

KINETIC CHARACTERIZATION OF hK6 INHIBITION BY PROTEASE INHIBITOR, SOYBEAN

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ABSTRACT

The kinetic characteristics, of interaction between hK6 (human Kallikrein) and soybean (BBI), protease inhibitor and antitumor agent, in the presence of substrate (Phenylalanine-Serine-Arginine)-(7-amino-4-methyl-coumarin) (FSR-AMC) were investigated. The hK6 were found to bind soybean in two reversible steps, by slow binding inhibition mechanism. The K_i of the first step binding was 13 nM and K_i^* of the second binding step was 1.6 nM. The microscopic rate constants were calculated as follows: $311 \text{ M}^{-1} \cdot \text{S}^{-1}$ for k_3 , $0.04 \times 10^{-6} \text{ M}^{-1} \cdot \text{S}^{-1}$ for k_{-3} , $0.2 \times 10^{-6} \text{ S}^{-1}$ for k_4 and $0.025 \times 10^{-6} \text{ S}^{-1}$ for k_{-4} respectively. The results suggested that the interaction mechanism between hK6 and soybean was like that of trypsin with this inhibitor but with rather lower inhibitory constants values.

Keywords: hK6, trypsin, soybean, kinetic, inhibition

INTRODUCTION

The human kallikrein gene family maps to chromosome 19^{q13.4} and has 15 members which are designated KLK1 to KLK15; and their respective proteins are known as hK1 to hK15 (1, 2). PSA (Prostate Specific Antigen), the best known member of this group is widely used for diagnosis and management of patients with prostate cancer (3, 4). Many other kallikreins are promising biomarkers for ovarian, prostate, testicular, and breast carcinomas (5). Kallikrein 6 is one of the newly identified genes which encode for hK6 (6,7) which is expressed in several tissues (6,8) and predominant expression of KLK6 as hK6 in brain cells is suggested to have potential involvement in development and expression of Alzheimers's disease (9).

The hK6 has trypsin like proteolytic activity which catalyses Arg-ending substrates, and is inhibited by serpins and soybeans (8, 10, 11). Soybean, Bowman-Birk Inhibitor (BBI) is a well known protease inhibitor (12, 13), with antitumor activity (14, 15). The inhibition mechanism and kinetic characteristics of elastase (16), cathepsin (17) and trypsin (18, 19) by soybean have been investigated. In this study inhibition kinetic constants of interaction between soybean (BBI) and hK6 was investigated and compared with kinetic characteristics of this enzyme with that of trypsin in relation to antitumor agent, soybean.

MATERIALS AND METHODS

Materials

Recombinant hK6 protein was produced and purified as reported previously (11,20). The synthetic peptide Phe-Ser-Arg-AMC (FSR-AMC) was purchased from BAHEM Bioscience (King of Prussia, PA), diluted in DMSO at a final concentration of 80 mM and stored at -20°C. 7-Amino-4-Methylcoumarin (AMC) was purchased from Sigma (St. Louis, MO). Soybean was from Sigma and lyophilized bovine trypsin was from Pierce Chemical Company (Rockford, IL). Stock solutions were prepared and stored according to the manufacturer's instructions.

Methods

The enzyme activity was measured as previously reported (11). Fluorescence was measured for 20 min on a Wallac Victor fluorometer (Perkin-Elmer, Wellesley, MA) set at 355 nm for excitation and 460 nm for emission, and Phe-Ser-Arg-AMC was used as substrate. Unless otherwise specified, 100 μl of the assay mixture contained 50 mM Tris, 0.1M NaCl, 0.5 mM substrate, 0-48 nM soybean, 12 nM hK6 of pH 7.4 which was incubated at 37°C. Both soybean and the substrate were incubated together with the buffer system at 37°C for 5 min before addition of the enzyme to start the reaction. Injection of 20 μl of hK6 solution initiated the reaction.

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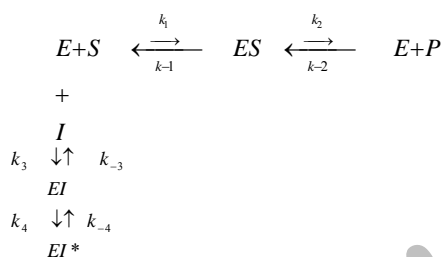
Table 1. Inhibition constants for hK6-soybean interaction (pH 7.4 , 37 °C)

Ki(nM)	Ki*(nM)	k ₃ (M ⁻¹ .S ⁻¹)	k ₋₃ (×10 ⁻⁶ M ⁻¹ .S ⁻¹)	k ₄ (×10 ⁻⁶ S ⁻¹)	k ₋₄ (×10 ⁻⁶ S ⁻¹)
13	-	311	0.04	-	-
-	1.6	k ₄ /Ki=1538	-	0.20	0.025

Enzyme-free reactions were used as a negative control and background fluorescence was subtracted from each value. All experiments were carried out in triplicates. A standard curve with known concentrations of AMC was used to calculate the rate of the product formation. The Plots progress curves for enzyme inhibition were fitted to equation and the Michaelis-Menten constants were calculated by non-linear regression analysis using the Enzyme kinetics Module 1.1 (Sigma plot, SSPS, Chicago, IL).

