for the endo-type subtilisin was smaller than the K_d for the exo-type carboxypeptidase P. This observation suggested that the endotype enzyme quickly finds the specific hydrolysis sites of its substrates (small K_d) and then slowly hydrolyzes the specific site (small k_{cat}). In

contrast, the exo-type enzyme did not find the terminus of the non-specific substrate quickly (large K_d) and hydrolyzed the terminal unit quickly and continuously before releasing the substrate (large k_{cat}) [37]. For CatD and Hb, the kinetic parameters obtained (K_d = 2.67 10^{-9} M and k_{cat} = 1.23 x 10^{-4} s^{-1}) suggest a high ability to bind to the substrate, as evidenced by the small K_d, and a slow hydrolysis rate, as evidenced by the small k_{cat}.

The hemoglobin hydrolysis was evaluated by varying the CatD concentration. The constructed calibration curve (y = a + bx) for CatD activity using concentrations of 0.76x10^{-8}, 2.17x10^{-8}, 4.57x10^{-8}, 7.61x10^{-8}, and 10.7x10^{-8} mol L^{-1} presented R^2 = 0.9774. The detection limit was found to be 0.12x10^{-8} mol L-1 for an S/n = 3 (see the supplementary data for detailed constructed curve and discussion).

In addition to monitoring human Hb cleavage by CatD using the real-time QCM technique, solution enzymatic assays using a UV detector were performed to obtain additional information, including the reaction using CatD with different concentrations of Hb substrate. The results obtained with the Hb reaction using a spectrophotometer confirmed that Hb hydrolysis by CatD occurred. Additionally, the UV method was employed to confirm the reaction carried out on the QCM without any inhibitor. After 5 hours of reaction with immobilized human Hb and CatD on the QCM, the solution was collected, and the absorbance was measured. The results confirm the Hb immobilized cleavage by CatD on QCM. (see the supplementary data).

3.4 QCM inhibitory assays

Traditional enzymatic assays are usually performed using spectrophotometric detection [15]. However, interference from compound autofluorescence due to the intrinsic fluorescent properties of some library compounds is observed routinely in our

laboratories. QCM was shown to be useful for performing inhibitory assays in which variations in frequency are used to determined enzyme inhibition.

Figure 5 presents 6 curves measured using crude hexane (A2CH) and dichloromethane (A2CD) extracts of *Almeidea* sp.; the pure compound evolitrine isolated from A2CD; the commercial pseudo-reversible inhibitor pepstatin A; a blank containing H_2O, DMSO and buffer; and a negative control containing the active enzyme.

Pepstatin A has been used as a positive control in inhibitory assays because it is known to be a potent inhibitor of CD. In QCM, the variation in frequency was small (-3 Hz) compared with that of the negative control (42 Hz) when pepstatin A was incubated with CatD, indicating the excellent inhibition of this compound.

Extract A2CH gave a negative result, as evidenced by the variation in frequency (54 Hz), indicating that it had no inhibitory properties. However, extract A2CD displayed a change in frequency of 12 Hz, suggesting an interaction between one or more compounds present in the crude extract and the enzyme. Further fractionation of this last crude extract to identify the possible inhibitor isolated the compound evolitrine, which gave a Δf of 12 Hz when tested against CatD and Hb on the QCM. Additionally, the evolitrine compound was tested against CatD using spectrophotometric technique and the results confirmed the reliability of the QCM methodology, identifying the evolitrine as an inhibitor, however with moderate activity. These data evince the QCM as an efficient technique that can be used to perform qualitative screening assays to investigate possible inhibitors from natural products against aspartic proteases such as Cathepsin D.