Data analysis

The progress curves were analyzed according to the slow-binding inhibitors mechanism (19). The scheme predicted for such mechanism is as follows:

**Scheme 1**

For such a two-step interaction between the inhibitor and enzyme, the formation of the reaction product as a function of time is obtained from the following equation (19).

$$[P]_{\text{obs}} = [P]_s - c_1 e^{-r_1 t} - c_2 e^{-r_2 t} \quad (\text{I})$$

Where $[P]_{\text{obs}}$ is the product concentration actually observed, and $[P]_s$ is the product concentration when it approaches infinity.

A plot of $\text{Log}([P]_s - [P]_{\text{obs}})$ against t should give a series of straight lines at different concentrations of the inhibitor with slopes of r_1 and r_2 (19). The values of r_1 and r_2 were found from each of straight lines. From the plots of $r_1 + r_2$ and $r_1 r_2$ against inhibitor, soybean (figure 3) the rate constants k_3 , k_{-3} , k_4 and

$$r_1 + r_2 = k_{-3} + k_4 + k_{-4} + \frac{k_3 k_m}{k_m + [S]} [I] \quad (\text{II})$$

$$r_1 r_2 = k_{-3} k_{-4} + (k_{-4} + k_4) \frac{k_3 K_m}{K_m + [S]} [I]$$

k_{-4} can be obtained. Plotting were based on the equation II.

RESULTS

Initial rate kinetics

The K_m and K_{cat} values were determined by measuring the initial rate of enzymatic hydrolysis for at least six different concentration of substrate (FSR-AMC). The data were fitted to the Lineweaver-Burk plot to of which K_m was equal to 0.41 mM and K_{cat} was equal to 38.3 min⁻¹.

The inhibition of hK6 by soybean.

The inhibition of hK6 by soybean in the presence of the substrate Phe-Ser-Arg-AMC(FSR-AMC) are shown in figure 1. There are a series of curves for FSR-AMC hydrolysis in the presense of different concentration of soybean. As it might be observed the rate of substrate hydrolysis decreased in two step processes, i.e. initial and steady-state velocities, respectively. The relatively rapid initial velocity decreased to a slower steady state and the curves approached steady-state values of product ($[P]_s$) by lengthening the reaction time.

Determination of the inhibition constants.

The mechanism of inhibition may be described as it is shown in scheme 1 in which E,S,P, and I represent the enzyme (hK6), substrate (FSR-AMC), product (AMC), and inhibitor (soybean), respectively. The k_3 , k_{-3} and k_4 , k_{-4} is association and dissociation rate constants for the first and second steps of enzyme-inhibitor complex formation respectively. The inhibition constant for the first step (Ki) is equal to k_{-3}/k_3 and the inhibition constant for the second step (Ki*) is equal to $k_{-4}/k_4/K_i$. A plot of $\text{Log}([P]_s - [P]_{\text{obs}})$ against t gives a curve (figure.2) that can be resolved in to two straight lines (figure is not shown) with slopes of r_1 and r_2 . The inhibition rates constant were calculated from the plots of $r_1 + r_2$ and $r_1 r_2$ respectively against inhibitor (soybean) (Fig.3), and are summarized in table 1. The inhibition constants of the first and second step inhibition, Ki and Ki*, were 13 and 1.6 nM, respectively. The values of k_3 , k_{-3} , k_4 and k_{-4} were 311 M⁻¹.S⁻¹, 0.04×10⁻⁶ M⁻¹.S⁻¹, 0.20×10⁻⁶ S⁻¹, and 0.025×10⁻⁶ S⁻¹, respectively (see table 1).

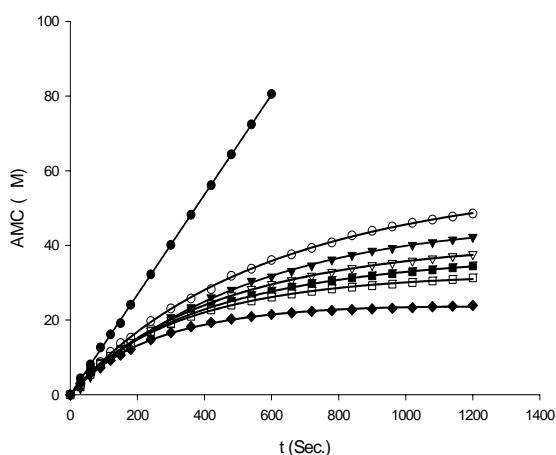


Fig. 1. Hydrolysis of 0.5 mM of FSR – AMC as substrate by hK6 (12 nM) in the absence ● and presence of different concentration of soybean . The concentrations of soybean were ○ (8 nM) , ▼(12 nM) , △ (16 nM) , ■ (20nM) , □(24 nM) and ♦ (32 nM) , respectively.