4. Conclusion

In this work, we described a novel approach for the inhibitor screening of natural products that was useful even for testing complex matrices such as plant extracts. The use of a QCM to perform assays with surface-immobilized Hb (substrate) and cathepsin D (target) eliminates the interference that is observed in spectrophotometric assays, commonly used, because it is based on real-time mass changes and not on the structural characteristics of the molecule. The methodology may be used to identify the hydrolysis of Hb by any enzyme that employs it as a substrate in solution assays without any labeling, demonstrating its multivariate uses in screening procedures to discover new hits against enzyme targets. Additionally, the kinetic parameters of the hydrolysis process were elucidated, K_d = 0.00267 10^{-6} M and k_{cat} = 1.23 x 10^{-4} s^{-1}, improving our understanding of the formation and decay of the ES complex and the steps of the catalytic hydrolysis reaction, which conventionally, only the hydrolysis product is followed in solution assays. Therefore, QCM can become a new tool to obtain kinetic parameters of proteases, because the catalytic hydrolysis reaction can be directly observed as low level of mass change.

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at

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Figure captions

Figure 1. Time course of Human Hb degradation by CatD detected by SDS-Tricine-PAGE gel. Hb represents time 0, i.e., no cleavage; 1, 2, 3 and 20 represent the time course (in hours) of the hydrolysis reaction. CD represents the profile of CatD.

Figure 2. Immobilization of human Hb on quartz crystal monitored by QCM. **Left)** Illustration of Hb immobilized on the quartz crystal and the QCM setup. **Right)** Typical frequency change for the self-assembled monolayer-functionalized QCM upon the addition of 1.5 mg mL^{-1} of human Hb (0.01 mol L^{-1} phosphate buffer and 0.5 mol L^{-1} NaCl, pH 7.2).

Figure 3. Illustration of Hb immobilization and enzymatic hydrolysis by CatD on QCM. The kinetic parameters (K_d *and* k_{cat}) of the reaction were obtained in this work.

Figure 4. Left) Typical frequency changes as a function of time for different CatD concentrations (0.76x10-8, 2.17x10-8, 4.57x10-8, 7.61x10-8, 10.7x10-8 mol L^{-1}). The QCM cell contained 924 µL of formate buffer (0.05 mol L^{-1}, pH 3.8), 45 µL of DMSO and 6 µL of BSA solution (2% w/v H2O) with human Hb immobilized at a concentration of 1.5 mg mL^{-1}. **Right)** Zoom of frequency changes curve of CatD (concentration of 2.17x10-8 mol L^{-1}).

Figure 5. Typical frequency changes as a function of time in the presence of crude extracts and natural and commercial inhibitors. Pepstatin A (red), A2CD (blue), A2CH (orange) and Evolitrine (green). Final concentrations = 5 µmol L-1, 125 µmol L^{-1}, 125 µmol L^{-1} and 546 µmol L^{-1}, respectively. The QCM cell contained 924 µL of formate buffer (0.05 mol L^{-1}, pH 3.8), 45 µL of DMSO and 6 µL of BSA solution (2% w/v H_2O)

with immobilized human Hb at 1.5 mg L^{-1}. The CatD concentration used was 2.17 x 10^{-8} mol L^{-1}.

Table 1. Data used for enzymatic assays on the QCM. The cell contained 924 µL of formate buffer (0.05 mol L^{-1}, pH 3.8) [43], 45 µL of DMSO and 6 µL of BSA solution (2% w/v H_2O) with immobilized human Hb at 1.5 mg L^{-1}

Table 1

Assays	Enzyme solution[1] (µL)	Formate Buffer[2] (µL)	H_2O (µL)	Pepstatin A solution[3] (µL)	Inhibitor solution[4] (µL)	DMSO (µL)
Blank	0	8.0	7.0	0	0	5
Negative Control	1.5	8.0	5.5	0	0	5
Positive Control	1.5	8.0	5.5	5	0	0
Inhibition Assays	1.5	8.0	5.5	0	5	0

[1] Concentration of CatD stock solution = 0.7 mg mL^{-1} in H_2O.

[2] Buffer = 0.05 mol L^{-1} sodium formate, pH 3.8.

[3] Concentration of Pepstatin A stock solution = 1 mmol L^{-1}.

[4] Final concentration of inhibitor solution = A2CH (125 µmol L^{-1}), A2CD (125 µmol L^{-1}) and Evolitrine (546 µmol L^{-1}).