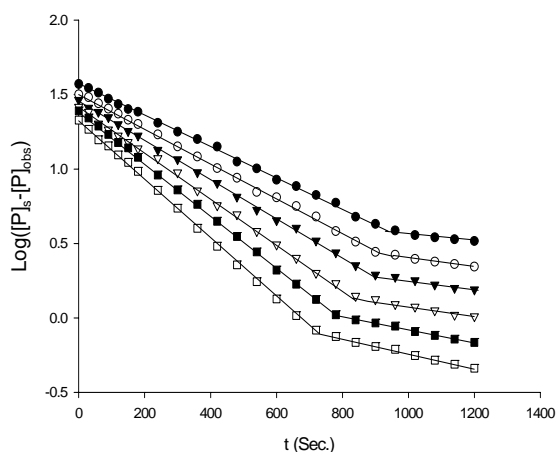


Fig. 2. Plot of $\text{Log}([P]_s - [P]_{\text{obs}})$ against t (Sec.) for the inhibition of hK6 by soybean . The soybean concentrations were ● (8 nM) , ○ (12 nM) , ▼ (16nM) , △ (20 nM) , ■ (24 nM) , □ (32 nM) , respectively . In fact data were taken from curves in fig . 1.

DISCUSSION

As shown in figure 1, in the absence of the inhibitor (soyben), hydrolysis of substrate (FSR-AMC) reached to the steady state velocity immediately. But, in the presence of soybean there was slow decrease in both (initial and steady-state) substrate hydrolysis rate, which varied as a function of the inhibitor concentration. The plots for the course of substrate (FSR-AMC) hydrolysis versus time (figure1), and $\text{Log}([P]_s - [P]_{\text{obs}})$ versus time (figure.2) which are

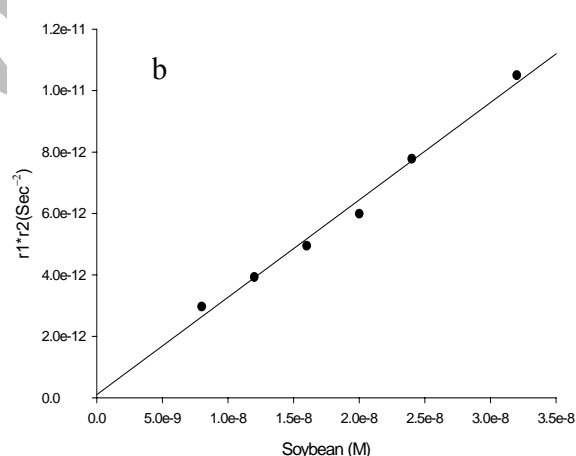
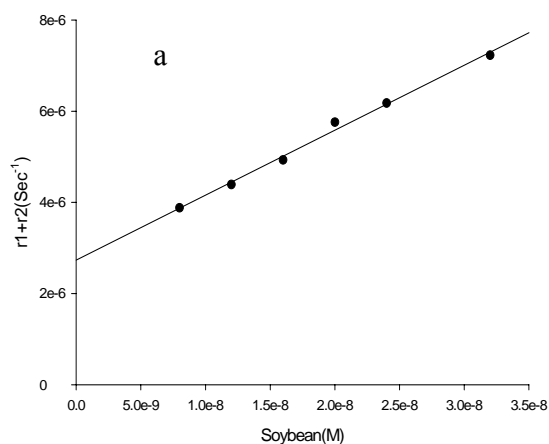


Fig. 3. The plots a) $r_1 + r_2$ b) $r_1 r_2$ against different concentrations of soybeans which were used . By using slopes and intersections of lines with y-axis , we calculated k_3 , k_{-3} , k_4 and k_{-4} respectively.

resolved into two straight lines with slopes of r_1 and r_2 suggested the formation of an inter-mediate resulting from binding of hK6 with soybean. From this result it is possible to conclude that the inhibition of hK6 by soybean could be described by the slow-binding inhibition mechanism presented in scheme 1, which have been predicted in analysis of interaction of this inhibitor with trypsin (19). Also this kind of the kinetic behaviour of substrate

hydrolysis by serine proteases in the presence of slow and tight binding inhibitor have been elucidated by other investigation (21-23). By increasing soybean concentration, the slope of straight line decreases and in fact lines with positive slopes are approached without reaching to the finite values of $[P]_{\infty}$ (figure 1). This result indicates that hK6-soybean association is reversible which is similar to those of trypsin-soybean association (19).

In the present investigation microscopic inhibitory rate constants for interaction of hK6 and soybean was calculated. The value of K_i and K_i^* were 13 and 1.6 nM, respectively. The K_i was rather small in comparison to that calculated for trypsin and soybean interaction (19). Also in our results the calculated value of k_3 was much higher than other

three rate constants of hK6 inhibition by soybean (see table1). However all calculated microscopic rate constants in this study were quite lower than the values reported for trypsin inhibition by soybean (19) which resembles to values which have been observed previously for the interaction of some other proteinases with soybean (16, 24).

It may be concluded that the stability of hK6-soybean association is lower than trypsin-soybean. Although, it has been demonstrated (22,25) that every proteinase-inhibitor pair has its own interaction peculiarities which might related to their chemical structures, it is possible by crystallographic analysis to gain further insight into the mode of interaction of hK6 with soybean.

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